DEPARTAMENTO DE LABORATORIO DE EPIGENÉTICA

EPIGENETIC TRANSCRIPTIONAL REPRESSION OF TUMOR SUPPRESOR GENES AND ITS REVERSION BY DRUGS

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1. Resumen

Introducción y objetivos

El comportamiento anormal de las células cancerosas es el resultado de la acumulación de numerosos defectos de dos tipos: alteraciones genéticas (mutaciones, amplificaciones y deleciones génicas, etc) y desregulación de los mecanismos epigenéticos. Estos fenómenos colaboran durante la iniciación y progresión del cáncer. A diferencia de los trastornos genéticos, los defectos epigenéticos son potencialmente reversibles, lo que ha suscitado en los últimos años la búsqueda de fármacos que selectivamente causen cambios en los patrones epigenéticos de las células tumorales, con la consiguiente diferenciación, muerte y/o parada de crecimiento de las mismas. Se han estudiado con especial interés dos tipos de estas sustancias: inhibidores de metiltransferasas de DNA (DNMTs) e inhibidores de desacetilasas de histonas (HDACs) dependientes de Zn(II). Entre otros efectos, ambos grupos de compuestos consiguen recuperar la expresión de genes supresores de tumores. Los inhibidores de DNMTs posibilitan la reactivación de aquellos genes silenciados mediante hipermetilación de la isla CpG de su promotor y los inhibidores de HDACs, de aquellos genes silenciados a través de la hipoacetilación de las histonas asociadas a su promotor.

A pesar de sus prometedores efectos en cultivos celulares, muchas de estas sustancias presentan inconvenientes que limitan su aplicación en quimioterapia: toxicidad, mutagenicidad, inestabilidad, etc.

Considerando estos antecedentes, se planteó como objetivo de esta tesis el estudio de compuestos cuya capacidad de alterar los patrones epigenéticos de las células tumorales no había sido analizada previamente. En concreto:

1. Se han determinado los efectos del anestésico procaína (4-aminobenzoato de 2-dietilaminoetilo, compuesto **15** en Fig.9) en la metilación de DNA genómico y en la proliferación celular.
2. Se ha realizado un estudio comparativo de siete inhibidores de HDACs dependientes de Zn(II): ácido butanoico (compuesto **23** en Fig.10), ácido valproico (compuesto **26** en Fig.10), MS-275 (compuesto **28** en Fig.10), tricostatina A (TSA, (compuesto **16** en Fig.10), SAHA (compuesto **17** en Fig.10), CX (compuesto **20** en Fig.10) y CY (compuesto **21** en Fig.10). Debido al reciente desarrollo de CX y CY, al iniciar este trabajo no había datos publicados sobre sus efectos en líneas celulares. En esta tesis se han analizado los efectos de estos siete compuestos *in vitro* y en cultivos de MCF7, especialmente las alteraciones originadas en el ciclo celular, los cambios inducidos en la acetilación de histonas y la expresión de ciertos genes.

Como modelo de células cancerosas se escogió la línea celular de cáncer de mama MCF7.

Resultados

Procaína: un nuevo agente desmetilante con efecto inhibidor del crecimiento en células cancerosas

El tratamiento de células MCF7 con procaína durante 3 días reduce la cantidad de 5-metilcitosina en DNA genómico proporcionalmente a la concentración del fármaco (ver Fig.15B y C). El máximo efecto se alcanza tras 24-48 h (ver Fig.17). Transcurridas 72 h, se observa la pérdida de 5-metilcitosina en el promotor de *RARβ2* (Fig.18A y B), que se encuentra hipermetilado en MCF7, y el consiguiente aumento de expresión del gen (Fig.18C). Procaína también provoca la parada del

ciclo celular durante la mitosis (Fig.19B y Fig.20) y reduce la proliferación celular (Fig.19A y B). El empleo de procainamida (compuesto **14** en Fig.9) en las mismas condiciones produce similares efectos (Fig.17, Fig.19B y C, Fig. 20), si bien en este caso la inhibición de la proliferación celular no es tan marcada. Sin embargo, 5-aza-2'-desoxicitidina (zdC, compuesto **4** en Fig.9) es mucho más potente y, aún cuando se utiliza en concentraciones dos órdenes de magnitud inferiores a las de procaína, en tratamientos de tres días causa mayores pérdidas de metilación que ésta (Fig.15B y C, Fig.16, Fig.17) y frena sensiblemente la proliferación celular (Fig.19A y C). La proporción de 5-metilcitosina en DNA global se reduce de manera continuada a lo largo de los tres días de tratamiento con zdC (Fig.17).

Estudio comparativo de siete inhibidores de desacetilasas de histonas.

Ensayos *in vitro* de actividad desacetilasa realizados con extractos nucleares de MCF7 muestran que todas las sustancias analizadas, inclusive CX y CY, bloquean la desacetilación de histonas aunque a distintas concentraciones (Fig.21): los ácidos hidroxámicos (TSA, SAHA, CX y CY) lo hacen dentro del rango de concentración micromolar y los ácidos carboxílicos de cadena corta (butanoico y valproico), a concentraciones milimolares. La concentración a la que MS-275 inhibe la desacetilación de histonas en este ensayo no se pudo encontrar puesto que cuando el compuesto está a concentración superior a 50 μM se forma un precipitado en la mezcla de reacción, mientras que a concentraciones menores a penas se aprecia inhibición.

El IC_{50} de cada sustancia para tratamientos de 24 h en cultivos de la línea MCF7 es del mismo orden de magnitud que la concentración necesaria para inhibir la actividad desacetilasa en el análisis *in vitro* (Fig. 22A). El compuesto que más se desvía de este comportamiento es MS-275, cuyo IC_{50} es 5 μM, una concentración que apenas afecta la desacetilación de histonas en el ensayo *in vitro*. Los cambios causados en el ciclo celular no son iguales en todos los casos (Fig.22B y C): los ácidos hidroxámicos incrementan la proporción de células en fase G2/M; los ácidos carboxílicos de cadena corta detienen la progresión del ciclo en G1/G0 y en G2/M y la

benzamida MS-275 para el ciclo en G1/G0 solamente. Además, de acuerdo con los análisis de ciclo celular realizados mediante citometría de flujo, la proporción de células que pudieran encontrarse en apoptosis es despreciable en cualquiera de estos tratamientos. Excepto que se indique lo contrario, todos los resultados mencionados de aquí en adelante fueron obtenidos al tratar MCF7 durante 24 h con el IC_{50} de cada sustancia.

En células vivas, estos siete inhibidores inducen un aumento similar en la acetilación de H3 (Fig.24A), siendo la forma diacetilada la especie predominante con todos los tratamientos (Fig.24C). También se incrementa de manera notable la acetilación de H4 (Fig.24B), pero mientras con CX, ácido valproico y SAHA la forma mayoritaria parece ser la monoacetilada, con MS-275 es la triacetilada y en los otros casos (TSA, ácido butanoico, CY) hay aproximadamente la misma proporción de formas mono-, di-, tri-, y tetraacetiladas (Fig.24D). Se escogieron tres sustancias, MS-275, ácido butanoico y CY, para estudiar los cambios en la acetilación de H4 al tratar las células con menores concentraciones de fármaco: un décimo del IC_{50} ($0,1 \cdot IC_{50}$), cuatro décimos del IC_{50} ($0,4 \cdot IC_{50}$) y siete décimos del IC_{50} ($0,7 \cdot IC_{50}$). Los resultados obtenidos con MS-275 y ácido butanoico son prácticamente idénticos (Fig.25). En ambos casos se observa que con $0,4 \cdot IC_{50}$ la distribución de H4 en no, mono-, di-, tri- y tetraacetilada es bastante similar a la obtenida cuando la concentración de estas sustancias es IC_{50}. Los cambios inducidos por CY son ligeramente distintos (Fig.25).

Se ha descrito que en ciertas líneas celulares algunos de estos compuestos alteran la expresión de las HDACs y/o inducen degradación de HDAC2. En las condiciones empleadas en este trabajo no se apreciaron cambios en la cantidad de proteína de las desacetilasas HDAC1 y HDAC2 (Fig.26).

El tratamiento con estos compuestos consigue incrementar la expresión del gen *CDKN1A* (Fig.28A). Todas las sustancias reactivan *JunD*, si bien CY parece ser menos eficiente que el resto, y todas ellas salvo MS-275 producen un aumento de la transcripción de *GADD45β* e *IGFBP3* (Fig.28A). Ninguno de los inhibidores parece alterar la expresión de *MT1X* y *MT2A* (Fig.28A). Los promotores de estos seis genes (*CDKN1A*, *IGFBP3*, *JunD*, *GADD45b*, *MT1X* y *MT2A*) carecen de metilación de DNA en la correspondiente isla CpG (Fig.28B), por lo que la ausencia de respuesta en

determinados casos no se puede atribuir a silenciamiento mediante hipermetilación aberrante de DNA. En los seis promotores el tratamiento con CY hace aumentar la proporción de H4 tetraacetilada y de H3 dimetilada en el residuo de lisina 4 (Fig.29), mientras parece que se reduce la presencia de HDAC2 (Fig.29). Asimismo, en los promotores de *MT1X* y *MT2A* se reduce la cantidad de H3 dimetilada en el residuo de lisina 9 (Fig.29).

Discusión

Procaína: un nuevo agente desmetilante con efecto inhibidor del crecimiento en células cancerosas

El mecanismo a través del cual procaína ejerce sus efectos en la metilación de DNA genómico es distinto al de zdC. Este último compuesto es un nucleósido que, una vez dentro de la célula, es fosforilado e incorporado al DNA en lugar de 2'-desoxicitidina durante la replicación. Una vez en el DNA, los residuos de 5-azacitosina inhiben de manera irreversible las metiltransferasas, lo que reduce la concentración de DNMTs activas en la célula. Debido a ello, en la siguiente ronda de replicación del DNA las hebras hijas no son metiladas con eficiencia y, por tanto, la proporción de 5-metilcitosina en DNA genómico baja. Cuantos más ciclos de replicación pasen con la acción de las metiltransferasas bloqueada, menor será la cantidad de 5-metilcitosina de DNA. Por el contrario, procaína no es un nucleósido y no se incorpora como tal en DNA, de manera que probablemente no necesite que transcurra ninguna ronda de replicación para poder comenzar a interferir en la metilación. Este hecho puede explicar por qué el máximo efecto de procaína se alcanza en 24-48 h, mientras zdC necesita más tiempo para lograr la máxima desmetilación. Hay varias posibles maneras a través las que procaína podría actuar. Por ejemplo, tanto procainamida como hidralazina forman complejos *in vitro* con DNA de doble hebra, favoreciendo transiciones de estructura B a estructura Z. Se ha sugerido que este fenómeno *in vivo* evita la acción de las DNMTs sobre su sustrato, con lo que se reduce la proporción de 5-metilcitosina en DNA genómico (Thomas and Messner 1986,

Zacharias and Koopman 1990). Por otra parte, hay resultados que parecen indicar que procainamida es un inhibidor competitivo de las DNMTs, aunque no está claro cuál(es) de estos enzimas se ven afectados (Scheinbart *et al.* 1991, Deng *et al.* 2003). Dado que procaína es un compuesto muy similar a procainamida, no sería de extrañar que alterase el funcionamiento de las metiltransferasas de la misma manera. Por último, no se deben excluir otros mecanismos de acción menos directos. Está descrito que hidralazina, un fármaco antihipertensivo, reduce la expresión de las metiltransferasas DNMT1 y DNMT3a, en consecuencia, la metilación de DNA (Deng *et al.* 2003).

A pesar de la fuerte pérdida de 5-metilcitosina que induce en el DNA genómico, zdC posee propiedades que limitan enormemente su utilización como fármaco: es una sustancia poco estable en disolución acuosa, en el organismo es rápidamente degradada por deaminasas de citidina, daña el DNA al unirse covalentemente a las DNMTs, etc. Incluso se considera que la gran bajada de la proporción de 5-metilcitosina en DNA inducida por esta sustancia puede originar, a largo plazo, la transformación de células normales (Ehrlich 2002). Por ello, las cualidades de procaína resultan interesantes. Procaína es estable en disolución acuosa y presenta baja toxicidad (de hecho, se utiliza como anestésico). Al no ser un nucleósido, cabe esperar que no dañe al DNA de la misma manera que zdC. Por último, procaína recupera la expresión de genes supresores de tumores a concentraciones a las que casi no altera la metilación global de DNA, lo que podría suponer la parada de crecimiento y/o muerte de las células tumorales con un bajo riesgo de inducir inestabilidad cromosómica en células sanas.

