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Etude des Histones Désacétylases (HDACs) comme cibles thérapeutiques dans le cancer gastrique

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SCIENTIFIC POPULARIZATION

“L’éducation est l'arme la plus puissante que vous pouvez utiliser pour changer le monde”

“Education is the most powerful weapon which you can use to change the world.”

Nelson Mandela

Résumé grand public

Reprogrammer la tumeur et traiter le cancer grâce aux modulateurs de l'épigénétique.

Notre corps est formé d'une multitude de cellules. Chacune de ces cellules contient un code barre : l'ADN. L'ADN possède une structure très régulée appelée chromatine qui varie au cours du temps. Les variations de cette structure rendent accessible ou non les informations (gènes) portées par l'ADN. Ces gènes sont nécessaires au devenir des cellules et au bon fonctionnement du corps humain. L'étude de la structure de l'ADN (donc la chromatine) et de l'accès aux gènes s'appelle l'épigénétique. De nombreux acteurs (famille de protéines) contrôlent l'épigénétique. Ces acteurs sont fréquemment dérégulés dans les cancers. C'est pourquoi dans les cancers, la structure de la chromatine est modifiée ayant pour conséquence une augmentation ou une diminution de l'expression de nombreux gènes favorisant le développement, et la progression des cancers et leur résistance aux traitements. Notre équipe s'intéresse au cancer de l'estomac qui est malheureusement résistant aux thérapies actuelles.

En outre, nous étudions les Histones Désacétylases (HDACs), une famille de protéines impliquées dans l'épigénétique qui régule la structure de la chromatine et l'expression des gènes. Nous déterminons leur rôle dans la progression et la résistance aux traitements du cancer gastrique et nous les utilisons comme de possibles cibles thérapeutiques. De plus, nous nous intéressons en particulier à un membre de cette famille ; HDAC4 dont l'expression modifie la réponse des cellules cancéreuses à la chimiothérapie. Par ailleurs, nous testons des inhibiteurs spécifiques des HDACs que nous combinons avec les chimiothérapies actuelles afin de lutter de manière plus efficace contre le cancer en fonction des HDACs exprimées.

La finalité de ce travail est d'augmenter l'efficacité des traitements chimio-thérapeutiques et d'améliorer la qualité de vie des patients atteints de cancer de l'estomac.

Summary to a non-scientific public

Reprogram the tumor and treat cancer with epigenetic modulators.

Our body is made up of a multitude of cells. Each of these cells contains a barcode: DNA. DNA has a highly regulated structure called chromatin that varies over time. The variations of this structure make the information (genes) carried by it accessible or not. In general, genes are necessary to direct the future of cells thereby assuring a proper functioning of the human body. The study of DNA structure (so the chromatin) and access to genes is called epigenetics. Many actors (family of proteins) control epigenetics. These actors are frequently deregulated in cancers. That's why, in cancers, the structure of the chromatin is modified resulting in an increase or a decrease in the expression of many genes favoring the development, and the progression of cancers and their resistance to treatments. Our team is interested in stomach cancer, which is unfortunately frequently resistant to current therapies.

In this respect, we are studying Histones Deacetylases (HDACs), a family of proteins involved in epigenetics that regulate chromatin structure and gene expression. We characterize their role in the progression and the resistance to gastric cancer treatments, and their use as possible therapeutic targets. In this respect, we are focusing on one member of this family; HDAC4 whose expression changes the response of cancer cells to chemotherapy. In addition, we test specific HDAC inhibitors that we combine with current chemotherapies to fight more effectively gastric cancers according to their HDAC expression profile.

Our final aim is to improve the effectiveness of current chemotherapeutic treatments and to improve the quality of life of patients with stomach cancer.

RESUME

“Vous me faites penser à mon ami le Dr. Watson qui a la fâcheuse habitude de raconter ses histoires en commençant par la fin.”

Sir Arthur Conan Doyle, *L'aventure de Wisteria Lodge*

Epidémiologie du cancer gastrique

Dans le Monde, le cancer gastrique (CG) est la 3ème cause de mort par cancer pour les hommes et la 5ème pour les femmes *(Michel P et al. 2017),* l'Asie et l'Europe de l'Est possédant les plus hauts taux d'incidence et de mortalité *(GLOBOCAN 2012).* En France, le CG n'est certes pas le plus fréquent, mais son **taux de survie global à 5 ans des patients est autour de 15%** *(CDU-HGE 2015).* Ce-dernier s'explique notamment par l'efficience du traitement du CG qui à l'heure actuelle, consiste principalement en une résection chirurgicale complète ou partielle complétée d'une chimiothérapie (Cisplatine, Oxaliplatine, 5-Fluorouracile) et/ou d'une radiothérapie. Cependant, il y a peu de cancers qui répondent aux traitements, en cause, **l'insensibilité aux traitements et/ou la sélection des cellules cancéreuses résistantes** *(Weichert W et al. 2008 ; Shi WJ and Gao JB. 2016)***.** De plus, le CG est diagnostiqué tardivement à cause de l'apparition des symptômes dans les phases avancées de la pathologie impactant une fois encore le taux de survie des patients *(Weichert W et al. 2008)*

Plusieurs facteurs de risque favorisent la tumorigenèse gastrique. Le style de vie comme une alimentation riche en sel, la consommation excessive d'alcool ainsi que le tabagisme augmentent le risque de CG. À l'inverse, une alimentation riche en fruits et légumes (antioxydants) diminue ce risque *(Lambert R. 2010, Quadri HS et al. 2017).* L'histoire infectieuse du patient par le virus d'Epstein-Barr (EBV) et par la bactérie *Helicobacter pylori* augmente également le risque de développer un CG. Ces dernières années, l'incidence du cancer gastrique a diminuée grâce à un contrôle de ces facteurs de risque, avec le plus important ; l'éradication d'*Helicobacter pylori (Michel P et al. 2017).* Certains facteurs génétiques sont également connus pour augmenter le risque de développer un CG. Cela concerne les formes héréditaires et représente 3% des cas de CG *(Quadri HS et al. 2017).*

Pathogenèse du cancer gastrique

L'infection par *Helicobacter pylori* favorise la transformation oncogénique des cellules épithéliales gastriques et l'apparition d'une atrophie gastrique chronique. L'association de l'infection par l'Epstein-Barr virus de même que l'association des facteurs de risque environnementaux (régime riche en sel, tabagisme, forte consommation d'alcool) et génétiques (syndrome de cancers associés, forme héréditaire de CG diffus) favorise la carcinogenèse gastrique *(Cross AJ et al. 2003 ; Cavanagh H and Rogers KMA. 2015 ;*

Boland CR and Yurgelun MB. 2017 ; Ma K et al. 2017 ; Quadri HS et al. 2017). Cette dernière est le résultat d'une atrophie gastrique chronique évoluant en métaplasie intestinale, puis en dysplasie et enfin dans en carcinome invasif *(Correa P and Piazuelo MB. 2012).* Le CG est histologiquement hétérogène. Par conséquent, plusieurs classifications ont été proposées. Une des plus utilisées est la classification de Lauren qui distingue le CG en trois grands types histologiques.

Le **type intestinal** désignant les cancers distaux également appelés cancer non-cardia. Ce type est le plus fréquent au monde. Il dépend de l'infection de l'épithélium gastrique par *H. pylori*, il se développe à un âge avancé et a une prépondérance masculine *(Correa P and Piazuelo MB. 2011).*

Le **type diffus** désignant les cancers du cardia et de la jonction œsophagienne-gastrique. Ce type est plus agressif avec un pronostique plus sombre *(Ma J et al. 2016).* Le type diffus est plus présent dans les pays *(Lambert R. 2010).* Son développement ne dépend pas de l'infection de l'épithélium gastrique par *H. pylori* cependant sa cancérogénèse n'est pas entièrement comprise *(Buas MF and Vaughan TL. 2013).*

Enfin le troisième type regroupant les cancers **indéterminés ou mixtes** *(Michel P et al. 2017).*

D'un point de vue moléculaire, **le CG possède une forte hétérogénéité** où de nombreuses voies cellulaires (MAP kinases, Wnt, YAP-TAZ, PI3K) sont altérées permettant l'apparition, la progression, l'invasion et la résistance aux traitements du CG. Parmi les mécanismes moléculaires altérées, on retrouve également les histones désacétylases (HDACs) *(Padmanabhan N et al. 2017 ; Ajani JA et al. 2017).*

Les Histones Désacétylases

Les histones sont modulées par plusieurs modifications post-traductionnelles parmi lesquelles l'acétylation. L'acétylation des lysines des histones est réversible et est contrôlée par l'équilibre entre les histones acétyltransférases (HATs) et les histones désacétylases (HDACs). L'acétylation des histones est associée à une décondensation de la chromatine et à une activation transcriptionnelle alors que la désacétylation des histones est associée à une condensation de la chromatine et à une répression transcriptionnelle. 18 HDACs sont décrites et sont séparées en quatre classes basées sur leur homologie de séquence avec la levure. La classe I homologue de la Rpd3 chez la levure qui contient les HDAC 1, 2, 3 et 8.

La classe II homologue de Hda1 qui est divisée en deux sous-classes : la classe IIa avec les HDAC 4, 5, 7 et 9 et la classe IIb avec les HDAC 6 et 10. La classe IV ne contient que l'HDAC11 présentant des similitudes avec les classes I et II. Les classes I et II sont dépendantes du zinc tandis que les enzymes homologues de Sir2 formant la classe III des Sirtuins qui sont des protéines NAD-dépendantes et/ou ADP ribosylases. En outre, plus de 50 protéines non histones sont également connues pour être acétylées ou désacétylées affectant leur stabilité, leur capacité à former des interactions protéiques, leur affinité pour l'ADN et leur activité transcriptionnelle. Par conséquent, les HDACs impactent de nombreuses voies cellulaires *(Li Y et Seto E. 2017).*

Il a été montré ces dernières années que le CG est sujet de modifications épigénétiques aberrantes *(Padmanahban N et al. 2017).* Différentes études ont montré notamment que des HDACs de classe I (HDAC 1, 2, 3) sont surexprimées *(Zhang J and Zhong Q. 2014),* et que des HDACs de classe II sont surexprimées (HDAC4), ou sous-exprimées (HDAC6) dans des cas de CG *(Kang ZH et al. 2014 ; He Q et al. 2017).* **Les altérations aberrantes des HDACs favorisent le développement, la progression et la survie des cellules cancéreuses** *(Zhang J and Zhong Q. 2014).*

De ce fait, **les inhibiteurs des histones désacétylases (HDACIs) présentent un intérêt thérapeutique dans la lutte contre le cancer** *(Zhang J and Zhong Q. 2014).* En général, les HDACIs induisent une augmentation globale de l'acétylation des histones et des protéines non-histones provoquant une décondensation de la chromatine. Les HDACs étant impliquées dans de nombreuses voies cellulaires, les HDACIs peuvent induire l'arrêt du cycle cellulaire, l'inhibition de la croissance, la différenciation, l'inhibition de l'invasion, l'autophagie, et stimuler une réponse immunitaire *(Mottamal M et al. ; 2015, Li Y et Seto E. 2017 ; Eckschlager T et al., 2017).*

Le SAHA (ou Vorinostat) est un inhibiteur des HDACs approuvé par la FDA en 2006 pour le traitement du lymphome cutané des cellules T. Il **possède des effets antiprolifératifs et pro-apoptotiques** dans différents modèles cancéreux (métastases cérébrales, mélanomes, cancers du poumon et colorectaux) *(Mottamal M et al. 2015).* Cependant, dans les essais cliniques, des effets secondaires tels que l'anémie, la fatigue, la diarrhée, et des décès ont été rapportés impliquant différents HDACIs dont le SAHA. Les études soulignent la **nécessité de déterminer le rôle des HDACs dans le CG et de minimiser l'effet toxique des HDACIs chez les patients** *(Mottamal M et al. 2015).* La toxicité pourrait s'expliquer par le rôle important des HDACs dans plusieurs processus biologiques et par la faible sélectivité des inhibiteurs. Pour résoudre ce problème, deux stratégies sont développées :

i) **Diminuer les doses des HDACIs utilisés en combinant les inhibiteurs avec d'autres thérapies** comme les chimiothérapies à base de dérivés de platine (PDC : Cisplatine, Oxaliplatine). En effet, la combination des HDACIs à de faibles doses a été proposée pour agir en synergie avec les PDC améliorant leur cytotoxicité *(Diyabalanage HVK et al. 2013 ; To KKW et al. 2017).*

ii) **Identifier plus précisément les fonctions des HDACs et développer des inhibiteurs plus spécifiques.** Mais la conception et la synthèse de tels inhibiteurs représentent un véritable défi *(Li Z and Zhu GT. 2014 ; Mottamal M et al. 2015).*

Au cours de ma thèse, j'ai travaillé sur ces deux stratégies afin d'apporter une réponse thérapeutique améliorant la qualité de vie des patients atteints de CG.

Problématique de la Thèse

Le but de ma thèse est d'étudier les HDACs comme cibles thérapeutiques dans le cancer gastrique. Mon **premier objectif** est de caractériser les HDACs nécessaires dans la progression et la résistance aux traitements du cancer gastrique. Les HDACs impactant la chimiorésistance, je teste un **traitement combinant un inhibiteur des HDACs** (le SAHA ou Vorinostat) **avec les dérivés de platine** utilisés en clinique (Cisplatine, Oxaliplatine) afin de promouvoir les effets cytotoxiques de ces chimiothérapies. En parallèle en **second objectif,** je détermine les **fonctions de HDAC4** dans le cancer gastrique et son **intérêt thérapeutique**. Plus précisément, je m'intéresse aux mécanismes cellulaires régulant son expression en réponse au Cisplatine. Puis j'inhibe HDAC4 avec des inhibiteurs pharmacologiques ; soit avec le SAHA (inhibiteur non spécifique de HDAC4) soit avec de **nouveaux HDACIs plus spécifiques** de HDAC4.

Etudes des HDACs et des traitements combinés HDACIs + dérivés de platine

Dans mon premier objectif de thèse, je détermine les histones désacétylases nécessaires dans la progression et la résistance aux traitements du CG.

J'ai analysé l'expression génique des HDACs par RT-qPCR dans les cellules cancéreuses gastriques **AGS (CG type intestinal)** et **MKN45 (CG type diffus, métastase hépatique)**. L'analyse des expressions relatives montre que les HDACs de Classe I (HDAC 1, 2 et 3) et HDAC7 (HDAC de classe II) sont les plus exprimées et que HDAC8 (HDAC de

Classe I) et les HDAC 9 et 6 (HDACs de Classe II) sont les moins exprimées. Globalement les deux lignées cellulaires ont le même profil d'expression des HDACs. De plus, **HDAC4** semble plus exprimée dans les cellules MKN45 que dans les cellules AGS. De manière intéressante, les HDAC 3, 4, 5, 7 et 9 sont les plus impactées par les traitements anticancéreux actuelles. Il est intéressant de noter que ces HDACs sont associées à l'agressivité ou à la résistance aux traitements dans différents cancers *(Ding JD et al. 2012 ; Colarossi L et al. 2014 ; Stronach EA et al. 2011 ; Kim MG et al. 2012 ; Li A et al. 2016).*

Les HDACs étant dérégulées dans différents cancers dont elles impactent la progression et la chimiorésistance *(Li Y and Seto E. 2017),* je teste des traitements combinant dérivés de platine (PDC) + HDACIs pour inhiber les HDACs et promouvoir l'effet cytotoxique des PDC *(Diyabalanage HVK et al. 2013 ; To KKW et al. 2017).* Je m'intéresse à la combinaison avec le **Cisplatine** la chimiothérapie de référence, et avec l'**Oxaliplatine** l'autre chimiothérapie à base de platine plus utilisée en clinique à l'heure actuelle. Au laboratoire, il est déjà établi une synergie entre le **SAHA** et le Cisplatine sur la survie des cellules AGS. Cette dernière a été réalisée par des tests de survie MTT complétés d'études isobologrammes *(Chou TC. 2006 ; 2010).* La synergie avec le SAHA est aussi retrouvée avec l'Oxaliplatine et cela à de faibles concentrations.

J'ai poursuivi ce travail en identifiant *in vitro* les voies cellulaires induites ou inhibées par les traitements combinés PDC + SAHA. Notamment sur la famille p53 ; une famille clef dans la réponse au stress cellulaire *(Reinhardt HC and Schumacher B. 2012).* En réponse aux traitements combinés PDC + SAHA, j'ai pu mettre en évidence un marqueur d'**apoptose** (Caspase 3 clivée par Western Blot), ainsi que la **diminution d'expression de protéines dites pro-survie** (HIF1α, Akt et pAkt en Western Blot) sur les cellules AGS. J'ai également observé une induction de *MEF2A*, un facteur de transcription partenaire des HDACs de Classe II *(Di Giorgio E et al. 2013)* ainsi qu'une baisse de l'expression de *CDX2* un marqueur intestinal *(Freund JN et al. 2015).* Dans le même temps, j'ai observé une baisse de l'expression de p53 dans ces mêmes cellules mais j'ai pu établir que le clivage de la caspase 3 est **p53 dépendant.** Bien qu'il y ait une baisse d'expression de p53, il y a une induction de ses gènes cibles comme ***p21*** et ***NOXA*** en réponse au SAHA. Cet effet p53 dépendant est corrélé avec la lignée cellulaire de cancer gastrique **KATOIII (délétée pour p53)** où les synergies n'induisent pas le clivage de caspase 3. Mais aussi avec les cellules **NUGC3** de cancer gastrique (**mutées pour p53** – Y220C) où la réactivation chimique de p53 permet l'augmentation du clivage de la caspase 3 en réponse aux traitements synergiques PDC + SAHA. **Le statut p53 des cellules impacte donc la réponse aux traitements.**

Par ailleurs, la baisse d'expression de p53 dans les cellules AGS ne semble pas être due à l'expression de microARNs ciblant p53 (miR25, 30-d, 125a, et 222) *(Rockavec M et al. 2014 ; Liu J et al. 2017).* Des immunomarquages de **Ki67** sur les cellules cancéreuses gastriques ont montré que les traitements synergiques diminuent la prolifération cellulaire (par le nombre de cellules) mais ne l'empêchent pas (marquage Ki67 toujours positif). Le laboratoire poursuit l'étude de la synergie en validant les traitements PDC ± SAHA *in vivo* dans un **modèle hétérotopique murin PDX** (Xénogreffes dans des souris NUDE de biopsies de cancers gastriques) en collaboration avec le CHU Hautepierre Strasbourg. D'ores et déjà, les résultats préliminaires montrent que la combinaison Cisplatine + SAHA diminue la croissance tumorale de manière plus importante que les traitements Cisplatine ou SAHA seuls.

Fonctions et régulation de l'expression de HDAC4 en réponse au Cisplatine dans le cancer gastrique

Le laboratoire a observé une expression aberrante de HDAC4 dans des biopsies de patients européens atteints de cancer gastrique (collaboration avec le CHU de Hautepierre). De plus, l'analyse des bases de données du TCGA *(www.cancergenome.nih.gov) montre* que l'expression de HDAC4 diffère selon les sous-types moléculaires du cancer gastrique. Elle est la plus importante pour le sous-type majoritairement retrouvé en Europe (CG de type diffus génomiquement stable) *(Spaety ME et al. En soumission).* De manière intéressante, le laboratoire a aussi établi que les patients avec une altération de HDAC4 ont un meilleur taux de survie globale. Nous avons corrélé *in vitro* l'expression de HDAC4 avec la **sensibilité des cellules AGS au Cisplatine.** Il a établi que le **Cisplatine diminue l'expression de HDAC4 via une boucle de régulation comprenant le miR140 et p53** *(Spaety ME et al. En soumission).* Des études supplémentaires sont nécessaires afin d'approfondir le rôle de HDAC4 dans les cellules cancéreuses gastriques.

Dans ce travail, ma contribution a été la réalisation d'expériences d'immunoprécipitation de chromatine (ChIP) afin d'affirmer ou d'infirmer une régulation directe de p53 sur HDAC4 et miR140. Les résultats que j'ai pu obtenir montrent que l'on n'a pas de régulation directe de p53 sur les régions promotrices de HDAC4 et de miR140.

De même, en m'appuyant sur la littérature *(Amodio N et al. 2016 ; Ma G et al. 2015 ; Kim HS et al. 2015)*, j'ai testé d'autres microARNs (miR-206, 29b et 125a) qui pourraient réguler HDAC4 en réponse au Cisplatine. Une grande difficulté rencontrée fut la sensibilité et

la variabilité de l'expression des microARNs. Le Cisplatine semble induire l'expression de manière dose dépendante des microARNs cités, après 36h de traitement et déjà après 24h pour le miR-206. Il reste à confirmer l'impact de ces microARNs dans la baisse importante de l'expression de HDAC4 en réponse au Cisplatine par une analyse fonctionnelle.

Etant donné que le Cisplatine diminue l'expression de HDAC4, nous avons déterminé *in sillico* les mécanismes moléculaires qui sont impactés ainsi que les gènes corrélés positivement et négativement avec l'expression de HDAC4 *(Spaety ME et al. En soumission)*.

J'ai validé par des expériences de perte de fonction de HDAC4 que plusieurs gènes candidats ont leur expression augmentée. Il s'agit des gènes ***BID, NOXA, BIK, CASPASE 8*** et ***AIFM1*** qui interviennent dans les voies apoptotiques, ***p21*** qui régule le cycle cellulaire et ***VAMP8*** et ***STXBP2*** qui ont un rôle dans le trafic vésiculaire. J'ai également pu établir une tendance pour le gène ***ASB1*** de la grande famille des Ankyrines. Ce travail montre donc **un rôle affin de l'axe p53/HDAC4 dans la réponse au Cisplatine.**

J'ai pu montrer que l'extinction de HDAC4 induit une apoptose des cellules AGS en réponse au Cisplatine. Je voulais inhiber plus spécifiquement HDAC4 dans les cellules AGS pour promouvoir l'effet cytotoxique du Cisplatine. Le SAHA est connu en clinique pour induire des effets secondaires sûrement dus à son activité pan-HDAC. Notre équipe collabore avec le Dr. Spencer (Université du Sussex) afin d'obtenir des inhibiteurs plus spécifiques des HDACs. J'ai testé le **JGS-038** néo synthétisé ciblant HDAC3 et en parallèle le **LMK-235** vendu comme inhibant plus spécifiquement les HDAC 4 et 5 *(Marek L et al. 2013)*.

J'ai pu établir que le **LMK-235 agit en synergie avec le Cisplatine** sur la survie des cellules AGS. J'ai pu montrer que le LMK-235 seul ou en combinaison avec le Cisplatine induit un **clivage de la caspase 3** (en Western Blot). **Ce résultat corrobore le rôle de HDAC4 dans la résistance au Cisplatine.** Par ailleurs, nos collaborateurs (Drs. Yanagihira et Okamoto, NCRC, Tokyo) ont également confirmé cette **synergie sur la croissance tumorale *in vivo*** dans un modèle murin.

Concernant le **JGS-038,** il agit en **synergie avec le Cisplatine** sur la survie des cellules NUGC3 mais pas des cellules AGS. Il nous reste à établir sa spécificité ainsi que les mécanismes cellulaires induits. D'ores et déjà le fait que le JGS-038 agit en synergie dans des cellules mutées pour p53 qui est un gène altéré dans plus de 50% des cancers gastriques, est très intéressant *(TCGA)*.

Par ailleurs, j'ai aussi montré que l'extinction de HDAC4 dans les cellules MKN45 n'induit pas un clivage de la caspase 3 en réponse au Cisplatine contrairement aux cellules AGS. Pourtant j'ai rapporté précédemment que HDAC4 est plus fortement exprimée dans les cellules MKN45 par rapport aux cellules AGS. A l'inverse, l'extinction de HDAC4 dans les cellules NUGC3 a augmenté le clivage de la caspase 3 en réponse au Cisplatine mais dans une plus faible mesure que dans les cellules AGS. Ainsi, il semblerait que la réponse au Cisplatine médiée par le niveau de HDAC4 est impacté par le type de GC et le statut p53 des cellules.

Conclusion et perspectives

Ma thèse met en évidence le **rôle des HDACs dans la réponse aux traitements** anticancéreux **à base de dérivés de platine** (PDC) dans le cancer gastrique (CG). Les résultats suggèrent que les HDACs, en particulier HDAC4, pourraient être des **marqueurs pronostiques prometteurs** et des cibles thérapeutiques dans le CG. Cependant, plusieurs aspects doivent encore être abordés avant de transférer nos résultats à la clinique.

Pour valider l'utilité de HDAC4 en tant que marqueur prédictif de la réponse au Cisplatine, nous devons effectuer une **étude clinique prospective** pour étudier le lien entre l'expression de HDAC4 et la survie globale chez les patients traités avec des composés de platine. De plus, il n'est pas entièrement établi comment HDAC4 contrôle la sensibilité du Cisplatine au niveau moléculaire. Par exemple, il reste à déterminer comment p53 impacte la réponse au Cisplatine via l'axe miR-140/HDAC4 en régulant des gènes spécifiques. Dans ce contexte, ***BIK*** semble être un candidat possible. De même, il sera intéressant d'étudier l'impact d'un régulateur de p53, **MDM2,** sur la boucle p53/miR-140/HDAC4. De plus, en se basant sur l'expression différentielle de HDAC4 dans les types de CG, il reste également à comparer l'impact de cette boucle dans le **CG diffus et intestinal.**

Concernant l'utilité des HDACs en tant que cibles thérapeutiques, Yoo C et ses collaborateurs ont mené une étude clinique sur l'association du Vorinostat (SAHA) avec la Capecitabine plus Cisplatine *(Yoo C et al. 2016)* qui n'a entraîné aucun gain sur la survie globale. Comme p53 semble être un modulateur impliqué dans la réponse aux traitements combinés PDC + SAHA chez les patients, il serait intéressant d'effectuer une **analyse rétrospective** de cette étude clinique pour établir **l'impact du statut p53** sur la réponse au Vorinostat. De plus, il reste à déterminer les **gènes impactés** par les traitements combinés dépendamment et indépendamment de p53. Cela pourrait nous aider à comprendre quel

mécanisme de mort cellulaire a lieu dans les cellules p53 -/- (par exemple KATOIII). Comme les types diffus et intestinal de CG ont des caractéristiques cliniques et pathologiques différentes, qui peuvent avoir un impact sur la chimiothérapie *(Ma J et al. 2016),* nous pouvons également comparer les voies cellulaires entre des cellules intestinales et diffuses de CG. Enfin, j'estime sincèrement que synthétiser une **nano-drogue PDC-HDAC inhibiteur**, dont les effets cytotoxiques seront indépendants de p53, pourrait être la prochaine référence en matière de traitement des patients. Ding JD et ses collaborateurs suggèrent que le SAHA induit une apoptose des lymphomes des cellules T via l'inhibition de HDAC3 *(Ding JD et al. 2012).* Comme le **JGS-038** semble être actif dans les cellules mutées pour p53, et parce qu'il cible HDAC3, il représente ainsi un bon candidat pour cette fusion chimique. Par ailleurs, comme nous voulons proposer un traitement à base de HDACIs, nous devons déterminer la spécificité de ces inhibiteurs dans nos combinaisons.

Plus généralement, les futurs résultats sur les marqueurs thérapeutiques HDAC4 et p53 pourraient nous aider à stratifier les patients dans le but de leur proposer un protocole de traitement personnalisé basé sur l'association entre les PDC et les HDACIs améliorant ainsi leur prise en charge et leur qualité de vie.

LIST OF ABBREVIATIONS

"The smallest things are by far the most important"

Sir Arthur Conan Doyle, *An Identity Case*

5-FU: 5-Fluorouracil

ABCC/MRP: ATP binding cassette or Multidrug resistance-associated protein

ACRG: Asian Cancer Research Group

ADP: Adenosine diphosphate

AID: Activation-induced cytidine deaminase

AIFM1: Apoptosis Inducing Factor Mitochondria Associated 1

AKT: RAC-alpha serine/threonine-protein kinase

AMPK: Adenosine monophosphate-activated protein kinase

ANOVA: Analysis of variance

antimir: Small interfering miRNA

AP: Apurinic or Apyrimidinic

APC: Adenomatous polyposis coli

AQP3: Aquaporin 3

ASB1: Ankyrin Repeat and Suppressor-of-cytokine-signalling Box Containing 1

ATCC: American Type Culture Collection

ATF4: Activating Transcription Factor 4

ATG: Autophagy related gene

ATM: Ataxia telangiectasia mutated

ATP: Adenosine triphosphate

ATP7A/B: Copper-transporting P-type ATPase Menkes' protein

AURKA: Aurora kinase A

Bab: Blood group antigen binding adhesin

BAD: Bcl-2-associated death promoter

BAK1: BCL2 Antagonist/Killer 1

BARF1: BamHI A rightward frame 1

BART: BamHI A rightward transcripts

BAX: BCL2 Associated X Protein

BCL-2: B-cell lymphoma 2

BCL2L1: BCL2 Like 1

BER: Base excision repair

BRCA1/2: Breast cancer 1/2

BSA: Bovine serum albumin

Cag: Cytotoxin-associated gene

CASP: Caspase

CDC2/CDK1: Cyclin Dependent Kinase 1

CDH1: E-cadherin

CDKN1A: Cyclin Dependent Kinase Inhibitor 1A or p21

CDKN1B: Cyclin Dependent Kinase Inhibitor 1B or p27

CDKN1C: Cyclin Dependent Kinase Inhibitor 1C or p57

CDX2: Caudal-related homeobox transcription factor 2

CHAC1: Cation transport regulator-like protein 1

ChIP: Chromatin immunoprecipitation

CHK1: Checkpoint kinase 1

CIN: Chromosomal instability

CISP: Cisplatin

CK1α: Casein kinase 1

CMTM2: Chemokine-Like Factor Superfamily Member 2

c-Myc: Avian Myelocytomatosis Viral Oncogene Homolog

CO_2: Carbon dioxide

COX-2: Cyclooxygenase-2

CpG: Cytosine-Guanine oligodeoxynucleotides

CSA/B: Cockayne syndrome A/B

Ctl/Ctrl: Control

CTNNB1/β-catenin: Catenin (Cadherin-Associated Protein), Beta 1

CYCS: Cytochrome C

DAPI: 4′,6-diamidino-2-phenylindole

DAPK: Death Associated Protein Kinase

DBD: DNA bindind domain

DIABLO: Second Mitochondria-Derived Activator of Caspase

DKK: Dickkopf-related protein

DMSO: Dimethylsulfoxyde

DNA: Deoxyribonucleic acid

DNMT: DNA methyltransferase

DO: Optic density

DSB: DNA Double-strand breaks

E2F: Retinoblastoma-associated protein

EBER: Epstein–Barr virus-encoded small RNA

EBNA: Epstein–Barr nuclear antigen 1

EBV: Epstein-Barr virus

ECL: Enterochromaffin-like cells

EDTA: Ethylenediaminetetraacetic acid

EGFR/ERB1/HER1: Epidermal growth factor receptor

EGTA: Egtazic acid

EMT: Epithelial-to-mesenchymal transition

ENDOG: Endonuclease G

EPHB3: Ephrin type-B receptor 3

ERCC1: DNA excision repair protein

ERK: Extracellular signal–regulated kinases

ER-stress: Endoplasmic-reticulum stress

f.e.: For example

FAK: Focal adhesion kinase

FAP: Familial adenomatous polyposis

FBS: Foetal bovine serum

FDA: Food and Drug Administration

FOLFOX: 5-FU, Folinic acid and Oxaliplatin combinatory treatment

FOXO: Forkhead box proteins family of transcription factors

GATA: Globin transcription factor

GC: Gastric cancer

GCC: Gastric cancer cells

GCF2: GC-rich sequence DNA-binding factor

GLI: Glioma-associated oncogene

Glut-1: Glucose Transporter Type 1

GS: Genomically stable

GSH: Glutathione

GSK3: Glycogen synthase kinase 3

GST: Glutathione S-transferase

HATs: Histone acetyltransferases

hCTR1/2: Human copper transporter

HDACIs: Histone deacetylase inhibitors

HDACs: Histone deacetylases

HDGC: Hereditary Diffuse Gastric Cancer

HEPES: 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid

HER2/ERB2: Human epidermal growth factor receptor

HIC1: Hypermethylated In Cancer 1

HIF: Hypoxia inducible factor

HLA: Human leukocyte antigen

HOXC10: Homeobox Protein C10

HOXD10: Homeobox Protein D10

HR: Homologous recombination

HSP: Heat shock proteins

HxKy(ac): (Acetylated) lysine y on histone x

IC_X: Concentration of drug reducing of x% the cell survival

IGF-1R: Insulin-like growth factor 1 receptor

IgG: Immunoglobulin G

IGSF8: Immunoglobulin superfamily member 8

JAK: Janus Kinase

JCRB: Japanese Collection of Research Bioresources

JNK: c-Jun N-terminal kinases

Ki67: Proliferation Marker Protein Ki67

KLF4: Kruppel Like Factor 4

KRAS: V-Ki-ras2 Kirsten rat sarcoma viral oncogene homolog

Ku70: X-Ray Repair Cross Complementing 6

LATS: Large Tumor Suppressor Kinase

LEF: Lymphoid enhancer-binding factor

LIG: DNA ligase

LMP2A: Epstein–Barr virus latent membrane protein 2

LncRNA: Long non-coding RNA

MAPK: Mitogen-activated protein kinases

MDM2: Mouse double minute 2 homolog

MEF2: Myocyte Enhancer Factor 2

MEKK: Mitogen-activated protein kinase kinase kinase

MET/HGFR: Tyrosine-protein kinase Met or Hepatocyte growth factor receptor

MGMT: O^6-alkylguanine DNA alkyl-transferase

mimic: Miming RNA

miRNA: Micro ribonucleic acid

MLH: MutL homolog 1, colon cancer, nonpolyposis type 2

MMP: Matrix metalloproteases

MMR: Mismatch repair

mRNA: Messenger ribonucleic acid

MSH: DNA mismatch repair protein

MSI: Microsatellite instability

MSS: Microsatellite stable

MST: Macrophage-Stimulating Protein

mTOR: Mechanistic target of rapamycin

MTT: 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide

MUC: Mucin

MyoD: Myogenic differentiation

NAD: Nicotinamide adenine dinucleotide

NCOR: Nuclear receptor co-repressor

NER: Nucleotide excision repair

NFκB: Nuclear factor-kappa B

NGS: Normal goat serum

NHEJ: Non-homologous end joining

NOXA/PMAIP3: Phorbol-12-myristate-13-acetate-induced protein 1

NRF2/NFE2L2: Nuclear factor (erythroid-derived 2)-like 2

NT: Untreated or un-transfected

O_2: Dioxygen

OCT: Octamer-binding transcription factor

OD: Oligomerization domain

OR: Odd ratio

OXA: Oxaliplatin

P/S: Penicillin/streptomycin

PALD1: Phosphatase Domain Containing Paladin 1

PARP: Poly [ADP-ribose] polymerase 1

PCNA: Proliferating cell nuclear antigen

PD-1: Programmed cell death 1

PDC: Platinum derivative compounds

PDCD4: Programmed cell death protein 4

PDK1: 3-phosphoinositide-dependent protein kinase-1

PDL1/2: Programmed death-ligand 1/2

PFA: Paraformaldehyde

PGC1α: Peroxisome Proliferator-Activated Receptor Gamma, Coactivator 1 Alpha

PGK1: Phosphoglycerate Kinase 1

PHD: Prolyl hydroxylases

PI3K: Phosphatidylinositol-4,5-bisphosphate 3-kinase

PI3KCA: Phosphatidylinositol 3-kinase

PIP2: Phosphatidylinositol-4,5-bisphosphate

PIP3: Phosphatidylinositol (3,4,5)-trisphosphate

PIPES: Piperazine-N,N′-bis(2-ethanesulfonic acid

PMS2: Mismatch repair endonuclease

Pol: Polymerase

PRIMA: P53 re-activation and induction of massive apoptosis

PTEN: Phosphatase and tensin homolog mutated in multiple advanced cancers 1

PUMA/BBC3: P53-upregulated modulator of apoptosis

pX: Phosphorylated X

qPCR: Quantitative polymerase chain reaction

RAF: Serine/threonine protein kinase

RASSF1: Ras association domain-containing protein 1

Rb: Retinoblastoma protein

RECK: Suppression Of Tumorigenicity 15 (Reversion-Inducing-Cysteine-Rich Protein With Kazal Motifs)

RelI: NF-Kappa-B subunit transcription factor

RFC: Replication factor C

RhoA: Ras homolog gene family member A

RNA: Ribonucleic acid

RNU6: Small Nuclear RNA U6

ROS: Reactive oxygen species

RPMI: Roswell Park Memorial Institute medium

RTK: Receptor tyrosine kinase

RT-qPCR: Reverse transcription and quantitative polymerase chain reaction

RUNX: Runt-related transcription factor

SAHA: Suberanilohydroxamic acid or Vorinostat

SBHA: Suberoyl bis-hydroxamic acid

SEM: Average deviation

SHH: Sonic hedgehog

SHP-2: Protein-tyrosine phosphatase 2C

siRNA: Small interfering RNA

SIRTs: Sirtuins

SMAD: Mothers against decapentaplegic homolog

SMO: Smoothened, Frizzled Class Receptor

SMRT: Silencing mediator for retinoid or thyroid-hormone receptors

SNORD: Small nucleolar RNA C/D box

SNP: Single nucleotide polymorphism

Src: Proto-oncogene tyrosine-protein kinase

SSB: Single-strand break

STAT3: Signal transducer and activator of transcription 3

STK11/LKB1: Serine/threonine kinase 11 or Liver kinase B1

STXBP2: Syntaxin Binding Protein 2

TA: Transactivation domain

TAZ: WW domain-containing transcription regulator protein 1

TBP: TATA-box binding protein

TCF: Transcription factor

TCGA: The Cancer Genome Atlas

TC-H 106: *N1*-(2-Aminophenyl)-*N7*-(4-methylphenyl)heptanediamide

TEAD: Transcriptional Enhancer Factor

TNM: Classification based on tumoral invasion, ganglionic invasion and presence of metastasis

TP53/63/73: Tumor suppressor protein 53/63/73

TSA: Trichostatin A

TXR1: Taxol resistance gene 1

Vac: Vacuolating cytotoxin

VAMP8: Vesicle Associated Membrane Protein 8

VEGF: Vascular endothelial growth factor

VHL: Von Hippel-Lindau

VPA: Valproic acid

WHO: World Health Organization

WNT: Wingless-type MMTV integration site family

WT: Wild-type

XIAP: X-linked inhibitor of apoptosis protein

XP: Xeroderma pigmentosum

XRCC1: X-ray repair cross-complementing protein 1

YAP: Yes-associated protein 1

YY1: Yin and yang 1 protein

ZEB: Zinc Finger E-Box Binding Homeobox

ΔN: Isoform without the transactivation domain

INTRODUCTION

“At this very moment, you are inflamed by the singularity of the moment and the hunter's instinct”

Sir Arthur Conan Doyle, *The Valley of fear*

Genetic factors	Environmental factors	Other factors
Sex (Men odd ratio: 1,7) Familial adenomatous polyposis Lynch syndrome Genetic polymorphisms for pro- and anti-inflammatory cytokines Peutz-Jeghers syndrome Hereditary diffuse gastric cancer (*CDH1* E-cadherin mutation) Polymorphisms for cell receptors of innate immune response	*Helicobacter pylori* infection resulting in chronic gastric atrophy Epstein-Barr virus Nitrites and salt rich-diet Heavy alcohol consumption Poor diet in fruits and vegetables (poor in antioxidants) Low intake of fibers Cigarette smoke	Biermer's disease Partial gastrectomy for infection Gastric ulcer Ménétrier's disease Gastric adenomatous polyps Barrett's esophagus Chronic atrophic Gastritis Gastric metaplasia Fundic gland polyps Hyperplastic polyps

Table 1: Risk factors of gastric cancer

Adapted from *Gomceli I et al. Gastric carcinogenesis 2012* and *CDU-HGE Abbreviated hepato-gastro-enterology and digestive surgery 3rd. edition Chap.27 2015*

Gastric cancer epidemiology

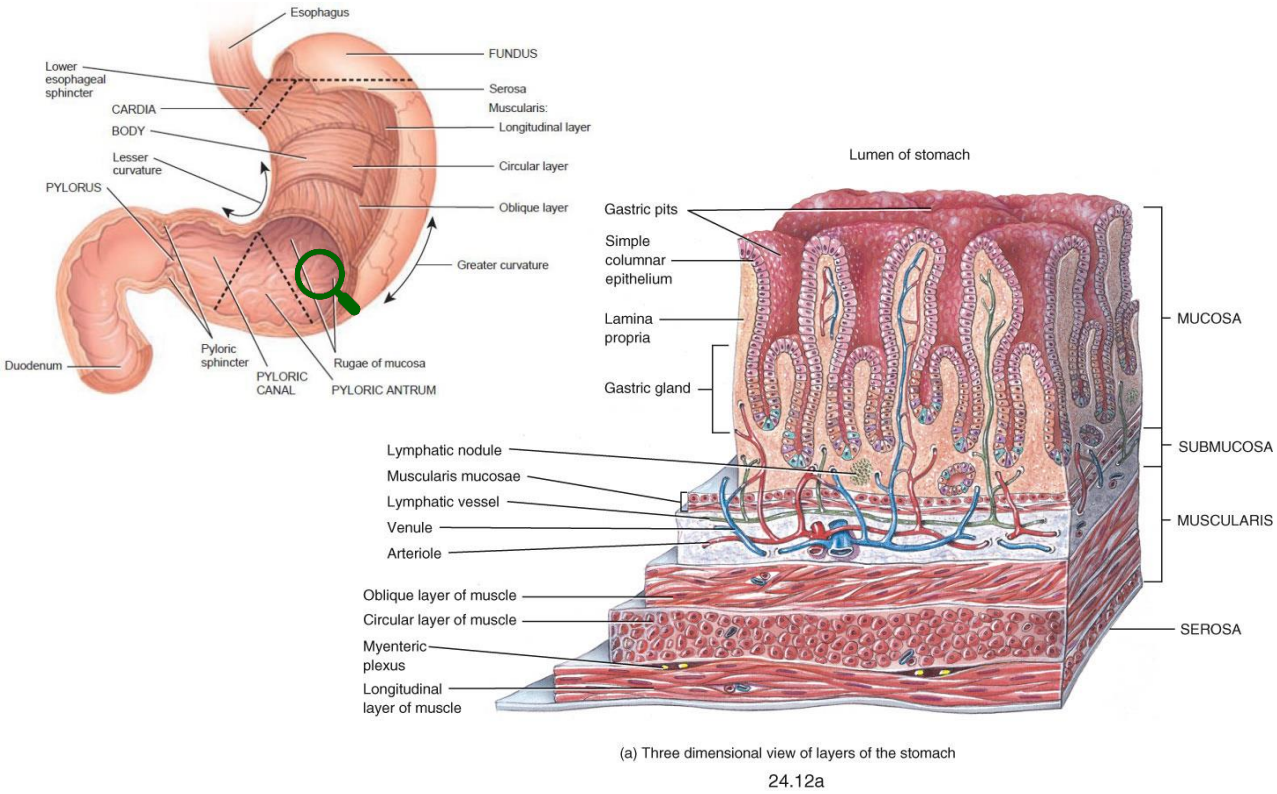
Gastric cancer (GC) is the 4th most common cancer for men and the 5th for women in the World. It represents the 3rd cause of cancer related death for men and the 5th for women *(Michel P et al. 2017).* The middle age to develop gastric cancer is 70 years old, and Asia and Eastern Europe regions possess the higher incidence and mortality levels *(GLOBOCAN 2012).* In France, cancers represent the first cause of death, besides GC is not the most common cancer, it is breast cancer for women and prostate cancer for men *(French National Institute of Cancer)*. Over the last years, the GC incidence decreased around 1.5% per year (out of gastro-esophageal junction and cardia cancers) *(Michel P et al. 2017).* In 2015, it was estimated about 6580 new cases of gastric cancer whose 66% in men *(French National Institute of Cancer)*. Unfortunately, the 5-year overall patient survival of GC is still low, around 15%, and as most GC are asymptomatic they are diagnosed tardily, which impacts on the survival rate making GC still a major health problem *(CDU-HGE 2015; Quadri HS et al. 2017)*.

Several risk factors promote gastric carcinogenesis **(Table 1).** Environmental factors and life style; like poorly preserved foods, pickled foods, salt rich-diet, nitrites, nitrates, heavy alcohol consumption and cigarette smoke increase gastric cancer risk, whereas a diet rich in fruits, vegetables and antioxidants decreases this risk *(Lambert R. 2010; Quadri HS et al. 2017).* The patient-infection-history by EBV (Epstein-Barr virus), and *Helicobacter pylori* increases also the risk to develop GC. The observed diminution of GC incidence is due to a better understanding and control to these risk factors with the most important; the eradication of *Helicobacter pylori (Michel P et al. 2017).* Besides environmental factors, some genetic factors are known to increase the risk of GC which concerns inherited forms and represents 3% of GC cases *(Quadri HS et al. 2017).*

Stomach histology

The stomach is composed of different regions, in the order from the esophagus and the proximal-distal axis, it is divided into the cardia, the fundus-corpus, and the antrum-pylorus region **(Figure 1a).** There are transitional zones, which separate the stereotypic corpus zone, the antrum and the pyloric epithelia. The stomach epithelium is composed of millions of glands. The gastric glands open into the bottom of the pits, on an average with 4 to 5 glands per pit.

(a)



(a) Three dimensional view of layers of the stomach

24.12a

(b)

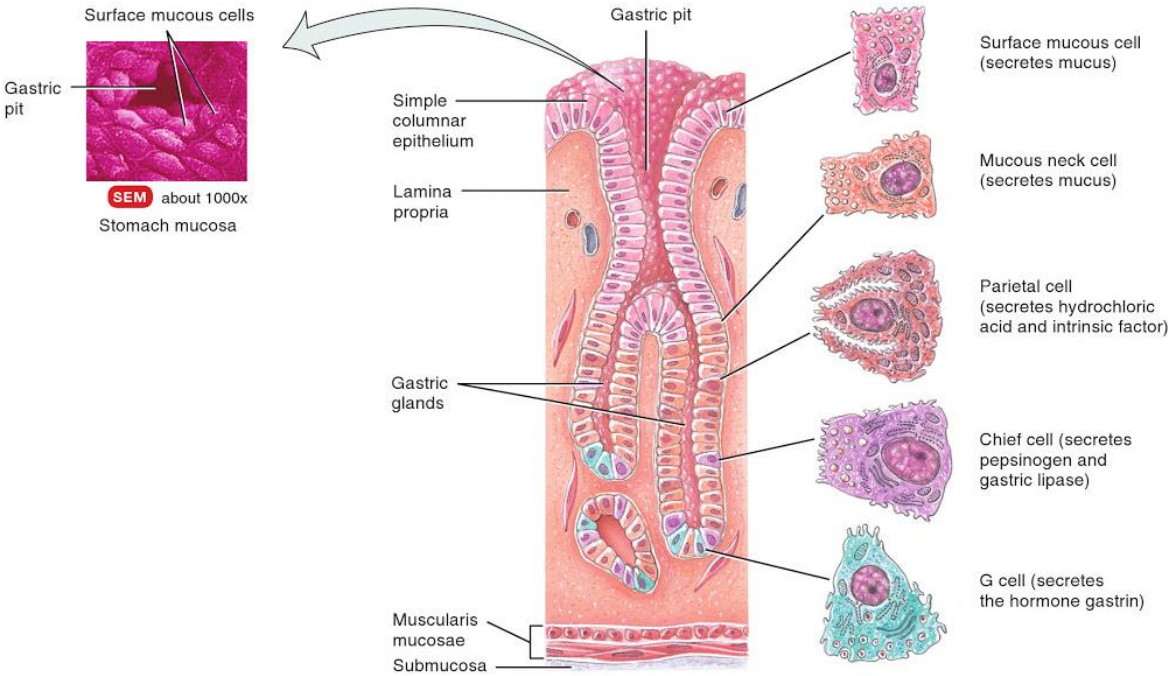


Figure 1: Stomach organization

(a) Stomach regions and three-dimensional view of stomach histology. **(b)** Gastric gland organization. Illustrations from *Tortora GJ and Derrickson B. Principles of Anatomy and Physiology John Wiley & Sons Edition Chap. 24, 2008*

In the corpus, glands are long and composed of several epithelial cell types **(Figure 1b).** From the surface to the base of the gland, we find the mucous cells secreting mucus and bicarbonate for stomach protection and lubrification. Second, the parietal cells secreting acids and intrinsic factors for protein digestion and vitamin B12 assimilation, respectively. Third, the zymogenic chief cells secreting pepsinogen and gastric lipase for protein and lipid digestion and finally, the hormone-secreting endocrine cells controlling acid secretion.

In the antrum, the glands are shorter and composed mostly of mucus-secreting cells and endocrine cells, which secrete hormones such as gastrin (stimulates acid secretion) and somatostatin (inhibits acid secretion). Fundic glands are straight, whereas antral glands are branched and coiled in their basal ends. Pits and glands between the fundic and antral region differ very much. Indeed, in the fundus, glands are composed of the mucous cells, the chief cells, the parietal cells, and the endocrine cells including the histamine-producing enterochromaffin-like cells (ECL) (stimulate acid secretion). The chief cells, the parietal cells and the ECL cells are also found in the corpus (body) of the stomach. In the antral region, gastric pits contain the mucous cells, the antral gland cells, the endocrine cells (mainly gastrin-producing G-cells, but also EC and somatostatin-producing D cells), and occasional parietal cells. In the pylorus, the gastric glands contain many more mucinous cells, any chief cells and few parietal cells *(Tortora GJ and Derrickson B. 2008; Gomceli I et al. 2012).*

Gastric cancer comes from epithelium precancerous lesion tumorigenesis resulting of several risk factors **(Table 1).** GC is histologically heterogeneous. Therefore, several classifications have been proposed.

Gastric cancer classifications

GC can be classified by immunohistochemical study of Mucins. Mucins (MUC) are the family of glycoproteins which form the protective gel of the gastric mucosa. Mucin family members present a different distribution in normal tissue compare to tumoral tissue *(Terada T. 2013).* That's why, an immunohistochemical classification categorized GC in three phenotypes; gastric phenotype (f.e. positive for antibodies against MUC5AC and MUC6), intestinal phenotype (f.e. positive for MUC2 and the intestinal marker CDX2), gastric-intestinal phenotype (mixed profile), and null profile (null type) *(Machado JC et al. 2000; Grabsch HI and Tan P. 2013).*

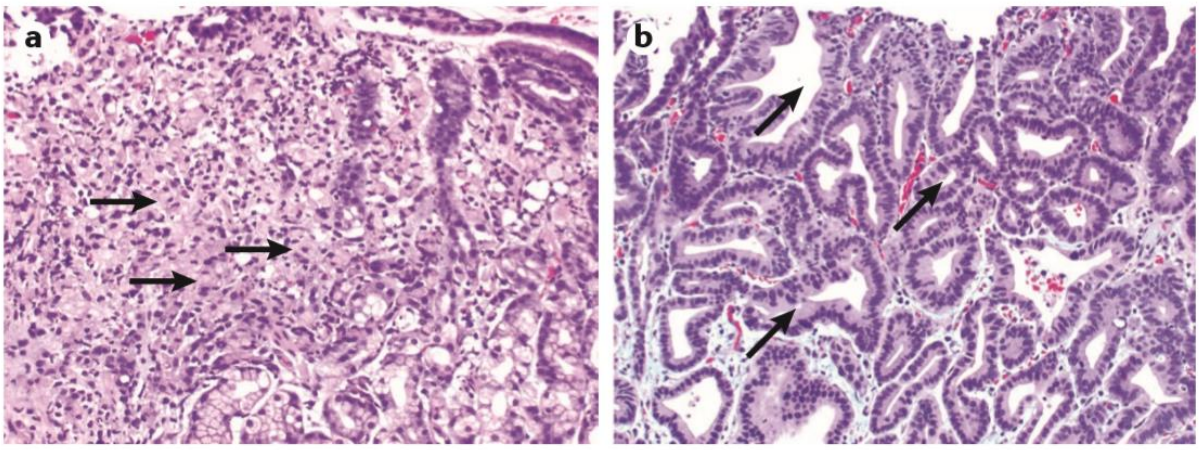


Figure 2: Histological classification of gastric cancer

Hematoxylin and eosin-stained slide of **(a)** diffuse type and **(b)** intestinal type of gastro adenocarcinoma according to Lauren classification. **(a)** Arrows point to signet ring cells, in which the nucleus is pushed out to the periphery of the cytoplasm. **(b)** Arrows are pointing to gland formation by tumor cells. Illustration from *Ajani JA et al. Nature reviews Disease primers 2017*

Another classification separates GC basing on tumor location, histological features, and clinical course. First, the proximal non-diffuse GC, which is in the gastric cardia and showing evidences of glandular dysplasia and chronic inflammation without atrophy. Second, the diffuse GC, which can be located anywhere in the stomach, and which has no glandular component, and no evidence of inflammation or atrophy. Third, the distal non-diffuse GC with chronic gastritis, atrophy, and intestinal metaplasia *(Shah MA et al. 2011; Grabsch HI and Tan P. 2013)*. However, the most commonly used classifications are the Lauren classification and the WHO classification.

The Lauren classification distinguishes GC into three histological types; the diffuse type also called cardia cancer (cancer of esophageal-gastric junction), the intestinal type also called non-cardia cancer (distal cancer), and the third class termed undetermined or mixed GC *(Michel P et al. 2017)*. Diffuse and intestinal types have different clinical-pathological characteristics and exhibit sensitivity to chemotherapy *(Ma J et al. 2016)*. The intestinal type represents the most common gastric cancer in the World associated with advanced age and male preponderance *(Correa P and Piazuelo MB. 2011)*. This type is more present in developing countries compare to the diffuse type *(Lambert R. 2010)*. In the intestinal type, tumor cells are arranged in glandular or tubular formations and present adhesion **(Figure 2)** *(Van Custem E et al. 2016)*, and it is associated with lymphatic or vascular invasion. The diffuse type of GC presents tumor cell populations less adherent infiltrating the stroma as small non-cohesive subgroups and tumors can show high mucus secretion **(Figure 2)** (*Ma J et al. 2016)*.

The WHO classification distinguishes GC in papillary, tubular, and mucinous adenocarcinoma, which correspond to the intestinal type of Lauren classification. The signet-ring cell carcinoma and other poorly cohesive carcinoma, which correspond to the diffuse type of Lauren classification, and the mixed carcinomas corresponding to indeterminate type of Lauren classification *(Hu B et al. 2012; Cislo M et al. 2018)*.

However, at the molecular level, GC possess intra-patient (f.e. metastases in the same organ) and inter-patient heterogeneity (f.e. genetic and tumor location) complicating the stratification of GC patients *(Padmanabhan N et al. 2017; Ajani JA et al. 2017)*. Recently, to improve the understanding of tumor heterogeneity and promote personalized medicine area, The Cancer Genome Atlas (TCGA) Research Network studied genetic alterations on 295 gastric primary tumors. They proposed a molecular classification based on multilevel "-omic" studies such as somatic copy number analysis, microRNA analysis, proteome, exome, and methylation analyses *(TCGA. 2014)*.

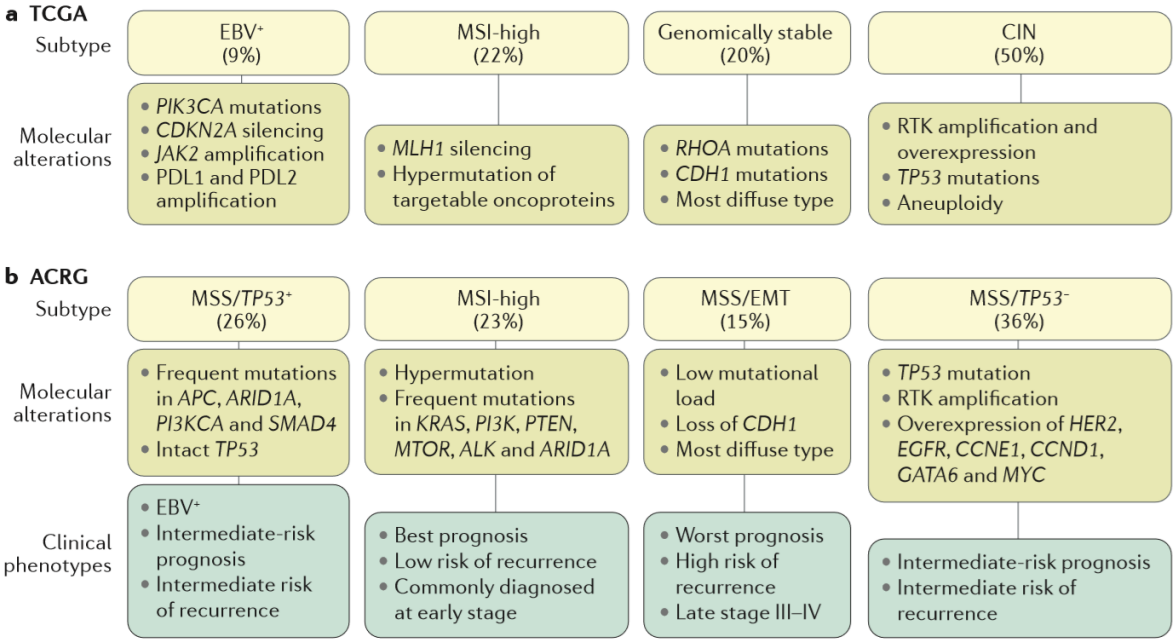


Figure 3: Molecular classification of gastric cancer

(a) The Cancer Genome Atlas (TCGA) Research Network identified four molecular subtypes of gastric cancer by several platform analysis; copy number variation, whole-exome sequencing, DNA methylation, RNA sequencing, microRNA sequencing and reverse phase protein array. Groups contain specific molecular alterations: Epstein-Barr virus positive tumours (**EBV+**); microsatellite instability high tumours (**MSI-high**); genomically stable (**GS**) tumours; and tumours with chromosomal instability (**CIN**). **(b)** The Asian Cancer Research Group (ACRG) identified four groups of gastric cancer based also on clinical information. Groups are microsatellite stable with intact TP53 (**MSS/TP53+**); MSI-high; microsatellite stable and expressing epithelial mesenchymal transition signatures (**MSS/EMT**) and microsatellite stable with TP53 mutations (**MSS/TP53−**). Illustration from *Ajani JA et al. Nature reviews Disease primers 2017*

The Asian Cancer Research Group (ACRG) practiced the same kind of analyses also based on clinical information of 300 primary tumors *(Ajani JA et al. 2017)*. Both studies class GC in fourth comparable classes **(Figure 3):**

EBV+: Epstein-Barr virus positive class regrouping tumors related to EBV infection. Some of their characteristics are the microsatellite stable, the low frequency of *TP53* mutations, and the frequent amplification of immune modulator *PD-L1* and *PD-L2* (Programmed death-ligand).

MSI-high: The microsatellite high instability class, which regroups tumors with hyper-mutations and frequent mutations in *KRAS, PI3K,* and *PTEN* for example.

GS: Genomically stable class, which is also microsatellite stable and characterized by *CDH1* loss of expression. This class expresses Epithelial-to-Mesenchymal Transition (EMT) markers and corresponds to the diffuse type of GC.

CIN: Chromosomal instability class showing a high frequency of *TP53* mutations and *HER2* overexpression.

Clinical diagnosis and patient care

Although, the Lauren and WHO classifications allow stratification of GC according to histological characteristics, a classification with practical use in clinic is the TNM classification **(Figure 4),** which is based on the parietal and ganglionic tumor extension. Stages are defined by association of tumoral invasion (T) of the mucous and adjacent structures, of ganglionic invasion (N) and the presence of metastasis (M) *(Michel P et al. 2017)*. When the diagnosis is established at early stage T1, the 5-year global survival rate is around 95% *(Ajani JA et al. 2017).* Unfortunately, GC can present a variety of non-specific and late symptoms such as peptic ulcer disease, gastroesophageal reflux, fatigue, diarrhoea, dyspeptic syndrome, and weight loss. As these symptoms are not specific to GC, the diagnosis of GC is easily overlooked or done only at advanced stages *(Quadri HS et al. 2017)* decreasing the median survival to 10 months *(Ajani JA et al. 2017).* The ultimate diagnosis rests on endoscopic examination with multiple biopsies and on the extension assessment of the tumor **(Figure 5).**

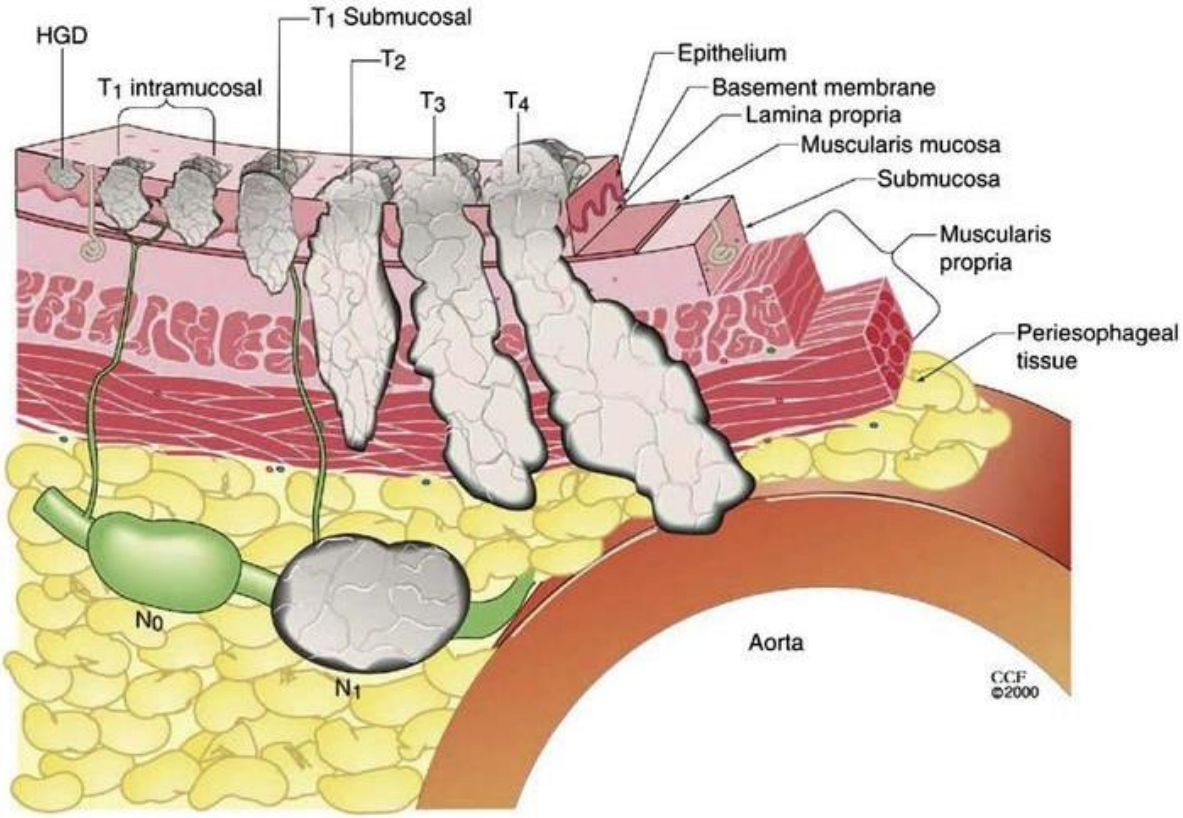


Figure 4: Gastric tumor stages

Tumor *in situ* or High-grade dysplasia (**HGD**). Tumoral invasion stage (**T**). **T1a:** the tumor extends to the chorion without reaching the lamina propria. **T1b:** the tumor extends to the submucosa while respecting the muscular. **T2:** tumor invading the muscular. **T3:** tumor invading the subserosa. **T4a:** tumor invading the serosa (visceral peritoneum) without invasion of adjacent structures. **T4b:** tumor invading adjacent structures. Node invasion stage (**N**). **N1:** invasion of 1 to 2 regional lymph nodes. **N2:** invasion of 3 to 6 regional lymph nodes. **N3a:** invasion of 7 to 15 regional lymph nodes. **N3b:** invasion of more than 15 regional lymph nodes. Presence of metastasis (**M**). **M0:** no metastasis. **M1:** distant metastasis. Illustration from *Cleaveland Clinical Cancer Center 2000.*

In France, the curative treatment rests on surgical excision combined with chemotherapy or radiotherapy. The size of excision depends on initial tumor location. All patients with a tumor superior to stage 1 have perioperative (pre- and postoperative) chemotherapy with Epirubicin, 5-Fluorouracil (5-FU) and Cisplatin or Oxaliplatin. The choice of chemotherapy depends on the age, general condition and HER status of the tumor *(CDU-HGE 2015; Michel P et al. 2017)*.

Epirubicin is a semisynthetic anthracycline cytotoxic antibiotic whose structure differs from doxorubicin to be less toxic and more efficient that doxorubicin. Epirubicin binds to nucleic acids resulting in inhibition of DNA and RNA synthesis and in promotion of DNA cleavage by topoisomerase II. Epirubicin generates also cytotoxic free radicals promoting cellular stress and cancer cell death *(Shaaban S et al. 2014)*.

5-FU (or its precursor: Capecitabine) is a heterocyclic aromatic compound (uracil analogue) which inhibits thymidylate synthase enzyme activity resulting in the increase of DNA damage. 5-FU is also incorporated in DNA and RNA instead of pyrimidine nucleotides and preventing therefore normal biosynthesis. Altogether, 5-FU promotes cell death *(Shaaban S et al. 2014)*.

Cisplatin is the historical platinum derivative anti-cancer compound (PDC). More recently, instate of Cisplatin, Oxaliplatin is used for the treatment of GC. PDC interact with nucleophilic sites present in RNA, DNA, and proteins such as glutathione (GSH), methionine and metallothioneins. Indeed, PDC bind to nitrogen atom N7 of purine residues forming DNA lesions: DNA intra-strand crosslinks, DNA inter-strand crosslinks and DNA-protein crosslinks. DNA intra-strand crosslinks are the dominant mechanism inducing DNA lesions. DNA inter-strand crosslinks are less important for Oxaliplatin, but they contribute to Cisplatin cytotoxicity. DNA-protein crosslinks impact on enzymes functions but are not reported to induce cell death. DNA adducts inhibit DNA replication and transcription causing cell cycle arrest and cell death unless the DNA is efficiently repaired *(Alcindor T and Beauger N. 2011; Dasari S and Tchounwou PB. 2014)*. More recently, Oxaliplatin is frequently associated with 5-FU (based of FOLFOX protocol used in clinic). Furthermore, Cisplatin affects polymerization of actin disrupting the cytoskeleton. In addition, Cisplatin interacts with mitochondrial DNA and promotes mitochondria malfunction inducing greater ROS formation increasing oxidative stress, DNA damage and finally cell death *(Diyabalanage HVK et al. 2013; Dasari S and Tchounwou PB. 2014; Shaaban S et al. 2014)*.

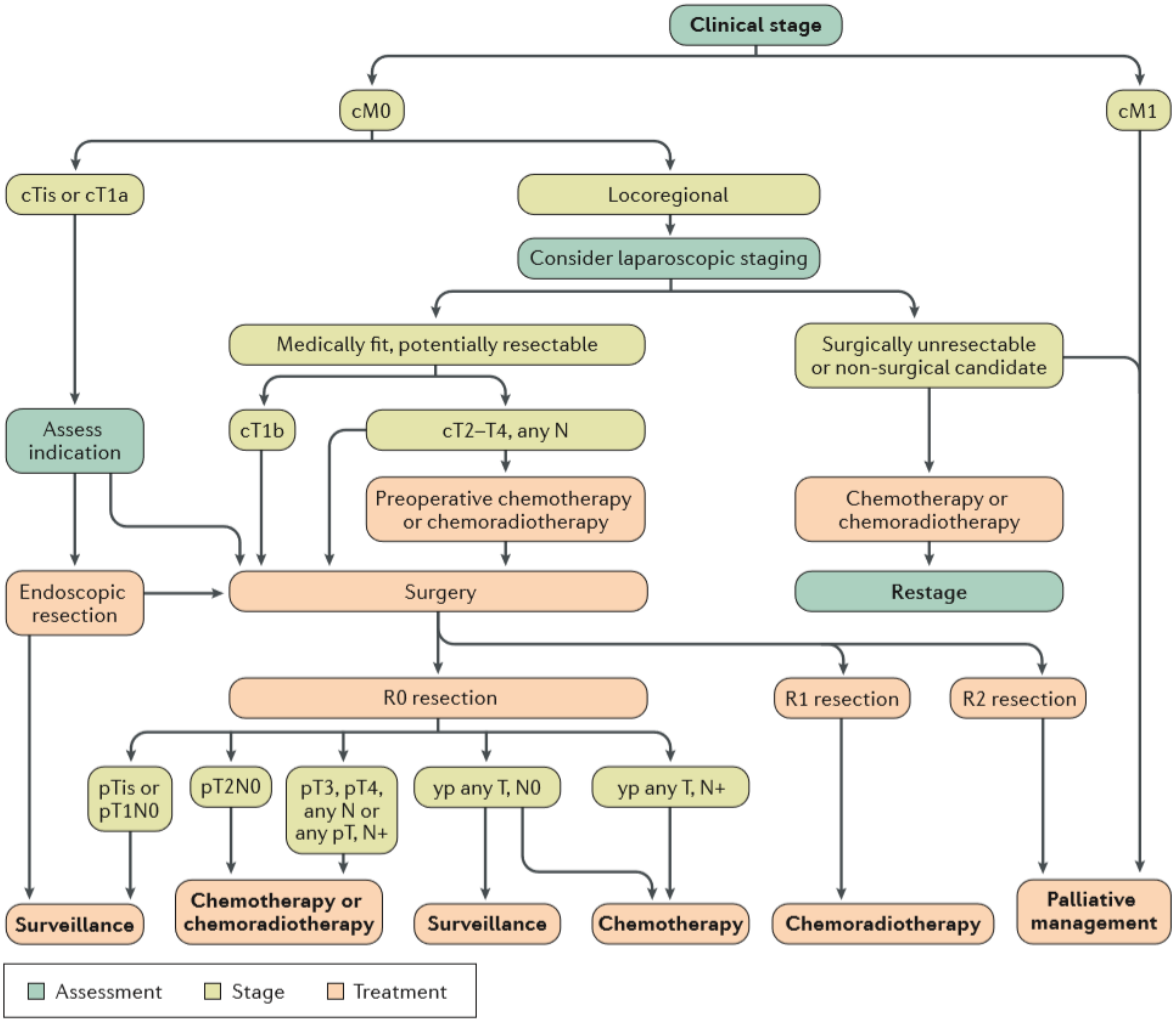


Figure 5: Representation of the therapeutic support for gastric cancer

The schema represents the US guidelines. TNM represent the different grade of the tumoral invasion **(T)** of the mucous and adjacent structures, the ganglionic invasion **(N)** and the presence of metastasis **(M). R:** Resection; **p:** Pathological staging; **yp:** Post-neoadjuvant therapy pathology classification. Illustration from *Ajani JA et al. Nature reviews Disease primers 2017*

In addition, 17,9% tumors with high expression of HER2 are targeted with Trastuzumab (anti-HER2; anti-cell proliferation) in a combinatory therapy with 5-FU and Cisplatin or Oxaliplatin *(Abrahao-Machado LF and Scapulatempo-Neto C. 2016; CDU-HGE 2015; Michel P et al. 2017).*

Patient prognosis and surveillance strongly depend on the TNM classification **(Figure 4).** After the curative surgical resection, the prognosis depends on ganglionic extension. In absence of ganglionic extension, the 5-year survival is about 60%, dropping down to 35% or 10% if ganglionic extension stage is N1 or N2, respectively. After curative treatment and for patients who can have another surgery or chemotherapy, a clinical surveillance is proposed for 5 years. It is based on physical examination, and abdominal ultrasound every six months and blood-count and thoracic x-ray every year. However, so far, no exhaustive study exists showing the impact on patient survival of a surveillance protocol. For patients with advanced GC stages (un-resectable or metastatic tumors), palliative treatment is proposed. It rests on surgery, chemotherapy, radiotherapy and treatments of symptoms to improve the quality of life of patients *(CDU-HGE 2015; Michel P et al 2017)*.

Treatment resistance

GC cells are frequently resistant or develop resistance to the current therapies. The key mechanisms of resistance are those regulating i) cellular drug concentration, ii) drug inactivation or sequestration, iii) response to oxidative stress, and iv) DNA repair **(Figures 6-10).**

PDC pass the cytoplasmic membrane via passive diffusion and by the human copper transporter (hCTR1/2) **(Figure 6).** It has been reported that up-regulation of hCTR1 is involved in Cisplatin resistance, which is not clearly established for Oxaliplatin. Thus, decrease of hCTR1 reduces Cisplatin cellular uptake in GC *(Shen DW et al. 2012; Martinez-Balibrea E et al. 2015; Shi WJ and Gao JB. 2016)*. Furthermore, in response to Cisplatin, cells express the GC-binding transcriptional factor 2 (GCF2), which inhibits the GTPase RhoA disrupting the cytoskeletal network causing defective membrane proteins resulting in the reduction of Cisplatin influx (*Shen DW et al. 2012).* On the opposite, several transporters such as ATP7A/B, OCT (solute carrier transporters organic cation transporters), the sodium pump Na, the K-ATPase and the ABCC subfamily (containing multidrug resistance associated proteins; MRP) are involved in PDC efflux.