Estudio comparativo de siete inhibidores de desacetilasas de histonas

Las siete sustancias estudiadas son capaces de inhibir la actividad HDAC *in vitro* y de inducir hiperacetilación de las histonas H3 y H4 en cultivos celulares. Se aprecia una clara relación estructura-actividad entre los compuestos, las concentraciones a las que inhiben la desacetilación en ensayos *in vitro* y el IC_{50} en la línea MCF7. En ambos casos, los ácidos carboxílicos de cadena corta son mucho

menos efectivos que los hidroxámicos. Este hecho se puede explicar considerando la estructura del centro activo de las desacetilasas dependientes de Zn(II) y la forma en que estas sustancias lo bloquean (Finnin *et al.* 1999, Vannini *et al.* 2004). Por una parte, los ácidos hidroxámicos de este estudio son moléculas de mayor tamaño que los ácidos carboxílicos, lo que les permite establecer mayor número de interacciones no covalentes con los aminoácidos de los enzimas. Por otra, el grupo funcional ácido hidroxámico es más afín por los cationes Zn(II) que el ácido carboxílico. Estos dos factores contribuyen a una mayor estabilidad de los complejos inhibidor-enzima en el caso de los ácidos hidroxámicos que en el de ácido butanoico o ácido valproico y por tanto la inhibición es más eficiente con los primeros. La benzamida MS-275 parece no ser eficaz *in vitro*, pero concentraciones micromolares de este compuesto inducen hiperacetilación de las histonas H3 y H4 en cultivos de MCF7 en extensión comparable a otras sustancias. A pesar de ser un dominio altamente conservado, la composición de aminoácidos del centro activo de las HDACs dependientes de Zn(II) varía ligeramente de unos enzimas a otros, de manera que no en todos los casos el cation metálico es accesible a un grupo voluminoso como la benzamida. De hecho, está descrito que la desacetilasa HDAC8, cuyo centro activo es más estrecho que el de otros enzimas de su familia, no es inhibida por MS-275 aunque sí por TSA y SAHA. Sin embargo, MS-275 es capaz de inhibir HDAC1 y HDAC3, otras desacetilasas dependientes de Zn(II) (Vannini *et al.* 2004). El hecho de que MS-275 no pueda alterar el funcionamiento HDAC8 y quizás el de alguna otra desacetilasa dependiente de Zn(II) explicaría la falta de eficiencia del compuesto *in vitro*, donde los enzimas no inhibidos catalizan la reacción en condiciones no naturales: el sustrato son histonas libres en lugar de estar incluidas en cromatina, pueden haberse perdido factores que actúen como reguladores de la actividad desacetilasa, etc. Sin embargo, en células vivas podría suceder que HDAC8 no sea capaz de reemplazar a las otras desacetilasas cuando estas son inhibidas, bien por ser mucho menos activa en cromatina que en histonas libres, bien por no ser reclutada a las mismas posiciones, etc. En ese caso, basta que MS-275 inhiba parte de las desacetilasas dependientes de Zn(II) para inducir hiperacetilación de histonas H3 y H4.

Las alteraciones producidas en el ciclo celular por estas siete sustancias también parecen estar relacionadas con su naturaleza química: MS-275 detiene el

ciclo en G1/G0, los ácidos hidroxámicos en G2/M y los ácidos carboxílicos, en ambas fases. No se puede olvidar que se han descrito 11 HDACs dependientes de Zn(II) humanas, algunas con variantes de *splicing*. A pesar de ser altamente homólogos entre sí, existen pequeñas variaciones entre estos enzimas, siendo probable que una misma sustancia no inhiba todas las desacetilasas con idéntica eficiencia. Además, dos compuestos diferentes no tienen por qué presentar la misma afinidad por el mismo enzima. Asimismo, hay que recordar que algunas desacetilasas también actúan *in vivo* sobre otras proteínas como α-tubulina o p53. Toda esta diversidad puede originar que los distintos inhibidores no tengan exactamente los mismos efectos en la célula viva. Tampoco hay que descartar la posibilidad de que alguna de estas sustancias interaccione con otras macromoléculas de la célula, alterando otras actividades metabólicas independientemente de la desacetilación de proteínas.

Los efectos de los siete compuestos en la acetilación de H3 y H4 de MCF7 parecen muy semejantes a nivel global. Sin embargo, la expresión génica no responde de la misma manera a todos ellos. Las siete sustancias consiguen reactivar el gen *CDKN1A* y todas salvo MS-275 incrementan la transcripción de *GADD45*β. Se ha propuesto que la sobreexpresión de *CDKN1A* debida al tratamiento con inhibidores de desacetilasas origina una parada de ciclo celular en G1/G0, mientras que si se reactiva *GADD45*β, el ciclo se detiene en G2/M (Hirose *et al.* 2003). Los resultados de este trabajo concuerdan con esa hipótesis, puesto que MS-275, el único compuesto que no altera la expresión de *GADD45*β, es la única sustancia que detiene el ciclo celular exclusivamente en G1/G0.

Ninguno de los inhibidores consigue alterar la expresión de *MT1X* y *MT2A*, a pesar de que el tratamiento con CY induce aumento de acetilación de H4 y de dimetilación del residuo 4 en H3 (dos marcas que se encuentran en cromatina transcripcionalmente activa) en sus respectivos promotores, de forma similar a lo que sucede con otros genes cuya expresión sí se incrementa. Curiosamente, sólo en los promotores de *MT1X* y *MT2A* se observa la presencia de H3 con la lisina 9 dimetilada, modificación que desaparece al tratar las células con CY. La metilación de Lys9 de H3 se considera una marca de heterocromatina y de genes silenciados y puede estar asociada a la hipermetilación de DNA. No obstante, el DNA de todos estos promotores carece de 5-metilcitosina.

Conclusiones

Procaína: un nuevo agente desmetilante con efecto inhibidor del crecimiento en células cancerosas

1. Procaína reduce la metilación de DNA en cultivos celulares, aunque no de una forma tan extensiva como 5-aza-2'-desoxicitidina. El máximo efecto de procaína se alcanza entre el primer y el segundo día de tratamiento.
2. Procaína es capaz de reactivar genes silenciados por hipermetilación (*RARβ2*) a concentraciones a las que reduce muy poco el contenido de 5-metilcitosina en DNA.
3. Procaína reduce la proliferación celular e induce parada del ciclo celular en mitosis.
4. Estas propiedades, junto con el hecho de que procaína no se incorpora en DNA hacen de procaína una sustancia potencialmente interesante para su estudio como posible terapia antitumoral.

Estudio comparativo de siete inhibidores de desacetilasas de histonas.

5. TSA, SAHA, CX, CY, MS-275, ácido butanoico y ácido valproico son inhibidores de HDACs dependientes de Zn(II) cuyos efectos en ensayos de inhibición *in vitro* y crecimiento celular muestran una clara relación estructura-actividad. Los ácidos carboxílicos son los compuestos menos eficaces.
6. Las siete sustancias afectan de forma comparable la acetilación global de H3 y H4 en cultivos de MCF7.
7. Ninguno de los inhibidores altera la cantidad de las proteínas de HDAC1 y HDAC2 en la célula.
8. Los diferentes efectos de los inhibidores de HDACs en los niveles de expresión de *CDKN1A* y *GADD45β* pueden estar relacionados con los distintos efectos en el

ciclo celular: MS-275 produce parada en G1/G0 y aumento de la transcripción de *CDKN1A* pero no de *GADD45β*. El resto de inhibidores producen parada en G2/M y aumento de la transcripción de CDKN1A y *GADD45β*.

9. A pesar de que todos estos compuestos inducen de manera similar la acetilación global de histonas, no todos inducen la reactivación de los mismos genes.
10. La insensibilidad de MT1X y MT2A a estos tratamientos no se debe ni a hipermetilación de DNA de sus respectivos promotores ni a la no acetilación de las histonas asociadas a ellos.
11. La inhibición de HDACs da lugar al aumento en la acetilación de la histona H4 asociada a los promotores de los seis genes estudiados.
12. La inhibición de HDACs produce acumulación de dimetil-lisina 4 y bajada en la proporción de dimetil-lisina 9 de H3 asociada a los promotores de los genes analizados en los que esta modificación está presente. Este hecho es una prueba más de la interrelación existente entre las distintas modificaciones post-traduccionales de las histonas.

2. Abbreviations

A_{220}	absorbance at 220 nm
A_{260}	absorbance at 260 nm
A_{280}	absorbance at 280 nm
APS	ammonium persulfate
ATRX	α-thalassemia, mental retardation syndrome X-linked protein
BSA	bovine serum albumin
bp	base pairs
CBP	CREB binding protein
CDKN1A	cyclin-dependent kinase inhibitor 1A
CREB	cyclic-AMP-response-element-binding protein
DAPI	2-(4-amidinophenyl)-6-indolecarbamidine dihydrochloride also named 4',6-diamide-2'-phenylindole dihydrochloride
DEPC	diethylpirocarbonate
DMEM	Dubelcco's modified Eagle's medium
DMSO	dimethylsulfoxide
DNMT	DNA (cytosine-5)-methyltransferase
dsDNA	double stranded DNA
DTT	(±)-*threo*-1,4-dimercapto-2,3-butanediol also named DL-dithiothreitol
ChIP	chromatin immunoprecipitation
EDTA	ethylenediaminetetraacetic acid
EGTA	ethylene glycol-bis(2-aminoethylether)-*N,N,N',N'*-tetraacetic acid
FBS	fetal bovine serum
GADD45β	growth arrest and DNA-damage-inducible, beta
GAPDH	glyceraldehyde-3-phosphate deshydrogenase

HAT	histone acetyltransferase
HDAC	histone deacetylase
HEPES	4-(2-Hydroxyethyl)piperazine-1-ethanesulfonic acid
HPCE	high-performance capillary electrophoresis
HPLC	high-performance liquid chromatography
IGFBP3	insulin-like growth factor binding protein 3
IP	immunoprecipitation
K4H3	lysine 4 in histone H3
K9H3	lysine 9 in histone H3
kbp	kilo base pairs
LAP2	lamina-associated polypeptide 2
MAPK	mitogen-activated protein-threonin kinase
MBD	methyl-CpG binding domain
mdC	5-methyl-2'-deoxycytidine
MSP	methylation-specific PCR
MT	metallothionein
NCoR	nuclear receptor corepressor
NLS	nuclear localization signal
NuRD	nucleosome remodelling and histone deacetylation complex
o/n	over night
PAGE	polyacrilamide gel electrophoresis
PARG	poly(ADP-ribose) glycohydrolase
PARP	poly(ADP-ribose) polymerases
PBS	phosphate-buffered saline
PCNA	proliferating cell nuclear antigen
PCR	polymerase chain reaction
PRMT	protein arginine methyltransferase
RAR	retinoic acid receptor
RIZ1	retinoblastoma interacting zinc-finger protein 1
RCF	relative centrifugal force
RNAi	RNA interference
PBS	phosphate buffered saline

RT-PCR	reversed transcription followed by PCR
SAHA	suberoylanilide hydroxamic acid
SAM	*S*-adenosyl-**L**-methionine
SDS	sodium dodecylsulfate
SUMO	small ubiquitin-related modifier
TBE	Tris-borate-EDTA buffer
TdT	terminal deoxynucleotidyl transferase
TFA	trifluoroacetic acid
TMEDA	*N,N,N′,N′*-tetramethylethylenediamine
TRD	target recognition domain
Tris	2-amino-2-(hydroxymethyl)-1,3-propanediol, also named tris(hydroxymethyl)aminomethane
TSA	trichostatin A
TUNEL	TdT-mediate dUTP nick end labelling
vol	volume
zdC	5-aza-2′-deoxycytidine

3. Introduction

3.1 Epigenetics in the context of chromatin

The word epigenetics defines the study of mitotically and/or meiotically heritable changes in gene expression that occur without alterations in the DNA sequence (Wolffe and Matzke, 1999). Epigenetic regulation is essential to ensure proper cell differentiation during the development of multicellular organisms, which are constituted by cells that are genetically homogeneous but structurally and functionally heterogeneous. In mammals, except for controlled genomic rearrangements like the ones of the immunoglobulin and T-cell receptor genes in lymphocytes, all other differentiation processes are initiated and maintained through epigenetic mechanisms, including genomic imprinting and X-chromosome inactivation. In addition, epigenetic regulation is thought to be responsible for the silencing of transposable elements in somatic cells of plants, fungi and animals (Martienssen and Colot 2001; Bird 2002).

Despite being inheritable, epigenetic modifications are reversible and can be affected by environmental stimuli such as the diet in animals or the temperature in plants (vernalization response). Moreover, epigenetic changes in mammals are associated with the aging process (reviewed in Jaenisch and Bird 2003; Liu *et al.* 2003). Alterations in the normal function of the epigenetic machineries are involved in the generation and progress of different diseases, like cancer, atherosclerosis, systemic lupus erythematosus, disorders related with errors in the imprinting of specific genes, syndromes like ICF (immunodeficiency, centromeric region instability and facial anomalies), ATRX (α-thalassemia, mental retardation syndrome X-linked)

and Rett and many others illnesses (for recent reviews, see Lund and van Louhizen 2004, Zaina *et al.* 2005; Januchowski *et al.* 2004; Waterland and Jirtle 2004; Ehlrich 2003; Tang *et al.* 2004; Kriaucionis and Bird 2003).

Mammalian cells have several known systems responsible for the epigenetic control of gene transcription and chromatin structure: DNA methylation, covalent modifications of the canonical core histones (H2A, H2B, H3 and H4), deposition of variant histone proteins into the nucleosomes, local chromatin rearrangement and non-coding RNA expression. These mechanisms are not independent but cooperate to ensure proper gene transcription and chromatin structure (reviewed in Lund and van Lohuizen 2004).

This Introduction is divided in three parts. The first one is an overview of the epigenetic mechanisms. The second part summarizes the epigenetic alterations that have been found in cancer cells and the third one reviews the drugs targeted to these abnormalities. All the three sections are focused on DNA methylation and histone deacetylation, since the reversion of these two modifications is the objective of this thesis.

3.1.1. The nucleosome

The context for the epigenetic regulation is the chromatin, a macromolecular structure which packs the genomic DNA in the eukaryotic cell. The building block of the chromatin is the nucleosome, an octamer of histone proteins (two copies of H2A, two of H2B, two of H3 and two of H4) with 146 bp of dsDNA wrapped around it in 1.65 turns. In average, a nucleosome is found every 160-240 bp in all the eukaryotic genomes, constituting the principal packaging element of DNA within the nucleus. Nucleosomes are further organized in compact higher-order structures (such as the 30 nm-chromatin fiber) which contain additional macromolecules, like the linker histone H1 or the high-mobility group proteins (HMG). The dynamic folding and unfolding of these putative superstructures regulates DNA accessibility and influences transcription, replication, DNA repair and recombination. The epigenetic machineries are involved in the control of the chromatin structure.

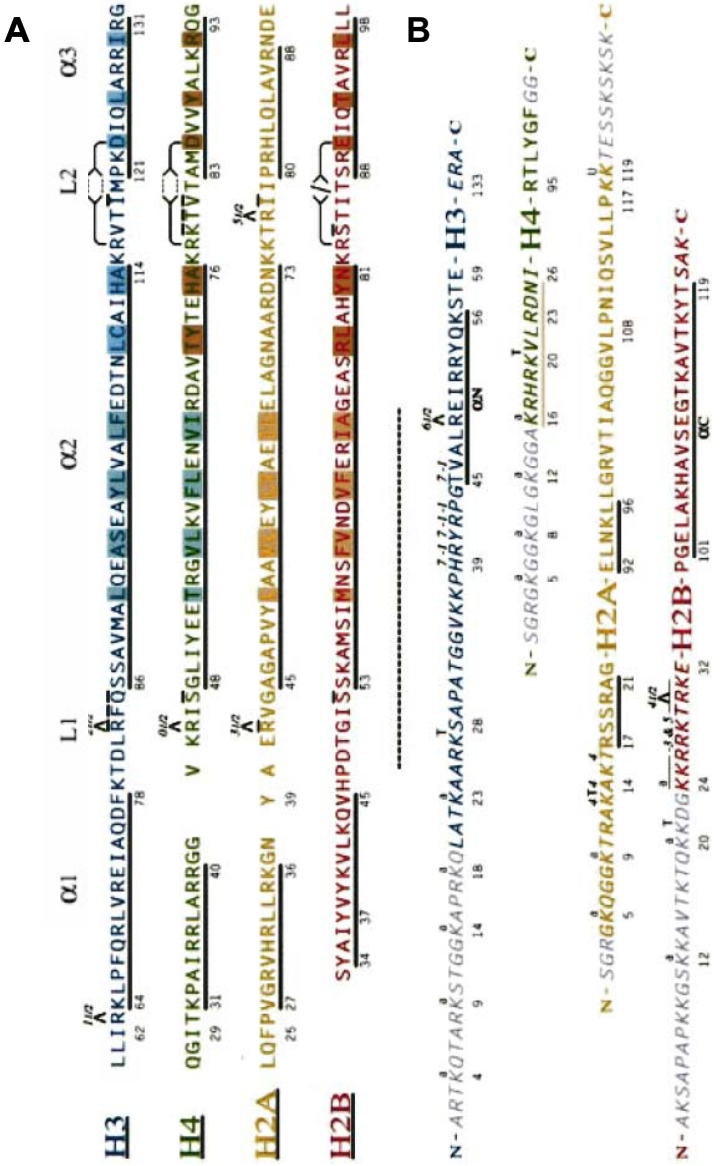


Fig. 1: Core histones sequences. **A**) The histone fold regions for H3, H4, H2A and H2B are aligned on the basis of their structured and labeled on the left (top). The α-helix (α1, α2, α3) and loop (L1, L2) secondary structural elements are labeled; α-helices are underlined and the overlying lines indicate β-strand hydrogen bonds between L1 and L2 loops. Some interaction sites are highlighted: H3-H4 α2-α2 (blue-green); H2A-H2B α2-α2 (orange); H3 4-helix bundle (blue); H4-H2B 4-helix bundle (brown). **B**) The histone fold extensions and tail (italics) regions are shown with the core histone name representing the histone fold. Sites of *in vivo* acetylation are indicated by the letter "a" and the site of ubiquitination is marked "U". In both **A** and **B**, Λ designates an arginine side chain that is inserted into the DNA minor groove. Taken from Luger *et al.* 1997.

Core histones (H2A, H2B, H3 and H4) are small basic proteins highly conserved, found in all eukaryotes. These polypeptides have in common the histone-fold, a structural domain characterized by three α-helices connected by two loops (see Fig. 1). The histone-fold acts as an assembly motif in the formation of histone heterodimers, that are further assembled to yield the histone octamer, around which the DNA superhelix is wrapped. The histone N- and/or C-termini protrude out of this compact structure (Luger *et al.* 1997). The structure based on the data obtained by Luger *et al.* is shown in Fig. 2.

Nucleosomes suffer directly the action of epigenetic regulators in four ways: DNA methylation, histone post-translational modifications (PTMs), deposition of histone variants and chromatin remodeling (reviewed in Khorasanizadeh 2004). All these changes take place in a coordinate way to specify downstream events (see section 3.1.7).

3.1.2 DNA methylation

One of the most studied epigenetic marks is the methylation of the position 5 of the cytosine ring in DNA. This feature is found in a wide scope of eukaryotic organisms, however the percentage of methylated cytosine, its distribution in the genome and the sequences where it is found vary between species.

In mammals, DNA methylation is associated with transcriptionally inactive chromatin, mainly transposable and retrotransposable repetitive sequences (such as Alu), inactive X-chromosome and imprinted genes. Cytosine methylation occurs simultaneously at both strands of the palindromic CpG dinucleotides (reviewed in Colot and Rossignol 1999; Bird 2002). However, in embryonic stem cells 15-20% of global 5-methylcytosine is not in CpG dinucleotides (Ramsahoye *et al.* 2000).

The proportion of CpG dinucleotides in the human genome is 1.2%, much smaller than the 4% expected from the abundance of cytosine and guanine (42% of the DNA bases). In addition, the distribution is not uniform: some areas, called CpG islands, are particularly rich in CpG whereas the rest of the genome is depleted in this dinucleotide (Takai and Jones 2002, Gardiner-Garden and Frommer 1987, Bird *et al.* 1985). CpG islands are mainly found in promoter regions. It is estimated that near

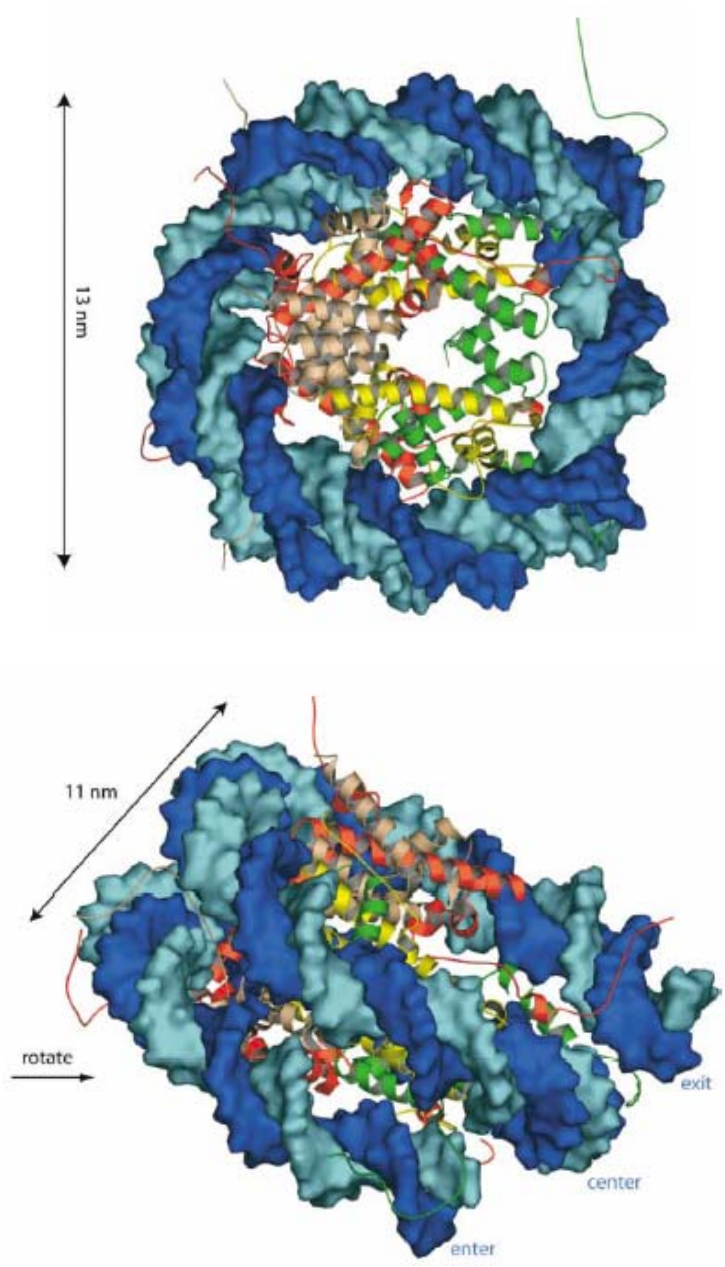


Fig. 2: The atomic structure of the nucleosome core particle. Each strand of DNA is shown in different shade of blue. The DNA makes 1.7 turns around the histone octamer to form an overall particle with a disk-like structure. Histone H3 is colored in green, H4 in yellow, H2A in red and H2B in pink. Taken from Khorasanizadeh 2004.

60% of the genes are associated to a CpG island (Lander *et al.* 2001, Venter *et al.* 2001, Antequera and Bird 1993). Some repetitive sequences, like the *Alu* family, have also a relatively high proportion of CpG dinucleotides (Takai and Jones 2002).

In healthy human somatic cells, 5-methylcytosine represents near 1% of the DNA base content, which means that most CpG dinucleotides are methylated, including those ones found in the body of the genes, intergenic regions and repetitive elements (Ehrlich *et al.* 1982). However, except for the X-inactive chromosome in females and the imprinted genes, CpG islands are devoid of 5-methylcytosine (reviewed in Bird 2002).

In mammals, DNA methylation patterns are established during embryonic development. First, after fertilization DNA demethylation takes place to erase the characteristic 5-methylcytosine distribution of the oocyte and the spermatocyte. After implantation, *de novo* methylation sets up the normal somatic DNA methylation patterns, whose maintenance is essential for the embryo development and differentiation (reviewed in Bird 2002, Bird and Jaenisch 2003, Li 2002).

3.1.2.1. DNA-methyltransferases

DNA (cytosine-5)-methyltransferases (DNMTs) are the enzymes which catalyze the transfer of the methyl group from *S*-adenosyl-L-methionine (SAM) to the position 5 in the cytosine ring. Up to now, four mammalian DNMTs have been identified (DNMT1, DNMT2, DNMT3A and DNMT3B). There is also a DNMT-like protein (DNMT3L). Human DNMT1 (Yen *et al.* 1992, Yoder *et al.* 1996), DNMT2 (Yoder and Bestor, 1998), DNMT3A and DNMT3B (Xie *et al.* 1999) have in common the DNA (cytosine-5)-methyltransferase domain. This domain, found in (cytosine-5)-methyltransferases from prokaryotes to human, is constituted by 10 conserved amino-acid sequence motifs (numbered from I to X) alternated with non-conserved regions. Of these sequence motifs, five (I, IV, VI, VIII and X) are highly conserved. The target recognition domain (TRD) resides in the variable region between motifs VIII and IX (Pósfai *et al.* 1989). DNMT3L lacks the motifs VII to X and the similarity to the DNMTs in motifs I, IV and VI is not complete (Aapola *et al.* 2000). The functional organization of the known human DNMTs is represented in Fig. 3.

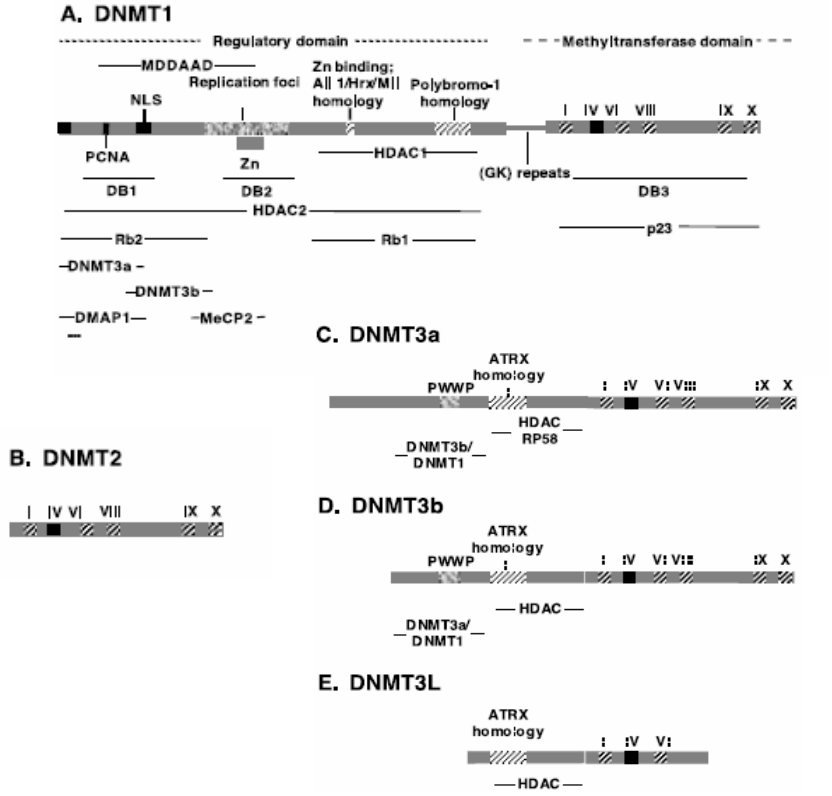


Fig. 3: Functional organization of mammalian DNA (cytosine-5) methyltransferases. **A**) Functionally mapped regions of the full-length human DNMT1 are illustrated along with the carboxy-terminal catalytic domain. Nuclear localization signal (NLS) (194–213), replication fork (RF) targeting peptide (320–567), Zn(II) binding region (652–670), and polybromo-1 homology regions are represented. The repressor domain shows homology to the mixed-lineage leukemia (trithorax homolog) protein (known as MLL or HRX or ALL1). Summaries of protein interaction regions are indicated. DNMT1 associated protein (DMAP1) binding (1–126), proliferative cell nuclear antigen (PCNA) binding (162–174), and histone deacetylase (HDAC1) binding (653–812) regions lie in the repressor domain. HDAC2 binds to the N-terminus region. Retinoblastoma gene product, Rb, binding regions (Rb1: 416–913, Rb2: 1–336) are shown. *De novo* methyltransferases DNMT3A and 3b also bind to the amino terminus of hDNMT1 (1–148 and 149–217). Methylated DNA dependent allosteric activation domain (MDDAAD) is identified at the amino terminal region of the enzyme (121–500). Methyl CpG binding protein (MeCP2) binding region (264–326) overlaps the RF region. Phosphoprotein p23, an associated factor for progesterone receptor, is bound with the catalytic region of DNMT1. Amino acid numbers representing different binding domains are in parentheses. Mapped DNA binding regions are indicated as DB1, DB2, and DB3. **B**) Architectural organization of DNMT2. The conserved motifs are indicated on top. Functionally mapped regions of the full-length hDNMT3A (**C**), DNMT3B (**D**) and DNMT3L (**E**) are illustrated along with the carboxy-terminal catalytic domain. Conserved motifs are indicated on top of the methyltransferase domain. Locations of interacting regions are on the bottom. HDAC, RP58 binding on DNMT3A and HDAC on DNMT3B and DNMT3L is indicated. PWWP and ATRX homology regions are indicated. Taken from Pradham and Estève, 2004.