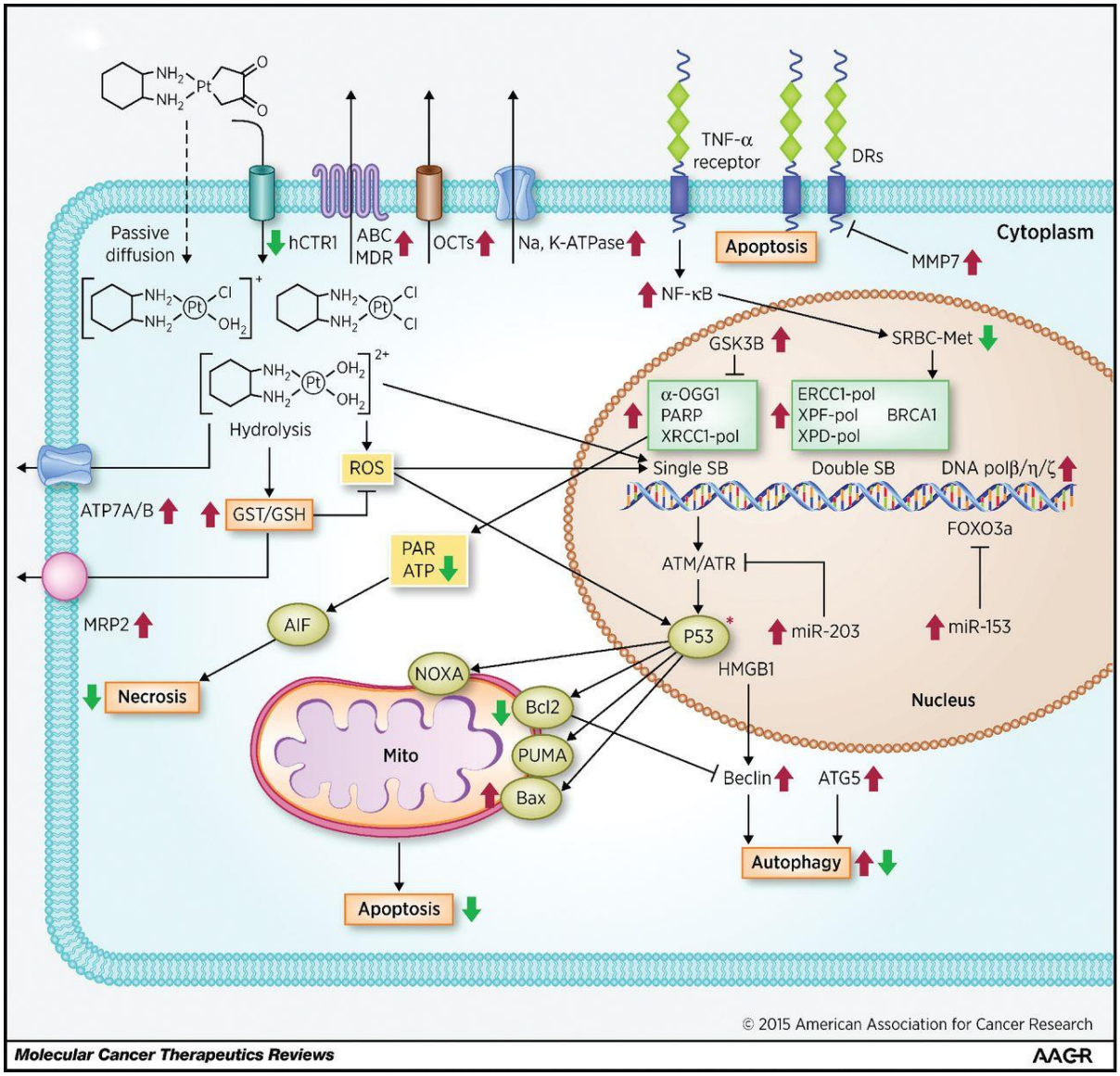


Figure 6: Oxaliplatin-associated resistance mechanisms

Mechanisms are closed to Cisplatin resistance. Green and red arrows represent increased and decreased expressions respectively. **–pol:** polymorphis; **-Met:** methylation; **DRs:** death receptors; **SB:** strand breaks. Illustration from *Martinez-Balibrea E et al. Molecular Cancer Therapeutics 2015*

These different proteins are frequently found to be up-regulated in cancers resulting in a reduction of PDC concentration in different PDC resistant cancer models including GC *(Martinez-Balibrea E et al. 2015; Shi WJ and Gao JB. 2016)*. Moreover, tumor cells might overexpress glutathione (GSH), Glutathione S-transferases (GST) methionine and metallothioneins to directly detoxify PDC and reduce ROS formation protecting cellular macromolecules from damage **(Figure 6).** Chaperones such as HSP60 and HSP10, which restore protein conformation reducing thereby cellular stress, are also overexpressed in PDC resistant cells *(Florea AM et al. 2011; Shen DW et al. 2012; Martinez-Balibrea E et al. 2015; Shi WJ and Gao JB. 2016)*. In addition, overexpression of anti-apoptotic proteins BCL-2 and XIAP are associated with increased GSH synthesis inducing also gastric cancer cells (GCC) PDC resistance *(Diyabalanage HVK et al. 2013; Shi WJ and Gao JB. 2016).* Expression of Taxol resistance gene 1 (*TXR1*) also modulates sensitivity to Cisplatin by controlling apoptosis. Indeed, in induced-Cisplatin-resistant GC cells *in vitro*, *TXR1* is overexpressed and its downregulation reverses drug resistance promoting apoptosis. Likewise, *TXR1* expression analysis in 18 GC patients showed its significant elevated by 2 expressions in the Cisplatin-resistant group compare to the sensitive-group *(Duan S et al. 2018).* Additional studies demonstrated that HIF-1α (Hypoxia inducible factor) overexpression increases the expression of *BCL-2* and decreases the expression of *BAX*, inhibiting apoptosis, and significantly induces the expression of *ABCB1* and *ABCC1*, promoting drug efflux *(Shi WJ and Gao JB. 2016)*. Likewise, the up-activation of the p38-MAPK and pAkt pathways are described to promote GCC multidrug resistance. Moreover, it was described that the p53 positive status is associated with an improved response in GC patients who received chemotherapy, indicating that p53 status might be a relevant predictive biomarker for response to chemotherapy in GC *(Shi WJ and Gao JB. 2016)*. Furthermore, it also has been shown that different miRNAs are implicated in the mechanisms influencing the resistance of cancers to different treatments. For example, miR-143, targeting *IGF1R* and *BCL2*, is down-regulated in GCCs and in 24 out of 30 GC patients promoting Cisplatin resistance of cancer cells *(Zhuang M et al. 2015).* In addition, miR-143 and miR-145 are reported to be significantly decreased in 30 and 27 of 43 GC patients, respectively, and in GCCs promoting 5-FU resistance *(Takagi T et al. 2009).*

Trastuzumab is successfully used for the treatment of HER-2-positive GC (around 17,9% out of gastric tumors). *(Abrahao-Machado LF and Scapulatempo-Neto C. 2016)* However, it is also susceptible to different resistance mechanisms, such as membrane target masking, inhibition of trastuzumab-mediated immune response or, the most described PI3K pathway activation (activating mutations in *PI3KCA* and *PTEN*) *(Ung M et al. 2015).*

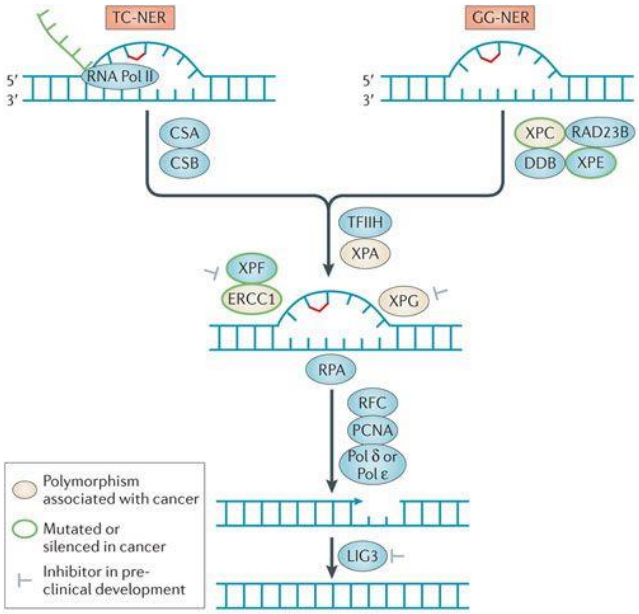


Figure 7: Nucleotide excision repair (NER)

TC-NER: Transcription-coupled nucleotide excision repair; **GG-NER:** Global NER; **CSA:** Cockayne syndrome WD repeat protein A; **XP**: Xeroderma pigmentosum; **DDB:** DNA damage-binding protein; **RPA:** Replication protein A; **Pol δ:** DNA polymerase-δ; **LIG3:** DNA ligase 3; **PCNA:** Proliferating cell nuclear antigen; **RFC:** replication factor C. Illustration from *Curtin NJ, Nature reviews Cancer 2012*

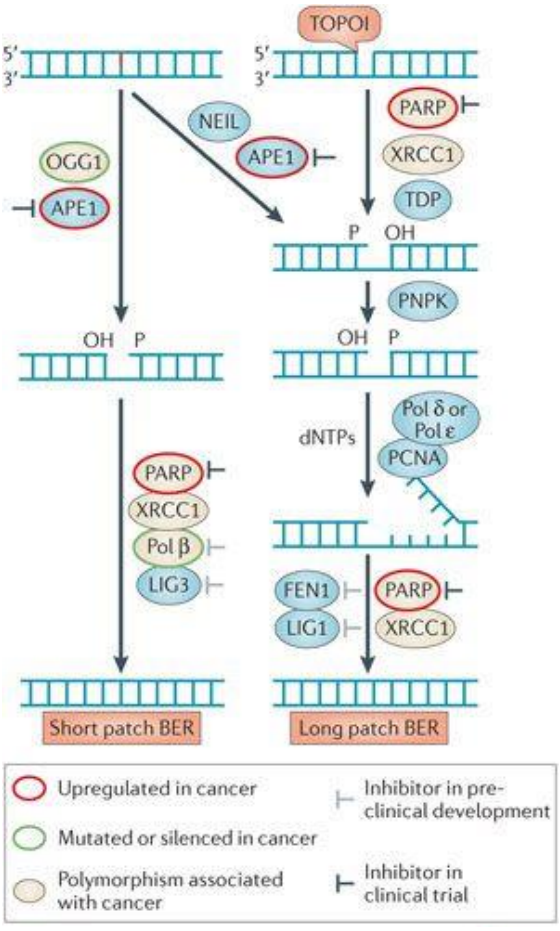


Figure 8: Base excision repair (BER)

OGG1: 8-oxoguanine DNA glycosylase; **NEIL:** Nei-like protein; **AP:** apurinic or apyrimidinic; **PKNP:** Polynucleotide kinase phosphatase; **PCNA:** Proliferating cell nuclear antigen; **FEN1:** Flap endonuclease 1; **Pol β:** DNA polymerase-β; **LIG3:** Ligase 3; **PARP:** Poly(ADP-ribose) polymerase 1; **TDP1:** Tyrosyl-DNA phosphodiesterase 1. Illustration from *Curtin NJ, Nature reviews Cancer 2012*

Furthermore, activating mutations or amplifications of EGFR*/MET/KRAS/PI3K/PTEN* (PI3K pathway) were associated and appeared as clinically useful to predict primary resistance to trastuzumab in patients with HER2-positive metastatic GC *(Pietrantonio F et al. 2018).*

5-FU GC resistance is also a major problem and it can be explained by an activation of the hedgehog, Wnt and Notch pathways. More preciously, N-87 GC cells followed by 5-FU treatment expressed hedgehog target genes *GLI1* and *GLI2*. GLI2 directly regulates the membrane transporter ABCG2 increasing 5-FU efflux and GC cells resistance *(Yu B et al. 2017).* Moreover, *NRF2* (or *NFE2L2* Nuclear Factor-Erythroid-derived 2-like 2), which is involved in oxidative stress response (endoplasmic reticulum stress, autophagy regulation, ROS detoxification) *(Ma Q. 2013)* and which is activated in several cancers, controls 5-FU resistance. Indeed, NRF2 silencing reduces cell resistance to cell death in response to 5-FU in GC cells. NRF2 was associated significantly in a cohort of 186 patients to 5-FU resistance and appears as a potential prognostic marker *(Hu XF et al. 2013).*

Finally, clinical resistance to 5-FU, radiotherapy and the most important mechanism to PDC resistance concern the DNA repair pathways **(Figures 7-10):** the nucleotide excision repair (NER), base excision repair (BER), the homologous recombination (HR) and non-homologous end-joining (NHEJ) pathways, that repair double-strand break (DSB), and mismatch repair (MMR) pathways, which are involved in repairing DNA single-strand break (SSB) *(Willers H et al. 2013; Basourakos SP et al. 2017).* Several proteins involved in DNA repair are found to be frequently up-regulated, silenced or mutated in cancers promoting cancer cells resistance to ROS and treatments *(Curtin NJ. 2012)*. Among the mechanisms involved in GC resistance, we will indicate the percentage of gene alterations found in a total of 393 stomach adenocarcinomas reported in the TCGA database *(http://www.cbioportal.org).*

Nucleotide excision repair. (Figure 7) Repair of lesions is done by transcription-coupled nucleotide excision repair (TC-NER) or by global NER (GG-NER). These pathways differ in their initial steps, TC-NER involves Cockayne syndrome WD repeat protein A (CSA) and CSB, whereas in GG-NER recognition is dependent on Xeroderma pigmentosum (XPC; 1.5% / XPE; 1.8%). Excision of the damaged oligonucleotide is done by XPG and ERCC1 (2.5%) and XPF, then re-synthesis of the intact oligonucleotide and ligation are accomplished by DNA polymerase-δ (Pol δ) or Pol ε and DNA ligase 3 (LIG3) *(Curtin NJ. 2012)*. Notice that in Oxaliplatin-acquired resistant cells, there is an up-regulation of XPD and ERCC1 *(Martinez-Balibrea E et al. 2015).* In addition, GC patients with low expression of ERCC1 demonstrate better response to Oxaliplatin-based adjuvant chemotherapy *(Liu YP et al. 2013; De Dosso S et al. 2013; Wang J et al. 2014).*

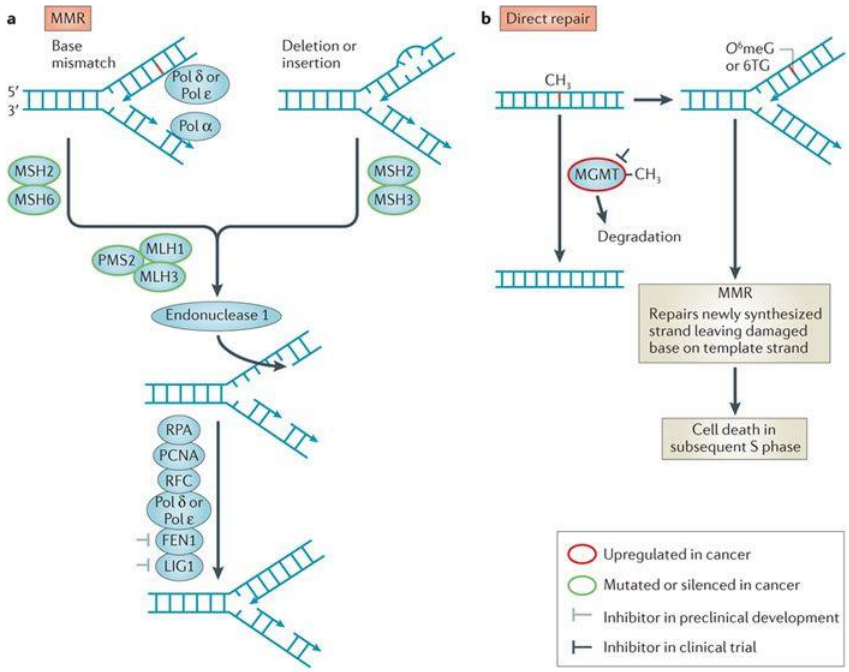


Figure 9: Mismatch repair (MMR) and direct repair

RPA: Replication protein A; **PCNA:** Proliferating cell nuclear antigen; **RFC:** Replication factor C; **Pol:** DNA polymerase; **FEN1:** Flap endonuclease 1; **MMR:** Mismatch repair; **O6meG:** O6-methylguanine; **6TG:** 6-thioguanine; **LIG1:** DNA ligase 1; **MGMT:** O6-methylguanine DNA alkyltransferase. Illustration from *Curtin NJ, Nature reviews Cancer 2012*

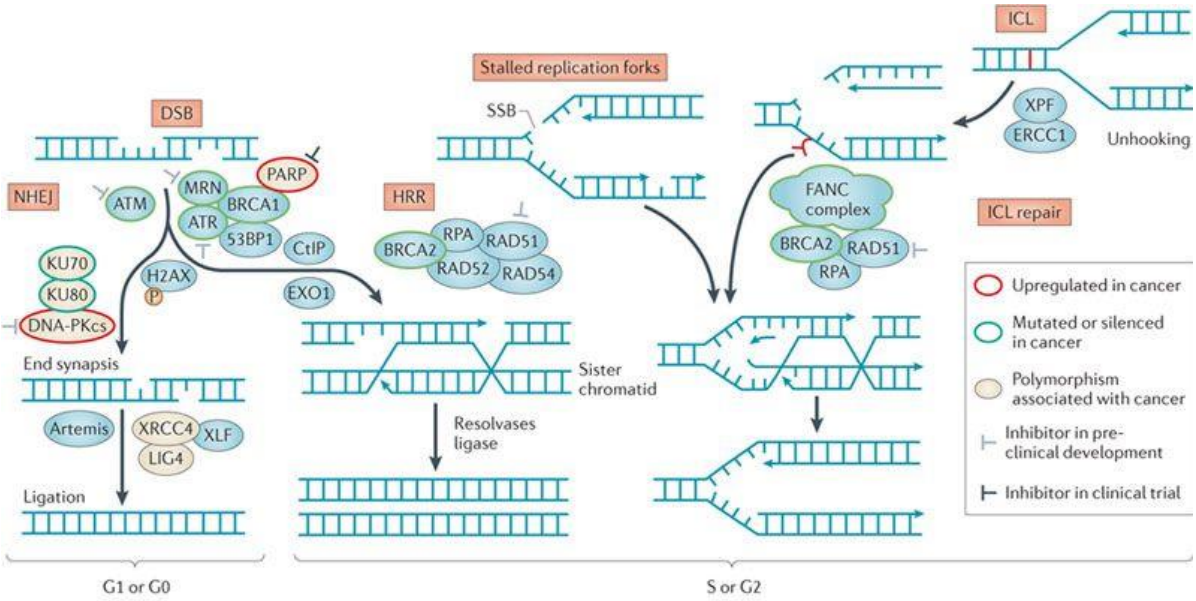


Figure 10: DNA double-strand break and inter-strand crosslink repair

DSB: DNA double-strand break; **NSB1:** Nijmegen breakage syndrome 1; **NHEJ:** Non-homologous end joining; **DNA-PKcs:** DNA-dependent protein kinase catalytic subunit; **LIG4:** DNA ligase 4; **XLF:** XRCC4-like factor; **HHR:** Homologous recombination repair; **PARP:** Poly(ADP) ribose polymerase 1; **CtIP:** CtBP-interacting protein; **EXO1**: Exonuclease 1; **ATM:** Ataxia-telangiectasia mutated; **53BP1:** p53 binding protein 1; **ATR:** Ataxia-telangiectasia; **ATRIP:** ATR-interacting protein: **FANC:** Fanconi anaemia; **ICLs:** Interstrand crosslinks. Illustration from *Curtin NJ, Nature reviews Cancer 2012*

Base excision repair. (Figure 8) Alkylated bases are removed by specific glycosylases like 8-oxoguanine DNA glycosylase (OGG1; 1.8%) or members of the Nei-like protein (NEIL1; 4%). The resulting apurinic or apyrimidinic (AP) site is then hydrolyzed by an AP endonuclease, such as APE1 (3%). In short patch repair, the single nucleotide is replaced by DNA polymerase-β (Pol β; 4%) and the ligation is done by ligase 3 (LIG3), and in long patch repair, up to 13 nucleotides are replaced by Pol δ or Pol ε and ligation is completed by LIG1. Poly(ADP-ribose) polymerase 1 (PARP1; 5%) and XRCC1 (X-ray repair cross-complementing protein 1; 4%) facilitate repair by recruiting repair enzymes *(Curtin NJ. 2012)*. Interestingly, SNP by amino acid changing 399Arg to Gln in XRCC1 is associated with a worse response to Oxaliplatin and FOLFOX treatments in gastric and colorectal cancers *(Martinez-Balibrea E et al. 2015).*

Mismatch repair. (Figure 9a) DNA mismatches result from the insertion of a mis-paired or fraudulent nucleotide. They are recognized by MSH2–MSH6 (4% / 6%) heterodimers, whereas deletions and insertions are recognized by MSH2–MSH3 (9%) heterodimers. Processing requires then PMS2 and MLH1–MLH3 (3% / 5%) heterodimers to recruit the same components of other DNA repair pathways such as endonuclease 1, replication protein A (RPA), proliferating cell nuclear antigen (PCNA), replication factor C (RFC), DNA polymerase-δ (Pol δ) or Pol ε and flap endonuclease 1 (FEN1) *(Curtin NJ. 2012)*. Mismatch repair (MMR) deficient phenotype via loss of ARID1A (AT-rich interactive domain 1A) is described especially for sporadic microsatellite instability-high (MSI-high) gastric cancers *(Kim KJ et al. 2015).*

Direct repair. (Figure 9b) O6-methylguanine (O6meG) or 6-thioguanine (6TG) damage can be repair by O6-methylguanine DNA alkyl-transferase (MGMT; 3%) *(Curtin NJ. 2012)*.

DNA double-strand break and inter-strand crosslink repair. (Figure 10) DNA double-strand break (DSB) repair, Homologous recombination repair (HRR) and Inter-strand crosslinks repair are recognized by MRN/BRCA1 (6%)/ATM (12%) complex, BRCA2 (13%) and XPF-ERCC1 respectively to recruit DNA reparation proteins *(Curtin NJ. 2012; Willers H et al. 2013)*.

In conclusion, so far, the mechanisms of PDC resistance described in GC are drug efflux, DNA damage repair and inhibition of apoptosis. However, it is extremely important to note that none of these genes, proteins or miRNAs have been yet established as relevant predictive markers for treatments administrated in clinic to patients. Meaning that patient care does not consider the molecular signature of the tumor.

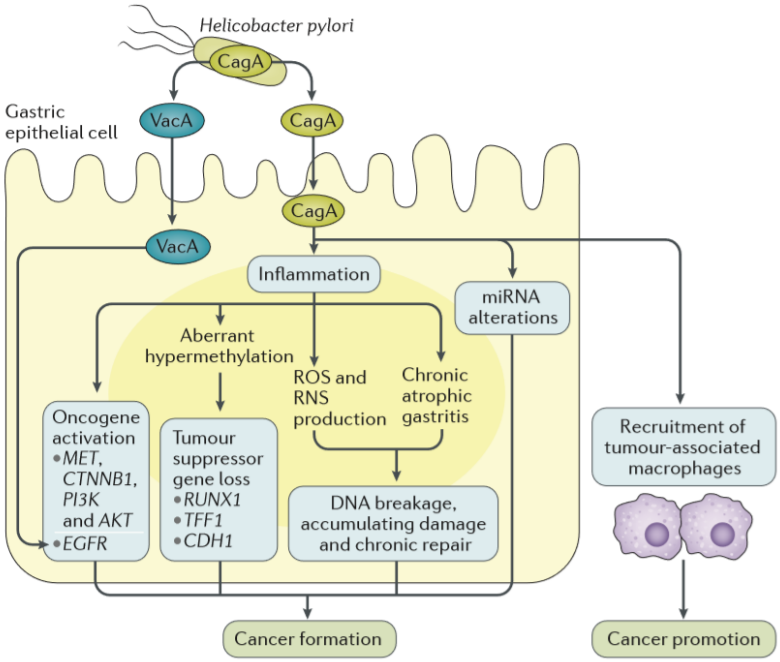


Figure 11: Pathogenicity of *Helicobacter pylori* on gastric epithelium which can result in gastric adenocarcinoma. Illustration from *Ajani JA et al. Nature reviews Disease primers 2017*

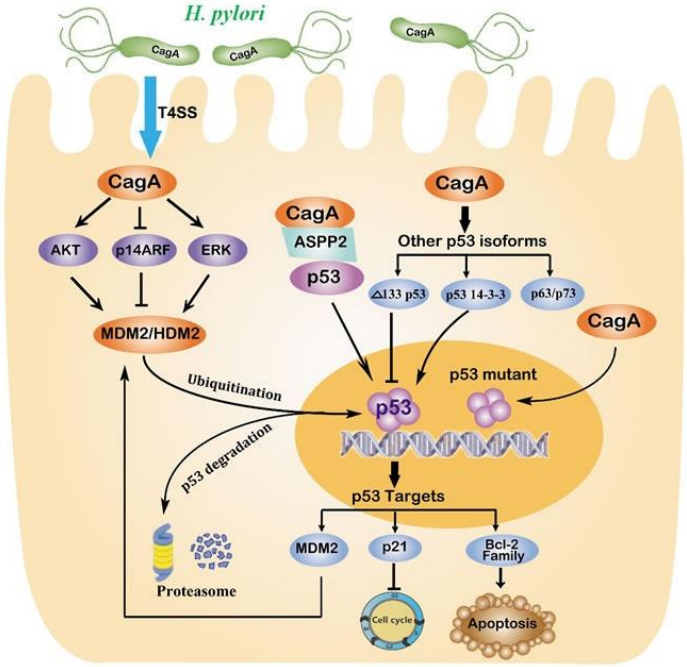


Figure 12: Regulation of p53 in gastric carcinogenesis induced by *H. pylori*

H. pylori injects the oncoprotein CagA into gastric epithelial cells which alters the tumour suppressor p53. Chronic H. pylori infection may cause an accumulation of mutant p53. *H. pylori* also increases MDM2 levels via multiple mechanisms that target the proteasome-mediated degradation of p53. The CagA protein also binds to the apoptosis-stimulating protein of p53 (ASPP), which prevents the interaction between ASPP2 and p53. Other isoforms and variants of the p53 family are involved in the regulation of p53 in response to *H. pylori* infection. Illustration from *Li N et al. Oncotarget 2016*

Gastric cancer pathogenesis

As described in Table 1, several risk factors promote precancerous lesions and gastric tumorigenesis.

Helicobacter pylori infection

Helicobacter pylori is a Gram-negative bacterium, which infects gastric epithelium. The infection activates several cellular pathways in gastric epithelial cells causing inflammation, playing a role in multistep carcinogenesis increasing the risk of gastric cancer **(Figure 11).** *H. pylori* virulence protein CagA (cytotoxin associated protein A), BabA2 (blood group antigen binding adhesin A2) and VacA (vacuolating cytotoxin A) alter cellular assembly and gastric homeostasis and promote tissue inflammation *(Jencks DS et al. 2018).* BabA2 protein allows *H. pylori* to interact to the cell surface via an unidentified receptor *(Dunne C et al. 2014)* and VacA protein alters the mitochondrial permeability and favors apoptosis *(Rivas-Ortiz CI et al. 2017).*

CagA forms with other Cag proteins (CagI, CagL, CagY) a core-system which targets the β-integrin receptor followed by CagA injection in the gastric epithelial cell *(Backert S et al. 2016).* Salt and nitrites rich diets increase *H. pylori* infection by promoting the bacteria colonization and its virulence factor CagA expression *(Fox JG et al. 1999; Loh JT et al. 2007).* CagA activity leads to the upregulation of oncogenic signaling, to the downregulation of tumor suppressor pathways, to a change of cell morphology and to the recruitment of immune-suppressing tumor-associated macrophages **(Figure 11)** *(Saadat I et al. 2007; Ajani JA et al. 2017).*

Indeed, CagA binds to the proto-oncogene Src homology 2-containing protein tyrosine phosphatase (SHP-2) resulting in aberrant activation of SHP-2 and consequently of the MAPK (mitogen-activated protein kinase) pathway promoting carcinogenesis *(Hatakeyama M. 2004).* CagA also induces an increased activation of AKT/MDM2 loop leading to a degradation of the tumor suppressor TP53 **(Figure 12)** *(Li N et al. 2016).* Furthermore, *H. pylori* promotes deregulation of the Hippo pathway **(Figure 13).** Indeed, *H. pylori* induces hyper-methylation of gene promoters like *RASSF1* and E-cadherin (*CDH1*) *(Zhang B et al. 2016)*. *H. pylori* also enhances the phosphatase activity on YAP/TAZ. Both mechanisms inhibit the Hippo pathway and induce nuclear localization and hyper-activation of the β-catenin promoting gastric tumorigenesis *(Hatakeyama M. 2004; Saadat I et al. 2007; Qiao Y et al. 2018).*

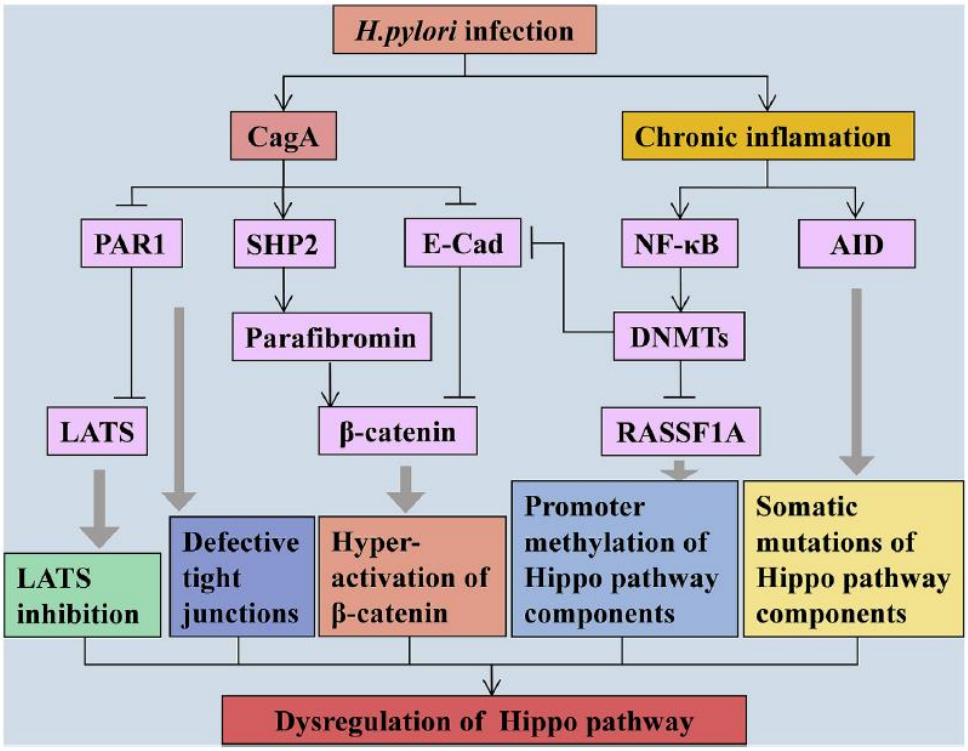


Figure 13: The signalling network connecting *Helicobacter pylori* infection with YAP/TAZ hyperactivation. Illustration from *Qiao Y et al. Cancer Letters 2018*

Moreover, *H. pylori* also enhances *EGFR* (Epidermal Growth Factor Receptor) expression which activation upregulates pAkt and pGSK3 in gastric cancer cell lines and consequently increases β-catenin activation and promotes cell proliferation *(Kim J et al. 2013).* *H. pylori* infection also induces NFκB activation **(Figure 13)** promoting tumorigenesis *(Sokolova O and Naumann M. 2017; Zandi S et al. 2018).* *H. pylori* infection and advanced stages of gastric tumorigenesis are also associated with a decrease of *HDAC6* expression in a cohort of 364 patients with GC and *H. pylori* promotes *HDAC1* and *HDAC4* expressions in infected cells inducing oncogenic transformation in gastric cancer *(He Q et al. 2017).* *H. pylori* infection promotes activation-induced-cytidine-deaminase (AID) upregulation which directly converts cytosine in uracil resulting in nucleotide alterations in several gene like TP53 in gastric cells **(Figures 12 and 13)** *(Matsumoto Y et al. 2007).*

DNA hypermethylation of CpG islands is achieved by DNA methyltransferases (DNMT). Interestingly, *DNMT1* and *DNMT3A* expression are increased in GC cells infected by *H. pylori* *(Yan J et al. 2011)* indicating that *H. pylori* infection promotes aberrant methylation of CpG islands. Aberrant methylation on tumor suppressor promoters of *p16, COX-2, RUNX3* are related to *H. pylori* infection promoting cancer initiation and progression *(Gigek CO et al. 2012).* *H. pylori* infection also contributes to upregulation and downregulation of several miRNAs. For example, it is associated with the expression of the oncogenic miRNA miR-21, which targets PDCD4 (regulator of proteins involved in cell cycle, tumor progression and differentiation) and PTEN (tumor suppressor). *H. pylori* infection is also associated with the downregulation of the tumor suppressor miRNAs miR-101 targeting COX-2 and let-7A targeting RAS *(Gigek CO et al. 2012; Nishizawa T and Suzuki H. 2015).*

In addition, *H. pylori* infection promotes ROS (Reactive Oxygen Species) formation in the epithelium and intracellular free radical activity in cells **(Figure 11)** *(Nishizawa T and Suzuki H. 2015).* ROS formation reduces Gastrokine 1 *(GKN1)* expression, which is involved in gastric mucosal defense promoting gastric tumorigenesis *(Yoon JH et al. 2014).* Moreover, *H. pylori* infection induces ER-stress (Endoplasmic-Reticulum stress) and expression of *CHAC1* (Cation transport regulator 1). In *H. pylori* infected GC cells, *CHAC1* reduces GSH level. High ROS formation participates also to the reduction of GSH, which consequently promotes DNA damage and mutations in *TP53* *(Wada W et al. 2018).* ROS formation also stabilizes HIF1α (Hypoxia Inducible Factor) in gastric *H. pylori* infected cells promoting gastric cell proliferation and protection from apoptosis. Interestingly, HIF1α protein expression was examined using immunohistochemistry on biopsies of normal mucosa (n=20), *Helicobacter pylori* associated gastritis (n=24), intestinal metaplasia (n=24), dysplasia (n=12) and intestinal (n=19) and diffuse (n=21) adenocarcinoma and was shown to be

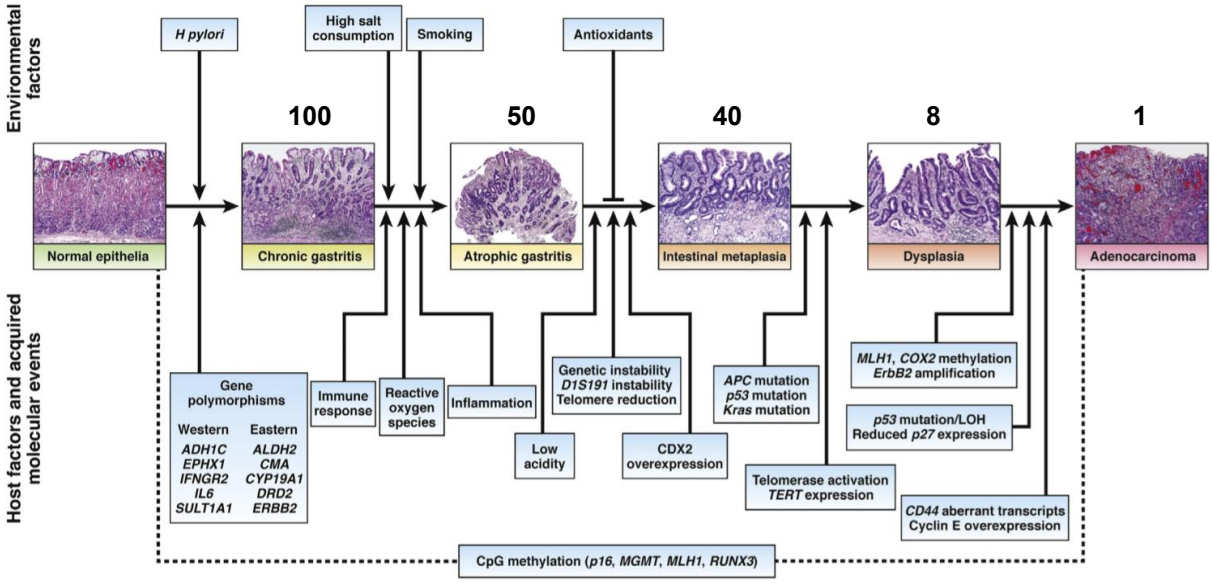


Figure 14: Pathogenesis of intestinal type of gastric cancer

Summary of several risk factors and genetic alterations of the cause and the pathogenesis of the intestinal type of gastric cancer. Illustration from *Tan P and Yeoh KG. Gastroenterology 2015.* Frequence start on 100 people *(data in The fundamentals of digestive pathology CDU-HGE Chap. 2, 2014)*

increased in density and intensity during *H. pylori* related cancerous cascade *(Griffiths EA et al. 2007).*

Altogether, *Helicobacter pylori* infection promotes gastric chronic atrophy, which is the first step of the precancerous cascade and promotes oncogenic transformation of gastric epithelial cells **(Figure 14).** Gastric carcinogenesis is the result of chronic gastric atrophy progressing in intestinal metaplasia, then in dysplasia and finally in invasive carcinoma *(Correa P and Piazuelo MB. 2012).* The gastric cancer intestinal type is highly correlated with intestinal metaplasia whereas the diffuse type is less associated with the inflammation cascade *(Jencks DS et al. 2018).*

Although *H. pylori* is a major risk factor of GC, its eradication does not eliminate the risk to develop GC. Indeed, *H. pylori* eradication only reduce the risk of GC around 40% because pre-neoplastic lesions (atrophic gastritis, intestinal metaplasia) have already been developed by the bacterium *(Lee YC et al. 2016; Cheung KS and Leung WK. 2018).* A patient monitoring with chemo-preventive treatments (Aspirin, Proton pump inhibitors, Metformin), life style change (antioxidant rich-diet) and endoscopic surveillance after *H. pylori* eradication, is studied to prevent of the occurrence of cancer *(Cheung KS and Leung WK. 2018).*

Epstein-Barr virus infection

Most patients with Epstein-Barr virus (EBV) positive GC are co-infected by *H. pylori* resulting in EBV-positive GC. It has been reported that the bacterium and the virus collaborate in the carcinogenic transformation of gastric cells *(Saju P et al. 2016)*. A characteristic of EBV positive GC is the amplification of the locus coding for *PD-L1* and *PD-L2.* PD-L1 and PD-L2 are ligands of PD-1, which is expressed on immune cells. Upon ligation with PD-L1 and PD-L2, PD-1 suppresses T cell proliferation *(Chia NY and Tan P. 2016)*. High PD-L1 expression levels (EBV-positive and MSI) in GC is associated with poor prognosis. In this respect, blocking the interaction between PD-1 and PD-L1/L2 could augment an antitumor response *(Gu L et al. 2017)*.

Multiple EBV viral genes and miRNAs promote gastric carcinogenesis **(Figure 15).** Indeed, the viral gene EBNA-1 impairs p53-dependent p21 expression and apoptosis, consequently promoting cell survival after DNA alterations. Another EBV viral gene, LMP-2A promotes *Cyclin E*, *DNMT1* and *DNMT3a* expressions, consequently leading to an increased cell proliferation and hypermethylation on promoters of several tumor suppressor genes (*p16, PTEN, APC, CDH1*), which participates to GC initiation and progression *(Chia NY and*

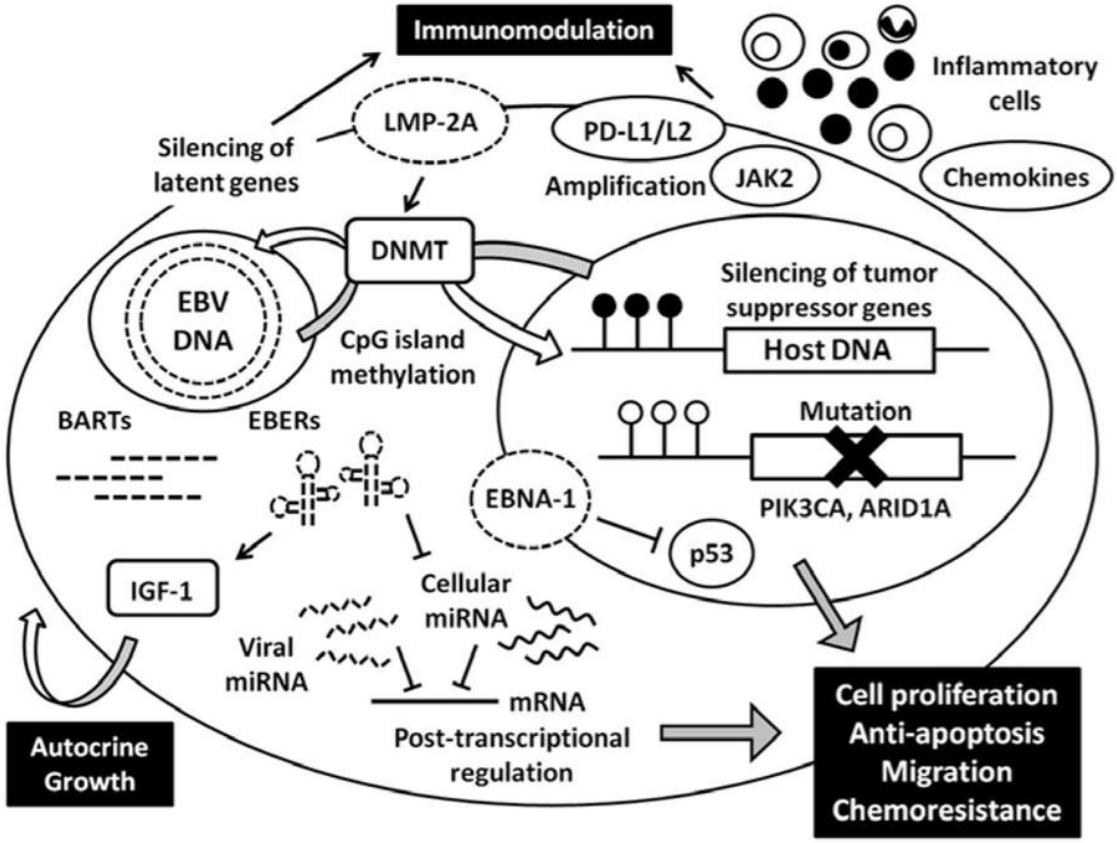


Figure 15: Epstein-Barr Virus related mechanisms promoting gastric carcinogenesis

Summary of oncogenic related EBV transcripts causing the pathogenesis of the gastric cancer EBV positive. Illustration from *Shinozaki-Ushik A et al. International Journal of Oncology 2015*

Tan P. 2016). TP73 gene promoter methylation is reported to be associated with GC EBV-positive (11/13) compared to EBV-negative GC (3/38) *(Ushiku T et al. 2007).*

Furthermore, the EBER viral transcripts suppress *CDH1* (E-cadherin) expression, a gene involved in cell proliferation and epithelial-to-mesenchymal transition (EMT), but also upregulate pFAK and downregulate the anti-metastatic protein RhoGD1. Thus, EBER promote GC cell migration *(Chia NY and Tan P. 2016).*

Moreover, BARTs are EBV multi-spliced transcripts to which BARF1 belongs. BARF1 increases *BCL2* expression, blocking apoptosis. BARF1 also increases *Cyclin D1* expression, reduces *p21* expression and up-regulates NFκB promoting cell proliferation. Finally, some EBV microRNA (miRNA) also impact many cellular pathways. For example, miR-BART5-5p inhibits *PUMA* expression repressing apoptosis, and the miR-BART9-3p inhibits *CDH1* expression promoting cell proliferation and migration *(Chia NY and Tan P. 2016; Polakovicova I et al. 2018).*

Life style

Salt rich diets produce N-nitroso, a nitrite derivative compound with a carcinogenic activity whereas antioxidant rich diets, like fruits and vegetables, have a protective effect on gastric epithelium *(Cross AJ et al. 2003).* Moreover, the risk to develop GC increases with duration of cigarette smoking and with amounts of cigarettes smoked per day, whereas stopping cigarette smoking decreases the risk which becomes like that of a never smoker 10 years after stopping *(Praud D et al. 2018).* Alcohol consumption also increases the risk of GC (OR = 1.39) suggesting that effective moderation of alcohol drinking may reduce the risk of GC *(Ma K et al. 2017).*

Inherited cancer related syndromes

Approximately 3% of GC are due to an autosomal dominant inherited cancer syndrome. For example, germline mutations in DNA damage reparation gene *BRCA1* or *BRCA2* (hereditary breast and ovarian cancer syndrome) increase the lifetime risk of developing stomach cancer by as much as 6-fold greater. Germline mutations of *APC* (Adenomatous Polyposis Coli which controls β-catenin concentration) cause familial adenomatous polyposis (FAP) increasing the risk of GC around 0.6%. Germline mutations in the tumor suppressor *TP53* (Li-Fraumeni syndrome) induce predisposition to many cancers (sarcoma, lung, breast and brain cancers), and increase the risk of GC development around 3%.

Germline mutations of mismatch DNA repair genes (*MSH2, MSH6, MLH1* or *PMS2)* cause Lynch syndrome promoting colorectal, ovarian and endometrial cancers, increasing up to 13% the risk of GC development. Peutz-Jeghers syndrome is another cancer predisposition syndrome caused by mutations in the AMPK pathway activator *STK11 (*or *LKB1),* which increases the risk to 29% to develop GC. Juvenile polyposis syndrome is another rare syndrome characterized by unique hamartomatous polyps (juvenile polyps) of the gastrointestinal tract caused by germline mutations in *SMAD4,* increasing the risk to 30% of GC. Next to germline mutations, the Sjörgen rheumatologic syndrome increases the risk of gastritis and gastric cancer development (OR = 2.53) *(Cavanagh H and Rogers KMA. 2015; Boland CR and Yurgelun MB. 2017)*.

Diffuse gastric cancer

The diffuse type of gastric cancer is further subdivided into two sub-types, the hereditary diffuse gastric cancer (HDGC) and the sporadic diffuse GC.

HDGC are due to an inherited cancer syndrome and occur in about 3% of all GC. HDGC are due to germline autosomal dominant mutations, which for example in the case of *CDH1* (E-cadherin) increases the risk to develop GC to 70% for men and 56% for women *(Quadri HS et al. 2017)*. Mutations of *CDH1* are present in about 30% of HDGC *(CDU-HGE 2015)*, but how the different germline autosomal dominant mutations contribute to the carcinogenesis of this HDCG is still largely unknown.

The sporadic diffuse GC comprising cardia cancers and gastroesophageal junction tumors *(Michel P et al. 2017; Lledo G et al. 2016).* The incidence of it 2.5-fold increased from 1973 to 1992 but stabilized these last twenty years in the USA, however patients are getting younger compare to the intestinal type of GC *(Buas MF and Vaughan TL. 2013).* The diffuse type of GC is more aggressive with a worse prognosis *(Ma J et al. 2016).* All the carcinogenesis is still not very well understood, but factors like salt-rich diets, alcohol consumption, obesity, and smoking increase the risk to develop it *(Buas MF and Vaughan TL. 2013)*. The gastroesophageal reflux diseases, such as Barrett's disease, increase tissue inflammation and alter the microbiome elevating also the risk for diffuse type of GC *(Yang L et al. 2009; Shi J et al. 2014).* Concerning the pathogenesis, two independent studies identified in 42.2% and 56.3% of sporadic diffuse GC patients, respectively, mutations in CDH1 or methylations of its promoter *(Cho SY et al. 2017; Machado JC et al. 2001).* In addition, comparison of 53 diffuse GC to 70 matched control tissues revealed that SNP 160A on *CDH1* is associated with a susceptibility to develop GC *(Humar B et al. 2002).*

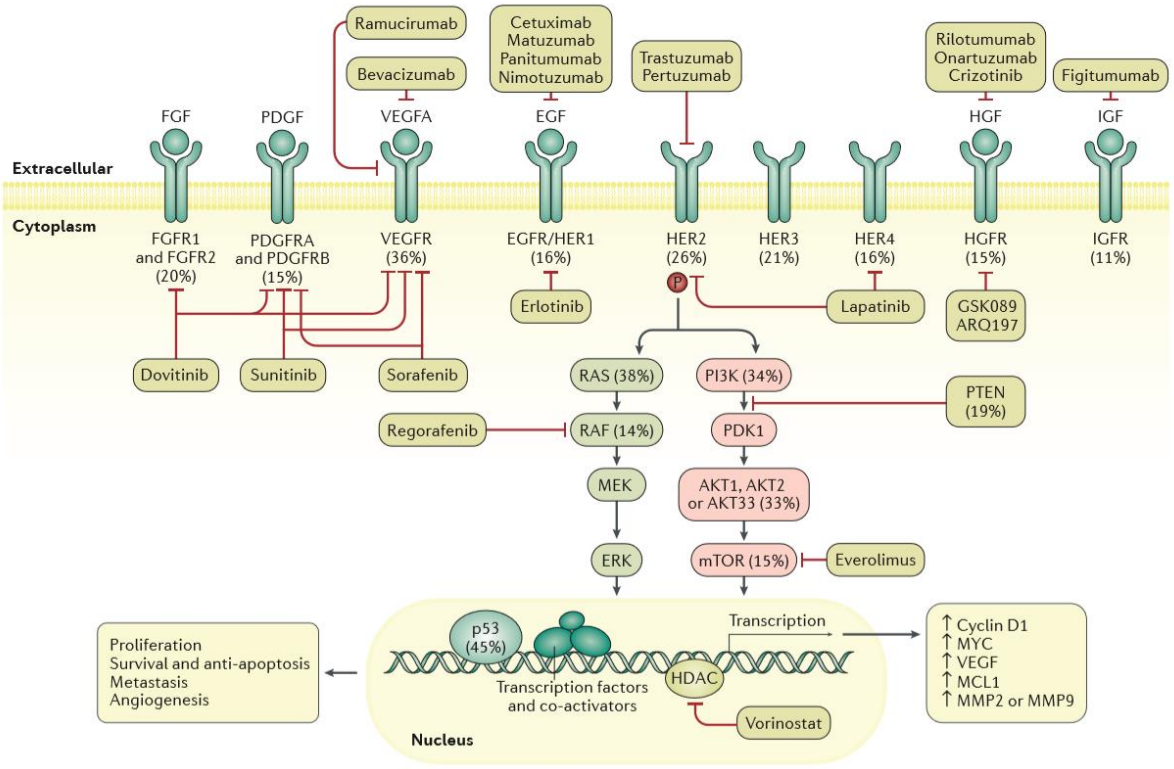


Figure 16: Molecular alterations in receptor tyrosine kinases and TP53 in gastric adenocarcinoma and potential target therapy

The frequency of the molecular alterations in the genes encoding each receptor tyrosine kinases shown here were obtained from The Cancer Genome Atlas database (n = 478) on gastric adenocarcinoma and included mutations and copy number alterations using the GISTIC (genomic identification of significant targets in cancer) algorithm and mRNA expression using RNA-Seq and protein expression Z-scores of >2 using reverse-phase protein array (www.cbioportal.org/). Illustration from *Ajani JA et al. Nature reviews Disease primers 2017*

To understand the role of *CDH1* in diffuse GC, Park and co-workers developed a mouse model harboring a loss of E-cadherin, p53 and Smad4. With this mouse model, they identified the Wnt pathway as a mechanism involved in diffuse type carcinogenesis, where Wnt mediates epithelial-to-mesenchymal transition (EMT) *(Park JW et al. 2018).* A proteomic study performed on diffuse GC, which was compared to genomic data analysis *(Ge S et al. 2018),* confirmed that *CDH1* mutations are associated with the activation (proteome) of the Wnt pathway. However, this study also showed that mutations in *CDH1* are associated with an activation of EGFR and Akt pathways and an inactivation of tumor suppressors EPHB3 and IGSF8. Importantly, according to their data Ge and co-workers regrouped diffuse GC into three proteomic based subtypes. i) Diffuse GC with enrichment of proteins involved in cell cycle regulation process. ii) Diffuse GC with enrichment of proteins involved in cell cycle regulation process and EMT process. iii) Diffuse GC with enrichment of proteins involved in immunological process. Interestingly, the type iii is associated with the worst prognosis. Cancer subtyping is associated with clinical outcome based solely on the altered cancer proteome irrespective of genetic background *(Ge S et al. 2018)* illustrating the interest to combine technologies for cancer studies. Next to the mutations found in *CDH1, TP53* and *RhoA,* a study on 23 cases of diffuse GC identified also mutations in *CMTM2.* Indeed, overexpression of *CMTM2* reduced GC cells proliferation but not cell invasion *in vitro* and low expression is associated to worse outcome in patients *(Choi JH et al. 2018)*. *CMTM2* was shown to play a role in human spermatogenesis due to its potential involvement in vesicular transport, but its potential role in GC is still unknown *(Liu G et al. 2007).*

Molecular pathways altered in gastric cancer

PI3 Kinase/Akt/mTOR pathway

(Figure 16) The PI3 Kinase/Akt/mTOR signaling pathway regulates cell proliferation and survival in response to growth factors. Basically, the activation of receptor tyrosine kinase (RTK) or the activation of G-protein-coupled receptor induces the phosphorylation cascade. Activated effector PI3 kinase phosphorylates PIP2 (phosphatidylinositol-4,5-bisphosphate) to produce PIP3 (Phosphatidylinositol (3,4,5)-trisphosphate), which phosphorylates PDK1(3-phosphoinositide-dependent protein kinase), which then phosphorylates Akt. pAkt induces the activation of mTOR, which promotes cell growth and protein synthesis. At the same time, pAkt inhibits many targets like p21, p27, BAD (Bcl2-associated agonist of cell death), GSK3 (Glycogen synthase kinase) to promote cell proliferation and to prevent apoptosis *(Matsuoka T and Yashiro M. 2014).*

It is reported that PI3K/Akt pathway is deregulated in GC. Indeed, an RTK, ERB-2 also called HER-2 is frequently overexpressed in intestinal type of GC but not in the diffuse type *(Shi J et al. 2012),* promoting pAkt activation *(Sukawa Y et al. 2012).* It is also described that *RUNX1,* a transcription factor involved in hematopoietic cell differentiation, positively regulates the HER2 signaling pathway. Indeed, suppression of *RUNX1* leads to dephosphorylation of HER2, and consequently suppressed the proliferation of GCC lines *(Mitsuda Y et al. 2018)*.

Immunohistochemistry and RT-qPCR studies of 120 GC shows HER2-HER3 co-expression is associated with pAkt and mTOR levels and their high expressions are positively related to the prognosis of patients *(Cao G et al. 2017).* Overexpression or amplification of *EGFR* are frequently found in 34 out of 82 GC (HER1/2) and in 79 out of 134 GC (HER3) and are associated with poor patient prognosis *(Hayashi M et al. 2008; Kandel C et al. 2014).* Another study identified amplifications of PIK3CA in 67% of GC and mutations of it in 8% out of 131, *(Shi J et al. 2012),* and showed that elevated *PIK3CA* expression is significantly correlated with tumor invasiveness, tumor phenotypes, and poor patient survival *(Jang SH et al. 2016).*

MAP Kinases pathway

(Figure 16) The MAPK pathway (mitogen-activated protein kinase) is a molecular cascade regulating several cellular responses as cell cycle regulation, cell adhesion, migration, differentiation, apoptosis and angiogenesis. Basically, growth factors and cytokines activate MAP3K (RAS and RAF isoforms, MEKK), which phosphorylate MAP2K (MEK1/2, MKK3/7), which phosphorylate MAPK (ERK, p38, JNK) promoting cellular response *(Yang M and Huang CZ. 2015)*.

The MAPK pathway is often deregulated in GC controlling tumor invasion and metastasis and *in vitro* inhibition of the p38/MAPK pathway reverses the EMT process of GC cells *(Yang M and Huang CZ. 2015). KRAS* presents 17% of alterations (on 393 GC, TCGA). Mutations in *KRAS* are frequently found in MSI subtype of GC *(Polom K et al. 2017)* and as well as mutations in *BRAF (Lee SH et al. 2003)* inducing a constitutive activation of the MAPK pathway.

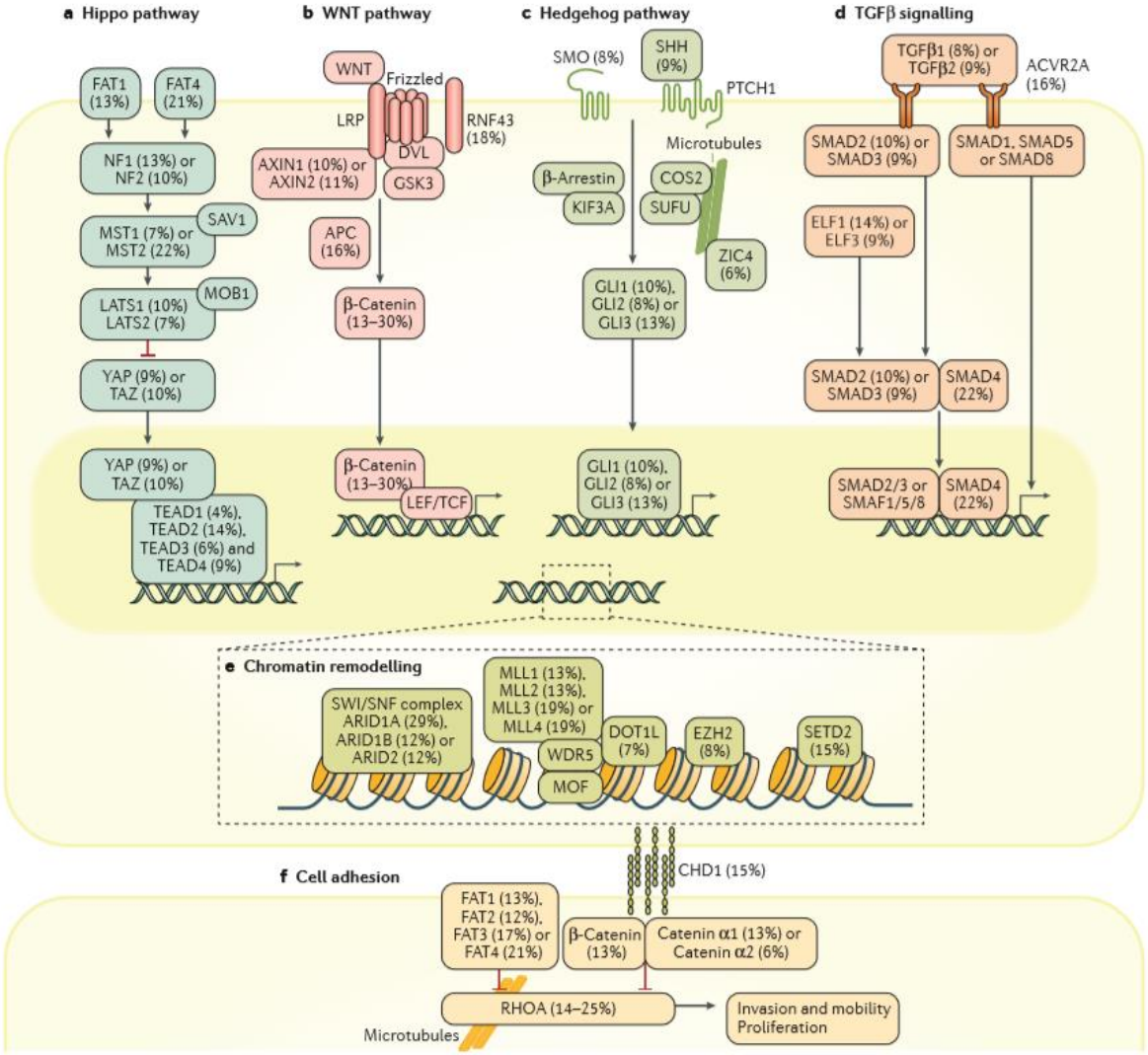


Figure 17: Molecular alterations common in gastric adenocarcinoma

The frequency of alterations in the corresponding recurrently mutated genes is shown in parentheses using data from The Cancer Genome Atlas database (TCGA; www.cbioportal.org), (n = 478 primary gastric adeno carcinomas) on **(a)** Hippo pathway, **(b)** WNT pathway, **(c)** Hedgehog pathway, **(d)** TGFβ signalling, **(e)** chromatin remodelling and **(f)** cell adhesion. Illustration from *Ajani JA et al. Nature reviews Disease primers 2017*

Wnt/β-catenin pathway

In the absence of Wnt ligands, the cytoplasmic β-catenin binds to the APC-GSK3β-CK1α complex, which phosphorylates β-catenin leading its degradation by the proteasome. The presence of a Wnt ligand on Frizzled receptor induces the membrane sequestration of the APC-GSK3β-CK1α complex thereby avoiding its degradation. The active β-catenin accumulates in the cytoplasm and is translocated to the nucleus where it acts as a transcriptional co-activator with the LEF-TCF proteins resulting in Wnt-responsive gene expression. Like the precedent molecular pathways, the Wnt/β-catenin signaling can be de-regulated in GC **(Figure 17)** by somatic mutations, promoter hyper-methylations and miRNA expressions *(Chiurillo MA. 2015).*

The *APC* gene was found to be mutated in some GC or has its expression lost by chromosome locus 5q21-22 deletion, promoter methylation or miRNA (miR-27) expression promoting Wnt signaling activation. Moreover, the tumor suppressor gene *RUNX3* inhibits the Wnt signaling pathway by forming a complex with TCF4/β-catenin, which prevents Wnt target gene expression. *RUNX3* expression was also sometimes found to be lost in GC due to methylation of its promoter, promoting EMT and maintenance of stem cell-like subpopulation.

However, up-regulation of factors in the Wnt pathway have also been identified in GC. Indeed, the β-catenin coding gene (*CTNNB1*) is mutated in a high proportion of GC but more commonly in the intestinal type resulting in an over-activation of β-catenin *(Ajani JA et al. 2017).* A study performed on a cohort of 180 GC samples, showed that *Wnt-1*, *β-catenin* and *E-cadherin* are overexpressed in 54.4%, 45.6%, 47.2%, respectively, promoting progression, invasion and metastasis of GCC *(Zhang H and Xue Y. 2008).* Yin Yang factor 1 (YY1) up-regulation is also present in several GCC lines promoting Wnt pathway over-activation. High expression of *YY1* was reported in 59.1% in total of 247 GC tissue samples *(Kang W et al. 2014). In vitro* and *in vivo* overexpression and inhibition of YY1 respectively increase and inhibit tumor growth via Wnt pathway *(Kang W et al. 2014).* Moreover, YY1 is inhibited and targeted by miR-584-3p which expression is decreased in GC (36/60 GC cases) promoting YY1 over-expression resulting in Wnt pathway over-activation facilitating growth and metastasis of GCC, and angiogenesis *(Zheng L et al. 2017).* Moreover, Aurora kinase A (*AURKA*), a centrosome associated cell cycle regulated serine/threonine kinase, was found to be amplified in 26 out of 393 gastric adenocarcinomas (TCGA; http://www.cbioportal.org). AURKA positively regulates pAkt and negatively the GSK-3β phosphorylation and activity.

Thereby, *AURKA* amplifications lead to APC-GSK3β-CK1α complex inhibition and pAkt up-regulation, which activates and promote the transcriptional activity the β-catenin/TCF complex respectively resulting in GC progression and invasion *(Fang D et al. 2007; Dar AA et al. 2009).* In addition, *MUC1* was found to be overexpressed in multiple cancers including gastric cancer *(Kufe DW. 2009; Saeki N et al. 2014).* MUC1 is described to interact with the β-catenin in the nucleus to stabilize the TCF4/β-catenin complex promoting Wnt target gene transcription in gastric cancer cells *(Kufe DW. 2009).*

Hippo/YAP-TAZ pathway

The Hippo pathway in part controls organ size, tissue homeostasis and tumor progression modulation. The central axis contains the phosphorylation cascade of MST1/2 (Macrophage-stimulating protein) then LATS1/2 (Serine/threonine-protein kinase) and finally YAP/TAZ in response to various stimuli as cell-cell contact, mechanical force (cadherin family member, f.e. FAT4), and hormones. Activation of the Hippo pathway leads to a phosphorylation of YAP/TAZ resulting in their cytoplasmic sequestration and their degradation. In contrast, un-phosphorylated YAP/TAZ translocates to the nucleus and promotes transcriptional expression of gene mediating cell proliferation and migration *(Qiao Y et al. 2018).*

According to the TCGA, there is an alteration of the Hippo pathway in 71% of reported GC cases **(Figure 17),** with alterations found in *MST1/2* (2.5 and 9% of 393 GC cases; TCGA), *LATS1/2* (6 and 5%; TCGA) and *RASSF1* (2.5%; TCGA). Promoter hyper-methylation of *RASSF1* induces Hippo pathway activation and in a meta-analysis of 1215 GC cases, is associated to an increased risk to develop GC (OR = 12.67) *(Shi DT et al. 2014).* MicroRNA profiling on 847 human GC showed more than 2-fold change for miR-138, which targets MST1 thereby inhibiting the Hippo pathway *(Yao Y et al. 2009; Qiao Y et al. 2018).* Moreover, low expression of *LATS1* and *LATS2* in 264 patients with gastric cancer are significantly associated with tumor stage, tumor invasion, lymph node metastasis, poor histological grade, and diffuse type of GC *(Son MW et al. 2017).* Recently, Choi and co-workers showed that activation of the YAP/TAZ in the pyloric stem cells of mice lead to the up-regulation of its target MYC and initiation of gastric tumorigenesis *(Choi W et al. 2018).*

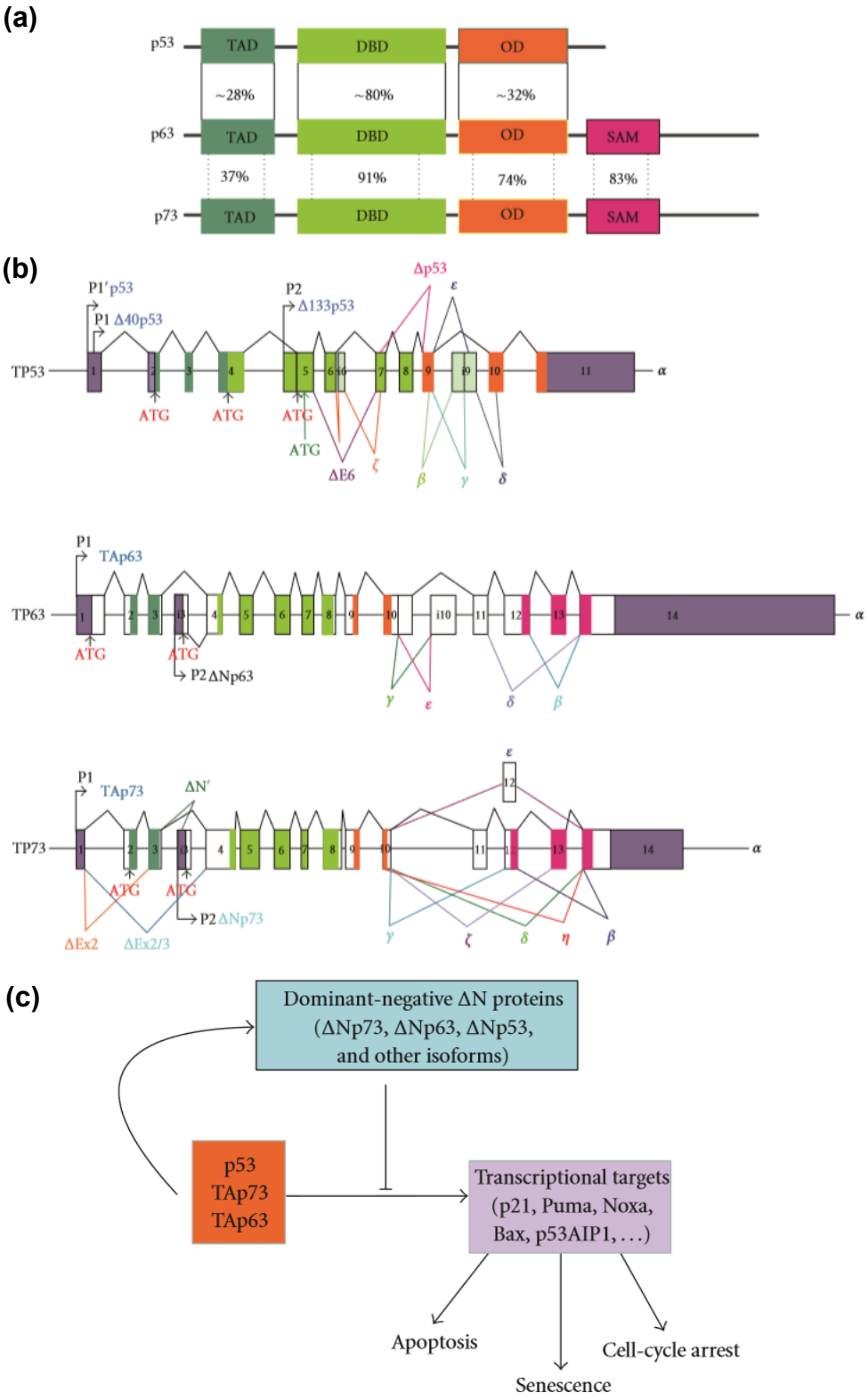


Figure 18: Architectures of human TP53, TP73, and TP63 genes

(a) TP53, TP73, and TP63 genes encode the transactivation (TAD), DNA-binding (DBD), and oligomerization (OD) domains. TP73 and TP63 encode additional SAM (Sterile Alpha Motif) domain. Percentages represent homologies of residues between p53, p63, and p73. **(b)** TP53, TP63, and TP73 genes have two promoters (P1 and P2). Splicing generates the different α, β, γ, ζ, ε, δ and η isoforms. **(c)** Interactions of p53 family members. Illustration from *Wei J et al. Journal of Nucleic Acids 2012*

NFκB pathway

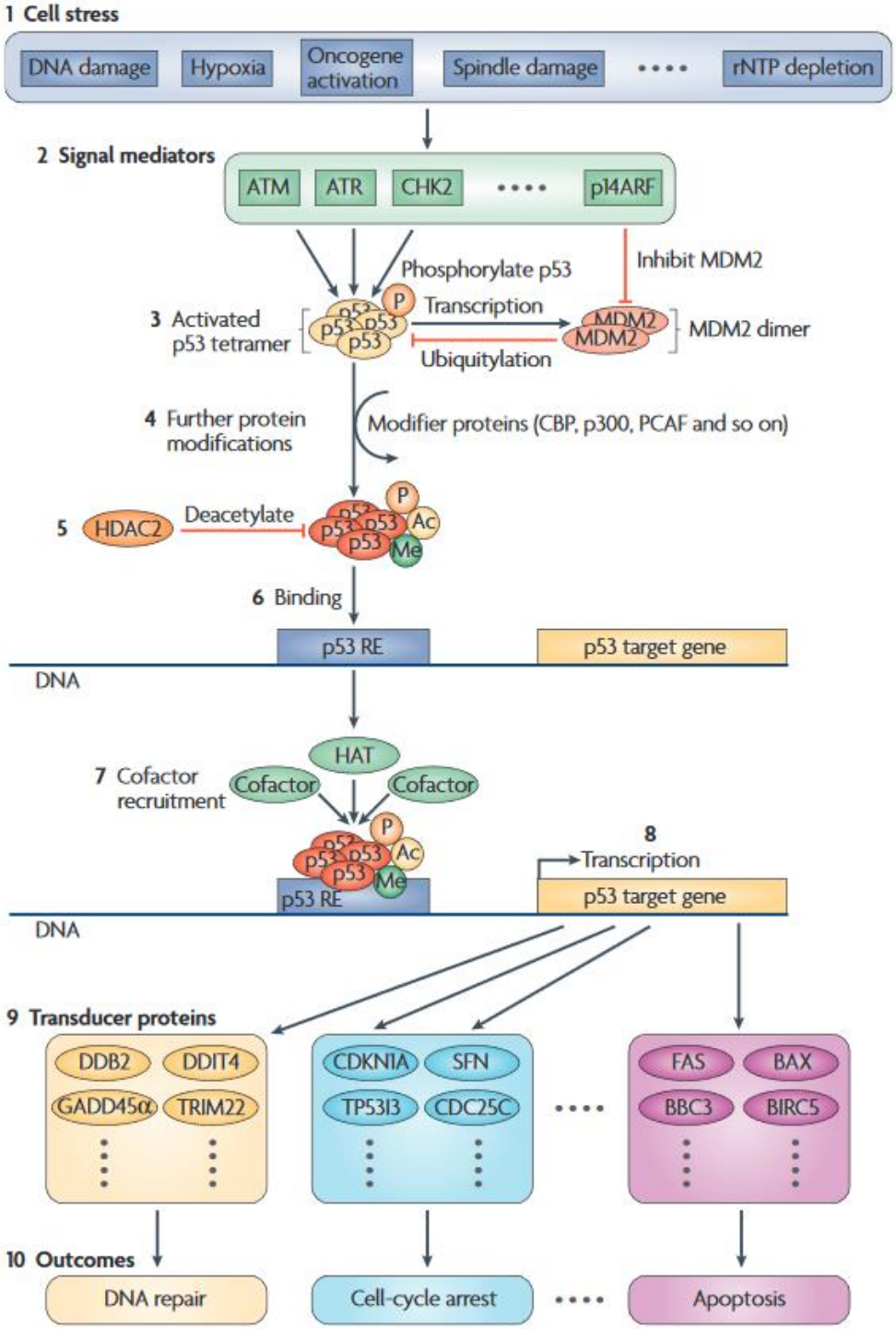
Nuclear factor kappa B (NFκB) refers to a group of transcription factors (RelA, RelB, c-Rel, NFκB1/p50, NFκB2/p52), which form homo- and heterodimers. In response to microbial compounds, hormones and growth factors, NFκB upregulates or suppresses the expression of several genes like cytokines/chemokines (IL-1, TNF), *VEGF*, *MMP* (Matrix metalloproteases) promoting GCC survival and invasion *(Sokolova O and Naumann M. 2017)* or Cyclooxygenase-2 (*COX2*) promoting cell growth and apoptosis inhibition *(Shi J et al. 2014).* According to the TCGA, on 393 GC reported cases, *RelA* is altered in 1.8%, *Relb* in 2.5%, *NFκB1* in 2.8%, and *NFκB2* in 5% with in majority gain function mutations or gene amplifications illustrating an over-activation of the pathway *(http://www.cbioportal.org).*

Sonic hedgehog pathway

(Figure 17) The Sonic hedgehog (SHH) signaling pathway is activated by SHH binding to the Patched (PTCH)-Smoothened (SMO) membrane-receptor complex. Upon activation, SMO promotes nuclear translocation of the GLI family of transcription factors (*Gli1, Gli2*, and *Gli3*) that subsequently activates target gene expression controlling cell cycle and invasion. In GC, SHH signaling pathway activation is directly correlated with GCC proliferation and tumor growth and SHH expression is associated with shorter survival in gastric cancer patients *(Katoh Y and Katoh M. 2005; Wan J et al. 2014; Ertao Z et al. 2016).*

The TP53 family

Human tumor protein TP53 is the founding member of the p53 transcription factor family, which contains beside p53, p63 and p73. They possess high homologies on their close structural elements; DNA binding domain (DBD), oligomerization domain (OD) and a transactivation domain (TA) **(Figure 18a).** TP63 and 73 have common target genes and functions to p53 like apoptosis control (response to cellular stress) but they also have distinct functions to p53 **(Figures 19 and 20)** *(Pflaum J et al. 2014)*. In addition, the presence of two promoters allows the transcription of two types of isoforms: TA, which has a complete N-terminal transactivation domain and ΔN, which has the transactivation domain truncated. In addition, the alternative splicing in the 3' region of the mRNA generates the different α, β, γ, ζ, ε, δ and η isoforms **(Figures 18bc)** *(Wei J et al. 2012).*


1 Cell stress
DNA damage
Hypoxia
Oncogene activation
Spindle damage
rNTP depletion
2 Signal mediators
ATM
ATR
CHK2
p14ARF
Phosphorylate p53
Inhibit MDM2
3 Activated p53 tetramer
Transcription
MDM2 dimer
Ubiquitylation
4 Further protein modifications
Modifier proteins (CBP, p300, PCAF and so on)
5 HDAC2
Deacetylate
6 Binding
p53 RE
p53 target gene
DNA
7 Cofactor recruitment
HAT
Cofactor
Cofactor
8
Transcription
p53 RE
p53 target gene
DNA
9 Transducer proteins
DDB2
DDIT4
GADD45α
TRIM22
CDKN1A
SFN
TP53I3
CDC25C
FAS
BAX
BBC3
BIRC5
10 Outcomes
DNA repair
Cell-cycle arrest
Apoptosis

← Figure 19: Mechanisms of p53 activation and regulation of downstream targets

① Stress which can eventually lead to cancer. ② Signal mediator proteins activate p53 by phosphorylating certain residues or inhibiting ubiquitylation by MDM2 (mouse double minute 2 homolog). ③ Both processes increase the half-life of p53 by inhibiting ubiquitylation resulting in higher levels of p53. ④ Further p53 modifications by acetyltransferases (CBP, p300, PCAF) and methyltransferases (sET9) can further stabilize the p53 protein and increase site-specific DNA binding. ⑤ For example, HDAC1/2 can inhibit p53 binding to DNA by deacetylating the protein. ⑥ The p53 tetramer binds to a p53 response element (RE) to regulate transcription of a nearby gene. ⑦ p53 also recruits cofactors such as histone acetyltransferases (HATs) and TATA-binding protein-associated factors (TAFs). ⑧ In this example, p53 mediates transactivation of its target gene, but p53 can also mediate transcriptional repression. ⑨ The p53 protein transactivates many genes, the protein products of which are involved in various pathways. ⑩ The most important pathways involved in tumour suppression that are activated by p53 lead to DNA repair, cell-cycle arrest, senescence and apoptosis.

ATM: ataxia telangiectasia mutated; **BAX:** BCl2-associated X protein; **BBC3:** BCl2-binding component-3; **BIRC5:** survivin; **CDKN1A:** cyclin-dependent kinase inhibitor-1A; **CHK2:** checkpoint kinase-2; **DDB2:** damage-specific DNA-binding protein-2; **DDIT4:** DNA-damage-inducible transcript-4; **Fas:** TNF receptor subfamily, member 6; **GADD45α:** growth arrest and DNA-damage inducible α; p14ARF; **sFN:** stratifin; **TP53I3:** tumour protein p53-inducible protein-3; **TRIM22:** tripartite motif containing-22.

Illustration from *Riley T et al. Nature reviews Molecular cell biology 2008.*

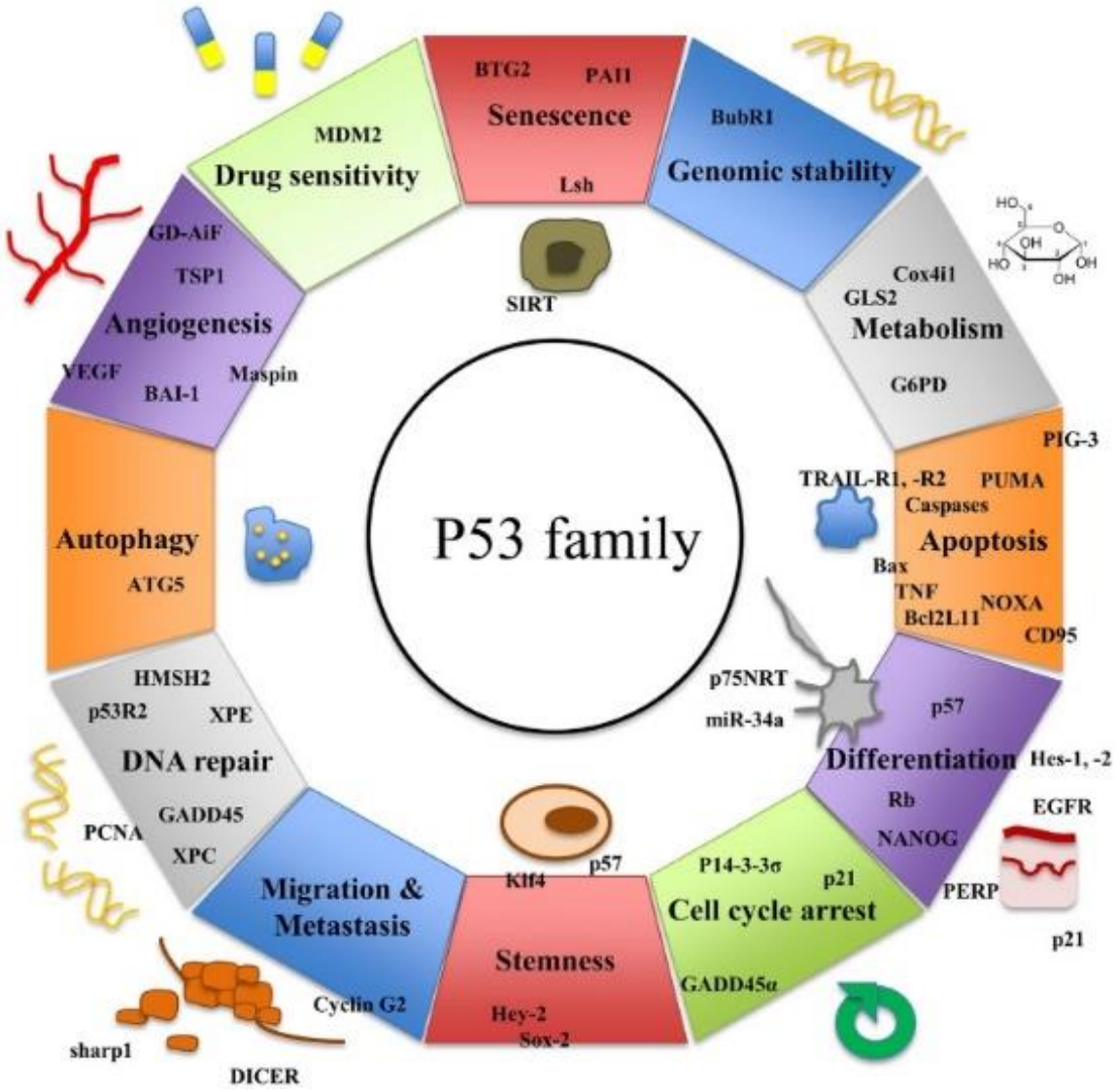


Figure 20: Functions of the p53 family

Functions of p53 and its homologs p63 and p73 and their target genes. Illustration from *Pflaum J et al. Frontiers in Oncology 2014*

TP53: TP53 knockout mice showed spontaneous tumors highlighting the role of TP53 as the "guardian of the genome" *(Pflaum J et al. 2014)*. According to the TCGA, p53 is mutated in around 50% of gastric cancer versus 4% for p63 and 1% for p73 *(http://www.cbioportal.org)*. In addition, the p53 status is different depend on GC subtypes. Indeed, TP53 is frequently found mutated in the intestinal chromosomal instable subtype *(http://www.cbioportal.org)*, whereas TP53 mutations are infrequent in EBV-associated GC (16 out of 46 GC reported) *(Ribeiro J et al. 2017)*. Moreover, in EBV-associated GC, there is a strong expression of p53 (present in 45 out of 46 cases) with a decrease of p53 mRNA levels compare to non-EBV-GC *(Ribeiro J et al. 2017).*

Besides mutations, TP53 level and activity is controlled by MDM2, an E3 ubiquitin ligase promoting p53 proteasomal degradation. In response to DNA damage, MDM2 is inhibited disrupting its binding to p53, which consequently accumulates and activates p53 **(Figure 19)** *(Wade M et al. 2010). MDM2* is overexpressed in 18 out of 43 GC tissues and *MDM2* gene amplification is more frequently found in diffuse GC. The upregulation of the MDM2 oncogene is accompanied of TP53 inactivation inhibiting cell cycle arrest and apoptosis promoting gastric tumorigenesis *(Günther T et al. 2000).* Interestingly, *TP53* mutations are detected in established GC whereas *TP53* expression is associated with high MDM2 expression in earlier tumor stages *(Busuttil RA et al. 2014).* This might be explained by *AURKA* overexpression in GC, which is reported to promote *MDM2* expression and consequently p53 degradation *(Sehdev V et al. 2013).*

Moreover, it is reported that p53 cannot induce apoptosis in response to DNA damage, without the presence of p63 or p73. Indeed, for example, p63 could bind to *BAX* and *NOXA* promoters in the absence of p53 in response to DNA damage, but these genes were not expressed *(Murray-Zmijewski F et al. 2006).* Besides, TP53 family members have common functions like apoptosis induction **(Figure 20).** In addition, p53 and TAp73 can bind to the ΔNp73 promoter inducing its transcription. ΔNp73, by a negative feedback, inhibits p53 and TAp73 activity *(Murray-Zmijewski F et al. 2006).* Thereby, it is interesting to determine how the p53 family member can interact and regulate each other expression or activity. A better understanding of the TP53 family interacting and regulation may increase the therapeutic benefits (*Wei J et al. 2012; Park S et al. 2016).*

TP63: The TAp63 isoforms can bind to DNA across p53 response element and activate transcription of target genes inducing cell cycle arrest or apoptosis (common functions to p53). However, mouse p63 knockout studies revealed that TP63 expression is necessary for limb and skin developments *(Moll UM and Slade N. 2004)* indicating that p63 proteins can bind DNA across specific p63 response elements inducing p63 but not p53 dependent

response *(Murray-Zmijewski F et al. 2006).* In addition, p63 knockout mice showed premature aging or cancer prone, highlighting an anti-tumoral role of p63, and studies of p63 mutated mice suggested that each p63 isoform has specific biological activities, which need to be clarified *(Moll UM and Slade N. 2004; Murray-Zmijewski F et al. 2006; Costanzo A et al. 2014).* Moreover, the TAp63 is reported to induce cellular senescence and inhibit cell proliferation, whereas the decrease of TAp63 expression increased cell proliferation and is associated with metastasis in breast cancer, suggesting an anti-tumoral activity of the TAp63 *(Moll UM and Slade N. 2004; Murray-Zmijewski F et al. 2006).* However, the ΔNp63 isoforms can bind DNA across p53 elements and can exert dominant-negative effects over p53, p73, and p63 by competing for DNA binding or by direct protein interaction **(Figure 18c).** In addition, several studies have reported that ΔNp63 isoforms have oncogenic proprieties, but they also suggest that ΔNp63 plays a dual role by promoting tumor development but reducing metastasis *(Moll UM and Slade N. 2004; Murray-Zmijewski F et al. 2006).* Thereby, TP63 isoforms play a role in cancers but additional studies are necessary to understand the regulation of p63 isoforms.

In GC, TP63 is altered in 9% out of 393 GC TCGA reported, which are in majority gene amplifications *(http://www.cbioportal.org/).* Tannapfel and co-workers studied p63 expression (TA and ΔN isoforms) in 68 GC patients. They remarked that p63 expression was higher in advanced diffuse type but TAp63 and ΔNp63 expression were not associated with the tumor stages, the prognosis or the mutational state of p53 in 32 patients out of 68 gastric adenocarcinomas *(Tannapfel A et al. 2001).* Whereas Song and co-workers found that p63 expression is higher in gastric tumoral tissues compare to normal tissues and positive p63 expression correlated significantly with shorter survival and lower 5-year survival rate in study of 101 GC tissues and 25 normal gastric mucosa tissues *(Song Y et al. 2015).* Likewise, Truond C and co-workers found high expression of p63 in 9 out of 200 GC. They concluded that p63 was specifically expressed in the sarcomatoid component of sarcomatoid carcinoma, but not in the adenocarcinoma component. They associated the overexpression of p63 with a poorer prognosis of GC *(Truong C et al. 2008).* Altogether, these studies highlight that the increase expression of p63 in less well differentiated GC might promote aggressive neoplastic growth *(Tannapfel A et al. 2001; Truong C et al. 2008).*

At the same time, studies in GCC revealed that high expression of ΔNp63 enhances GATA6 expression, increasing cell proliferation and colony formation whereas suppression of ΔNp63 expression suppresses cell proliferation, reduces GATA6 expression and induces apoptosis of MKN28 GC cells *(Wang H et al. 2012)* illustrating a pro-tumoral role of ΔNp63

in GC. Interestingly, *GATA6* (GATA-binding factor 6) is overexpressed in GC (amplified in 36/393 reported GC cases; TCGA). Its role is not completely clear, but it seems to promote cell proliferation and intestinal differentiation *(Sulahian R et al. 2014)*. According to Ashton Acton Q. and co-workers' work, study a link between GATA6 and ΔNp63 may be interesting to validate the correlation in GC patients.

TP73: As TP63, the TAp73 isoforms **(Figure 18b)** can bind to DNA across p53 response elements activating target gene transcription inducing cell cycle arrest or apoptosis and inducing also specific p73 response. The ΔNp73 isoforms can bind DNA across p53 elements and can exert dominant-negative effects over p53, p63 and p73 activities **(Figure 18c)** *(Murray-Zmijewski F et al. 2006).* Mouse p73 knockout studies revealed that TP73 expression is necessary for neurogenesis of specific neural structures such as the hippocampus, for pheromonal signaling, and for normal fluid dynamics of cerebrospinal fluid *(Moll UM and Slade N. 2004).* However, ΔNp73 knockout mice showed altered neuronal development and high sensitivity to DNA damaging agents and elevated p53-dependent apoptosis whereas TAp73 knockout mice were cancer prone and had a defective meiosis suggesting that ΔNp73 and TAp73 can activate isoform specific gene *(Murray-Zmijewski F et al. 2006; Rufini A et al. 2011).*

In GC, TP73 is altered in 3% out of 393 GC TCGA reported cases with in majority deep deletions *(http://www.cbioportal.org/).* High expression of p73 is described in 37 out of 39 GC patients *(Wei J et al. 2012).* Likewise, but more specifically, Vilgelm AE and coworkers showed that ΔNp73 expression is increased in a cohort of 185 patients with GC and is associated with poor patient survival; the median survival time for patients with increased ΔNp73 is 20 months whereas for patients with a negative/weak expression it is 47 months. Interestingly, Vilgelm AE and co-workers showed that HIC1 (Zinc finger and BTB domain-containing protein 29 or Hypermethylated in cancer 1) targets *ΔNp73* promoter reducing its expression. HIC1 is hypermethylated in gastric cancer promoting the up-regulation of ΔNp73 *(Vilgelm AE et al. 2010)* which may inhibit p53 and TAp73 activities and promotes gastric tumorigenesis. Thereby ΔNp73 seems to have pro-tumoral role in GC.

According to the literature previously cited, TAp73 can recuse cell proliferation and induce apoptosis, thereby can have anti-tumoral activity. However, TAp73 overexpression promotes *Cyclin D* expression, leading to cell proliferation and Doxorubicin resistance of GC cells *(Ji ZP et al. 2017)* and it reduces the apoptosis induced by chemotherapeutic drugs, which is dependent on p53 in several GCC lines (SNU-1, SNU-3, and AGS) *(Qiang L et al. 2018).* Thereby, TAp73 overexpression promotes drug resistance and seems to have pro-tumoral role in GCC highlighting dual role of p73 in tumorigenesis.