Several experiments, including crystallographic and NMR studies performed on the bacterial enzyme M.HhaI, confirmed the mechanism proposed by Santi and colleagues in 1983 for the action of DNMTs (for reviews, see Christman 2002 and Jeltsch 2002). Essentially, in a first stage, the enzyme flips out the target base from dsDNA by rotating the adjacent phosphodiester bonds in the sugar backbone (Klimašauskas *et al.* 1994, O'Gara et al 1996, Klimašauskas *et al.* 1998, O'Gara *et al.* 1998, Vilkaitis *et al.* 2000). Only the π stacking interactions between the cytosine and the contiguous bases as well as the hydrogen bonds between cytosine and the guanine in the opposite strand are broken in that process. Then, the methyl group transfer takes place in several steps. Initially, the 6 position in the base ring is attacked (1,4 addition) by the thiolate of the cysteine in motif IV. Glutamic acid in motif VI helps by stabilizing the charge on the ring. In this point, enzyme and DNA are covalently linked to each other. Then, carbon 5 in the cytosine ring attacks the methyl group in SAM, and finally, a β-elimination releases the enzyme at the same time that restores the π-bond between positions 5 and 6 in the base (O'Gara *et al.* 1996, Sheikhnejad *et al.* 1999, Zhou *et al.* 2002). The scheme is shown in Fig. 4A.

Human DNMT1 is the largest DNA-methyltransferase found in this species. The catalytic domain is in the C-terminal region, like in DNMT3A and DNMT3B, separated from the N-terminal regulatory region by a series of GK repeats (see Fig. 3). The catalytic domain becomes inactive if large parts of the N-terminal region are deleted. The N-terminal part of the enzyme contains a nuclear localization signal (NLS) and a DNA replication foci-targeting peptide (RF), together with sequences that bind diverse proteins, like DNA-methyltransferase associated protein 1 (DMAP1), proliferating cell nuclear antigen (PCNA), retinoblastoma protein (Rb), DNMT3A or DNMT3B (see Fig. 3). There is also a methylated DNA dependent allosteric activation domain (MDDAAD), a Zn(II) binding region with homology to the mixed lineage leukaemia protein (MLL or HRX or ALL1) and another sequence homologous to the chicken protein polybromo-1 (Pradhan and Estève, 2003).

In vitro, DNMT1 shows preference for the hemimethylated DNA (i.e., only one of the DNA strands is methylated) over the unmethylated substrate and *in vivo* colocalizes with the replication foci during S phase (Leonhardt *et al.* 1992). For this reason DNMT1 is considered to be the DNMT responsible of conserving the

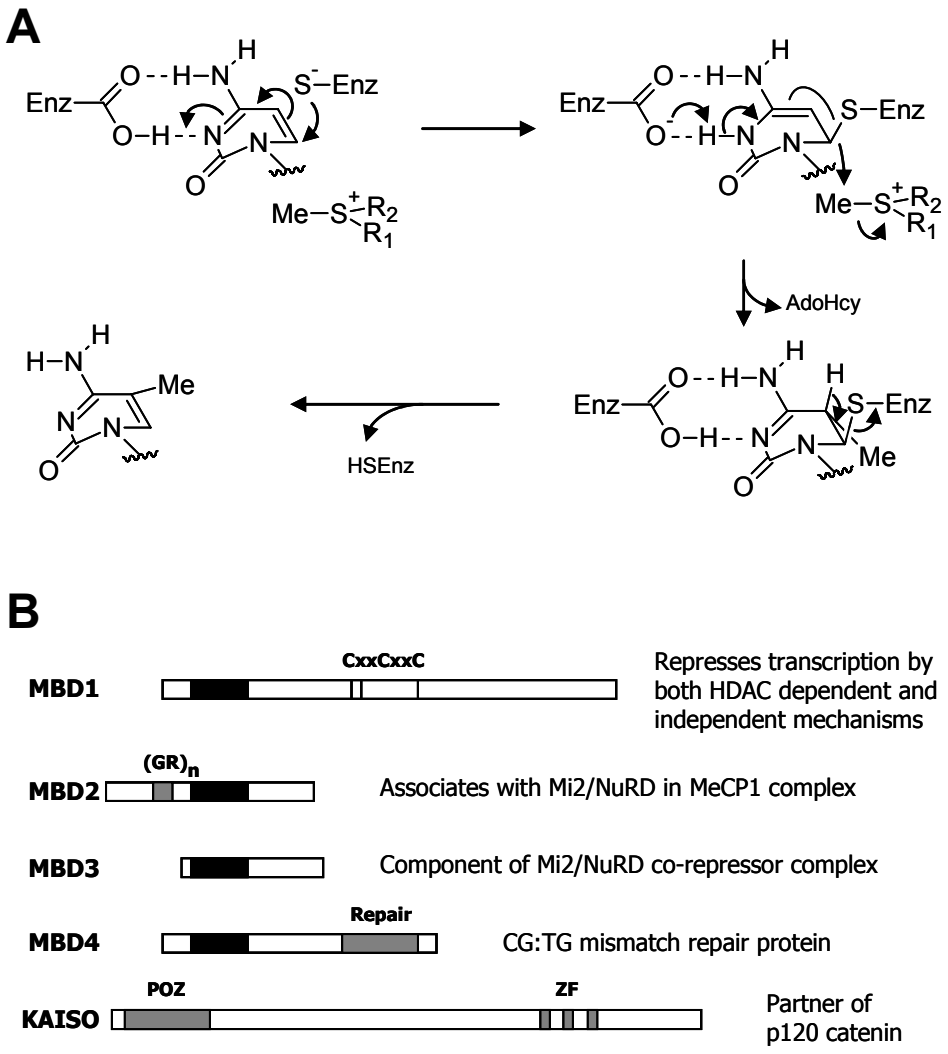


Fig. 4: **A**) Scheme showing the reaction pathway of the DNMTs based on the crystallographic data obtained with the bacterial enzyme M.Hha I by Zhuo and coworkers (Zhuo *et al.* 2002). Once the base is flipped out from DNA and inserted in the DNMT catalytic pocket, the 6-position carbon is attacked by a cystein residue from the enzyme ($EnzS^-$). An acidic residue in the enzyme helps to stabilize the negative charge of the base ring (EnzCOOH). Then the methyl group is transferred from SAM ($MeS^+R_1R_2$) to the 5 position in the base and the resulting intermediate suffers then a *syn*, β-elimination to release the methylated cytosine and the enzyme (EnzSH). AdoHcy stands for *S*-adenosylhomocysteine. **B**) Methyl-CpG binding proteins in mammals. The black boxes indicate the position of the MBD domain. Taken form Prokhortchouk and Hendrich, 2002.

methylation pattern over cell division, the "maintenance DNMT". However, partially overlapping functions with other DNMTs cannot be excluded (Rhee *et al.* 2002). On the one hand, overexpression of DNMT1 leads to *de novo* methylation in cell lines, suggesting a possible role not only maintaining but also in generating methylation patterns (Vertino *et al.* 1996). On the other hand, in cell lines lacking DNMT1 the loss of 5-methylcytosine in DNA is not as dramatic as it would be expected if this enzyme would be the only responsible for maintenance methylation (Rhee *et al.* 2000, Espada *et al.* 2004).

DNMT1 is the predominant DNA-methyltransferase in human tissues (Robertson *et al.* 1999). In somatic cells, two splicing variants, DNMT1a and DNMT1b, have been detected. Both proteins are identical except for a 16 amino acids insertion in the N-terminal region of DNMT1b (Hsu *et al.* 1999). Both transcripts are present in comparable amounts, but DNMT1a is more abundant than DNMT1b (Bonfils *et al.* 2000). In mouse, two gamete-specific splicing variants have been described, one present only in oocytes and another one in spermatocytes and skeletal muscle cells (Mertineit *et al.* 1998, Ratnam *et al.* 2002, Aguirre-Arteta *et al.* 2000).

Very little is known about mammalian DNMT2. The human enzyme has 391 amino acids and its only domain is the catalytic one. A wide range of eukaryotic organisms, including *Drosophila melanogaster* and *Schizosaccharomyces pombe*, has DNMT2 homologs, all of them with a conserved sequence of 41 amino acids between motifs VIII and IX (Dong *et al.* 2001). The methylating activity of this enzyme has been controversial for several years, since it shows very low activity *in vitro*, mice lacking it are viable with no major defects (Hermann *et al.* 2003) and *Dnmt2*-deficient mouse stem cells appear to be normal (Okano *et al.* 1998). However, recent experiments demonstrate that endogenous DNMT2 can bind DNA in human living cells, suggesting that it has methyltransferase activity *in vivo* (Liu *et al.* 2003).

Both DNMT3A and DNMT3B proteins belong to the same family of DNA methyltransferases. In their N-terminal region there is a PWWP domain followed by a cystein rich region similar to ATRX zinc finger. The PWWP domain, also found in a variety of chromatin-related proteins like WHSC1 (Wolf-Hirschhorn syndrome candidate 1) or MSH6 (mutS (*E. coli*) homolog 6), is able to bind DNA *in vitro*, but *in*

vivo it rather seems to be involved in protein-protein interactions. In mouse embryonic cells, the PWWP domain is required for the targeting and the activity of Dnmt3a and Dnmt3b (Stec *et al.* 2000, Qiu *et al.* 2002, Chen *et al.* 2004). The ATRX-homology domain of murine Dnmt3a, whose sequence is 98% identical to human DNMT3A, interacts with HDAC1 (Fuks *et al.* 2001). In mice, both Dmt3a and Dnmt3b can repress transcription also by other mechanisms different to DNA methylation and both enzymes are essential for *de novo* methylation during development (Dodge *et al.* 2005; Kaneda *et al.* 2004, Chen *et al.* 2003; Okano *et al.* 1999, Bachman *et al.* 2001, Fuks *et al.* 2001, reviewed in Li 2002). In humans, there are different splicing variants of both genes, but their function is not clear (Weisenberger *et al*. 2002, Xie *et al.* 1999, Robertson *et al.* 1999). Mutations in different regions of DNMT3B are responsible of the ICF (immunodeficiency, centromeric region instability and facial abnormalities) syndrome in near 60% of patients. The most characteristic hallmark of this disease is the centromeric instability, which correlates with a severe hypomethylation of satellites 2 and 3. The patients with mutations in DNMT3B also have hypomethylated alpha satellites and the females, also hypomethylated X-inactive chromosome (Jiang *et al.* 2005).

As mentioned before, DNMT3L has a truncated methyltransferase catalytic domain, so it is not enzymaticaly active. The N-terminal part of the protein is similar to that of DNMT3A and 3b, having also an ATRX homology domain (Aapola *et al.* 2000). Despite the inability to methylate DNA, DNMT3L is directly involved in epigenetic repression by recruiting HDAC activity (Aapola *et al.* 2002, Deplus *et al.* 2002), stimulation of DNMT3A activity in living cells (Chédin *et al.* 2002), and stimulation of both murine Dnmt3a and Dnmt3b *in vitro* (Gower at al. 2005, Suetake *et al.* 2004). Moreover, the phenotype of *Dnmt3a* conditional knockout mice and *Dnmt3L* knockout mice are indistinguishable (Kaneda *et al.* 2004). Dnmt3l is also implicated in the establishment of genomic imprinting in mice and spermatogenesis (Bourc'his *et al.* 2001, Webster *et al.* 2005, Bourc'his and Bestor 2004, reviewed in Li 2002).

3.1.2.2. Demethylation

As mentioned above, the establishment of DNA methylation patterns during embryonic development involves genome wide demethylation. Demethylation also takes place during normal gametogenesis (reviewed in Bird 2002, Li 2002, Bird and Jaenisch 2003). Two different models have been proposed to explain how mammalian cells reduce the amount of 5-methylcytosine present in their DNA: passive demethylation and active demethylation (Jeltsch 2002).

Passive demethylation is produced when the cell goes trough a second replication round before maintenance methylation has been completed. This yields one of the daughter dsDNA molecules completely unmethylated and the other one, hemimethylated. Passive demethylation is slow and requires cell cycle progression.

The active demethylation requires enzyme(s) or complex(es) that either remove only the methyl group in the position 5 of the cytosine or replace the whole 5-methylcytosine ring for unmethylated cytosine in a repair-like way. Battacharya and coworkers found that MBD2, one of the methyl-CpG binding domain (MBD) proteins (see 3.1.2.3), hydrolyses the 5-methyl group in the cytosine ring without affecting DNA base sequence; however this result remains controversial (Battacharya *et al.* 1999). Another protein containing the MBD, MBD4 (also known as MED1) is a thymine glycosylase. MBD4 binds to the mCpG·TpG mismatch due to 5-methylcytosine deamination and removes the thymine ring. The resulting is an abasic site on DNA is later filled with unmethylated cytosine by other enzymes and complexes (Hendrich *et al.* 1999). MBD4 has also a small 5-methylcytosine glycosylase activity *in vitro* (Zhu *et al.* 2000) and even can remove other modified bases from DNA (reviewed by Parsons, 2003). However, it seems that the main biological function of MBD4 is rather the protection of the genome against the possible mutations caused by 5-methylcytosine deamination than the demethylation itself (reviewed in Hendrich and Tweedie 2003). Another tymine glycosylase (TDG) without MBD has been found to act on C/T mismatches in CpG sequences (Neddermann *et al.* 1996).

Appart from thymine glycosylases and MBD2, no other protein or complexes with DNA demethylating activity have been described.

3.1.2.3. Sensors of DNA methylation

The presence of 5-methylcytosine residues on DNA influences the recruitment of different enzymes, transcription factors and repressors to the affected DNA sequence. Several proteins, like Kaiso and the proteins containing the MBD, recognize methylated DNA and recruit different repressor complexes to their target sequence (see next paragraph). Also, DNA methylation interferes with the binding of certain transcription factors, such as CREB (cyclic-AMP-response-element-binding protein) or SP1, or proteins involved in the establishment of genomic imprinting, such as CTCF (CCCTC binding factor) or BORIS (brother of the regulator of the imprinted sites) (Mancini *et al.* 1999, Klenova *et al.* 2002, reviewed in Lewis and Murrell 2004, Jaenisch and Bird 2003). On the other hand, there are some proteins that selectively bind unmethylated DNA sequences, like CGBP (CpG-binding protein), and that may protect CpG islands from methylation (see below).

There are 5 known human proteins containing the MBD: MeCP2, MBD1, MBD2, MBD3 and MBD4 (Fig. 4B). Three of them, MBD1, MBD2 and MBD3, have splicing variants. Except for MBD3, all these proteins (MeCP2, MBD1, MBD2 and MBD4) bind methylated CpG dinucleotides in DNA through the MBD motif. Even though MBD3 does not bind specifically methylated DNA, it is a member of the repressor complex NuRD/Mi2, interacting through the MBD domain with MTA2 (metastasis-associated gene family, member 2) and HDAC1 (Hendrich and Bird 1998, Saito and Ishikawa 2002). The involvement of MeCP2, MBD1 and MBD2 in transcriptional repression of methylated DNA sequences is widely reported (for reviews, see Ballestar and Wolffe 2001, Jaenisch and Bird 2003, Hendrich and Tweedie 2003). This fact does not exclude the participation of these proteins (for instance, MBD1) in silencing of non-methylated DNA through other complexes (Jørgensen *et al.* 2004). As already mentioned, MBD4 is involved in DNA mismatch repair and not in transcriptional repression.

Kaiso, a protein belonging to the BTB/POZ family of transcription factors, does not have the MBD domain (Fig. 4B) but binds selectively DNA sequences with at least two methylated CpG dinucleotides (Prokhortchouk *et al.* 2001). Kaiso recruits the repressive NCoR complex to certain methylated promoters (Yoon *et al.* 2003).

However, Kaiso has also methylation-independent DNA binding capacity, at least *in vitro* (Daniel *et al.* 2002).

MBD3-like protein 1 (MBD3L1) and MBD3-like protein 2 (MBD3L2) are two proteins with homology to MBD2 and MBD3 but with no MBD domain (Jiang *et al.* 2002). The first one, MBD3L1, associates with components of the MeCP1 and NuRD/Mi2 complexes (Jiang *et al.* 2004). The other one, MBD3L2, also interacts with NuRD/Mi2, but the resulting MBD3L·NuRD complex is either involved on methylation-independent transcriptional repression or not repression at all (Jin *et al.* 2005).

CpG-binding protein (CGBP) binds specifically unmethylated CpG motifs *in vitro* and acts as an activator of genes whose promoters reside within CpG islands (Voo *et al.* 2000). *In vivo*, this protein colocalizes with euchromatin and is essential for embryonic development in mouse (Lee and Skalnik 2002, Carlone and Skalnik 2001). It has been proposed that CGBP might protect CpG islands from methylation (Bird 2002).

3.1.3. Histone post-translational modifications

The N-terminal portion of the four core histone proteins is flexible, rich in basic amino acids, and highly conserved across eukaryotes. These tails protrude out of the nucleosome, and therefore are accessible for interaction with other proteins, including contacts with adjacent nucleosomes, as well as for different enzymatic machineries that cause several types of covalent post-translational modifications: acetylation of lysine residues, methylation of lysine and arginine side-chains, phosphorylation of serine and threonine, ADP-ribosylation of glutamic acid residues, ubiquitylation, sumoylation and biotinylation of lysine side-chains. Not only the N-termini but also some residues in the histone fold, like lysine 79 in H3, and the C-tails are exposed to interactions with other macromolecules and suffer post-translational modifications. The sequence of the tails of the canonical core histones and the best documented post-translational modifications are shown in Fig. 5. Such post-translational histone modifications correlate with various nuclear activities, including replication, chromatin assembly and transcription (Khorasanizadeh 2004).

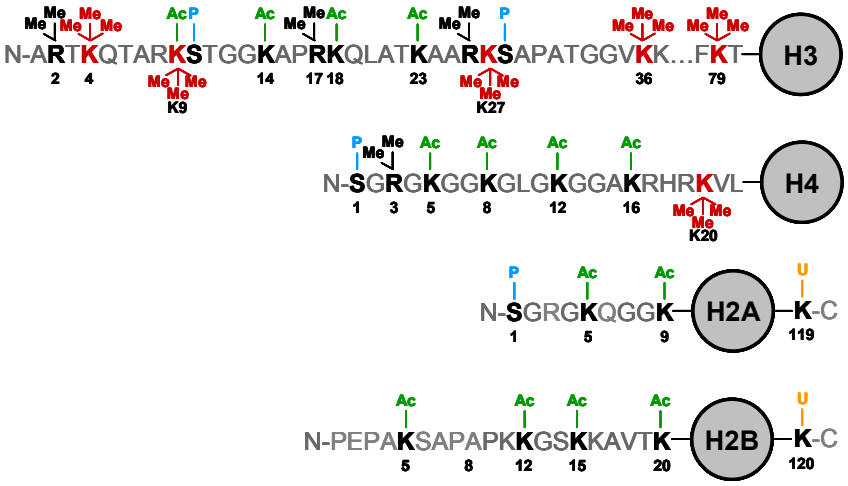


Fig. 5: The best known post-translational covalent modifications of the core histones (H2A, H2B, H3 and H4). Lysine methylation (Me) is represented in red. Acetylation (Ac), phosphorylation (P) and ubiquitination (Ub) are indicated in green, blue and orange, respectively. Arginine methylation is represented in black. Modifications shown above each amino acid correlate with activation, whereas the lysine methylation shown below correlates with repression. The highest degree of methylation possible for each residue is displayed. Taken from Sims III *et al.* 2003.

3.1.3.1. Lysine acetylation

The ε-amino group of certain lysine residues in the core histone tails (see Fig. 5) is acetylated under certain circumstances. In general, it is assumed that histone acetylation associates with transcriptional activation, whereas the silent and more compact chromatin is enriched in deacetylated histones. Two non-exclusive models try to explain this fact. The first one proposes that acetylation interferes in the contact between histone tails and DNA and/or between histones and the adjacent nucleosomes, thus altering the higher-order chromatin structure and allowing the access of transcription factors to DNA. The second model considers that acetylation and deacetylation of histone tails are recognized by different proteins that recruit transcription activators or repressors, respectively (reviewed in Hasan and Hottiger 2002).

Histone acetylation is reversible and highly dynamic. Two major sets of enzymes with opposite activity, histone acetyltransferases (HATs) and histone deacetylases (HDACs), are responsible for the establishment and maintenance of

histone acetylation patterns. Histone acetylation and deacetylation equilibrium is thought to be involved in the regulation of different nuclear processes, like global basal transcription, replication timing, repair of DNA double-strand breaks and gene-specific transcription, including both initiation and elongation (reviewed in Kurdistani and Grunstein 2003, Hasan and Hottiger 2002).

Histone acetylation happens both in the cytoplasm and in the nucleus. In the cytoplasm, recently synthesized histones become modified by B-type HATs enzymes. The importance of this acetylation for histone deposition in nucleosomes is not well understood (reviewed in Verreault 2000). In the nucleus, A-type HATs modify histones that are packed in nucleosomes. According to the amino acid sequence and the homology of the different regions, A-type HATs can be grouped into different families (see Table 1), conserved from yeast to human. All of them share the catalytic domain, which transfers the acetyl group from acetyl-CoA to the ε-amino group of the target lysine. *In vivo* most of the A-type HAT proteins are part of multisubunit complexes that regulate the activity and substrate specificity of the enzymes. Moreover, some of the A-type HATs can acetylate protein others than histones, like the high-mobility group proteins (HMGs), general transcription factors, the ATPase BRM, Werner helicase, other HATs or p53 (reviewed in Yang 2004, Kurdistani and Grunstein 2003, Hasan and Hottiger 2002, Marmorstein 2001).

There are three known classes of human HDACs, named class I (similar to yeast Rpd3), class II (similar to yeast Hda1) and class III (similar to yeast Sir2). As in the case of the HATs, some HDACs deacetylate several proteins other than histones, such as p53, E2F, or α-tubulin. Class I and II enzymes (HDAC1-11), also known as the classical family of HDACs, share the deacetylase core, a region of about 390 amino acids that contains the catalytic site. The catalytic site, conserved in eukaryotic cells, consists of a tubular pocket with hydrophobic walls and a Zn(II) cation in the bottom. Once the acetylated lysine residue is inserted the pocket, the metallic cation facilitates the hydrolysis of the amide by orientating and polarizing the carbonyl group in the N-acetylamide, as represented in Fig. 6 (Finnin et al. 1999, Vannini et al. 2004). Most of these enzymes associate with other proteins which can target and modulate the HDACs (deRuijter et al. 2003, Yang and Seto 2003).

Table 1: Human A-type HATs

Family	Enzyme	Substrate (1)	Established role
Gcn5/PCAF	PCAF	H3/H4, TFs, E1A, TAT	transcriptional coactivator
	GCN5L	H3/H4, TFs	transcriptional coactivator
P300/CBP	CBP	histones, TFs, E1A	transcriptional coactivator
	P300	histones, TFs, E1A, TAT	transcriptional coactivator
MYST	TIP60	H3/H4, androgen receptor	transcriptional co-regulator, DNA repair and apoptosis
	MOF	H4	transcriptional coactivator
	HBO1	H3/H4	DNA replication, transcriptional co-repressor
	MOZ	H3/H4	transcriptional coactivator
	MORF	H3/H4	transcriptional coactivator
p160	SRC-1	H3/H4	transcriptional coactivator
	ACTR	H3/H4	transcriptional coactivator
CIITA	CIITA	H4	transcriptional coactivator
$TAF_{II}250$	$TAF_{II}250$	H3/H4	transcription initiation, kinase and ubiquitin ligase
TFIIIC	TFIIIC subunits	H3/H4	transcription initiation
CDY	CDY	H4	
	CDYL	H4	histone-to-protamine transition during spermatogenesis

(1) TFs stands for transcription factors, E1A is the adenoviral oncoprotein E1A and TAT stands for HIV TAR RNA-binding protein

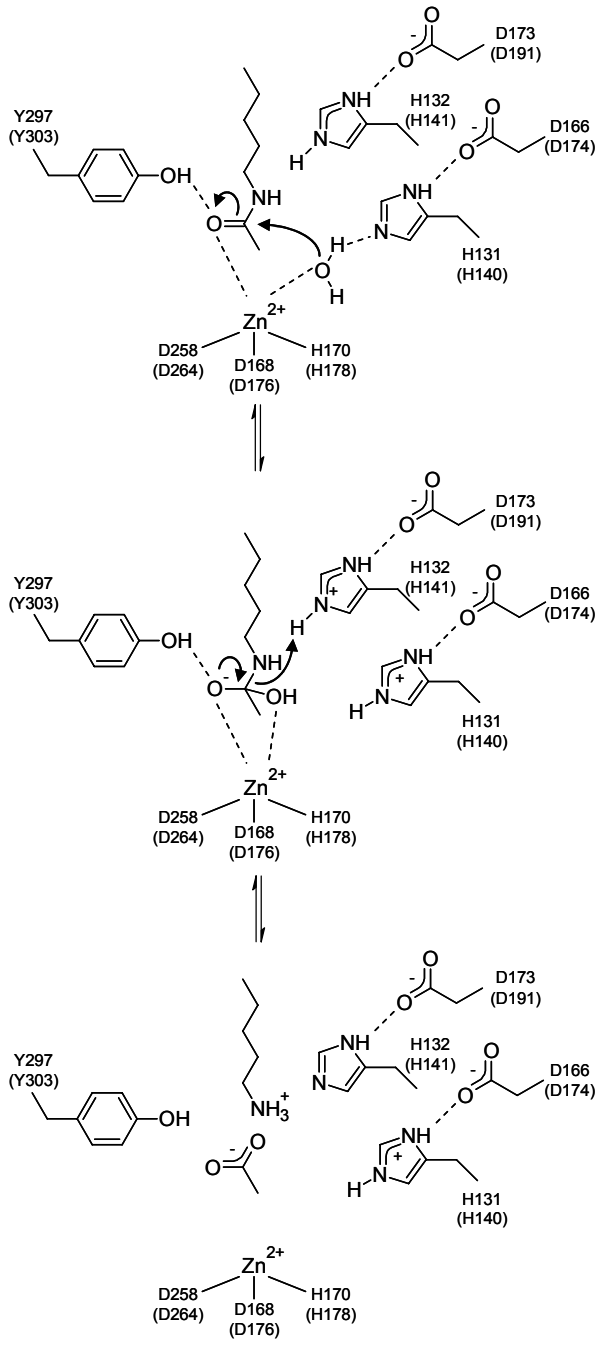


Fig. 6: The proposed catalytic mechanism for the deacetylation of acetylated lysine. HDLP (histone deacetylase-like protein, from Aquifex aeolicus) active-site residues and their proposed HDAC1 counterparts (in parenthesis) are labelled. From Finnin et al. 1999.