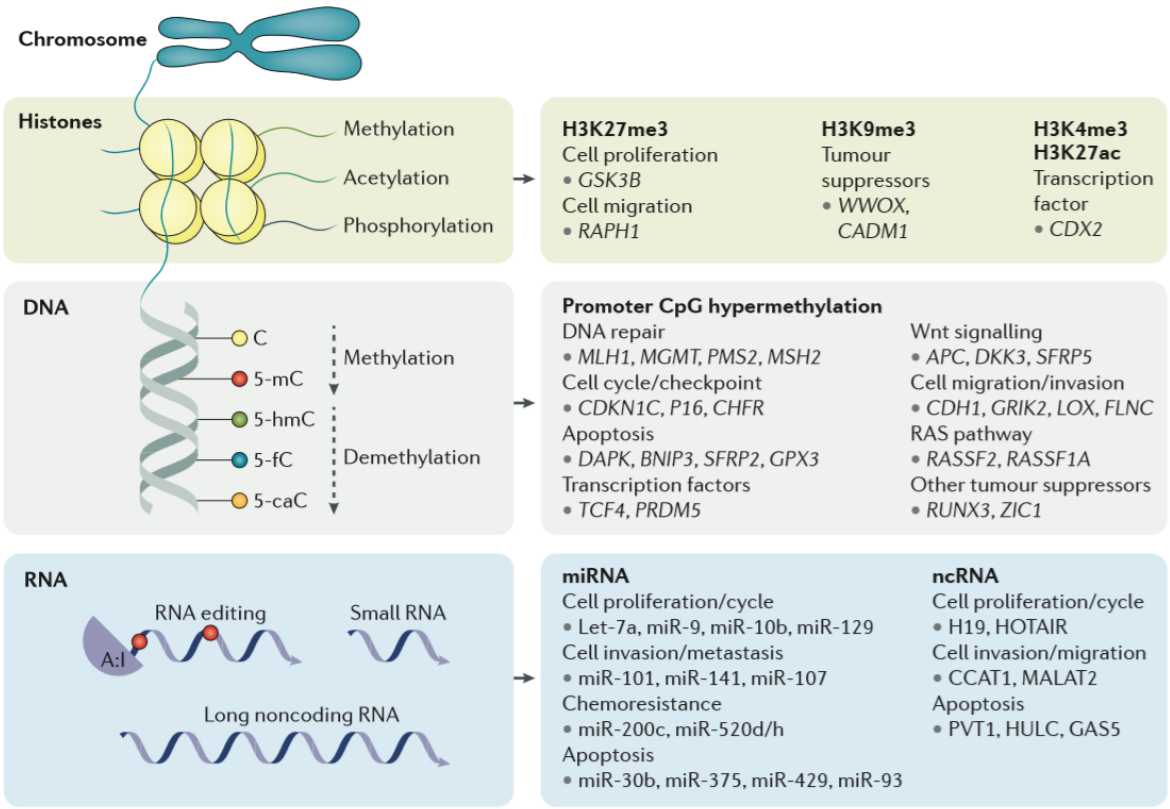


Figure 21: Epigenetic marks in gastric cancer

A: adenosine; **C:** cytosine; **5-caC:** 5-carboxycytosine; **5-fC:** 5-formylcytosine; **5-hmC:** 5-hydroxymethylcytosine; **I:** inosine; **5-mC:** 5-methylcytosine; **miRNA:** microRNA; **ncRNA:** noncoding RNA. Acetylated H3K27 (**H3K27ac**) and trimethylated H3K27 (**H3K27me3**) are generally associated with active regulatory and inhibitive regulatory respectively. Illustration from *Padmanabhan N et al. Nature reviews Gastroenterology & Hepatology 2017*

Epigenetic alterations

Tumor epigenetic include aberrant DNA methylations, histone modifications, non-coding RNA and RNA editing **(Figure 21).** Environmental factors such as life-style (diet, physical activity, smoking) and infections (*H. pylori* and EBV) are correlated with DNA methylation changes *(Li Y et al. 2015)*. DNA methylations are early found in pre-cancerous lesions indicating their roles in gastric carcinogenesis initiation *(Padmanabhan N et al. 2017).* Several promoters were reported to be hyper-methylated in GC and were associated with the TNM stage or the Lauren classification, and were associated with metastasis or poor patient survival. For example, compare to normal tissue, *MGMT,* implicated in DNA repair, has its promoter 4.6-fold methylated promoting treatment resistance, *p16,* involved in cell cycle regulation, has its promoter 1.3-fold methylated promoting gastric tumor growth, and *CDH1* promoter is 2.33-fold methylated promoting GCC invasion. Likewise, HoxD10 promoter is 85.7-fold methylated promoting GC invasion and aggressiveness, *APC* promoter is 1.4-fold methylated promoting Wnt pathway over-activation, and *RASSF2* promoter is 2-fold methylated inducing RAS pathway over-activation, tumor growth and invasion *(Qu Y. et al. 2013)*.

Another example, *CASPASE8* promoter methylation is significantly increased in gastric tumor samples compare to normal tissue (comparison of 30 normal and 30 pathologic cases) inhibiting apoptosis *(Azarkhazin F and Tehrani GA. 2018).* Interestingly, the methylation of gene promoters in cancerous tissue seems to correlate with the location of the gastric tumor. Indeed, the frequencies of methylation out of 81 gastric cancer tissues from the upper, middle, and lower thirds of the stomach were 11.1%, 23.1%, and 45.4 %, respectively, for *MLH1*; 22.2%, 30.8%, and 57.6 %, respectively, for *MGMT*; and 44.4%, 48.7%, and 51.5 %, respectively, for *DAPK (Kupcinskaite-Noreikiene R et al. 2016).* Alterations (mutations, amplifications) in epigenetic regulatory genes are also reported to have a crucial role in aberrant epigenetic events and carcinogenesis. For example, *TET1* (Ten-Eleven Translocation 1) promoting CpG islands demethylation, has its expression commonly lost in MSI subgroup of GC maintaining DNA hyper-methylations *(TCGA; Padmanabhan N et al. 2017).* On the contrary, the DNA methyltransferase *DNMT3A* is high expressed in 70.4% of 54 patients with GC promoting aberrant methylations and it is associated with tumor stage and lymph node metastasis *(Yang J et al. 2011).*

(a) (b)

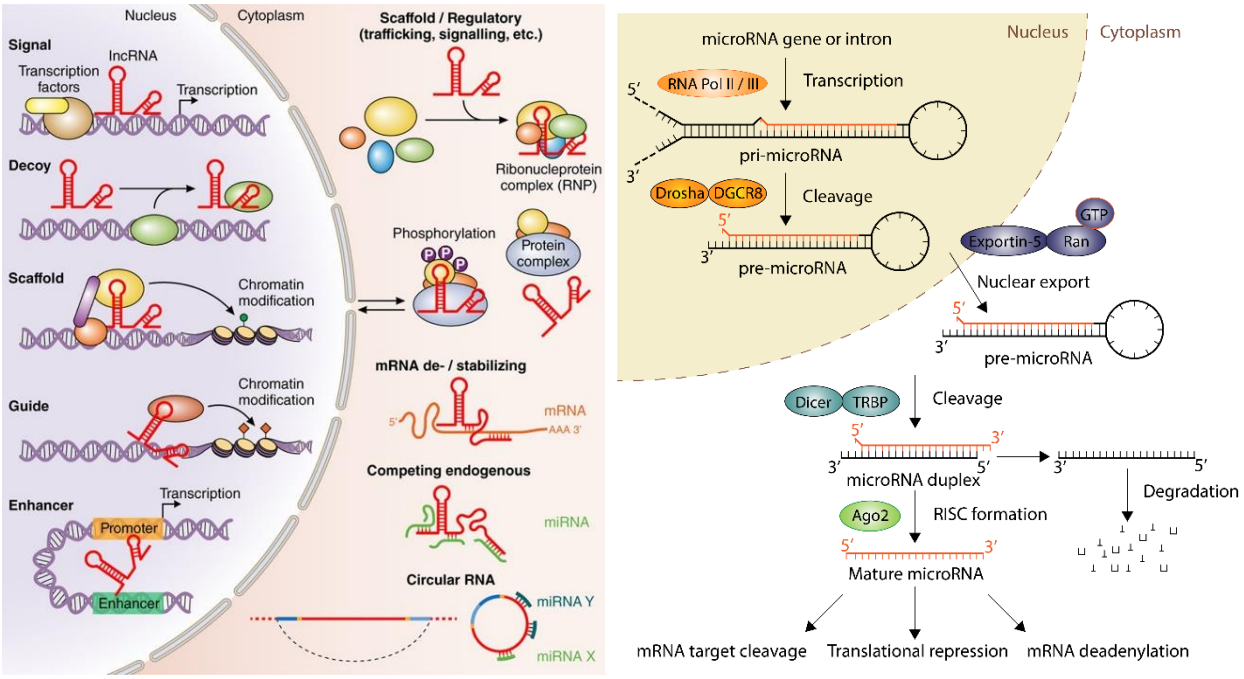


Figure 22: Long non-coding RNAs (lncRNAs) and microRNAs (miRNAs) in human cells

(a) LncRNAs are transcribed by RNA polymerase II or III may be multiexonic, 5′-capped, and polyadenylated. Nuclear-localized lncRNAs regulate gene expression in various modes such as in a response to stimuli (signal), sequester transcription factors/protein complex (decoy), bring together multiprotein complexes (scaffold) or guide transcription factors/protein complex to specific target site (guide) to activate or repress transcription and induce chromosomal looping to increase association between enhancer and promoter region (enhancer). Cytoplasmic lncRNAs can stabilize ribonucleoprotein complexes, regulate mRNAs stability or sponge miRNAs, thus controlling translational events. Further regulatory functions may involve protein signaling (eg, phosphorylation status) and trafficking. Illustration from *Bär C et al. Circulation 2016*

(b) miRNA genes are usually transcribed by RNA polymerase II. They are initially transcribed as long RNA stem-loop called primary miRNA (pri-miRNA). A single pri-miRNA may contain one to six miRNA precursors. The double-stranded RNA structure of the hairpins in a pri-miRNA is recognized by a nuclear protein DGCR8. DGCR8 associates with the enzyme Drosha to cleave RNA and product a pre-miRNA (precursor-miRNA). pre-miRNAs are exported from the nucleus by Exportin-5. In cytoplasm, the pre-miRNA is cleaved by the RNase III enzyme Dicer forming a miRNA duplex about 22 nucleotides in length. One strand of the mature miRNA is incorporated into the RNA-induced silencing complex (RISC) where the miRNA and its mRNA target interact and results in translational repression or target degradation. Illustration from *www.vectorbiolabs.com*

Non-coding RNAs

Non-coding RNAs such as microRNAs (miRNAs) and long non-coding RNAs (lncRNAs) are also involved in gastric tumorigenesis **(Figure 21).** Non-coding RNAs are RNA sequences, which do not code for a protein. LncRNAs are sequences of 200 nucleotides to 100 kilobases controlling gene expression **(Figure 22a).** LncRNAs often function as important cis and trans-acting modulators to a specific chromatin locus by recruiting chromatin-remodeling complex to control protein-coding gene expression (epigenetic modifications) *(Bhat SA et al. 2016; Bär C et al. 2016).* For example, LncRNA FEZF1-AS1 level is upregulated in 82 GC samples compared to adjacent histologically normal tissue, and its upregulation is positively associated with tumor size, stage and poor survival of GC patients. *In vitro* study revealed that LncRNA FEZF1-AS1 recruits and binds to the demethylase LSD1 mediating H3K4me2 demethylation on *p21 (CDKN1A)* promoter inhibiting its expression and thereby contribute to tumor proliferation, which supports tumor size observations in GC patients *(Liu Y W et al. 2017).*

Moreover, LncRNAs can complex with transcription factors and transcriptional co-factors to guide them to a specific target sequence and they can also sequester miRNAs thereby regulating messenger RNA stability. *(Bhat SA et al. 2016; Bär C et al. 2016).* For example, LncRNA FER1L4 sequesters miR-106a-5p, which targets PTEN, promoting abundance of PTEN and thereby inhibits cancer cell growth. Inversely, downregulation of LncRNA FER1L4 liberates miR-106a-5p, inhibiting PTEN, and promoting cell proliferation *(Xia T et al. 2015),* corroborating the low expression of LncRNA FER1L4 observed in 56 out of 61 GC patients positively associated with tumor size and stage *(Liu Z et al. 2014).*

miRNAs are sequences around 25 nucleotides, which are a part of the RISC/DICER/AGO complex to target specific mRNAs leading to their degradation or inhibiting their translation **(Figure 22b)** *(Catanalotto C et al. 2016).* Several miRNAs are reported to be deregulated in GC **(Table 2)** promoting cancer progression *(Ishiguro H et al. 2014; Tsai MM et al. 2016)*.

In addition, a study of 365 GC cases associates single nucleotide polymorphism (SNP) with the risk to develop GC subtype specific. SNP in miR-29a/miR-29b-1 cluster are associated with diffuse subtype (OR = 1.72), SNP of the miR-363/miR-92a-2/miR-19b-2/miR-20b/miR-18b/miR-106a cluster is associated with non-cardia GC (OR = 1.41) *(Espinosa-Parrilla Y et al. 2014)*. And miR-196a was significantly associated with poor differentiated GC *(Mu YP et al. 2014; Tsai MM et al. 2016).*

miRNAs	Expression	GC samples	Clinical applications	Cell functions	Targets
OncomiRNAs					
miR-221	Up-regulated	GCC 82 GC 82 HC 46 Dys	Poor survival Diagnosis	Radio-resistance Cell cycle	*CDKN1A* *CDKN1B* *CDKN1C* *PTEN*
miR-222	Up-regulated	GCC 114 GC 36 CAG 56 HC 30 GCT	Diagnosis TNM stages Poor survival Poor survival	Radio-resistance Cell cycle	*CDKN1A* *CDKN1B* *CDKN1C* *PTEN*
miR-21	Up-regulated	GCC GCT	LNM Prognosis Poor survival Venous invasion Diagnosis	Cell proliferation Invasion Cell cycle Metastasis	*PDCD4* *PTEN* *CDKN1A* *CDKN1C*
miR-21	Up-regulated	174 GC 39 HC	Diagnosis	ND	*ND*
miR-21	Up-regulated	103 GC 103 HC	Diagnosis Prognosis	ND	*ND*
miR-21	Up-regulated	69 GC 42 Pre 42 Post	LNM Prognosis Poor survival Venous invasion Diagnosis	Cell proliferation Invasion	*PTEN* *RECK*
miR-148a	Up-regulated	GCC GCT	Invasion Metastasis Prognosis	Metastasis Organ invasion	*DNMT1*
miR-196a/196b	Up-regulated	109 GC	Invasion LNM Metastasis Prognosis TNM stages Lauren subtype (Intestinal) Poor survival	Metastasis Invasion Migration	*RADIXIN*
Tumor suppressor miRNAs					
miR-15b	Down-regulated	GCC GCT	ND	Chemoresistance	*BCL-2*
miR-181c	Down-regulated	GCC GCT	ND	Transcriptional activation	*KRAS*

miRNAs	Expression	GC samples	Clinical applications	Cell functions	Targets
Tumor suppressor miRNAs					
Let7a	Down-regulated	GCC	ND	Cell cycle Cell proliferation	*KRAS* *HRAS* *NRAS*
miR-218	Down-regulated	GCC GCT 40 GC	LNM Prognosis Advanced GC	Metastasis/Invasion Transcriptional activation	*COX2* *NFκB* *ROBO1*
miR-125	Down-regulated	70 GC 87 GC	TNM stages Invasion Metastasis Poor prognosis	Cell proliferation	*ERB2*
miR-143	Down-regulated	43 GC	ND	Chemoresistance	*ND*
miR-143*	Down-regulated	GCC	ND	Cell proliferation	*DNMT3A*
miR-145	Down-regulated	43 GC	ND	Chemoresistance	*ND*

Table 2: Examples of miRNAs altered in gastric cancer

CAG: Chronic atrophic gastritis; **Dys:** Dysplasia; **GC:** Gastric cancers; **GCC:** Gastric cancer cells; **GCT:** Gastric cancer tissues; **HC:** Healthy controls; **LNM:** Lymph node metastasis; **Pre:** pre-operative; **Post:** post-operative; **ND:** Non-determined

Adapted from *Tsai MM et al. International Journal of Molecular Sciences 2016* and *Ishiguro H. et al. World Journal of Gastroenterology 2014. * Zhang Q et al. 2017*

These results indicated that miRNAs and their genetic variations contribute to the molecular mechanisms of gastric carcinogenesis *(Espinosa-Parrilla Y et al. 2014)* but further analysis is necessary to determine miRNA regulation in GC and to characterize miRNAs relevant for clinical diagnosis and prognosis.

Histone Deacetylases

Histone function is modulated by multiple post-translational modifications. Next to methylation, there is reversible acetylation of the lysines on histones **(Figure 23).** Acetylation is controlled by the balance between histone acetyltransferases (HATs) and histone deacetylases (HDACs). Histone acetylation is associated with a chromatin de-condensation and transcriptional activation whereas histone deacetylation is associated with a condensation of chromatin and transcriptional repression.

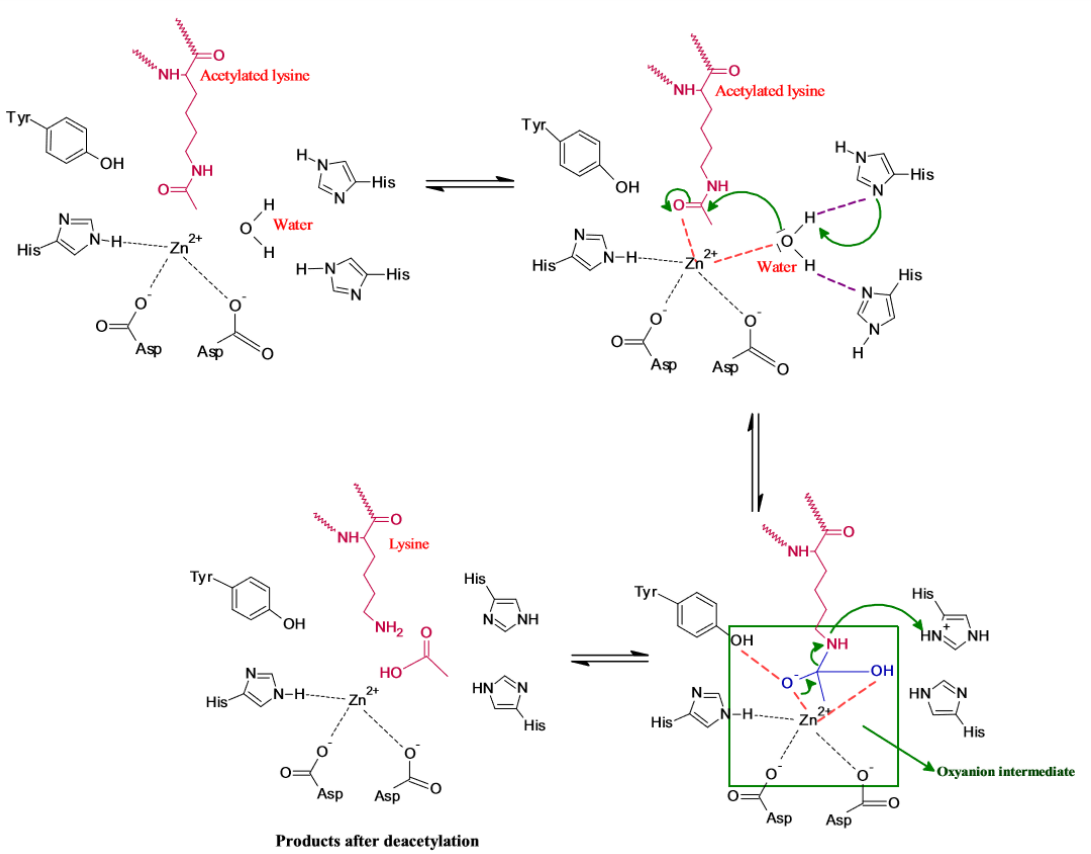


Figure 23: Mechanism for HDAC catalysis

Illustration from *Manal M et al. Bioorganic Chemistry 2016*

Class	Isoform	Yeast counterpart	Size	Co-factor	Location	Expression	Catalytic domain
(I)	HDAC1	RPD3	58	Zn^{++}	N	Ubiquitous	482 aa
	HDAC2		59		N		488 aa
	HDAC3		50		N, C		428 aa
	HDAC8		44		N		377 aa
(IIa)	HDAC4	HDA1	120	Zn^{++}	N, C	Specific	1084 aa
	HDAC5		130		N, C		1122 aa
	HDAC7		110		N, C		952 aa
	HDAC9		160		N, C		1011 aa
(IIb)	HDAC6	HDA2	160	Zn^{++}	N, C	Specific	1215 aa
	HDAC10		70		N, C		(Inactive) 669 aa
(III)	SIRT1	Sir2	120	NAD^{+}	N	Variable	NAD 747 aa
	SIRT2		45		C		NAD 389 aa
	SIRT3		28		M		NAD 399 aa
	SIRT4		35		M		NAD 314 aa
	SIRT5		36		M		NAD 310 aa
	SIRT6		39		N		NAD 355 aa
	SIRT7		48		N		NAD 400 aa
(IV)	HDAC11	RPD3/HDA1	39	Zn^{++}	N	Ubiquitous	347 aa

TRENDS in Microbiology

Figure 24: Histone deacetylase and sirtuin family

Location N, C and M are for nuclear, cytoplasmic and mitochondrial respectively. Illustration from *Shirakawa K et al. TRENDS in Microbiology 2013*

18 HDACs are described and are separated into four classes based on their sequence homology and cellular sub-location **(Figure 24).** Class I Rpd3-like group comprises HDAC 1, 2, 3 and 8. Class II Hda1-like enzymes is sub-divided into two sub-classes: Class IIa with HDAC 4, 5, 7 and 9 and Class IIb with HDAC 6 and 10. Class IV contains only HDAC11 which possesses similarities to Class I and II. Class III Sir2-like enzymes containing Sirtuins, which are NAD-dependent and/or ADP ribosylase proteins, in contrast to Class I and II, which activity depends on zinc *(Li Y and Seto E. 2017).* Beside their classically known target histones, they are also known to de-acetylate other proteins impacting thereby on their stability, their capacity to form protein interactions, their affinity to DNA and their transcriptional activity **(Table 3).** Consequently, HDACs impact on multiple biological process and their de-regulations in cancers promote tumor development, tumor progression, and metastasis formation **(Figure 25).**

Class I HDACs: Weichert and co-workers studied 2617 tissue microarray spots from 143 GC patients and 606 tissues slides from 150 GC showing that 52 GC out of 143 and 32 GC out of 150 patients expressed Class I HDACs (HDAC 1, 2 and 3), 60 GC out of 143 and 65 GC out of 150 expressed one or two of these Class I members, and 31 GC out of 143 and 53 GC out of 150 were negative for these three HDACs. Interestingly, the authors showed that patients highly expressing all Class I HDACs and HDAC2 have the poorest prognosis *(Weichert W et al. 2008; Weichert W. 2009).* This is supported by Wisnieski F and co-workers showing also HDAC2 upregulation in 50 GC tissues *(Wisnieski F et al. 2014).* In vitro HDAC2 knockdown restores p16 activity and reduces *Cyclin D1* expression inducing cell cycle arrest, and promoting autophagic process *(Kim JK et al. 2013),* thereby HDAC2 overexpression promote GCC proliferation explaining the poor outcome in GC patients.

In addition, 127 GC patients whose 69% high expressed HDAC1 and 85% high expressed HDAC2, but neither HDAC1 expression nor HDAC2 expression are associated with overall survival *(Mutze K et al. 2010)* contrary to the study of Weichert and co-workers, in which GC patients highly expressing all Class I HDACs and HDAC2 have the poorest prognosis *(Weichert W et al. 2008; Weichert W. 2009).* Likewise, Wisnieski F and co-workers studied 50 matched pairs of GC showing a significantly decrease of HDAC1 expression in tumoral tissues compare to adjacent tissue *(Wisnieski F et al. 2014)* contrarily once again to Weichert and co-workers' study. At the molecular level, Wisnieski F and co-workers associated the down-regulation of *HDAC1* with the decrease of *CDKN1A* expression promoting cell proliferation *(Wisnieski F et al. 2014).* However, CDKN1A is a target of HDAC1 and HDAC2 *(Yamaguchi T et al. 2010; Li S et al. 2017)* and *HDAC2* were upregulated in the 50 GC tissues *(Wisnieski F et al. 2014),* thereby HDAC2 may be involved

Proteins	HATs	HDACs	Acetylation effects	References
p53	p300/CBP, PCAF	HDAC1, SIRT1	Increase protein stability, DNA affinity and transcriptional activity	*Reed SM and Quelle DE. 2014*
YY1	p300/CBP	HDAC1, HDAC2, HDAC3	Decrease DNA affinity	*Yao YL et al. 2001*
STAT3	p300/CBP	HDAC3	Increase DNA affinity, transcriptional activity and protein interactions	*Zhuang S. 2013*
C-Myc	PCAF/GCN5, TIP60	SIRT1, SIRT2	Increase protein stability	*Patel JH et al. 2004; Romeo MM et al. 2015 ; Bosch-Presegué L and Vaquero A. 2011; Kulic A et al. 2014.*
GATA	CBP (GATA1), p300 and PCAF (GATA2)	HDAC3 (GATA2), HDAC4 (GATA6)	Increase DNA affinity and transcriptional activity	*Boyes J et al. 1998; Ozawa Y et al. 2001; Kim GR et al. 2016*
MyoD	PCAF, CBP and p300	HDAC1	Increase transcriptional activity	*Mal A et al. 2001; Duquet A et al. 2006*
E2F/Rb	PCAF, CBP and p300	HDAC1	Increase protein stability	*Martinez-Balbas MA et al. 2000; Markham D et al. 2006*
NFκB	P300/CBP	HDAC3, SIRT1	Promote protein-protein interactions	*Greene WC and Chen LF. 2004; Pejanovic N et al. 2012*
HIF1α	ARD1	HDAC4, SIRT1	Decrease transcriptional stability	*Geng H et al. 2011; Joo HY et al. 2015*
Smad7	P300	HDAC1	Increase protein stability	*Simonsson M at al. 2005*
α-Tubulin	αTAT1	HDAC6, SIRT2	Promote protein-protein interaction	*Li L and Yang XJ. 2015*
Importin-α	CBP, p300	-	Promote protein-protein interaction	*Bannister AJ et al. 2000*
Ku70	PCAF, CBP	SIRT1	Disrupts protein-protein interaction	*Cohen NY et al. 2004*
Hsp90	-	HDAC6	Disrupts protein-protein interaction	*Kovacs JJ et al. 2005*
β-catenin	PCAF, p300	SIRT1, HDAC6	Promote protein-protein interaction and transcriptional activity	*Lévy L et al. 2004*
Survivin	CBP	HDAC6	Promote protein-protein interaction	*Wang H et al. 2010*
FOXO1	CBP/p300	SIRT1	Decrease DNA affinity and transcriptional activity	*Matsuzaki H et al. 2005*
FOXO3a	CBP/p300	SIRT1, SIRT2 and SIRT3	Decrease DNA affinity and transcriptional activity	*Wang X et al. 2017*
PGC1-α	GCN	SIRT1	Decrease transcriptional activity	*Lerin C et al. 2006*

Table 3: Examples of non-histone targets of HATs and HDACs

Adapted from *Kim E et al. Current Topics in Medicinal Chemistry 2015*

in CDKN1A down-regulation. The size of the precedent studies may be criticized, indeed, a comparison of 28 studies shows that *HDAC1* expression is higher in GC compared to normal tissues, especially in tumor lymph-node metastasis and it is negatively associated with the overall patient's survival *(Cao LL et al. 2017).* Alongside, suppression of HDAC1 inhibits metastasis potential of gastric cancer cell lines *in vitro* by expressing miR-34a targeting CD-44 involved in cell invasion and proliferation *(Lin L et al. 2015).* Consequently, we can assume that *HDAC1* expression is instead increased in GC promoting GCC proliferation.

In addition, Xu G and co-workers reported *HDAC3* overexpression in 60 GC patients compare to normal tissues *(Xu G et al. 2018).* Interestingly, studies on GCC explained that *in vitro HDAC3* overexpression increases cell growth, and inversely HDAC3 knockdown reduces GCC viability *(Xu G et al. 2018).* Moreover, HDAC3 decreases the interaction of p53 with PUMA promoter reducing apoptosis of GCC *in vitro (Feng L et al. 2013),* consequently the programmed cell death 5 (PDCD5)-mediated HDAC3 dissociation from p53 results in HDAC3 proteasomal degradation and GCC apoptosis. Interestingly, PDCD5 expression is reduced in 88 GC patients out of 102 *(Yang YH et al. 2006)* promoting gastric tumorigenesis *in vitro* and *in vivo* and may explain the observed overexpression of HDAC3 in patients GC *(Choi HK et al. 2015; Xu G et al. 2018).*

At last, *HDAC8* is overexpressed in several GCC lines compare to immortalized normal gastric epithelial cell line GES-1, which corroborates with 47 GC patients out of 51 overexpressing *HDAC8.* Likewise, HDAC8 suppression *in vitro* inhibits gastric cancer cells proliferation and induces apoptosis *(Song S et al. 2015).*

Thereby Class I HDAC upregulations promote tumor progression and may represent promising therapeutic targets.

Class II HDACs: Low expression of HDAC10 was described in 179 GC paraffin samples (51.4% in tumoral tissues versus 87.3% in adjacent tissues) to be positively correlated with gender (male preponderance), tumor size (>5cm), histological grade (poorly differentiated cells), tumor invasion, lymph node metastasis status and tumor stage, but not with Lauren classification *(Jin Z et al. 2014).* Interestingly, Osada and co-workers supported this study in 72 lung cancer patients which also low expressed HDAC10 in tumoral tissue *(Osada H et al. 2004).* Moreover, in *vitro* HDAC10 inhibition induces thioredoxin-interacting protein expression and causes accumulation of ROS (Reactive oxygen species) in SNU-620 GCC altering ROS signaling *(Lee JH et al. 2010)* which may lead to constant expression of HIF1α under normoxia conditions and expression of its target genes such as VEGF *(Park JH et al.*

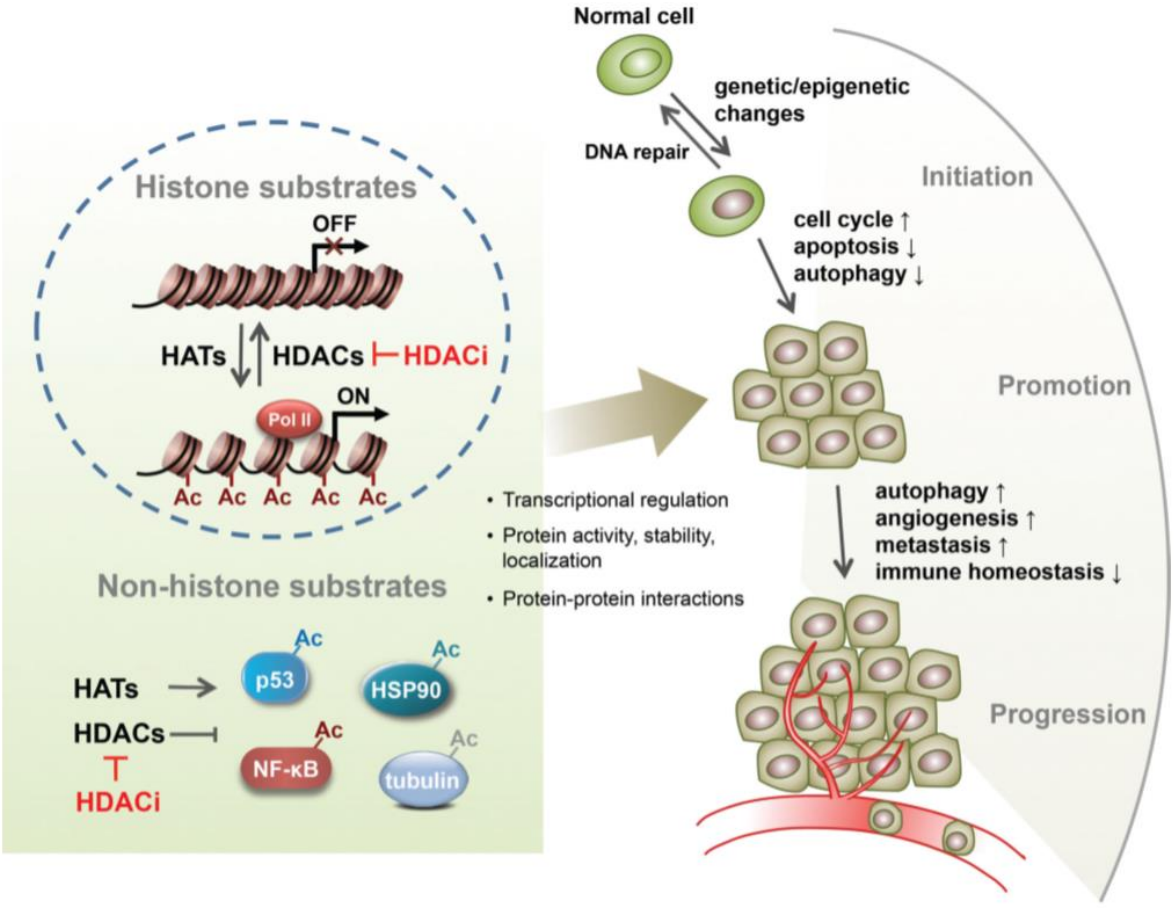


Figure 25: Different functions of HDACs and their therapeutic interest

HDACs and HDACIs regulate different stages of cancer through multiple mechanisms impacting different biological processes. Illustration from *Li Y and Seto E. Cold Spring Harbor Perspectives in Medicine 2017*

2003). Likewise, HDAC10 overexpression suppresses MMP 2 and 9 expressions (Matrix metalloproteinase), indicating that low expression of HDAC10 promotes cervical cancer metastasis *(Song C et al. 2013).* In addition, low HDAC10 expression promotes multi-model cancer cell proliferation via Let-7–HMGA2–Cyclin A2 pathway *(Li Y et al. 2015).* Thereby, *in vitro* studies suggest that low expression of HDAC10 may promote cancer cells proliferation and invasiveness which may explain the association between TNM stage and low HDAC10 expression in GC patients.

Moreover, Kang and co-workers and Colarossi L and co-workers, respectively, observed an overexpression of *HDAC4* in 29 and 10 GC tissues (compared to normal gastric tissues). They drew a parallel, *in vitro,* with the overexpression of *HDAC4* in GCC *(Kang ZH et al. 2014; Colarossi L et al. 2014).* Interestingly, it promotes SGC-7901 GCC progression via p21 repression whereas HDAC4 down-regulation inhibits cell growth, induces autophagy and apoptosis *(Kang ZH et al. 2014)* and HDAC4 inhibition, by a HDAC4 chemical inhibitor (MC1568), synergistically acts with docetaxel improving its cytotoxicity promoting SNU-16 GCC apoptosis *(Colarossi L et al. 2014).*

Moreover, Yu and co-workers showed that HDAC7 is down-regulating in 54 out of 86 GC tissues compared to non-tumoral tissue. However, they remarked that overexpression of HDAC7 correlates with distant metastasis, and poor prognosis. In addition, *in vitro* suppression of HDAC7 reduces GCC proliferation, migration and invasion *(Yu Y et al. 2017).* Thereby, HDAC7 overexpression seems to appear during gastric carcinogenesis favorizing GC migration and invasion.

He Q and co-workers studied 364 GC patients showing that HDAC6 expression is reduced in GC tissue compare to normal and premalignant gastric lesions and low level of HDAC6 is associated with advanced TNM stages. Likewise, the authors significantly correlated low HDAC6 expression with *Helicobacter pylori* infection in patients. Interestingly, He Q and co-workers showed that *H. pylori* infection decreases HDAC6 expression in mice and in gastric epithelial immortalized GES1 cells *(He Q et al. 2017).* Alongside, Jung KH and co-workers showed that HDAC6 overexpression reduces tumor cell growth and promotes autophagic cell death, *in vitro* and in mouse xenograft model *(Jung KH et al. 2012)* supposing a tumor suppressor role of HDAC6. Thereby, the reduction of HDAC6 expression in GC patients seems to be induced by *H. pylori* infection and may be involved in early step of gastric tumorigenesis.

Another study showed increase expression of HDAC9 in biopsies of 100 GC patients compare to adjacent tissues, but it does not seem to correlate with tumor stage or prognosis *(Wu T et al. 2016).* However, the authors remarked that low HDAC9 expression in adjacent para-carcinoma tissue is associated with worse survival after 100 months (34.1% VS 11.1%, P=0.002) indicating that HDAC9 has different biological functions in tumoral and in adjacent tissues. The expression of HDAC9 in gastric cancer tissues (GCT) seems to promote cell proliferation whereas the expression of HDAC9 in para-carcinoma tissues could prevent tumor metastasis development impacting on patient overall survival *(Wu T et al. 2016).* All these results indicate the need to better understand the functions of HDACs under normal condition and in the tumor microenvironment.

Interestingly, enzymatic activity of Class II HDACs is dependent of multiprotein complex containing a Class I HDAC; HDAC3 *(Fischle W et al. 2002)* highlighting once again the role of HDACs Class I in GC, and the interest to inhibit them.

Class III HDACs: The role of Sirtuins in GC is discussed. Indeed, SIRT1 and STAT3 co-overexpression is associated with poor survival outcome in 83 Asian patients with gastric cancer *(Zhang S et al. 2017)* and SIRT1 overexpression in 51 GC cases (Western-Blot) and in 557 GC cases (immunohistochemistry) is associated with poor prognostic and prognosis *(Noguchi A et al. 2014).* In another cohort of 50 GC, SIRT1 expression is associated with tumor grade *(Mohammadi-Saravle S et al. 2018).* But other studies on 221 GC patients with SIRT3 expression have a better prognosis than those without *(Huang KH et al. 2014)* and a study of 1065 GC associated high expression of SIRT1 with favorable overall survival and high expressions of SIRT2-4 and SIRT6-7 with poor overall survival *(Shen X et al. 2017).* Likewise, another study reported low SIRT1 expression in 112 GC tissues and that *in vitro* overexpression of SIRT1 inhibits GCC growth and proliferation *(Zhang Y et al. 2015).* Consequently, SIRT1 may act as a tumor suppressor or promoter in GC. A better understanding of the functions of HDACs in GC is once again highlighted.

The different Sirtuin members are reported to have dual role in cancers *(Bosch-Presegué L and Vaquero A. 2011; Kulic A et al. 2014).* Indeed, SIRT1 knock-out mice exhibit more chromosomal aberrations and impaired DNA repair compare to WT mice. In addition, transgenic mice overexpressing SIRT1 exhibit less DNA damage and are partially protected from cancers *(Bosch-Presegué L and Vaquero A. 2011),* suggesting anti-tumoral effect of SIRT1. However, more evidence of oncogenic SIRT1 activity were reported. For example, it is described that SIRT1 can deacetylate FOXO, promoting DNA repair gene expression and inhibition of apoptosis-related genes inducing thereby drug resistance in breast cancer cells.

Likewise, SIRT1 can deacetylate p53 and p73, causing inhibition of their functions such as apoptosis and SIRT1 can also deacetylate DNA repair proteins such as XPA and Ku70, which can be also deacetylated by SIRT3, promoting DNA repair *(Bosch-Presegué L and Vaquero A. 2011; Kulic A et al. 2014).* Likewise, dual role is reported for SIRT2. Indeed, SIRT2 may function as a tumor suppressor by maintaining microtubule mitotic integrity explaining its low expression in gliomas whereas SIRT2 is upregulated in leukemia and pancreatic cell lines for example and may inhibit p53 and p73 activity in response to DNA damage promoting drug resistance *(Bosch-Presegué L and Vaquero A. 2011; Kulic A et al. 2014)*. Thereby, anti- or pro-tumoral activity of Sirtuins in GC is an opened question. SIRT3 knockout mice exhibit spontaneous tumorigenesis in mammary glands suggesting tumor suppressor role of SIRT3 *(Kulic A et al. 2014).* In GCC (AGS, SGC-7901 and BGC-823), Wang and co-workers showed a lower expression of SIRT3 compared to normal immortalized GES-1 cells and that overexpression of SIRT3 inhibits cell proliferation through down-regulation of Notch-1 *(Wang L et al. 2015).* This corroborates an anti-tumoral effect of SIRT3, supporting the better prognosis observed by *Huang KH et al.* in 221 GC patients *(Huang KH et al. 2014).*

In addition, Zhang S and co-workers observed an overexpression of SIRT7 positively associated with tumor stage, lymph node involvement, metastasis and worse patient overall survival *(Zhang S et al. 2015)*, correlating with study of Shen X and co-workers with the same conclusion *(Shen X et al. 2017)*. Interestingly, Zhang S and co-workers observed that SIRT7 overexpression in GCC promotes cell growth and inhibits apoptosis by epigenetically inhibiting miR-34a and SIRT7 suppression reverses these effects *in vitro* and *in vivo (Zhang S et al. 2015),* explaining probably the outcome on patient's survival. Finally, we previously reported that high SIRT2-4 expressions are associated with a poor patient's overall survival out of 1065 GC *(Shen X et al. 2017).* However, another study on 86 pairs of GC tissues and adjacent normal tissue showed a low expression of SIRT4 negatively correlated with tumor size, pathological grade, and lymph node metastasis, predicting thereby a poor prognosis *(Sun H et al. 2018).* Likewise, SIRT4 is reported to have its expression increased promoting f.e. breast cancer proliferation, and inversely has its expression decreased f.e. in colorectal cancer promoting cancer suppression, highlighting once again a dual role in cancers *(Huang G and Zhu G. 2018).* In GCC (MKN-45 and HGC-27), SIRT4 expression is low and its overexpression inhibit cell proliferation, migration and invasion, describing SIRT4 acting as a tumor-suppressor in GC *(Sun H et al. 2018).* As certain Sirtuins may have dual role, further analyzes are necessary to understand the pro- or antitumoral activity of Sirtuins in GC.

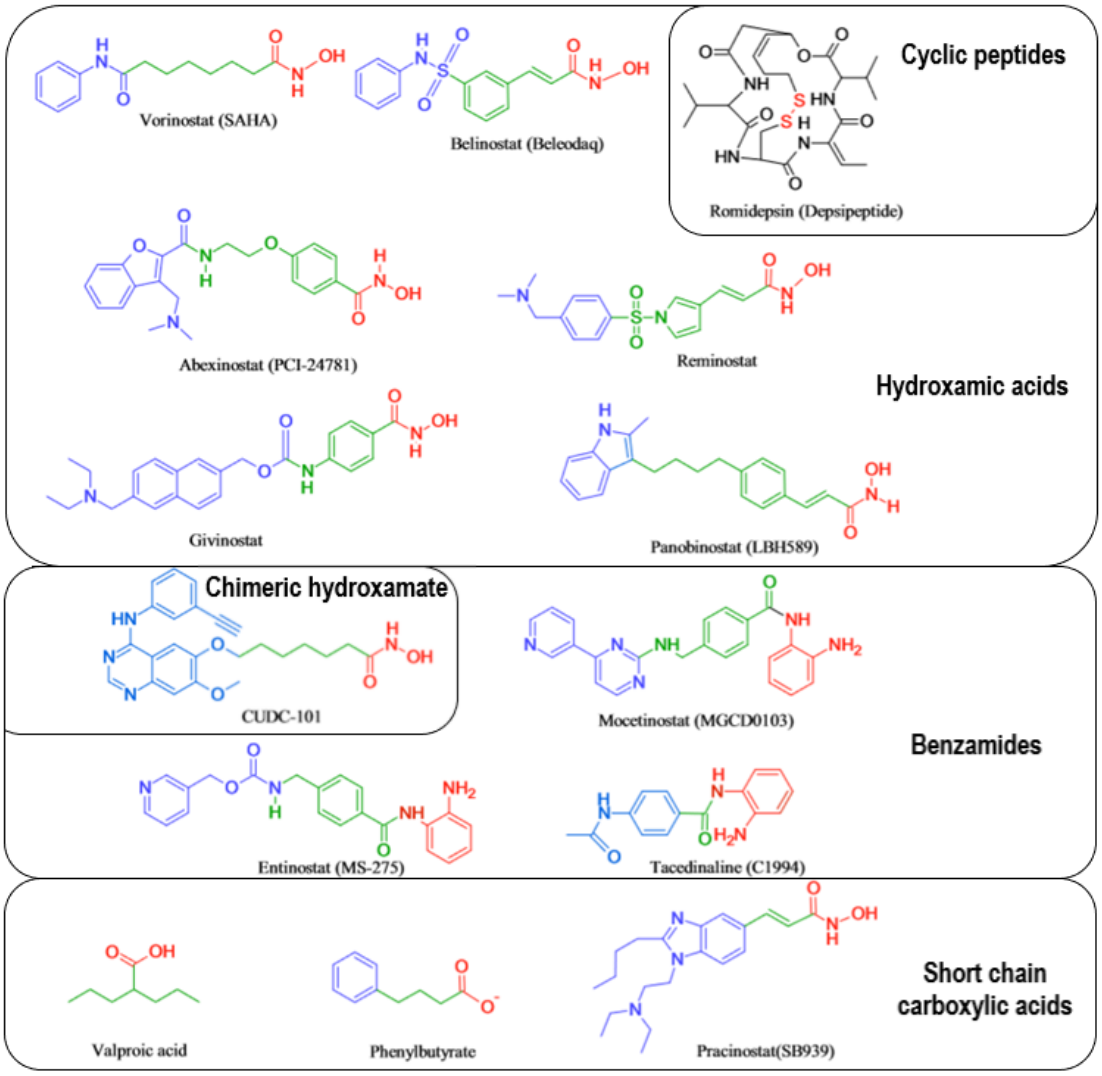


Figure 26: Class and structure of HDAC inhibitors

The cap, linker and the Zn^{2+} binding group are represented in blue, green and red, respectively.

Adapted from *Manal M et al. Bioorganic Chemistry 2016*

Altogether, alterations of HDACs play an important role in gastric carcinogenesis and inhibition of HDACs might be a promising therapy.

New areas for gastric cancer therapy

In France, the curative treatment of GC rests on surgical excision combined with radiotherapy or chemotherapy with Epirubicin, 5-Fluorouracile (5-FU), Cisplatin and/or Oxaliplatin. The choice of the chemotherapy depends on the age, general condition and HER status of the tumor. Indeed, HER2 is currently the only therapeutic marker in GC targeted by monoclonal antibody therapy (Trastuzumab) *(CDU-HGE 2015; Michel P et al. 2017)*. Unfortunately, HER2 positive GC represent about 17.9% of all GC *(Abrahao-Machado LF and Scapulatempo-Neto C. 2016)* and GCC are frequently resistant or develop resistance to current treatment. Thereby, the 5-year overall patient survival of GC is still low, around 15%.

Thereby, we need to better understand the mechanisms of GC aggressiveness and resistance to therapy with the aim to determine relevant therapeutic or prognostic markers and to propose a new protocol of treatments for GC patients by identifying the patients who will answer best (possessing these markers).

Based on molecular studies, multiple pathways are involved in gastric tumorigenesis, thereby multiple protein can be considered as promising therapeutic targets. For example, recently, an Asiatic phase III study tested the Nivolumab (anti-PD-1) showing a significant difference in favor of the anti-PD-1 with median overall survival of 5,26 months in the nivolumab group (330 GC patients) and 4,14 months in the placebo group (130 patients) *(Kang YK et al. 2017).*

Histone deacetylase inhibitors

Histone acetylation is an important mechanism controlling the transcription of approximately 2-10% of genes *(Mariadason JM et al. 2000).* Acetylation on histone and non-histone protein, such as p53 or HIF1α, impact several signaling pathway involved in tumorigenesis; cell cycle, apoptosis, DNA repair damage, angiogenesis, metastasis and autophagy. In addition, multiple HDACs are overexpressed in several cancers including GC promoting tumor progressions. For this reason, HDAC inhibitors (HDACIs) are developed to counteract the pro-oncogenic activity of HDACs *(West AC and Johnstone RW. 2014; Li Y and Seto E. 2017).* HDACIs are pharmacologic inhibitors regroup in six classes of chemical compounds **(Figure 26);** hydroxamic acids, short chain fatty aliphatic acids, benzamides,

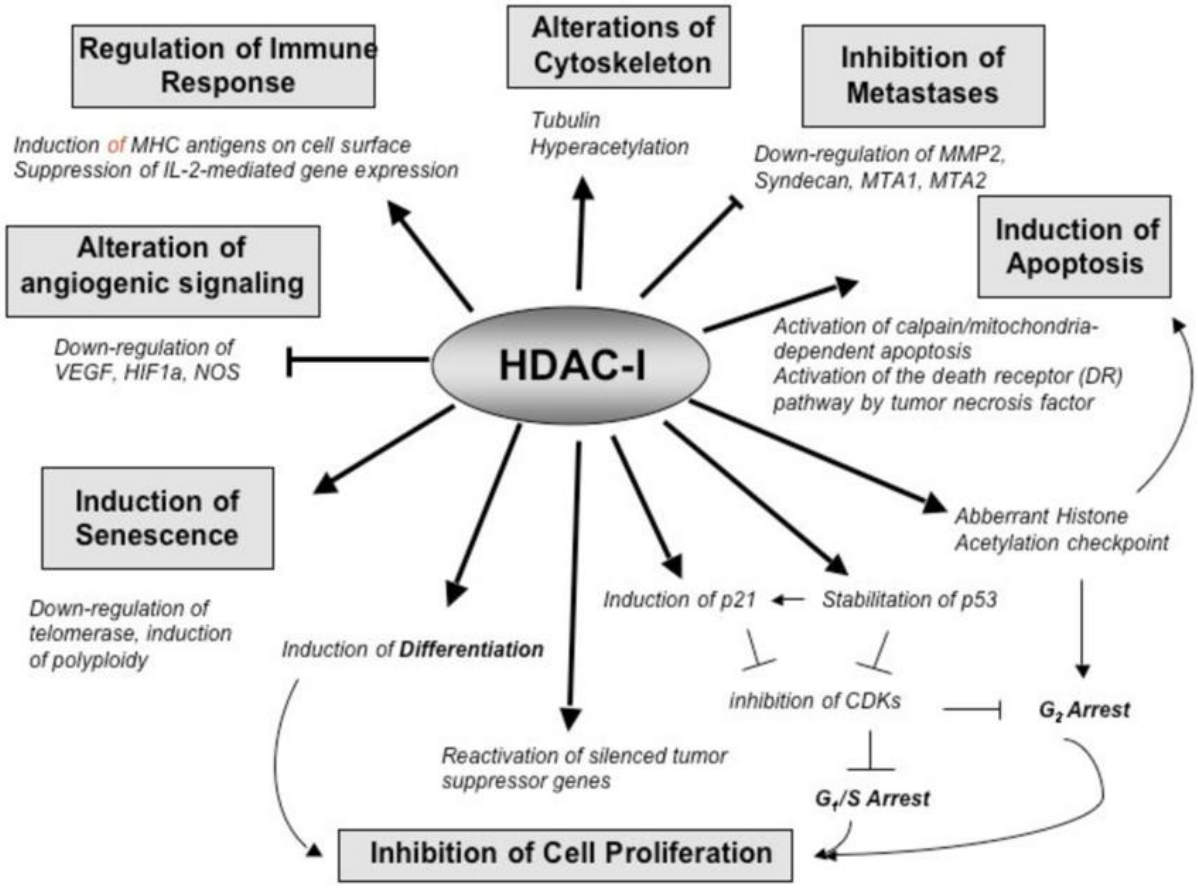


Figure 27: Effects of HDAC inhibitors in cancer cells. Illustration from *Budillon A et al. Recent patents on anti-cancer drug discovery 2007*

Class	HDAC Inhibitor	Target HDAC Class	Clinical Status
hydroxamic acids	Trichostatin A	pan	preclinical
	SAHA	pan	approved for cutaneous T-cell lymphoma
	Belinostat	pan	approved for peripheral T-cell lymphoma
	Panabiostat	pan	approved for multiple myeloma
	Givinostat	pan	phase II clinical trials—relapsed leukemia and multiple myeloma
	Resminostat	pan	phase I and II clinical trials—hepatocellular carcinoma
	Abexinostat	pan	phase II clinical trial—B-cell lymphoma
	Quisinostat	pan	phase I clinical trial—multiple myeloma
	Rocilinostat	II	phase I clinical trial—multiple myeloma
	Practinostat	I, II and IV	phase II clinical trial—prostate cancer
	CHR-3996	I	phase I clinical trial—advanced/metastatic solid tumors refractory to standard therapy
short chain fatty acids	Valproic acid	I, IIa	approved for epilepsia, bipolar disorders and migraine, phase II clinical trials—several studies
	Butyric acid	I, II	phase II clinical trials—several studies
	Phenylbutyric acid	I, II	phase I clinical trials—several studies
benzamides	Entinostat	I	phase II clinical trials—breast cancer, Hodgkin´s lymphoma, non-small cell lung cancer, phase III clinical trial—hormone receptor positive breast cancer
	Tacedinaline	I	phase III clinical trial—non-small cell lung cancer and pancreatic cancer
	4SC202	I	phase I clinical trial—advanced hematological malignancies
	Mocetinostat	I, IV	phase II clinical trials—Hodgkin´s lymphoma
cyclic tetrapeptides	Romidepsin	I	approved for cutaneous T-cell lymphoma
sirtuins inhibitors	Nicotinamide	all class III	phase III clinical trial—laryngeal cancer
	Sirtinol	SIRT 1 and 2	Preclinical
	Cambinol	SIRT 1 and 2	Preclinical
	EX-527	SIRT 1 and 2	cancer preclinical, phase I and II clinical trials—Huntington disease, glaucoma

Table 4: Overview of HDAC and Sirtuins inhibitors. Table from *Eckschlager T et al. International Journal of Molecular Sciences 2017*

chimeric hydroxamate, cyclic tetrapeptides and Sirtuin inhibitors *(Mottamal M et al. 2015).* Generally, HDACIs induce an overall increase of histone and non-histone protein acetylations and induce a chromatin de-condensation. As HDACs are involved in several cellular pathways, HDACIs can promote **(Figure 27)** cell cycle arrest, growth inhibition, differentiation, invasion inhibition, autophagy, increase radio- and drug-sensitivity and stimulate immune response, which are described in various cancer cell lines *(Mottamal M et al. 2015; Li Y and Seto E. 2017; Eckschlager T et al. 2017).*

For example, TSA (Trichostatin A) induces apoptosis of GCC line BGC-823 *(Zou XM et al. 2008)* and of SGC-7901 cell line by a caspase-independent pathway *(Wu Z et al. 2007).* In GC cell lines, TSA down-regulates histone modifier genes *HDAC1, HDAC2, HDAC3, GCN5,* and *PCAF* expressions, reduces *MYC* and promotes *CDKN1A* expressions inhibiting cell growth. HDAC inhibition down-regulating *HDAC* and *HAT* expressions, suggesting that HDACs and HATs control their own and each other's gene expression *(Wisnieski F et al. 2014).*

Suberoylanilide Hydroxamic Acid (SAHA or Vorinostat) is a hydroxamic acid-based pan-HDAC inhibitor approved by the FDA in 2006 for treatment of advanced primary cutaneous T-cell lymphoma in which it reduces cell proliferation and induces cell apoptosis *(Zhang C et al. 2005).* However, it so far does not have the FDA approval for the treatment of other cancers but findings in different *in vitro* and *in vivo* models for other cancers suggests that it also might be beneficial for the treatment of glioblastomas, lung, colorectal and gastric cancers. For example, SAHA induces a radio-sensitization in glioblastoma cell lines and in xenograft models of colorectal cancers. It possesses anti-proliferative and pro-apoptotic effects in brain metastasis, melanoma, advanced solid tumors, lung and colorectal cancers *(Mottamal M et al. 2015; Li Y and Seto E. 2017).* SAHA upregulates *RUNX3* tumor suppressor expression, inhibiting GCC growth *(Huang C et al. 2007).* SAHA acts synergistically with MG132 *in vitro* and *in vivo* cytotoxicity, suppresses proliferation and invasion, and induces apoptosis of GCC but has hepatic and peripheral blood cells toxicity *(Lu H et al. 2016).*

In clinical trials **(Table 4),** Vorinostat in combination with Capecitabine plus Cisplatin as first-line chemotherapy for patients with metastatic or unresectable GC showed that the median follow-up of 14.1 months, and the median progression-free survival and overall survival were 7.1 months and 18.0 months, respectively, in phase I study *(Yoo C et al. 2014).* However, in phase II, Vorinostat does not enhance treatment efficiency *(Yoo C et al. 2016).* Nevertheless, a phase I study showed that Vorinostat can be combined with radiotherapy for gastrointestinal carcinoma *(Ree AH et al. 2010).*

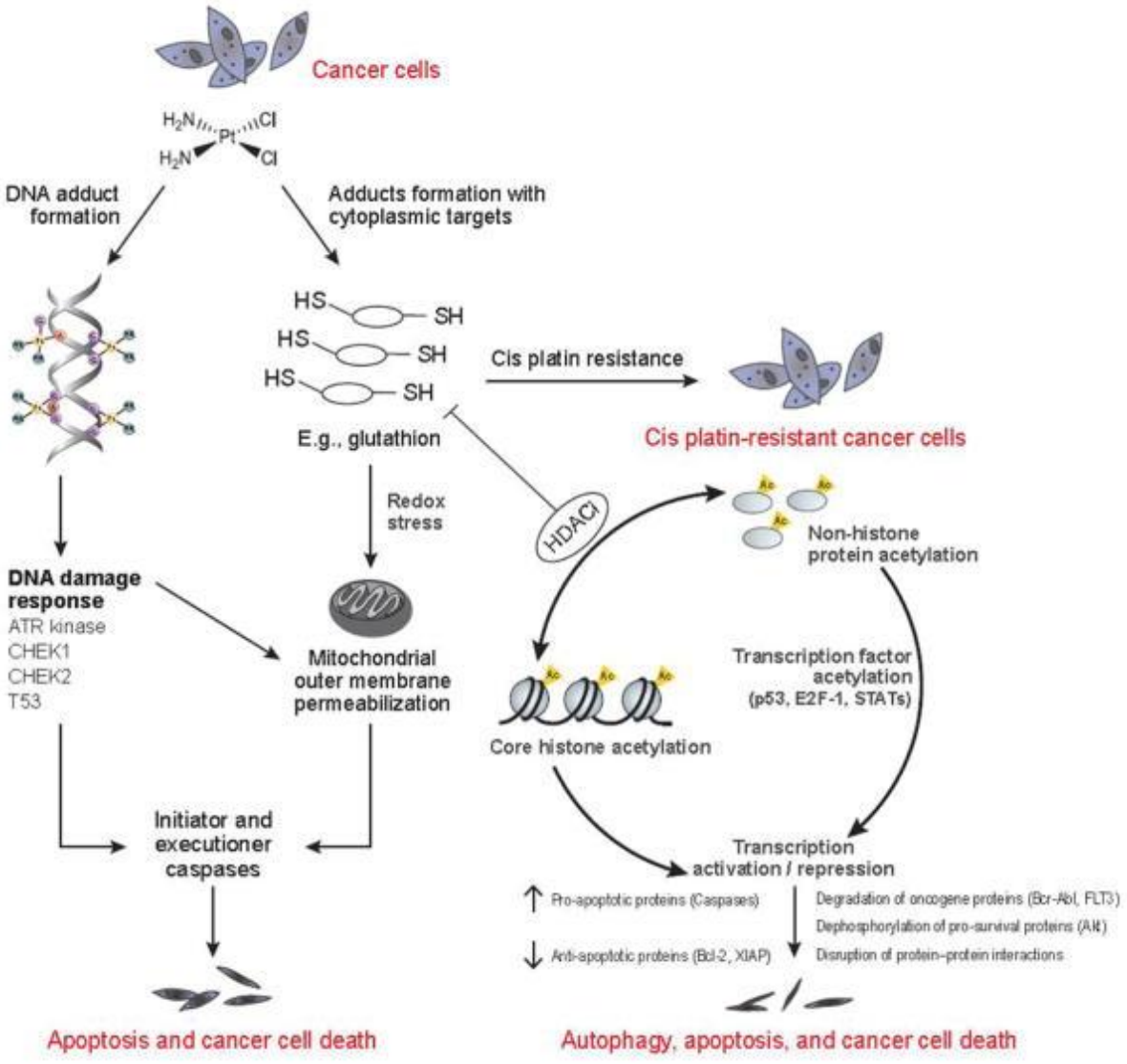


Figure 28: Anticancer activities of histone deacetylase inhibitors and cisplatin combined therapy. Illustration from *Diyabalanage HVK et al. Cancer Letters 2013*

In another phase II study, VPA (Valproic acid), another HDACI, was added or not to Paclitaxel treatment for advanced GC but there was no statistically significant difference between Paclitaxel alone treatment or with VPA *(Fushida S et al. 2016).*

In clinical trials, HDACIs may induce side effects like anemia, fatigue, diarrhea, neutropenia, thrombocytopenia, and deaths *(Mottamal M et al. 2015).* All these studies point the necessity to determine the role of HDACs in GC and to minimize the toxic effect of HDACIs in patients *(Mottamal M et al. 2015; Yoo C et al. 2016).* The toxicity could be explained by the important role of HDACs in several biological process and by the low selectivity of HDAC inhibitors so far used in clinics. To solve this problem two strategies are developed.

First, decrease HDACI-used doses by combining HDACIs with other therapies such as cytotoxic drugs, immunotherapy, and radiotherapy *(Falkenberg KJ and Johnstone RW. 2014; Li Z et Zhu WG. 2014; Abdelfatah E et al. 2016).* As we descript above, GC frequently develop resistance to their current standard treatments Cisplatin and Oxaliplatin. The use of HDAC inhibitors have been proposed to sensitize GCC and to synergistically improve the cytotoxicity of Cisplatin and Oxaliplatin at low doses **(Figure 28)** *(Diyabalanage HVK et al. 2013; To KKW et al. 2017).* Indeed, SAHA enhances the antitumor activity of oxaliplatin in GCC SGC-7901 and MKN-28 *(Zhou C et al. 2014)* and *in vitro* combination between SAHA and Cisplatin shows synergistic effects irrespective of the initial Cisplatin sensitivity *(Mutze K et al. 2010).* Combinatory treatments PDC and SAHA may represent an alternative treatment for GC. Nonetheless, we cited before the phase II study about combinatory treatments Vorinostat with Capecitabine plus Cisplatin as first-line chemotherapy for patients with GC *(Yoo C et al. 2016)*. In this study, the authors concluded that the combination with Vorinostat possesses higher toxicities than standard treatment protocol. They considered that further clinical trials on the current study combination do not seem to be warranted for GC patients *(Yoo C et al. 2016)*. However, they made the study in patients with metastatic or unresectable GC, and they highlighted the possibility to use another chemotherapeutic drug *(Yoo C et al. 2016)* like Oxaliplatin. Thus, study PDC + SAHA combinations in GCC will allow us to characterize the molecular pathways and the markers involved in the activity and/or in the resistance of the treatments. Consequently, we could propose combinatory treatments to the patients with the better response.

Second, identify more precisely HDAC functions and develop more specific HDACIs to improve treatment efficiencies. However, HDACI design and synthesis represent a real challenge *(Li Z et Zhu WG. 2014; Mottamal M et al. 2015).* For example, Negmeldin AT and co-workers proposed a SAHA analogues modified at the C2 position which displays HDAC6/8 selectivity (0.6-2μM versus 180μM for HDAC1/2) *(Negmeldin AT et al. 2017).* A chemical complex PDC-Belinostat (HDACI) possesses considerable cytotoxicity against ovarian cancer cells and exhibits favorable cyto-selectivity relative to cisplatin and Belinostat *(Parker JP et al. 2013).* And Dong J and co-workers designed and synthesized new compound specifically targeting HDAC6 which induces cell cycle arrest and apoptosis in GCC lines *(Dong J et al. 2018).*

Importantly, if we want to develop specific and more efficient HDAC inhibitors, we need to know more about the role of genetic and epigenetic alterations in gastric tumorigenesis and in treatment resistance. This work will lead to establish new prevention, prognostic and therapeutic marker or targets, to improve the diagnosis and treatment efficiencies. Futures, the aim is to improve the patient care and the quality of life of patients.

Objectives

Studies agree, a better understanding of the molecular pathways and the genetic alterations involved in gastric cancer tumorigenesis may lead to new diagnostic, new therapeutic and new preventive approaches to the disease and an increase in patient quality of life. Histone deacetylases are known to promote tumorigenesis *(Li Y and Seto E. 2014).* In this area, the aim of my thesis project is to study the histone deacetylases as therapeutic targets in gastric cancer.

The laboratory recently showed that Cisplatin impacts gene expression involved in epigenetic regulation on U87 glioblastoma cells *(Licona C et al. 2017).* One of them, HDAC4 (HDAC Class II) has its expression decreased after 24h of Cisplatin treatment. Interestingly, we obtained the same effect in AGS gastric cancer cells and we determined that HDAC4 level impacts AGS sensitivity to Cisplatin *(Spaety ME et al. In submission)*

In a first objective, I determined HDAC4 functions and its therapeutic interest in gastric cancer. More precisely, I continued the laboratory project by focusing on the regulation of HDAC4 expression in response to Cisplatin. Then, by loss of function experiments, I validated candidate gene, which expression was correlated with HDAC4 in the TCGA database. And finally, I inhibited HDAC4 using pharmacologic inhibitor (HDACI) to promote Cisplatin cytotoxic effect.

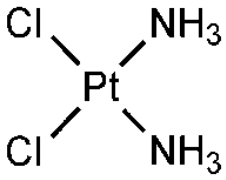
HDACs impact chemosensitivity and HDAC inhibitors are proposed to synergistically improve PDC cytotoxicity *(Diyabalanage HVK et al. 2013; To KKW et al. 2017).* In a second objective, I tested a combinatory treatment between platinum derivative compounds (PDC) usually used in clinic (Cisplatin, Oxaliplatin) and a pan-HDAC inhibitor approved by the FDA (SAHA or Vorinostat) to increase their cytotoxic effects. I characterized the mechanisms involved in the synergistic treatment response.

Finally, I studied HDAC expression in gastric cancer models to characterize HDACs required to cancer progression and chemoresistance. To provide an answer to this problem, I tested new designed HDACIs.

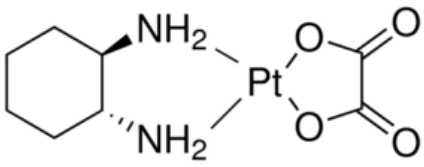
MATERIALS & METHODS

“You know my methods. Apply them”

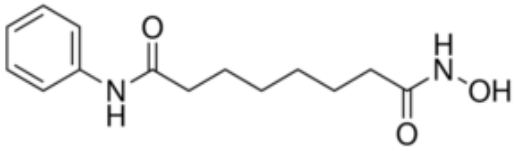
Sir Arthur Conan Doyle, *The Sign of Four*



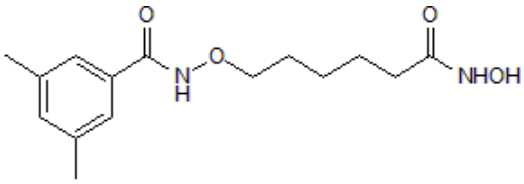
Cisplatin



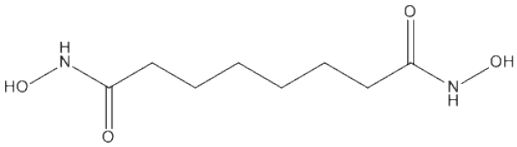
Oxaliplatin



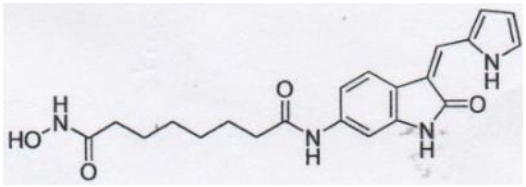
SAHA



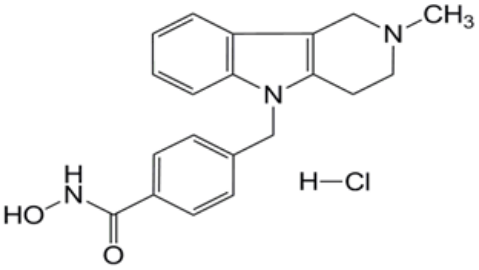
LMK-235



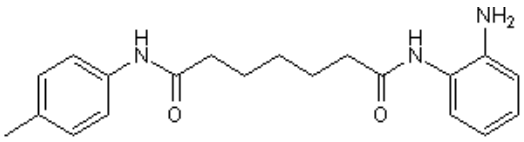
SBHA



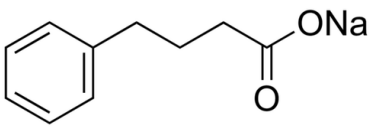
JGS-038



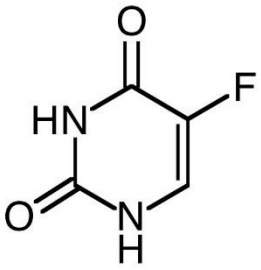
Tubastatin A



TC-H 106



Sodium 4 Phenylbutyrate



5-Fluorouracil

Figure 29: Chemical structure of platinum derivative compounds, 5-Fluorouracil and HDAC inhibitors

N.B.: In the result part, will be underlined the figures showing my participation to STREINTH Lab projects.

Survival analysis

Overall survival comparing patients with wild type and altered *HDAC4* was estimated using Kaplan-Meier curves. Survival time was defined as the interval between the date of treatment and the date of the date of death for any reason (uncensored), or the last date when the patient was known to be alive (censored). A log rank test was used to evaluate the differences between subgroups and was considered as statistically significant when $p<0.05$ in two-sided tests.

Cell culture

AGS cells (ATCC CRL-1739™), KATOIII cells (ATCC HTB-103™), NUGC3 cells (JCRB Cell Bank, JCRB0822), and MKN45 cells (JCRB Cell Bank, JCRB0254) are grown in RPMI (Roswell Park Memorial Institute medium; Dominique Dutscher) with 10% fetal bovine serum (FBS; Gibco, Life technologies) and 1% penicillin/streptomycin (P/S; PAN-Biotech) at 37°C in a humidified atmosphere and 5% CO_2. Mycoplasma contamination has been tested negatively using PlasmoTest (Invivo gene).

Cellular treatments

We used 5-Fluorouracil (Sigma) and platinum derivative compounds (PDC) usually prescribed in clinic: Cisplatin (Accord Health Care) and Oxaliplatin (Hospira). We tested different Histone Deacetylase Inhibitors (HDACIs) solubilized at 20mM in DMSO (VWR Chemicals): pan-HDAC inhibitors; SAHA (Vorinostat; Tocris), SBHA (Tocris) and Sodium 4 Phenylbutyrate (Tocris), a HDAC Class I more specific inhibitor; TC-H 106 (Tocris), a HDAC4/HDAC5 more specific inhibitor; LMK-235 (Tocris) *(Marek L et al. 2013),* a HDAC6 more specific inhibitor; Tubastatin A (Tocris) and a possible HDAC3 inhibitor new synthetized; JGS-038 (Dr. John Spencer, Sussex University, UK) **(Figure 29).**

MTT survival test

10.000 cells (AGS, MKN45) or 20.000 cells (KATOIII, NUGC3) were seeded per wells in 96-well plates (Falcon Multiwell) 24h prior to any treatment. Drugs (PDC, HDACI) are diluted in RPMI+FBS+P/S and 100µL/well are deposited on cells for 48h. MTT survival test is realized by replacing the culture medium with 100µL/well of new culture medium completed with 5mg/mL of MTT product (Sigma Aldrich) for 1h30 at 37°C. Then, cells are lysed, and the colored formazan product is solubilized in 100µL/well of DMSO. Coloration is an indicator of cellular viability. Optic density (DO) is measured at 590nm with Tristar² Mutlimode Reader® (Berthold Technologies). Experiments were done with four technical replicates and realized at least in three independent times. Cellular viability is evaluated by taking the untreated cells (NT) as control. IcX represents the concentration where we observed a reduction of cellular viability of X%. It is determined graphically with Prism Graphpad 5 software; curves are generated as log(inhibitor) vs. response -- Variable slope (four parameters).

Isobologram assay

10.000 cells (AGS, MKN45) or 20.000 cells (KATOIII, NUGC3) were seeded per wells in 96-well plates (Falcon Multiwell) 24h prior to any treatment. Combined treatments (PDC + HDACI), or each concentration of drug alone are diluted in RPMI+FBS+P/S and 100µL/well are deposited on cells for 48h. MTT survival tests are realized as described before. Cellular viability and treatment efficiencies are evaluated by taking the untreated cells (NT) as control (100% of cellular viability; 0% of treatment efficiency). Combined treatment efficiencies are compared to drug alone efficiencies with Compusyn program software (ComboSyn, Inc) which determines mathematically the combination indexes *(Chou TC. 2006 and 2010).* We arbitrarily and conservatively considered antagonist effect on cell survival between the drugs when combination indexes correspond to value superior to 1.20, additive effect between 0.80 and 1.20 and synergistic effect when combination indexes are inferior to 0.80. For each drug or combined treatment, experiments were done in height technical replicates and at least realized in three independent times.

Transfection

Silencing RNA

250.000 cells were seeded per well in 6-well plates (Falcon, Multiwell) 24h prior any treatment in an antibiotic free RPMI+FBS medium. Transfections are performed with the Lipofectamine® RNAiMAX Reagent. SiRNA and 4µL of Lipofectamine® RNAiMAX Reagent are diluted separately in Opti-MEM® Medium (respectively 150µL/product for 1 well). Diluted siRNA is added to the diluted Lipofectamine® RNAiMAX Reagent and the mix is incubated 5min at room temperature. The 300µL of the mix (siRNA-lipid complex) are added to cells (drip throughout the well) with a total volume of 2mL/well (1700µL of fresh RPMI+FBS medium + 300µL siRNA-lipid complex) for 7h at 37°C. Then, the culture medium is replaced with fresh medium RPMI + FBS. After 48h, cells are treated with the different described experimental conditions by replacing the culture medium (always no antibiotics). The different siRNA used are: **sip53** (30nM, Eurogentech) 5'-GGA AAC UAC UUC CUG AAA A-3'; **Control siRNA duplex pGL3 luciferase** (30nM, Eurgoentech, SR-CL011-005); **siHDAC4** (HSS114673, Stealth siRNA, Thermo Fisher Scientific); **Control siRNA Stealth** β-lactamase reporter control RNAi (Stealth siRNA, Thermo Fisher Scientific); **mimic miR-140** (100nM, Sigma) 5'-CAG UGG UUU UAC CCU AUG GUA G-3'; **antimiR-140** (30nM, Sigma) 5'-CUA CC AUA GGG UAA AAC CAC UG-3'

Plasmid expression vectors

250.000 cells were seeded per well in 6-well plates (Falcon, Multiwell) 24h prior any treatment. Expression vectors for p53, TAp73 and HDAC4 (Addgene) were transfected by polyethylenimmine (PEI)-based or JetPrim® (Polyplus) as described by the provider and detailed in *(Benosman S et al. 2011).*

Microarrays analysis

ECL files from microarray experiments (GEO accession number: GSE66493) were first analyzed individually using AltAnalysis software. Deregulated genes were identified based on 2-fold change expression and t test p-value < 0.05. Deregulated genes were then analyzed by GO-Elite with Prune Ontology term using Z score (cutoff 1.96, p-value 0.05) and Fisher Exact Test for ORA (2000 permutation) for over-representation in selected biological processes in several resources: Gene Ontology, MPhenoOntology, Disease Ontology,

GOSlim, PathwayCommons, KEGG, Transcription Factor Targets, miRNA Targets, Domains, BioMarkers, RVista Transcription Sites, DrugBank, BioGrid.

Quantitative PCR

500.000 cells were seeded per well in 6-well plates (Falcon, Multiwell) 24h prior any treatment. Then cells were treated with the described drugs at indicated concentrations and times by replacing the culture medium. Total RNA was extracted with TRIzol® Reagent.

For mRNA relative quantification:

RNA reverse transcription is performed with the High Capacity cDNA Reverse Transcription Kit® (Applied Biosystems) in a total reaction mix of 20µL as described by the provider during 2h at 37°C. qPCR are performed with the FastStart Universal SYBR Green PCR Master Mix® or the FastStart Universal Probe Master Mix TaqMan® (Roche) in 20µL total volume per reaction containing 4µL of diluted cDNA, 10 µL of FastStart Reagent and 500nM of primers. qPCR are carried out in 7500 Real Time PCR System® (Applied Biosystems). Relative expressions are normalized with TBP according to the method $2^{(-\Delta\Delta Ct)}$ *(Livak KJ and Schmittgen TD. 2001)*.

For miRNA relative quantification:

RNA reverse transcription is performed with the RT miScript mix Hi Spec reagent (Qiagen) during 1h at 37°C. qPCR are performed as described by the provider in 25µL total reaction mix miScript SYBR Green PCR Kit (Qiagen) containing 2.5µL of cDNA diluted and 500nM of primers. qPCR are carried out in LightCycler® 480 (Roche) or in 7500 Real Time PCR System® (Applied Biosystems) according to the provider's advices. Relative expressions are normalized with RNU6 or an arbitrary Ct according to the method $2^{(-\Delta\Delta Ct)}$.

Human tissue analysis:

Healthy tissue samples, gastric tumor biopsies and distant normal gastric tissues (n= 26), and gastric cancer-derived cell lines were obtained from the Digestive Surgery department of Hautepierre Hospital (Strasbourg, France, authorization number: NCT02491840) or the National Cancer Research Center (Tokyo, Japan). All samples were obtained with informed consent according to the Declaration of Helsinki and approved by the Human Ethics Committee of the Strasbourg University Hospital (CHU), France. Written informed consent was obtained from all participants.

List of primers:

FastStart Universal SYBR Green PCR Master Mix (Roche)		
Human primer	**Right (reverse) sequence 5' to 3'**	**Left (forward) sequence 5' to 3'**
HDAC1	TGG TCC AAA GTA TTC AAA GTA GTC A	CGG TGC TGG ACA TAT GAG AC
HDAC2	CCT TTT CCA GCA CCA ATA TCC	CAG ATC GTG TAA TGA CGG TAT CA
HDAC3	GAA CTC ATT GGG TGC CTC TG	GAC CTA TGA CAG GAC TGA TGA GG
HDAC4	GAC CAC AGC AAA GCC ATT C	GTG GTA GAG CTG GTC TTC AAG G
HDAC5	CAA GGC AGC ACA GCA TAC AT	CTG AAT ACC ACA CCC TGC TCT
HDAC6	CAC AGC AGC ACC ATT CAGA	TAT CTG CCC CAG TAC CTT CG
HDAC7	CCA GAG GAA GCA GCA CAG T	CTC CAG CAG CAC CCT CAG
HDAC8	TTT CCG TCG CAA TCG TAA TA	GCA CTG CAT AAG CAG ATG AGA
HDAC9	GGC CCA TTG TTT GGT GAA	TCC CCC TTC GAA AAA CTG A
GLUT-1	CAG ATA GGA CAT CCA GGG TAG C	GGT TGT GCC ATA CTC ATG ACC
VEGF	CCA CTT CGT GAT GAT TCT GC	CCT TGC TGC TCT ACC TCC AC
AQP3	AGG AGT GGG GAC ACG ATG	GGG GCT CTG CAG TCT TCA C
p53	GTG TGG AAT CAA CCC ACA GCT	CAG CCA AGT CTG TGA CTT GCA
TAp63	CTG TGT TGT AGG GGC TGG TGG AC	TGT ATC CGC ATG CAG GAC T
TAp73	GCA GAT TGA ACT GGG CCA TG	GCA CCA CGT TTG AGC ACC TC
CYCLIN B2	TGT GGG TTT ATG GAC TGC AA	GAA GAT TGG GAG AAC CCT CA
CDC2 (CDK1)	CAA TCC CCT GTA GGA TTT GG	TGG ATC TGA AGA AAT ACT TGG ATT CTA
NRF2 (NFE2L2)	TTT GGG AAT GTG GGC AAC	GAG ACA GGT GAA TTT CTC CCA AT
ASB1	CAG ATG AAG CCG GTG TTT G	TGA GGC CTT GCA GGT CTT T
PALD1	CCG CAC ACA GAA GAT GAC AC	CCA GCC TCT CAG GGT TCA

VAMP8	TCA TTT CCT CCA CCT TCA CTG	CTA GGC GAA TTC ACT TAC TGA CC
STXBP2	CCT TGA TCT TCT CCC CCT CT	CGG TGG AGA AGC TGT GTA GTG
BID	GGA ACC GTT GTT GAC CTC AC	GCA CAG TGC GGA TTC TGT C
CASP 6	CAC AGT TTC CCG GTG AGA ATA	GAT GCA GCC TCC GTT TAC A
BAK1	CGG AAA ACC TCC TCT GTG TC	AGA CCT GAA AAA TGG CTT CG
CASP 3	TCC AAA AAT TAT TCC TTC TTC ACC	TGG AAT TGA TGC GTG ATG TT
AIFM1	CGC CTC CTT CCC AAC TTT AT	GCT ACA AGC ACG CTC TAA CAT C
ENDOG	ATT TCC CAT CAG CCT CTG TC	CGC AGC TAC CAA AAC GTC TA
CYCS	CCT CCC TTT TCA ACG GTG T	TGT GCC AGC GAC TAA AAA GA
CASP 8	TTT CTG CTG AAG TCC ATC TTT TT	GGT CACT TGAA CCT TGG GAA T
DIABLO	GCG GTT ATA GAG GCC TGA TCT	TGA CTG CAG TTG GTC TTT CAG
BIK	GGC TCA CGT CCA TCT CGT	CCC TAT GGA GGA CTT CGA TTC
BCL2L1	AGC GGT TGA AGC GTT CCT	AGC CTT GGA TCC AGG AGA A
MEF2A	TGG AAC TGT GAC AGA CAT TGA A	TGA TGC GGA ATC ATA AAA TCG
TBP	CGC TGG AAC TCG TCT CAC TA	GCC CAT AGT GAT CTT TGC AGT
miScript SYBR Green PCR Kit (Qiagen)		
miR-29b-1-3p	TAG CAC CAT TTG AAA TCA GTG TT	
miR-125a-5p	TCC CTG AGA CCC TTT AAC CTG TGA	
miR-206	TGG AAT GTA AGG AAG TGT GTG G	
miR-222-3p	AGC TAC ATC TGG CTA CTG GGT	
miR-15a-5p	TAG CAG CAC ATA ATG GTT TGT G	
miR-22-3p	AAG CTG CCA GTT GAA GAA CTG T	
miR-25-3p	CAT TGC ACT TGT CTC GGT CTG A	
miR-30d-5p	TGT AAA CAT CCC CGA CTG GAA G	
miR-1-3p	TGG AAT GTA AAG AAG TAT GTA T	

miR-140-5p	CAG TGG TTT TAC CCT ATG GTA G
RNU6	Hs_RNU6-2_11 (QIAGEN)
SNORD61	Hs_SNORD61_11 (QIAGEN)
SNORD95	Hs_SNORD95_11 (QIAGEN)
FastStart Universal Probe Master Mix TaqMan (Roche)	
p21	Hs 00355782_m1 (TaqMan GEA Applied)
p57	Hs 00175938_m1 (TaqMan GEA Applied)
CDX2	Hs 00230919_m1 (TaqMan GEA Applied)
NOXA (PMAIP1)	Hs 00560402_m1 (TaqMan GEA Applied)
PUMA (BBC3)	Hs 00248075_m1 (TaqMan GEA Applied)
BAX	Hs 00180269_m1 (TaqMan GEA Applied)
TBP	Hs 00427620_m1 (TaqMan GEA Applied)

Chromatin immunoprecipitation

1.000.000 of AGS cells were seeded in culture boxes 24h prior any treatment. Then, cells were treated with Cisplatin 6h or were untreated (NT) by replacing the culture medium. Chromatin immunoprecipitation is realized with Magna ChIp G 17-611/17-409 Kit® (Millipore). Beforehand, non-specific sites of magnetic beads were blocked twice 10min under agitation with provided PBS1x added of 2.5mg/mL of pure BSA (AM2616, Ambion). Then, beads were separated in two experimental conditions and were incubated 7h at 4°C under agitation with respective antibodies diluted in provided PBS1x added of 2.5mg/mL of pure BSA. Firstly, the control condition with **normal mouse IgG** (sc-2025, Santa Cruz Biotechnology, 4µg). Secondarily, the condition with antibodies against **p53** (mouse anti-p53 1C12, #2524 Cell Signaling, 1:200). Antibodies-beads complexes are used for the chromatin immunoprecipitation as described by the provider. Chromatin purification is done with QIAquick PCR Purification® Kit (Qiagen) and chromatin is eluted in 32µL of elution buffer. DNA relative quantifications are performed with qPCR as described before using

2µL/reaction of purified DNA and the FastStart Universal SYBR Green PCR Master Mix® (Roche) in the 7500 Real Time PCR System® (Applied Biosystems). Enrichment in %input is calculated with the method %Input = $2^{(Ct_{input}-6,64-Ct_{ChIp})}$ x100.

Human primer	Right (reverse) sequence 5' to 3'	Left (forward) sequence 5' to 3'
Promoter p21	CTG AAA ACA GGC AGC CCA AG	GTG GCT CTG ATT GGC TTT CTG
Promoter miR-140	CCA GCT CAA AAG GAG CAA TC	CGC TGT CTA GCC CAG TGC
Promoter HDAC4	TTC CCT CAT GTT TCT GTC TTC A	GTA TGG GCA AAA GGC AAA GA

Western blot

500.000 cells were seeded per well in 6-well plates (Falcon Multiwell), 24h prior to any treatment. Then, cells were treated to indicated drugs and times by replacing the culture medium. Adherent and supernatant cells are lysed with Laemmli® 1x (Biorad) added of Dithiothreitol 50mM (DTT; Sigma-Aldrich) at the rate of 100µL/condition. Total proteins are sonicated and denatured before being put on an SDS-PAGE gel (10-20µL/sample). Western blots are performed using Polyvinylidene fluoride (PVDF) membranes and primary antibodies directed against **p53** (mouse anti-p53 DO-1, sc-126, Santa Cruz, 1:1000 in PBS-Milk1%-Tween1‰ [Fisher Bioreagents]), **CLEAVED CASPASE 3** (rabbit anti-cleaved caspase 3, #9661, Cell Signaling, 1:1000 in PBS-Milk1%-Tween1‰), **LC3B** (rabbit anti-LC3B, NB100-2220, Novus Biological, 1:1000 in TBS [152,3mM Tris-HCl; 46,2mM Tris-Base; 1,5M NaCl [Sigma] pH 7.6)-BSA5%-Tween1‰]), **ATF4** (rat anti-ATF4, Creb-2, W16016A, Biolegend, 1:500 in PBS-Milk1%-Tween1‰), **Acetylated H3K9** (rabbit anti-histone H3 [Acetyl K9], ab61231, Abcam, France, 1:500 in PBS-Milk1%-Tween1‰), **HISTONE H3** (rabbit anti-histone H3, #4499, Cell Signaling, 1:1000 in PBS-Milk1%-Tween1‰), **HDAC4** (rabbit anti-HDAC4, 607702, Biolegend, 1:500 in PBS-Milk1%-Tween1‰), **Phosphorylated AKT** (rabbit anti-phospho-Akt [Ser 473], #4060, Cell Signaling, 1:2000 in TBS-BSA5%-Tween1‰), **AKT** (rabbit anti-Akt, #9272, Cell Signaling, 1:1000 in TBS-BSA5%-Tween1‰), **HIF1 ALPHA** (anti-HIF1-alpha, BD Biosciences, 1:1000 in PBS-Milk1%-Tween1‰), **Acetylated ALPHA TUBULIN** (rabbit anti-acetyl [Lys40] alpha tubulin, #5335, Cell Signaling, 1:1000 in TBS-BSA5%-Tween1‰) and **ALPHA TUBULIN** (rabbit anti-alpha tubulin, #2144, Cell Signaling, 1:1000 in TBS-BSA5%-Tween1‰). Secondary antibodies **anti-rabbit NA934V**, **anti-rat NA935** and **anti-mouse NXA931V** (Horseradish linked, ECL GE Healthcare, 1:10.000) are incubated 1h at room temperature. Protein levels are normalized to **ACTIN** (mouse anti-actin Clone C4, Chemicon, 1:10.000). Western blot revelations are done with ECL reagent® (GE

Healthcare) and observed with PXi® Syngene. Finally, relative quantification to ACTIN is carried out with Genetools software (Syngene). Western blots were performed three times independently.

Immunocytology

250.000 cells were seeded per well in 12-well plates on lamella (Falcon Multiwell), 24h prior any treatment. Beforehand, lamellas were incubated with 0.1mg/mL of poly-D-lysine (Sigma-Aldrich) 3h at 37°C and washed with EDTA (PAN-Biotech) Cells were treated to indicate drugs by replacing the culture medium. After 24h, cells are fixed 20min at 37°C with a PHEM-PFA solution (60mM PIPES [Sigma-Aldrich]; 25mM HEPES [Sigma-Aldrich]; 10mM EGTA [PAN-Biotech]; 3% PFA [Electron Microscopy Science]; pH 6,9). Then, cells are permeabilized 30min at room temperature with PBS1x-Triton0.5% (Euromedex). After, non-specific sites are blocked 30min at room temperature with PBS1x-NGS5% (Normal Goat Serum, Sigma Aldrich). Afterward, cells are incubated 1h at 37°C in a humidified atmosphere with primary antibody against **Ki67** (rabbit anti-Ki67, RM-9106-S, Thermo Scientific, 1:400 in PBS1x-NGS5%). Then, and always in dark, cells are incubated 1h at 37°C in a humidified atmosphere with secondary antibody **anti-rabbit** (goat anti-rabbit Alexa 488, Life technologies, 1:1000). Between each step, cells are washed with PBS1x and PBS1x-NGS5%. After, cells are incubated 10min at room temperature with DAPI (Sigma-Aldrich, 1:20.000). Finally, cells are washed with purified water and the lamellas are mounted on blades with Mounting medium FluorSafe reagent® (Calbiochem). The blade-lamella assemblies are left to polymerize at room temperature overnight and then stored at 4°C. Observations are done with the ApoTome 2® microscope (Zeiss) and Zen Blue® software.

Xenografts

Tumors were implanted into BALB/c male nude mice (aged 6–8 weeks, Charles River) by intradermal subcutaneous injection in the lower flank using 5×10^6 cells. Tumors could grow up to 150mm^3 before starting the treatment. Two hundred microliters of vehicle or Cisplatin (10mg/Kg) or LMK-235 (5mg/Kg) or a combination of both was administrated intraperitoneally. Tumor volume was measured with calipers until day 28. All experiments were conducted in compliance with project and personal licenses issued under the French and Japanese Animals Committee guidelines for the welfare of animals in experimental procedures. The work was approved by a local ethical review committee.

Graphical representation and statistical tests

Box plots and histograms represent sample fold inductions compared to the indicated control. Box plots and histograms are obtained by the quantification of three technical replicates of *n* biological replicates. Normality distribution was checked by Shapiro-Wilk test and homoscedasticity was checked by Bartlett test. Statistical differences were calculated with ANOVA and Tukey post-test or with Mann-Whitney test with a p-value corrected using Prism Graphpad 5 software. * $p< 0.05$; ** $p< 0.01$; *** $p< 0.001$

RESULTS

"A problem without solution may interest a student but would not fail to annoy the casual reader"

Sir Arthur Conan Doyle, *The Thor Bridge problem*

Article A: A HDAC4-miR-140-p53/p73 auto-regulatory loop controls gastric cancer response to chemotherapy and impacts on patient prognosis

A HDAC4-miR-140-p53/p73 auto-regulatory loop controls gastric cancer response to chemotherapy and impacts on patient prognosis

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Abstract

Purpose: Gastric cancer (GC) remains a health issue due to low efficiency of standard therapies, such as Cisplatin. This unsatisfactory situation highlights the necessity of finding factors impacting gastric cancer aggressiveness and resistance to therapy.

Experimental design: Biopsies of GC patients and cell lines (AGS; KATOIII) were analyzed for variations in histone deacetylase 4 (HDAC4) expression and their correlation with patients' survival and sensitivity to Cisplatin, and for molecular mechanisms of HDAC4 regulation and activity.

Results: We report that deregulations of *HDAC4* impacts on GC cells sensitivity to Cisplatin and on patient's prognosis. Indeed, Cisplatin inhibits *HDAC4* expression via miR-140, which itself is repressed by proteins of the p53 family (p53, TAp73). This HDAC4-miR-140-P53/P73 auto-regulatory loop is supported by the correlation between the p53 mutational status and expression of *HDAC4* and miR-140 in GC patients. Alteration in *HDAC4* is clinically relevant as *HDAC4* expression is elevated in certain GC molecular subgroups and that mutations or deletions of it favors patients' survival. Furthermore, overexpression of HDAC4 protects GC cells from Cisplatin, whereas inhibiting *HDAC4* favors Cisplatin-induced apoptosis and Cisplatin-inhibited tumor growth. This anti-apoptotic role of HDAC4 is supported in GC patients by a correlation of HDAC4 expression with inhibition of several molecular mechanisms, including apoptosis. HDAC4 regulated protein levels of p53 and its homologue TAp73, and the expression of the pro-apoptotic p53 target gene PMAIP1 (NOXA).

Conclusion: Altogether these results reveal an auto-regulatory loop involving p53/TAp73, miR-140 and HDAC4 to control the response of gastric cancer cells to Cisplatin and to impact on patients' survival.

Keywords: miR-140, HDAC4, p53, p73, Gastric cancer, Cisplatin

Introduction

Gastric cancer (GC) is the fifth most common cancer and represents the second highest incidence of cancer-related death worldwide *(Mihmanli M et al. 2016).* The first line of treatment is a surgical resection combined with perioperative chemotherapy using organometallic platinum-based compounds (Cisplatin, Oxaliplatin). Unfortunately, only a limited number of tumors respond to the treatment due to intrinsic or acquired resistance *(Florea AM et al. 2011).* In addition, the lack of early prognosis markers leads to a late diagnosis often occurring at locally advanced or metastasis stage, with a median survival time of only 10 months.

In GC, resistance mechanisms are not well understood, but examples of activation of DNA repair and decrease of the apoptotic response have been reported. One of the Cisplatin resistance mechanisms in GC cells is an overexpression or amplification of HER2, which leads to the initiation of epithelial-mesenchymal transition (EMT) correlating with an unfavorable outcome for patients *(Huang D et al. 2016).* In addition, patients treated with Cisplatin can exhibit an overexpression of ERCC1 and BRCA1, two enzymes implicated in the nucleotide excision repair pathway, correlating also with a worse prognosis *(Pietrantonio F et al. 2013).* Furthermore, one major actor of the apoptosis pathway after DNA damage is the p53 protein. p53 is a known tumor suppressor, which is inactivated in more than 60% of GC *(Grabsch HI and Tan P. 2013; Mahu C et al. 2014)* and whose expression is related to the sensitivity of cells to Cisplatin *(Rivlin N et al. 2011; Osman AA et al. 2015; Oren M et al. 2010).* Part of the inhibitory impact of p53 mutants on cell death is mediated by their interaction with the two other members of the p53 family: p63 and p73 *(Gaiddon C et al. 2001).* These three genes encode two classes of isoforms, either containing a transactivation domain in the N terminus (p53, TAp63, TAp73) or not (∆p53, ∆Np63, ∆Np73). It has been reported that these proteins are involved in many aspects in digestive cancers' progression and aggressiveness *(Arrowsmith CH. 1999; Zaika AI and El-Rifai W. 2006).* For instance, altered expression of TA/∆Np73 isoforms has been observed in gastric cancers and expression of the ∆Np73 isoform correlates with poor prognosis *(Tomkova K et al. 2004; 2006; Vilgelm AE et al. 2010).*

Another resistance mechanism to chemotherapies involves epigenetic modifications (histone acetylation/deacetylation, histone/DNA methylation) and post-transcriptional regulations (microRNAs) *(Ellis L et al. 2009; Hong L et al. 2013).* HDAC enzymes are aberrantly expressed in various cancer types including GC *(Hagelkruys A et al. 2011).* The HDAC family is composed of 4 classes: class I (HDAC 1, 2, 3 and 8), IIa (HDAC 4, 5, 7 and 9), IIb (HDAC 6 and 10) and IV (HDAC11) that are Zn^{2+} dependent, and the class III (Sirtuins)

that is NAD^+ dependent. HDACs remove the acetyl group of lysine residues from histone and non-histone substrates, leading to chromatin compaction and decreased gene transcription *(De Ruijter AJ et al. 2003).* HDAC1/2 are overexpressed in advanced GC and their expression level is correlated with poorer patient prognosis *(Mutze K et al. 2010).* HDAC4 is also overexpressed in GC cell lines and has been implicated in cell growth and apoptosis arrest *(Kang ZH et al. 2014).* Some of the functions of HDACs in cancer progression can be explained by their interaction with p53. Indeed, HDAC1 can interact with p53, reducing its binding capacity to the promoter of the pro-apoptotic gene *BAX*, thus favoring cancer cell survival *(Juan LJ et al. 2000).*

Micro (mi)RNAs are small non-coding RNA of approximately 22 nucleotides in size, which regulate gene expression through target mRNA translation inhibition or destabilization. Numerous deregulations of miRNA have been described in gastric cancers, but their functions are not always clear *(Riquelme I et al. 2016).* For instance, the oncomiR miR-21 is overexpressed in 92% of GC leading to the inhibition of the PTEN tumor suppressor expression *(Sekar D et al. 2016).* Inversely, genomic loss of the tumor suppressor miR-101 is implicated in cancer progression through EZH2 overexpression *(Ishiguro H et al. 2014).* miRNA often organize in clusters and share common functions. Thus, miR-222-221 and miR-106b-25 are shown to be upregulated in GC tissues, increasing the G1/S transition through the activation of CDK2 *(Kim YK et al. 2009).* In addition, it has also been shown that miRNA can act on GC cells chemosensitivity. For instance, miR-143, miR-144 and miR-145 are good prognosis markers for the effectiveness of the chemotherapy *(Takagi T et al. 2009; Akiyoshi S et al. 2012).* Finally, miR-15b and miR-16 are downregulated in multidrug resistant GC cell lines and their ectopic expression can help to chemo-sensitized GC cells through the inhibition of the anti-apoptotic gene *BCL2 (Xia L et al. 2008).*

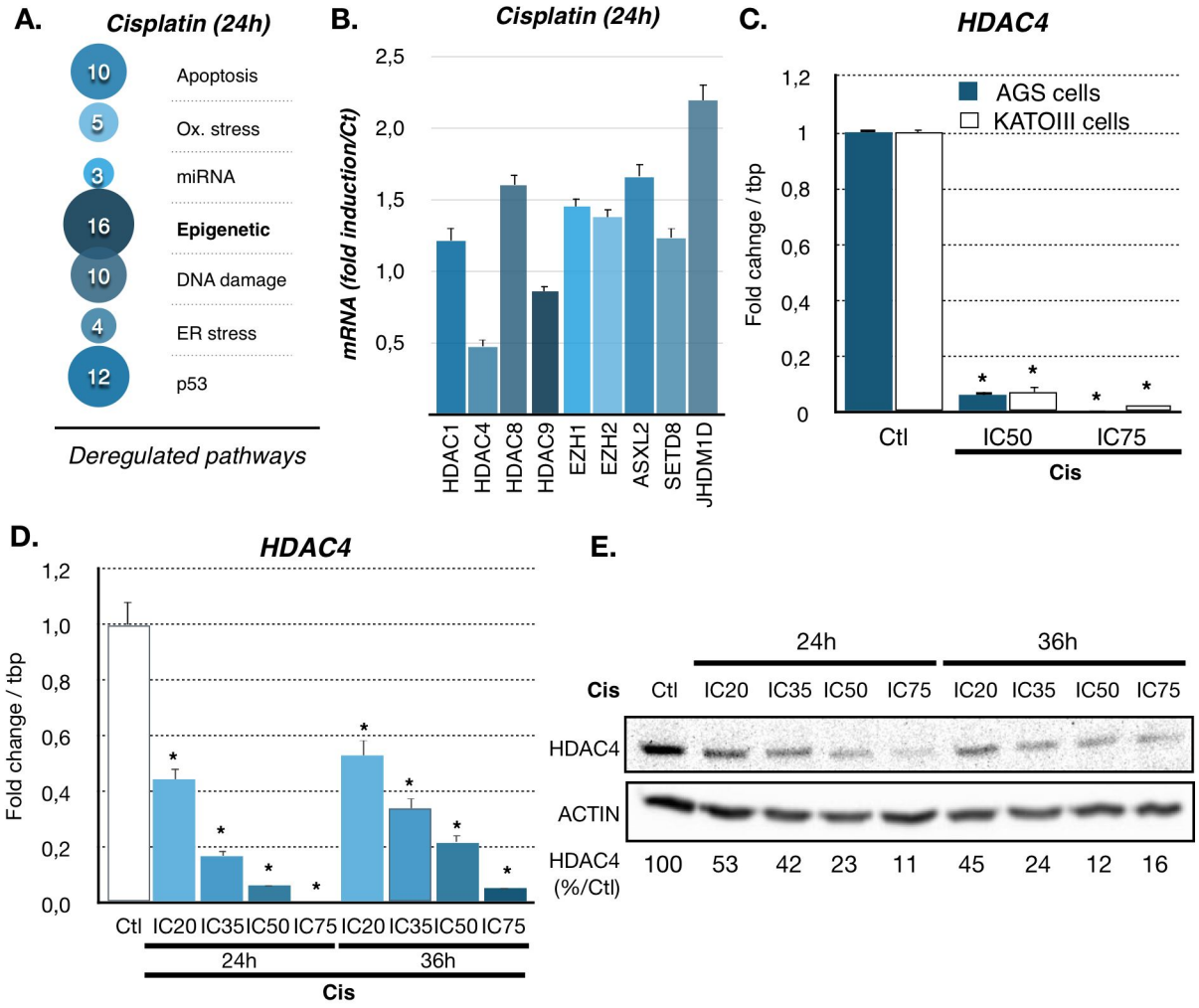
Here, we have investigated the response of cancer cells and healthy digestive tissues to chemotherapies to understand the molecular mechanisms underlying chemo-resistances and side effects caused by these therapies. To this end, we performed a microarray analysis to identify genes deregulated by Cisplatin in cancer cells and identified HDAC4 as a gene inhibited by Cisplatin. Strengthened by the finding of Kang *et al.* that HDAC4 is overexpressed in gastric cancer cell lines *(Kang ZH et al. 2014),* we decided to focus our attention on the role of HDAC4 and the underlying molecular mechanisms that are put in place in response to Cisplatin in GC cancer.

Results

Loss of HDAC4 facilitates Cisplatin cytotoxicity on gastric cancer cells

Platinum-based compounds (Cisplatin, Oxaliplatin) are frequently used to treat different types of cancer. We previously performed a microarray-based transcriptomic analysis on U87 cancer cells treated with Cisplatin for 6h and 24h *(Licona C et al. 2017).* Unsupervised bioinformatics pathways analyses showed that several genes involved in epigenetic regulations were deregulated after 24 hours of treatment **(Figure 1A).** Amongst them, *HDAC4* was significantly repressed by Cisplatin at 24h compared to other HDAC or epigenetic regulators **(Figure 1B).** Based on this observation, we chose to investigate whether the expression of *HDAC4* was also regulated in gastric cancer cells, since Cisplatin-based therapy is considered a gold standard for this type of cancer. We used two different gastric cancer cell lines (AGS and KATOIII cells). The response of these cell lines to Cisplatin was first assessed by monitoring their survival using a MTT assay after 48 hours of treatment with increasing concentrations of Cisplatin **(Supplementary figure 1A).** From these curves, we extrapolated IC_{20}, IC_{35}, IC_{50}, IC_{75}, which are concentrations of Cisplatin that respectively induced 20%, 35%, 50% and 75% of loss of cell viability. To validate the impact of Cisplatin on HDAC4 expression in gastric cancer cells, we treated AGS and KATOIII cells with Cisplatin at two doses (IC_{50} and IC_{75}) for 24 hours. Cisplatin treatment drastically diminished *HDAC4* mRNA level in both cell lines after 24 hours of treatment **(Figure 1C).** In both cases, the effect of Cisplatin was dose-dependent. We then examined in more details the regulation of *HDAC4* expression in AGS cells. Dose-dependent and time-dependent analyses of *HDAC4* mRNA **(Figure 1D)** and protein levels **(Figure 1E)** were performed. Cisplatin-dependent down-regulation of *HDAC4* expression was detected already with low doses (IC_{20}, IC_{35}) of Cisplatin and lasted up to 36 hours after treatment. Thus, Cisplatin treatment can reduce the quantity of both HDAC4 proteins and mRNA levels in gastric cancer cells. Since it appeared that Cisplatin reduces *HDAC4* expression, we set to determine whether modulating *HDAC4* expression prior to treatment could impact on the response of gastric cancer cells to Cisplatin.

Figure 1


A.
Cisplatin (24h)
10
Apoptosis
5
Ox. stress
3
miRNA
16
Epigenetic
10
DNA damage
4
ER stress
12
p53
Deregulated pathways
B.
Cisplatin (24h)
mRNA (fold induction/Ct)
2,5
2,0
1,5
1,0
0,5
HDAC1
HDAC4
HDAC8
HDAC9
EZH1
EZH2
ASXL2
SETD8
JHDM1D
C.
HDAC4
Fold cahnge / tbp
1,2
1
0,8
0,6
0,4
0,2
0
AGS cells
KATOIII cells
Ctl
IC50
IC75
Cis
D.
HDAC4
Fold change / tbp
1,2
1,0
0,8
0,6
0,4
0,2
0,0
Ctl IC20 IC35 IC50 IC75 IC20 IC35 IC50 IC75
24h
36h
Cis
E.
24h
36h
Cis
Ctl
IC20
IC35
IC50
IC75
IC20
IC35
IC50
IC75
HDAC4
ACTIN
HDAC4 (%/Ctl)
100
53
42
23
11
45
24
12
16

← Figure 1: Regulation of *HDAC4* expression in gastric cancer in response to cisplatin

A. Major pathways and molecular mechanisms deregulated in response to cisplatin treatment. Microarrays analysis of U87 cells treated for 24 hours with cisplatin (IC_{50}). Deregulated genes identified by statistical difference ($p< 0.05$) were analyzed by bioinformatics for unsupervised pathway and mechanism clustering. Numbers indicate the number of genes found deregulated in each pathway or molecular mechanism.

B. Expression level of selected epigenetic regulators found deregulated in the microarray analysis. Graph represents means with standard deviation (n=3). Ct, control.

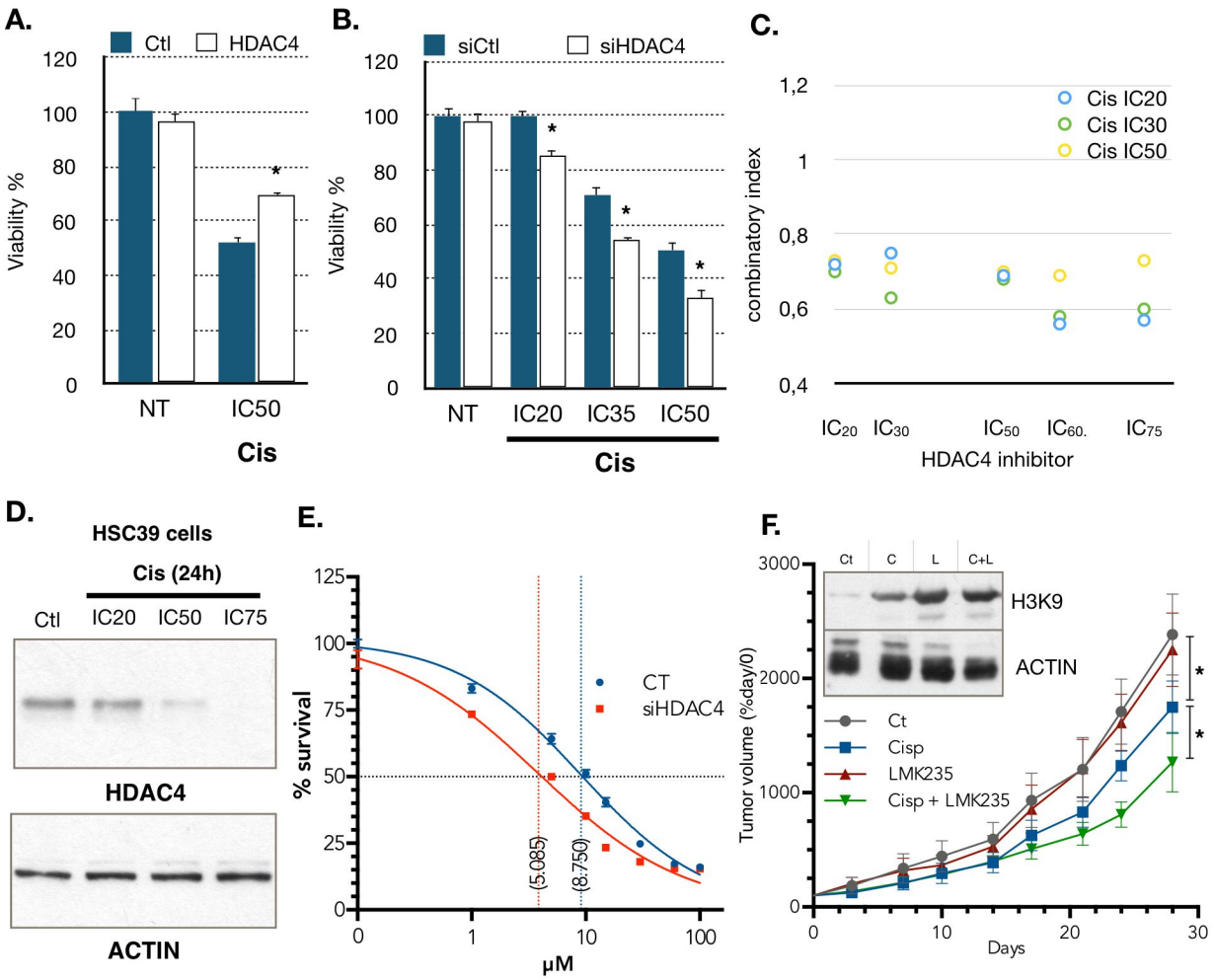
C. Expression of HDAC4 in gastric cancer cell lines treated with cisplatin. HDAC4 mRNA level was assayed in AGS or KATOIII cells by RT-qPCR. Cells were treated at the IC_{50} and IC_{75} of cisplatin (Cis) for 24h. Bars are means of fold induction versus the control (Ctl) and the indicated cisplatin concentration (µM). * p< 0,001 (n=3), compared with the control, as calculated by one-way ANOVA test followed by Tukey test.

D. Expression of HDAC4 in AGS cell line treated with cisplatin for 24h and 36h. HDAC4 mRNA level was assayed in AGS cells by RT-qPCR. Bars are means of fold induction versus the control (Ctl). * p< 0,001 (n=3), compared with the control, as calculated by one-way ANOVA test followed by Tukey test.

E. Proteins from AGS cells treated or not (Ctl) for 24h and 36h with the indicated concentrations of cisplatin (IC_{50}, IC_{75}) were separated on a SDS PAGE gel and propped with an HDAC4 specific antibody. Numbers at the bottom state in % the quantification of HDAC4 expression under cisplatin treatment compared to not treated AGS cells, normalized to actin expression (%Ct).

To this end, we used gain and loss of function experiments. AGS cells were transfected for 48 hours with a plasmid that allows the expression of HDAC4 **(Figure 2A),** or with *HDAC4* siRNA that silenced *HDAC4* expression by about 80% **(Figure 2B).** After 48 hours of transfection, cells were then treated with different doses of Cisplatin for 48 hours and cell survival was assessed using MTT assay. Overexpression of HDAC4 resulted in partial protection of AGS cells from Cisplatin toxicity **(Figure 2A).** Reciprocally, silencing of HDAC4 resulted in further decreased cell viability caused by Cisplatin **(Figure 2B).** We then used a chemical inhibitor of HDAC4, LMK235 *(Marek L et al. 2013),* and investigated its possible synergy with Cisplatin treatment. Combinatory experiments associating increasing doses of Cisplatin with increasing doses of LMK235 were performed and the survival of AGS gastric cancer cells were monitored by MTT. Results were analyzed to establish combinatory indexes **(Figure 2C, supplementary table 1)** *(Chou TC. 2006).* The analysis revealed that LMK235 and Cisplatin were acting in synergy to induce cytotoxicity at lower dose of both drugs ($<IC_{50}$), further supporting the pro-resistance role of HDAC4 in gastric cancer inhibitor

Figure 2


A.
Ctl
HDAC4
Viability %
120
100
80
60
40
20
0
NT
IC50
Cis
B.
siCtl
siHDAC4
Viability %
NT
IC20
IC35
IC50
Cis
C.
Cis IC20
Cis IC30
Cis IC50
combinatory index
1,2
1
0,8
0,6
0,4
IC20
IC30
IC50
IC60.
IC75
HDAC4 inhibitor
D.
HSC39 cells
Cis (24h)
Ctl
IC20
IC50
IC75
HDAC4
ACTIN
E.
% survival
125
100
75
50
25
0
CT
siHDAC4
(5.085)
(8.750)
0
1
10
100
µM
F.
Ct
C
L
C+L
H3K9
ACTIN
Tumor volume (%day/0)
3000
2000
1000
0
Ct
Cisp
LMK235
Cisp + LMK235
0
10
20
30
Days
*
*

(Figure 2A, B). *In vitro* experiments using MC1568, a controversial and poorly soluble inhibitor of HDAC4 *(Duong V et al. 2008),* confirmed that a combinatory treatment of an HDAC4 and Cisplatin produces a synergistic response in gastric cancer cells (data not shown). Similar results were obtained on the diffuse type gastric cancer cell line HSC39. Cisplatin reduced HDAC4 protein levels and silencing of HDAC4 or inhibition using LMK235 favored Cisplatin cytotoxicity *in vitro* **(Figure 2D, E, supplementary figure 5).** Furthermore, LMK235 improved the ability of Cisplatin in reducing tumor growth in xenografted HSC39 tumors in nude mice **(Figure 2F).**

← Figure 2: HDAC4 function in cisplatin response

A. AGS cells were plated in 96-wells plates, transfected with a plasmid (0,05µg) encoding for HDAC4 (pHDAC4) or an empty vector (pcDNA3, Ctl) for 24h and treated for 48h with the indicated concentration of cisplatin. Viability of the cells was evaluated using a MTT test. * p< 0,001 (n=4), compared with the control, as calculated by one-way ANOVA test.

B. AGS cells were plated in 96-wells plates and transfected with a siRNA (10nM) against HDAC4 or luciferase (10nM, siCtl) for 48h and treated for 48h with the indicated concentrations of cisplatin. Viability of the cells was evaluated using a MTT test. * p< 0,001 (n=4), compared with the control, as calculated by one-way ANOVA test followed by Tukey test.

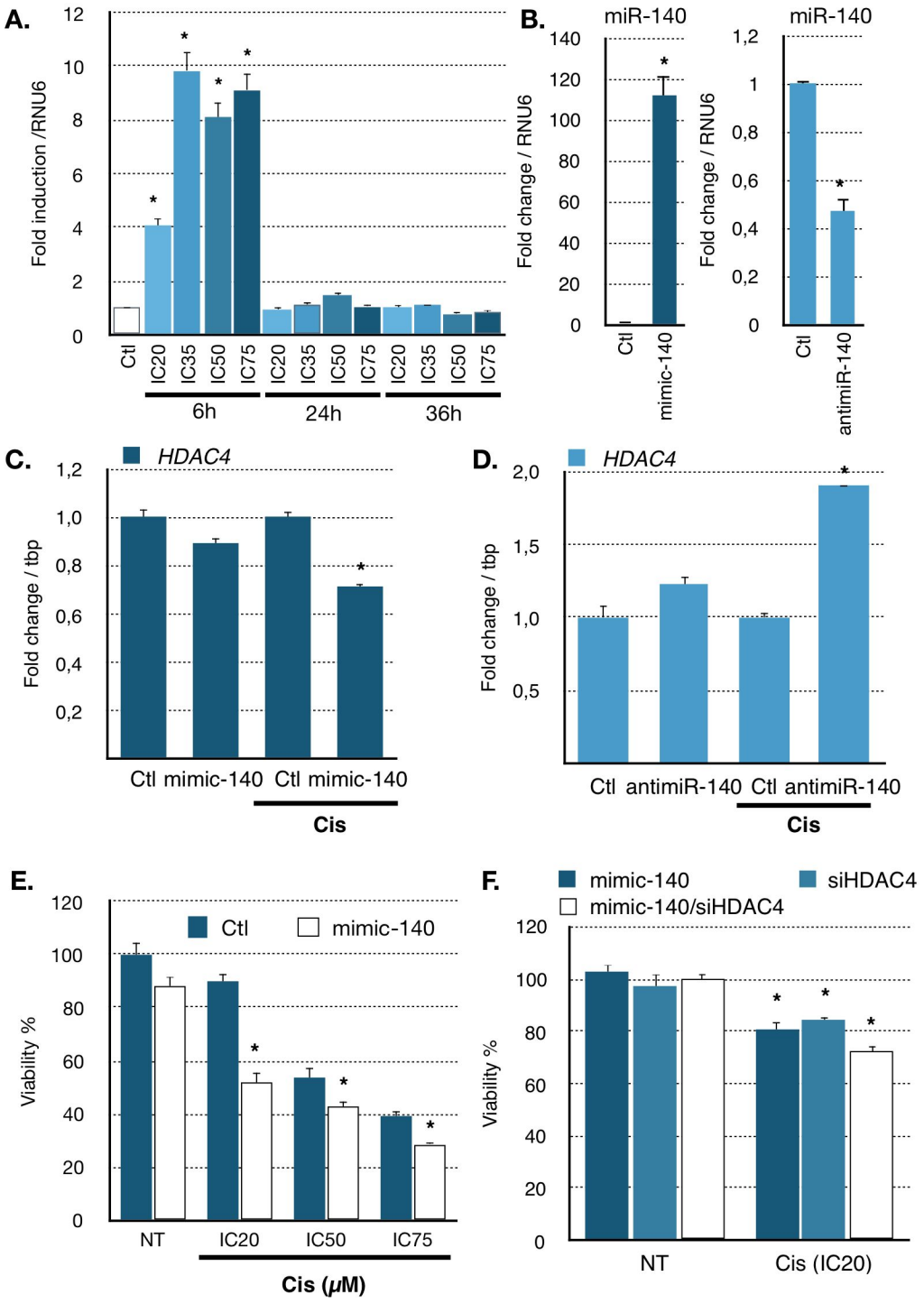
C. Combinatory indexes of treatment with LMK-235 and cisplatin. AGS cells were treated with a combination of increasing concentration of LMK-235 and cisplatin and the cytotoxicity was evaluated by MTT after 48h of treatment. Graph represents combination indexes for cisplatin concentration of IC_{20}, IC_{30} and IC_{50} combined with IC_{20}, IC_{30}, IC_{50}, IC_{60}, IC_{75} of LMK-235. Combination indexes are inferior to 0.80 indicating a synergistic effect between LMK-235 and cisplatin on AGS cell survival.

D. Proteins from HSC39 cells treated or not (Ctl) for 24h with the indicated concentrations of cisplatin ($IC_{20,}$ IC_{50}, IC_{75}) were separated on a SDS PAGE gel and propped with an HDAC4 specific antibody. Numbers at the bottom state in % the quantification of HDAC4 expression under cisplatin treatment compared to not treated AGS cells, normalized to actin expression (%Ct).

E. HSC39 cells were plated in 96-wells plates and transfected with a siRNA (10nM) against HDAC4 or luciferase (10nM, siCtl) for 48h and treated for 48h with the indicated concentrations of cisplatin. Viability of the cells was evaluated using a MTT test. IC50 were statistically different, p = 0,016 (n=5), compared with the control, as calculated by *t* test.

F. HSC39 cells were implanted intradermal in nude mice. Mice were treated when tumor reached 150mm^3 with cisplatin (10mg/Kg) or LMK235 (5mg/Kg) or a combination of both once a week. Tumor size was monitored twice a week using a caliper. * indicate p< 0.05 as calculated by *t* test. *Inset*: Western blot of histone 3 acetylated a lysine 9 and actin performed on tumor extracts at 28 days. Ct: Control; C: Cisplatin; L: LMK235.

Figure 3


A.
Fold induction /RNU6
Ctl
IC20
IC35
IC50
IC75
6h
24h
36h
B.
miR-140
Fold change / RNU6
Ctl
mimic-140
antimiR-140
C.
HDAC4
Fold change / tbp
Ctl mimic-140 Ctl mimic-140
Cis
D.
HDAC4
Fold change / tbp
Ctl antimiR-140 Ctl antimiR-140
Cis
E.
Ctl
mimic-140
Viability %
NT
IC20
IC50
IC75
Cis (μM)
F.
mimic-140
siHDAC4
mimic-140/siHDAC4
Viability %
NT
Cis (IC20)

← Figure 3: miR140-dependent regulation of HDAC4 expression in gastric cancer cells treated with Cisplatin

A. Expression of miR-140 in AGS cells treated with Cisplatin over time at increasing concentrations. miR-140 level was assayed by RT-qPCR. Bars are means of fold induction versus the control (Ctl) and the indicated Cisplatin concentrations. * p< 0,001 (n=3), compared with the control, as calculated by one-way ANOVA test.

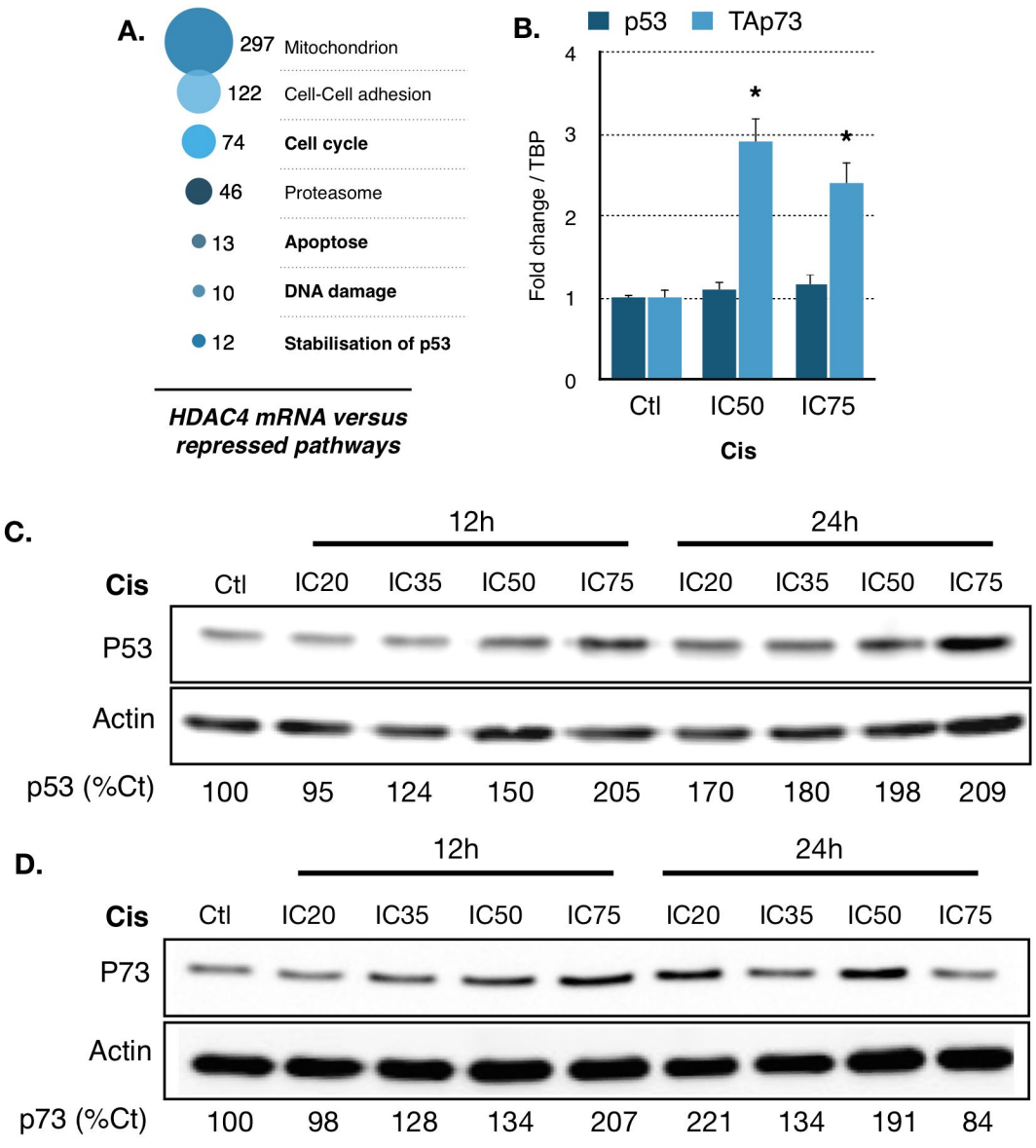
B-D. Expression of *HDAC4* in AGS cells transfected 48h with a miR-140 specific mimic (mimic-140; 100nM) or antimiR (antimiR-140; 30nM) specific for miR-140 and treated or not with Cisplatin (25µM, 12h). miR-140 and *HDAC4* RNA level was assayed by RT-qPCR. Bars are means of fold induction versus the control (Ctl). * p< 0,001 (n=3), compared with the control mimic, and the control antimiR, as calculated by one-way ANOVA test.

E-F. AGS cells were plated in 96-wells plates and transfected with a mimic specific for miR-140 (mimic-140; 100nM), or mimic-140 with siHDAC4, and their respective control, for 48h and treated or not for 48h with the indicated concentration of Cisplatin. Viability of the cells was evaluated as described in Figure 2C.

miR-140 partly mediates Cisplatin-induced HDAC4 repression

Considering the rapid and efficient loss of HDAC4 mRNA by Cisplatin treatment, we hypothesized that it might be mediated by miRNA. It was previously reported that *HDAC4* expression is regulated by miR-140 in osteosarcoma cells *(Song B et al. 2009).* Therefore, we analyzed whether miR-140 might be involved in the regulation of *HDAC4* expression by Cisplatin. First, RT-qPCR analyses revealed that Cisplatin strongly stimulated (up to 10- fold) miR-140 expression level 6 hours after treatment, reverting to the basal level after 24 hours **(Figure 3A).** Induction of miR-140 level was already occurring at low doses of Cisplatin (IC_{20}, IC_{35}). To further characterize the relationship between HDAC4 and miR-140 we used mimics and anti-miRNA oligonucleotides to overexpress or block miR-140 to assess its importance in *HDAC4* regulation. AGS cells were transfected for 48 hours with miR-140 mimics or anti-miRNA and then treated with Cisplatin (IC_{50}) for 24 hours before *HDAC4* mRNA levels were measured by RT-qPCR. We first verified that the mimics and anti-miRNA oligonucleotides had the expected effect on miR-140 expression **(Figure 3B).** Transfection of miR-140 mimics slightly reduced *HDAC4* mRNA level in control condition and more significantly (0.7 fold) in presence of Cisplatin **(Figure 3C).** Reciprocally, anti-miRNA oligonucleotides directed against miR-140 increased *HDAC4* mRNA level in control condition and even more significantly (1.9 fold) after Cisplatin treatment **(Figure 3D).** It is noteworthy that the anti-miRNA experiment did not completely abolish miR-140 expression **(Figure 3B),** which might explain why it did not completely restore *HDAC4* RNA level after Cisplatin treatment.

Figure 4


A.
297 Mitochondrion
122 Cell-Cell adhesion
74 Cell cycle
46 Proteasome
13 Apoptose
10 DNA damage
12 Stabilisation of p53
HDAC4 mRNA versus repressed pathways
B.
p53
TAp73
Fold change / TBP
Ctl
IC50
IC75
Cis
C.
12h
24h
Cis
Ctl
IC20
IC35
IC50
IC75
P53
Actin
p53 (%Ct)
100 95 124 150 205 170 180 198 209
D.
12h
24h
Cis
Ctl
IC20
IC35
IC50
IC75
P73
Actin
p73 (%Ct)
100 98 128 134 207 221 134 191 84

Altogether, these results indicated that miR-140 is at least partly involved in the regulation of HDAC4 RNA level caused by Cisplatin treatment. To further evaluate the importance of miR-140 in Cisplatin cytotoxicity, we transfected AGS cells with miR-140 mimic and measured cell survival by MTT assay after treatment with Cisplatin. MiR-140 mimics significantly reduced cell survival in cells treated with increasing doses of Cisplatin **(Figure 3E)** and this effect was additive when combined with HDAC4 siRNA **(Figure 3F).** These results further support the involvement of a miR-140-HDAC4 axis in the response of gastric cancer cells to Cisplatin.

HDAC4 regulates pro-apoptotic pathway, including p53 and TAp73 expression in gastric cancer cells

Since HDAC4 expression impacts on the Cisplatin response of gastric cancer cells, we examined potential molecular mechanisms that might account for this. First, we analyzed the TCGA data for gastric cancers by sorting for genes whose expression correlates positively or negatively with *HDAC4* expression. An unsupervised bioinformatics pathway analysis *(DAVID: https://david.ncifcrf.gov; https://reactome.org)* revealed that several cellular mechanisms are altered **(Figure 4A; supplementary figure 3B).**

← Figure 4: HDAC4 expression correlates with deregulation of cell cycle and proapoptotic pathways regulated by protein of the p53 family

A. Deregulated signaling pathways and cellular processes that correlated negatively with HDAC4 expression in gastric cancer data of the TCGA. Gene expression data of gastric cancer tumors from the TCGA were analyzed to identify genes with an expression that correlates negatively (Pearson correlation coefficient < -0.3) with the expression of HDAC4 (cBioportal.com). Then, the list of genes was subjected to unsupervised pathways analyses *(DAVID: https://david.ncifcrf.gov; https://reactome.org).* Graphic represents the most relevant deregulated pathways.

B. Expression of p53 and TAp73 in AGS cell lines treated or not (Ctl) with indicated concentrations of cisplatin for 24h. mRNA level of p53 and TAp73 was assayed by RT-qPCR. Bars are means of fold induction versus the control (Ctl) and the concentrations of cisplatin. * p< 0,001 (n=3), compared with the control, as calculated by one-way ANOVA test.

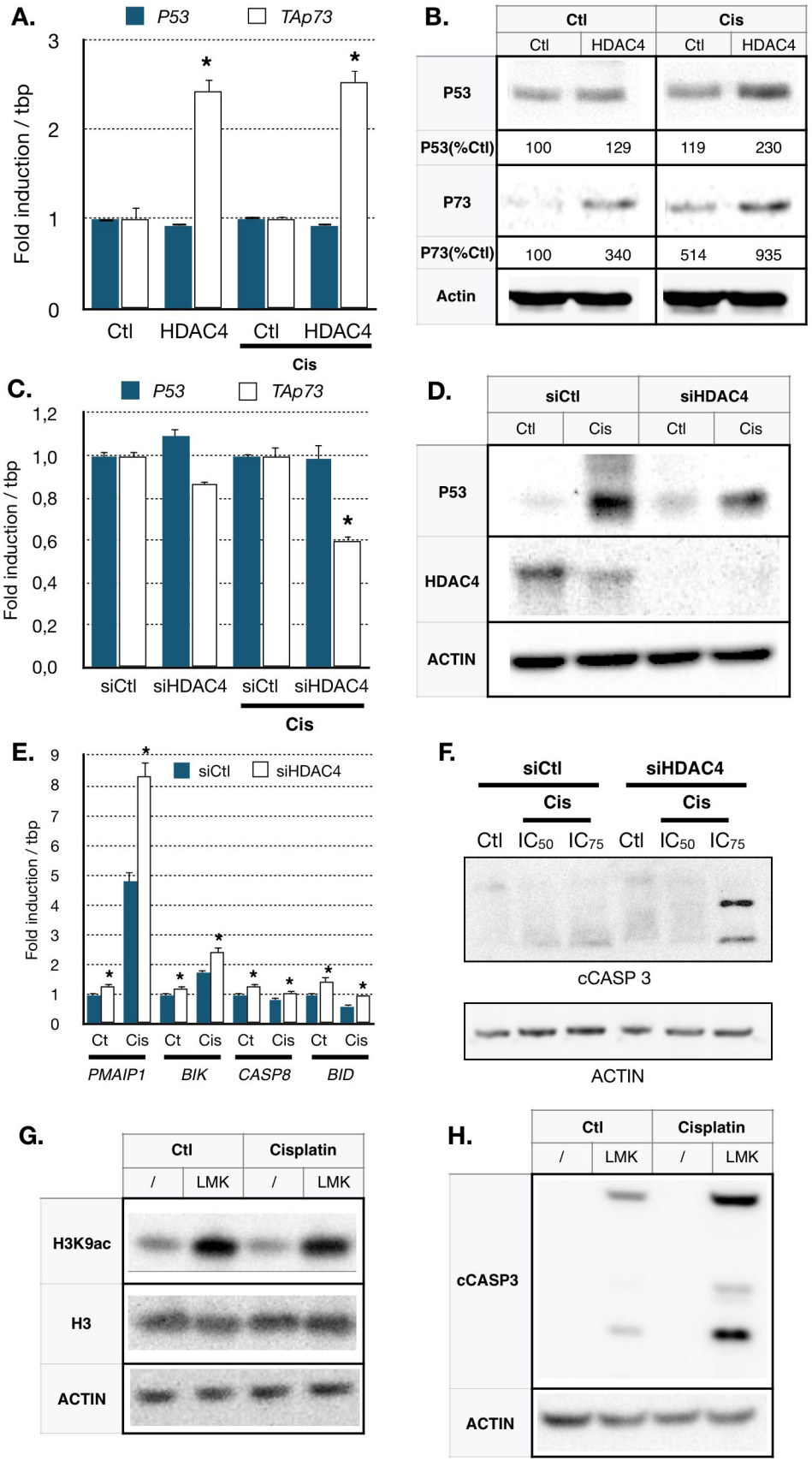
C, D. Proteins from AGS cells treated or not (Ctl) with the indicated concentrations and time of cisplatin were separated on a 10% SDS PAGE gel. Western blot experiment was performed using an antibody against p73 or p53. Numbers at the bottom state in % the quantification of HDAC4 expression under cisplatin treatment compared to not treated AGS cells, normalized to actin expression (%Ct).

This approach gave us an overview on the top ranked pathways potentially affected by the expression level of *HDAC4* in patients with gastric cancer. For instance, the genes whose expressions are negatively correlated with *HDAC4* expression are related to a relatively large variety of pathways/mechanisms **(Supplementary figure 3B).**Amongst the top 7 ranked pathways/mechanisms anti-correlating with a high expression of *HDAC4* are the cell cycle, apoptosis, DNA damage and stabilization of p53 **(Figure 4A).** These pathways are known to be some of the main targets of the P53 and TAp73 transcription factors, which themselves are key factors in Cisplatin response *(Tomkova K et al. 2004; 2006; Vigelm AE et al. 2010).* Therefore, we analyzed their expression levels in AGS cells upon Cisplatin treatment. As expected, p53 mRNA was only modestly induced by Cisplatin **(Figure 4B)** but p53 protein levels were induced in a dose- and time-dependent manner by Cisplatin **(Figure 4C).** In contrast, both TAp73 mRNA levels and proteins were induced by Cisplatin **(Figure 4B, D).** We then analyzed, by RT-qPCR, the expression of known p53 and TAp73 target genes that regulate cell survival. We choose the pro-apoptotic gene *PMAIP1* (*NOXA*) and the cell cycle regulator genes *CDKN1* (p21), *P57* and *AQP3*. All four genes were induced by Cisplatin **(Supplementary figure 4),** but only *CDKN1* and *AQP3* were already significantly induced 12 hours after the treatment. Interestingly, *P57* and *PMAIP1* were only induced after 24 hours of treatment.

Altogether, these results indicated that both p53 and TAp73 are induced by Cisplatin in AGS cells, which could lead to the activation of pro-apoptotic genes such as *PMAIP1*.

We then assessed whether HDAC4 impacts on *P53* and *P73* expression. AGS cells were transfected for 24 hours with an expression vector encoding HDAC4. Cells were then treated with Cisplatin (IC_{50}) for another 24 hours before *P53* and TAp73 isoform RNA and protein levels were measured by RT-qPCR and Western blot, respectively. Overexpression of HDAC4 in AGS cells had no effect on p53 mRNA level **(Figure 5A),** but induced p53 protein level by two-fold after Cisplatin treatment **(Figure 5B).** Interestingly, HDAC4 induced TAp73 expression both at mRNA and protein levels and in both control and in Cisplatin-treated conditions **(Figure 5A, B).** Reciprocally, HDAC4 siRNA reduced TAp73 mRNA level without significantly affecting p53 mRNA levels **(Figure 5C).** The effect at the protein level also confirmed the previous experiment in that HDAC4 siRNA reduced p53 protein levels after Cisplatin treatment **(Figure 5C).**

Figure 5


A.
P53
TAp73
Fold induction / tbp
Ctl
HDAC4
Ctl
HDAC4
Cis
B.
Ctl
Cis
Ctl
HDAC4
Ctl
HDAC4
P53
P53(%Ctl)
100
129
119
230
P73
P73(%Ctl)
100
340
514
935
Actin
C.
P53
TAp73
Fold induction / tbp
siCtl
siHDAC4
siCtl
siHDAC4
Cis
D.
siCtl
siHDAC4
Ctl
Cis
Ctl
Cis
P53
HDAC4
ACTIN
E.
siCtl
siHDAC4
Fold induction / tbp
Ct
Cis
Ct
Cis
Ct
Cis
Ct
Cis
PMAIP1
BIK
CASP8
BID
F.
siCtl
siHDAC4
Cis
Cis
Ctl
IC50
IC75
Ctl
IC50
IC75
cCASP 3
ACTIN
G.
Ctl
Cisplatin
/
LMK
/
LMK
H3K9ac
H3
ACTIN
H.
Ctl
Cisplatin
/
LMK
/
LMK
cCASP3
ACTIN

← Figure 5: HDAC4 expression correlates with deregulation of cell cycle and proapoptotic pathways regulated by protein of the p53 family

A, B. AGS cells were transfected with a plasmid encoding for HDAC4 (1µg) or an empty vector (pcDNA3, Ctl) for 24h and then treated for 12h with cisplatin. Expression of p53 and TAp73 was assayed by RT-qPCR (**A**) and Western blot (**B**). * p< 0,001 (n=3), compared with the control as calculated by one-way ANOVA followed by a Tukey posttest.

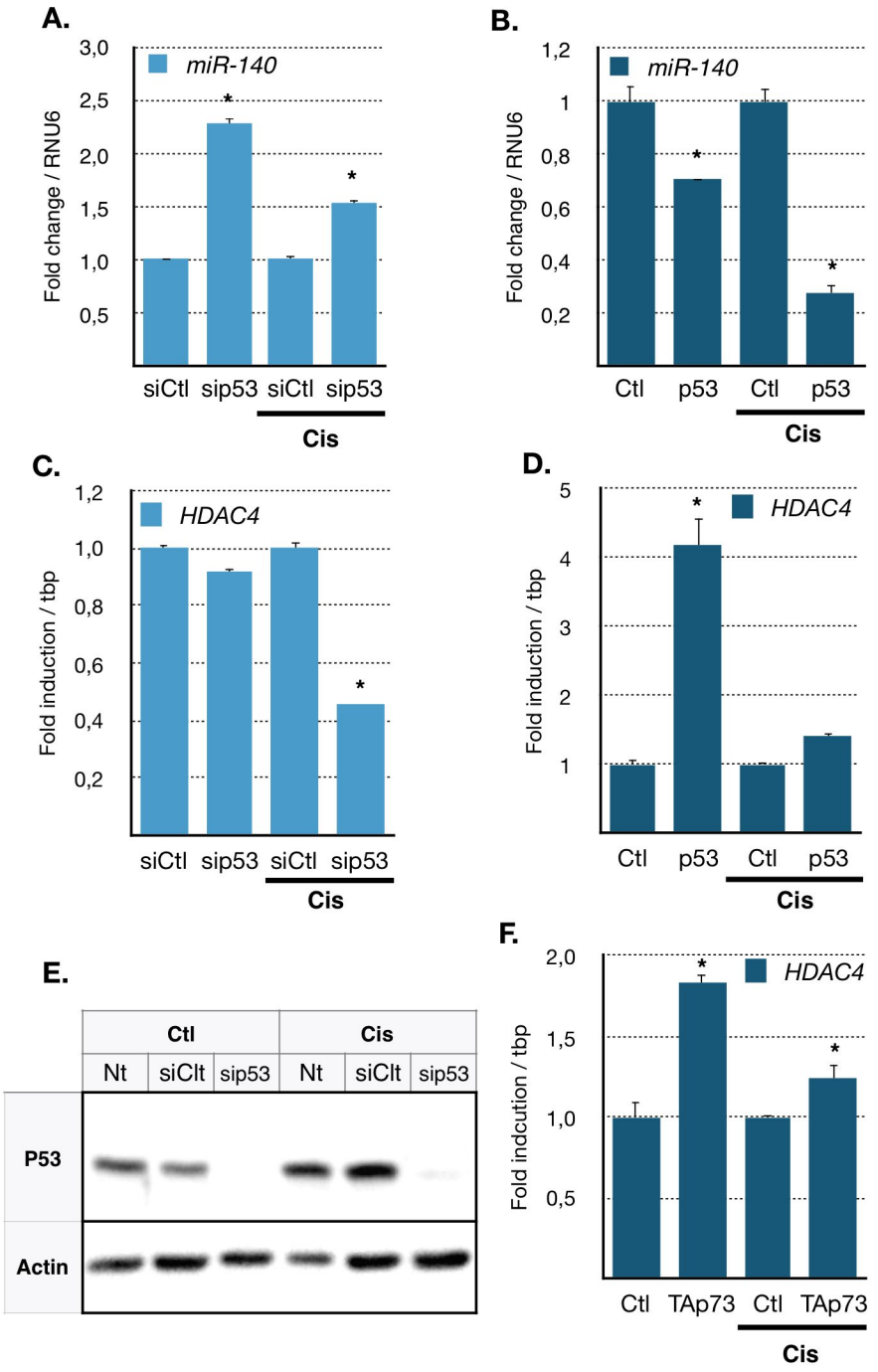
C-E. AGS cells were transfected with a siRNA (10nM) against HDAC4 or luciferase (10nM, siCtl) for 48h and treated with cisplatin for 12h. Expression of p53 and TAp73 was assayed by RT-qPCR (**C**) and Western blot (**D**). Likewise, expression of p53/TAp73 target genes (PMAIP1, BIK) and additional pro-apoptotic genes (CASP8, BID) was assayed by RT-qPCR. * p< 0,001 (n=3), compared with the siCtrl as calculated by one-way ANOVA followed by a Tukey posttest.

F. Proteins from AGS cells transfected with a siRNA (10nM) against HDAC4 or luciferase (10nM, siCtl) for 48h and treated for 24h with the indicated concentrations of cisplatin treated or not (Ctl) with the indicated concentrations (IC50, IC75) of cisplatin were separated on a SDS PAGE gel. Western blot experiment was performed using an antibody against cleaved caspase 3 as described in Figure 1D.

G, H. Proteins from AGS cells treated with LMK235 (IC50) or/and cisplatin (IC50) 24h were separated on a SDS PAGE gel. Western blot experiments were performed using an antibody against H3, acetylated H3K9, cleaved caspase 3 (cCASP3) and ACTIN as described in Figure 1D.

We then investigated the expression levels of p53/TAp73 targets genes related to cell cycle or apoptosis. Interestingly, whereas the reduced p53 protein level by HDAC4 silencing, had no effect on the expression of *P21* and *CYCLIN B2*, it reduced expression of the cell cycle inhibitor *P57* and stimulated the expression of several pro-apoptotic genes *PMAIP1, BIK, BID and CASP8* **(Figure 5E; supplementary figure 4E)** *(Lopez I et al. 2017; Li Y and Seto E. 2017).* Moreover, the higher cytotoxicity of Cisplatin when HDAC4 was silenced correlated with an induction of caspase 3 cleavage at 24h post treatment **(Figure 5F).** This is also illustrated by the chemical inhibition of HDAC4 with the LMK235 *(Marek L et al. 2013).* The HDAC inhibition activity of the LMK235 increased the acetylation level of H3K9 and promoted a cleavage of caspase 3, which was increased by the synergistic combinatory treatment with Cisplatin **(Figure 5G, H).**

Figure 6


A.
miR-140
Fold change / RNU6
3,0
2,5
2,0
1,5
1,0
0,5
siCtl sip53 siCtl sip53
Cis
B.
miR-140
Fold change / RNU6
1,2
1
0,8
0,6
0,4
0,2
Ctl p53 Ctl p53
Cis
C.
HDAC4
Fold induction / tbp
1,2
1,0
0,8
0,6
0,4
0,2
siCtl sip53 siCtl sip53
Cis
D.
HDAC4
Fold induction / tbp
5
4
3
2
1
Ctl p53 Ctl p53
Cis
E.
Ctl
Cis
Nt siClt sip53 Nt siClt sip53
P53
Actin
F.
HDAC4
Fold indcution / tbp
2,0
1,5
1,0
0,5
Ctl TAp73 Ctl TAp73
Cis

These results indicated that part of HDAC4 function in gastric cancer involves the regulation of apoptosis and the activity of proteins of the p53 family. However, it involves a complex mechanism as the silencing of HDAC4 reduced the expression of p53, but only selectively impacts a subset of p53 target genes to favor apoptosis.

← Figure 6: p53 and TAp73 regulates HDAC4 expression via miR-140

B. AGS cells were transfected with a siRNA against p53 (30nM) or luciferase (30nM, siCtl) for 72h and treated with Cisplatin (12h). Nt indicated expression in cells that were not transfected. Western blot experiments were performed on a SDS PAGE gel, using an antibody against p53, as described in Figure 1E.

C. AGS cells were transfected with a siRNA against p53 (30nM), or pGL3-luciferase (Ctl) 72h and treated with Cisplatin (12h). Expression of miR-140 was assayed by RT-qPCR as described in Figure 3.

D. AGS cells were transfected with a plasmid encoded for p53 (1µg) or the empty vector (pcDNA3, Ctl) for 24h and treated with Cisplatin for 12h. Likewise, AGS cells were transfected with siRNA against p53 (30nM) or pGL3-luciferase (30nM, siCtl) for 72h and treated with Cisplatin for 12h. Expression of miR-140 was assayed by RT-qPCR as described in Figure 3.

E. AGS cells were transfected with a siRNA against p53 (30nM), or pGL3-luciferase (Ctl) 72h and treated with Cisplatin (12h). Expression of HDAC4 was assayed by RT-qPCR as described in Figure 3.

F. AGS cells were transfected with 1µg of TAp73 or pcDNA3 (Ctl) for 24h and treated with indicated Cisplatin dose for 12h. Expression of HDAC4 was assayed by RT-qPCR. Bars are means of fold induction versus the empty vector (Ctl). * $p < 0{,}0001$ (n=3), compared with the control, as calculated by one-way ANOVA test.

The miR-140-HDAC4 pathway is regulated by proteins of the p53 family in gastric cancer

TP53 is mutated in more than 50% of gastric tumors. Hence, to understand the mechanisms contributing to the miR-140-*HDAC4* pathway in gastric cancer, we analyzed whether p53 activity contributes to mir-140 and/or HDAC4 expression. Therefore, AGS cells were transfected for 24h with a siRNA directed against p53 and treated with Cisplatin for another 24 hours before quantifying miR-140 levels by RT-qPCR **(Figure 6A, E).** Silencing of p53 significantly increased the level of miR-140, both in the control and in the Cisplatin-treated condition. Conversely, overexpression of p53 repressed miR-140 level **(Figure 6B).** These results suggested that p53 might induce HDAC4 expression by reducing miR-140 levels.

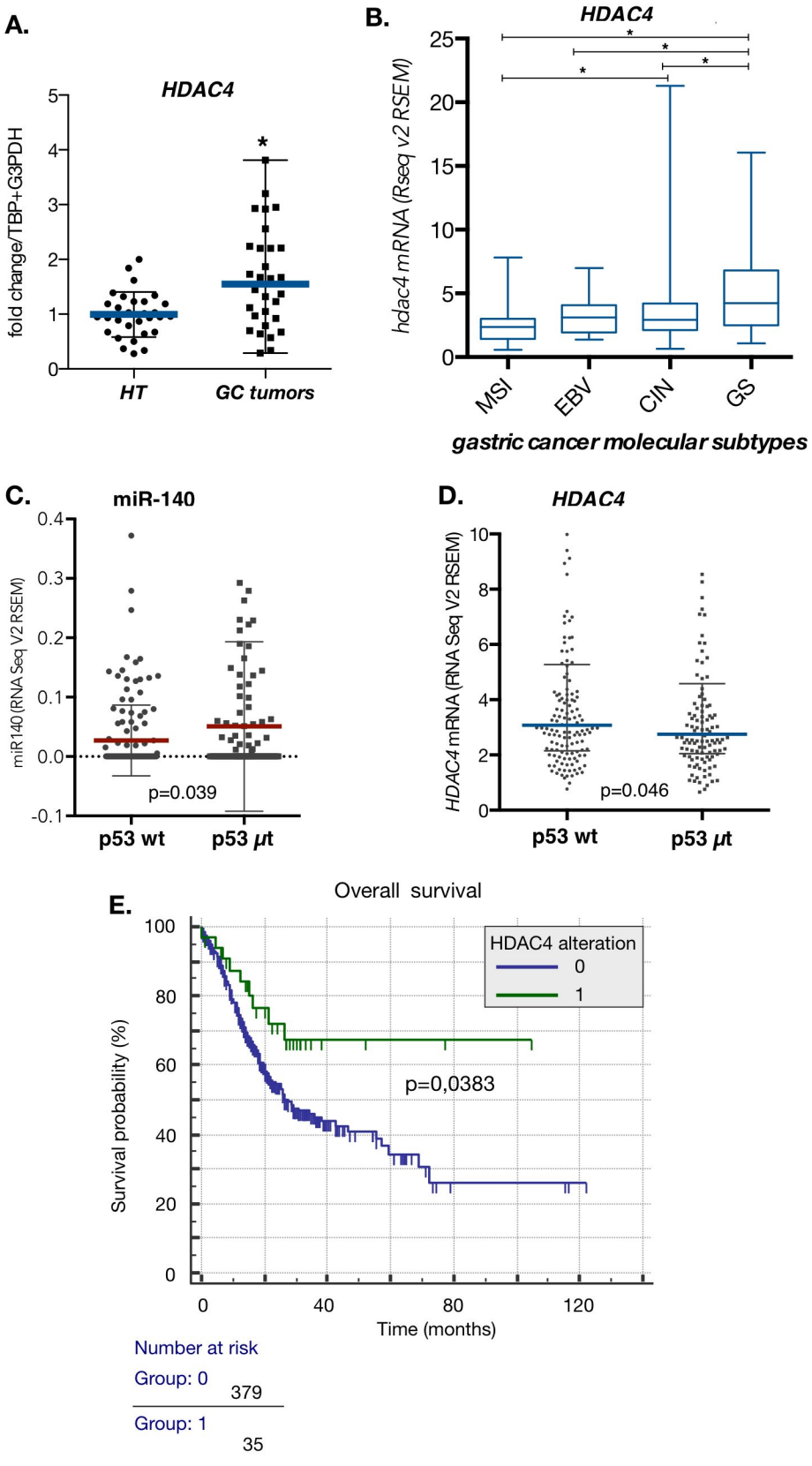
To establish the impact of p53 on *HDAC4* we measured its expression in the samples described above. We found that transfection of siRNA against p53 in AGS cells decreased *HDAC4* mRNA level **(Figure 6C).** However, the effect of p53 siRNA was stronger on Cisplatin-treated cells, reducing *HDAC4* mRNA level by about 55%. Reciprocally, overexpression of p53 led to a robust 4-fold increase in *HDAC4* mRNA level in control condition as measured by RT-qPCR **(Figure 6D).** Importantly, in Cisplatin-treated cells the impact of p53 overexpression on *HDAC4* expression was strongly reduced when compared to Cisplatin treatment only. This is most likely due to the activation of endogenous p53 by the Cisplatin treatment and a further overexpression of p53 did not lead to a measurable effect. We also measured the effect of a homologue of p53, namely TAp73, on *HDAC4* mRNA level by transfecting AGS cells with an TAp73 expression vector **(Figure 6F).** Similarly, to p53, TAp73 overexpression induced *HDAC4* mRNA level, having a stronger effect in the control condition than in presence of Cisplatin. However, the induction caused by TAp73 (1.75 fold) was less intense compared to p53 (4 fold).

Altogether these results suggested that protein of the p53 family (p53 and TAp73) might induce HDAC4 expression by reducing miR-140 level. This mechanism contributes to an auto-regulatory loop that tightly controls the expression level of HDAC4 in gastric cancer upon treatment with Cisplatin.

Gastric cancer harbors HDAC4 de-regulations that impact on patient survival

To further characterize the role of HDAC4 in gastric cancer, we analyzed by RT-qPCR *HDAC4* mRNA levels in both tumor and adjacent healthy tissue biopsies from a cohort of 31 gastric cancer patients. Although the *HDAC4* expression pattern showed a strong variation in healthy and tumor tissues, the HDAC4 mRNA level was significantly higher in gastric cancer biopsies (GC tumors) compared to the adjacent healthy tissues (HT) **(Figure 7A).** We then analyzed *HDAC4* expression data of the TCGA data base for gastric cancers and found that HDAC4 expression varied depending on the molecular subgroup of gastric cancer **(Figure 7B).**

Figure 7


A.
HDAC4
fold change/TBP+G3PDH
HT
GC tumors
B.
HDAC4
hdac4 mRNA (Rseq v2 RSEM)
MSI
EBV
CIN
GS
gastric cancer molecular subtypes
C.
miR-140
miR140 (RNA Seq V2 RSEM)
p=0.039
p53 wt
p53 µt
D.
HDAC4
HDAC4 mRNA (RNA Seq V2 RSEM)
p=0.046
p53 wt
p53 µt
E.
Overall survival
HDAC4 alteration
0
1
Survival probability (%)
p=0,0383
Time (months)
Number at risk
Group: 0
379
Group: 1
35

For instance, the genetic stable (GS) subgroup showed the highest level of HDAC4 expression, while the chromosome unstable (CIN) showed the lowest. However, no statistically relevant difference in patient survival was observed when considering the expression level of HDAC4 (data not shown). We then assessed the influence of the p53 status on the expression of miR-140 and HDAC4 in gastric tumors. We found that the expression level of miR-140 is higher in tumors with mutated p53 proteins, which suggested that p53 might directly or indirectly repress miR-140 expression **(Figure 7C).** Conversely, HDAC4 mRNA level is higher in tumors with wild type p53 proteins **(Figure 7D),** supporting the results obtained *in vitro* **(Figure 6C, D).** Interestingly, besides the elevated expression of *HDAC4* we observed in some gastric tumors, 5.4 % of gastric cancers investigated in the TCGA harbored point mutations, frame shift deletion or deep deletion in *HDAC4* gene **(Supplementary figure 3A).** The exact impact of these alterations on HDAC4 properties remains to be established. However, a Kaplan-Meyer analysis showed that the presence of these alterations favors patients' overall survival **(Figure 7F).**

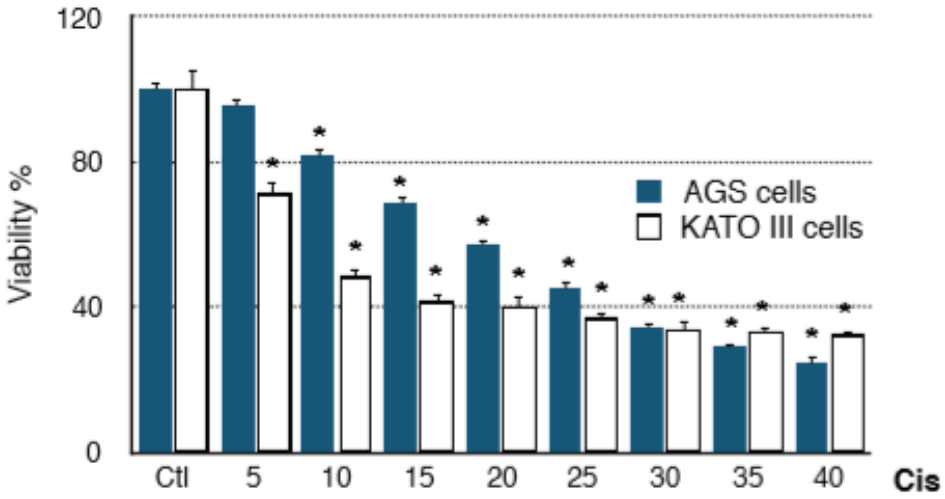
← Figure 7: HDAC4 expression and role in gastric cancer patient survival

A. Expression level of HDAC4 in gastric cancers. RNA was extracted from gastric cancer biopsies and adjacent healthy tissue samples. RT-qPCR for HDAC4 was performed and the results were normalized against TBP and G3PDH. Graph represents medians with ranges (n=26). **A.** Expression level of HDAC4 in gastric cancers. RNA was extracted from gastric cancer biopsies and adjacent healthy tissue samples. RT-qPCR for HDAC4 was performed and the results were normalized against TBP and G3PDH. Graph represents medians with ranges (n=26). $p < 0.0069$, paired *t* test.

B. *HDAC4* expression level in the gastric tumors of the TCGA based on the molecular subgroups. Expression data for *HDAC4* in gastric tumor were extracted from the TCGA data library and analyzed based on the molecular subgroup (MSI n=58; EBV n=24, CIN n=126, GS n=50). Graph represent mean with standard deviation. * when $p < 0.05$ as determined by ANOVA followed by a Tukey test. Similar results were obtained with Holms-Sidak test or Kruskal-Wallis non-parametric test.

C, D. miR-140 and *HDAC4* expression level in the gastric tumors of the TCGA based on the p53 mutational status. Expression data for miR-140 and *HDAC4* in gastric tumor were extracted from the TCGA data library and analyzed based on the mutational status of p53 (p53 wt n=141; p53 µt n=117). Graph represent mean with standard deviation and analyzed by ANOVA followed by an unpaired t test.

E. Kaplan–Meier analysis of patients' overall survival of the 379 patients with wildtype *HDAC4* and 36 patients with either mutated or deleted *HDAC4* and stratified according to HDAC4 mutational status: 0 = no mutation, 1 = mutation or deletion. Mutation or deletion of HDAC4 was a predictor of tumor recurrence ($p = 0.0383$).



Supplementary figure 1: AGS cells or KATOIII cells were treated in 96-wells plates 48h with the indicated concentration (micromolar) of cisplatin. Viability of the cells was evaluated using a MTT test. * p < 0,001, compared with the control, as calculated by one-way ANOVA test.

AGS cells					
LMK-235 / Cisplatin (µM)	IC_{20}	IC_{30}	IC_{50}	IC_{60}	IC_{75}
IC_{20}	**0,72**	**0,70**	**0,73**	*0,89*	*0,91*
IC_{30}	**0,75**	**0,63**	**0,75**	**0,74**	*0,81*
IC_{50}	**0,69**	**0,59**	**0,69**	**0,73**	*0,88*
IC_{60}	**0,56**	**0,58**	**0,69**	**0,76**	*0,81*
IC_{75}	**0,57**	**0,60**	**0,73**	**0,79**	*0,92*

Table S1: Combinatory indexes of treatment with LMK-235 and cisplatin

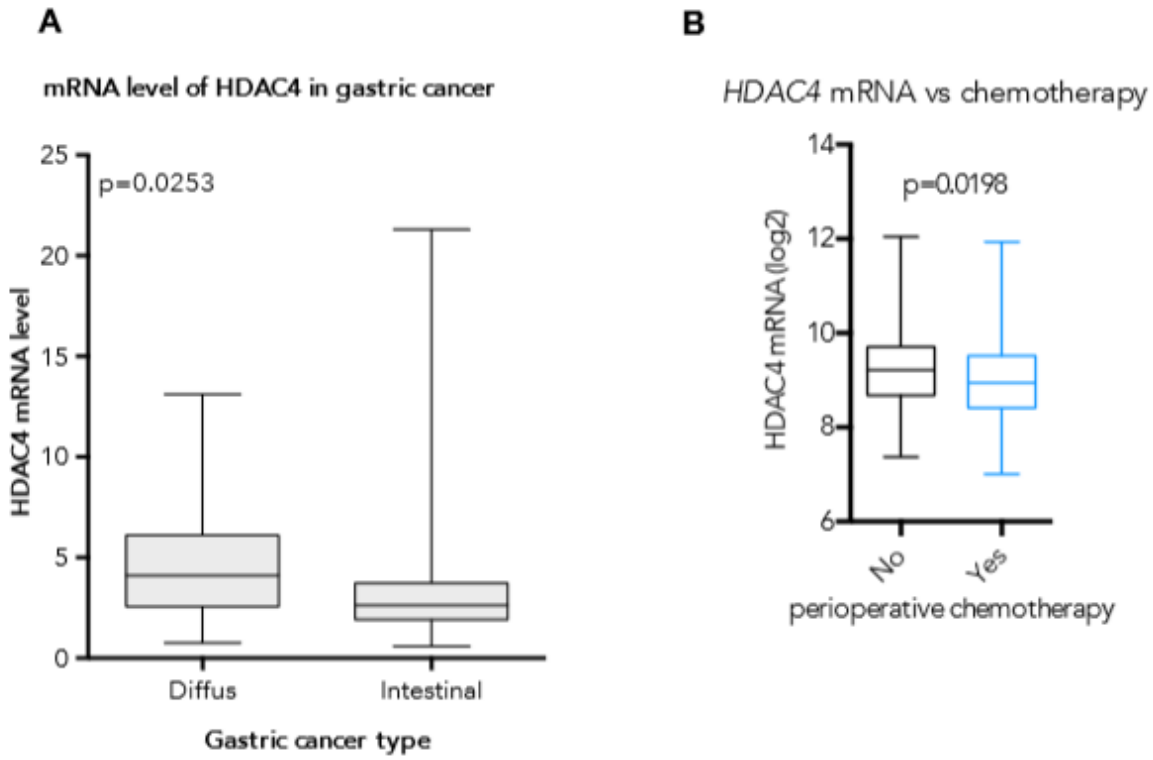
AGS cells were treated with a combination of increasing concentration of LMK235 and cisplatin and the cytotoxicity were evaluated by MTT after 48h of treatment. Combination indexes are in majority inferior to 0.80 (bold) indicating a synergistic effect between LMK-235 and Cisplatin on AGS cell survival. Isobologram assay 10.000 AGS cells were seeded per well in 96_well microplates (Falcon Mutliwell), 24h prior to any treatment. Combination of cisplatin and LMK-235 or each different concentrations of drug alone were applied for 48h in fresh medium. MTT assay was performed as previously described by replacing the medium with fresh medium containing 10% of MTT (Sigma) 5mg/L for 1h30 *(Gaiddon et al. 1999).* Cells were lysed, and the formazan product was solubilized with 100% DMSO. Measurments were performed at 550nm with the Tristar² Mutlimode Reader (Berthold Technologies). Treatment efficiencies were compared to individual treatment control efficiencies with Compusyn program (ComboSyn, Inc) which determined combination index (Chou 2006). In the present study, when the combination index is superior or equal to 1.20 indicates an antagonist effect, between 0.80 to 1.20 an additive effect, and when the index is inferior to 0.80 it suggests synergic effects between LMK-235 and Cisplatin on cell survival.

Altogether, these results indicate that molecular subgroups of gastric tumor harbors elevated level of HDAC4 expression based on the p53 status. It also shows that deregulation of HDAC4 impact on patients' overall survival.

Discussion

Gastric cancer remains a worldwide important health issue, including in western countries, due to a low 5-year survival rate (bellow 30%). Part of its unfavorable prognosis is due to the poor and variable sensitivity of advanced gastric tumors to perioperative chemotherapy protocols, especially platinum-based therapies. We have gathered molecular and clinical evidences indicating that histone deacetylase 4 (*HDAC4*) is a good candidate to explain some of the features leading to a resistance mechanism to Cisplatin in gastric cancer. In addition, we have identified a complex regulatory loop in which HDAC4 functionally interacts with a post-transcriptional regulator, miR-140, and tumor suppressor genes p53 and TAp73.

The role of HDAC4 in the resistance of gastric cancer cells to Cisplatin-based chemotherapy is suggested by several observations. Firstly, *HDAC4* expression is elevated in gastric cancer biopsies compared to normal tissues, showing differences between molecular subgroups. HDAC4 expression is elevated in the genetic stable (GS) and chromosome instable (CIN) subgroups, as well as in the diffuse versus the intestinal histological subgroup **(Supplementary figure 2A).** Importantly, mutation or deletion of *HDAC4* in gastric cancer favors patient survival. Secondly, the silencing of *HDAC4* favors Cisplatin cytotoxicity. Similarly, inhibition of HDAC4 activity with LMK235 synergizes with Cisplatin to induce cytotoxicity *in vitro* and favors the anticancer activity of Cisplatin *in vivo*. Reciprocally, *HDAC4* overexpression counteracts the activity of Cisplatin. Thirdly, in gastric cancer cells treated with Cisplatin at a toxic dose, *HDAC4* expression goes down dramatically, which is supported by clinical data of the TCGA showing that HDAC4 expression is lower in tumors that have been treated with chemotherapy (supplementary data 2B). The role of *HDAC4* in Cisplatin resistance is further supported by additional studies showing that *HDAC4* high expression reduces docetaxel activity *(Colarossi L et al. 2014)* and favors the growth of these cells in the absence of treatment *(Kang ZH et al. 2014).* Furthermore, *HDAC4* is overexpressed in esophageal carcinomas and breast cancers and is associated with a poor prognosis *(Zeng LS et al. 2016; Cohen AL et al. 2013).* Similarly, high expression of HDAC4 is a bad prognosis factor in selected glioblastomas sub-types (pro-neuronal, mesenchymal) *(Cohen AL et al. 2013).*

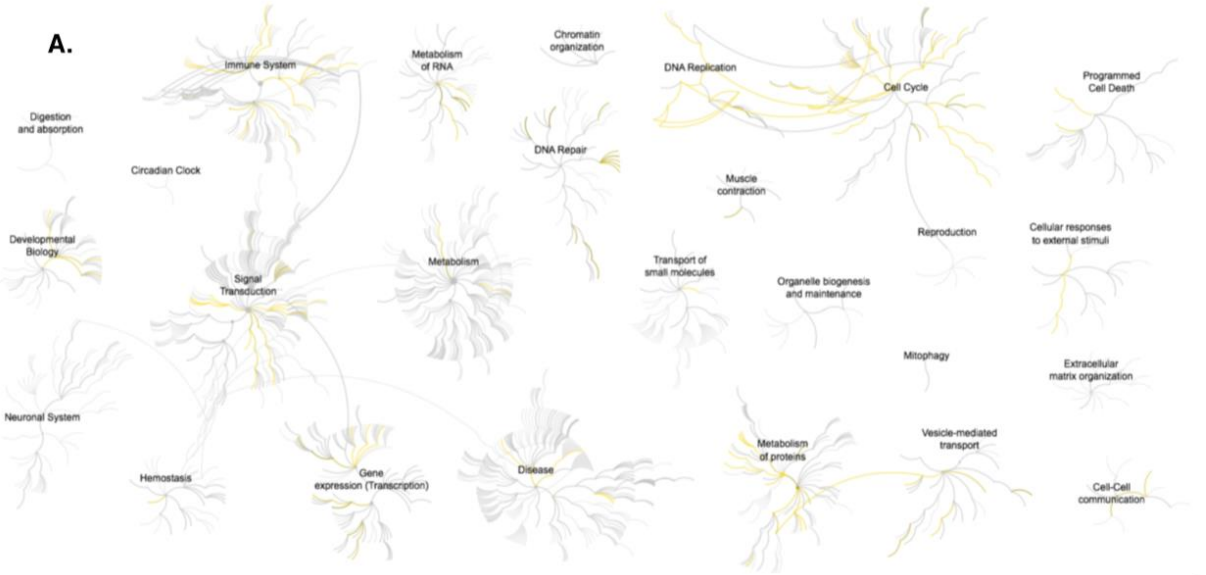


Supplementary figure 2: Expression of HDAC4 mRNA in gastric cancers of the TGCA established by RNAseq. Data were downloaded from the CBioprotal web site and analysed using Prism. Unpaired t test indicated a statistical significant difference between groups. *indicates $p<0.05$). Histological subtypes and perioperative chemotherapy are indicated.