Class I enzymes (HDAC1, HDAC2, HDAC3, HDAC8) localise into the nucleus and are ubiquitously expressed. HDAC1, HDAC2 and HDAC3 are part of repressor complexes such as SIN3, NuRD/Mi2, CoREST (REST corepressor), NCoR and SMRT (silencing mediator of retinoic acid and thyroid hormone receptor), but, to date, no interaction partner or endogenous complex for HDAC8 has been found. Moreover, it is not clear that HDAC8 deacetylates histones *in vivo* (Waltregny *et al.* 2005, Hu *et al.* 2003).

The expression of class II HDACs is in general tissue dependent. This group of proteins can be subdivided in class IIa (HDAC4, HDAC5, HDAC7 and HDAC9) and class IIb (HDAC6 and HDAC10) enzymes. Class IIa HDACs share the amino-terminal region that includes domains for interaction with transcription factors such as the ones in MEF2 (myocyte enhancer factor 2) family and CtBP (C-terminal binding protein), etc. All the class IIa HDACs shuttle between cytoplasm and nucleus and all of them interact through their C-termini with SMRT and NCoR complexes. Class IIb enzymes have duplicated catalytic domains and both are primarily cytoplasmic. These enzymes only translocate to the nucleus in response to certain stimuli (reviewed in Verdin *et al.* 2003).

Human HDAC11 shares features of both groups, so its inclusion in one or another family is not clear.

The human HDACs belonging to class III have been recently discovered. It is reported the existence of 7 proteins (Sirt1-7) in this group, but the biological role of most of them is not yet known. Moreover, it is unclear whether any of these proteins have catalytic activity (reviewed in Blander and Guarente 2004). Unlike class I and class II HDACs, these enzymes are NAD+ dependent. The reaction subproducts are nicotinamide and *O*-acetyl-ADP-ribose (Jackson and Denu 2002, Tanner *et al.* 2000).

Acetylation of lysine residues influences the interaction of histone with other macromolecules. On the one hand, the positive charge of the lysine side-chain disappears with acetylation and the hydrogen bonds in which the ε-amino group is involved become different. Therefore, acetylation interferes with the contact of histone with DNA, RNA or certain proteins. On the other hand, acetylation represents a docking site for other molecules, especially proteins containing the bromodomain.

The bromodomain is a region of roughly 110-amino-acid, conserved in eukaryotes. It functions as an acetyl-lysine-recognition module, binding not only histones but also other acetylated proteins, such as p53. This domain is commonly found in diverse transcription-regulatory factors, like HATs and chromatin-remodelling proteins. These factors are usually part of multisubunit complexes with diverse transcription-related functions (reviewed in Yang 2004; Marmorstein 2001).

3.1.3.2. Lysine methylation

Methylation of histone lysine residues is involved in the regulation of specific gene expression at euchromatic loci and in the organization of bulk eu- and heterochromatin. To date, six lysine residues in the canonical core histones have been found to be methylated in different contexts: lysines 4, 9, 27, 36 and 79 in H3 (K4H3, K9H3, K27H3, K36H3 and K79H3 respectively) and lysine 20 in H4 (K20H4). In addition, each ε-amino group can be modified with 1, 2 or 3 methyl groups, resulting in a high number of theoretically possible combinations. In contrast to acetylation, methylation does not alter the charge of the side-chain but increases its bulkiness and hydrofobicity (for recent reviews see Peters and Schübeler 2005, Sims *et al.* 2003, Lachner and Jenuwein 2003).

Methylation of K9H3, K27H3 and K20H4 correlate with transcriptional silencing. Di- and trimethylation of K9H3 and K20H4 as well as trimethylation of K27 are modifications involved in silencing the inactive X chromosome in mammalian females (Chadwick and Willard 2004, Rougeulle *et al.* 2004, Plath *et al.* 2003). Trimethylation of K9H3 and monomethylation of K27H3 colocalize with pericentric heterochromatin and also methylation of K20H4 has been found in heterochromatic domains (Peters *et al.* 2003, Rice *et al.* 2003, Schotta *et al.* 2004; Kourmouli *et al.* 2004). Di- and monomethylation of K9H3 accumulate in silent areas of euchromatin (Rice *et al.* 2003). Moreover, dimethylation K9H3 is sufficient to repress transcription in a reporter gene in *Xenopus laevis* oocytes (Stewart *et al.* 2005).

On the other hand, the transcription start of expressed genes is enriched in di- and trimethyl K4H3 not only in higher eukaryotes, but also in yeast (Kouskouti and Talianidis 2005, Liang *et al.* 2004, Schneider *et al.* 2004, Ng *et al.* 2003a). These

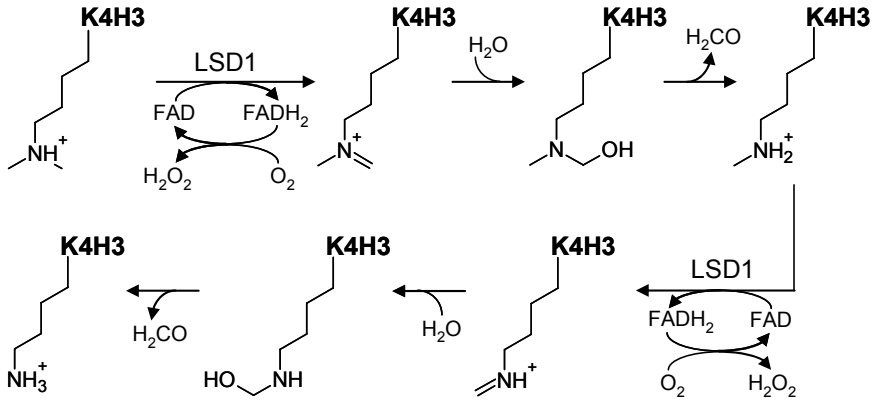


Fig. 7: Possible mechanism for LSD1-catalyzed demethylation of dimethyl K4H3 is shown, but the proposed reactions are also compatible with monomethylated lysines or methylated arginines. Taken from Shi *et al.* 2004.

modifications may constitute the molecular memory for recent transcription events (Ng *et al.* 2003a). Also, the 3' region of active genes contains more di- and trimethyl K36H3 compared to the constitutively inactive ones. It seems that methylation in this position is coupled to elongation and maybe RNA processing (Bannister *et al.* 2005, Kizer *et al.* 2005, reviewed in Hampsey and Reinberg 2003). Finally, dimethylation of K79H3 appears at the transcription start of active genes, like methylation of K4H3, and it is not present in heterochromatin (Kouskouti and Talianidis 2005, Ng *et al.* 2003).

All the known lysine methyltransferases use SAM as methyl-donor cofactor. Except for DOT1L (the enzyme that methylates K79H3) and its homologues all the other histone lysine methyltransferases share a conserved catalytic module, the SET domain, found in a large number of eukaryotic proteins and even in bacteria. The SET domain can be subdivided in 3 regions, the amino terminal area (SET-N), the carboxy terminal module (SET-C) and a highly variable region in between (SET-I). It is thought that at least part of the substrate specificity of these enzymes is owed to the SET-I module, whereas the post-SET domain determines the number of methyl groups (one, two or three) that the enzyme can add. The histone lysine methyltransferases can be grouped in several families according the amino acid

sequences surrounding the SET domain: SUV, SET1, SET2, EZ and RIZ (reviewed in Cheng *et al.* 2004, Xiao *et al.* 2003, Kouzarides 2002).

For a long time it was thought that lysine methylation was the most stable histone modification because no strong experimental evidences supported the existence of lysine demethylases, despite several theoretical possibilities and putative enzymes were suggested (Trewick *et al.* 2005). It was proposed that only the removal of the modified tail (proteolytic cleavage) or the replacement of the whole

histone for an unmodified version could revert methylation (reviewed in Bannister *et al.* 2002). However, recently a homolog of the amine oxidases, LSD1 (lysine specific demethylase 1), was found to catalyse the demethylation of di- and monomethylated lysine amino groups *in vitro* and in human cultured cells. According to the postulated mechanism, first the lysine amino group is oxidized, to yield an imine that is further hydrolysed to release formaldehyde. The cofactor FAD is required for the oxidation (see Fig. 7). Database search allowed the identification of another human protein similar to LSD1 as well as different LSD1-like proteins present in other species, like *Drosophila melanogaster*, *Arabidopsis thaliana* or *Schizosaccaromyzes pombe* (Shi *et al.* 2004, Forneris *et al.* 2005).

Certain methylated lysine residues in core histones are specifically recognized by the chromodomain of some proteins. For instance, HP1α (heterochromatin protein 1α) chromodomain binds selectively methylated K9H3 and Polycomb chromodomain binds methylated K27H3. To now, no other domain has been reported to bind selectively histones methylated at lysine residues, but not all the proteins containing a chromodomain show affinity for methylated histones. There are 11 families of chromodomains and the interaction partner of many of them is not yet clear. Moreover, it is reported that some of these domains can bind non-histone proteins, DNA or RNA (reviewed in de la Cruz *et al.* 2005; Brehm *et al.* 2004; Sims *et al.* 2003).

3.1.3.3. Arginine methylation

Histone arginine methylation is related with both transcriptional activation and repression. Arginine residues 8 and 17 in H3 (R8H3, R17H3) and 3 in H4 (R3H4) have been found to be methylated in cells. Additionally, PMRT1 is able of methylate

in vitro arginine residue 26 in H3 (R26H3), but this modification has not been found *in vivo*. (Pal *et al.* 2004, reviewed in Cheng *et al.* 2004, Kouzarides 2002).

In humans, seven protein arginine methyltransferases (PRMT) have been described, but not all of them are fully characterized and not all of them act on histones. It is known that the major substrate *in vivo* for PMRT4 (also known as CARM1) is R17H3, whereas PMRT1 prefers R3H4. These methyltransferases use SAM as source for methyl groups and have a conserved PMRT core, consisting in a methyltransferase domain, a β-barrel and a dimerization domain. It seems that dimerization is necessary for activity. There are two families of PMRTs, type I and type II. Both catalyze the formation of monomethylarginine, but type I PRMTs (PRMT1-4, PRMT6) also form asymmetric dimethylarginine and type II PRMTs (PRMT5 and PRMT7) form symmetric dimethylarginine (Lee *et al.* 2005, reviewed in Cheng *et al.* 2004, Kouzarides 2002).

No proper "arginine demethylases" are known. However, two laboratories have found independently that human PAD4 (peptidylarginine deiminase-4) converts a monomethylated arginine residue in a citrulline one, releasing monomethylamine (reviewed in Denman 2005).

3.1.3.4. Serine and threonine phosphorylation

Phosphorylation of the core histones is a post-translational modification related with mitosis and transcriptional activation, although its function is not clear. In human cells, phosphorylation of serine 10 and 28 in H3 (S10H3, S28H3), serine 1 in H4 (S1H4), serine 1 in H2A (S1H2A) and threonine 11 (T11H3) takes place in late G2 phase and/or different stages of the mitosis (Goto *et al.* 2002, Barber *et al.* 2004, Preuss *et al.* 2003, reviewed in Nowak and Corces 2004). Aditionally, phosphorylation of serine 14 in H2B (S14H2B) is related with chromatin condensation during apoptosis and phosphorylation of threonine 3 in H3 (T3H3) has been found in avian cells during prophase and metaphase (Cheung *et al.* 2003, Polioudaki *et al.* 2004). In any case, the most studied is phosphorylation of S10H3, which is not only involved in mitosis but also plays a role in gene activation through the MAPK (mitogen-activated protein-threonin kinase) pathway (Dunn and Davie 2005, reviewed in Nowak and Corces 2004, Prigent and Dimitrov 2003).

3.1.3.5. Poly(ADP-ribosyl)ation

Poly(ADP-ribosyl)ation is the attachment of a poly(ADP-ribose) polymer to a protein, most likely via the γ-carboxy groups of glutamic acid residues. The ADP-ribose units are linked to each other through glycosidic bonds 1''->2'. The chain can be either linear or branched. This modification is found in the core histones (mainly H2B, but also H3 and H4) as response to DNA damage. Also, poly(ADP-ribosyl)ation is important for the transcriptional activity of certain inducible genes and, in other cases, it is involved in silencing.

In vivo, poly(ADP-ribosyl)ation has a fast turnover. The poly(ADP-ribose) polymerases (PARPs) are the enzymes which catalyze the successive transfer of ADP-ribose units to protein acceptors, using NAD+ as substrate, to produce linear and/or branched polymers of ADP-ribose. The poly(ADP-ribose) glycohydrolase (PARG) hydrolyses the bond between two consecutive units of ADR-ribose and the poly(ADP-ribose) lyase hydrolyses the bond between the monomer directly attached to the protein and the glutamic acid residue (reviewed in D'amours *et al.* 1999, Kraus and Lis 2003).