In epithelial ovarian, colon and myeloid cancer cells, HDAC4 increases proliferation and migration *(Shen YF et al. 2016; Amodio N et al. 2016; Vallabhapurapu SD et al. 2015; Wilson AJ et al. 2008).* Although these data suggest that increased HDAC4 expression could be a resistance mechanism in different types of cancers and for several sorts of chemotherapeutic drugs, it is clearly not the case. Indeed, in the colon cancer cell line HCT116, HDAC4 participates in the resistance to 5-FU (5-fluorouracil) but not to methotrexate *(Song B et al. 2009)*.

Therefore, the role of HDAC4 in cancer progression and sensitivity to chemotherapy seems complex and additional information on HDAC4 partners or targets are required to explain how HDAC4 expression can differently impact cancer cells behavior. The results we obtained clearly established that Cisplatin treatment had a very significant effect on *HDAC4* expression in gastric cancer cells. Cisplatin-mediated repression of HDAC4 expression occurs at the RNA level and can reach up to 90% at the protein level with high doses of Cisplatin (IC_{75}). This reduction of HDAC4 level is functionally relevant as an artificial overexpression of HDAC4 counteracts Cisplatin activity. Reciprocally, an amplified inhibition of *HDAC4* expression using siRNA accentuates Cisplatin cytotoxicity. The Cisplatin-induced regulation of HDAC4 expression in gastric cancer cells involves complex and balanced mechanisms. One of them involves miR-140. Indeed, miR-140 is rapidly induced by Cisplatin (within 6 hours) before returning to basal levels after 24 hours of treatment. Artificial overexpression of miR-140 level inhibits HDAC4 expression, whereas anti-miRNAs against miR-140 counteract the negative effect of Cisplatin on HDAC4 level by even doubling it. In addition, miR-140 expression significantly impacts on gastric cancer cell behavior, as miR-140 mimics favor Cisplatin cytotoxicity.

Although, the mechanisms leading to miR-140 induction are not known yet, the downregulation of miR-140, including the one occurring after its initial induction at later time points of Cisplatin treatment, is partially mediated by p53 and TAp73. Indeed, silencing of p53 using siRNA increases miR-140 level in control condition, and partially restores its level during Cisplatin treatment. By doing so, p53 and TAp73 help maintaining *HDAC4* expression to a minimal level, even under Cisplatin condition. These *in vitro* data are confirmed by analyses of the clinical data of the TCGA showing that the expression level of HDAC4 and miR-140 are different depending on the p53 mutational status in gastric tumors. Altogether, it suggests that HDAC4 expression might be necessary to ensure some of p53 functions upon Cisplatin treatment. For instance, previous works have suggested that HDAC4 was involved in the p53-dependent repression of G2/M regulators upon DNA damage, such as Cdc2 and Cyclin B2 *(Imbriano C et al. 2005; Basile V et al. 2006).*



Supplementary figure S3A. Schematic representation of the result of unsupervised pathway analysis performed in "reactome" (https://reactome.org). The genes analysed are negatively correlated (Pearson r = -0.45 to 0.2) with HDAC4 expression in tumors of gastric cancer patients referenced in by TGCA (http:// www.cbioportal.org/).

Sample ID	Cancer Type	Protein Change	Mutation Type	MS
TCGA-HU-8602-01	Diffuse Type Stomach Adenocarcinoma	K282Sfs*116	Frame_Shift_Del	Somatic
TCGA-HU-A4GX-01	Diffuse Type Stomach Adenocarcinoma	A552T	Missense_Mutation	Somatic
TCGA-F1-6177-01	Stomach Adenocarcinoma	M902Wfs*55	Frame_Shift_Del	Somatic
TCGA-BR-8361-01	Stomach Adenocarcinoma	M902Wfs*55	Frame_Shift_Del	Somatic
TCGA-BR-8368-01	Stomach Adenocarcinoma	M902Wfs*55	Frame_Shift_Del	Somatic
TCGA-BR-8487-01	Stomach Adenocarcinoma	M902Wfs*55	Frame_Shift_Del	Somatic
TCGA-D7-A4YY-01	Stomach Adenocarcinoma	M902Wfs*55	Frame_Shift_Del	Somatic
TCGA-HU-A4GN-01	Tubular Stomach Adenocarcinoma	M902Wfs*55	Frame_Shift_Del	Somatic
TCGA-BR-4361-01	Stomach Adenocarcinoma	M902Wfs*55	Frame_Shift_Del	Somatic
TCGA-BR-4361-01	Stomach Adenocarcinoma	Q1006R	Missense_Mutation	Somatic
TCGA-B7-5816-01	Diffuse Type Stomach Adenocarcinoma	D977N	Missense_Mutation	Somatic
TCGA-BR-8372-01	Stomach Adenocarcinoma	R601Q	Missense_Mutation	Somatic
TCGA-BR-8591-01	Stomach Adenocarcinoma	A1045T	Missense_Mutation	Somatic
TCGA-BR-A4QL-01	Stomach Adenocarcinoma	A373T	Missense_Mutation	Somatic
TCGA-CG-4442-01	Stomach Adenocarcinoma	L663V	Missense_Mutation	Somatic
TCGA-CG-4442-01	Stomach Adenocarcinoma	A440T	Missense_Mutation	Somatic
TCGA-CG-5733-01	Stomach Adenocarcinoma	P228L	Missense_Mutation	Somatic
TCGA-F1-6874-01	Stomach Adenocarcinoma	T648Pfs*14	Frame_Shift_Del	Somatic
TCGA-HF-7132-01	Mucinous Stomach Adenocarcinoma	E1070K	Missense_Mutation	Somatic
TCGA-HJ-7597-01	Signet Ring Cell Carcinoma of the Stomach	R200C	Missense_Mutation	Somatic
TCGA-HU-8602-01	Diffuse Type Stomach Adenocarcinoma	D234N	Missense_Mutation	Somatic
TCGA-HU-A4G9-01	Tubular Stomach Adenocarcinoma	A909V	Missense_Mutation	Somatic
TCGA-HU-A4GQ-01	Tubular Stomach Adenocarcinoma	A37V	Missense_Mutation	Somatic
TCGA-HU-A4GT-01	Tubular Stomach Adenocarcinoma	G299R	Missense_Mutation	Somatic
TCGA-HU-A4H3-01	Diffuse Type Stomach Adenocarcinoma	T355M	Missense_Mutation	Somatic
TCGA-BR-6452-01	Stomach Adenocarcinoma	M902Hfs*5	Frame_Shift_Ins	Somatic
TCGA-CD-A4MG-01	Diffuse Type Stomach Adenocarcinoma	X996_splice	Splice_Site	Somatic
TCGA-CD-A4MG-01	Diffuse Type Stomach Adenocarcinoma	E585*	Nonsense_Mutation	Somatic
TCGA-CG-5725-01	Stomach Adenocarcinoma	Y225Rfs*23	Frame_Shift_Del	Somatic
TCGA-VQ-A8P2-01	Mucinous Stomach Adenocarcinoma	S741P	Missense_Mutation	Somatic

Supplementary figure S3B. Mutations and deletion present in tumors of gastric cancer referenced in the TGCA. Mutations or deletion are present in 5.4% of the 478 tumors (http://www.cbioportal.org/).

In addition, HDAC4 has been shown to play a critical role in cell survival by interacting with p53BP1 during G2/M cell cycle check point *(Kao GD et al. 2003)*. Hence, p53 and TAp73 might contribute to maintain a minimal HDAC4 protein level to ensure in some cells a functional G2/M cell cycle check point via the repression of miR-140. We were not able to confirm that HDAC4 regulates *CDC2* or *CYCLIN B2* in gastric cancer cells. However, we showed that HDAC4 modulates the expression of two other target genes of the p53 and TAp73 proteins. Several genes inhibited by HDAC4, such as *PMAIP1, BID, BIK* and *CASP8* are pro-apoptotic, which could contribute to the resistance mechanism initiated by HDAC4 high expression.

In addition to its participation in the regulation of selected p53-target genes, we also showed in this study that HDAC4 is involved in the expression of p53 and TAp73 in gastric cancer cells in response to Cisplatin. Indeed, silencing of HDAC4 reduces the induction of both p53 and TAp73 by Cisplatin. This regulation occurs at the mRNA level for TAp73, but not for p53. The exact nature of the mechanism allowing HDAC4 to favor the increase of p53 protein is yet to be identified, but it does not seem to involve a direct interaction, since we did not see any co-immunoprecipitation between the two proteins under control or Cisplatin conditions.

Altogether, this study highlights a complex regulatory loop linking the tumor suppressor genes p53 and TAp73 with the epigenetic regulators HDAC4 and miR-140 that control in part the response to Cisplatin in gastric cancer cells and how it might impact on patient survival. This p53/TAp73-miR140-HDAC4 regulatory loop may play a critical role in gastric cancer response to therapy, as both HDAC4 and P73 are often expressed at an elevated level in this type of cancer. In addition, p53 are mutated in more than 70% of metastatic gastric cancers. The clinical relevance of this regulatory loop is highlighted by the fact that mutation or deletion of HDAC4 favors survival of patients with gastric cancer. Hence, the use of selective inhibitor of HDAC4, such as LMK235, in combination with Cisplatin may represent a promising therapeutic alternative.

Acknowledgments

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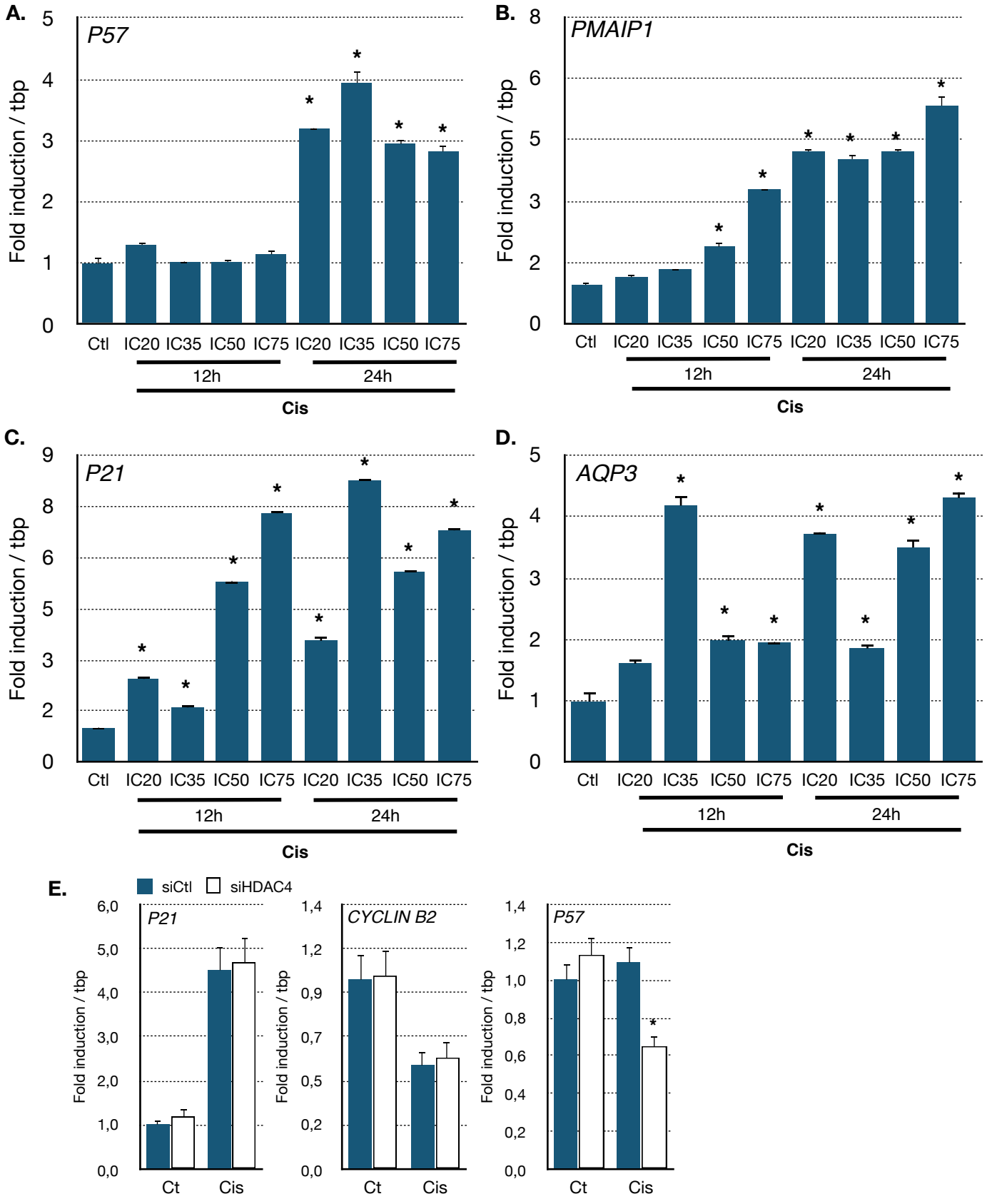


Cisplatin induces several p53 and TAp73 target genes in gastric cancer cells

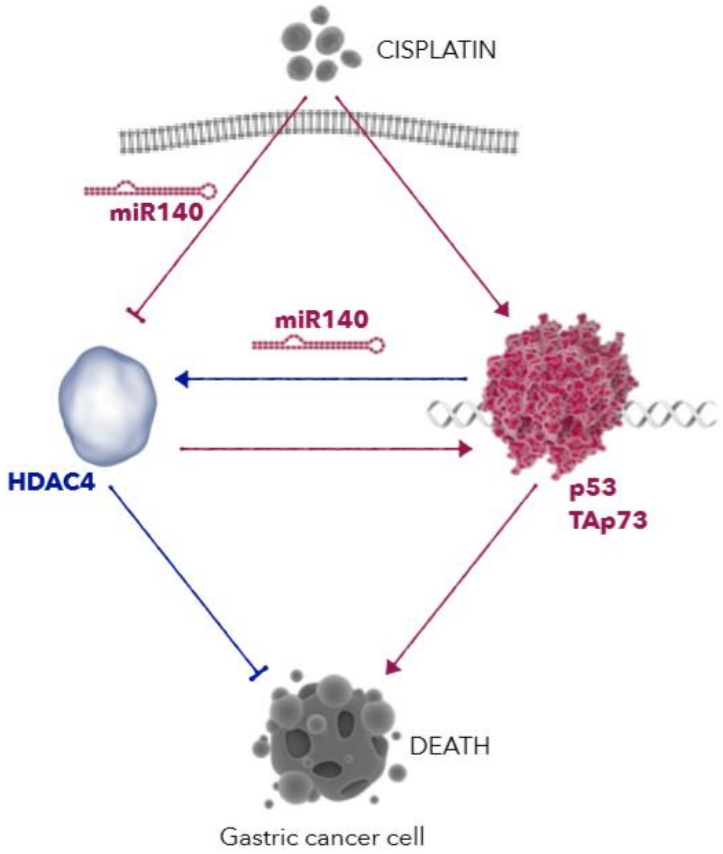
Figure S4A-B-C-D-E. Expression of p53 family members' target genes in AGS cells treated or not (Ctl) with indicated concentrations of cisplatin for 12h or 24h. *P57*, *P21*, *CYCLINB2,* RNA levels were assayed by RT-qPCR.

(UdS) through the project “synergie” are thanked for partial support of this work. We are also thankful for the technical support of E. Martin.

HSC-39 cells					
LMK-235 / Cisplatin (µM)	IC_{20}	IC_{30}	IC_{50}	IC_{60}	IC_{75}
IC_{20}	0.84	**0.79**	0.82	0.87	1.01
IC_{30}	**0.76**	**0.72**	**0.75**	**0.78**	0.98
IC_{50}	**0.72**	**0.67**	**0.73**	**0.74**	0.99
IC_{60}	**0.61**	**0.59**	**0.68**	**0.72**	0.93
IC_{75}	**0.62**	**0.55**	**0.65**	**0.80**	0.94

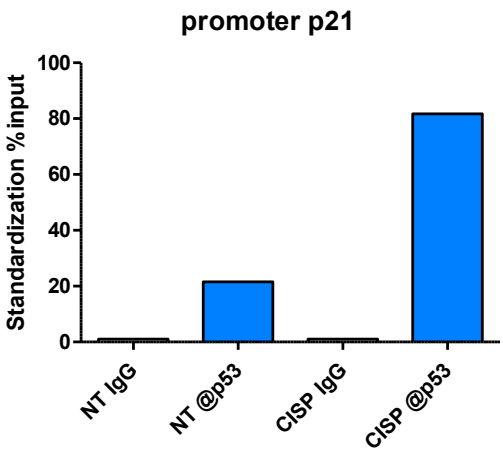
Supplementary table 2: Combinatory indexes of treatment with LMK-235 and cisplatin

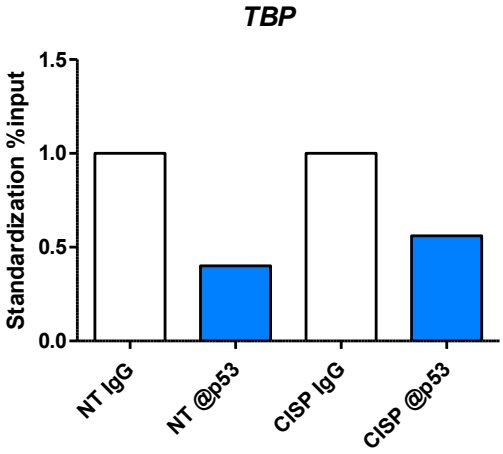
HSK-39 cells were treated with a combination of increasing concentration of LMK235 and Cisplatin and the cytotoxicity was evaluated by MTT after 48h of treatment. Combination indexes are in majority inferior to 0.80 (bold) indicating a synergistic effect between LMK-235 and Cisplatin on AGS cell survival.

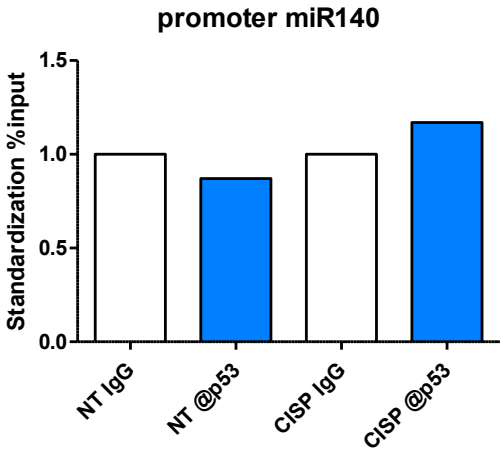


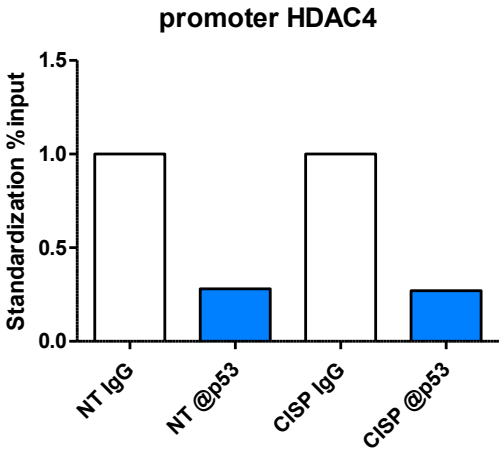
Supplementary figure 5: Schematic representation of the regulatory loop involving HDAC4, p53/p73 and miR-140 in the response of gastric cancer cells to cisplatin

Complementary results on Article A









Determined fixation site on HDAC4 promoter:

ATGCCTGGCTGTAATATCAGCACAGAGAACGTGATAGACAGCTGTATCACTAGAGGTTC**GTATGG**
GCAAAAGGCAAAGAAAAACAACGGCAAGG**AAGCATGTCA**GAATGTTACCAA**GGGCTTGTCT**GTG
TGTGATGGCATCATGGATGACTTTTTAAATACTTAACCTGTGATGCGCATGTATTGCTTTTATAAGC
AGAAAATGAAAACCTGAGAAAATGGTCATTA**TGAAGACAGAAACATGAGGGAA**CTATTTT

Determined fixation site on miR-140 promoter:

CGATCATGCCCCTCTCCAGCCAGGGCCC**CGCTGTCTAGCCCAGTGC**GCTCTGCACACTTTCTTG
GGCTAGGCTTCCCACCCATGCCTGCC**ACACATGCCT**GACATCCAATTC**TGGCATGTTT**CAGAATT
GGATGGAATCTGGTCTTTCACCACATTTCAGA**GATTGCTCCTTTTGAGCTGG**GCCCAAAAGATTT

<u>Complementary figure A1</u>: p53 does not target HDAC4 and miR140 promotor sequences

AGS cells were treated 6h with Cisplatin (40µM) or not (NT). Chromatin immunoprecipitation were performed using an antibody against p53 (@p53) or with mouse normal IgG as control to carry out a qPCR. Enrichment was calculated with the method %Input = $2^{(Ct_{input}-6{,}64-Ct_{ChIp})}$ x100. The promotor sequence of *p21* gene is used as a positive control, and TBP mRNA as a negative control. Are presented the possible p53 fixation sites using p53-scan. In red are the fixation sites, and in green the sequences to qPCR primer design.

TP53 and interaction on miR-140 and HDAC4 promoters

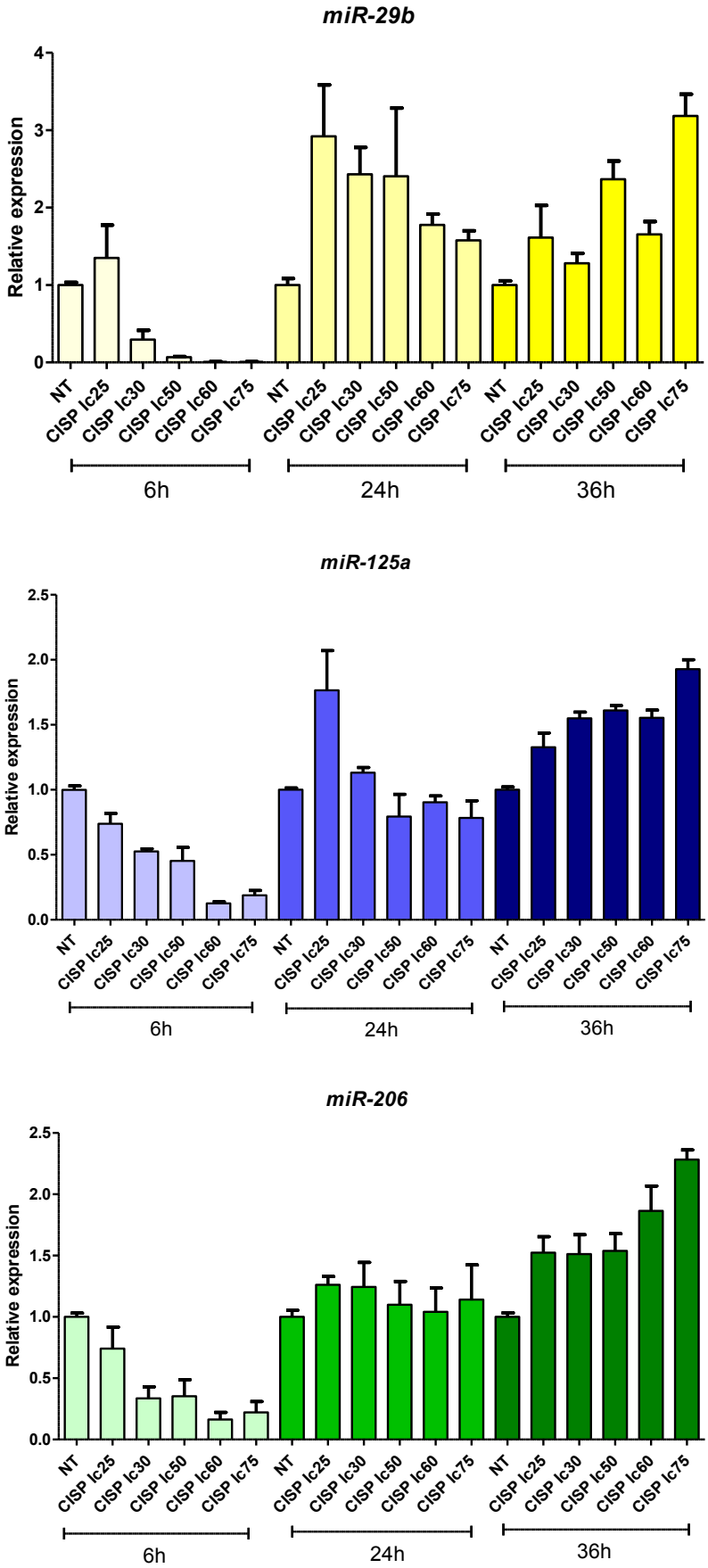
TP53 impacts miR-140 and *HDAC4* expressions. We hypothesized that TP53 binds directly on miR-140 and *HDAC4* promoter sequences. To determine p53 fixation sites, we looked for TP53 consensus sequence on promoters. We found one possible site of fixation on miR-140 and *HDAC4* promoter, thereby we generated the couple of primers specific to these genic regions to perform a chromatin immunoprecipitation followed by a qPCR **(Complementary figure A1).** We practiced the same analysis on *p21* promoter sequence that we used as a positive control and we used the common TBP primers for aspecific negative control. Unfortunately, in response to Cisplatin, there were no enrichment of miR-140 and *HDAC4* promoter sequence after p53 immunoprecipitation. Thereby, TP53 does not bind on the miR-140 and *HDAC4* promoter corresponding to genic region tested.

Other miRNAs targeting HDAC4 are induced by Cisplatin

Cisplatin reduces HDAC4 expression in AGS cells via miR-140 **(Figure 1D),** which is expressed by a dose-dependent manner only at early time (6h) **(Figure 3A).** HDAC4 mRNA level is still reduced at long time (24 and 36h) of Cisplatin treatment independently of miR-140 expression. We hypothesized that Cisplatin induces other miRNAs targeting HDAC4 expression taking over miR-140. We tested reported miRNAs: miR-29b, miR-125a and miR-206 *(Amodio N et al. 2016; Ma G et al. 2015; Kim HS et al. 2015)*. Interestingly, Cisplatin seemed to induce this three-miRNA expression by a dose-dependent manner after 36h of treatment and more importantly after 24h for miR-206 **(Complementary figure A2).** It remains to confirm the impact of these miRNAs in the significant decrease of the expression of HDAC4 in response to Cisplatin by a functional analysis.

HDAC4 suppression does not always induce apoptosis in response to Cisplatin

HDAC4 suppression promotes the cleavage of caspase 3 in response to Cisplatin, inducing AGS cell apoptosis **(Figure 5F).** Thereby, HDAC4 level controls in part AGS cell sensitivity to Cisplatin **(Figure 2A, B).** We wanted to test this hypothesis in other gastric cancer cell lines. We used intestinal type cancer cell lines; NUGC3, which are p53 mutated (Y220C), and we used diffuse type cancer cell lines; MKN45, which are bi-allelic WT/p53 mutated (R110C) **(Complementary figures A3 and A4).**



<u>Complementary figure A2</u>: Cisplatin induces miRNA targeting HDAC4 expression

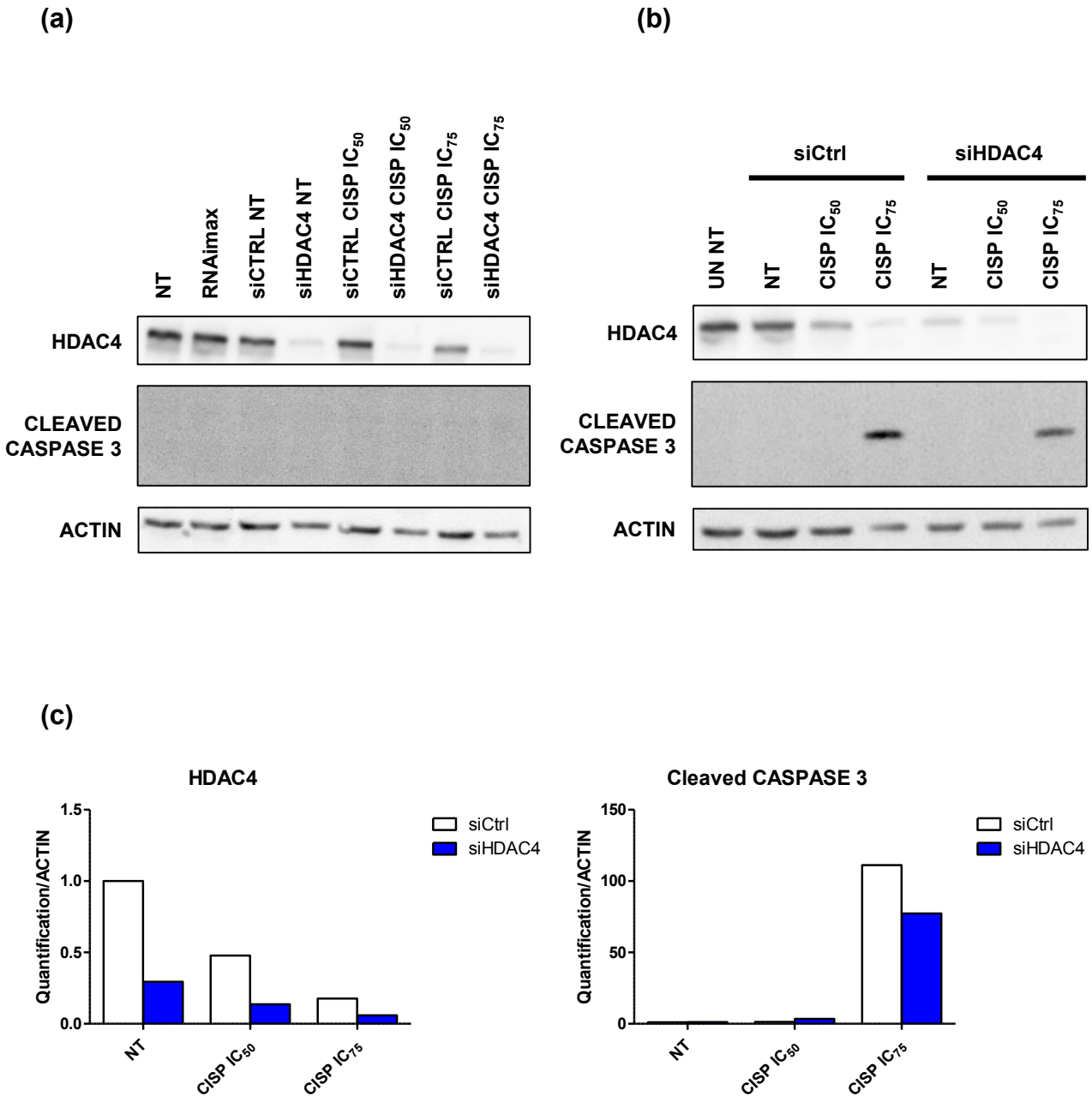
AGS cells were treated or nor (NT) with the different concentrations of Cisplatin (CISP) at the different times. microRNA levels were analyzed by RT-qPCR. Histograms represent the fold induction of miRNAs (Mean + SEM) for each condition normalized to an arbitrarily Ct.

We observed that Cisplatin did not induce a cleavage of caspase 3 at 24h but did at 48h of treatment indicating MKN45 cells apoptosis. Whereas Cisplatin promoted caspase 3 cleavage already at 24h, so apoptosis in NUGC3 cells. However, HDAC4 suppression seemed to decrease the level of cleaved caspase 3 in response to Cisplatin in MKN45 cells whereas it seemed to increase it in NUGC3 cells, although, the induction observed in NUGC3 cells is of less importance than in AGS cells **(Figure 5F).** These results suggest that HDAC4 suppression increases Cisplatin cytotoxicity in intestinal type of gastric cancer cells whereas it has the opposite effect in diffuse type of gastric cancer cells. Likewise, the result highlight that HDAC4 suppression increases more importantly Cisplatin efficiency in p53 WT than in p53 mutated intestinal type of GCC.

Unsupervised pathway analysis

The unsupervised pathway analysis (**Supplementary figure 3;** *DAVID: https://david.ncifcrf.gov; https://reactome.org)* showed that one of the pathway which gene expressions are negatively correlated with HDAC4 expression is apoptosis. This pathway is known to be one of the main targets of the TP53 and TAp73 transcription factors, which themselves are key factors in Cisplatin response *(Tomkova K et al. 2004; 2006; Vigelm AE et al. 2010).* The different genes obtained by the analysis are *BID, CASP6, BAK1, CASP3, AIFM1, CASP7, BAX, ENDOG, CYCS, CASP8, DIABLO, BIK, BCL2L1,* and *PMAIP1 (NOXA)*. We validated the negative correlation between HDAC4 and these related genes in our model of AGS cells **(Complementary figures A5-A8).**

HDAC4 suppression promoted AGS cell apoptosis in response to Cisplatin at 24h of treatment (Cleavage of caspase 3 by Western blot) **(Figure 5F).** Thereby, we analysed the expression of apoptotic related gene at an earlier time (8h). As previously observed, Cisplatin strongly reduced HDAC4 expression (90% reduced) **(Complementary figure A5).** Among gene negatively correlated with HDAC4, we significantly observed an induction (1,2-fold increased) of *BID, NOXA, BIK, CASPASE8* and *AIFM1* expressions when we supressed HDAC4 (siHDAC4) compare to the silencing control RNA (siCtrl) **(Complementary figures A5-A8).** In addition, Cisplatin reduced *CASPASE 8, AIFM1* and *BID* expressions but the suppression of HDAC4 less diminished CASPASE 8, AIFM1 and BID mRNA levels in presence of Cisplatin. Likewise, Cisplatin increased NOXA and BIK mRNA level and the suppression of HDAC4 enhance *NOXA* (2-fold increased) and *BIK* (1,4-fold increased) expressions in presence of Cisplatin by a dose-dependent manner. Moreover, we observed that HDAC4 suppression 1,3-fold increased *p21* expression suggesting a reduction of AGS



<u>Complementary figure A3</u>: HDAC4 suppression does not promote apoptosis in response to Cisplatin in MKN45 cells

(a) MKN45 cells were transfected with 50nM of a scramble siRNA (siCtrl) or of a siRNA against HDAC4 (siHDAC4) for 48h. Then cells were treated 24h or not (NT) with Cisplatin (CISP) at IC_{50} (5,8µM) or IC_{75} (20,5µM). **(b)** MKN45 cells were transfected with 50nM of a scramble siRNA (siCtrl) or of a siRNA against HDAC4 (siHDAC4) for 24h or not (UN). Then cells were treated 48h with Cisplatin (CISP) at IC_{50} (5,8µM) or IC_{75} (20,5µM) or not (NT). **(a-b)** Western Blots were performed using antibodies against HDAC4, Cleaved caspase 3 and Actin. **(c)** Graphics represent the relative quantification normalized to Actin of Western Blot in b.

cell proliferation, which correlates with the reported HDAC4 overexpression promoting *p21* repression and thereby SGC-7901 GC cells progression *(Kang ZH et al. 2014)*. However, we did not observe a significant difference of p21 mRNA level between siHDAC4 and siCtrl conditions in presence of Cispaltin suggesting that Cisplatin has dominant effects on *p21* expression, which is 4-fold increased.

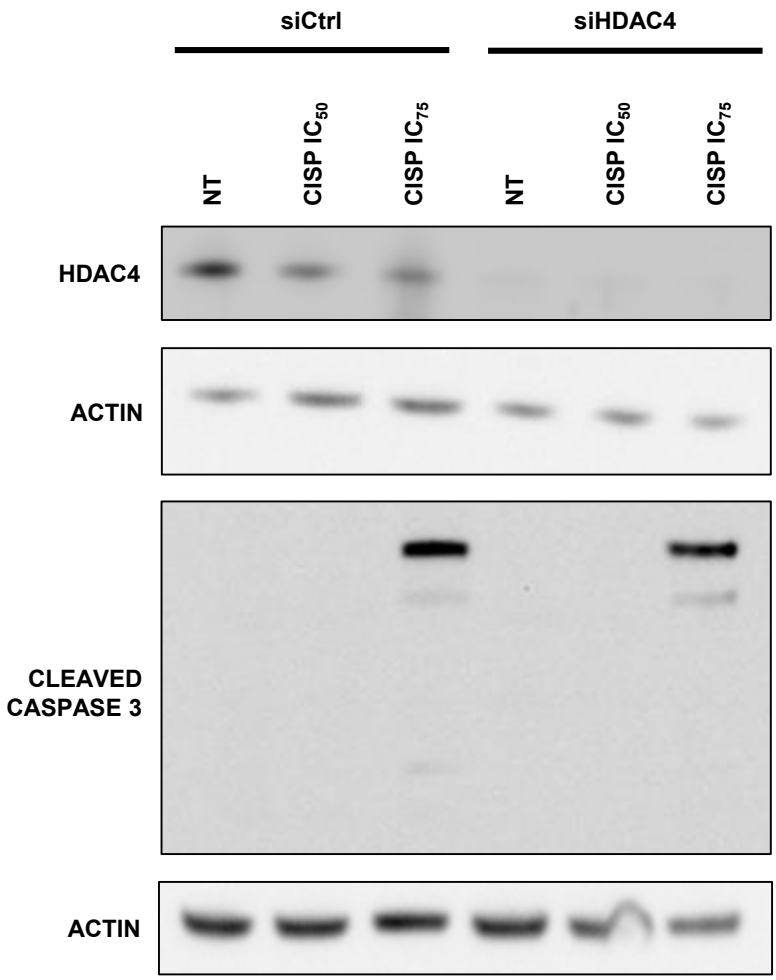
On the contrary, HDAC4 suppression did not impact significantly *BAX, BCL2L1, CASPASE 6, DIABLO* and *ENDOG* expressions. In addition, Cisplatin reduced *DIABLO* (10-20% decreased), *BCL2L1* (20-40% decreased) and *CASPASE 6* (10-20% decreased) expressions. Whereas Cisplatin enhanced 1,3-fold and 1,1-fold *BAK1* and *CASPASE 3* expressions, respectively. In addition, we did not observe a significant difference of *BAK1* expression between siCtrl and siHDAC4 conditions, but an expression decreasing trend was observed with HDAC4 suppression in absence or in presence of Cisplatin at IC_{50}. Moreover, we showed that HDAC4 suppression 10% decreased *CYCS* expression compare to silencing control RNA condition in presence Cisplatin at IC_{50}.

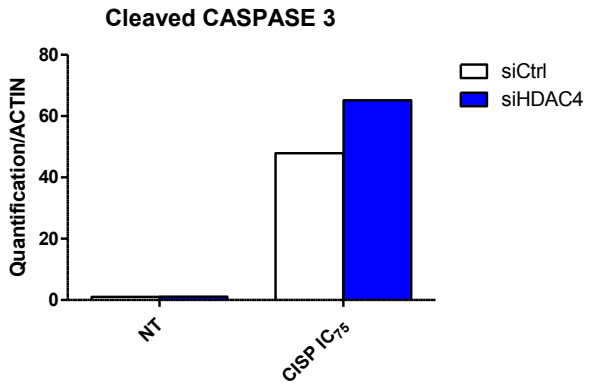
Altogether, we validated the negative correlation between *HDAC4* and *p21, BID, AIFM1, CASPASE 8, BIK* and *NOXA* expressions. We showed that Cisplatin reduced anti-apoptotic gene *BID, AIFM1, CASPASE 8,* and *BCL2L1* expressions, and that it increased cell cycle controller gene *p21* and pro-apoptotic gene *CASPASE 3, BIK* and *NOXA* expressions. Interestingly, we determined that *BIK* and *NOXA* expressions are potentialized with HDAC4 suppression correlating with the observed cleavage of caspase 3 and thereby with the apoptosis in response to Cisplatin.

HDAC4 correlated genes

Co-expression analysis in TCGA database *(http://www.cbioportal.org)* positively correlates PALD1 and ASB1 mRNA levels with HDAC4 expression with 0.82 and 0.75 Pearson's correlation, respectively, and negatively correlates VAMP8 and STXBP2 mRNA levels with HDAC4 expression with -0.51 and -0.47 Pearson's correlation, respectively, on a total of 2345 genes. We validated the positive and negative correlations between HDAC4 and these genes in our model of AGS cells **(Complementary figure A9).**

HDAC4 suppression 1,15-fold increased VAMP8 and 1,12-fold increased STXBP2 (1,12-fold increased) mRNA level. Moreover, Cisplatin treatment reduced these gene expressions and the differences were no longer visible between siCtrl and siHDAC4 conditions. In addition, HDAC4 suppression did not change *ASB1* and *PALD1* expressions in





<u>Complementary figure A4</u>: HDAC4 suppression does not promote apoptosis in response to Cisplatin in NUGC3 cells

NUGC3 cells were transfected with 20nM of a scramble siRNA (siCtrl) or of a siRNA against HDAC4 (siHDAC4) for 48h. Then cells were treated 24h with Cisplatin (CISP) at IC_{50} (2,5µM) or IC_{75} (56µM) or not (NT). Western Blots were performed using antibodies against HDAC4, Cleaved caspase 3 and Actin. Graphic represents the relative quantification of the cleaved caspase 3 normalized to Actin.

absence or in presence of Cisplatin, although Cisplatin reduced their expressions. However, we showed an increasing expression trend of ASB1 with HDAC4 suppression, whereas we showed a decreasing expression trend with HDAC4 suppression in presence of Cisplatin at IC_{50}. VAMP8 and STXBP2 are involved in vesicle membrane traffic pathway *(Messenger SW et al. 2014).* Interestingly, *STXBP2* was found to be upregulated (3.06-fold-change) in multidrug gastric cancer cell EPG85-257RDB *(Heim S and Lage H. 2005)* but the role of *STXBP2* and *VAMP8* in GC are not yet established.

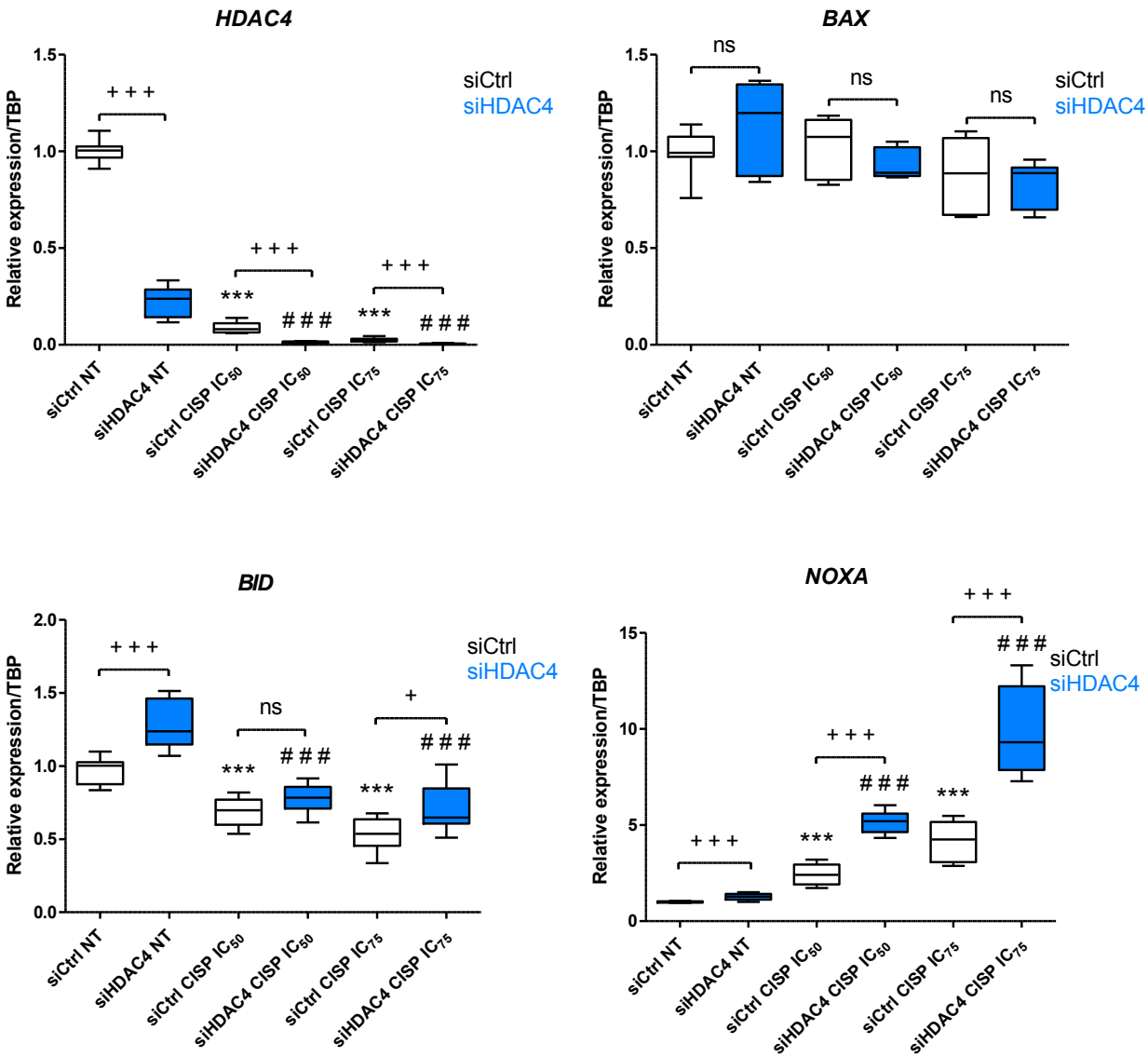
Altogether, we only validate the negative correlation between *HDAC4* expression and *VAMP8* and *STXBP2* expressions.

TP53 but not HDAC4 suppression impacts CYCLIN B2 expression

According to the literature, in response to DNA damage, p53 represses *CYCLIN B2* expression via recruiting HDAC4 at the promoter region in colon cancer cells inhibiting G2/M progression *(Basile V et al. 2006).* We wanted to test the depending p53/HDAC4 complex on *CYCLIN B2* expression in our model of AGS cells.

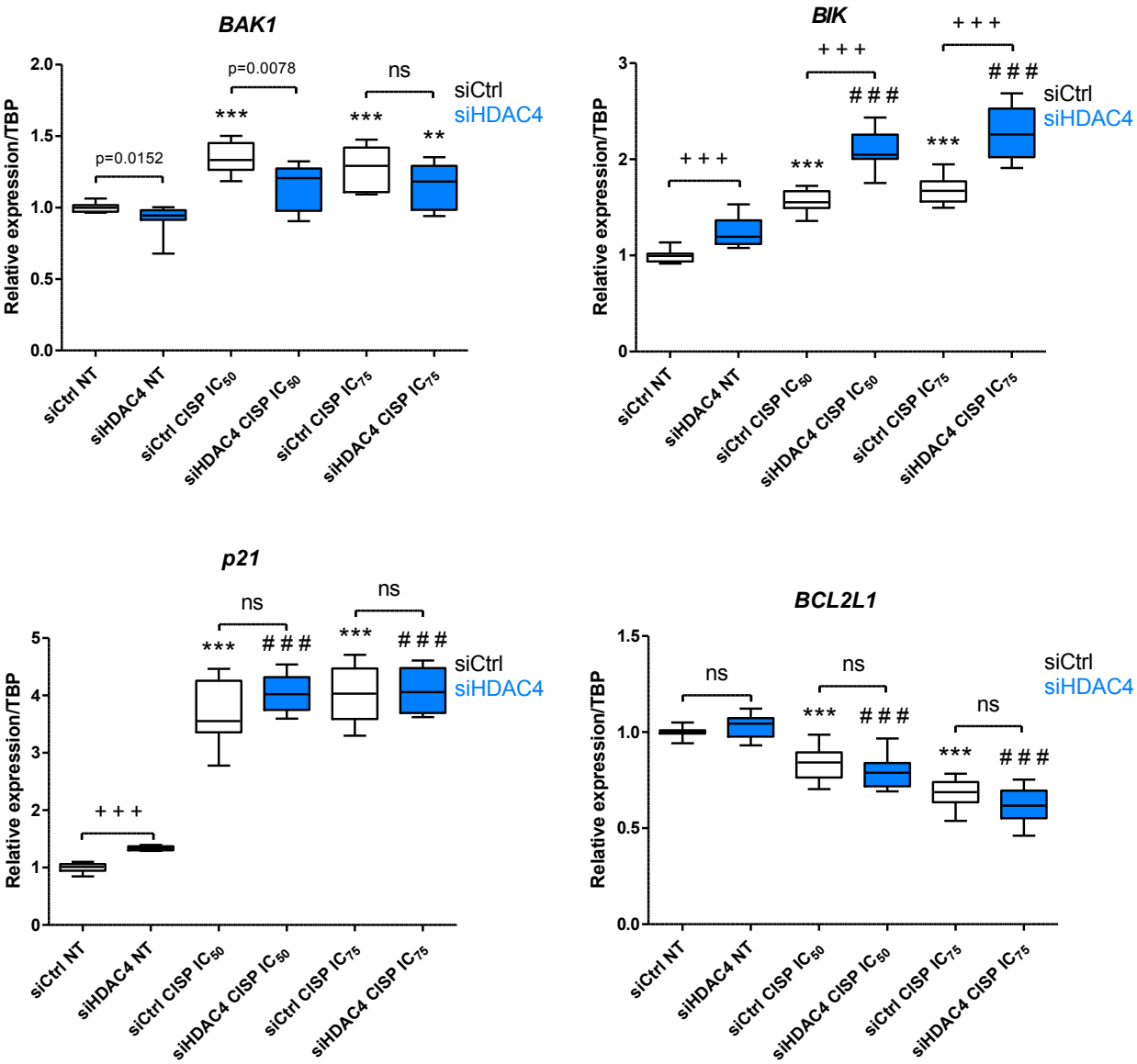
TP53 suppression 1,4-fold increased CYCLIN B2 mRNA level compare to siCtrl condition in absence or in presence of Cisplatin treatment **(Complementary figures A10-A11).** In addition, Cisplatin reduced CYCLIN B2 expression around 50%, which correlated with p21 induction described previously, and reducing cell proliferation. Interestingly, p53 suppression significantly 1,3-fold increased CDC2 mRNA level in absence of Cisplatin whereas in its presence CDC2 expression was 1,4-fold increased, but there were no significant differences with p53 suppression. On the contrary, HDAC4 did not impact *CYCLIN B2* and *CDC2* expressions. However, Basile and co-workers remarked that the suppression of HDAC4 is insufficient to invert the *CYCLIN B2* repression *(Basile V et al. 2006)* because HDAC4 is not the only HDAC associated with p53 in response to DNA damage, there are also HDAC1 before HDAC4 and HDAC5 *(Imbriano C et al. 2005).*

Our results may confirm the role of p53 in the G2/M progression and that suppression of HDAC4 does not impact *CYCLIN B2* and *CDC2* expressions *(Imbriano C et al. 2005; Basile V et al. 2006).*



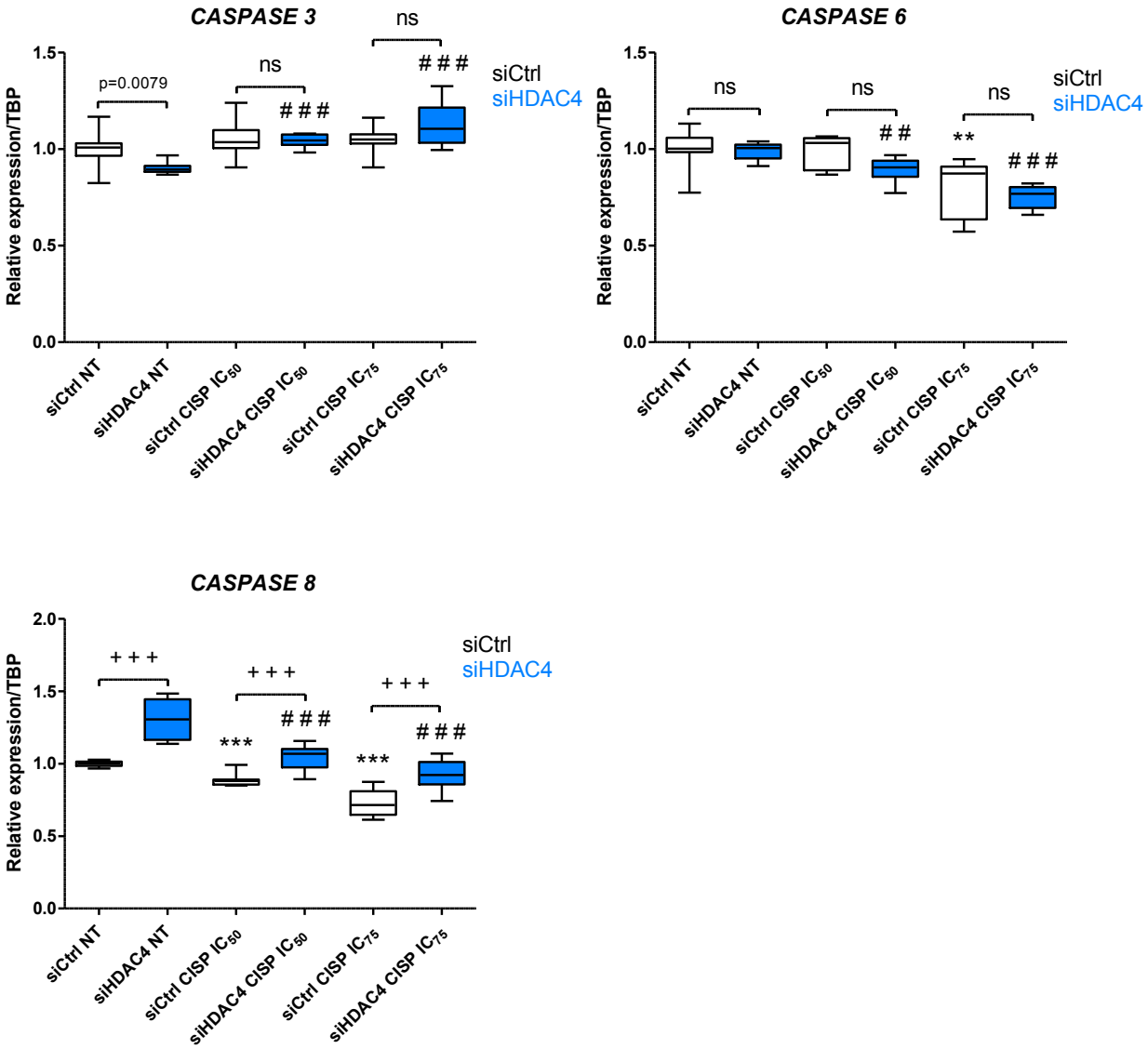
<u>Complementary figure A5</u>: Impact of HDAC4 on apoptosis pathway

AGS cells were transfected with 10nM of a scramble siRNA (siCtrl) or of a siRNA against HDAC4 (siHDAC4) for 48h. Then, cells were treated 8h or not (NT) with Cisplatin (CISP) at IC_{50} (25µM) or IC_{75} (40µM). HDAC4, BID, NOXA (n=4) and BAX (n=3) mRNA levels were analyzed by RT-qPCR. Box plots represent the fold induction of mRNA for each condition normalized to the control (siCtrl NT). Statistical differences were calculated by Mann-Whitney test with a correction as p-value limit p=0,0071. * vs siCtrl NT ; # vs siHDAC4 NT ; + siCtrl vs siHDAC4. For *BID*, statistical differences were calculated with ANOVA and Tukey post-test (Normal distribution and homoscedasticity were checked). * p< 0,05 ; ** p< 0,01 ; *** p< 0,001



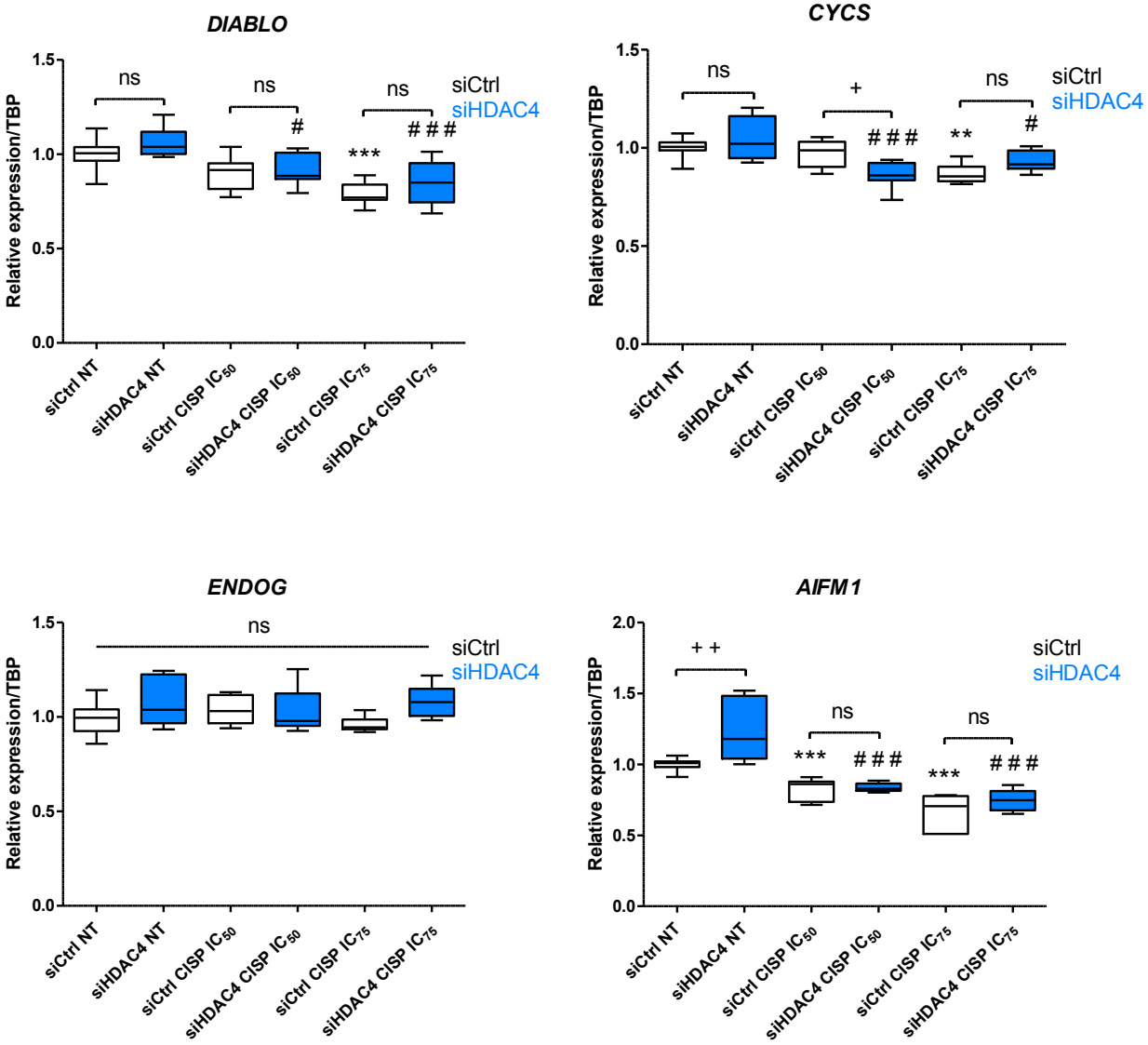
<u>Complementary figure A6</u>: Impact of HDAC4 on apoptosis pathway

AGS cells were transfected with 10nM of a scramble siRNA (siCtrl) or of a siRNA against HDAC4 (siHDAC4) for 48h. Then, cells were treated 8h or not (NT) with Cisplatin (CISP) at IC_{50} (25µM) or IC_{75} (40µM). BAK1, p21 (n=3), BIK and BCL2L1 (n=4) mRNA levels were analyzed by RT-qPCR. Box plots represent the fold induction of mRNA for each condition normalized to the control (siCtrl NT). Statistical differences were calculated by Mann-Whitney test with a correction as p-value limit p=0,0071. * vs siCtrl NT ; # vs siHDAC4 NT ; + siCtrl vs siHDAC4. ** p< 0,0071 ; *** p< 0,001



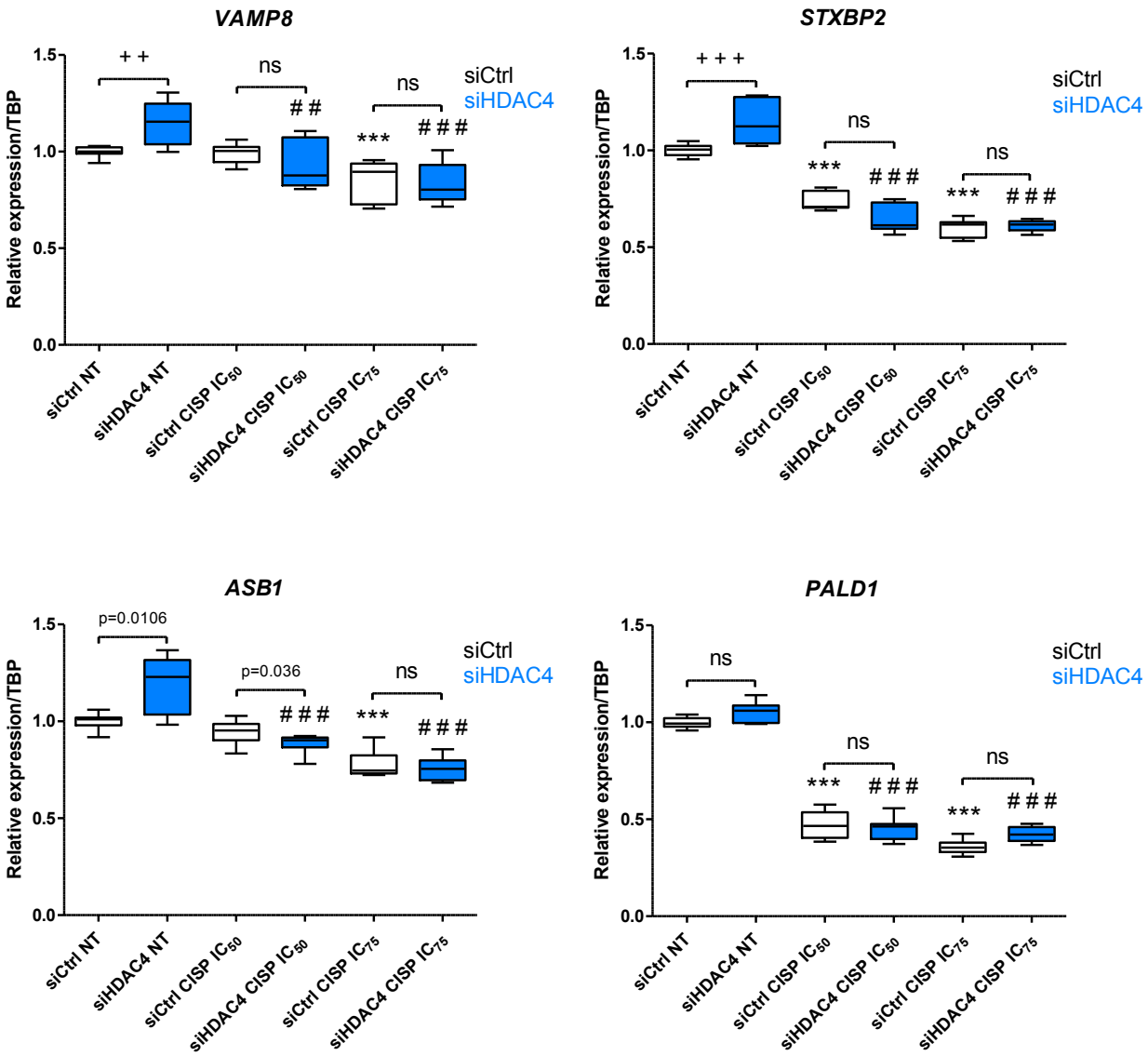
<u>Complementary figure A7</u>: Impact of HDAC4 on apoptosis pathway

AGS cells were transfected with 10nM of a scramble siRNA (siCtrl) or of a siRNA against HDAC4 (siHDAC4) for 48h. Then, cells were treated 8h or not (NT) with Cisplatin (CISP) at IC_{50} (25µM) or IC_{75} (40µM). CASPASE 3, CASPASE 6 (n=3) and CASPASE 8 (n=4) mRNA levels were analyzed by RT-qPCR. Box plots represent the fold induction of mRNA for each condition normalized to the control (siCtrl NT). Statistical differences were calculated by Mann-Whitney test with a correction as p-value limit p=0,0071. * vs siCtrl NT ; # vs siHDAC4 NT ; + siCtrl vs siHDAC4. ** p< 0,0071 ; *** p< 0,001



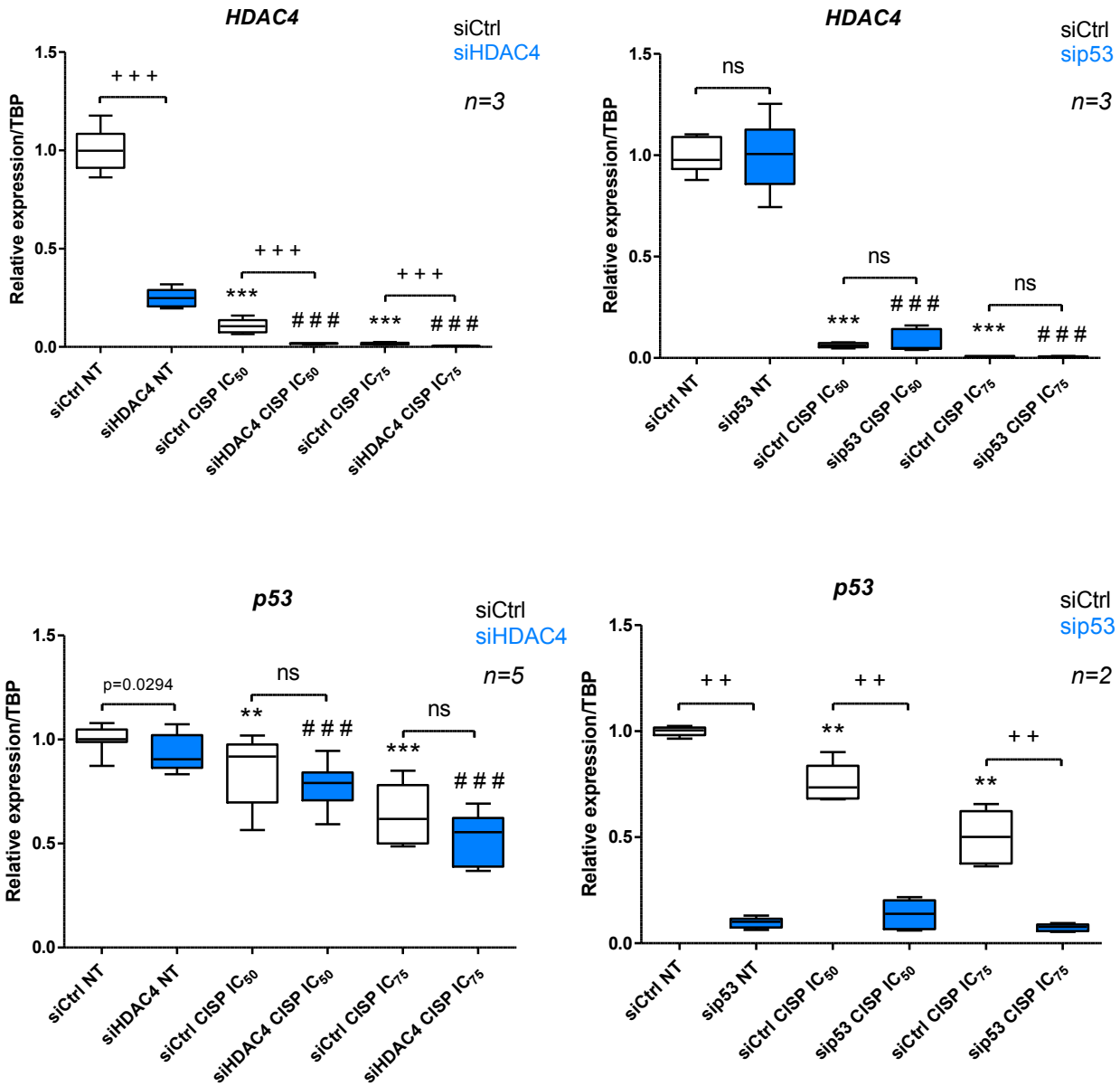
<u>Complementary figure A8</u>: Impact of HDAC4 on apoptosis pathway

AGS cells were transfected with 10nM of a scramble siRNA (siCtrl) or of a siRNA against HDAC4 (siHDAC4) for 48h. Then, cells were treated 8h or not (NT) with Cisplatin (CISP) at IC_{50} (25µM) or IC_{75} (40µM). DIABLO, CYCS and ENDOG (n=3) mRNA levels were analyzed by RT-qPCR. Box plots represent the fold induction of mRNA for each condition normalized to the control (siCtrl NT). Statistical differences were calculated with ANOVA and Tukey post-test (Normal distribution and homoscedasticity were checked). * vs siCtrl NT ; # vs siHDAC4 NT ; + siCtrl vs siHDAC4 ; * p< 0,05 ; ** p< 0,01 ; *** p< 0,001 For AIFM1, statistical differences were calculated by Mann-Whitney test with a correction as p-value limit p=0,0071. **p < 0,0071 ; ***p < 0,001



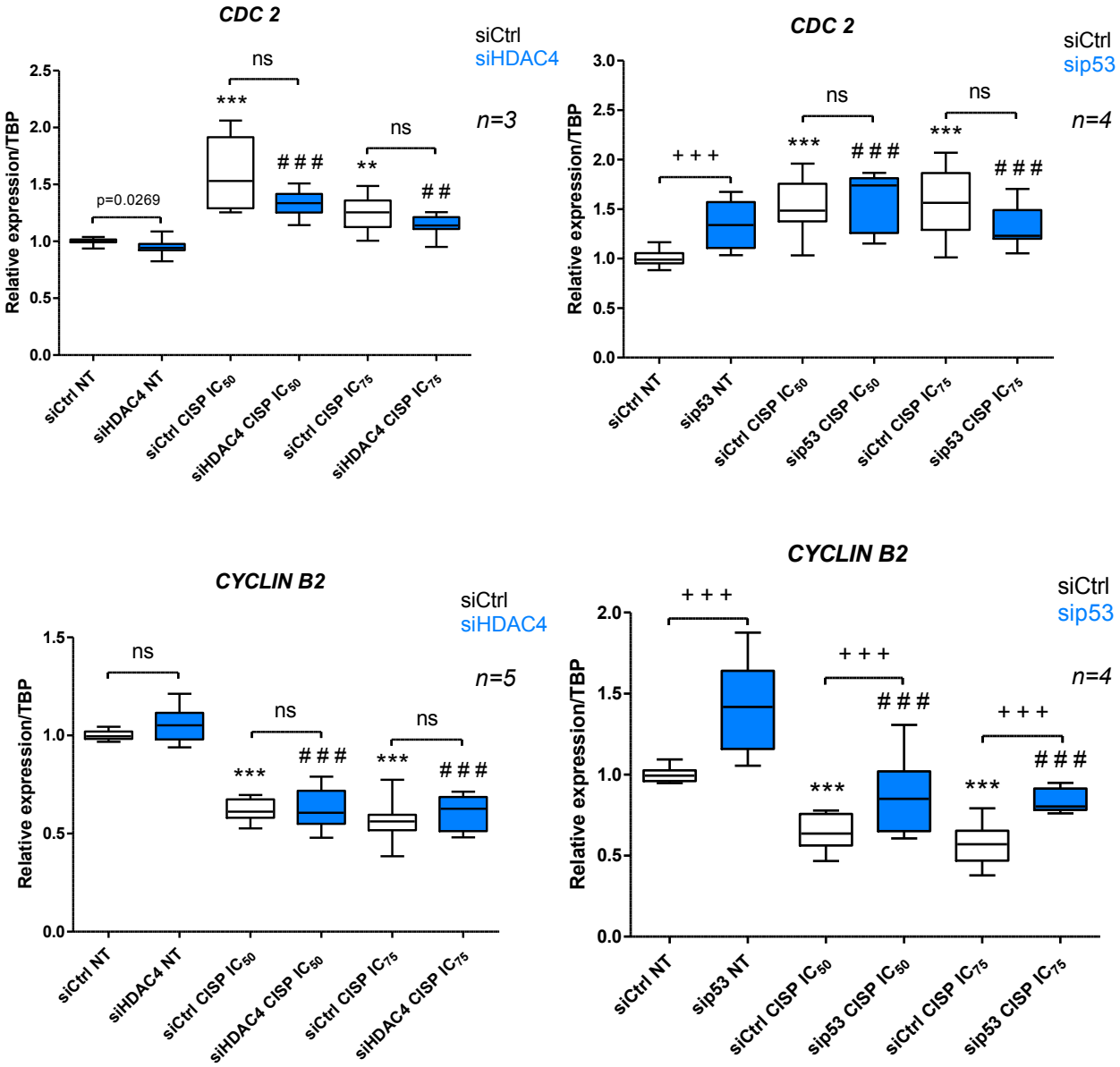
<u>Complementary figure A9</u>: Candidates of HDAC4 related genes

AGS cells were transfected with 10nM of a scramble siRNA (siCtrl) or of a siRNA against HDAC4 (siHDAC4) for 48h. Then, cells were treated 8h or not (NT) with Cisplatin (CISP) at IC_{50} (25µM) or IC_{75} (40µM). VAMP8, STXBP2, ASB1 and PALD1 (n=3) mRNA levels were analyzed by RT-qPCR. Box plots represent the fold induction of mRNA for each condition normalized to the control (siCtrl NT). Statistical differences were calculated by Mann-Whitney test with a correction as p-value limit p=0,0071. * vs siCtrl NT ; # vs siHDAC4 NT ; + siCtrl vs siHDAC4. For PALD1, statistical differences were calculated with ANOVA and Tukey post-test (Normal distribution and homoscedasticity were checked). * p< 0,05 ; ** p< 0,01 ; *** p< 0,001



<u>Complementary figure A10</u>: Impact of HDAC4 and p53 expression on each other

AGS cells were transfected with 10nM of a scramble siRNA (siCtrl) or of a siRNA against HDAC4 (siHDAC4) for 48h. Then, cells were treated 24h or not (NT) with Cisplatin (CISP) at IC_{50} (25µM) or IC_{75} (40µM). HDAC4 and p53 mRNA levels were analyzed by RT-qPCR. Box plots represent the fold induction of mRNA for each condition normalized to the control (siCtrl NT). Statistical differences were calculated by Mann-Whitney test with a correction as p-value limit p=0,0071. * vs siCtrl NT ; # vs siHDAC4 NT ; + siCtrl vs siHDAC4 ; ** p< 0,0071 ; *** p< 0,001



<u>Complementary figure A11</u>: Impact of HDAC4 and p53 on CDC2 and CYCLIN B2 expression

AGS cells were transfected with 10nM of a scramble siRNA (siCtrl) or of a siRNA against HDAC4 (siHDAC4) for 48h. Then, cells were treated 24h or not (NT) with Cisplatin (CISP) at IC_{50} (25µM) or IC_{75} (40µM). CDC2 and CYCLIN B2 mRNA levels were analyzed by RT-qPCR. Box plots represent the fold induction of mRNA for each condition normalized to the control (siCtrl NT). Statistical differences were calculated by Mann-Whitney test with a correction as p-value limit p=0,0071. * vs siCtrl NT ; # vs siHDAC4 NT ; + siCtrl vs siHDAC4 ; ** p< 0,0071 ; *** p< 0,001

Article B: The HDAC inhibitor SAHA (Vorinostat) inhibits p53 expression but synergies with platinum compounds to program gastric cancer cells into apoptosis via a p53-dependent pathway

The HDAC inhibitor SAHA (Vorinostat) inhibits p53 expression but synergies with platinum compounds to program gastric cancer cells into apoptosis via a p53-dependent pathway

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Abstract

In personalized medicine, the developments of combinatory therapies associating drugs that target different pathways, specifically deregulated in patient's tumors, drives hopes to treat resistant cancers while reducing side effects. The prerequisite to optimize this strategy is a precise understanding of the molecular basis of combinatory therapies, which can be different from monotherapies. In this study, we analyzed the molecular mechanisms involved in combinatory treatments of gastric cancer cells, associating the Cisplatin or Oxaliplatin-based standard treatment with a histone deacetylase inhibitor (HDACI) the suberoylanilide hydroxamic acid (SAHA). We showed that SAHA potentiated the cytotoxicity of both Cisplatin and Oxaliplatin in gastric cancer cells by reprograming the cellular response toward apoptosis as indicated by Caspase 3 cleavage. However, SAHA counteracted the induction of the tumor suppressor gene *TP53* at protein levels, through an inhibition of its transcription. In addition, SAHA impacted on the transcription of two other members of the p53 family: TAp63 and TAp73. Importantly, functional studies using siRNA showed that despite having its expression diminished, p53 was necessary for the induction of the cleavage of caspase 3 by SAHA and platinum drugs combined. In addition, results indicate that combined treatments impact HDAC4, MEF2 and CDX2 expressions which were reported to play a role in gastric cancer aggressiveness. Altogether, these results indicate that combinatory therapies associating SAHA and platinum drugs are promising strategies in gastric cancer treatment, but efficiency might be dependent upon the presence of functional p53 proteins.

Keywords: HDACI, SAHA, Vorinostat, p53, p63, p73, gastric cancer, Cisplatin, Oxaliplatin, apoptosis, autophagy, MEF2A, CDX2, HDAC4

Introduction

Gastric cancer (GC) is the fourth most common cancer for men, the fifth for women in the World, and represents the third cause of cancer-related death worldwide *(Michel P et al. 2017).* Surgical resection combined with perioperative chemotherapy using platinum-based compounds (PDC: Cisplatin, Oxaliplatin) is the cornerstone of current treatments. Although the use of chemotherapy has improved the survival rate of patients, the outcome stays unfavorable with a 30 to 40% response-rate to treatment and a median survival of only 6-9 months in advanced GC**.** Furthermore, significant side effects of platinum derivatives (f.e. nephrotoxicity, emetic activity, polyneuropathy) associated with the frequent development of resistances (*de novo* or acquired), limit their use and effectiveness *(Florea AM et al. 2011).* All in all, the identification of early prognosis markers and the development of alternative therapeutic strategies currently represent an absolute necessity.

One of the mechanisms of resistance to chemotherapies involves epigenetic modifications (histone acetylation/deacetylation, histone/DNA methylation) and post-transcriptional regulations (microRNAs). Fraga and co-workers have reported a loss in acetylation of the histone H4K16 and trimethylation H4K20 at repeated DNA sequences of tumor cells at early stage in tumorigenesis (*Fraga MF et al. 2005)*. Histone deacetylase enzymes (HDACs) are aberrantly expressed in various cancers such as gastric or colon cancer. The HDAC family is composed of 4 classes: class I (HDAC 1, 2, 3 and 8), IIa (HDAC 4, 5, 7 and 9), IIb (HDAC 6 and 10) and IV (HDAC11) which are Zn^{2+} dependent and class III (Sirtuins) which is NAD^{+} dependent *(Li Y and Seto E. 2017).* HDACs remove the acetyl group of lysine residues from histone and non-histone substrates, leading to chromatin compaction and decreasing gene transcription. They can also target non-histone substrates, such as the tumor suppressor gene p53, the Hypoxia Inducible Factor (HIF1α) and the α-tubulin, impacting on DNA affinity, transcriptional activity, protein stability and protein interactions, thus having a key role in different cellular pathways *(Zhang J and Zhong Q. 2014; Li Y and Seto E. 2017).* Moreover, HDAC expression is known to be linked to carcinogenesis *(Marks P et al. 2001).* HDAC1 can interact with p53, which reduces its binding capacity to the promoter of the pro-apoptotic gene *BAX*, thus favoring cancer cell survival, which is correlated with poor prognosis (*West AC et al. 2014).* For this reason, HDAC inhibitors (HDACIs) have been developed to counteract the pro-oncogenic activity of HDACs *(West AC et al. 2014; Li Y and Seto E. 2017).*

The HDACIs are classified in five groups, according to their chemical structures. These anticancer agents can induce different phenotypes including growth arrest, differentiation and apoptosis *(Li Y and Seto E. 2017).*

Among them, one of the most promising HDACI, a pan-inhibitor targeting class I-II-IV of HDACs called SAHA was approved by the FDA in 2006 for the treatment of cutaneous T-cell lymphoma. SAHA treatment shows an anticancer activity in a variety of tumor cell lines and tumor-bearing animals, with relatively no side effects on normal cells *(Kelly WK et al. 2005).* SAHA has not a massive effect on gene expression, as less than 2% of genes are hyperacetylated *(Komatsu N et al. 2006).* However, it has a specific action on genes involved in apoptosis, cell cycle, tumor suppression and differentiation *(Rikiishi H et al. 2007).* SAHA induces a p53-independent apoptosis through the mitochondria pathway *(Vrana JA et al. 1999)* but acts synergistically with p63/p73 *(Shim SH et al. 2010)* and is thought to overcome multidrug resistance in various cancer cell lines *(Lee MJ et al. 2008).* A gene signature analysis performed by Claerhout and co-workers suggests that SAHA is a good potential drug candidate in GC treatment, as it inhibits cellular growth and induces cellular death in gastric cancer cell lines *(Mutze K et al. 2010; Claerhout S et al. 2011; Yoon C et al. 2014),* involving RUNX3 *(Huang C et al. 2007),* MYC *(Labisso WL et al. 2012)* or SRC *(Zhou C et al. 2014).* In addition, the use of SAHA potentiated the activity of taxane anticancer drugs in gastric cancer cells *(Chang H et al. 2010).*

Cisplatin cytotoxicity depends on the formation of DNA adducts, which results in cell cycle arrest and the induction of apoptosis *(Florea AM et al. 2011).* The formation of these adducts requires a DNA accessibility, which can be altered by DNA-associated proteins such as histones. HDACIs are described as chemosensitizers for Cisplatin, through a reduction of chromatin condensation and therefore an improvement of DNA platination *(Kim MS et al. 2003; Lin CT et al. 2008; Davies NP et al. 2000).* The use of a combination of HDACI with currently-used chemotherapies, such a SAHA + Cisplatin / Oxaliplatin has been described in various types of cancers such as oral squamous cell carcinoma (OSCC), ovarian cancer or osteosarcoma *(Kim MS et al. 2003; Ong PS et al. 2012, Pettke A et al. 2016; Rikiishi H et al. 2007; Sato T et al. 2006).* In OSCC, the combination of SAHA and Cisplatin was associated with increased expression of markers (Caspase-4/12, eIF2a) involved in the endoplasmic reticulum stress pathway *(Suzuki M et al. 2009).* Some of the molecular mechanisms involved in the synergistic effect of these two drugs - especially regarding their combined influence on p53 - are still controversial. For instance, in gastric cancer cells, the combination of SAHA with Oxaliplatin reverses the Oxaliplatin-induced activation of the tyrosine kinase Src and decreases the phosphorylation of AKT, which is a major factor for cellular growth and survival *(Zhou C et al. 2014).* More recently, a phase I trial of SAHA combined with Cisplatin and capecitabine in patients with advanced gastric cancer showed an increased median overall survival time of 18 months, with manageable side effects *(Yoo C et al. 2014).*

Name	Characteristics*	Drugs	IC_{25} (µM)	IC_{30} (µM)	IC_{50} (µM)	IC_{60} (µM)	IC_{75} (µM)
AGS	Adenocarcinoma Diploid p53 WT MDM2 mutated/WT (bi-allelic) APC silent Kras mutated/WT (bi-allelic) CDH1 (G579fs9) homozygous CTNNB1 mutated/WT (bi-allelic) PIK3CA mutated/WT (bi-allelic)	SAHA	2	2,75	3,5	6	10
		Oxaliplatin	0,5	2,5	5	30	40
		Cisplatin	10	15	25	30	40
NUGC3	Adenocarcinoma Hypotriploid p53 mutated (Y220C)	SAHA	3	3,5	4	9,5	13
		Oxaliplatin	2,7	3,7	14	24	85
		Cisplatin	1	1,5	2,5	10	56
KATOIII	Pleural effusion Hypotetraploid p53 deleted K-sam amplification c-met amplification ERK amplification Cyclin E amplification APC deletion	SAHA	1	2	3	5	10
		Oxaliplatin	0,5	2	10	15	20
		Cisplatin	5	7,5	10	20	40

<u>Table S1:</u> SAHA and platinum derivative compounds (PDC) impact gastric cancer cell survival

Gastric cancer cells were seeded in 96-wells and treated 48h with cisplatin, oxaliplatin or SAHA (Vorinostat). Viability of the cells was evaluated using MTT tests. The table summarizes the different concentrations for each drug graphical determined (Figure 1). For example, IC_{25} represents the concentration where we have 25% of the total effect on cell survival. **ATCC ; Yokozaki H. Pathology International, 2000 ; Cancer Cell Lines, Part 2, Human Cell Culture, 2002 ; portals.broadinstitute.org/ccle*

The promising findings for the combination of SAHA with Cisplatin/Oxaliplatin suggest that it might be used as an alternative treatment in gastric cancer. However, the use of such a combinatory treatment requires a precise understanding of the mechanisms involved in this synergistic response and the identification of precise prognosis markers, allowing a better selection of responsive patients. Therefore, the aim of our study was to investigate the anticancer effect of the combination between SAHA and platinum-based compounds (Cisplatin or Oxaliplatin) in gastric cancer cell (GCC) lines and to explore the molecular pathways involved.

Results

Platinum derivative compounds and SAHA impact gastric cancer cell survival

The gold standard for the treatment of gastric cancer is chemotherapy using platinum derivative compounds (PDC: Cisplatin, Oxaliplatin). We studied the PDC effect on cancer cells for a better understanding of cellular resistance to treatment, to optimize it. To this end, we performed a microarray analysis between cancer cells treated with Cisplatin compared to a control condition, showing an impact of Cisplatin on the expression of epigenetic-related genes, especially HDACs *(Licona C et al. 2017).* We then investigated more precisely HDAC4, which appeared to be markedly deregulated in the response of gastric cancer cells to Cisplatin treatment. Indeed, HDAC4 protein level partially determined the sensitivity of gastric cancer cells to Cisplatin. We also show that patients presenting HDAC4 alterations have a better overall survival compare to patient without HDAC4 alterations *(Spaety et al. In submission; http://www.cbioportal.org)*. We performed the present study on GCC of the same intestinal cancer type; AGS (p53 WT), NUGC3 (p53 mutated Y220C) and KATOIII (p53 deleted) **(Table S1).** The response of cell lines to platinum compounds and HDAC inhibitor (SAHA) was first assessed by monitoring their survival using an MTT assay after 48 hours of treatment with increasing concentrations of Cisplatin or Oxaliplatin **(Figure 1a).** Cellular viability curves allowed us to determine the different IC_{25}, IC_{30} IC_{50} IC_{60}, and IC_{75} which are drug concentrations that respectively induced 25%, 30%, 50%, 60% and 75% of complete loss of cell viability and are reported in Supplementary table 1. AGS cells are more sensitive to Oxaliplatin than to Cisplatin whereas NUGC3 are more sensitive for Cisplatin and possess higher IC_{50} for Oxaliplatin. KATOIII cells are considered sensitive to Cisplatin and Oxaliplatin because they possess an IC_{50} inferior or equal to 10µM.

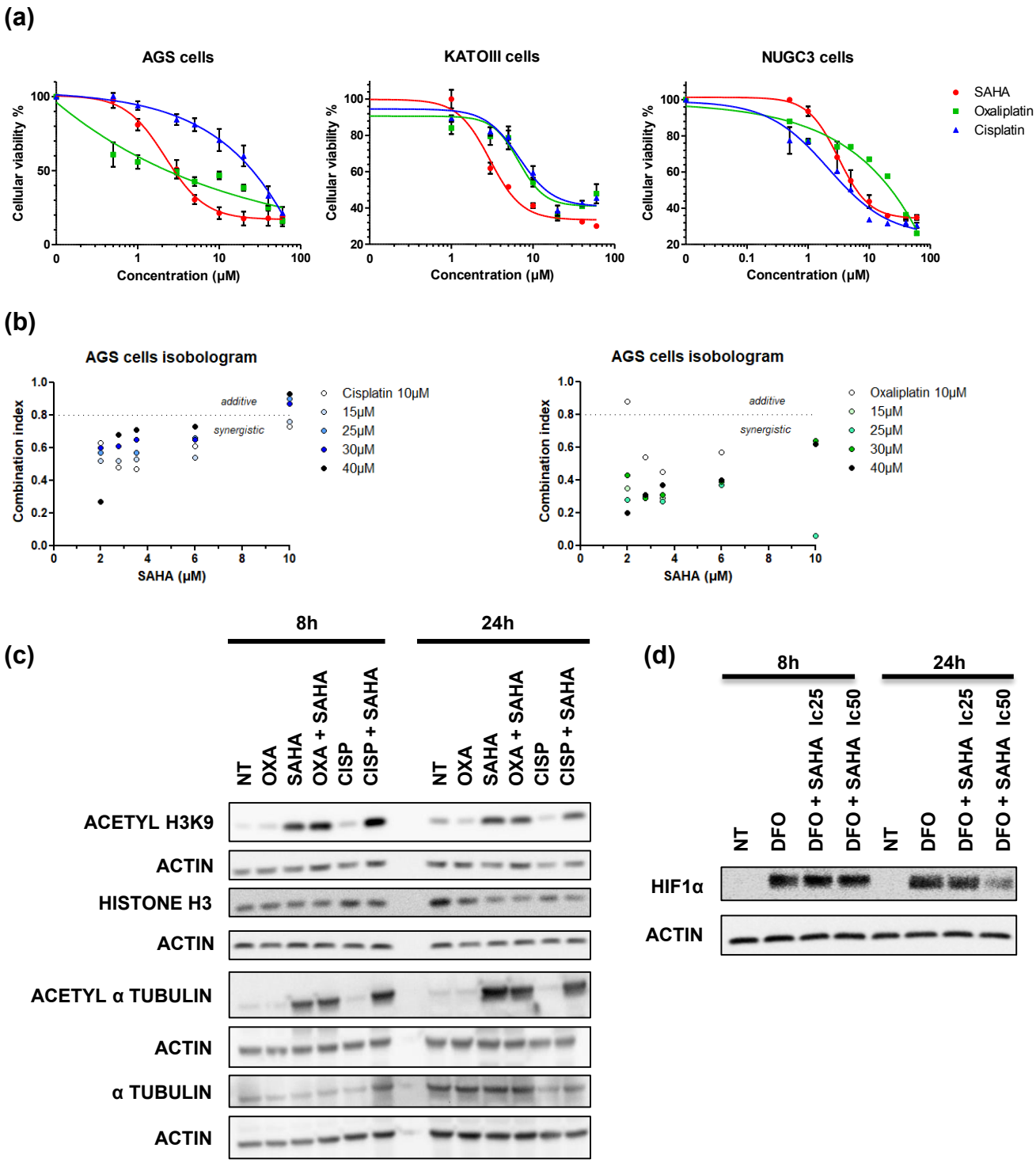


Figure 1: SAHA and platinum compounds impact gastric cancer cell survival

(a) Gastric cancer cells were seeded in 96-wells and treated 48h with Cisplatin, Oxaliplatin or SAHA (Vorinostat). Viability of the cells was evaluated using MTT tests. Curves represent the log (inhibitor) vs. response -- Variable slope (four parameters). Data are represented in % compared to the non-treated condition (n =3). **(b)** AGS cells were treated with Cisplatin (CISP) or Oxaliplatin (OXA) and SAHA (Vorinostat) for 48h followed by an isobologram assay to determine mathematically the combination index. We arbitrarily and conservatively considered antagonist effect between the drugs correspond to value > 1.20, additive effect between 0.80 and 1.20 and synergistic effect <0.80. **(c)** AGS cells were treated or not (NT) with the synergistic concentrations of cisplatin (CISP), oxaliplatin (OXA) and SAHA for 8h and 24h. Western blot experiments were performed using antibodies against acetylated Lysine K9 on Histone H3, against Histone H3 protein and antibodies against acetylated-alpha Tubulin and alpha Tubulin. **(d)** AGS cells were treated with Deferoxamin (DFO; 100µM) to mimic hypoxia condition and stabilize HIF1α. Additive treatement with SAHA was put for 8h or 24h or was not (NT). Western blot was performed with antibodies against HIF1α.

Synergy between platinum derivative compounds and SAHA

First, to establish the impact of HDACI on the cytotoxicity of platinum-based drugs in gastric cancer cells, we performed isobologram assays *(Chou TC. 2006; 2010).* AGS cells were treated for 48h with different concentrations of either Cisplatin or Oxaliplatin, combined with SAHA, followed by an isobologram assay to mathematically determine the combination index **(Figure 1b).** The combined treatments with both PDC and SAHA had in majority combinatory index below 1. These results indicated that combined treatments had synergistic effect on AGS cell survival **(Figure 1b).** Synergistic activities between Cisplatin and SAHA were obtained in others GCC KATOIII and NUGC3 **(Supplementary figure 1).** However, in KATOIII cells, the results were more heterogeneous with Oxaliplatin showing antagonistic effects when combined with SAHA except for IC_{50}. In NUGC3 cells, synergistic effects between Cisplatin + SAHA and between Oxaliplatin + SAHA were only observed at low concentrations of both drugs **(Supplementary figure 1).**

To verify the correct HDAC inhibition activity of SAHA in our models, we analyzed the acetylation status of the lysine 9 of histone H3 and on non-histone targets α-tubulin and HIF1α. The acetylation level of H3K9 is controlled by HDAC3 status *(Bhaskara S et al. 2010; Vecera J et al. 2018)* and is necessary for gene expression *(Gates LA et al. 2017).* As expected, SAHA induced a strong increase of H3K9 acetylation **(Figure 1c),** reflecting its HDAC inhibitor activity. Interestingly, PDC alone or combined with SAHA had not strong influence on H3K9 acetylation levels. Moreover, HDAC6 is required for EGFR-induced β-catenin nuclear localization *(Li Y et al. 2008),* HDAC6 controls autophagosome maturation *(Li JY et al. 2010)* and α-tubulin acetylation level *(Li G et al. 2011).* As expected, SAHA induced a strong increase of α-tubulin acetylation **(Figure 1c),** reflecting its HDAC inhibitor activity on HDAC6. Interestingly, PDC alone or combined with SAHA had not strong influence on α-tubulin acetylation levels as well. Furthermore, HIF1α is an important transcription factor involved in tumor metabolic switch, invasion and drug resistance *(Kitajima Y and Miyazaki K. 2013; Rohwer N and Cramer T. 2011).* HIF1α is regulated among other by HDAC enzymes modifying its stability and its activity (*Geng H et al. 2011; Joo HY et al. 2015).* Indeed, HDAC1 and HDAC3 enhance HIF1α stability and its transactivation function in hypoxic conditions and inhibition of these HDACs decreases HIF1α protein level and its transcriptional activity in human and mouse tumor cell lines *(Kim SH et al. 2007).* HDAC4 controls HIF1α stability and activity, indeed, HDAC4 inhibition (shRNA) inhibits HIF1α protein stability *(Geng H et al. 2011).* The position of lysine acetylation is important because acetylation at lysine 709 increases HIF1α protein stability *(Geng H et al. 2012).* As expected, SAHA reduced HIF1α stability promoting HIF1α protein level reduction **(Figure 1d).**

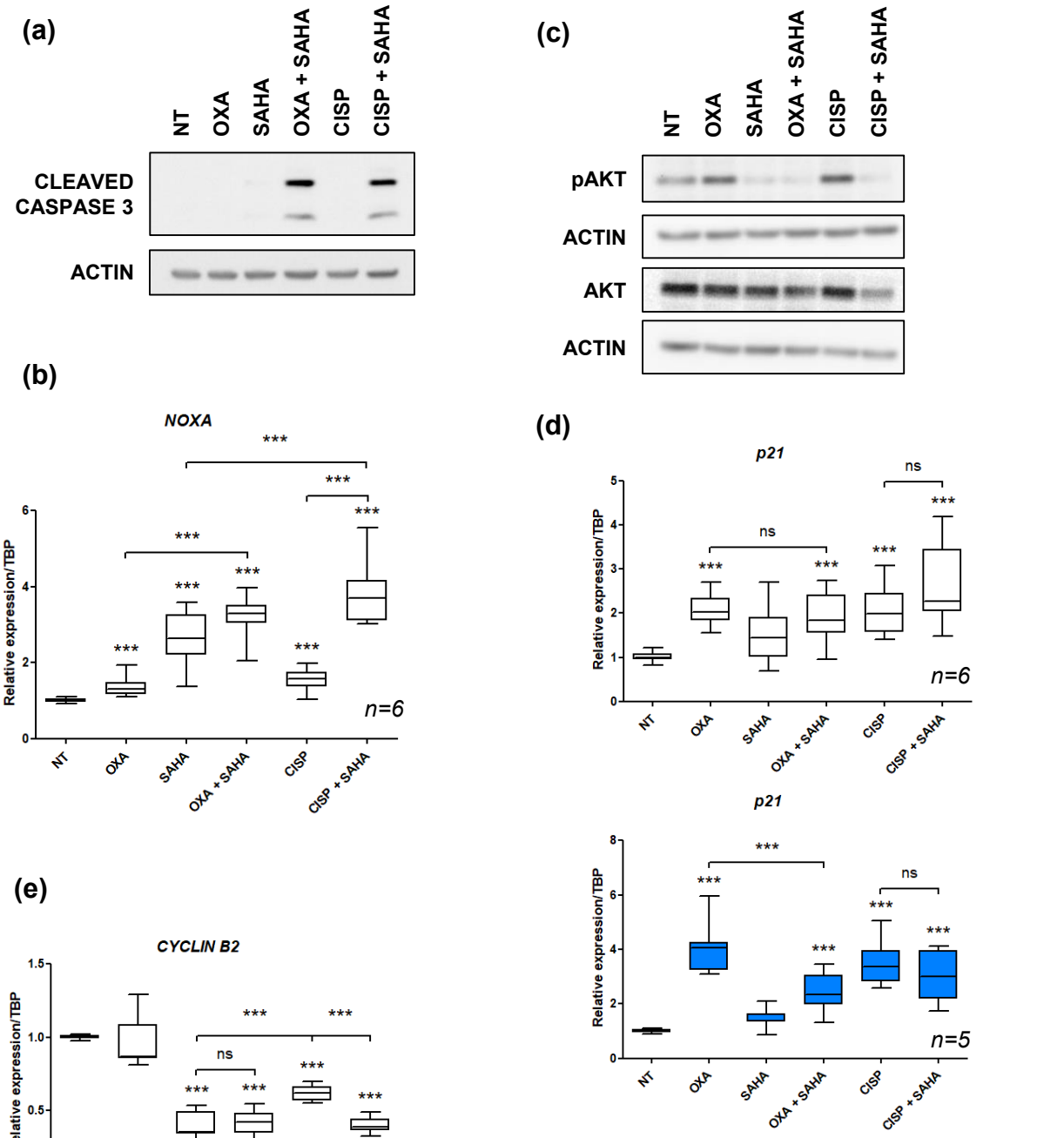


Figure 2: Combinatory treatments reduce AGS cell survival and proliferation and induce an apoptosis

AGS cells were treated or not (NT) with the synergistic concentrations of Cisplatin (CISP), Oxaliplatin (OXA) and SAHA (Vorinostat). **(a)** Western blot experiment was performed using antibodies against the cleaved Caspase 3 after 24h of treatment. **(b)** RT-qPCR were assayed after 8h of treatment. Box plots represent the fold induction of NOXA mRNA for each condition versus the control (NT). **(c)** Western blot experiment was performed after 24h of treatment using antibodies against the Akt protein or the phosphorylated Akt protein at serine 473 (pAkt). **(d-e)** RT-qPCR were assayed after 8h (white) or 24h (blue) of treatment. Box plots represent the fold induction of p21 and CYCLIN B2 mRNA normalized to the control (NT). *** p< 0.001 are calculated by Mann-Whitney test (p-value limit = 0,0033).

And SAHA decreased HIF1α activity on its target genes *VEGF* and *GLUT-1* **(Supplementary figure 2).**

Altogether, these results indicated that SAHA inhibits HDAC enzymes and that SAHA acts synergistically with PDC on GCC survival, but this depends on the PDC and the concentrations used in the different cell lines.

Combinatory treatments PDC + SAHA reduce AGS cell proliferation and promote apoptosis

To understand the molecular basis of the synergistic activity of SAHA combined with PDC in GCC, we analyzed the activation of markers for cellular death and cellular survival pathways. Thus, we looked at the level of cleaved Caspase 3, which reflects apoptosis **(Figure 2a).** AGS cells were treated for 8h and 24h, with SAHA, Cisplatin and Oxaliplatin used individually or combined at a synergistic concentration (combinatory index = 0.5 corresponding to Cisplatin = 15µM; Oxaliplatin = 0.5µM and SAHA = 2.75µM). Individual doses of Oxaliplatin or Cisplatin did not have a significant effect on the cleavage of Caspase 3 and SAHA alone only induced a very small increase of Caspase 3 cleavage after 24h of treatment. On the contrary, the combination of SAHA with either Cisplatin or Oxaliplatin strongly induced the cleavage of Caspase 3, suggesting that combinatory treatments favored a pro-apoptotic program in AGS cells **(Figure 2a).**

For a better understanding of the molecular mechanisms involved in the stimulation of apoptosis by the combination of SAHA and platinum-based compounds, we focused on survival, autophagic and apoptotic pathways **(Figure 2b-d).** We analyzed the expression of *NOXA, PUMA* and *BAX* involved in autophagy and apoptosis *(Shibue T et al. 2003; Yee KS et al. 2009; Liu YL et al. 2014).* We looked the protein level of phosphorylated Akt *(Matsuoka T and Yashiro M. 2014),* and expression of *p21* and *CYCLIN B2* involved in cell cycle regulation *(Ogasawara T. 2013)*.

Each drug increased NOXA mRNA level at 8h of treatment **(Figure 2b).** Oxaliplatin 1,2-fold increased, Cisplatin 1,5-fold increased and SAHA 2,6-fold increased *NOXA* expression. Combinatory treatments more enhanced NOXA mRNA levels. Indeed OXA + SAHA 3,2-fold increased and CISP + SAHA 3,6-fold increased *NOXA* expression correlating with the cleavage of caspase 3 observed at 24h of treatment. We had not a significantly induction of *NOXA* expression at 24h **(Supplementary figure 3).** Surprisingly, SAHA alone or combined with PDC reduced other pro-apoptotic gene *PUMA* and *BAX* expression at 8h of treatment **(Supplementary figure 3).**

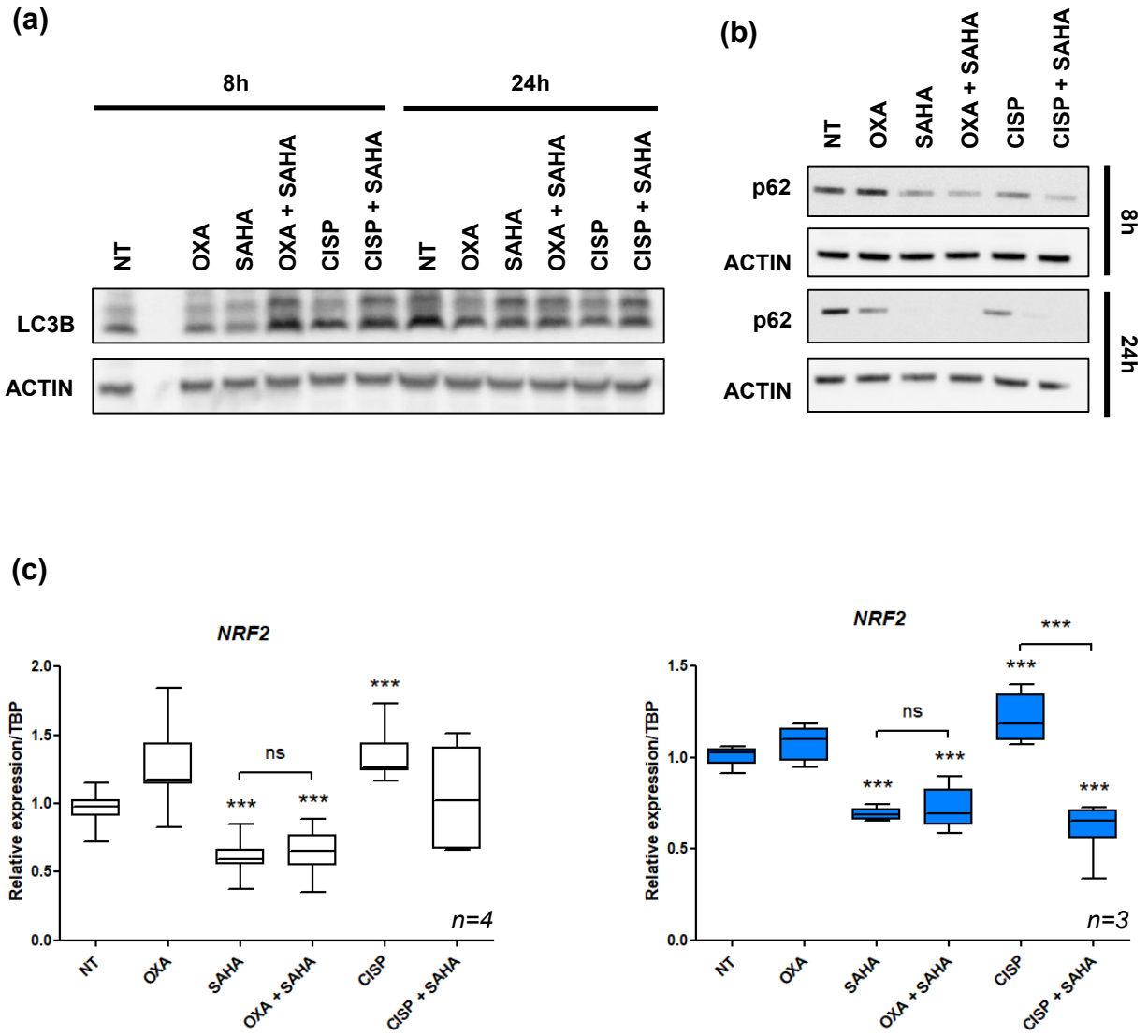


Figure 3 : Combinatory treatments do not promote autophagy in AGS cells

AGS cells were treated or not (NT) with the synergistic concentrations of Cisplatin (CISP), Oxaliplatin (OXA) and SAHA (Vorinostat). **(a)** Western blot experiment was performed after 8h or 24h of treatment using antibodies against the LC3B protein. The active form of LC3B (LC3B-II) is the bottom band and its quantification is represented in supplementary data. **(b)** Western blot experiment was performed after 8h or 24h of treatment using antibodies against p62. **(c)** RT-qPCR were assayed after 8h (white) or 24h (blue) of treatment. Box plots represent the fold induction of NRF2 mRNA normalized to the control (NT). *** p< 0.001 are calculated by Mann-Whitney test (p-value limit = 0,0033).

SAHA 50% reduced PUMA expression and Cisplatin alone or combined with SAHA 30% reduced it. Cisplatin also 30% reduced BAX mRNA level whereas it enhanced *NOXA* expression. SAHA had dominant effect on *BAX* expression and 70% reduced its expression.

Furthermore, SAHA alone or combined with PDC reduced pAkt level suggesting a decrease of pro-survival signaling pathway in AGS cells **(Figure 2c).** This correlated with the study of Zhou and co-workers indicating that AKT was downregulated when using combinatory treatment Oxaliplatin and SAHA in GCC *(Zhou C et al. 2014).* The reduction of pAkt correlated with the induction of *p21* expression (approximately 2-fold increased) with PDC treatments and their combinations with SAHA at 8h and 24h **(Figure 2d).** By the same, SAHA 50% reduced CYCLIN B2 mRNA level at 8h in AGS cells and the combination CISP + SAHA more significantly more decreased *CYCLIN B2* expression. At 24h, PDC and SAHA alone reduced *CYCLIN B2* expression (~ 50%) and combinatory treatments significantly more decreased Cyclin mRNA level (~ 70%) suggesting a reduction of AGS cell proliferation **(Figure 2e).** Otherwise, CDC2 which is associated with CYCLIN B during the cell cycle *(Guadagno TM and Newport JW. 1996)*, had its lowest expression level observed at 24h of combinatory treatment Cisplatin and SAHA (~ 50% decreased) **(Supplementary figure 3).** To illustrate the impact of combinatory treatments on cell proliferation we performed an immunocyto-staining of the proliferation marker Ki67. Combined treatments PDC + SAHA also strongly reduced the proliferation marker Ki67 in AGS cells and the number of cells (less observation of DAPI signal) **(Supplementary figure 4).** These results indicated that PDC + SAHA reduce AGS cell proliferation and induce apoptosis reducing AGS cell survival. A part of cells can still proliferate (are KI67 positive).

We were interested in the potential role of autophagy in treatment resistance or apoptosis induction. Indeed, autophagy can play pro-survival and pro-death role in gastric cancer *(Qian HR and Yang Y. 2016).* In our model of AGS cells, we looked ATG8 (LC3B) which is involved in the biosynthesis of autophagosomes, and p62 which is in a ubiquitin binding complex that serves as a selective autophagy substrate **(Figure 3ab)** *(Qian HR and Yang Y. 2016).* We also looked NRF2 expression which is involved in endoplasmic reticulum stress response and crosstalk to promote autophagy *(Digaleh H et al. 2013).* Combined treatments PDC + SAHA increased the protein level of LC3B active form at 8h **(Figure 3a; Supplementary figure 5).** However, we remarked that SAHA alone or combined with PDC reduces *p62* and *NRF2* expression **(Figure 3bc).** At the opposite, Cisplatin alone increased *NRF2* expression but SAHA had dominant effect on PDC. These results showed that we had not autophagy.

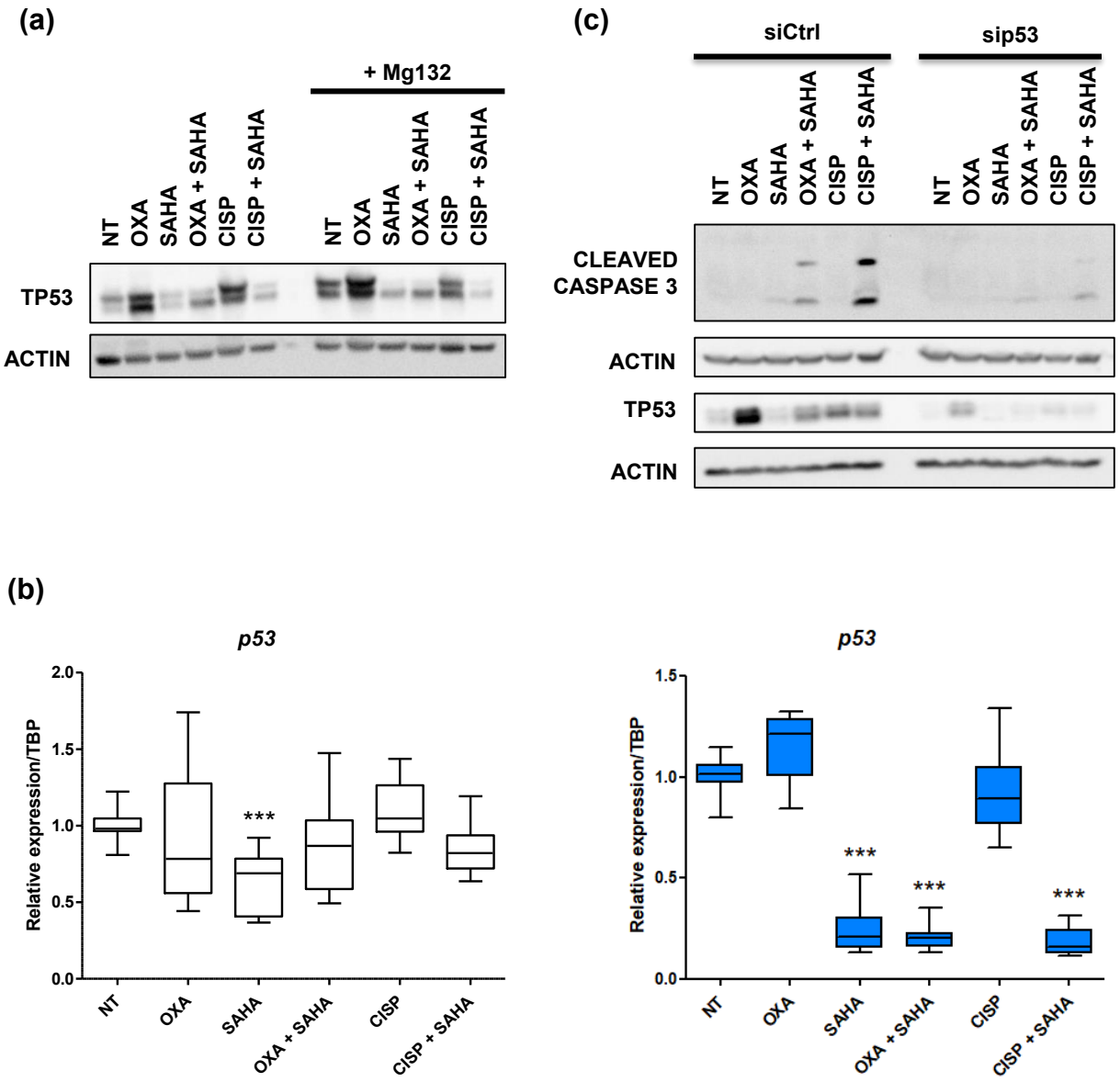


Figure 4: The induced apoptosis is p53 dependent in AGS cells

AGS cells were treated or not (NT) with the synergistic concentrations of Cisplatin (CISP), Oxaliplatin (OXA) and SAHA (Vorinostat). **(a)** Cells were treated 24h with synergistic combinations and with or without 10µM of Mg132 a proteasome inhibitor during 6h. Western blot experiment was performed using antibodies against p53. **(b)** RT-qPCR were assayed after 8h (white) or 24h (blue) of treatment. Box plots represent the fold induction of p53 mRNA for each condition versus the control (NT) (n=5). *** p< 0,001 are calculated by Mann-Whitney test (p-value limit = 0,0033). **(c)** AGS cells were transfected with control siRNA (siCtrl) or siRNA directed against p53 (sip53) for 48h. Cells were then treated or not (NT) with the synergistic concentrations of Cisplatin (CISP), Oxaliplatin (OXA) and SAHA (Vorinostat) for 24h. Western blot experiment was performed using antibodies against p53 and Cleaved Caspase 3.

Synergy reduces *TP53* expression, but the synergy-related apoptosis is p53 dependent in AGS cells

The tumor-suppressor gene *TP53* often mutated in gastric cancer, is a key regulator of cellular death and Caspase 3 activation *(Grabsch HI and Tan P. 2013; Mahu C et al. 2014)* and is known to be induced in response to Cisplatin *(Oren M et al. 2010; Osman AA et al. 2015; Rivlin N et al. 2011).* We analyzed *p53* expression in AGS cells to verify if the increased apoptosis caused by the combinatory treatment was mediated by p53. AGS cells were treated with the synergic concentrations of the different drugs for 24h. As expected, Cisplatin and Oxaliplatin induced p53 at protein level **(Figure 4a).** SAHA used alone slightly reduced the protein level of p53, but surprisingly, the combination of SAHA with Cisplatin or Oxaliplatin strongly inhibited the platinum-induced induction of p53 at protein level. TP53 expression is mainly regulated at post-translational level through ubiquitination and proteasome degradation. Therefore, we used the proteasome inhibitor MG132, to understand if this repression was due to a destabilization of p53 proteins. Unexpectedly, MG132 did not restore p53 protein levels in the presence of SAHA (alone or combined). Hence, we analyzed p53 mRNA levels using RT-qPCR. AGS cells were treated for 8h and 24h under the same experimental conditions **(Figure 4b).** mRNA levels for *TP53* were not affected by Cisplatin and Oxaliplatin. On the contrary, SAHA reduced *TP53* mRNA level at 8h and strongly decreased *TP53* mRNA level after 24h of treatment, a repression which was also observed with the combined treatment. These results indicated that the loss of p53 proteins with the combinatory treatments, involved a transcriptional regulation of the *TP53* gene.

We hypothesized that the apoptosis induced by PDC + SAHA combined treatments are p53 dependent. For a clarification of the role of p53 in the activation of caspase 3 induced by combinatory treatment using SAHA and platinum drugs in gastric cancer, we used siRNA directed against TP53 (sip53) or siRNA control (siCtrl) and assessed the protein level for p53 and the cleavage of Caspase 3 **(Figure 4c).** Surprisingly, the extinction of p53 expression strongly reduced the level of cleaved caspase 3 suggesting that even if combinations PDC + SAHA reduced p53 expression, p53 was necessary to induce AGS cell apoptosis.

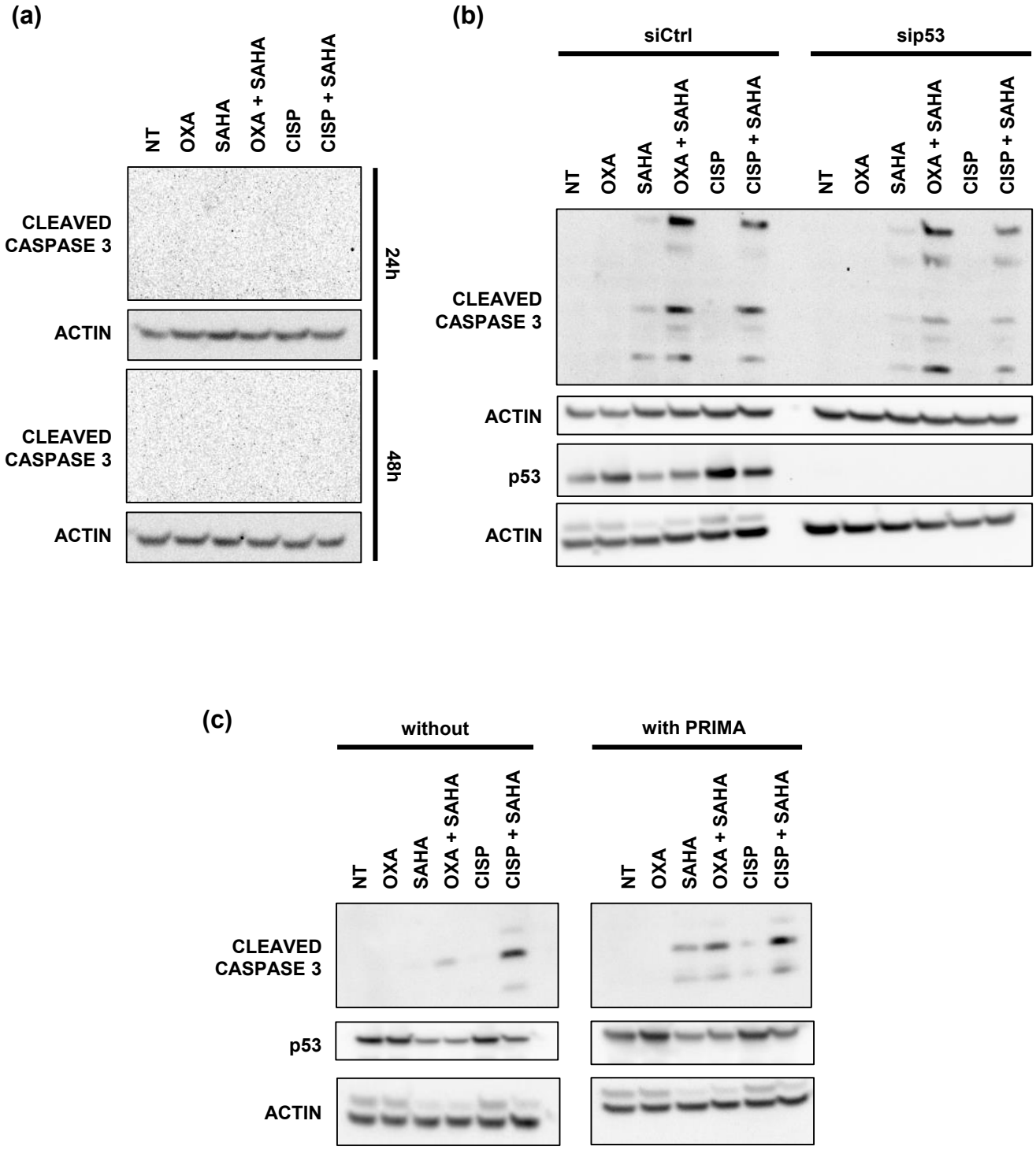
TP53 mRNA level was downregulated by SAHA alone at 8h. We hypothesized that the reduction of p53 mRNA level was due to miRNAs targeting p53 expression. According to miRTarBase *(http://mirtarbase.mbc.nctu.edu.tw/php/index.php)* and the literature *(Ishiguro H et al. 2014; Rokavec M et al. 2014; Liu J et al. 2017)* we analyzed the expression of potential miRNAs targeting p53 (miR-25, miR-30d, miR-125a, and miR-222) and a miRNA targeted by

p53 (miR-22) *(Tsuchiya N et al. 2011; Lin J et al. 2014).* To quantify miRNA expressions by the method $2^{(-\Delta\Delta Ct)}$ we tested the expression of reference miRNA (RNU6, SNORD65, and SNORD95) **(Supplementary figure 6).** Unfortunately, none of the reference miRNAs could be used because each treatment strongly reduced their expressions. We decided to use arbitrarily an identic Ct to calculate the relative expression **(Supplementary figure 7).** We didn't find a miRNA targeting p53 induced by SAHA alone at 4h, which would explain the reduction of *TP53* level at 8h **(Figure 4b).** At 8h, we saw variation in miRNA expression and it seemed that any miRNA candidate was induced with SAHA alone explaining the reduction of *TP53* mRNA level **(Supplementary figure 7).** miR-22 a target of p53 was induced at 4h of treatment of Oxaliplatin but was reduced with other treatments or combinations **(Supplementary figure 6).** At 8h, miR-22 seemed to be induce by all treatments but a variation was still present **(Supplementary figure 7).**

Altogether, these results indicated that combinatory treatments PDC + SAHA reduced *TP53* expression but the remaining p53 proteins are required for a full activation the apoptotic pathway. The p53 reduction seemed to be not due to the expression of miR-25, miR-30d, miR-125a and miR-222.

TP53 status drives apoptotic pathway in combined treatments in gastric cancer cells

As previously observed, Oxaliplatin and Cisplatin acts synergistically with SAHA on GCC survival **(Supplementary figure 1).** Previous results also indicated that combined treatments induced a p53-mediated apoptosis. As p53 is often mutated in gastric cancer, we wanted to study the effects of the combined treatment on KATOIII cells with a p53 deletion and on p53 mutated NUGC3 cells ($Y220C^{+/+}$) *(Liu X et al. 2013).* We first performed an isobologram assay on both cell lines to determine the synergistic concentration for platinum derivative compounds and SAHA **(Supplementary figure 1).** We found a strong synergy of the combination of SAHA with Cisplatin on cell survival in KATOIII cells and more heterogeneous effects when combined with Oxaliplatin. We were interested in the mechanisms involved in the reduction of KATOIII cell survival. Again, we focused study on synergistic combined treatments PDC + SAHA (combinatory index = 0.5). KATOIII cells were treated 24h or 48h with Cisplatin = 10µM; Oxaliplatin = 10µM and SAHA = 3µM. As hypothesized, we didn't observe a cleavage of caspase 3 neither at 24h nor at 48h of treatment in p53-deleted KATOIII cells **(Figure 5a)** validating our hypothesis. Then, we looked on proliferation marker Ki67 **(Supplementary figure 8)** and LC3B autophagic marker **(Supplementary figure 9).** Observations of Ki67 at 24h of treatment didn't clearly indicate a decrease of cell number (DAPI intensity) or Ki67.



<u>Figure 5</u>: Combined treatments PDC + SAHA have different impact on apoptosis in gastric cancer cells

(a-b) Gastric cancer cells were treated or not (NT) with the synergistic concentrations of Cisplatin (CISP) or Oxaliplatin (OXA) and SAHA (Vorinostat). **(a)** KATOIII cells were treated 24h or 48h. Then, western blots were performed using antibodies against Cleaved Caspase 3. **(b)** NUGC3 cells were transfected 48h with a siRNA directed to p53 (sip53) or with a scramble siRNA (siCtrl). Then, cells were treated 24h with the synergistic combined treatments and western blot experiments were performed using antibodies against Cleaved Caspase 3 and p53. **(c)** NUGC3 cells were pretreated or not with PRIMA-1^{MET} (44µM) during 8h. After, cells were treated with Cisplatin (CISP) or Oxaliplatin (OXA) and SAHA (Vorinostat) for 24h at the synergistic concentrations. Western blot experiments were performed using antibodies against Cleaved Caspase 3 and p53.

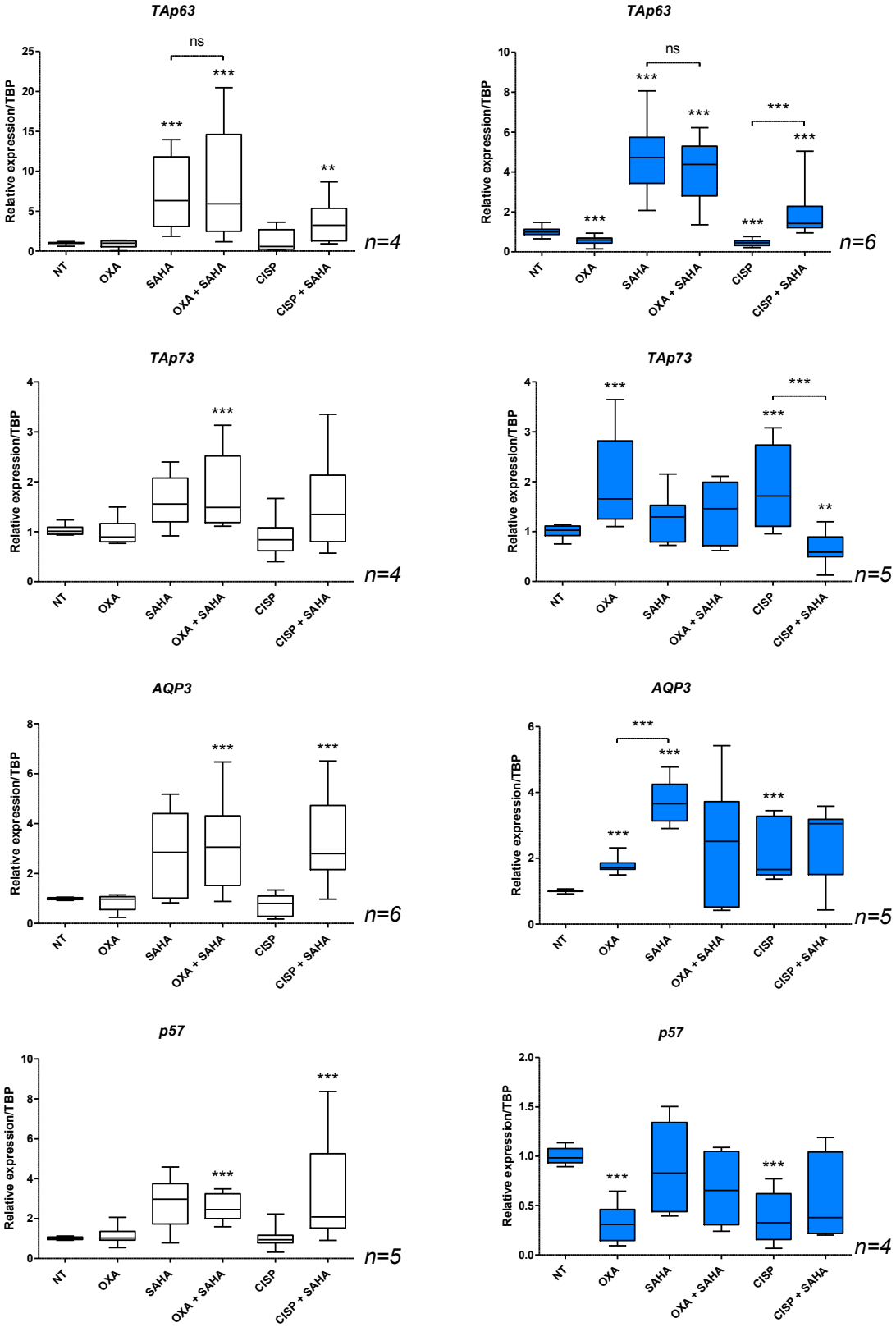
Ki67 intensity seemed to increase with Oxaliplatin or SAHA alone **(Supplementary figure 9).** Otherwise, SAHA neither combined to Oxaliplatin nor to Cisplatin induced the autophagic active form of LC3B (bottom band) **(Supplementary figure 10).** These results indicate that in KATOIII cells (p53 deleted), the combinatory treatments did not induce a caspase-dependent apoptosis but reduce cell survival by an undetermined pathway.

Afterwards, we compared the effects of combinatory treatments between AGS p53 WT cells and NUGC3 p53-mutated cells **(Figure 5b).** Again, we focused study on synergistic combined treatments PDC + SAHA (combinatory index = 0.5). NUGC3 cells were treated 24h with Cisplatin = 2.5µM; Oxaliplatin = 14µM and SAHA = 3.5µM. As expected, SAHA alone induced a cleavage of caspase 3, which was stronger when combined to platinum compounds, as we have observed on AGS cells. Unexpectedly, the inhibition of p53 expression by the siRNA did not suppress the cleavage of caspase 3 **(Figure 5b)** which is the opposite effect of AGS cells **(Figure 4c).** To determine the role of p53 in the induction of apoptosis, we used PRIMA-1 compound to reactivate p53 mutant *(Lambert JMR et al. 2009).* We performed the same experiment with combined drug concentrations for 24h with or without PRIMA-1 pre-treated cells during 8h **(Figure 5c).** We used a PRIMA-1 concentration of 44µM corresponding to IC_{50} determined by MTT survival test (data not shown). As expected, the p53 restauration mediated by PRIMA-1 compound increased the level of cleaved caspase 3 suggesting a reactivation of p53-mediated apoptosis in NUGC3 cells. Moreover, we also performed an immunostaining on Ki67 showing SAHA reduced cell number (less DAPI signal) and cell proliferation (less Ki67 signal) alone or combined with PDC illustrating the synergistic activity of combined treatments on NUGC3 cells **(Supplementary figure 10).**

Altogether, these results indicated that combined treatments reduce GCC survival by p53 dependent and independent manners. TP53 status drives the apoptotic pathway in response to combined treatments in GCC.

Combined treatments impact TP53 family expression in AGS cells

TP53 is the founding member of the p53 family that also contains p63 and p73, which are also known to induce apoptosis *(Arrosmith CH, 1999; Pietsch EC et al. 2008).* We have previously shown that these transcription factors are induced and can mediate the response to anticancer drug *(Benosman S et al. 2011; Von Grabowiecki Y et al. 2016).* These three genes encode two classes of isoforms either containing a transactivation domain in the N terminus (p53, TAp63, TAp73) or not (Δp53, ΔNp63, ΔNp73). These proteins are thought to be involved in many aspects of the digestive system cancers *(Zaika AI and El-Rifai W. 2006)*



<u>Figure 6:</u> Combinatory treatments impact p63 and p73 expression and p73 target gene expression in AGS cells

AGS cells were treated or not (NT) with the synergistic concentrations of Cisplatin (CISP), Oxaliplatin (OXA) and SAHA (Vorinostat). RT-qPCR were assayed after 8h (white) or 24h (blue) of treatment. Box plots represent the fold induction TAp63, TAp73, AQP3 and p57 mRNA normalized to the control (NT). ** p< 0,0033; *** p< 0.001 are calculated by Mann-Whitney test (p-value limit = 0,0033).

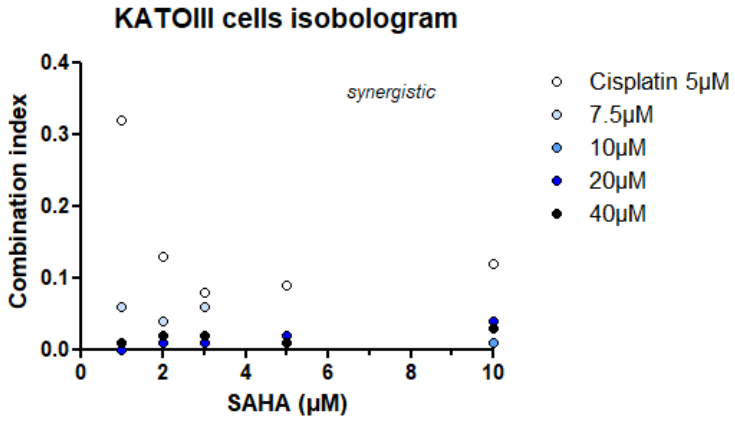
and TAp63 and TAp73 isoforms are commonly seen as pro-apoptotic transcription factors *(Wei J et al. 2012)*.

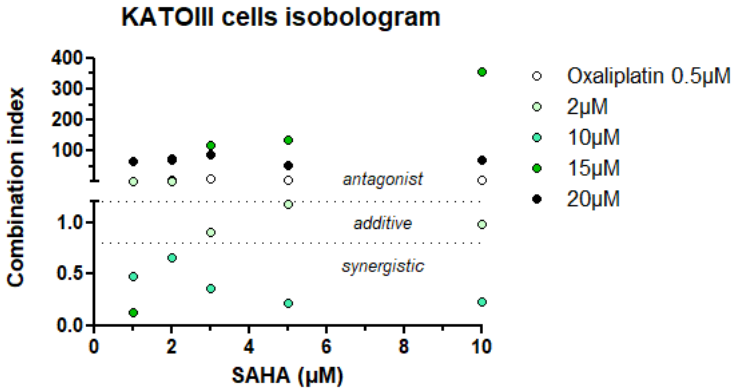
Thus, we decided to assess whether the combinatory treatment SAHA/platinum drugs would impact on the expression of these isoforms in AGS cells. At 8h of treatment, Cisplatin and Oxaliplatin alone did not induce TAp63 and TAp73 mRNA levels in AGS cells **(Figure 6).** On the contrary (compared to its effect on p53) SAHA increased *TAp73* mRNA level when combined with Oxaliplatin after 8h of treatment, while the combination with Cisplatin seemed but did not significantly impact on p73 mRNA expression level. SAHA alone and combined with Oxaliplatin and Cisplatin induced TAp63 mRNA level after 8h of treatment, while the single use of Cisplatin did not significantly impact on p63 mRNA expression level. At 24h of treatment the same effects were observed for *TAp63*, whereas for *TAp73* we observed an increase with platinum compounds alone, but none with SAHA and a decrease with Cisplatin + SAHA combination **(Figure 6).** As expected, TAp73 target genes *AQP3* and *p57* had their expression increased when *p73* expression were higher. Interestingly, the combination Cisplatin with SAHA significantly up-regulated *AQP3* and *p57* while it was not the case for p73. SAHA alone induced AQP3 expression at 24h **(Figure 6).**

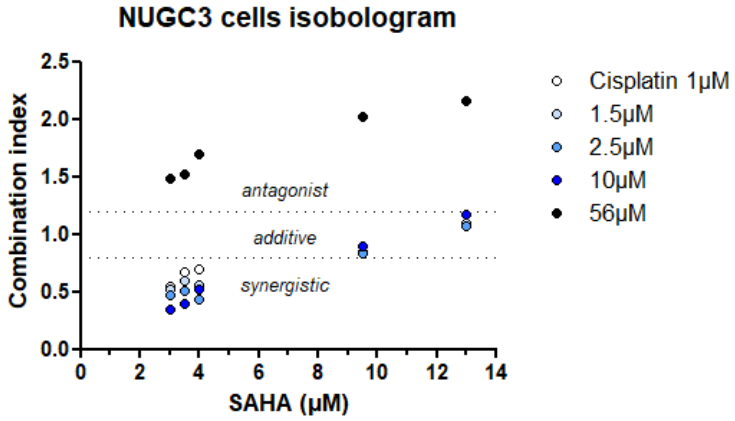
Altogether, these results indicated that Cisplatin, Oxaliplatin, SAHA and the combined treatments of SAHA and platinum compounds had different impacts on the expression of the various members of the p53 family which may promote apoptosis.

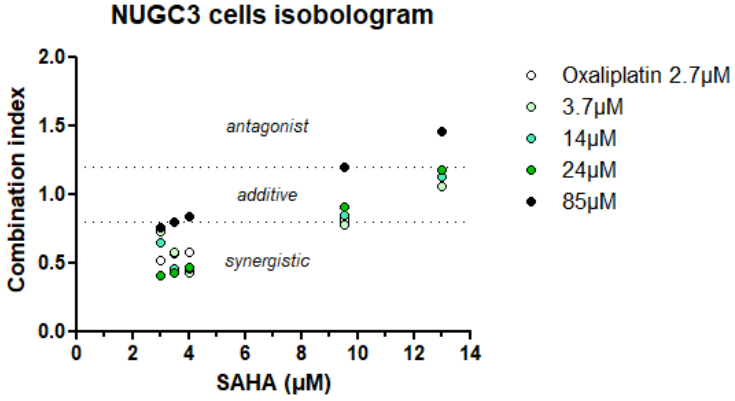
Combined treatments impact HDAC4, CDX2 and MEF2 expressions

In a previous study, we determined the role of HDAC4 level in Cisplatin sensitivity of AGS cells *(Spaety ME et al. Submitted).* Cisplatin reduced HDAC4 mRNA level and SAHA did not change this **(Supplementary figure 11a).** Oxaliplatin and SAHA alone or combined did not change HDAC4 expression in AGS cells. Nonetheless, at 24h, treatments reduced HDAC4 protein level, but the higher effect was observed with combination CISP + SAHA **(Supplementary figure 11b).** According to our previous study, this important reduction of HDAC4 protein level may explain the reduction of AGS cell survival *(Spaety ME et al. Submitted).* Previously, we determined an auto-regulatory loop in response to Cisplatin between p53 and HDAC4 *(Spaety ME et al. Submitted).* We wanted to know if the reduction of *HDAC4* expression is due to miRNA. miR-22 is targeted by p53 *(Tsuchiya N et al. 2011; Lin J et al. 2014)* and interestingly HDAC4 is a target of miR-22 *(Lu W et al. 2015).* But it was not induced neither at 4h nor 8h with combined treatment CISP + SAHA **(Supplementary figures 6 and 7)** so it is not involved in *HDAC4* expression reduction.









Supplementary figure 1: Combinatory treatments impact differently gastric cancer cells

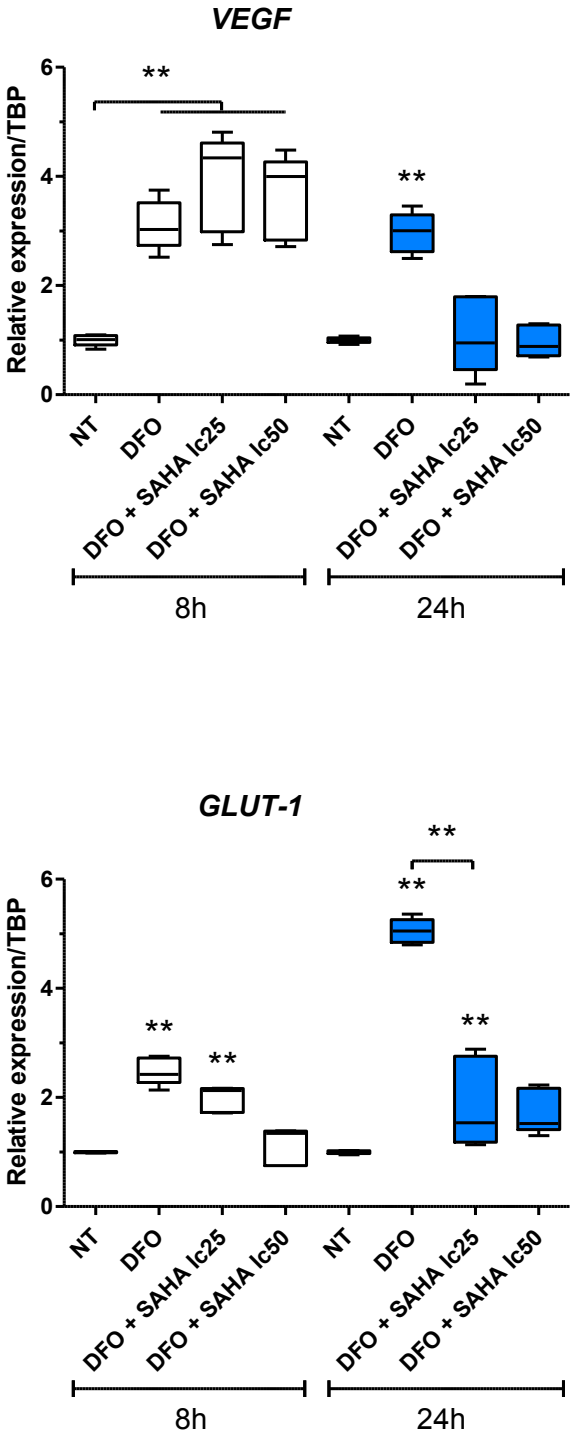
Gastric cancer cells were treated at the indicated concentrations of Cisplatin (CISP) or Oxaliplatin (OXA) and SAHA (Vorinostat) for 48h followed by an isobologram assay to determine mathematically the combination index. We arbitrarily and conservatively considered antagonist effect between the drugs correspond to value > 1.20, additive effect between 0.80 and 1.20 and synergistic effect <0.80.

Then, we looked two markers which can be associated to the aggressiveness of GC; CDX2 and MEF2. *CDX2* is a homeobox gene coding for a transcriptional factor involved in intestinal identity and morphogenesis *(Freund JN et al. 2015).* *CDX2* acts as a tumor suppressor when it is expressed in the intestine *(Gross I et al. 2008; Platet N et al. 2017)* whereas its ectopic expression in gastric cells induces gastric intestinal metaplasia in transgenic mice *(Silberg DG et al. 2002).* Moreover, *CDX2* overexpression promotes gastric cancer cells multidrugs resistance *(Yan LH et al. 2014)* describing *CDX2* as a prognostic marker in GC *(Masood MA et al. 2016).* In AGS cells, we showed that SAHA alone or combined to PDC and Cisplatin alone reduced significantly (~ 50%) CDX2 mRNA level **(Supplementary figure 11c).**

The myocyte factor 2 (MEF2) is a family of transcriptional factors containing four members MEF2A, MEF2B, MEF2C and MEF2D which play crucial role in cell proliferation, survival and differentiation. MEF2 can activate or repress transcription through interactions with co-activators like p300 (HAT) or co-repressors respectively. Co-repressors are the four Class IIa HDACs: HDAC 4, 5, 7 and 9 *(Di Giorgio E et al. 2018).* The role of MEF2 is controversial in cancers, indeed its transcriptional activity mediated by the different protein complexes can act as oncogene or tumor suppressor. In the TCGA database, MEF2A is altered in 5%, MEF2B in 1.5%, MEF2C in 2.8%, and MEF2D in 4% of 393 reported cases of stomach adenocarcinomas *(http://www.cbioportal.org).* In our model, PDC 1,4-fold increased *MEF2A* expression. SAHA 1,7-fold enhanced *MEF2A* expression and addition of PDC didn't change this increase **(Supplementary figure 11d).**

Discussion

Alterations affecting HDAC expression and histones acetylation have been described in various types of cancers, including gastric cancers. These deregulations are thought to be related to cancer progression as well as resistance against cytotoxic anticancer drugs, thus resulting in a poorer outcome. The chemical pan-inhibitors of HDAC have been developed and two HDACI drugs recently received FDA-approval for routine-usage: SAHA (Vorinostat, 2006) in cutaneous T cell lymphoma and hydroxamic acid variants (f.e. Farydak, Panobinostat 2015) in multiple myeloma. In addition, studies started to explore the interest of a combination of these inhibitors with classical gold-standard therapies to bypass potential resistance mechanisms and to reduce side effects. However, the combinatory treatment synergic effect on cancer cells is dependent on the molecular characteristics of the tumor, and combinatory treatment might even lead to antagonistic effect in some cases.



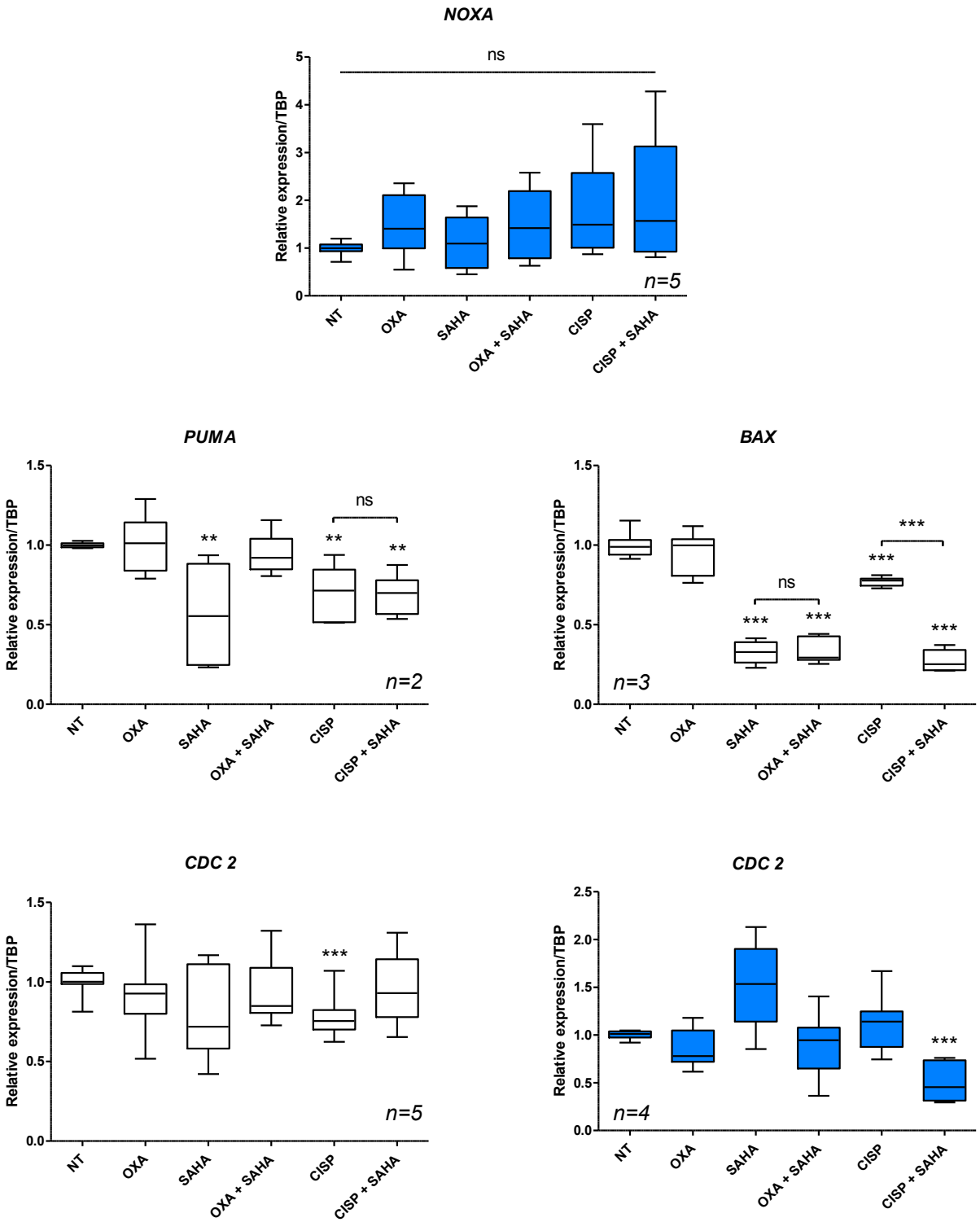
<u>Supplementary figure 2</u>: SAHA reduces HIF1alpha protein level and its activity on gene expression in AGS cells

AGS cells were treated or not (NT) with Deferoxamine (DFO; 100µM) added or not with SAHA (Vorinostat). RT-qPCR were assayed after 8h (white) or 24h (blue) of treatment. Box plots represent the fold induction of VEGF, and GLUT-1 mRNA for each condition versus the control (NT) (n=2). ** p< 0,0083 are calculated by Mann-Whitney test (corresponding to p-value limit).

In this study, we investigated the interest of combinatory treatment in gastric cancer cells using the standardly-used in clinical routine platinum-based drugs (Cisplatin or Oxaliplatin) in combination with SAHA. We have identified some of the molecular mechanisms involved in the synergistic effect of combinatory treatment on AGS cells, which highlights the necessity for a better characterization of the molecular specificities of the tumors treated with these combinations.

The synergistic effect of SAHA on cell survival is observed in association with Cisplatin or Oxaliplatin on the AGS cells at almost all tested doses. This combination triggered a marked cleavage of Caspase 3 and a shift in LC3 variants. Interestingly, no significant activation of Caspase 3 was observed in AGS cells treated with Cisplatin or Oxaliplatin in monotherapies at these concentrations and only a weak activation was observed with SAHA alone. This demonstrated that the SAHA/platinum combination induced a reprogramming, leading to cellular death by apoptosis. It is likely that higher doses of Cisplatin, Oxaliplatin and SAHA will also induce Caspase 3 activation and cellular death, as previously described. When combined, these different drugs are used at lower doses, which might reduce the risk of side effects *(Florea AM et al. 2011; Manal M et al. 2016).* Moreover, autophagy can play a dual role in GC. Indeed, restauration of autophagic genes (*Atg6* and *Klotho*) induces autophagy and GCC apoptosis *(Xie B et al. 2013; Wang Y et al. 2017)* but autophagy is also described to promote cisplatin resistance *(Zhang HQ et al. 2013).* In AGS cells, SAHA was not able to induce an autophagy. This is the contrary of a previous study by Vrana *et al.* showing that Cisplatin-induced apoptosis is stimulated in renal tubular epithelial cells in presence of autophagy inhibitors *(Vrana JA et al. 1999).*

The apoptosis stimulation by combinatory SAHA/platinum treatments was correlated to the absence of an increase of p53 at protein levels, especially as the pro-apoptotic properties of cytotoxic anticancer drugs often are associated with increased p53 expression *(Florea AM et al. 2011).* In our study, we observed an uncoupling between the p53 expression level and the stimulation of apoptosis. The SAHA-mediated inhibition of p53 expression involved a reduction of p53 transcription and similarly a caspase-dependent apoptosis. Previous studies have shown contradictory results regarding the role of p53 in the cellular response to the combination of SAHA and platinum drugs. For instance, *Dong G et al.* showed that SAHA had a cytoprotective effect on renal tubular cells treated with Cisplatin, through the suppression of p53 activation *(Dong G et al. 2010).* On the contrary, *Hacker S et al.* suggested that the synergistic activity of SAHA with anticancer drugs in medulloblastoma cells was mediated by p53 *(Hacker S et al. 2011).*

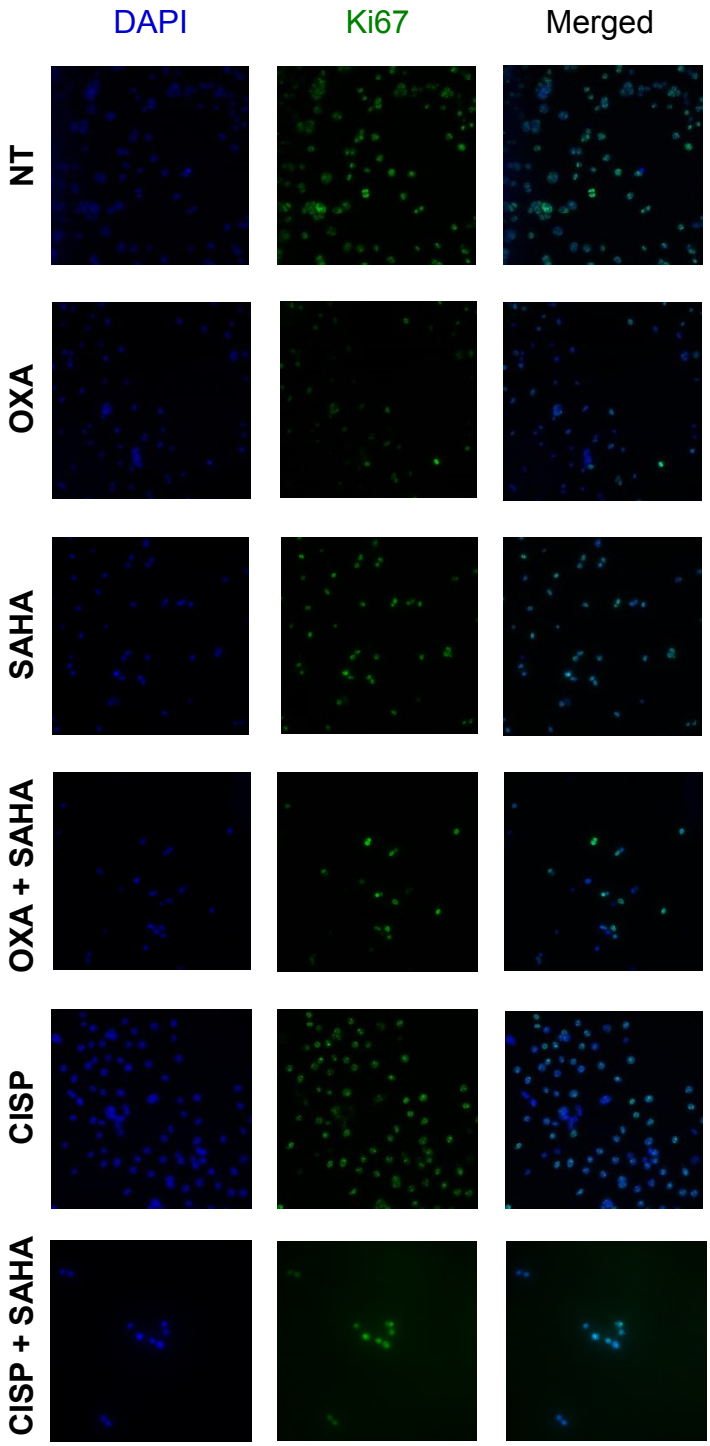


<u>Supplementary figure 3</u>: Combinatory treatments impact apoptosis and proliferation pathways in AGS cells

AGS cells were treated or not (NT) with the synergistic concentrations of Cisplatin (CISP), Oxaliplatin (OXA) and SAHA (Vorinostat). RT-qPCR were assayed after 8h (white) or 24h (blue) of treatment. Box plots represent the fold induction of NOXA, PUMA, BAX and CDC2 mRNA normalized to the control (NT). ** p< 0,0033 ; *** p< 0.001 are calculated by Mann-Whitney test (p-value limit = 0,0033).

In addition, SAHA induced the expression of TAp63 in gastric cancer cells, suggesting that this p53 family member is somehow involved in the anticancer mechanism of SAHA in gastric cancer, which has only been described in head and neck cancer in the literature *(Finzer P et al. 2004).* However, in the absence of a TAp63 antibody, the nature of the observed isoform (TAp63 versus ΔNp63) remains to be established before a conclusion could be drawn.

If the exact roles of p63 in the apoptosis induced by combinatory treatments remained to be demonstrated, we showed that the cleavage of Caspase 3 is strongly dependent on the presence of these p53-family proteins in the AGS gastric cancer cells. Indeed, Shim *et al.* enhanced SAHA anticancer effects by adding p63/p73 in head and neck squamous cell carcinoma *(Shim SH et al. 2010).* The role of p53 in the synergistic activity of SAHA with Cisplatin is also supported by the fact that the combination of SAHA with Oxaliplatin appeared less synergistic in p53-mutated KATOIII gastric cancer cells, which does not seem to be the case for Cisplatin. On the contrary, in NUGC3 p53-mutated cells Cisplatin and Oxaliplatin both induced a cleavage of caspase 3 which seemed to be p53 independent. These complex results illustrate well the contradictory documented results of the influence of HDACI (such as SAHA) in monotherapy or combined with platinum derivate compounds on p53. Early studies showed that cellular growth arrest and induction of p21 mediated by SAHA were independent from p53 *(Huang L et al. 2000; Vrana JA et al. 1999)* and another study by *Sonneman et al.* demonstrates that SAHA did not require p53 for its anticancer effects *(Sonnemann J et al. 2014). Li et al.* also showed that SAHA appeared more active on cancer cells with a p53 mutation *(Li D et al. 2011).* However, these observations are balanced by other studies based on different cells lines, indicating the important role of p53 for the biological activity of SAHA *(Henderson C et al. 2003).* Similarly, to the use of SAHA in monotherapy, the implication of p53 in combinatory treatments, which associate SAHA with DNA-damaging anticancer drugs, is also unclear. As stated above, SAHA appeared protective against Cisplatin in renal tubular cells through a suppression of p53 activation *(Dong G et al. 2010)* and SAHA acted synergistically with doxorubicin in colon cancer cells independently of p53 *(Alzoubi S et al. 2016).* Inversely, SAHA combined with anticancer drugs in medulloblastoma cells *(Hacker S et al. 2011)* and combined with doxorubicin on cervical cancer cells *(Lee SJ et al. 2014)* appeared to act synergistically on p53. Therefore, it seems that the contribution of p53 in the synergistic activity of SAHA with DNA damaging drugs, such as Cisplatin and Oxaliplatin, might be dependent on the cellular context, including the cancer type and especially the molecular markers, as well as the exact mode of action of the individual drugs.



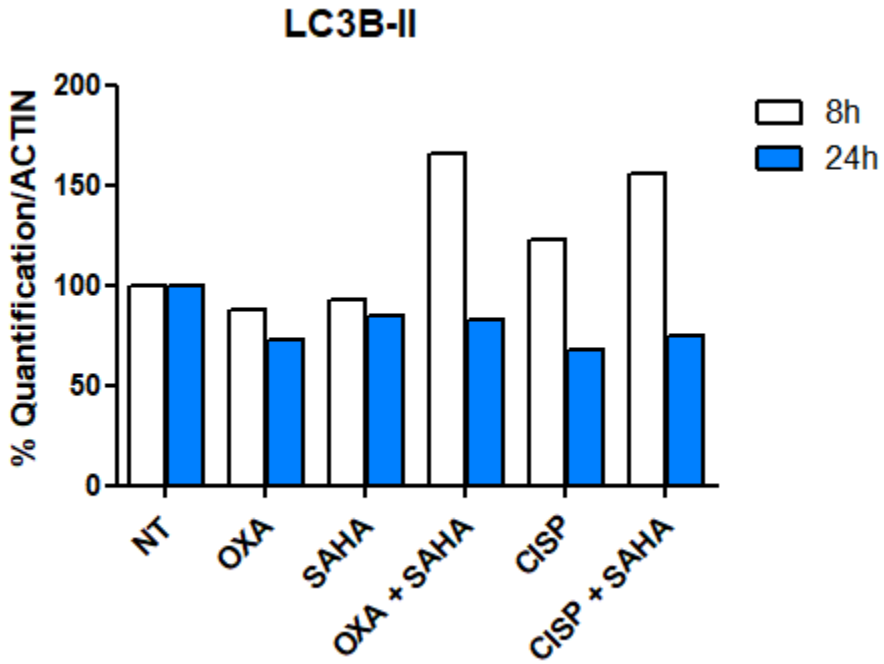
<u>Supplementary figure 4</u>: Impact of combinatory treatments on AGS cell proliferation

Immunocytology on AGS cells were performed after 24h of treatment to reveal Ki67 (Alexa 488 – Green) and DNA (DAPI – Blue). Cells were treated or not (NT) with the synergistic concentrations of Cisplatin (CISP), Oxaliplatin (OXA) and SAHA (Vorinostat). Observations are done with the ApoTome 2® microscope (Zeiss) and Zen Blue software x40.

Otherwise, PDC and SAHA combinations reduce CDX2 mRNA level in AGS intestinal type of GC. *CDX2* is overexpressed in GC and is involved in early-stage of tumorigenesis *(Xiao ZY et al. 2012),* but *CDX2* expression decreases with tumor stage *(Liu Q et al. 2007)* makes tumor cells poorly differentiated promoting metastasis. Interestingly, *CDX2* overexpression promotes gastric cancer cells multidrug resistance *(Yan LH et al. 2014).* Thus, the reduction of CDX2 level observed with PDC + SAHA might participate to treatment cytotoxic effects.

Furthermore, *HDAC4* is overexpressed in GC *(Kang ZH et al. 2014; Spaety ME et al. In submission).* Inhibition of HDAC4 increases docetaxel cytotoxicity in SNU-16 GC cells *(Colarossi L et al. 2014)* and increases AGS GCC sensitivity to Cisplatin *(Spaety ME et al. In submission).* In our study, *HDAC4* has its expression strongly decreased with CISP + SAHA combination which enhances Cisplatin cytotoxicity. Moreover, HDAC4 is a co-repressor of MEF2 *(Di Giorgio E et al. 2018).* Interestingly, gene repressed by HDAC4 may be also MEF2 targets *(Di Giorgio E et al. 2013).* In our model, *MEF2A* has its expression increased by combinatory treatments PDC + SAHA. This may indicate an activation of MEF2 target gene expressions. Moreover PI3K/Akt pathway can represses MEF2 transcriptional activity *(Di Giorgio E et al. 2013).* In our model of AGS cells, pAKT is strongly reduced with PDC + SAHA combinations which may promote MEF2 activity on its target genes such as *KLF4* which can suppress tumor formation in colon and gastric tumors *(Ma y et al. 2014; Ghaleb AM et al. 2016).* The role of MEF2 is controversial in cancers, indeed its transcriptional activity mediated by the different protein complexes can act as oncogene or tumor suppressor. As examples, p38 can phosphorylates MEF2A in GC cells to promote GLUT-4 expression and so enhance glucose uptake and tumor cell growth *(Liu J et al. 2015).* At the same time, MEF2A can promote *p21* and *CYCLIN D1* expression reducing GCC proliferation *(Ma Y et al. 2014).* Further, MEF2D promotes tumorigenicity in malignant glioma cells *(Zhao Y et al. 2016)* and mir-19 expression is reduced in 260 GC reported cases which promotes *MEF2D* expression enhancing the Wnt/β-catenin pathway so GC cells proliferation and survival *(Xu K and Zhao YC. 2016).* In our model of AGS cells, MEF2A seems to have an anti-tumoral effect but further analysis is necessary to determine HDAC4/MEF2 signaling pathway in gastric cancer.

This study illustrates the importance of the development of combinatory therapies associating standard Cisplatin/Oxaliplatin drugs with HDACIs such as SAHA, for the perioperative treatment of gastric cancers. It also pinpoints the necessity a better characterization of the molecular specificities of the treated tumor for an optimized choice and use of drug combinations based on selected markers, such the expression of a



<u>Supplementary figure 5</u>: LC3B quantification in response to combinatory treatments in AGS cells

Quantification normalized to ACTIN of the Western blot of LC3B presented in Figure 3.

functional p53 protein. Another important point which should be considered is the differences observed between the first (Cisplatin) and the second (Oxaliplatin) generations of platinum drugs, as the newly developed HDAC inhibitors might act differently depending on the associated drug. These results could open new treatment possibilities especially in resistant cancers, with a reduction of side effects.