3.1.3.6. Lysine ubiquitination and sumoylation

Ubiquitin and the small ubiquitin-related modifier (SUMO) family are peptides that can be covalently attached to core histones. Despite all these peptides can form polymeric chains, ubiquitin and SUMO peptides conjugated to histones are usually monomers. In higher eukaryotes, lysine 119 in H2A (K119H2A) and lysine 120 in H2B (K120H2B) are the main ubiquitinated residues within core histones. Some studies show that mammalian ubiquitinated H2A is involved in telomeric silencing, X chromosome inactivation and in the formation of XY bodies during spermatogenesis (Smith *et al.* 2004; Baarends *et al.* 2005). However, according to other reports, histone ubiquitination is located in nuclease-sensitive sites and transcriptionally active chromatin. In any case, the levels of ubiquitinated H2A change during the cell cycle: this modified histone disappears during G2->M phase transition and is restored in the M->G1 transition. Regarding SUMO peptides, mammalian H4 has been reported to be modified by SUMO-1 and SUMO-3 in a

higher extent than H2A, H2B and H3. This modification seems to be associated with transcriptional repression (Shiio and Eisenman 2003).

The machinery that attaches ubiquitin to proteins is very similar to that one which links SUMO. In both cases, three enzymes are necessary. The activating enzyme E1 forms a thioester bond with the corresponding modifying peptide. The conjugating enzyme E2 takes the peptide from E1 and the ligase E3 drives the formation of an isopeptide bond between the C-terminal group in the peptide and the ε-amino group of a lysine residue in the target protein. Specific isopeptidases remove ubiquitin and the different SUMO peptides from the proteins (for reviews see Gill 2004, Zhang 2003, Jason *et al.* 2002).

3.1.3.7 Lysine biotinylation

In humans, all the canonical core histones can be biotinylated. In H4, this modification takes place in positions K8 and K12 (Camporeale *et al.* 2004). Biotinylation of histones is more abundant in proliferating than in quiescent cells and associates with silent chromatin (Stanley *et al.* 2001, Peters et al. 2002). Additionally, UV light has been found to increase the proportion of biotinylated histones in Jurkat cells (Peters *et al.* 2002). Holocarboxylase synthetase could be the enzyme (or one of the enzymes) that catalyses these modifications (Narang *et al.* 2004).

3.1.4. Histone variants

As mentioned above, the exchange of the different core histones isoforms constitutes part of the epigenetic regulation. In humans, it has been reported the existence of variants for all the histones except for H4. The best characterized ones are represented in Fig. 8.

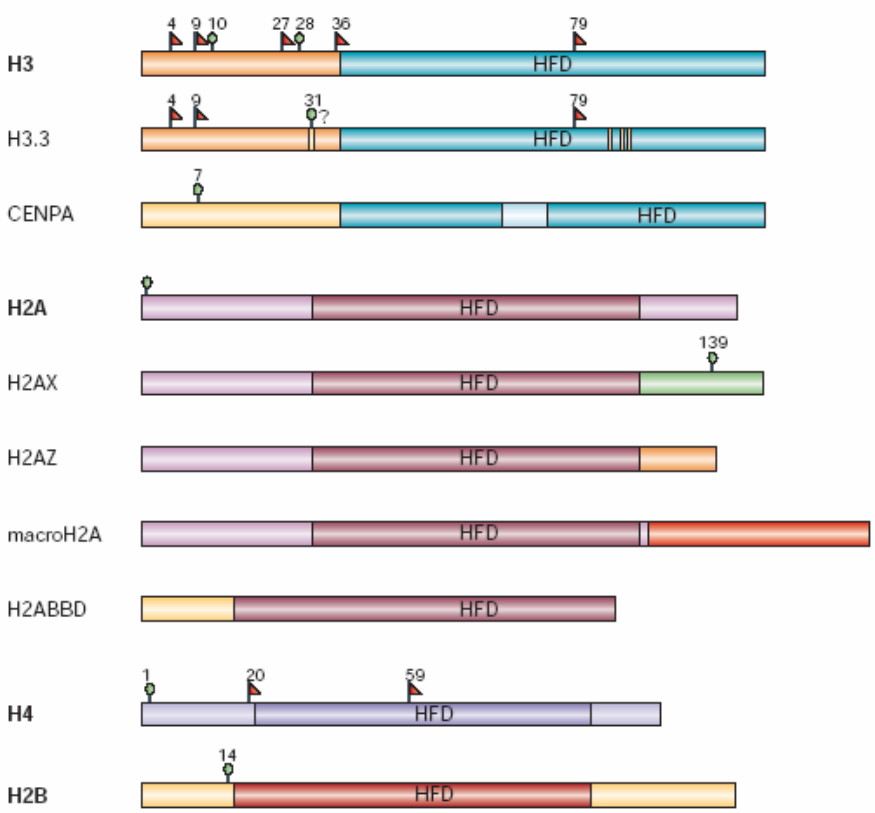


Fig. 8: Canonical core histones and their best characterized variants. The major core histones contain a conserved histone-fold domain (HFD). In addition, they contain N- and C-terminal tails that harbour sites for various post-translational modifications. For simplicity, only well-established sites for lysine methylation (red flags) and serine phosphorylation (green circles) are shown (other types of modifications, such as ubiquitylation, are not shown). In the histone H3.3 variant, the residues that differ from the major histone H3 (also known as H3.1) are highlighted in yellow. Three of these residues are contained in the globular domain and one resides in the N terminus. This N-terminal residue (Ser31) has been speculated to be a potential site for phosphorylation on H3.3. The centromeric histone CENPA has a unique N terminus, which does not resemble other core histones. Two sites of phosphorylation have been identified in this region, of which Ser7 phosphorylation has been shown to be essential for completion of cytokinesis. The region in the globular domain that is required for targeting CENPA to the centromere is highlighted in light blue. Histone H2A variants differ significantly from the major core H2A in their C terminus. The C terminus of H2AX harbours a conserved serine residue (Ser139), the phosphorylation of which is an early event in response to DNA double-strand breaks. A short region in the C terminus of H2AZ is essential for viability in *Drosophila melanogaster*. MacroH2A has an extended C-terminal macro domain, the function of which is unknown. Finally, the H2ABBD is the smallest of the H2A variants and contains a distinct N terminus, which lacks all of the conserved modification sites that are present in H2A. The C terminus is also truncated and lacks the docking domain that is found in other H2A species. The histones H4 and H2B are also shown, including their known methylation and phosphorylation sites. Taken from Sarma and Reinberg, 2005.

Among the several isoforms of H3, the best known are H3.3 and CENPA (centromere protein A). The first one, H3.3 is found in transcriptionally active loci. It is deposited into chromatin in both replication dependent and replication independent ways, in contrast to the canonical H3 (H3.1), which is only placed into nucleosomes during S-phase. CENPA, located in centromeres, is essential for proper chromosome segregation during mitosis.

Human histone H2A has four known variants. H2AX is scattered all over the genome, and its phosphorylation during the early steps of DNA repair is necessaryfor an efficient response to DNA damage. H2AZ may be involved in the maintenance of facultative heterochromatin and in preventing the spreading of heterochromatin. MacroH2A is only found in vertebrates and, despite been enriched in the inactive X chromosome, is not essential for the maintenance of its silencing. The C-terminal domain of macroH2A is a leucine zipper, which could be involved in the establishment of inter-nucleosome interactions and so promoting the compaction of chromatin. The mammalian specific H2A-Barr-body-deficient (H2ABBD) localizes in autosomes and the active X chromosome, being excluded of the inactive X in females.

Histone H2B has a testis-specific variant (TSH2B or HIST1H2BA) whose function is not yet know (Li *et al.* 2005).

These variants can also suffer post-translational modifications, like the canonical core histones (for reviews, Sarma and Reinberg 2005, Kamakaka and Biggins 2005).

3.1.5. Chromatin remodeling

Nucleosome remodeling defines a large number of ATP-dependent alterations of the canonical nucleosome structure. These perturbations of the DNA-histone interactions may lead to the relocation of histone octamers from a particular DNA fragment to available acceptor DNA in *cis* or *trans*, establishing a "fluid" state of chromatin in which the overall packaging of DNA is maintained, but individual sequences are transiently exposed to interacting factors (Becker and Hörz 2002). The remodeling is involved in all major reactions that occur on chromatin, including gene

transcription and repression, positioning of the nucleosomes in the replication origin, DNA repair, etc.

The common feature to the diversity of chromatin remodeling complexes is the presence of an ATPase from the Snf2-like family. According to the existence of different domains, the Snf2-like ATPases are grouped in several subfamilies. In humans, there are five subfamilies. The SNF2 (sucrose nonfermenting protein 2) subfamily proteins, like BRG1 (BRM/SWI2-related gene 1, also named SMARCA4) or BRM (also named SMARCA2), contain a bromodomain. The members of the ISWI (imitation of Swi) subfamily, such as SNF2H (sucrose nonfermenting protein 2 homolog, also known as SMARCA5), SNF2L (sucrose nonfermenting, yeast, homolog-like 1, also named SMARCA1) or CHRAC1 (chromatin accessibility complex 1), have a SANT domain. The proteins of the CHD1 (chromodomain helicase DNA binding protein 1) subfamily have a chromodomain. The other subfamilies are CSB (Cockayne syndrome group B) and RAD54, which contains ATRX. For reviews see Eberharter and Becker 2004, Längst and Becker 2004, Lusser and Kadonaga 2003, Becker and Hörz 2002, Narlikar *et al.* 2002.

3.1.6. Non-coding RNAs

The synthesis of non-coding RNA molecules is also involved in the epigenetic control of gene expression. The best known example in mammals, including humans, is the transcription of *XIST* (X inactive-specific transcript), the only gene expressed in the inactive X chromosome in females. The coating of the inactive X chromosome with *XIST* RNA is necessary and sufficient to initiate and spread the silencing. In order to maintain the X chromosome inactivated, *XIST* RNA coordinates chromosome-wide chromatin modifications, including remodeling, changes in histone post-translational modifications, deposition of histone variants and DNA methylation. Together with another non-coding RNA, *TSIX*, *XIST* controls the choice of the active X chromosome (reviewed in Plath *et al.* 2002).

Another sort of RNAs involved in regulation of gene expression are the microRNAs (miRNA), RNA molecules of about 22 nucleotides in length found in metazoan. The mechanism by which these nucleic acids act is not completely

understood. It is known that miRNA molecules are not fully complementary to their mRNA targets. Depending on the degree of mismatch, miRNA inhibit the translation of the targeted transcript or induce its degradation (see Meister and Tuschl 2004; Lippman and Martienssen 2004, Bartel 2004). In human cells, transfection with exogenous RNA fully complementary to its target causes not only mRNA degradation, but also DNA methylation at the promoter of the targeted gene (Morris *et al.* 2004). Furthermore, chicken cells with defective machinery to process miRNA show heterochromatin defects (Fukugawa *et al.* 2004).

3.1.7. A cross-talk between epigenetic modifications

All the features above mentioned (DNA methylation, histone post-translational modifications, histone variants, chromatin remodeling and non-coding RNA) act in an orchestrated manner to control gene expression and chromatin structure throughout the cell cycle. These marks are dependent on each other and interconnected through various mechanisms, resulting in a very complex network that allows the cell to store and inherit non-DNA-encoded information.

Theoretically, each modification can exert its action through "loss of function" and/or "gain of function" mechanisms. In the first case, the modification interferes with the binding of proteins and factors that recognize the unmodified species, whereas in the second case, the modification generates a docking site that is specifically recognized by certain protein modules (reviewed by Yang 2004). For instance, DNA methylation prevents the binding of CGBP protein and CTCF to their target sequences ("loss of function"), whereas it is recognized for the MBD proteins ("gain of function"). Other examples for "gain of function" are the interaction between bromodomain-containing proteins and acetylated histones or between some chromodomains and histones methylated at certain lysine residues.

Three models have been suggested to explain how the combination of modifications of chromatin, especially histone post-translational modifications, is translated. The first one, known as the "histone code" proposes that multiple histone modifications, acting in a combinatorial or sequential fashion on one or multiple histone tails, specify unique downstream functions (Strahl and Allis 2000). This

concept can be extended to a higher level, assuming that the distinct domains in higher-order chromatin structure are dependent on the local concentration and combination of differentially modified nucleosomes (Jenuwein and Allis 2001). However, other researchers consider that the combinatorial aspect of the histone modifications is not supported by the experimental data and so the histone code is not a valid model (Kurdistani and Grunstein 2003, Schreiber and Bernstein 2002). The second model, known as the "signaling network model of chromatin", postulates that there are parallelisms between other signaling pathways in the cell (such as the receptor tyrosine kinases) and the chromatin function. Among the common features of these signalling networks, the most relevant are the robustness, resulting from feedback loops and redundant mechanisms to propagate the signal; the bistability or switch-like behaviour, due to feedback loops; and the adaptability. Despite these parallelisms, chromatin function has unique characteristics, such as inheritance through cell division (Schreiber and Bernstein 2002). Finally, another alternative to the "histone code" is the "histone surface" model. Given that covalent modifications of histones are not independent of each other and that histone marks do not function in a combinatorial way, it has been suggested that different sets of modifications on a nucleosome generate different binding surfaces that can interact with various proteins. Diverse combinations of modifications can generate a similar surface and so be recognized by the same proteins (Kurdistani and Grunstein 2003).