Acknowledgments

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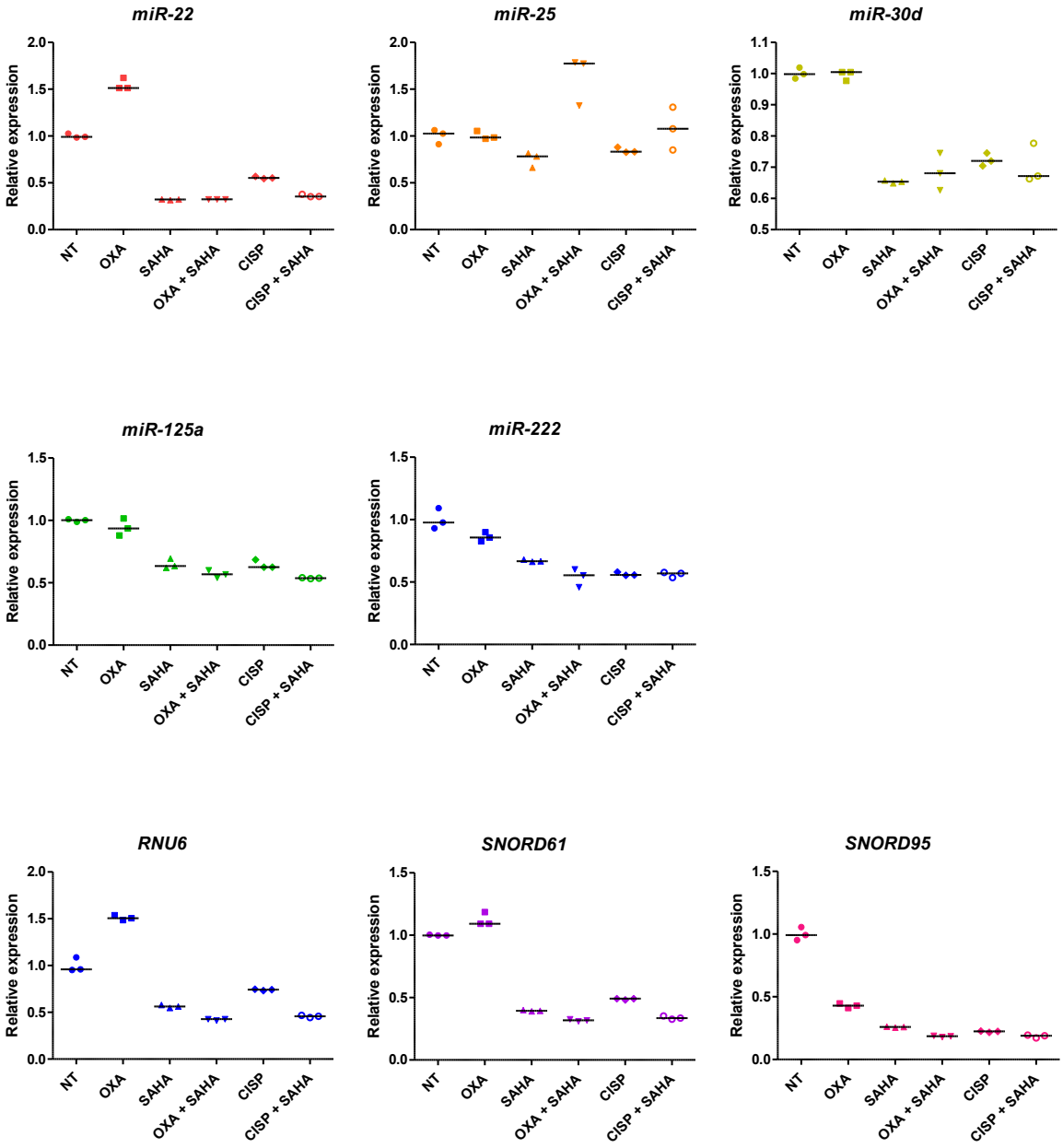
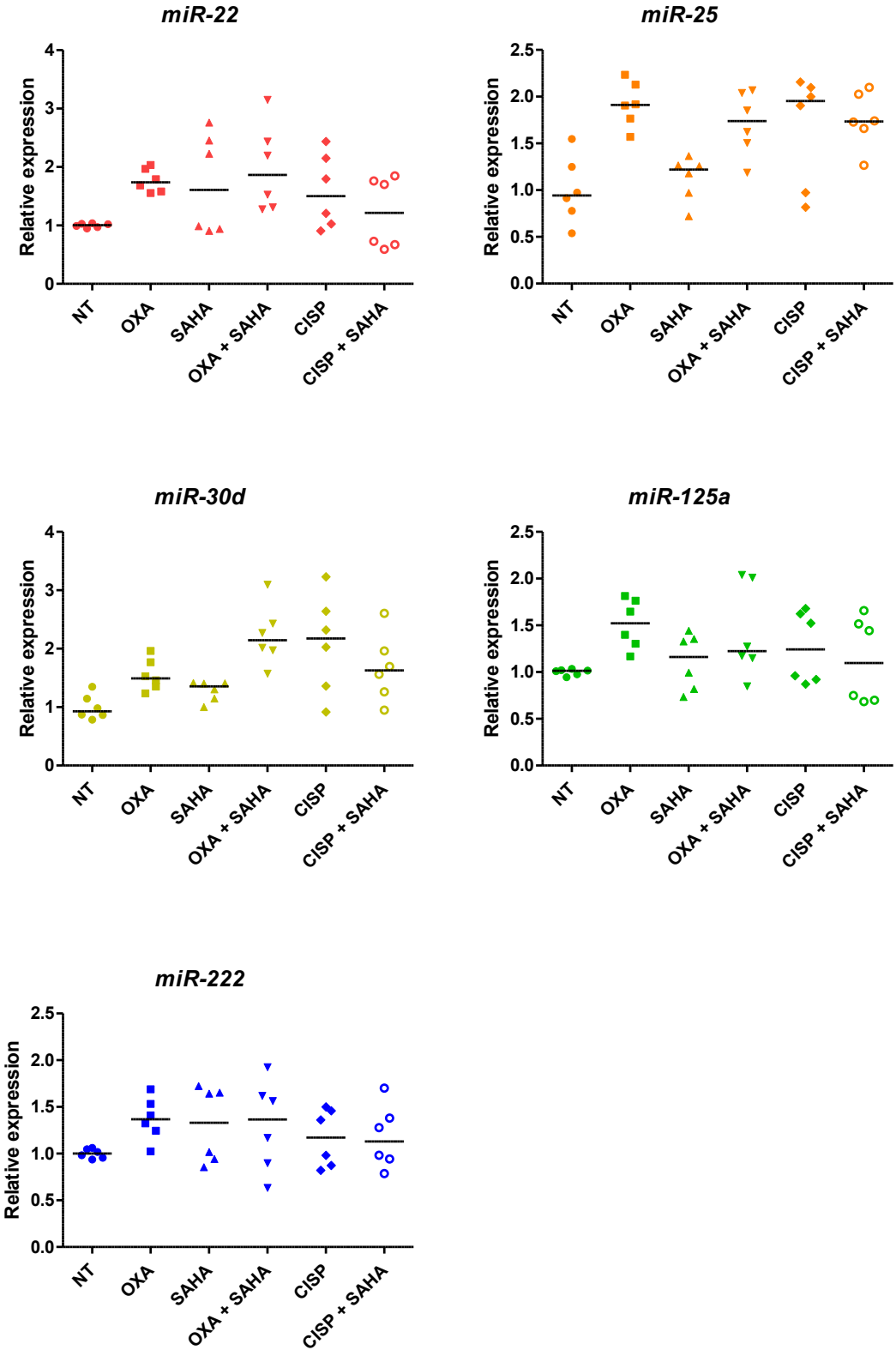


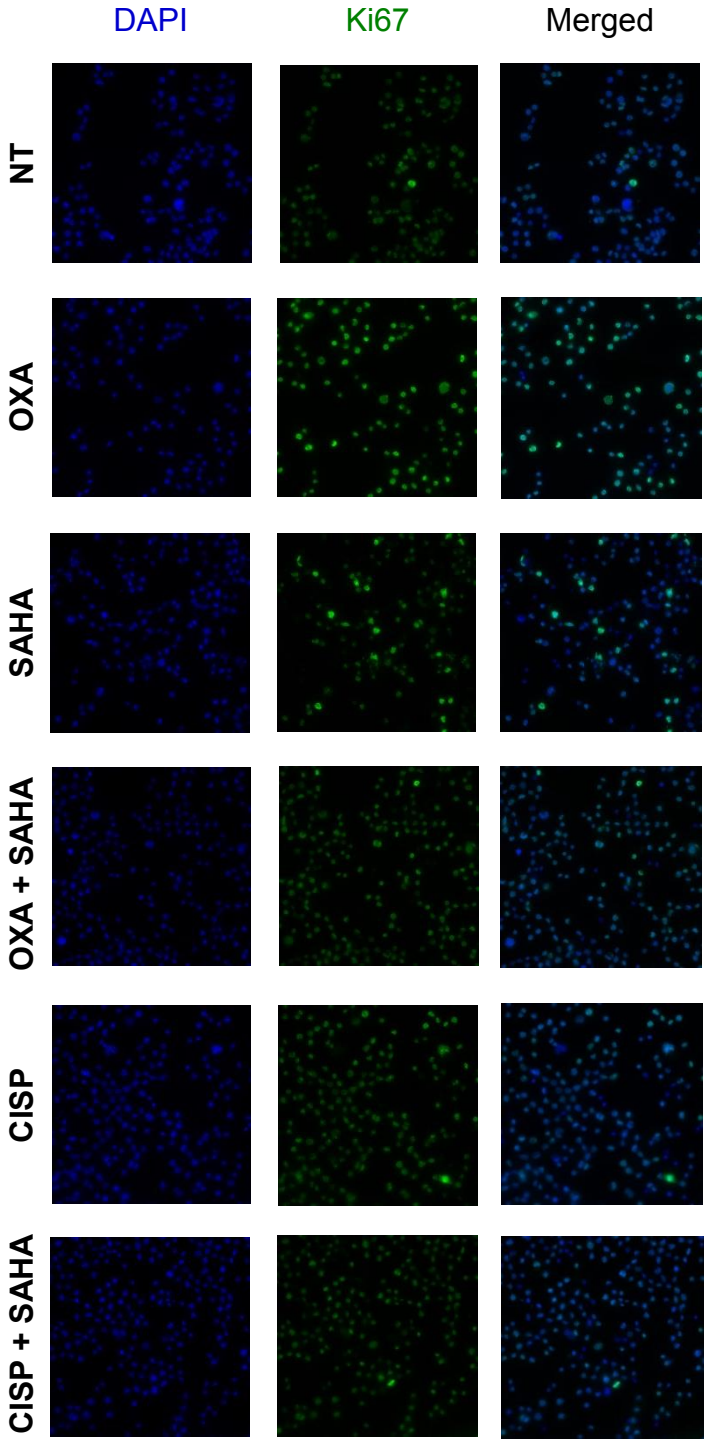
<u>Supplementary figure 6</u>: PDC and SAHA impact miRNA targeting and targeted by p53 expression

AGS cells were treated 4h with Oxaliplatin (OXA; 0,5µM), Cisplatin (CISP; 15µM), SAHA (Vorinostat ; 2,75µM) alone or combined or were untreated (NT). microRNA level were analyzed by Rt-qPCR. Points represent relative expression of each technical replicates. RNU6, SNORD61, SNORD95 are reference miRNA for quantification. Because of treatment impact on their expressions, the relative quantification were done with an arbitrarily Ct and calculated with $2^{(-\Delta\Delta Ct)}$.



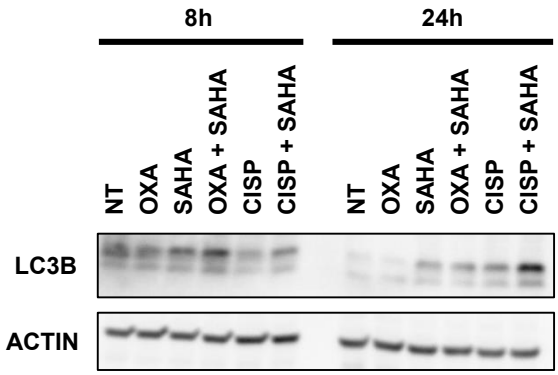
<u>Supplementary figure 7</u>: PDC and SAHA impact miRNA targeting and targeted by p53 expression

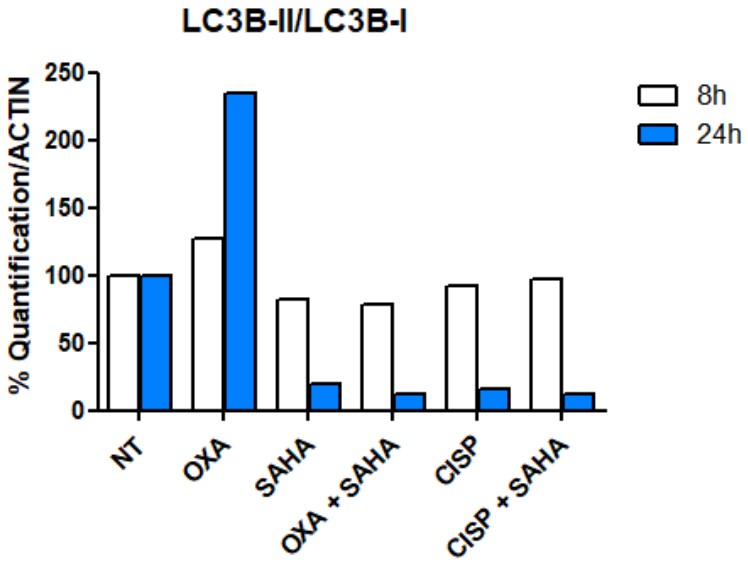
AGS cells were treated 8h with Oxaliplatin (OXA; 0,5µM), Cisplatin (CISP; 15µM), SAHA (Vorinostat ; 2,75µM) alone or combined or were untreated (NT). microRNA level were analyzed by Rt-qPCR. Points represent relative expression of each technical replicates (n=2). The relative quantification were done with an arbitrarily Ct and calculated with $2^{(-\Delta\Delta Ct)}$.



<u>Supplementary figure 8</u>: Impact of combinatory treatments on KATOIII cell proliferation

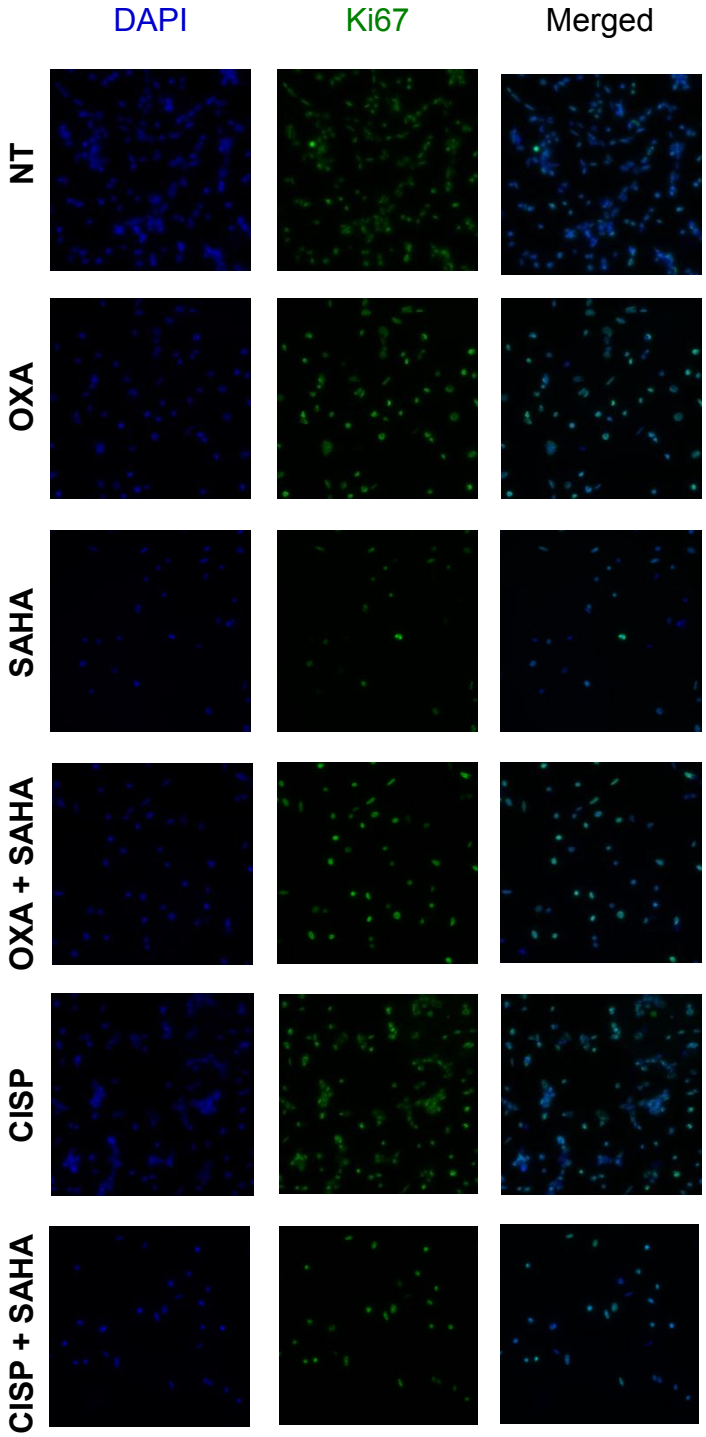
Immunocytology on KATOIII cells were performed after 24h of treatment to reveal Ki67 (Alexa 488 – Green) and DNA (DAPI – Blue). Cells were treated or not (NT) with the synergistic concentrations of Cisplatin (CISP), Oxaliplatin (OXA) and SAHA (Vorinostat). Observations are done with the ApoTome 2® microscope (Zeiss) and Zen Blue software x40.





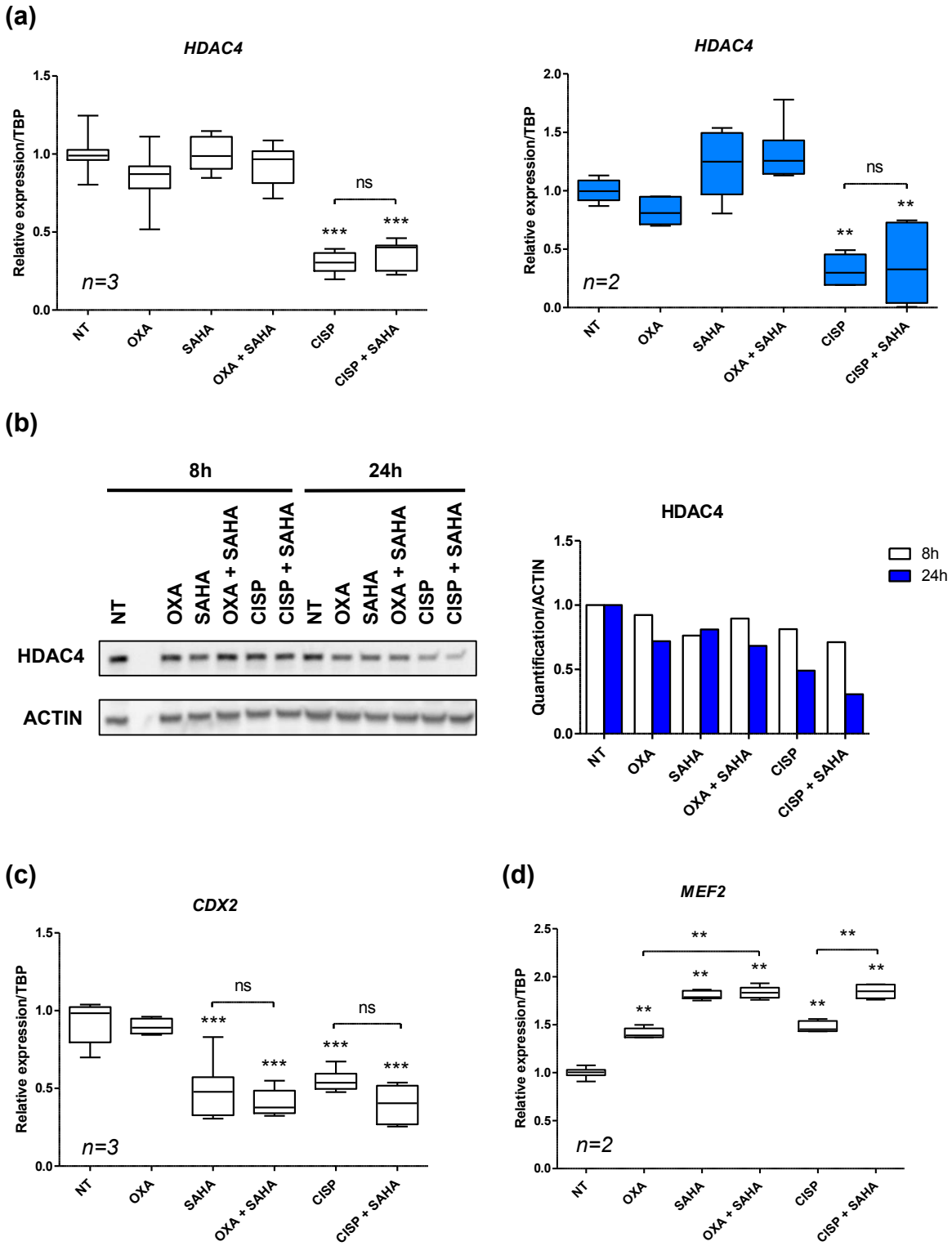
<u>Supplementary figure 9</u>: Combinatory treatments do not induce autophagy in KATOIII cells

KATOIII cells were treated or not (NT) with the synergistic concentrations of Cisplatin (CISP), Oxaliplatin (OXA) and SAHA (Vorinostat) for 8h or 24h. Western blot was performed using antibodies against LC3B. The active form of LC3B (LC3B-II) is the bottom band. The relative quantification of the ratio LC3B-II/LC3B-I (active/non-active form) are represented and obtained by a normalization to Actin.



<u>Supplementary figure 10</u>: Impact of combinatory treatments on NUGC3 cell proliferation

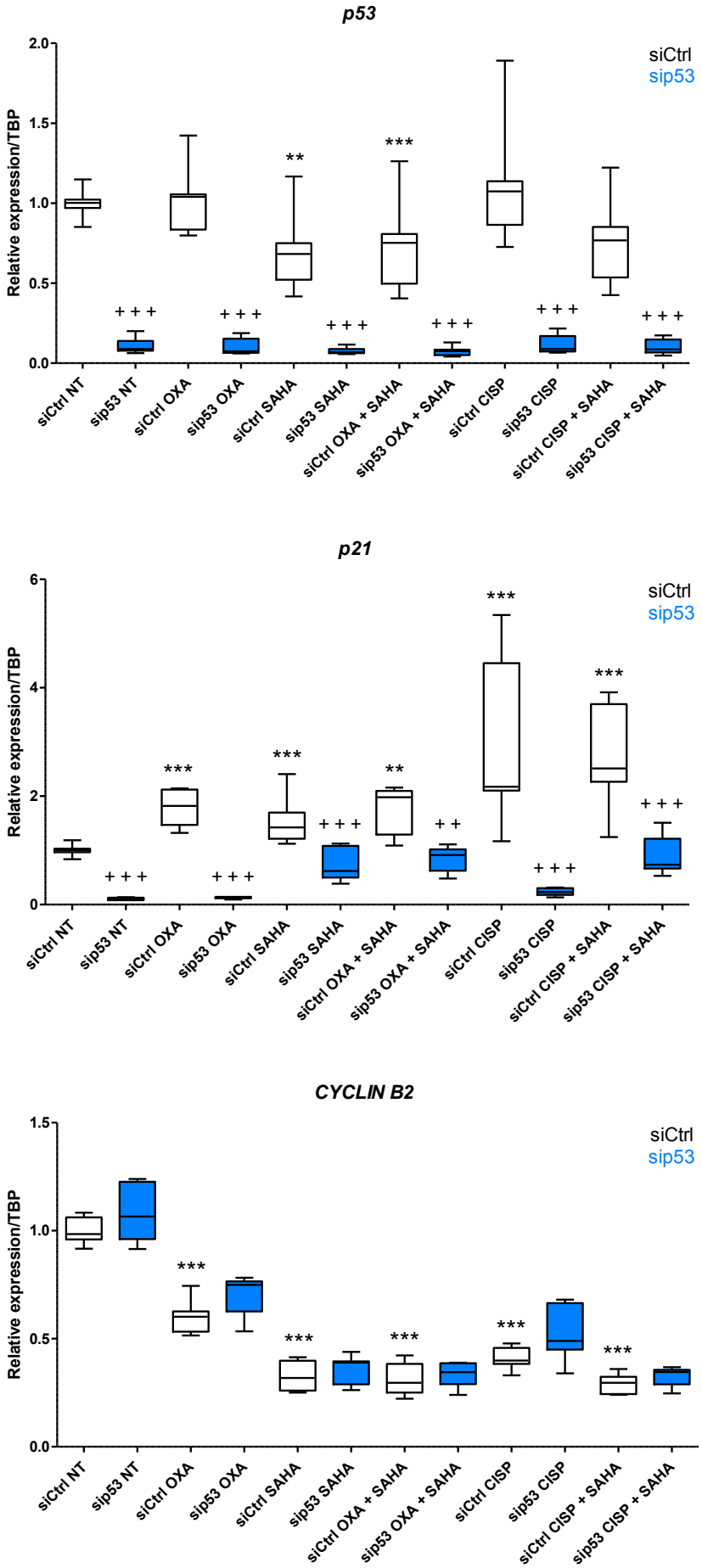
Immunocytology on NUGC3 cells were performed after 24h of treatment to reveal Ki67 (Alexa 488 – Green) and DNA (DAPI – Blue). Cells were treated or not (NT) with the synergistic concentrations of Cisplatin (CISP), Oxaliplatin (OXA) and SAHA (Vorinostat). Observations are done with the ApoTome 2® microscope (Zeiss) and Zen Blue software x40.



<u>Supplementary figure 11</u>: Combinatory treatments impact HDAC4, CDX2 and MEF2 expression in AGS cells

AGS cells were treated or not (NT) with the synergistic concentrations of Cisplatin (CISP), Oxaliplatin (OXA) and SAHA (Vorinostat). **(a)** RT-qPCR were assayed after 8h (white) or 24h (blue) of treatment. Box plots represent the fold induction of HDAC4 mRNA normalized to the untreated (NT) control. **(b)** Western blot was performed using antibodies against HDAC4. The relative quantification was normalized to Actin. **(c-d)** RT-qPCR were assayed after 8h of treatment. Box plots represent the fold induction of CDX2 and MEF2 mRNA normalized to the control (NT). ** p< 0,0033 ; *** p< 0.001 are calculated by Mann-Whitney test (p-value limit = 0,0033).

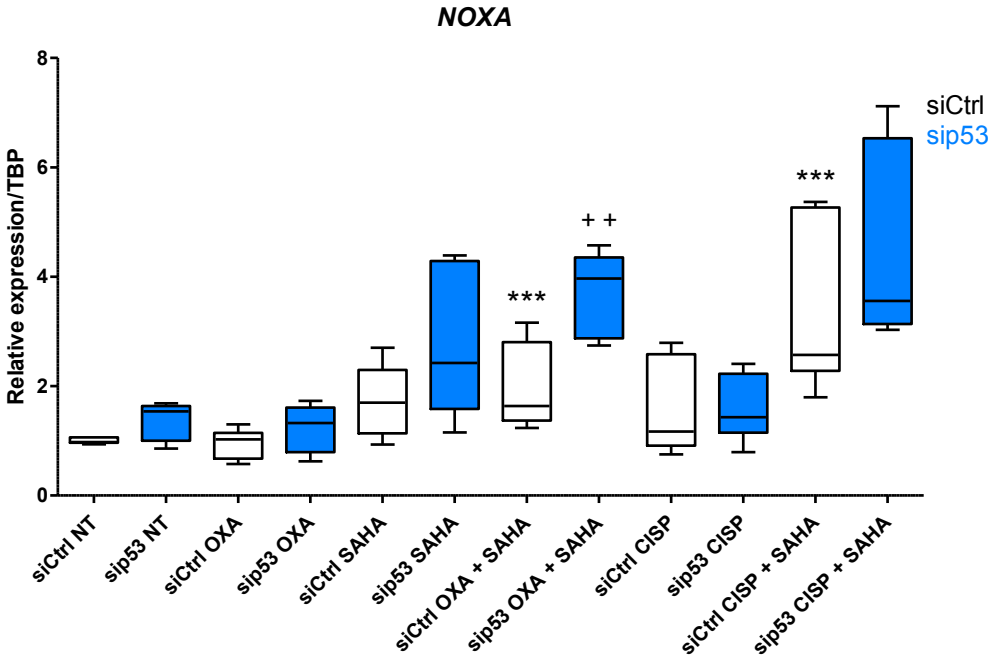
Complementary results on Article B

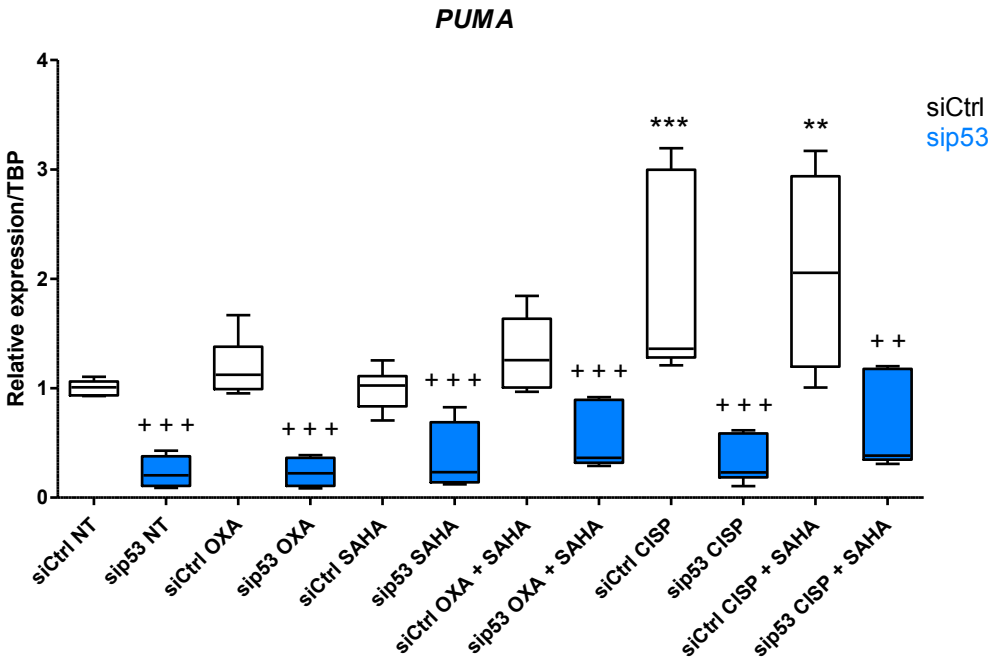


Complementary figure B1: Impact of p53 on apoptotic pathway

Impact of TP53 on the induction of the synergy PDC + SAHA related apoptosis

As previously described, synergistic treatments PDC + SAHA promote a p53 dependent apoptosis in AGS cells, and TP53 suppression inhibited the cleavage of caspase 3 in response to combinatory treatments. We wanted to know the impact of TP53 on pro-apoptotic gene in response to the combinatory treatments. Thereby, we suppressed TP53 by using a silencing RNA and we treated AGS cells with the same combination of treatments for 8h **(Complementary figure B1).** As previously observed **(Figures 2bde and 4b),** SAHA decreased *p53* expression, and SAHA and PDC alone or combined approximately 2-fold increased *p21* expression, and 3-fold decreased *CYCLIN B2* expression. In addition, *PUMA* expression was 1,2-fold increased with combinatory Oxaliplatin + SAHA treatment. Surprisingly, Cisplatin alone or combined with SAHA 2-fold increased *PUMA* expression **(Complementary figure B1),** whereas precedingly SAHA and Cisplatin alone or combined 30% decreased *PUMA* expression **(Supplementary figure 3),** suggesting that the transfection impacted PUMA mRNA level. Nonetheless, as expected, suppression of p53 inhibited the induction of its target genes *p21* and *PUMA*. Moreover, *NOXA* expression was 1,6-fold and 2,5-fold increased with the combinations Oxaliplatin + SAHA and Cisplatin + SAHA, respectively, correlating with the cleavage of the caspase 3 **(Figure 2a).** Unexpectedly, p53 suppression significantly promoted *NOXA* expression with Oxaliplatin + SAHA treatment, indicating that p53 might inhibit *NOXA* expression. Moreover, we showed that Oxaliplatin 1,8-fold, SAHA 2,5-fold and the combination 3,8-fold increased *BIK* expression. We also observed that Cisplatin 1,2-fold and the combination with SAHA 3,7-fold increased *BIK* expression. We remarked that the suppression of p53 significantly decreased the induction of *BIK* expression with PDC treatments, and we might see a negative trend with Oxaliplatin and SAHA combination. However, we observed a variation in the relative expression. In addition, we only observed a significant induction of *DIABLO* expression with combined treatments PDC + SAHA correlating with the observed cleaved caspase 3. Nonetheless, TP53 suppression did not significantly changed *CYCLIN B2* and *DIABLO* expressions. Thereby p53 seems to not be involved in their expressions in response to our treatments. However, as we just note, we had contrary results concerning *CYCLIN B2* and *PUMA* expressions. In addition, it is difficult to conclude yet for *BIK* expression. Further experiments are necessary to check the impact of p53, and as they are induced, of p63 and p73 also on these genes.

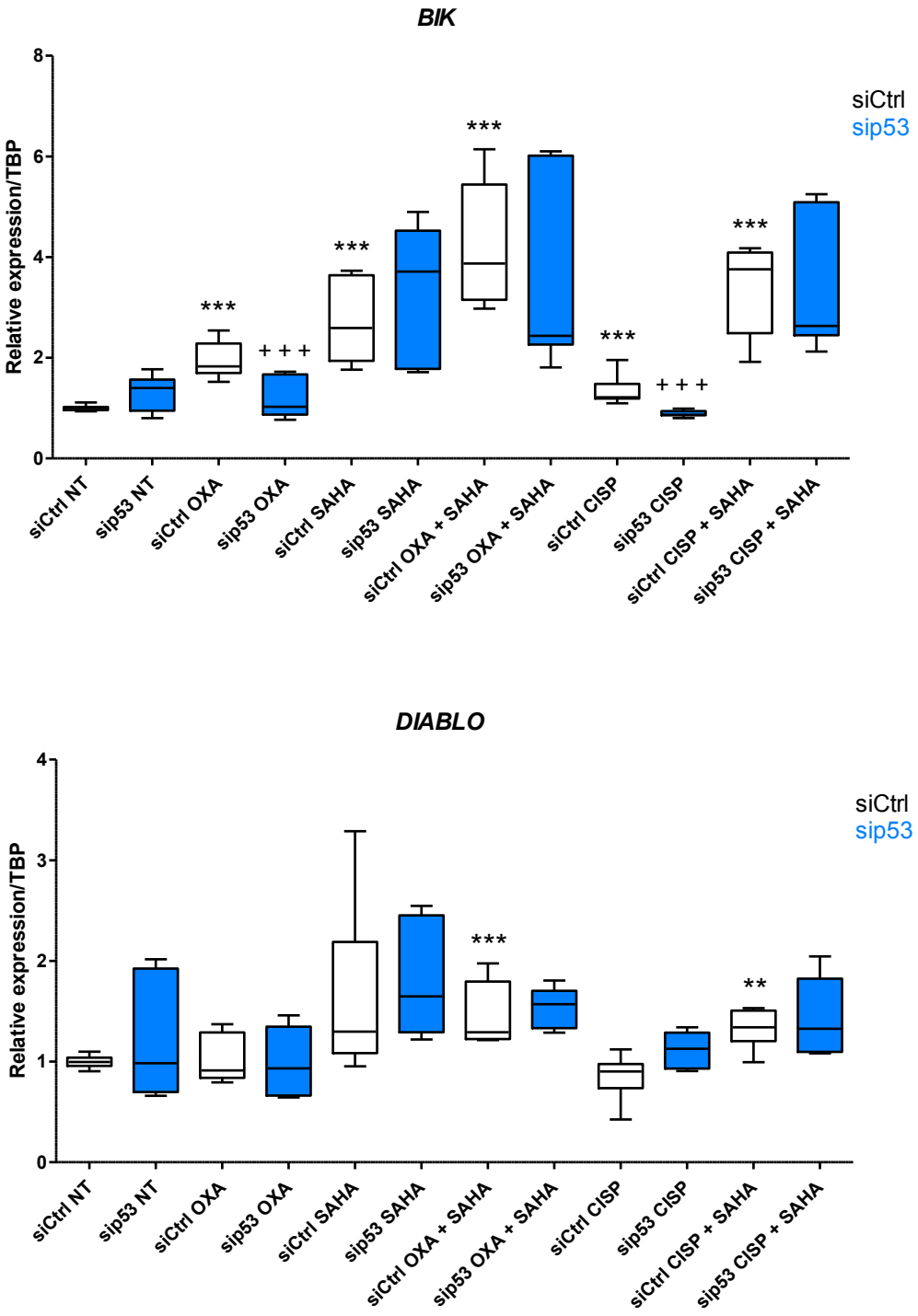




Continuation complementary figure B1: Impact of p53 on apoptotic pathway

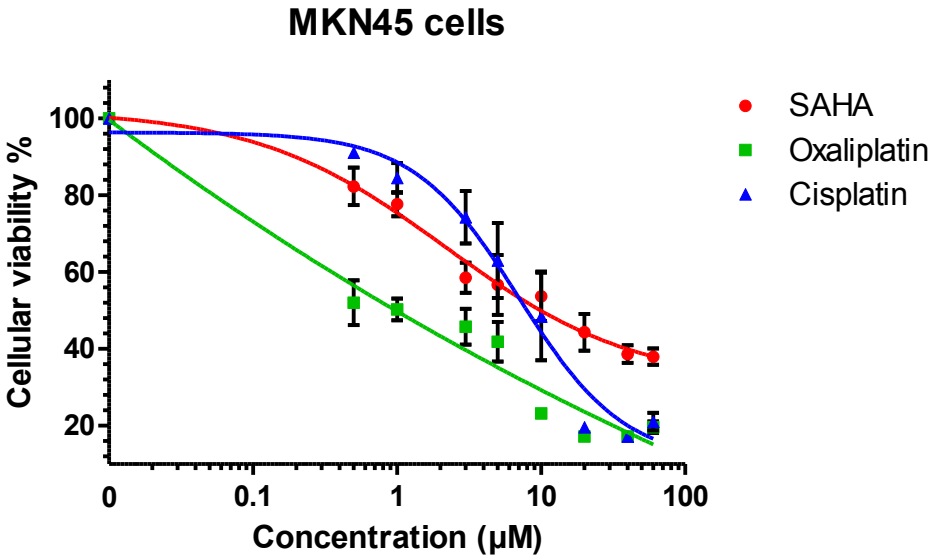
Study of combined treatments PDC + SAHA on MKN45 cells

We were interested to study the combinatory treatments PDC + SAHA on a diffuse type of GC model. Thereby, we used MKN45 cells, and we determined Oxaliplatin, Cisplatin and SAHA activity on cell survival by MTT as previously **(Complementary figure B2).** Likewise, we determined the effect of combined treatments on MKN45 cell survival by isobologram assays **(Complementary figure B3).** SAHA showed only synergistic effect with combination with Oxaliplatin especially with low concentrations of both drugs. However, neither at 24h nor at 48h, PDC + SAHA treatments did not induce a cleavage of caspase 3, suggesting that they did not induce an apoptosis **(Complementary figure B4).** Nonetheless, the combined treatments seemed to reduce cell proliferation and density by looking on Ki67/DAPI immunocytology explaining the synergistic activity of the combined treatment on the cellular survival **(Complementary figure B5).** Thereby, the synergistic activity of SAHA combined with PDC may depend on the cancer type, suggesting that the response may depend on specific molecular characteristics which are to be determined, besides the p53 status in the intestinal type of GC.



Continuation complementary figure B1: Impact of p53 on apoptotic pathway

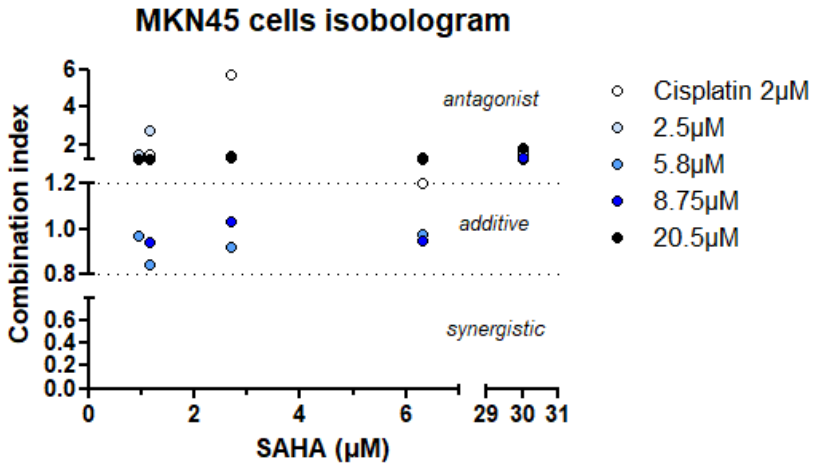
AGS cells were transfected with 30nM of a scramble siRNA (siCtrl) or of a siRNA against TP53 (sip53) for 48h. Then, cells were treated 8h or not (NT) with SAHA (Vorinostat), Oxaliplatin (OXA) and Cisplatin (CISP) at synergistic concentrations. TP53, CYCLIN B2, p21, PUMA, NOXA, BIK, and DIABLO (n=3) mRNA levels were analyzed by RT-qPCR. Box plots represent the fold induction of mRNA for each condition normalized to the control (siCtrl NT). Statistical differences were calculated by Mann-Whitney test with a correction as p-value limit p=0,0045. * vs siCtrl NT ; + siCtrl vs siHDAC4. ** p< 0,0045 ; *** p< 0,001

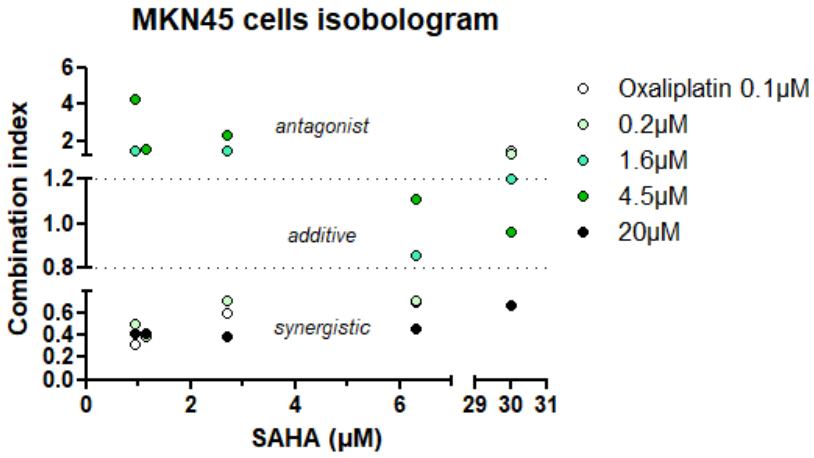


Name	Characteristics*	Drugs	IC_{25} (µM)	IC_{30} (µM)	IC_{50} (µM)	IC_{60} (µM)	IC_{75} (µM)
MKN45	Liver metastasis Near diploid p53 WT/mutated (R110C) bi-allelic c-met amplification ERK mRNA amplification Cyclin E amplification	SAHA	0,95	1,15	2,7	6,30	30
		Oxaliplatin	0,1	0,20	1,6	4,5	20
		Cisplatin	2	2,5	5,8	8,75	20,5

<u>Complementary figure B2</u>: SAHA and platinum compounds impact MKN45 cell survival

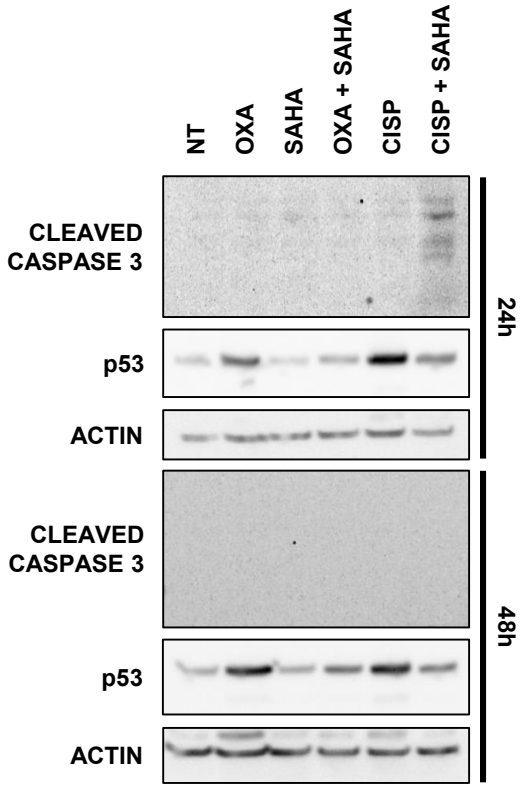
MKN45 cells were seeded in 96-wells and treated 48h with Cisplatin, Oxaliplatin or SAHA (Vorinostat). Viability of the cells was evaluated using MTT tests (n=3). The table summarizes the different concentrations for each drug graphical determined. For example, IC_{25} represents the concentration where we have 25% of the total effect on cell survival. **ATCC ; Yokozaki H. Pathology International, 2000 ; Cancer Cell Lines, Part 2, Human Cell Culture, 2002 ; portals.broadinstitute.org/ccle*





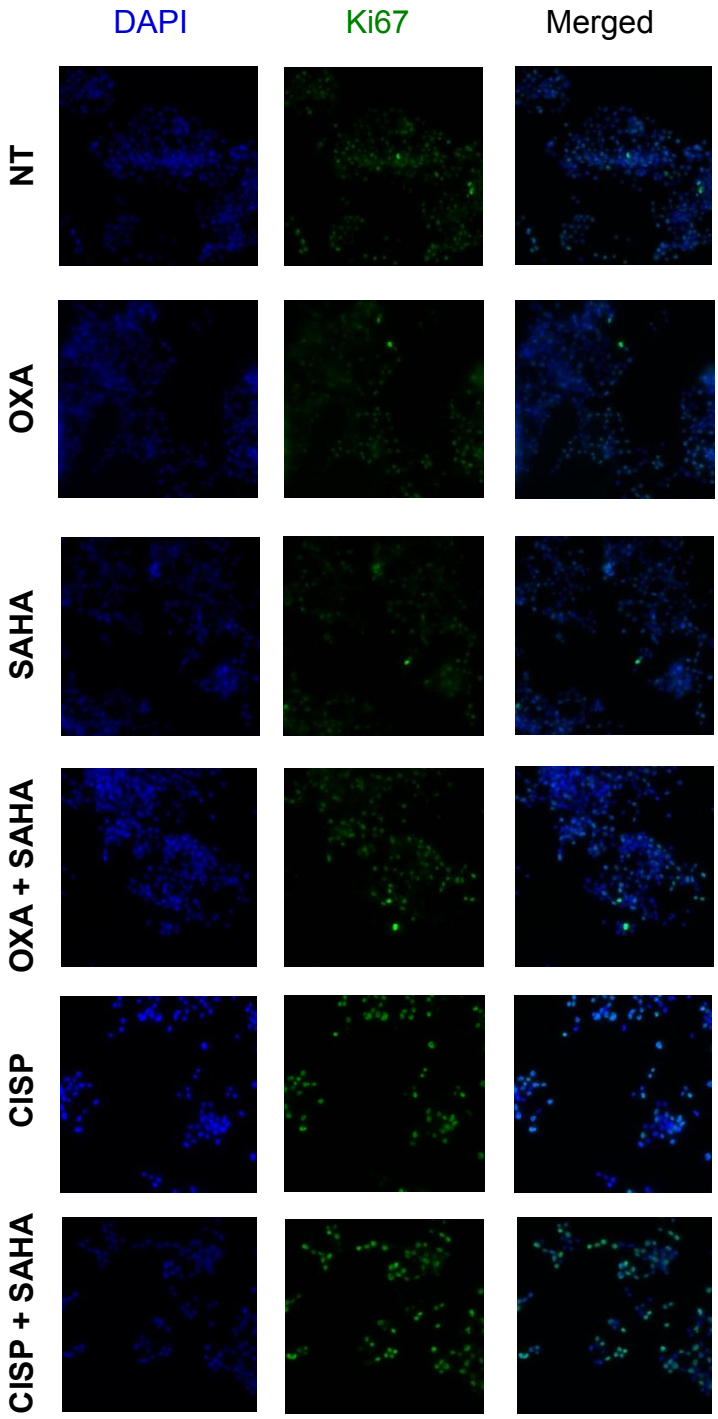
<u>Complementary figure B3</u>: Combinatory treatments impact differently MKN45 cells

MKN45 cells were treated at the indicated concentrations of Cisplatin (CISP) or Oxaliplatin (OXA) and SAHA (Vorinostat) for 48h followed by an isobologram assay to determine mathematically the combination index. We arbitrarily and conservatively considered antagonist effect between the drugs correspond to value > 1.20, additive effect between 0.80 and 1.20 and synergistic effect <0.80.



<u>Complementary figure B4</u>: Combined treatments PDC + SAHA do not induce a caspase dependent apoptosis in MKN45 cells

MKN45 cells were treated or not (NT) for 24h or 48h with Cisplatin (CISP ; 5,8µM) or Oxaliplatin (OXA ; 0,1µM) and SAHA (Vorinostat ; 1,15µM). Then, Western blots were performed using antibodies against Cleaved Caspase 3 and p53.



<u>Complementary figure B5</u>: Impact of combinatory treatments on MKN45 cell proliferation

Immunocytology on MKN45 cells were performed after 24h of treatment to reveal Ki67 (Alexa 488 – Green) and DNA (DAPI – Blue). Cells were treated or not (NT) with the synergistic concentrations of Cisplatin (CISP ; 5,8µM), Oxaliplatin (OXA ; 0,1µM) and SAHA (Vorinostat ; 1,15µM). Observations are done with the ApoTome 2® microscope (Zeiss) and Zen Blue software x40.

Article C: Histone deacetylase expression in gastric cancer; study of more specific inhibitors

Histone deacetylase expression in gastric cancer; study of more specific inhibitors

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Abstract

Gastric cancer (GC) cells have intrinsic or develop resistance to chemotherapy based on platinum derivative compounds (Cisplatin, Oxaliplatin). It is established that HDACs impact chemosensitivity in cancers but not much is known about which HDACs are found to be mainly altered in gastric cancers and how alterations of these correlate with the overall patient's survival. For this, we analyzed the TCGA (The Cancer Genome Atlas) for alteration of HDACs and correlated these with the overall patient survival. This showed that alterations in HDAC4 is associated with a better overall patient survival (logrank Test P-value = 0.107). In addition, to determine possible HDACs involved in gastric cancer drug resistance, we analyzed how current standard treatment protocols for GC impacts on the expression of different HDAC family members. We showed that Class I HDACs (HDAC 1, 2 and 3) and HDAC7 (Class II HDAC) are highly expressed in AGS and MKN45 cells, and that HDAC4 (Class II HDAC) is more expressed in MKN45 cells. In addition, we showed that Class II HDACs (HDAC 4, 5, 7 and 9) and HDAC3 are in majority more impacted by the current treatments. Interestingly, these HDACs are reported to be associated with different cancer aggressiveness, which suggests that a combinatory treatment with an HDAC inhibitor (HDACI) might be beneficial for the treatment of GC. However, utilization of the FDA-approved HDACI SAHA (suberanilohydroxamic acid) induces adverse side effects in clinical trials due to its pan-HDAC activity. Thereby, it appears necessary to develop new more specific HDACIs. We tested different Class I or II HDAC more specific inhibitors, and we observed that HDACIs with higher effect on gastric cancer cell survival was the new designed HDAC3 inhibitor; JGS-038. Moreover, Class II HDAC enzymatic activities were reported to depend on a multiprotein complex containing HDAC3. Thereby, we hypothesized that by inhibiting HDAC3 we might indirectly inhibit HDAC4 activity and synergistically decreases cell survival in combination with platinum based anticancer drug as expected by a previous study. We observed that JGS-038 acts synergistically with certain concentrations of Cisplatin on p53-mutated GC NUGC3 cell survival and not in p53-WT AGS GC cell survival. Because p53 is often mutated in GC, JGS-038 may represent a promising therapeutic alternative but before further mechanistic studies are necessary.

Keywords: HDAC, HDACI, JGS-038, SAHA, Gastric cancer, Cisplatin, Oxaliplatin

Introduction

Gastric cancer (GC) is the 4th most common cancer for men and the 5th for women in the World. It represents the 3rd cause of cancer related death for men and the 5th for women *(Michel P et al. 2017).* The middle age to develop GC is 70 years old, and Asia and Eastern Europe regions possess the higher incidence and mortality levels *(GLOBOCAN 2012).* In France, these last years, the gastric cancer incidence (out of cardia cancer) decreased around 1.5% per year *(Michel P et al. 2017).* In 2015, it was estimated about 6580 new cases of GC with a masculine preponderance *(French National Institute of Cancer)*. Unfortunately, the 5-year overall patient survival of GC is still low, around 15%, and as most GC are asymptomatic they are diagnosed tardily, which impacts on the survival rate making GC still a major health problem *(CDU-HGE 2015; Quadri HS et al. 2017)*. Environmental factors and life style like poorly preserved foods, salt rich-diets, heavy alcohol consumption and cigarette smoking increase the risk to develop GC. On the contrary, antioxidant rich-diets (fruits and vegetables) decrease this risk *(Lambert R. 2010; Quadri HS et al. 2017).* The patient-infection-history by EBV (Epstein-Barr virus), and *Helicobacter pylori* increases also the risk to develop GC. The observed diminution of GC incidence is due to a better understanding and control to these risk factors with the most important; the eradication of *Helicobacter pylori (Michel P et al. 2017).* In addition, different genetic factors are known to increase the risk of GC. It concerns inherited forms and represents 3% of GC cases *(Quadri HS et al. 2017).* Usually, gastric cancers are classified according to Lauren classification which distinguishes GC in three histological types. First, the intestinal type also called non-cardia cancer (distal cancer) promoted by *H. pylori* infection. Second, the diffuse type also named cardia cancer (cancer of esophageal-gastric junction) not associated with *H. pylori*. And the third class concerning undetermined or mixed GC *(Buas MF and Vaughan TL. 2013; Michel P et al. 2017).*

Several pathways are deregulated in GC, promoting cancer cell proliferation, invasion, metastasis and resistance to current treatments mostly with Cisplatin or Oxaliplatin (Platinum compounds; PDC). One of these mechanisms is the alterations of histone deacetylases (HDACs) expression and activity on histone and non-histone protein targets *(Li Y and Seto E. 2017).* 18 HDACs are described and are separated into four classes based on sequence homology to yeast. HDAC Class I contains HDAC 1, 2, 3 and 8, Class II which is divided into two sub-classes: Class IIa containing HDAC 4, 5, 7 and 9 and Class IIb containing HDAC6 and 10, and Class IV which contains HDAC11. Class I and II are zinc dependent whereas the Class III which contains Sirtuins, is NAD-dependent *(Li Y and Seto E. 2017).*

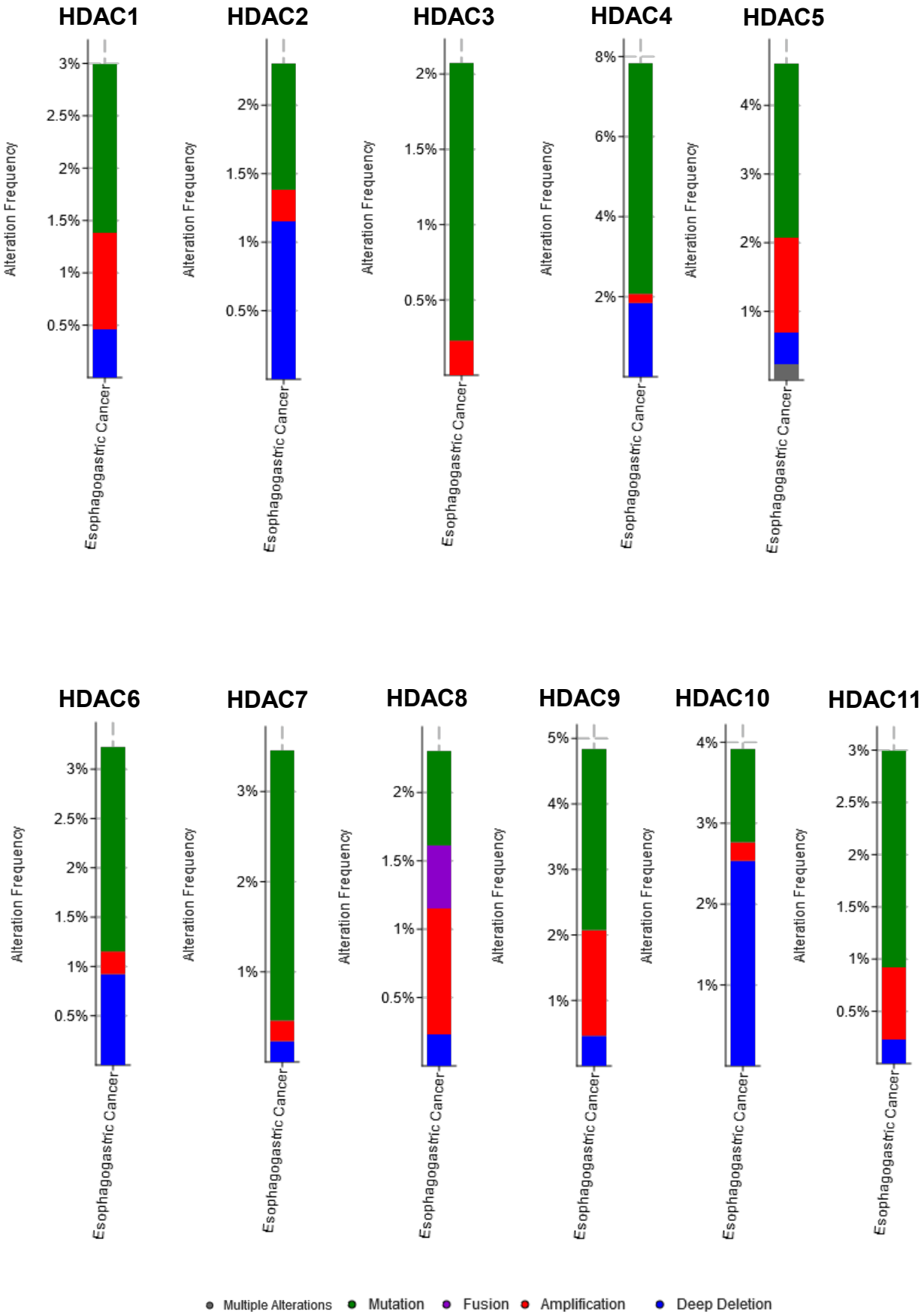


Figure 1: HDAC alterations in stomach adenocarcinomas

Percentages represent HDAC alterations on 434 total stomach adenocarcinomas of the TCGA database. http://www.cbioportal.org/

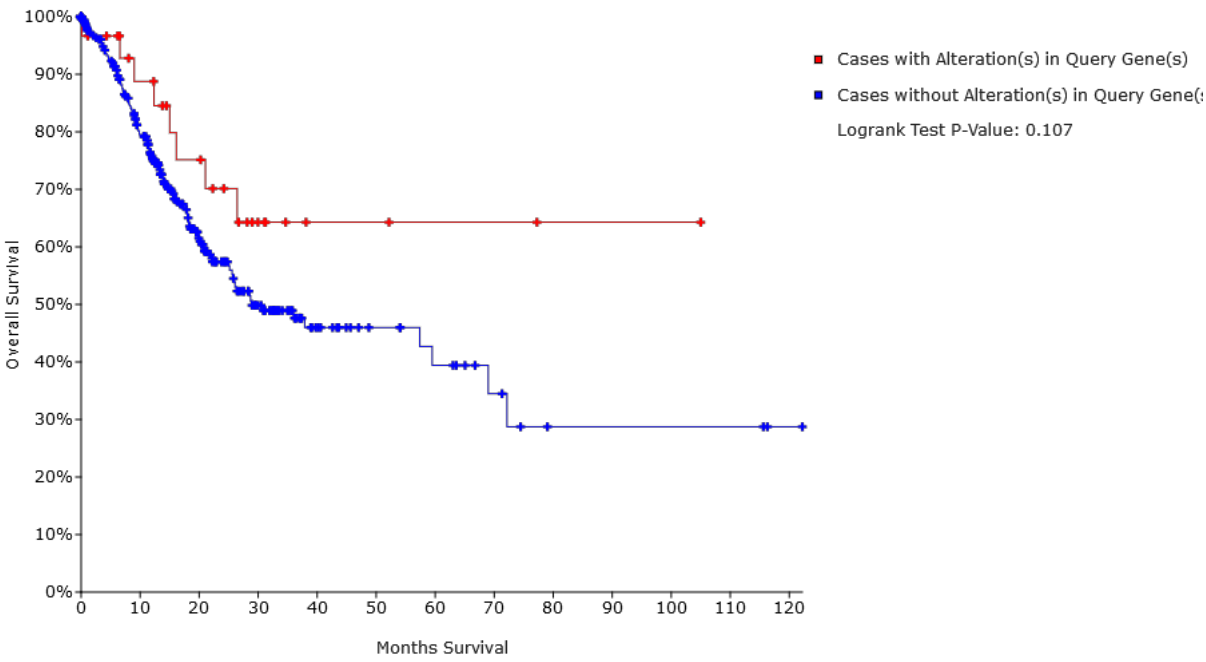
Histone function is modulated by the balance between histone acetyltransferases (HATs) and histone deacetylases (HDACs). Histone acetylation is associated with a chromatin de-condensation and a transcriptional activation whereas histone deacetylation is associated with a condensed chromatin and a transcriptional repression *(Li Y and Seto E. 2017).* Moreover, different non-histone proteins are also known to be acetylated like the tumor suppressor TP53, chaperone protein HSP90 or the Hypoxia Inducible Factor HIF1α whose acetylation levels may impact their stability, their capacity to form protein interactions, their affinity to DNA and their transcriptional activity *(Kim E et al. 2015).*

Consequently, HDACs impact multiple biological process involved in cancer progression and resistance to treatments. Several HDACs have been described to be over-expressed and associated to poor outcome for patients such as Class I HDACs (HDAC 1, 2 and 3) in two German cancer centers where 21% of a cohort of 150 patients with 606 samples of tissue expressed these three HDACs. HDAC expression was higher in metastasis and HDAC2 expression was associated with poor prognosis *(Weichert W et al. 2008; Weichert W. 2009).* In addition, HDACs have been reported to control cancer cell sensitivity to current platinum based anticancer therapies. For example, HDAC1 and 3 and HDAC2 and 4 are overexpressed in different ovarian resistant cells to Cisplatin *(Kim MG et al. 2012).* Another example, high HDAC6 expression is associated with Cisplatin resistance in non-small cell lung cancer cells and HDAC6 inhibition sensitizes the cells to Cisplatin *(Wang L et al. 2012),* and high HDAC2 expression promotes Oxaliplatin resistance in colorectal cancer cells *(Alzoubi S et al. 2016).* Thereby, histone deacetylase inhibitors (HDACIs) appear to be a promising therapy to restore the sensitivity to platinum anticancer drug inducing cancer cell death *(Diyabalanage HVK et al. 2013)*. For example, SAHA (Vorinostat) is a pan-HDACI approved by the FDA 2006 for the treatment of cutaneous T-cell lymphoma. Nonetheless, in clinical trials, there are side effects like anemia, fatigue, diarrhea, neutropenia and thrombocytopenia, or deaths which were reported involving different HDACI including SAHA *(Mottamal M et al. 2015)*. The toxicity could be explained by the important role of HDACs in several biological process and by the low selectivity of HDAC inhibitors. Thereby, it is necessary to develop new more specific HDAC inhibitors. Likewise, it is important to determine the group of patients in which HDAC inhibitors will have the better response to minimize the side effects. In the present study, we wanted, first, to characterize the expression of HDACs in the different subtypes of GC, and to determine how the current treatment protocols impact on their expressions. Second, we studied combined treatments with more specific HDACIs and platinum anticancer drugs with the aim to propose personalized treatment according to the HDAC expression profile.

HDAC	Mutation	Amplification	Deep deletion	Fusion	Multiple alterations
HDAC1	7	4	2	-	-
HDAC2	4	1	5	-	-
HDAC3	8	1	-	-	-
HDAC4	25	1	8	-	-
HDAC5	11	6	2	-	1
HDAC6	9	1	4	-	-
HDAC7	13	1	1	-	-
HDAC8	3	4	1	2	-
HDAC9	12	7	2	-	-
HDAC10	5	1	11	-	-
HDAC11	9	3	1	-	-

Table 1: HDAC alterations in stomach adenocarcinomas

Table summarizing HDAC alterations on 434 total stomach adenocarcinomas of the TCGA database (Figure1).



	Number of Cases, Total	Number of Cases, Deceased	Median Months Survival
Cases with Alteration(s) in Query Gene(s)	**32**	**8**	**NA**
Cases without Alteration(s) in Query Gene(s)	**354**	**131**	**28.94**

Figure 2: Patient overall survival according to HDAC4 alterations in gastric cancer

The Cancer Genome Atlas: *http://www.cbioportal.org/*

Results

HDAC expression and treatment response in gastric cancer

To establish, which HDACs are altered in GC and, how these alterations correlate with the overall survival of patients and may represent a possible therapeutic target, we analyzed the alteration of HDACs and patient survival in 434 GC of the TCGA (The Cancer Genome Atlas) informatic base *(http://www.cbioportal.org).* These analyzes showed that in general HDACs are altered in 121/434 (28%) reported cases of GC, with HDAC4 being the most frequently altered HDAC 34/434 GC **(Figure 1 and Table 1).** Importantly, patients with HDAC4 alterations seem to have a better prognosis than patients without (log rank Test P-value = 0.107) **(Figure 2).** This result suggests that in GC certain HDACs might be potential therapeutic targets, and a combinatory treatment with HDAC inhibitors (HDACIs) might be beneficial the overall patient's survival.

However, not much information exists on how standard treatment protocols for GC impact on the expression of the different HDAC family members and neither on the respective effect of HDACIs. For this reason, we treated AGS cells, which is an intestinal type of gastric cancer cell (GCC) line, and MKN45 cells, which is a diffuse type of GCC line, with drugs frequently used for the treatment of GC. AGS cells and MKN45 cell were treated for 24h with Oxaliplatin, 5-FU, Oxaliplatin + 5-FU and SAHA, a pan-HDACI approved by the FDA 2006 for the treatment of cutaneous T-cell lymphoma, or a combinations Oxaliplatin + SAHA. RNA was extracted an analyzed then for the expression of the different HDAC family members by RT-qPCR **(Figure 3).** We observed that Class I HDAC members, HDAC 1, 2 and 3, and HDAC7 (Class II HDAC) are most expressed compare to HDAC8 and other Class II HDAC members, HDAC9 and 6, which are the lowest expressed HDACs. Globally, the expression profile of HDACs is similar between the AGS and the MKN45 cells. Interestingly, we observed that HDAC4 expression level is higher in MKN45 cells compare to its relative level in AGS cells. This result supports a previous study *(Spaety ME et al. In submission),* in which we described an overexpression of HDAC4 in patients with GC and more importantly in the diffuse subtype.

In AGS cells, current protocol treatments Oxaliplatin, 5-FU and their combination (base of FOLFOX treatment) did not change *HDAC1* expression **(Figure 4).** Interestingly, SAHA decreased HDAC1 mRNA level (~ 30%) and addition of Oxaliplatin did not modify this. Moreover, Oxaliplatin decreased around 20% *HDAC2* expression, and SAHA around 50%. Interestingly, the combination Oxaliplatin + SAHA reduced more importantly *HDAC2* expression, whereas 5-FU alone or combined with Oxaliplatin had not effect.

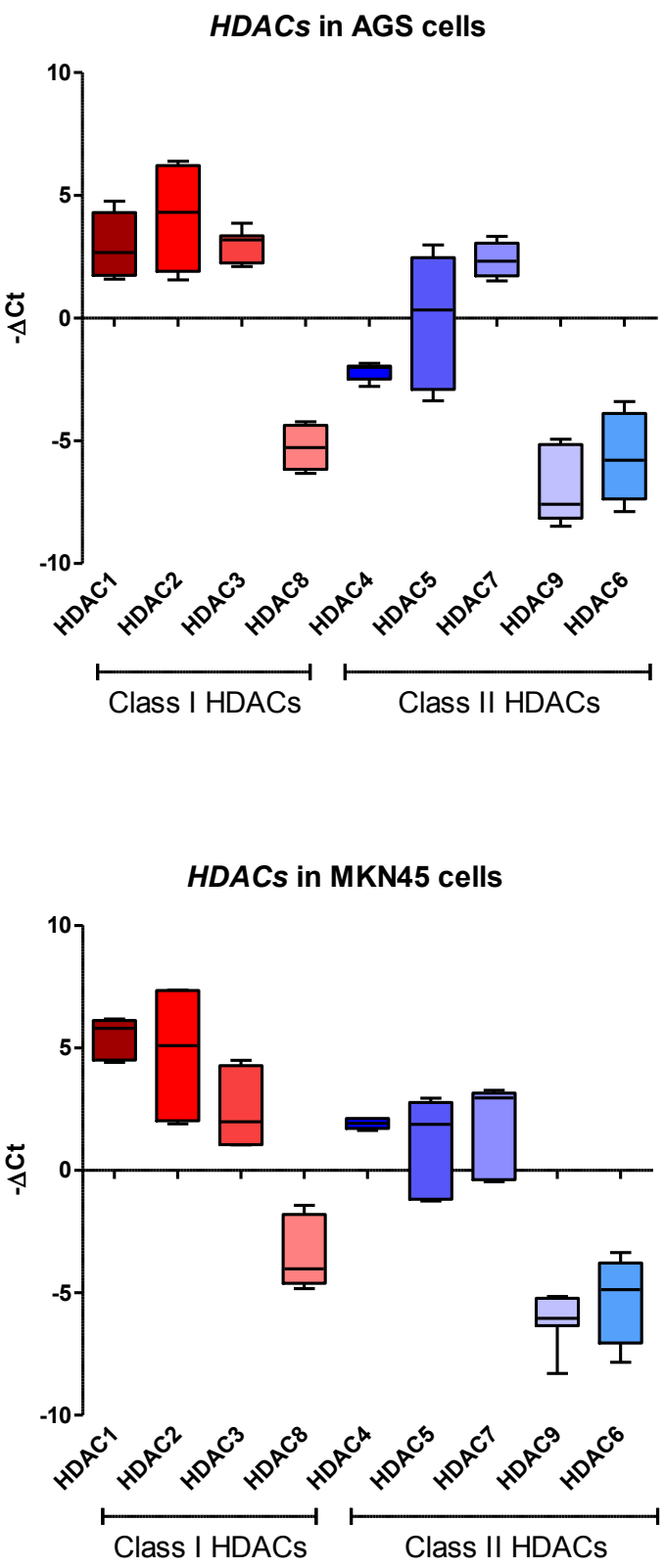


Figure 3: HDAC basal expression in AGS and MKN45 cells

HDAC mRNA levels were analyzed by RT – qPCR. Box plots represent the -1ΔCt (Ct_{HDAC} - Ct_{TBP}) for each HDAC.

In addition, SAHA was the only treatment increasing *HDAC3* expression (1,7-fold) but its combination with Oxaliplatin suppressed this effect. Finally, the last HDAC Class I member, HDAC8, has its expression 50% reduced by Oxaliplatin added or not with 5-FU, and SAHA had no effect on HDAC8 mRNA level and it did not change the effect of Oxaliplatin in combination. Likewise, Oxaliplatin and 5-FU alone 70% reduced *HDAC4* expression, and their combination synergistically more decreased HDAC4 mRNA level (around 90%) whereas SAHA didn't impact *HDAC4* expression and did not change Oxaliplatin effect in combination. It was the complete opposite for HDAC5. Indeed, Oxaliplatin 1,5-fold increased HDAC5 mRNA level, SAHA 2,6-fold and the combination synergistically 3,1-fold increased its expression. Moreover, Oxaliplatin 30% reduced *HDAC6* expression whereas in combination with 5-FU, Oxaliplatin 1,4-fold increased HDAC6 mRNA level. Neither SAHA nor 5-FU alone significantly changed *HDAC6* expression. In addition, Oxaliplatin 15% reduced *HDAC7* expression and 5-FU around 30%. Interestingly, SAHA practically suppressed *HDAC7* expression and combination with Oxaliplatin did not change this effect. Finally, Oxaliplatin, 5-FU and their combination 5 to 8-fold increased *HDAC9* expression. Interestingly, SAHA did not change HDAC9 mRNA level and more importantly suppressed the effect of Oxaliplatin in combination **(Figure 4).**

In MKN45 cells, Oxaliplatin did not change HDAC1 mRNA level whereas 5-FU reduced around 30% this HDAC expression. Interestingly, Oxaliplatin and 5-FU combined acted synergistically and reduced more HDAC1 level around 40%. SAHA alone decreased around 40% HDAC1 expression and combination with Oxaliplatin did not change the relative expression. Moreover, Oxaliplatin and 5-FU alone had no effects on *HDAC2* expression, whereas their combination 30% reduced HDAC2 mRNA level. SAHA 50% decreased *HDAC2* relative expression and addition of Oxaliplatin did not change this. In addition, SAHA had dominant effect on Oxaliplatin and 2.7-fold increased HDAC3 mRNA level whereas 5-FU did not impact HDAC3 expression. 5-FU decreased *HDAC8* expression (~ 20%) as well as SAHA, however we were not able to see a statistical difference compare to the untreated (NT) condition with the combination Oxaliplatin + 5-FU, and Oxaliplatin did not change SAHA's effect on HDAC8 mRNA level. Moreover, Oxaliplatin and 5-FU respectively 70% and 60% reduced *HDAC4* expression. Interestingly, their combination had synergistic effect and decreased around 90% HDAC4 expression. SAHA also reduced HDAC4 mRNA level around 70% and acted synergistically with Oxaliplatin to decrease to 80% *HDAC4* expression. Oxaliplatin and 5-FU alone or combined approximately 3-fold increased *HDAC5* expression whereas SAHA 7-fold increased its expression. In addition, we saw a significant decrease of *HDAC6* expression with Oxaliplatin combined with 5-FU (~ 70%).

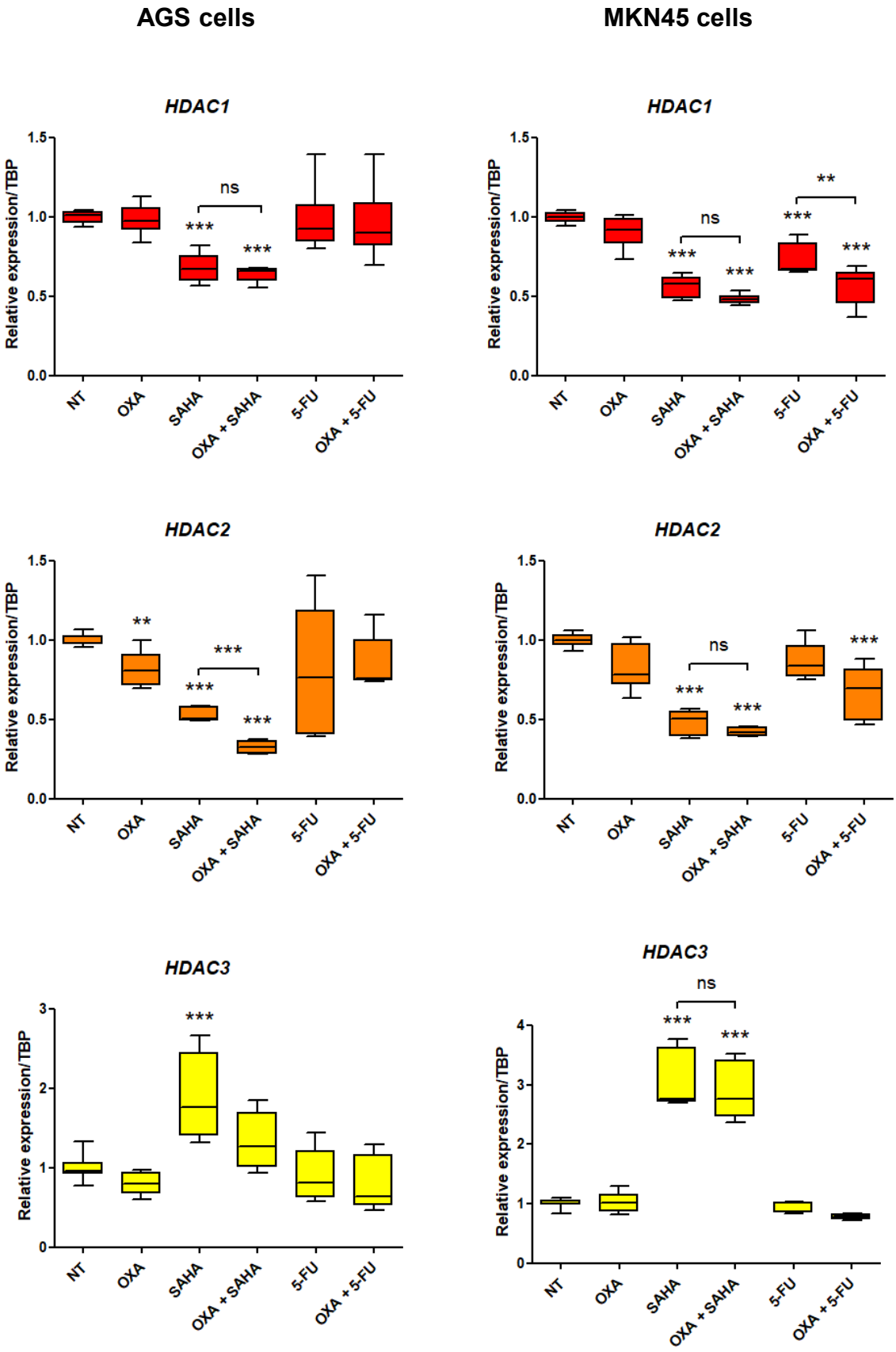


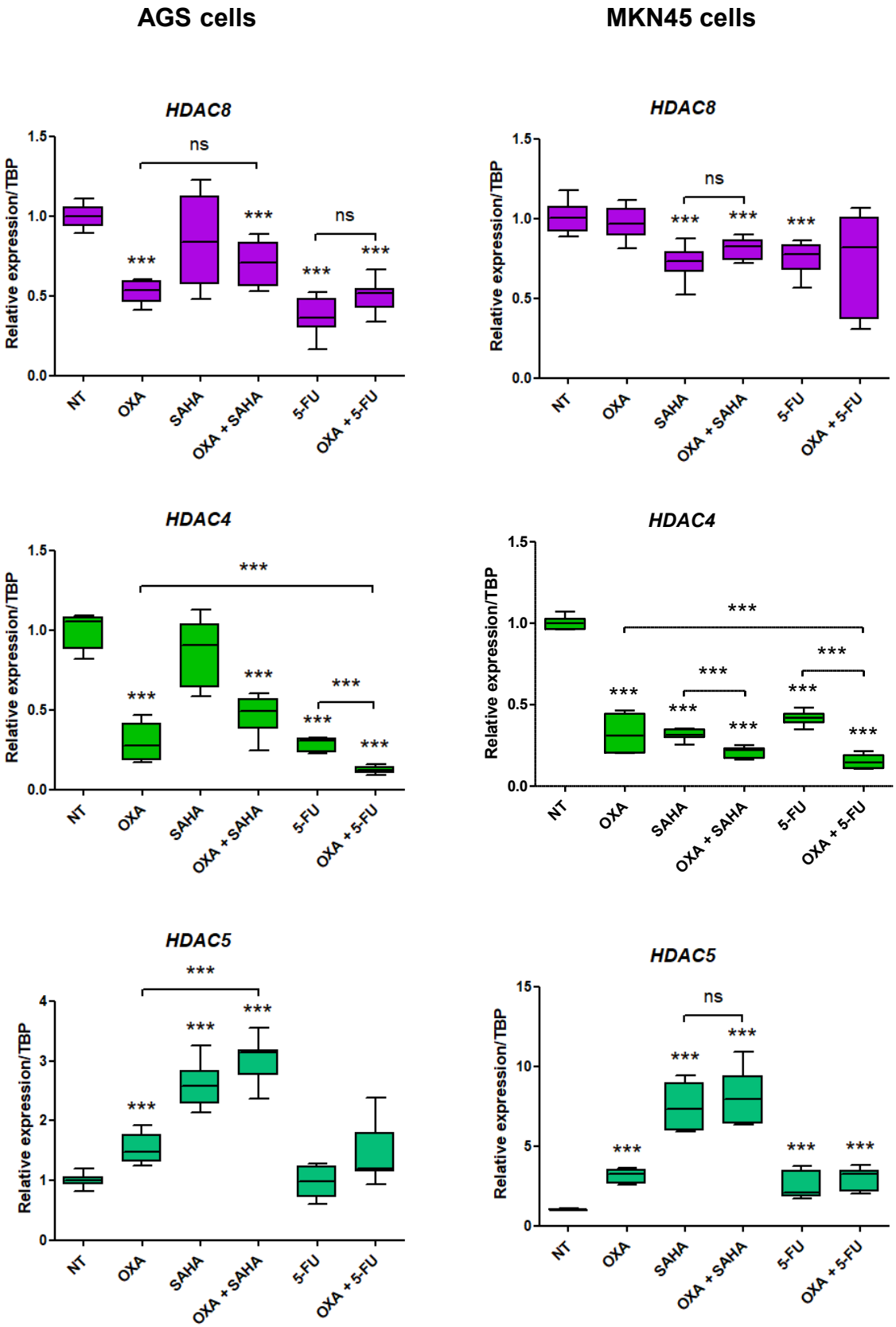
Figure 4: **Oxaliplatin and SAHA or 5-FU combined treatment effects on HDAC expression in gastric cancer cells**

Likewise, 5-FU alone or combined with Oxaliplatin 60% reduced HDAC7 mRNA level, and SAHA alone or combined practically suppressed HDAC7 expression. Finally, Oxaliplatin and 5-FU alone 3,1-fold increased *HDAC9* expression but with their combination we were not able to see a statistical difference compare to the untreated (NT) condition. SAHA 4-fold increased *HDAC9* expression. Interestingly, Oxaliplatin and SAHA seemed to have antagonist effect because their combination suppressed the induction of HDAC9 mRNA level **(Figure 4).**

SAHA alone or combined with Oxaliplatin decreased *HDAC1* expression and more importantly *HDAC2* expression level in both GCC lines AGS and MKN45. The combination OXA + 5-FU reduced both HDAC expressions in MKN45 cells only. On the contrary, 5-FU combined with Oxaliplatin reduced *HDAC8* expression in AGS cells and 5-FU alone decreased *HDAC8* expression in both cell lines. Moreover, SAHA reduced *HDAC8* expression in MKN45 cells alone or in combination with Oxaliplatin whereas, in AGS cells, *HDAC8* expression was reduced with Oxaliplatin alone or combined with SAHA. Interestingly, HDAC3 was the only Class I HDAC member which expression was induced with SAHA treatment in both cell lines. In addition, HDAC4, the most altered HDAC according to the TCGA, had its expression reduced with the different treatments in AGS and MKN45 cells, and more importantly with combined treatments. Moreover, Oxaliplatin, SAHA and their combination increased *HDAC5* expression in both GCC, and 5-FU also but only in MKN45 cells. Concerning *HDAC6* expression, the effects were different between the two cell lines. Indeed, Oxaliplatin decreased *HDAC6* expression in AGS cells, and the combination with 5-FU increased its expression whereas in MKN45 cells the Oxaliplatin + 5-FU decreased HDAC6 mRNA level. Likewise, 5-FU decreased *HDAC7* expression in GC cells but SAHA had the strongest effect. Finally, Oxaliplatin and 5-FU alone or combined increased HDAC9 mRNA level in AGS cells whereas in MKN45 cells we only had a significant increase with the different drugs alone **(Figure 4).**

Test of HDAC inhibitors in gastric cancer cells

In a previous work, we described that HDAC4 controls in part AGS GCC sensitivity to Cisplatin, and that HDAC4 is overexpressed in GC, and more in the diffuse type of GC, which is more present in developed regions like Europe *(Spaety ME et al. In submission).* We confirmed this observation in our model of diffuse type of GC, MKN45 cells **(Figure 3).** In addition, in the TCGA, we showed that HDAC4 alterations are correlated to a better patient overall survival **(Figure 2).** Thus, inhibition of HDAC4 may be an alternative therapeutic option. In addition, we characterized a higher expression of Class I HDACs, HDAC1, 2 and 3 in GCC.

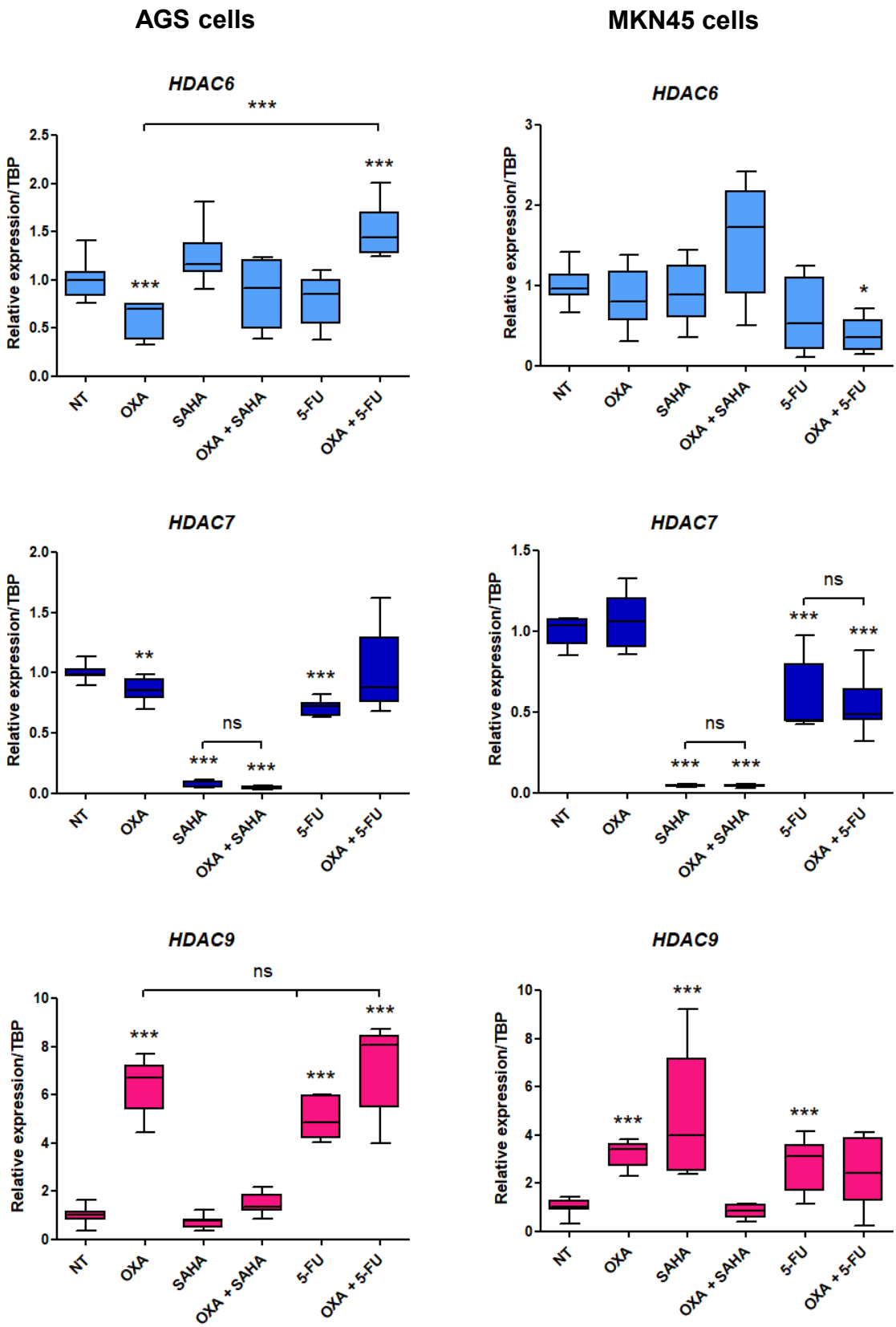


<u>Continuation figure 4:</u> Oxaliplatin and SAHA or 5-FU combined treatment effects on HDAC expression in gastric cancer cells

Interestingly, Class I HDACs are reported to be overexpressed in patient's tumoral gastric tissues *(Weichert W et al. 2008; Weichert W. 2009; Choi HK et al. 2015; Xu G et al. 2018).* Moreover, Class II HDAC enzymatic activities depend on multiprotein complex containing HDAC3 and SMRT/NCOR co-factors *(Fischle W et al. 2002),* and HDAC4 and HDAC9 are reported to form a complex with HDAC1 acting as a co-repressor on Cyclin D1 promoter *(Micheli L et al. 2017).* Thereby, it appears interesting to inhibit more specifically Class I and Class II HDACs. In addition, pan-HDAC inhibitors such as SAHA possess adverse side effects in clinical trials. Thus, we need to characterize more specific HDAC inhibitors.

We tested several HDACIs specific to a Class or a HDAC enzyme. We used pan-HDAC inhibitors; SAHA, SBHA and Sodium 4 Phenylbutyrate. We tested also a HDAC Class I more specific inhibitor; TC-H 106, a HDAC4/HDAC5 more specific inhibitor; LMK-235, a HDAC6 more specific inhibitor; Tubastatin A, and a new designed HDAC3 inhibitor; JGS-038. We used two intestinal GCC lines: AGS cells (p53 WT) and NUGC3 cells (p53 mutated Y220C) to compare the possible effect of p53 in the drug response. In our cell lines, we also compared the platinum derivative anticancer compound (PDC): Cisplatin, which is the historical chemotherapy **(Figure 5).** IC_{50} represents the concentration where we have 50% of the maximal effect on cell survival. AGS cells are less sensitive to Cisplatin compared to NUGC3 cells, the IC_{50} are respectively 25µM and 4,4µM. In both cell lines, S4PB, TC-H 106, Tubastatin A and SBHA have the lowest effect on GC cell survivals. Indeed, they didn't reach the IC_{50}, or reached it at concentrations widely superior to 10µM. On the contrary, LMK-235 influenced cell survivals similarly to the SAHA with a low IC_{50}. Interestingly, in both cell lines; the new synthetized inhibitor of HDAC3 had the lowest IC_{50} so the biggest effect on cell survival compare to all tested HDACI and PDC **(Figure 5).** For JGS-038, we determined IC_{50} of 1,4µM and 1.9µM for AGS and NUGC3 cells, respectively.

In GC, chemotherapy is used to reduce the risk of recurrence and metastasis in patients with localized tumor after surgery and to relieve symptoms in patients with unresectable tumors. Nonetheless, GC have intrinsic or acquired chemoresistance *(Shi WJ and Gao JB. 2016).* HDACs impact chemosensitivity and HDAC inhibitors are proposed to synergistically improve PDC cytotoxicity *(Diyabalanage HVK et al. 2013; To KKW et al. 2017).* JGS-038 possesses the lowest IC_{50} and HDAC3 is known to form complex to HDAC4 *(Fischle W et al. 2002),* and we know that *HDAC4* expression makes cell less sensitive to platinum drugs, thereby, we tested the combination between the JGS-038 with the historical PDC; Cisplatin to increase it cytotoxic effect **(Figure 6).** The hypothesis was that by inhibiting HDAC3 we might indirectly inhibit HDAC4 activity and observed similar results as our previous study, where HDAC4 inhibition synergistically decreases cell survival in combination



<u>Continuation figure 4:</u> Oxaliplatin and SAHA or 5-FU combined treatment effects on HDAC expression in gastric cancer cells

AGS cells were treated 24h or not (NT) with Oxaliplatin (OXA ; 7,4µM), SAHA (Vorinostat ; 3,5µM), 5-Fluorouracil (5-FU ; 28,6µM). MKN45 cells were treated 24h or not (NT) with Oxaliplatin (OXA ; 1,6µM), SAHA (Vorinostat ; 2,7µM), 5-Fluorouracil (5-FU ; 172,4µM). HDAC mRNA level were analyzed by RT –qPCR. Box plots represent the fold induction of mRNA for each condition normalized to the control (NT) (n=3). Statistical differences were calculated by Mann-Whitney test with a correction as p-value limit p=0,0033. ** p< 0,0033 ; *** p< 0,001

with Cisplatin *(Spaety et al. In submission).* Unfortunately, JGS-038 compound didn't act synergistically with Cisplatin on AGS cells where the sensitivity to Cisplatin was the lowest. Whereas JGS-038 acted synergistically for certain concentration of Cisplatin in NUGC3 cells.

Discussion

Alterations affecting HDAC expression and histone acetylation have been described in various types of cancers, including gastric cancers. These deregulations are thought to be related to cancer progression as well as resistance against cytotoxic anticancer drugs, thus resulting in a poorer overall survival for patients. For this reason, HDACs are proposed as promising therapeutic targets. However, in clinical trials, several sides were reported like anemia, fatigue, diarrhea, and neutropenia involving different HDAC inhibitors (HDACIs) including SAHA. Consequently, it is necessary to minimize the toxic effect of HDACI in patients. This toxicity may be explained by the low selectivity of the currently used HDACIs like SAHA. In the present study, our first objective was to characterize the HDAC expression in gastric cancer, how their expressions are altered by the standard treatment protocols and how they are affected by a HDACI, SAHA an pan-HDACI approved by the FDA 2006 for the treatment of cutaneous T-cell lymphoma. Our second objective was then to test several newly developed inhibitors of HDACs, some of them being more specific for a certain HDAC subfamily or specific HDAC for their toxicity on different GC cell lines. And our third aim was then to establish a combinatory treatment protocol with a more specific HDACI.

We determined that in gastric cancer cell (GCC) lines MKN45 and AGS, there is a high expression of Class I HDACs (HDAC 1, 2 and 3), corroborating with the literature describing Class I HDAC overexpression in GC *(Weichert W et al. 2008; Weichert W. 2009; Choi HK et al. 2015; Xu G et al. 2018),* and associating HDAC2 expression with poor prognosis *(Weichert W et al. 2008; Weichert W. 2009).* In addition, these HDACs are described to control cancer cell sensitivity to current therapies. For example, overexpression of HDAC1 and 3 and overexpression of HDAC2 is associated with Cisplatin resistance in different ovarian cancer cells *(Kim MG et al. 2012)* and high expression of HDAC2 promotes 5-FU and Oxaliplatin resistance in colorectal cancer cells *(Alzoubi S et al. 2016).*

Moreover, in our study, we showed that HDAC4 is more expressed in diffuse type MKN45 GC cells than in intestinal type AGS GC cells, corroborating with a previous work, in which we observed overexpression of HDAC4 in GC and more importantly in diffuse type of

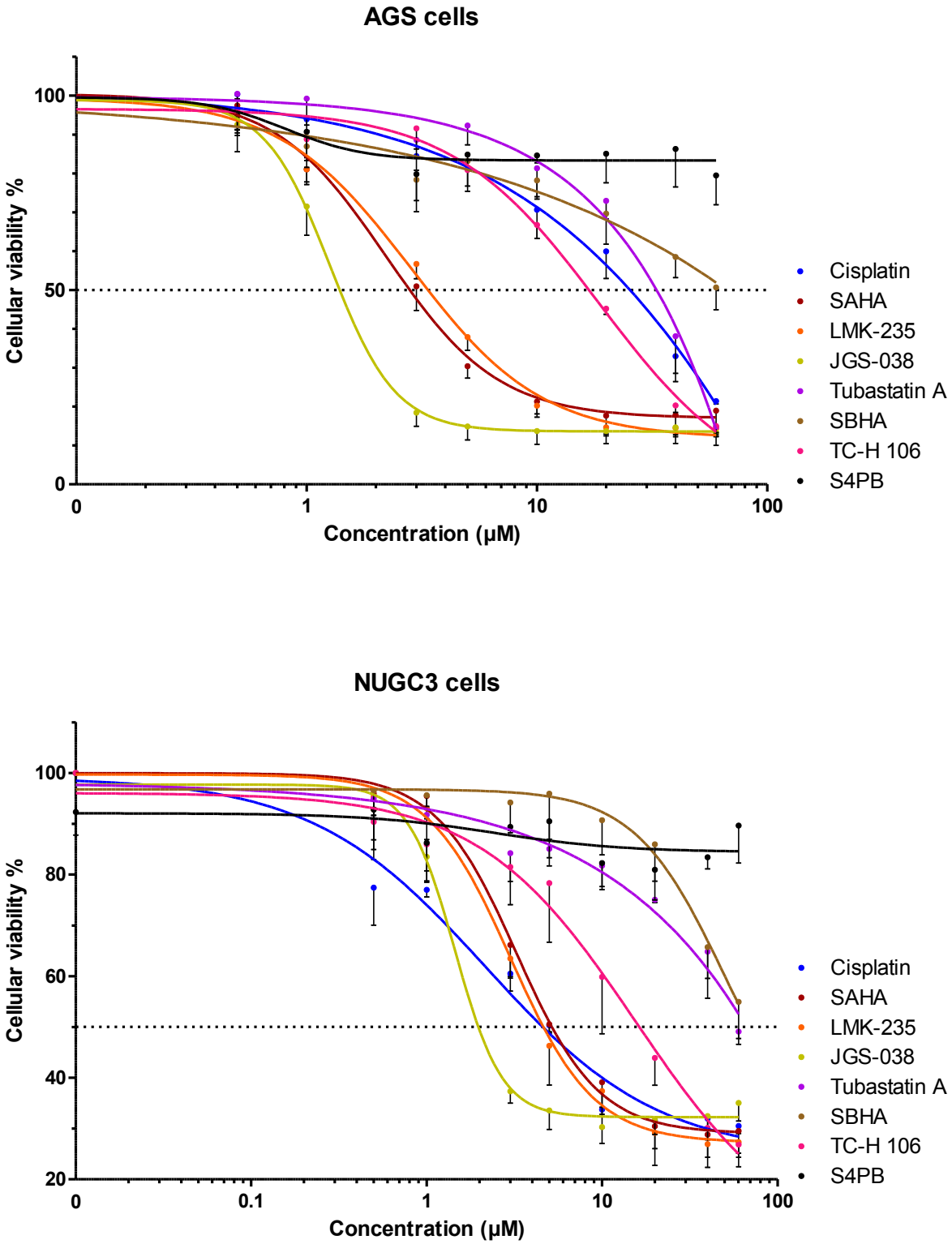


Figure 5: SAHA and platinum compounds impact gastric cancer cell survival

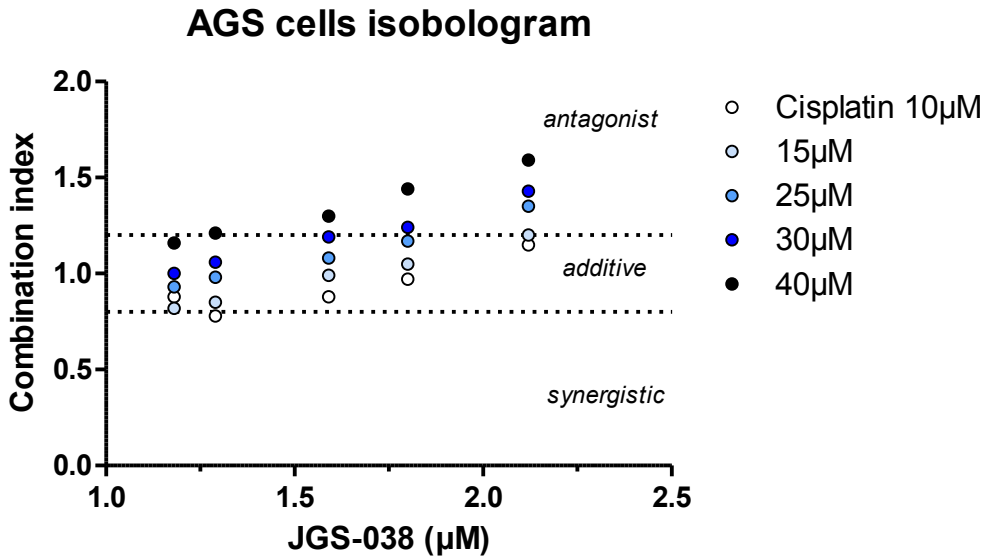
Gastric cancer cells were seeded in 96-wells and treated 48h with platinum derivative compound (Cisplatin) or HDAC inhibitors (SAHA; LKM-235; JGS-038; Tubastatin A; SBHA; TC-H 106; sodium 4 phenylbutyrate [S4PB]). Viability of the cells was evaluated using MTT tests. Curves are generated as log(inhibitor) vs. response -- Variable slope (four parameters) and represent the mean + SEM of three independent experiments.

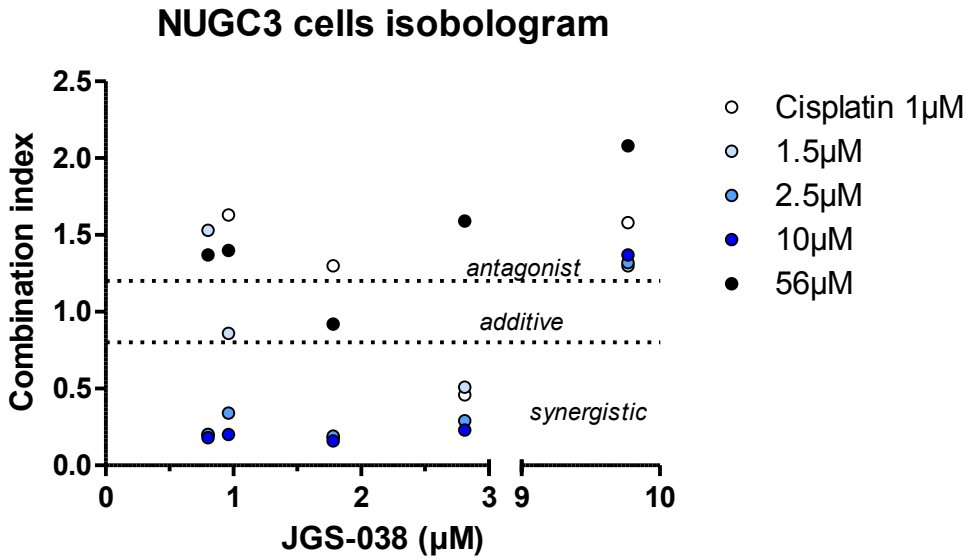
GC *(Spaety ME et al. In submission).* Besides, we remarked in the TCGA database, that patients with GC possessing alterations in HDAC4 have a better overall survival. *In vitro* study revealed that HDAC4 level controls in part Cisplatin sensitivity of AGS cells *(Spaety ME et al. In submission),* suggesting that HDAC4 activity is involved in GC aggressiveness. This is supported in the literature, indeed, in ovarian cancer cell lines, HDAC4 overexpression enhances Cisplatin resistance increasing cellular viability *(Kim MG et al. 2012)* whereas HDAC4 silencing promotes Cisplatin sensitivity *(Stronach EA et al. 2011),* and HDAC4 inhibition increases docetaxel cytotoxicity in SNU-16 GCC *(Colarossi L et al. 2014).*

In addition, Class II HDAC enzymatic activity is dependent on multiprotein complex containing Class I HDAC. Indeed, HDAC7 activity is associated to HDAC3 *in vivo (Fischle W et al. 2001)* and HDAC4 and HDAC9 can form a complex with HDAC1 to act as co-repressors on Cyclin D1 promoter *(Micheli L et al. 2017).* In addition, we determined, in our models, that Class I HDACs is higher expressed, among which HDAC3, which is essential for the NCOR/SMRT axis to maintain chromatin structure and DNA damage repair *(Bhaskara S et al. 2010).* Interestingly, Ding JD and co-workers suggest that SAHA (suberanilohydroxamic acid), a pan-HDACI approved in 2006 by the FDA for the treatment of advanced primary cutaneous T-cell lymphoma, induces apoptosis depending on the inhibition of HDAC3 *(Ding JD et al. 2012).* We throughout showed that Class I HDACs (HDAC1, 2 and 3) and Class II HDACs (HDAC4 and 7) are the most expressed HDACs in GCC, and to a greater degree for Class I. We also showed that Class II HDACs (HDAC 4, 5, 7 and 9) and HDAC3 are in majority more impacted by the current treatments. Interestingly, these HDACs are reported to be associated with cancer aggressiveness as previously cited *(Ding JD et al. 2012; Colarossi L et al. 2014; Stronach EA et al. 2011; Kim MG et al. 2012).* Another example from Li A and co-workers, showed that *in vitro* HDAC5 inhibition reduces cell growth and induces apoptosis of breast cancer cells *(Li A et al. 2016).*

Altogether, it suggests that a combinatory treatment with an HDAC inhibitor (HDACI) might be beneficial for the treatment of cancers. Nonetheless, utilization of pan-HDAC inhibitors such as SAHA may lead to adverse side effects in clinical trials *(Mottamal M et al. 2015).* For example, SAHA (Vorinostat) possesses higher toxicities than standard treatment protocol in phase II study on patients with unresectable or metastatic GC *(Yoo C et al. 2016)*. Thereby, it is necessary to study more specific HDAC inhibitors.

We tested different HDACIs more specific for a certain HDAC subfamily or specific HDAC for their toxicity on different GC cell lines. Our results pointed that the pan-HDACI SAHA, the HDAC4/HDAC5 more specific inhibitor LMK-235 and the HDAC3 inhibitor JGS-





<u>Figure 6:</u> Isobologram assays on GCC

AGS and NUGC3 cells were treated with Cisplatin (CISP) or Oxaliplatin (OXA) and JGS-038 for 48h followed by an isobologram assay to determine mathematically the combination index. We arbitrarily and conservatively considered antagonist effect between the drugs correspond to value > 1.20, additive effect between 0.80 and 1.20 and synergistic effect <0.80.

038 possess the lower IC_{50} so the higher effects on AGS and NUGC3 GCC survival. Likewise, HDAC inhibitors have been proposed to sensitize cancer cells to platinum anticancer drugs (PDC) increasing their cytotoxicity *(Diyabalanage HVK et al. 2013; To KKW et al. 2017).* JGS-038 possessing the lowest $IC_{50,}$ HDAC3 forming complex with HDAC4 *(Fischle W et al. 2002),* and HDAC4 level controlling cell sensitivity to PDC, we tested the combination between the JGS-038 with the historical PDC; Cisplatin to increase it cytotoxic effect. Unfortunately, JGS-038 compound didn't act synergistically with Cisplatin on AGS cells, in which the sensitivity to Cisplatin was the lowest, whereas JGS-038 acted synergistically for certain concentration of Cisplatin in NUGC3 cells. These results may indicate that inhibition of HDAC3 does not indirectly inhibit HDAC4 activities in AGS cells. It is probably not a HDAC4/HDAC3 complex which regulates PDC sensitivity in AGS cells. However, JGS-038 acts synergistically with certain concentrations of Cisplatin in NUGC3 p53 mutated cells, suggesting that it is p53 is not involved in the decrease of cell survival in response to JGS-038. Recently, a comparative effect of different HDACIs on 60 cell lines revealed that, besides p53, additional molecular mechanisms cause HDACI efficiencies *(McClure JJ et al. 2018),* which is the case in NUGC3 cells. In addition, JGS-038 did not act synergistically just with low or high concentrations of Cisplatin, and we did not observe specific and logical combinations of drug concentrations. Further experiments are necessary to determine the mode of action of the JGS-038 and its specificity, and maybe to test the combination with Oxaliplatin, the other PDC more used in current treatment, before performed *in vivo* analysis to determine if JGS-038 may represent a promising therapeutic alternative.

DISCUSSION

“The ideal logician, he remarked, should be able when he has been shown only once a particular fact in all its aspects, to deduce from it not only all the succession of events which led to it, but also all the consequences that will ensue.”

Sir Arthur Conan Doyle, *The five Orange Pips*

These last years, the incidence of gastric cancer (GC) (out of gastroesophageal junction and cardia cancers) has decreased around 1.5% *(Michel P et al. 2017).* However, the 5-year overall survival is still low, around 15% making GC a health problem *(CDU-HGE 2015).* Current treatments rest on surgery completed with chemotherapy based on platinum compounds and 5-Fluorouracile and/or radiotherapy. Unfortunately, a limited number of tumors responds to the therapies owing to insensitivity to the treatments and/or selection of resistant gastric cancer cells *(Shi WJ and Gao JB. 2016)*. All the mechanisms involved in gastric carcinogenesis including the resistance to therapies are not totally known. Thereby, to improve the patient care and their quality of life, we need to better understand the molecular pathways and the genetic alterations involved in gastric carcinogenesis and tumor aggressiveness. This work will lead to the identification of new diagnostic and therapeutic markers, to adapt current treatments and certainly to propose new therapies.

In the first objective of the present thesis, we studied HDAC4 functions and its therapeutic interest in GC. More precisely, we described an auto-regulatory loop p53/miR-140/HDAC4 in response to Cisplatin, and we highlighted the role of HDAC4 in platinum derivative anticancer drug (PDC) sensitivity. In a second objective, we determined that HDAC4, like other HDACs, are highly expressed in gastric cancer cells (GCC) and that they are associated with cancer cell resistance to current treatments. Thereby, in a third objective, we tested different HDAC inhibitors (HDACIs) specific to a HDAC subclass or enzyme, which we combined with PDC to improve their chemotherapeutic effects with the aim to propose personalized treatments according to the HDAC expression profile.

The auto-regulatory loop p53/miR-140/HDAC4

We showed that overexpression of p53 reduces miR-140 expression and that inversely, suppression of p53 is associated with an increase of miR-140 level. Moreover, we showed that miR-140 targets HDAC4, thereby, p53 can modulates *HDAC4* expression via miR-140. In addition, we observed a decreasing trend of p53 expression when we suppressed HDAC4, and we observed that Cisplatin reduces HDAC4 expression via miR-140. Consequently, we described a functional auto-regulatory loop between p53, miR-140 and HDAC4 modulated by Cisplatin. Interestingly, we found that HDAC4 level controls in part AGS cells sensitivity to Cisplatin treatment, suggesting that HDAC4 might be a therapeutic marker. Likewise, we described an overexpression of HDAC4 in gastric tumoral tissue from patients, and mostly in the genetic stable and in the chromosome instable subgroup (TCGA), as well as in the diffuse versus the intestinal histological subgroup. In addition, we observed

that miR-140 expression is higher and HDAC4 expression is lower in patients with GC p53 mutated. Besides, we hypothesized that HDAC4 overexpression increases Cisplatin resistance, explaining the frequent resistance of GC to the current treatments, and on the contrary, high expression of miR-140 decreases HDAC4 level, increasing Cisplatin sensitivity and reducing GC aggressiveness. Interestingly, Fang and co-workers described that 97 out of 144 patients with GC highly expressing miR-140 is associated with a better overall survival (Log-rank p=0.0084) *(Fang Z et al. 2017),* supporting thereby our hypothesis. Furthermore, our hypothesis also corroborates with the reported HDAC4 overexpression, which promotes tumor progression and is associated with poor prognosis in esophageal carcinoma *(Zeng LS et al. 2016).*

Then, we tested our hypothesis on other GCC lines. Because HDAC4 expression is lower in patients with GC p53 mutated, and because we described an auto-regulatory loop containing p53 and HDAC4 in response to Cisplatin, we compared the intestinal type p53-WT AGS GC cells, with the p53-mutated (Y220C) NUGC3 GC cells. Moreover, because diffuse and intestinal types of GC have different clinical-pathological characteristics, which may impact the chemotherapy *(Ma J et al. 2016),* we also used diffuse type of GC model, the MKN45 cell line.

We observed that HDAC4 suppression increased the cleavage of caspase 3 in response to Cisplatin in AGS and in NUGC3 cells, but it was to a lesser extent in NUGC3 cells than in AGS cells. Thereby, in p53 mutated cells, the reduction or the suppression of HDAC4 level does not promote the apoptotic response to Cisplatin treatment. This result may indicate that the p53 status play a role in Cisplatin response. Interestingly, Tahara and co-workers observed that patients with GC mutated for TP53 possess a worse overall survival *(Tahara T et al. 2016)* corroborating with our observations. Moreover, in osteosarcoma U-2 OS (p53 WT) and colon cancer HCT116-p53 WT cells, ectopic expression of miR-140 increases p53 and p21 expressions, in part through the suppression of HDAC4. But ectopic expression of miR-140 does not increase p21 expression in osteosarcoma MG63 (p53 mutated) and in HCT116-p53 null cells, indicating that miR-140 exerts its functions in a p53 dependent manner *(Song B et al. 2009).*

According to our hypothesis, high expression of miR-140, reduces HDAC4 expression increasing Cisplatin sensitivity and according to Fang and co-workers GC highly expressing miR-140 are associated with a better overall survival *(Fang Z et al. 2017).* However, we observed that HDAC4 suppression reduced the level of cleaved caspase 3 in MKN45 cells, which may indicate that HDAC4 has opposite effects in response to Cisplatin in diffuse type of GC. Interestingly, Zhang and co-workers described miR-140 overexpression in cancer

stem cell-like MKN45 subpopulation cells *(Zhang HH et al. 2015).* The hypothesized it that these cancer stem cells are involved in the high mortality of gastric cancer by the initiation of relapse and metastasis in GC *(Zhang HH et al. 2015).* Thereby, the miR-140/HDAC4 axis might have different effect between intestinal and diffuse type of GC.

In addition, in the literature, low expression of miR-140 is reported to promote chemoresistance in osteosarcoma cells *(Meng Y et al. 2017),* but also cancer stem cell formation in basal-like early stage breast cancer *(Li Q et al. 2014)* and liver cancer progression and development via NFκB activation *(Takata A et al. 2013).* On the contrary, up-regulation of miR-140 is reported to inhibit the EMT, invasion and metastasis in colorectal cancer *(Yu L et al. 2016; Li J et al. 2018)* and it is described to directly suppress PD-L1 expression inhibiting PD-L1/Cyclin E pathway and reducing non-small cell lung cancer proliferation *(Xie WB et al. 2018).* Thereby, miR-140 seems to have anti-tumoral activities.

Altogether, we can distinguish cellular pathways which may be controlled by the miR-140/p53/HDAC4 loop involves in drug response, tumor growth and maybe in immunotherapy response. In GC, the activity of miR-140 seems to depend on cancer subtype and the p53 status. A better understanding of the miR-140 expression regulation and functions of its target genes, such as HDAC4, will improve the understanding on cancer resistance and progression and may lead to a better therapeutic approach.

HDAC4 related genes

In our study, HDAC4 suppression promotes apoptosis in response to Cisplatin in AGS cells. We wanted to determine which genes are negatively expressed compared to HDAC4 and which are involved in Cisplatin response. For this, we performed an unsupervised pathway analysis *(DAVID: https://david.ncifcrf.gov; https://reactome.org)* and we also performed a co-expression analysis out of 2345 genes from the TCGA database *(http://www.cbioportal.org).* In absence of Cisplatin treatment, we confirmed the negative expression correlation between *HDAC4* and *BID, AIFM1, CASPASE 8, BIK* and *NOXA* involved in apoptotic pathway, *p21* involved in cell cycle regulation, and *VAMP8* and *STXBP2* involved in vesicular traffic. In response to Cisplatin, HDAC4 suppression thwart the reduction of *BID, AIFM1,* and *CASPASE 8* mRNA levels, and interestingly, HDAC4 suppression 1,4-fold and 2-fold induces *BIK* and *NOXA* expressions, respectively, correlating with apoptosis (cleaved caspase 3 in Western Blot). Di Giorgio and co-workers described that genes which expression is controlled by HDAC4 may be or not also MEF2 targets *(Di*

Giorgio E et al. 2013). When looking in the literature, MEF2 promotes *p21, CYCLIN D1* and *KLF4* expressions reducing cancer cells proliferation *(Ma Y et al. 2014; Rowaland BD and Peeper DS. 2005).* Moreover, the transcriptional factor KLF4 promotes also *p21* expression in response to DNA damage in HCT116 cells via p53 *(Yoon HS et al. 2003),* thereby KLF4 can suppress tumor formation in colon and gastric tumors *(Ma y et al. 2014; Ghaleb AM et al. 2016).* Interestingly, KLF4 is a target of miR-140 *(Li Q et al. 2014),* as well as p38 MAPK, which can reduce MEF2 expression *(Chen X et al. 2017),* thereby KLF4 and MEF2 may be other genes regulated by the p53/miR-140/HDAC4 loop. However, the literature appears contradictory because miR-140 may inhibits KLF4 expression reducing tumor progression, and as the same time, may promotes MEF2 expression, which may induce *p21* and *KLF4* expressions. In addition, *KLF4* as *MEF2* roles in cancers are controversial *(Wang B et al. 2015; Liu J et al. 2015; Zhao Y et al. 2016; Xu K and Zhao YC. 2016).* Indeed, KLF4 is reported to induce apoptosis and inhibit tumorigenic progression and as the same time it is reported to participate in the metastasis and invasion in different model of breast cancer cells *(Wang B et al. 2015).* Likewise, MEF2A member promotes *GLUT-4* expression enhancing glucose uptake and gastric cancer cell growth *(Liu J et al. 2015),* and at the same time, MEF2A is reported to promote *p21* and *CYCLIN D1* expression reducing GCC proliferation *(Ma Y et al. 2014).* Further analyzes to determine the HDAC4/MEF2 signaling pathway in gastric cancer, as well as KLF4 and other miR-140 targets may be an interesting research axis.

HDAC expression and drug sensitivity

We determined that, in MKN45 and AGS GCC lines, there is a high expression of Class I HDACs (HDAC 1, 2 and 3), corroborating with several studies describing Class I HDAC overexpression in GC *(Weichert W et al. 2008; Weichert W. 2009; Choi HK et al. 2015; Xu G et al. 2018).* Moreover, we showed that HDAC4 is more expressed in diffuse type MKN45 GC cells than in intestinal type AGS GC cells, corroborating with the precedent part, in which we observed overexpression of HDAC4 in GC and more importantly in diffuse type of GC. In addition, we remarked in the TCGA database, that patients with GC possessing alterations in HDAC4 have a better overall survival. As our i*n vitro* study revealed that HDAC4 level controls in part Cisplatin sensitivity of AGS cells (precedent part), it highlights that HDAC4 activity is involved in GC aggressiveness. This is supported in the literature, indeed, in ovarian cancer cell lines, HDAC4 overexpression enhances Cisplatin resistance increasing cellular viability *(Kim MG et al. 2012)* whereas HDAC4 silencing promotes Cisplatin sensitivity *(Stronach EA et al. 2011).*

However, Kim MG and co-workers also observed that different HDAC enzymes are associated with Cisplatin resistance and they suggested that HDAC enzymes may confer drug resistance via several mechanisms *(Kim MG et al. 2012).* Indeed, overexpression of HDAC1, 2 or 3 is associated with Cisplatin resistance in different ovarian cancer cells *(Kim MG et al. 2012)* and high expression of HDAC2 promotes 5-FU and Oxaliplatin resistance in colorectal cancer cells *(Alzoubi S et al. 2016).* Likewise, high HDAC6 expression is associated with Cisplatin resistance in non-small cell lung cancer cells and its inhibition sensitized cells to Cisplatin *(Wang L et al. 2012).* Moreover in *vitro* HDAC5 inhibition reduces cell growth and induces apoptosis of breast cancer cells *(Li A et al. 2016).* In our models, we also showed a high expression of HDAC7, which expression is correlated to a poor prognosis in patients with GC *(Yu Y et al. 2017).* Finally, we showed that Class II HDACs (HDAC 4, 5, 7 and 9) and HDAC3 are globally more impacted by the current treatment protocols.

Interestingly, these HDACs are reported to be associated with cancer aggressiveness as just discussed. Suggesting that combinatory treatment with HDAC inhibitors (HDACIs) and current treatments might enhance the anticancer drug efficiencies.

Combinatory treatments between HDACIs and platinum compounds

Combinatory treatments PDC + SAHA

We showed that SAHA (suberanilohydroxamic acid), a pan-HDACI approved in 2006 by the FDA for the treatment of advanced primary cutaneous T-cell lymphoma, acts synergistically with platinum derivative anticancer compounds (PDC: Cisplatin, Oxaliplatin) on GCC survival. However, we observed synergistic effects between Cisplatin and SAHA in AGS (p53 WT), NUGC3 (p53 mutated) and KATOIII (p53 deleted) cells but not in MKN45 (p53 mutated) cells whereas we observed synergistic effect between Oxaliplatin and SAHA in all these cell lines. Thereby, treatment responses depend on the PDC used in cancer cells. Moreover, we only observed apoptosis (cleavage of caspase 3 by Western Blot) induced by combinatory treatments PDC + SAHA in AGS and NUGC3 cells.

One of the mechanisms explaining this difference in our cell lines is the p53 status. Indeed, in AGS cells, although combinatory treatments decrease p53 expression, the suppression of p53 (with siRNA) inhibits the apoptosis (cleavage of caspase 3 suppressed). In addition, in NUGC3 cells, pharmacologic reactivation of p53 increase the apoptosis

(cleavage of the caspase 3 enhanced). However, in KATOIII cells, we didn't observe an apoptosis (cleavage of caspase 3 by Western Blot), neither in MKN45 cells.

In the literature, the impact of p53 in SAHA-mediated response is controversial. Indeed, Sonnemann J and co-workers demonstrated that SAHA does not require p53 for its anticancer effects *(Sonnemann J et al. 2014).* Li D and co-workers also showed that SAHA appeared more active on cancer cells p53 mutated *(Li D et al. 2011)* and Alzoubi S and co-workers showed that SAHA acted synergistically with doxorubicin in colon cancer cells independently of p53 *(Alzoubi S et al. 2016).* Nonetheless, p21 induction reducing cell proliferation is induced by p53-dependent and p53-independent pathways *(Lee JH et al. 2012).* SAHA combined with anticancer drugs in medulloblastoma cells *(Hacker S et al. 2011)* and combined with doxorubicin on cervical cancer cells *(Lee SJ et al. 2014)* appeared to act synergistically on p53. Interestingly, McClure JJ and co-workers screened 60 cell lines sensitivity to HDAC inhibitors showing lethality in p53 WT cells. However, they noted that not all p53 WT cell lines are sensitive to the HDAC inhibitor treatments, indicating that besides p53, additional molecular mechanisms cause HDACI efficiency *(McClure JJ et al. 2018),* which concern in our study KATOIII and MKN45 cells.

Another possibility which may explain the differences observed between our cell lines in response to combined treatments is the molecular characteristic of the cells. More precisely, MKN45 cells are diffuse type of GC compare to AGS, KATOIII and NUGC3 cells which are intestinal type of GC. In addition, MKN45, KATOIII and NUGC3 cells are derived from metastasis whereas AGS cells are from a primary tumor site which might impact the aggressiveness and the drug response.

Concerning the molecular pathways involved in combinatory treatments response, in AGS cells, we showed that Cisplatin + SAHA combination decreases more importantly HDAC4 level. According to the precedent part, this increase the cellular sensitivity to Cisplatin. Likewise, in our cells, combinatory treatments PDC + SAHA increased MEF2A expression. We also showed that they decrease pAkt level which may promote MEF2A activation *(Di Giorgio E et al. 2013)* suggesting expression of MEF2 target genes and hypothesized that MEF2 play anti-tumoral functions in GC. Moreover, PDC + SAHA combinations reduced CDX2 mRNA level in AGS GCC. CDX2 is overexpressed in GC and is involved in early-stage of tumorigenesis *(Xiao ZY et al. 2012),* but CDX2 expression is reported to decrease with tumor stage *(Liu Q et al. 2007)* makes tumor cells poorly differentiated promoting metastasis. Interestingly, CDX2 overexpression promotes GCC multidrug resistance *(Yan LH et al. 2014).* Thereby, the observed reduction of CDX2 may participate to treatment cytotoxic effects.

In addition, SAHA induced the expression of TAp63 in GCC, suggesting that this p53 family member is somehow involved in the anticancer mechanism of SAHA in GC, which has only been described in head and neck cancer in the literature *(Finzer P et al. 2004).* Whereas TAp73 were induced with Oxaliplatin + SAHA combination at 8h only and decreased at 24h with Cisplatin + SAHA combination. TP53 family members have common functions like apoptosis induction and can interact or regulate their expression each other. Because of TP53 status impacts the response to combined treatments, and because p53 is mutated in around 50% of GC (TCGA), we need to better understand the TP53 family interaction and regulation to identify therapeutic benefits (*Wei J et al. 2012; Park S et al. 2016).*

At last, Xu S and co-workers synthetized a Cisplatin-SAHA nanodrug, which interestingly possesses higher toxicity than Cisplatin and SAHA alone in Cisplatin sensitive and resistant non-small cell lung cancer cell lines *in vitro* and *in vivo.* Especially, the nanodrug increases accumulation at the tumor site, enhances cellular uptake and DNA binding efficacy, and down-regulates Cisplatin resistance-related proteins *(Xu S et al. 2017).* Although, Xu S and co-workers highlight the interest to merge chemically Cisplatin and SAHA, their study supports our results and the interest of the synergistic combinations between PDC and SAHA as anticancer protocol.

Combinatory treatment Cisplatin + LMK-235

Utilization of pan-HDAC inhibitors such as SAHA may lead to adverse side effects in clinical trials *(Mottamal M et al. 2015).* For example, SAHA (Vorinostat) possesses higher toxicities than standard treatment protocol in phase II study on patients with unresectable or metastatic GC *(Yoo C et al. 2016)*. Thereby, it is necessary to study more specific HDAC inhibitors. Previously, we described HDAC4 suppression increases Cisplatin sensitivity in AGS cells. We highlighted the clinical relevance of the p53/miR-140/HDAC4 regulatory loop by inhibiting more specifically HDAC4 in *vitro* and *in vivo* with LMK-235, which significantly increased Cisplatin cytotoxicity. Therefore, the use of selective inhibitor of HDAC4, such as LMK-235, in combination with Cisplatin may represent a promising therapeutic alternative.

Combinatory treatment Cisplatin + JGS-038

We tested several HDACIs specific to a Class or a HDAC enzyme. Interestingly, in AGS and NUGC3 cell lines, the new synthetized inhibitor of HDAC3, JGS-038, has the lowest IC_{50} so the biggest effect on cell survival compare to all tested HDACI, with IC_{50} of 1,4µM and 1.9µM for AGS and NUGC3 cells, respectively. Because of Class II HDAC enzymatic activities is dependent on multiprotein complex containing HDAC3 and

SMRT/NCOR co-factors *(Fischle W et al. 2002),* we hypothesized that by inhibiting HDAC3 we might indirectly inhibit HDAC4 activity and observed similar results as our previously, where HDAC4 inhibition synergistically decreases cell survival in combination with Cisplatin. Unfortunately, JGS-038 compound didn't act synergistically with Cisplatin on AGS cells where the sensitivity to Cisplatin was the lowest. Whereas JGS-038 acted synergistically for certain concentration of Cisplatin in NUGC3 cells. Further experiments are necessary to determine the mode of action of the JGS-038 and its specificity, and maybe to test the combination with Oxaliplatin, the PDC commonly used in current treatment, before performed *in vivo* analysis to determine if JGS-038 may represent a promising therapeutic alternative. However, the fact that JGS-038 acts synergistically with Cisplatin in p53 mutated cells, knowing that p53 is frequently mutated in GC, is interesting.

CONCLUSION & PERSPECTIVES

My thesis highlights the role of HDACs in platinum derivative anticancer drug (PDC) response in gastric cancer (GC). The results suggest that HDACs, in particular HDAC4, might be promising prognostic markers and therapeutic targets in GC. However, several aspects are yet to be addressed before transferring our findings into the clinic.

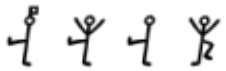
To validate the usefulness of HDAC4 as a predictive marker for Cisplatin response, we need to perform a clinical prospective study to investigate the link between HDAC4 expression and the overall survival in patients treated with platinum compounds. In addition, it is not fully understood how HDAC4 controls Cisplatin sensitivity at the molecular level. For instance, it remains to determine mechanistically how p53 impacts the response to Cisplatin via the miR-140/HDAC4 axis by regulating specific genes. In this context, *BIK* appears to be a possible candidate. Likewise, it will be interesting to study the impact of a regulator of p53, MDM2, on the p53/miR-140/HDAC4 loop. Moreover, based on the differential expression of HDAC4 in GC subtypes, it remains also to compare the impact of this loop in diffuse and intestinal type of GC.

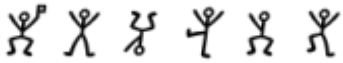
On the usefulness of HDACs as therapeutic targets, Yoo C and co-workers led a clinical study on Vorinostat (SAHA; HDAC inhibitor) combination with Capecitabine plus Cisplatin treatment *(Yoo C et al. 2016)* that led to no gain on the overall survival in GC patients. As p53 seems to be a modulator involved in PDC + SAHA combined treatment response in patients, it would be interesting to perform a retrospective analysis of this study to establish the impact of the p53 status on Vorinostat response. In addition, it remains to determine the genes impacted by the combinatory treatments dependently and

independently of p53. This could help us to understand what kind of cell death processing are taking place in p53-/- cells (f.e. KATOIII). As diffuse and intestinal types of GC have different clinical-pathological characteristics, which may impact the chemotherapy *(Ma J et al. 2016),* we can also compare the key pathways between intestinal and diffuse type of GC cells and study the treatment synergy *in vivo*. Finally, I truly believe that synthetize PDC-HDACI nanodrug, which cytotoxic effects will be p53 independent, could be the next gold standard for treating patients. Ding JD and co-workers suggest that SAHA induces T-cell lymphoma apoptosis depending on the inhibition of HDAC3 *(Ding JD et al. 2012).* As JGS-038 seems to be active in p53 mutated cells, and because it targets HDAC3, thereby it represents a good candidate for this chemical fusion. In addition, as we want to propose a treatment with HDACIs, we must determine the specificity of these inhibitors in our combinations.

Altogether, the future findings on HDAC4 and p53 as therapeutic markers might help us to make a patient stratification with the aim to propose a personalized protocol of treatments based on the association between PDC and HDACIs improving the patient care and the quality of life of patients.

APPENDICES





Iron(III) pincer complexes as a strategy for anticancer studies

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Communication

Fe Pincer Anticancer Complexes

CLUSTER ISSUE

Iron(III) Pincer Complexes as a Strategy for Anticancer Studies

Aldo S. Estrada-Montaño,[a] Alexander D. Ryabov,[b] Alexandre Gries,[c,d] Christian Gaiddon*[c,d] and Ronan Le Lagadec*[a]

Abstract: $[Fe(NCN)_2]PF_6$ (**1**·PF_6) [NCHN = 1,3-di(pyridin-2-yl)benzene] was readily obtained by a transmetalation reaction between $[Fe_3(CO)_{12}]$ and Hg(NCN)Cl followed by a metathesis reaction with KPF_6. X-ray diffraction, electron paramagnetic resonance spectroscopy, and cyclic voltammetry studies confirmed the proposed structure. Cytotoxic assays in human colon cancer (HCT-15), lung cancer (SKLU), and gastric cancer (AGS, KATOIII) cells were performed, and the IC_{50} data obtained for all cell lines showed that **1**·PF_6 has a much higher activity than cisplatin.

Introduction

Cisplatin and carboplatin are two of the most successful anticancer agents, and they are used to treat ovarian, testicular, and lung cancers. Unfortunately, therapy with these platinum-based drugs is limited by dose-dependent toxicity and acquired resistance. For those reasons, a variety of other metal-based anticancer compounds are being investigated.[1] In particular, numerous group 8 metal complexes have been studied as potential anticancer agents. The most representative coordination ruthenium-based complexes are KP1019 and NAMI-A, which have entered phase II clinical trials.[2–7] The structures of organometallic ruthenium compounds are more diverse. Among them, a series of arene–ruthenium complexes such as RM175, RAPTA-C, RAED-C, and UNICAM-1 have shown very promising therapeutic effects.[8–12] Additionally, a class of cyclometalated ruthenium complexes known as RDCs (ruthenium-derived compounds) has shown particularly good activity as anticancer agents, for both in vitro and in vivo studies.[8,13] Various reports have shown that the mechanisms by which phenylpyridine derivatives exert their anticancer effects are presumably not through DNA interaction, as in the case of cisplatin, but in part through redox reactions with oxidoreductases.[14,15] Furthermore, cyclometalated osmium compounds (ODCs) have displayed good to excellent in vitro activities, for instance, against A172 (human glioblastoma) cell lines, and have shown IC_{50} values in the nanomolar range. As for RDCs, the activity of ODCs is related both to their lipophilicity, which favors cell entry, and to their reduction potentials.[16] The development of iron-based drugs is highly relevant owing to the lower intrinsic toxicity of iron. However, examples of iron complexes with anticancer properties are scarce relative to the number of examples available for ruthenium and osmium complexes, even if a steadily growing number of publications has been observed in recent years.[17] A few coordination complexes bearing polypyridine and pincer ligands have been reported, but their cytotoxic activity is relatively modest.[18,19] The most interesting results have been obtained with ferrocene derivatives, and in particular with the ferrocifen-type complexes.[17,20–25] We recently prepared an iron(II) cyclometalated compound, $[Fe(CO)_2(phpy)_2]$ (phpyH = 2-phenylpyridine), and found that its activity on HTC116 and AGS cell lines was relatively moderate (IC_{50} in the 25 μM range), presumably as a result of its instability in solution.[26] To stabilize the complex, while maintaining the advantages of the carbon–metal σ-bond, we considered that the use of pincer ligands would be of interest.[27] Even if the use of pincer ligands can be seen as an advantage over bidentate cyclometalated phenylpyridine, to the best of our knowledge, only one iron complex with am NCN monoanionic pincer ligand, $[Fe(CO)_2(NCN)Br]$ for which NCN = bis(oxazolinyl)phenyl, has been synthesized, but no anticancer activity has been reported.[28] Ruthenium and osmium complexes with one terpyridine ligand and one monoanionic NCN pincer ligand have been prepared and studied against several cancer cell lines, and they showed good cytotoxic activities with IC_{50} < 3 μM.[16,29] Therefore, tridentate ligands derived from polypyridines seem to be a good option for the stabilization of metalated iron compounds with potential cytotoxic properties. Additionally, the thriving interest in such iron pincer compounds is reflected in the fact that their structures have recently been modeled and various computational studies have been performed. For instance, the photophysical properties of iron(II) complexes bearing tridentate 1,3-di(pyridyn-2-yl)benzene (NCN), 6-phenyl-2,2′-bipyridine (CNN), and 2-(1,1′-biphenyl-3-yl)pyridine (CCN) pincer ligands have

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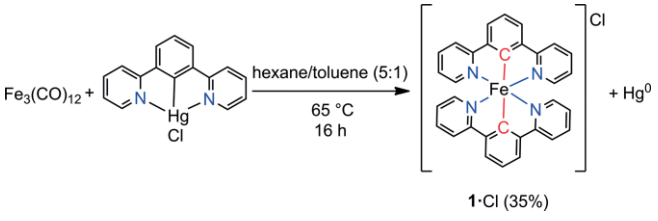
been predicted.[30,31] However, no synthetic methods have been developed.

In this communication, we wish to report on the straightforward synthesis of $[Fe(NCN)_2]PF_6$ as a bis(pincer)iron(III) compound and its cytotoxic activity on human colon cancer (HCT-15), lung cancer (SKLU), and gastric cancer (AGS, KATOIII) cell lines.

Results and Discussion

A transmetalation reaction between $Fe_3(CO)_{12}$ and Hg(NCN)Cl in a hexane/toluene mixture led to the formation of $[Fe(NCN)_2]Cl$ (**1**·Cl) as an orange precipitate in 35 % yield with the concomitant formation of Hg^0. A small amount (ca. 7 %) of the highly reactive iron(II) $[Fe(NCN)_2]$ (**2**) blue complex was also isolated from the solution. However, compound **2** was readily oxidized to the orange Fe^{III} complex, which precluded its biological assays. Intents to grow crystals were unsuccessful (Scheme 1).



Scheme 1. Synthesis of compound **1**·Cl.

Crystals of **1**·Cl were obtained from the slow solvent evaporation of a concentrated $CHCl_3$ solution. Unfortunately, the X-ray diffraction data obtained from those crystals were of poor quality and not good enough for publication. To obtain suitable crystals of **1**, a metathesis reaction with KPF_6 was performed in CH_2Cl_2, and crystals were obtained by slow solvent evaporation of a $CHCl_3$ solution. The molecular structure is shown in Figure 1. The cation moiety in **1**·PF_6 is a mononuclear complex with two pincer N–C–N ligands coordinated to an Fe^{III} center.

The geometry around the iron atom is pseudooctahedral. Two N–C–N ligands are orthogonal, and the angle between the N–C–N planes is 89.21°. The Fe–C and Fe–N bond lengths in **1**·PF_6 and $[Fe(CO)_2(NCN)Br]$[28] are similar.

The X-band electron paramagnetic resonance (EPR) spectrum of a frozen solution of **1**·PF_6 in CH_2Cl_2 is shown in Figure 2. A very broad signal suggests the existence of two species of different spins. The pseudoisotropical signal centered at 318.29 mT (g = 2.002) is assigned to an octahedral low-spin Fe^{III} complex (S = 1/2). The signal at 148.2 mT (g = 4.3504) is ascribed to a distorted octahedral high-spin Fe^{III} complex (S = 5/2). The relative intensity between these signals indicates that **1**·PF_6 consists mainly of a complex with S = 1/2.

The redox properties of **1**·PF_6 were studied by cyclic voltammetry in acetonitrile. Three quasireversible features can be observed. One wave at 0.996 V (vs. Ag/AgCl) can be attributed to the oxidation of iron(III) to iron(IV). A second wave at –0.401 V can be assigned to the reduction of iron(III) to iron(II), whereas the feature at –1.404 V is likely due to the Fe^{I}/Fe^{II} transition (Figure 3).

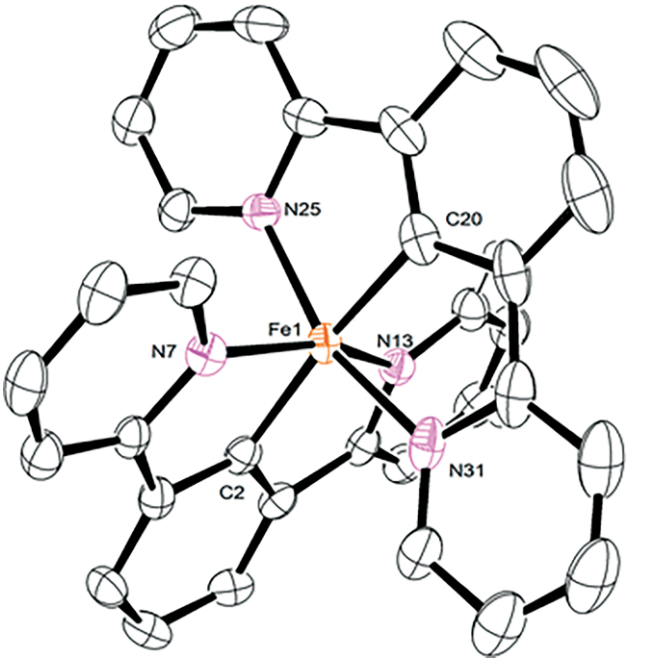


Figure 1. ORTEP representation of **1**·PF_6 with 50 % probability ellipsoids. The hydrogen atoms and PF_6^- anion are omitted for clarity. Selected bond lengths [Å] and angles [°]: Fe–C20 1.920(4), Fe–C2 1.920(3), Fe–N31 2.043(3), Fe–N25 1.986(3), Fe–N7 1.998(3), Fe–N13 2.007(3); C20–Fe–C2 173.75(15), N7–Fe–N13 158.50(12), N25–Fe–N31 159.36(12), C20–Fe–N13, 100.12(14), N13–Fe–N31 92.83(11), N25–Fe–N7 89.63(12).

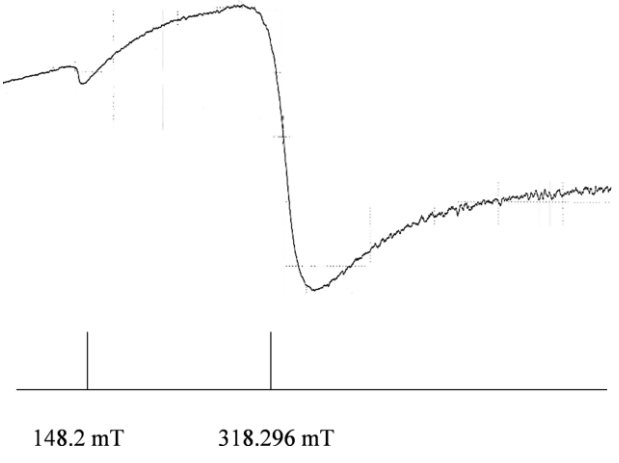


Figure 2. X-band EPR spectrum of **1**·PF_6 (9.02377 GHz) in CH_2Cl_2 at 77 K.

Prior to evaluating the activity of **1**·PF_6 against cancer-cell lines, we studied its stability by UV/Vis spectroscopy. Solutions of **1**·PF_6 in pure DMSO (40 μM) and in water containing 1 % of DMSO (40 μM) were found to be stable for at least 24 h under ambient conditions, as the spectra showed almost no differences (Figures 4 and 5).

The cytotoxic activity of $[Fe(NCN)_2]PF_6$ was tested in cell lines of different origins: colon (HCT-15), lung (SKLU), and stomach (AGS, KATOIII) cancers. AGS cells express a wildtype form of the p53 tumor-suppressor gene. The metastatic gastric KATOIII cancer cells have a deletion of p53. The IC_{50} data of these cell lines for cisplatin and **1**·PF_6 are shown in Table 1. An example of

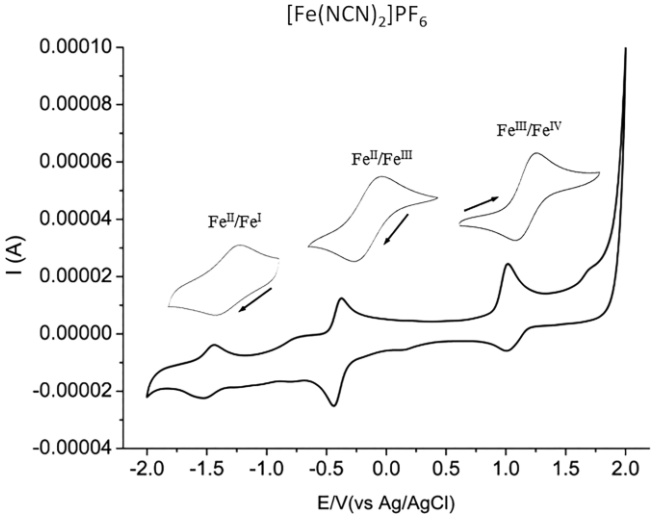


Figure 3. Cyclic voltammogram of **1**·PF_6 in MeCN (0.1 M nBu_4NPF_6, 100 mV s^{-1}, glassy carbon, Ag/AgCl, 25 °C).

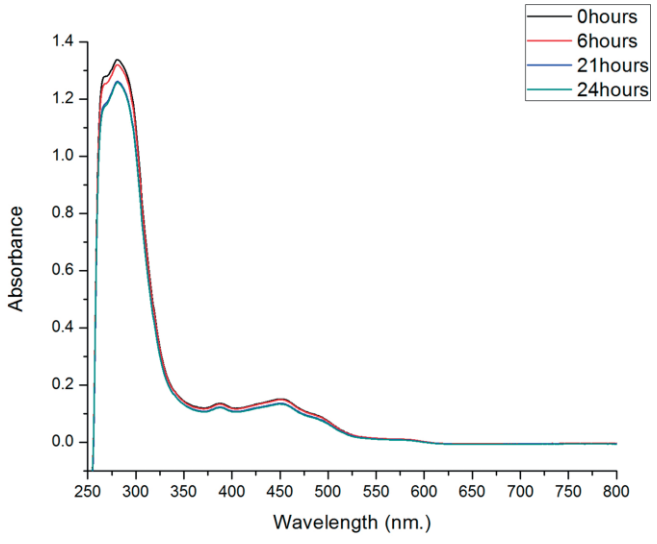


Figure 4. UV/Vis spectra of **1**·PF_6, 40 μM DMSO solution.

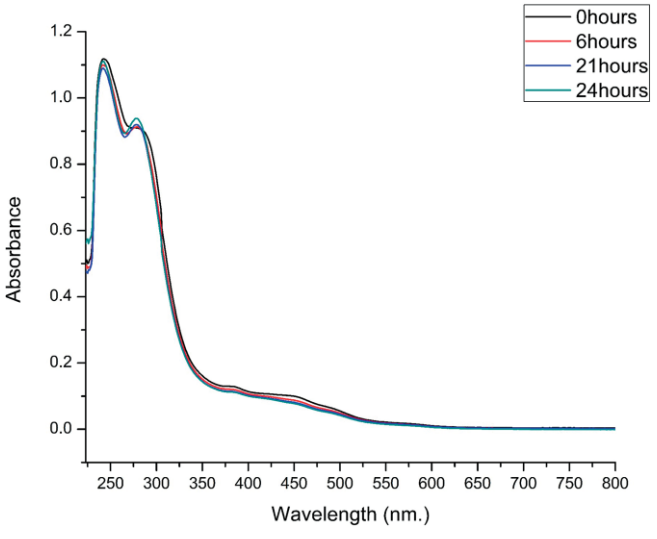


Figure 5. UV/Vis spectra of **1**·PF_6, 40 μM in water containing 1 % of DMSO.

the survival curves (survival vs. concentration [μM]) obtained for **1**·PF_6 with AGS cells is shown in Figure 6.

Table 1. Cytotoxicity of cisplatin and **1**·PF_6 on gastric (AGS, KATOIII), lung (SKLU-1), and colon (HCT15) cell lines at 48 h.

Compound	IC_{50} [μM]			
	AGS	KATOIII	HCT15	SKLU-1
1·PF_6	0.7 ± 0.1	0.8 ± 0.1	0.20 ± 0.07	0.50 ± 0.03
Cisplatin	29.0 ± 1.3	11.0 ± 0.9	30.5 ± 1.4	3.4 ± 0.5

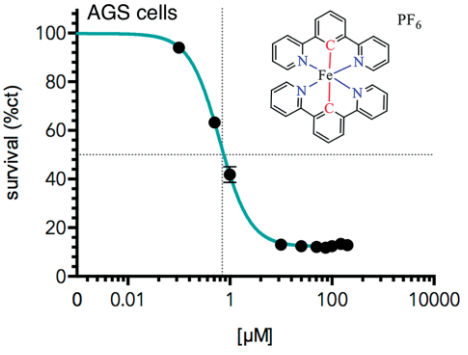


Figure 6. Cytotoxicity of **1**·PF_6 at different concentrations on the AGS cell line. The green line represents the extrapolation curve used to estimates the IC_{50} value (see the Experimental Section for conditions).

The data in Table 1 indicate that **1**·PF_6 is significantly more active than cisplatin in all the cell lines tested. In particular, the AGS and HCT15 cells, which respond poorly to cisplatin (IC_{50} > 20 μM), are highly sensitive to **1**·PF_6 (IC_{50} < 1 μM). In addition, it is noteworthy that the absence of p53 expression in KATOIII cells does not affect the activity of **1**·PF_6, and the IC_{50} values are not significantly different between the wildtype and mutated expressing cell lines. Observation under the microscope indicated that after 48 h of treatment with **1**·PF_6, the AGS cells were reduced in number and featured characteristics of cell death (shrinking and detached) (Figure 7). Similar results were obtained with KATOIII cells in the presence of cisplatin.

To characterize the cell death induced by **1**·PF_6 further, we analyzed markers of apoptosis. Western blot analysis showed that **1**·PF_6 did not induce cleavage of caspase 3 (caspase 3*), a well-documented effector of apoptosis. In contrast, cisplatin caused clear cleavage of caspase (Figure 8). Similarly, the tumor suppressor and proapoptotic gene p53 was induced by cisplatin in AGS cells, but not by **1**·PF_6. These results suggest strongly that **1**·PF_6 induced cell death through a p53 and caspase 3 independent pathway.

We then investigated alternative pathways leading to cell death. Previous work showed that metal-based compounds containing ruthenium could induce endoplasmic reticulum (ER) stress.[32,33] In addition to apoptosis, ER stress can induce autophagy and caspase-independent cell death.[33] To assess the activity of ER stress, we performed Western blot on ATF4 (activating transcription factor 4) (Figure 9). Interestingly, **1**·PF_6 strongly induced ATF4 in AGS cells, whereas cisplatin had no effect. We then correlated the expression of ATF4 with the expression profile between two forms of LC3, a marker for autophagy. Complex **1**·PF_6 induced a diminution in the ratio between the two

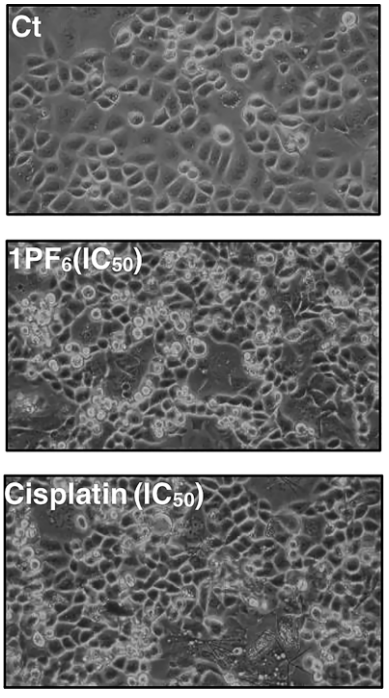


Figure 7. Bright light microscopy images showing rounded and floating AGS cells following treatment with **1**·PF_6 and cisplatin at IC_{50} after 48 h of treatment.

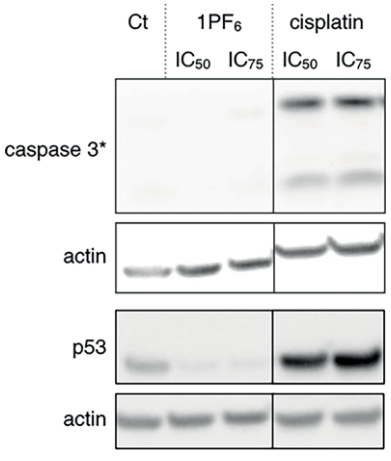


Figure 8. Cleavage of caspase 3 and expression of p53 in AGS cells following treatment with **1**·PF_6 and cisplatin (48 h of treatment at IC_{50} and IC_{75}).

forms of LC3 (LC3I/LC3II), which was indicative of a change in the autophagy process. These results suggested that **1**·PF_6 caused cell death through a caspase-independent pathway but through one involving ER stress and autophagy.

The cytotoxicity of **1**·PF_6 is comparable to that observed for the structurally close ruthenium and osmium derivatives, such as $[M(NCN)(NNN)]^+$ (M = Ru, Os; NNN = 2,2′;6′,2″-terpyridine). For instance, $[Ru(NCN)(NNN)]PF_6$ showed IC_{50} = 0.7 μM against HCT-116 (human colon cancer), whereas $[Os(Me\text{-}NCN)(NNN)]PF_6$ [Me-NCN = 3,5-di(2-pyridyl)toluene] showed IC_{50} = 0.3 μM against A172 (human glioblastoma). Such values are in the same range as those obtained for **1**·PF_6.[16,29] In addition, as

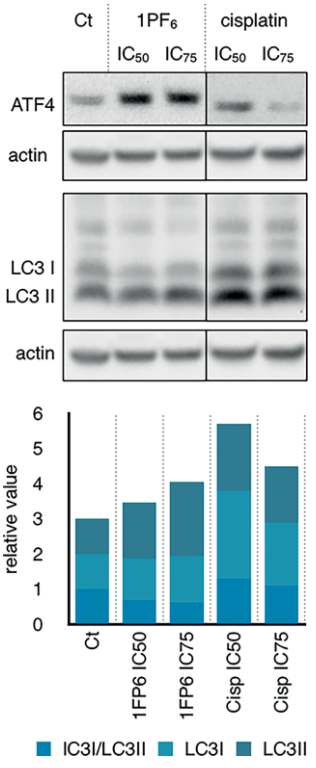


Figure 9. Expression of ATF4 and LC3 in AGS cells following treatment with **1**·PF_6 and cisplatin (48 h of treatment at IC_{50} and IC_{75}).

indicated above, **1**·PF_6 also induces the ER stress pathway in a manner similar to that of several ruthenium complexes.

Conclusions

The facile preparation of a bis(pincer)iron(III) compound from readily available $Fe_3(CO)_{12}$ was reported, and cytotoxic studies demonstrated that the new complex displays a significantly higher activity than cisplatin against several cancer cell lines of different origins (colon, lung, and gastric cancers). Albeit preliminary, the results are very encouraging and could lead to the development of a new family of cytotoxic organometallic iron complexes that bypass the need for caspase-dependent apoptosis. We are currently preparing a series of complexes with substituted NCN ligands and are studying their cytotoxicity on other cell lines and evaluating in more detail how they can induce ER stress.

Experimental Section

Experimental Details: Synthesis of the iron complexes was performed under argon by using a double vacuum/inert-gas line. Toluene and hexane were distilled from sodium, and $CHCl_3$, CH_2Cl_2, and acetonitrile were distilled from P_2O_5. $[Fe_3(CO)_{12}]$, mercury acetate, and anhydrous diethyl ether were purchased from Sigma–Aldrich and were used as received. 1,3-Di(pyridin-2-yl)benzene was prepared according to the literature.[34] The infrared spectra were recorded with an Alpha ATR spectrometer from Bruker Optics and were analyzed with OPUS software. Elemental analysis was performed by the corresponding facilities at the Instituto de Química,

UNAM. Electrospray ionization mass spectrometry (ESI-MS) was performed by using a Bruker Esquire spectrometer. EPR measurements were made with a Jeol JES-TE300 spectrometer. Electrochemical measurements were performed with a PC-interfaced potentiostat–galvanostat AUTOLAB PGSTAT 12. A three-electrode setup was used with a BAS working glassy carbon electrode, Ag/AgCl reference electrode, and auxiliary Pt electrode. Before each measurement, the working electrode was polished with a diamond paste and rinsed with acetone and distilled water. Crystalline red prisms of **1**·PF_6 were grown by slow solvent evaporation from a saturated $CHCl_3$ solution. The X-ray intensity data were measured at 150(2) K with a Bruker Smart Apex CCD diffractometer by using standard Mo-K_α radiation (λ = 0.71073 Å). A multiscan absorption correction procedure was applied. The integration of data was done by using a monoclinic unit cell to yield a total of 35179 reflections to a maximum 2θ angle of 52.36°, of which 6108 [R(int) = 0.0921] were independent. Structure solution was performed by using SHELXS-2012 and refinement (full-matrix least squares) was performed by using the SHELXS-2014/7 program.[35] Hydrogen atoms were placed in calculated positions and were allowed to ride on the atoms to which they are attached. Crystal structure parameters and experimental data on the structure solution and refinement are given in Table S1 (Supporting Information). CCDC 1515206 (for **1**·PF_6) contains the supplementary crystallographic data for this paper. These data can be obtained free of charge from The Cambridge Crystallographic Data Centre.

Synthesis of Hg(NCN)Cl: A mixture of 1,3-di(pyridin-2-yl)benzene (280 mg, 1.2 mmol) and mercury acetate (380 mg, 1.2 mmol) was heated to reflux in ethanol (30 mL) for 24 h. The mixture was cooled to room temperature, and a solution of LiCl (90 mg, 2.2 mmol) in methanol (10 mL) was added. The mixture was heated to reflux for 15 min and then cooled to room temperature. The solution was poured into H_2O (100 mL), and the precipitate was filtered and washed with H_2O and methanol at 0 °C. The white solid was dried under vacuum. Yield: 320 mg (58 %). ^{1}H NMR ($CDCl_3$): δ = 8.73 (ddd, $^3J_{H,H}$ = 5.1 Hz, $^4J_{H,H}$ = 2.1 Hz, $^5J_{H,H}$ = 1.0 Hz, 2 H), 7.98 (d, $^3J_{H,H}$ = 7.5 Hz, 2 H), 7.87 (m, 2 H), 7.81 (ddd, $^3J_{H,H}$ = 7.8 Hz, $^4J_{H,H}$ = 7.5 Hz, $^5J_{H,H}$ = 2.1 Hz, 2 H), 7.52 (t, $^3J_{H,H}$ = 7.5 Hz, 1 H), 7.35 (ddd, $^3J_{H,H}$ = 7.5 Hz, $^4J_{H,H}$ = 5.1 Hz, $^5J_{H,H}$ = 1.2 Hz, 2 H) ppm. ^{13}C NMR ($CDCl_3$): δ = 158.1, 149.0, 143.8, 137.4, 129.0, 128.1, 123.0, 121.59 ppm. MS (DART+): m/z = 469.0. $C_{16}ClH_{11}HgN_4$ (495.33): calcd. C 41.12, H 2.37, N 5.99; found C 40.99, H 2.34, N 6.07.

Synthesis of [Fe(NCN)$_2$]Cl (1·Cl) and [Fe(NCN)$_2$] (2): In a Schlenk tube, [$Fe_3(CO)_{12}$] (54 mg, 0.107 mmol) was dissolved in hexane/toluene (5:1; 30 mL), and then [Hg(NCN)Cl] (50 mg, 0.107 mmol) was added, and the mixture was heated to reflux for 16 h. After cooling to room temperature, an orange precipitate was observed as well as a few drops of mercury. The mixture was filtered through porous glass, and the remaining blue solution was concentrated to dryness under vacuum to give compound **2** as a blue solid (4 mg, 7 %). ^{1}H NMR ($CDCl_3$): δ = 8.66 (d, $^3J_{H,H}$ = 4.7 Hz, 4 H), 8.56 (s, 2 H), 8.0 (dd, $J_{H,H}$ = 6.0 Hz, $J_{H,H}$ = 1.8 Hz, 4 H), 7.72 (m, 8 H), 7.53 (t, $^2J_{H,H}$ = 7.8 Hz, 2 H), 7.17 (m, 2 H) ppm. MS (ESI+): m/z = 518.1. The orange precipitate was dissolved in CH_2Cl_2 (20 mL) and filtered through Celite to eliminate the remaining mercury derivatives and Hg^0. The solution was concentrated to dryness under vacuum to give **1**·Cl as an orange solid (21 mg, 35 %). MS (ESI+): m/z = 518.2. $C_{32}H_{22}ClFeN_4$·3$CHCl_3$ (912.0): calcd. C 46.09, H 2.76, N 6.14; found C 46.14, H 3.03, N 6.16.

Synthesis of [Fe(NCN)$_2$]PF$_6$: In a Schlenk tube, **1**·Cl (20 mg, 0.036 mmol) was dissolved in CH_2Cl_2 (20 mL), and KPF_6 (13 mg, 0.071 mmol) was added. The mixture was stirred at room temperature for 24 h. The solution was filtered through Celite, and the solvents were evaporated to dryness under vacuum. The residue was dissolved in CH_2Cl_2 (10 mL) and filtered again through Celite to remove all residual salts. Concentration under vacuum gave pure **1**·PF_6 as an orange solid. Crystals were obtained by slow solvent evaporation of a saturated $CHCl_3$ solution. IR (ATR): $\tilde{\nu}$ = 834 (s, PF_6) cm^{-1}. MS (ESI+): m/z = 518.3. $C_{32}H_{22}F_6FeN_4P$·0.5$CHCl_3$ (723.1): calcd. C 53.99, H 3.14, N 7.75; found C 54.3, H 3.02, N 7.70.

Study of the Stability of 1·PF$_6$: The stability studies were performed by using a 2600 Shimadzu UV/Vis instrument at 20 °C with 40 μM solutions of **1**·PF_6 in DMSO and in water containing 1 % of DMSO (prepared from 20 mM stock solution in DMSO). Spectra were recorded every 2 h for 24 h.

Cell Line Culture and Culture Medium: HCT-15 (human colorectal adenocarcinoma) and SKLU-1 (human lung adenocarcinoma) cell lines were cultured in RPMI-1640 medium supplemented with 10 % fetal bovine serum (FBS), 2 mM L-glutamine, (Gibco) antibiotic–antimycotic, and 1 % nonessential amino acids (Gibco). They were maintained at 37 °C under a humidified atmosphere with 5 % CO_2. The viability of the cells used in the experiments exceeded 95 %, as determined with trypan blue. The cells were removed from the tissue culture flasks by treatment with trypsin and diluted with fresh media. Of these cell suspensions, an aliquot (100 μL containing 10000 cells $well^{-1}$) was pipetted into 96-well microtiter plates (Costar), and the material was incubated at 37 °C under a 5 % CO_2 atmosphere for 24 h. Subsequently, a solution (100 μL) of the compound obtained by diluting the stocks was added to each well. The cultures were exposed to the compound (0.1–3.1 μM) and cisplatin (1–3 μM) for 48 h. After the incubation period, the culture was washed with buffer, and 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT; 100 μL) was added. The plates were incubated at 37 °C for 4 h. The human gastric adenocarcinoma (AGS) and human gastric carcinoma (KATOIII) cell lines were acquired from ATCC (Manassa, VA). AGS cells were cultured in RPMI 1640 (Roswell Park Memorial Institute medium) containing 10 % of FBS and 1 % of penicillin/streptomycin. KATOIII cells were cultured in RPMI 1640 medium containing 20 % of FBS and 1 % of penicillin/streptomycin. All cell lines were grown at 37 °C under a humidified atmosphere of 95 % air and 5 % CO_2. Experiments were performed on cells within 20 passages.

Cell Viability: The antiproliferation activities on cancer cells were determined by using the MTT assay. Cells were seeded at 10000 cells $well^{-1}$ (100 μL) in Cellstar 96-well plates (Greiner Bio-One) and were incubated for 24 h. Thereafter, cancer cells were exposed to drugs at different concentrations in media for 48 h. Then, the MTT assay was performed as described previously.[36] Inhibition to cell viability was evaluated with reference to the IC_{50} value, which is defined as the concentration needed for 50 % reduction in survival based on the survival curves. IC_{50} values were calculated from the dose/response curves (cell viability vs. drug concentration). IC_{50} calculation and statistical analyses were performed using Prism (GraphPad software).

Western Blot: Cells or tissue were lysed with LB (125 mM Tris-HCl, pH 6.7, 150 mM NaCl, 0.5 % NP40, 10 % glycerol). Proteins (20 μg) were denatured and deposited directly (75 μg of proteins) onto a sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) gel. Western blotting was performed by using antibodies raised against p53 (rabbit anti-p53, FL-393, Santa Cruz, CA), AFT4 (Santa Cruz, CA), caspase 3 cleaved (Cell Signaling), and LC3 (Cell Signaling). Secondary antibodies (anti-rabbit, anti-mouse: Sigma, MA) were incubated at 1:1000. Loading was controlled with actin (rabbit anti-β-actin, 1:4000, Sigma).[37]

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List of publications

The HDAC inhibitor SAHA inhibits p53 expression but synergies with platinum compounds to program gastric cancer cells into apoptosis via a p53-dependent pathway. Gries A., Spaety M-E., Venkatasamy A., Lony C., Benoit R., Pfeffer S., Mellitzer G., and Gaiddon C. **In redaction**

Reactivity and biological activity of bis-cyclometalated 2-phenylpyridine iron(II) complex. Estrada-Montaño A.S., Oviedo-Fortino J.A., Torres-Gutierrez C., Marcial-Hernández R., Gries A., Gaiddon C., Ryabov A.D., and Le Lagadec R. **In redaction**

Histone deacetylase expression in gastric cancer; study of more specific inhibitors. Gries A., Spencer J., Mellitzer G., and Gaiddon C. **In preparation**

A HDAC4-miR140-p53/p73 auto-regulatory loop controls gastric cancer response to chemotherapy and impacts on patient prognosis. Spaety M-E., Gries A., Venkatasamy A., Jung A., Okamoto K., Yanagihira K., Benoit R., Mellitzer G., Pfeffer S., and Gaiddon C. **In submission**

Iron(III) pincer complexes as a strategy for anticancer studies. Estrada-Montano A.S., Ryabov A.D., Gries A., Gaiddon C., and Le Lagadec R. **Eur J Inorg Chem. 2017**; 12:1673-1678

List of presentations

Expression regulation, functions, and therapeutic interest of the Histone Deacetylase HDAC4 in gastric cancer. (Oral communication) Spaety M-E., Gries A., Venkatasamy A., Romain B., Yanagihira K., Okamoto K., Jung A., Mellitzer G., Pfeffer S., and Gaiddon C. Translational Medicine Federation of Strasbourg (FMTS) – Strasbourg, France April 2018

Expression regulation, functions, and therapeutic interest of the Histone Deacetylase HDAC4 in gastric cancer. (Oral communication) Spaety M-E., Gries A., Venkatasamy A., Romain B., Yanagihira K., Okamoto K., Jung A., Mellitzer G., Pfeffer S., and Gaiddon C. 36th meeting of the Digestive Epithelial Cell Study Club (CECED) – Marseille, France 29-30 March 2018

Génétique et épigénétique des cancers; de la genèse des cancers à la thérapie. (Oral communication of scientific popularization) Gries A. Jardin des Sciences – Molsheim, France 8 February 2018

Génétique et épigénétique des cancers; de la genèse des cancers à la thérapie. (Oral communication of scientific popularization) Gries A. Jardin des Sciences – Niederbronn-les-Bains, France 29 November 2017

Design and Characterisation of Histone Deacetylase Inhibitors (HDACI) in Gastric Cancer Therapy. (Poster communication) Gries A., Spaety M-E., Mellitzer G., and Gaiddon C. Gordon Research Conference – Cancer Genetics and Epigenetics – Lucca di Barga, Italy 23-28 April 2017

Design et caractérisation des Inhibiteurs des Histones Désacétylases (HDACI) dans la thérapie du cancer gastrique. (Oral communication) Gries A., Spaety M-E., Mellitzer G., and Gaiddon C. 10th Grand-East Cancer pole Meeting – Nancy, France 24-25 November 2016

Histone Deacetylases and Cancers. (Oral communication) Gries A., Spaety M-E., Mellitzer G., and Gaiddon C. European Cancer Center – Strasbourg, France 13 May 2016

Synergy between Platinum Derivative Compounds and SAHA an Inhibitor of Histone Deacetylases in AGS cancer cells. (Poster communication) Gries A., Spaety M-E., Mellitzer G., and Gaiddon C. Translational Medicine Federation of Strasbourg (FMTS) – Strasbourg, France April 2016

Fonctions de l'Histone Désacétylase HDAC4 dans le cancer gastrique. (Oral communication) Spaety M-E., Gries A., Mellitzer G., and Gaiddon C. 34th meeting of the Digestive Epithelial Cell Study Club (CECED) – Lille, France 10-11 March 2016

Contractual mission

The 30th March 2017, 161 middle school students from Strasbourg met to participate to their first scientific congress organized in partnership with the *Jardin des sciences* of the University of Strasbourg, the International Space University of Illkirch-Graffenstaden, and with the coordination of the Space city in Toulouse. With two PhD students, we made scientific coordination and popularization and we involved in the congress logistics. During several months, we performed documentary research, we helped the young students to prepare poster and oral presentations about “Life in Space”, and we trained them. Like scientific researchers, the 161 middle school students presented their works and answered to the questions from the public. Finally, they had the opportunity to meet a former astronaut Jean-Jacques Favier and to discuss by videoconference to former astronauts Claudie Haigneré in Paris, Michel Tognini in Vaulx-en-Velin, Philippe Perrin in Toulouse and to an engineer in Germany in the astronaut training center.

http://www.isunet.edu/news/latest-news/scientific-congress-for-kids-another-stem-initiative-at-isu/453

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"If you are collecting the facts, maybe others will find the explanation."

Sir Arthur Conan Doyle, *The Thor Bridge Problem*

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Alexandre GRIES

Etude des Histones Désacétylases (HDACs) comme cibles thérapeutiques dans le cancer gastrique

Résumé

En raison de l'efficience des traitements, le taux de survie globale à 5 ans des patients avec un cancer gastrique (CG) est d'environ 15%. A l'heure actuelle, il n'existe pas de stratifications des patients permettant de prescrire un protocole de traitements efficace.

Durant ma thèse, j'ai établi le rôle de HDAC4 dans la sensibilité des cellules de CG au Cisplatine. J'ai montré que cette réponse semble dépendre du type de CG (intestinal ou diffus) et du statut p53 des cellules cancéreuses. J'ai souligné l'intérêt de combiner un inhibiteur des HDACs (SAHA) avec les chimiothérapies à base de dérivés de platine (PDC : Cisplatine, Oxaliplatine) afin de promouvoir leurs effets cytotoxiques. De manière intéressante, j'ai observé que la réponse aux traitements combinés est différente suivant le statut p53 des cellules cancéreuses.

Ces résultats permettent d'ouvrir de nouvelles perspectives dans l'utilisation des traitements combinés PDC + SAHA dans la thérapie du CG. En particulier, le facteur p53 qui est souvent muté dans les CG, pourrait être un marqueur thérapeutique pour un tel protocole de traitement.

Mots-clefs : HDAC, HDACI, miRNA, p53, platine, cancer gastrique

Abstract

Due to the efficiency of treatments, the 5-year overall survival rate for patients with gastric cancer (GC) is approximately 15%. Currently, there is no stratification of patients to prescribe an effective treatment protocol.

During my thesis, I established the role of HDAC4 in the sensitivity of GC cells to Cisplatin. I have shown that this response seems to depend on the type of GC (intestinal or diffuse) and the p53 status of cancer cells. I emphasized the interest of combining an HDAC inhibitor (SAHA) with platinum derivative chemotherapies (PDC: Cisplatin, Oxaliplatin) to promote their cytotoxic effects. Interestingly, I observed that the response to combination treatments is different depending on the p53 status of the cancer cells.

These results open new perspectives in the use of PDC + SAHA combination therapies in GC. The p53 factor, which is often mutated in GC, could be a therapeutic marker for a such treatment protocol.

Keywords: HDAC, HDACI, miRNA, p53, platin, gastric cancer