In any case, some relationships between the different epigenetic mechanisms are documented (reviewed in Fischle *et al.* 2003). Some enzymes recognize certain epigenetic modifications and catalyze the addition of a different one in the same region. The ATPases in the SNF2 subfamily are targeted through their bromodomain to chromatin areas rich in acetylated histones. There, these ATPases take part in the acetylation-dependent nucleosome assembly and remodeling (reviewed in Yang 2004). On the other hand, some enzymes are inhibited by certain modifications, like p300, which cannot acetylate H3 if the same tail is methylated on K9H3, or Suvar3-9 histone lysine methyltransferases, which cannot methylate K9H3 if the same tail is methylated on K4 (reviewed in Fischle *et al.* 2003). The existence of complexes containing different epigenetic regulator proteins constitutes another way of connecting the epigenetic marks. For instance, MBD1 recruits SETDB1 to

methylated DNA, establishing a link between DNA methylation and K9H3 methylation (Sarraf and Stancheva 2004). The association of MBDs with HDACs in complexes, such as MeCP1, links DNA methylation and histone deacetylation (reviewed in Hendrich and Tweedie 2003). Finally, there are some data about the cascade of modifications in a few cases, like X chromosome inactivation in females or the activation of certain promoters. The events involved in the silencing of the inactive chromosome in females begin with *XIST* RNA expression. Once the coating of the inactive X chromosome by *XIST* RNA starts, in the already coated regions K9H3 becomes methylated at the same time that K4H3 methylation decreases. Then, the affected genes become silenced and their replication timing moves to the late S-phase. Also H4 becomes hypoacetylated. Next, deposition of macroH2A takes place onto the inactive chromosome and finally, DNA is methylated. DNMTs and HDACs collaborate with *XIST* to maintain the inactive X chromosome silenced (reviewed in Plath *et al.* 2002). Another example is the time-sequence of modifications on an exogenous pS2 promoter after estrogen stimulation of human cells. In this case, the stimulation induces the acetylation of K18 in H3 at the promoter. Next, PRMT4 is recruited, methylates R17H3 and then CBP acetylates K23H3 (Daujat *et al.* 2002).

Despite all the research done in the last few years, the interplay between the different epigenetic modifications is far from been completely known and understood.

3.2. Role of epigenetic alterations in cancer

The abnormal behaviour of cancer cells reflects the qualitative and quantitative alterations in their gene expression profile. Changes in DNA sequence and deregulation of the epigenetic mechanisms collaborate for the tumor initiation and progression. Epigenetic aberrations are directly involved in silencing tumor-suppressor genes, reactivation of genes that can promote cancer development and generation of chromosomal instability. The changes in DNA methylation patterns are the best documented epigenetic abnormalities of the cancer cell. During the last few years, alterations in histone acetylation have also been characterized and it is expected that, in the future, the accumulation of data about other epigenetic mechanisms will lead to a better understanding of the epigenetic deregulations related to cancer (reviewed in Laird 2005; Lund and van Louhizen2004).

3.2.1. Causes of the epigenetic alterations in cancer cells

The origin of the altered epigenetic patterns in cancer cells is not yet well understood, but it is known that errors in recruitment of the epigenetic machineries, expression of non-functional alleles, inadequate transcription levels of epigenetic related proteins and environmental factors underlie the aberrant epigenetic control of gene expression.

3.2.1.1. Errors in targeting epigenetic mechanisms

The best known examples of errors in the recruitment of epigenetic mechanisms are found in leukemias. Diverse chromosomal translocations generate chimera proteins that, in response to certain stimuli, target repressor complexes to the promoters of genes that should be activated. One of the many examples is the AML-ETO fusion protein. This chimera contains the DNA binding domain of AML1 (acute myeloyde leukemia 1 gene, also named RUNX1), a protein which can act as transcriptional activator, fused to ETO (eight twenty one), a protein that binds to the

repressor complexes NCoR and Sin3A. As result, genes that should be activated by AML1 become repressed (reviewed in Lund and van Louhizen 2004).

3.2.1.2. Expression of non-functional epigenetic regulator proteins

Certain epigenetic regulator proteins are inactivated due to mutations in a variety of cancers. Some examples are the histone methyltransferase RIZ1, the histone acetyltransferases p300 and CBP, and the subunits of chromatin remodelling complexes BRG1 and SNF5. The alterations in gene expression caused directly by these mutant proteins have not been studied in detail, but experiments in mice and/or cultured cells show the tumor-suppressor properties of these enzymes (reviewed in Lund and van Louhizen 2004).

3.2.1.3. Changes in the expression of epigenetic regulator proteins.

Some of the proteins involved in epigenetic regulation are overexpressed in cancer cells. That is the case of the histone methyltransferase EZH2 (enhancer of zestes homolog 2), overexpressed in breast, prostate and liver cancer (Sudo *et al.* 2005), or HDAC2, overexpressed in gastric cancer (Song *et al.* 2005). The way by which these alterations contribute to cancer development is not clear. For EZH2, some data suggest that the excess of protein could have dominant negative effects in the functions of the Polycomb repressor complex 2 (PRC2), but other results do not support this hypothesis (reviewed in Lund and van Louhizen 2004).

Other enzymes, such as RIZ1, p300, CBP or the remodelers BRG1 and BRM are not transcribed in certain types of cancer due to deletions and/or promoter DNA hypermethylation. In addition, the downregulation of some microRNA genes in tumors may affect chromatin structure and lead to chromosomal instability (reviewed in Lund and van Louhizen 2004).

3.2.1.4. Environmental factors

Some diet components and virus infections are known to influence the epigenetic patterns. For instance, cadmium inhibits DNMT activity, inducing global DNA hypomethylation. Also, mice fed with diets poor in methionine and choline suffer a decrease in DNA methylation and hepatocellular carcinoma.

Fermentation of the dietary fibre in the intestine yields diverse short chain fatty acids, including butanoic acid, a substance that inhibits HDACs (see 3.3.2.2). It has been suggested that butanoic acid protects colon against cancer, probably due to its effects on HDACs, but these chemopreventive properties remain controversial (reviewed in Lupton 2004, Hassig *et al.* 1997).

3.2.2. Direct consequences of epigenetic alterations in cancer cells

3.2.2.1. Silencing of tumor-suppressor genes

Tumor-suppressor genes are defined as the genes whose products are required for the normal cell function and whose loss of function contributes to the initiation or the development of the cancer. Two hits, one affecting each allele, are necessary for the complete loss of function of a tumor-suppressor gene in a cell (Knudson's hypothesis). Each one of these two hits can be either a genetic change, like mutation or deletion, or an epigenetic alteration. The best known epigenetic deregulations involved in the silencing of tumor-suppressor genes in cancer cells are DNA hypermethylation and histone hypoacetylation. Both alterations lead to the recruitment of repressor complexes that reduce or completely abolish the expression of the affected genes.

DNA hypermethylation is the increase in the proportion of 5-methylcytosine in a given DNA sequence. In cancer cells, DNA hypermethylation silences tumor-suppressor genes whose promoter is a CpG island. Some examples are *CDKN2A* (cyclin-dependent kinase inhibitor 2A, also $p16^{ARF/INK4a}$), *MLH1* (mutL homolog 1), *VHL* (von Hippel-Lindau tumor suppressor), E-cadherin and the retinoblastoma gene (reviewed in Esteller 2005, Feinberg and Tycko 2004, Jones and Laird 1999).

Histone hypoacetylation is the reduction in the number of acetyl groups attached to the histones in a given chromatin region. In cancer cells, the expression of certain tumor-suppressor genes, such as *CDKN1A* (cyclin-dependent kinase inhibitor 1A, also named $p21^{WAF/Cip}$), is often lost because of the hypoacetylation of histones at their promoters (reviewed in Johnstone and Licht 2003).

3.2.2.2. Derepression of genes that promote tumor growth

Changes in the epigenetic patterns can also lead to the reactivation of genes which are silent in the normal somatic cell. The 5-methylcytosine content at the promoters of oncogenes such as *H-RAS* or *BCL2*, testis-specific genes such as the melanoma antigen family of genes (MAGE) and imprinted genes such as *IGF2* (insulin-like growth factor-II) is diminished in many human cancers, resulting in an increase of their expression that contributes to tumor growth and development (reviewed in Ehrlich 2002, Feinberg and Tycko 2004, Lund and van Louhizen 2004).

3.2.2.3. Chromosomal instability

Karyotypic instability allows cancer progression by facilitating genome variability and so the emergence of mutant cells with selective advantages. Alterations in the epigenetic mechanisms can promote chromosomal instability. The best studied phenomenom is the hypomethylation of repetitive DNA sequences, especially pericentromeric satellites. These repetitive elements in the normal somatic cells are usually heavily methylated, associated with histone hypoacetylation and packed in heterochromatic regions, but in several cancers these satellites loss 5-methylcytosine. This fact is thought to reduce the compaction of the chromatin, resulting in a more accessible DNA for interaction with other DNA sequences and thus favouring the recombination. Indeed, satellite recombination is thought to be responsible for the translocations t(1;16) found in Wilms tumors and in some ovarian and breast carcinomas (reviewed in Ehrlich 2002, Feinberg and Tycko 2004). Retrotransposable sequences, like LINE-1, are also heavily methylated in normal somatic cells, probably to repress their transcription. However, in cancer cells (prostate, bladder, liver, lung and breast cancer among others), these elements are hypomethylated, which may cause an increase in their activity thus leading to genomic instability (Florl *et al.* 2004, Chalitchagorn *et al.* 2005, reviewed in Esteller 2005, Feinberg and Tycko 2004).

3.3. Epigenetic drugs for cancer therapy

In contrast to genetic alterations, epigenetic modifications are reversible, therefore the correction of aberrant epigenetic patterns is theoretically possible. This assumption has driven, during the last few years, a vast research to find novel anticancer drugs able of restoring, at least partially, the normal gene activity of the cells. Most of the efforts have been devoted to the development of chemicals that recover the expression of tumor-suppressor genes silenced by either promoter DNA hypermethylation and/or histone hypoacetylation. Inhibitors of the enzymatic activity of DNMTs or class I and class II HDACs have been or are currently tested for their use as cancer chemotherapy drugs (see 3.3.1 and 3.3.2). Inhibitors of DNMTs cause a drop in global DNA methylation and so are able to revert promoter hypermethylation. Inhibitors of Zn(II)-dependent HDACs induce an increase of histone acetylation by disrupting the balance between HATs and HDACs activity. It is expected that the increase in the knowledge of epigenetic mechanisms will allow the development of drugs interfering with other chromatin modifying enzymes, such as histone methyltransferases.

Despite being the most studied and known epigenetic drugs, the antitumor activity of DNMTs and Zn(II)-dependent HDACs inhibitors is not fully understood. Two models, not mutually excluding, are suggested to explain their anticancer properties. One of the models considers that the reactivation of tumor-suppressor genes silenced in the cancer cells triggers mechanisms of apoptosis and cell cycle control. The other model proposes that inhibition of DNMTs or HDACs avoids proper chromatin condensation during G2 and M phases of the cell cycle, causing aberrant chromosomal segregation and/or mitosis that lead to cell cycle arrest and apoptosis (Knox *et al.* 2000; Rhee *et al.* 2002, reviewed in Haaf 1995, Johnstone and Licht 2003).

Another feature that remains unclear is the selective toxicity of these drugs towards cancer cells. Since the chemicals are inhibitors of enzymes present in all kind

of cells, it could be expected that normal cells would suffer their effects. Indeed, one of the major disadvantages of these treatments is the possibility that these drugs have mutagenic effects in the long term. The deregulation of chromatin structure induced by these compounds in normal cells can increase retrotransposons expression and the rate of genomic DNA recombination (reviewed in Ehrlich 2003).

The properties of the known epigenetic drugs as well as their effects in normal and cancer cells will be discussed in the next pages.

3.3.1. Substances altering DNA methylation

As pointed out, the most studied epigenetic alterations in cancer cells are the changes in DNA methylation patterns. Tumor cells suffer global DNA hypomethylation at the same time that some CpG island-conatining gene promoters become aberrantly hypermethylated. The use of chemicals able of reducing the presence of 5-methylcytosine in DNA has been explored in order to reactivate the expression of tumor-suppressor genes silenced through hypermethylation in cancer cells. According to their mechanism, these substances can be classified in three big groups: drugs that reduce DNMTs expression, drugs that inhibit the DNMT enzymes and drugs that alter DNA methylation interfering with other cell functions (such as signalling cascades) or by non identified mechanisms.

3.3.1.1. Inhibitors of DNMTs expression

Reduction of DNMT1 expression by triggering the RNAi response is one the way of decreasing the 5-methylcytosine content in genomic DNA of cancer cells. In fact, MG98 and other antisense oligonucleotides directed against DNMT1 diminish the proportion of 5-methylcytosine in DNA by decreasing both DNMT1 mRNA levels and DNMT activity in murine models (Ramchandani *et al.* 1997). MG98 is a DNMT1 antisense oligonucleotide with a non-natural backbone that increases its *in vivo* stability: the phosphate groups are replaced by phosphorothioate groups and some hydroxyl groups in certain ribose moieties are methylated (reviewed in Szyf 2002). Acording to a pharmacokinetic study performed on tumor-bearing nude mice, saturation of plasma proteins with phosphorothioate oligonucleotides allows their

distribution in most tissues, preferentially accumulating in kidney and liver. At high doses, also an appreciable concentration of the synthetic oligonucleotide is found in the tumors (Qian *et al.* 1997). The use of MG98 as chemotherapy for solid tumors is currently evaluated in a phase II clinical trials (Stewart *et al.* 2003, Davis *et al.* 2003).

3.3.1.2. Inhibitors of DNMT enzymatic activity

DNMT1, DNMT3a and DNMT3b catalytic domain have two binding sites, one for the cytosine residue and another one for SAM. Chemicals that tightly bind any of these pockets inhibit DNMTs activity and the result is a decrease of the genomic DNA methylation levels. Three classes of substances are included in this group: DNA analogues, cytidine (compound **1** in Fig.9) and 2'-deoxycitidine (compound **2** in Fig.9) analogues and SAM-related compounds.

3.3.1.2.1. DNA analogues

Oligonucleotides containing a phosphorothioate backbone and CpG sequences have been tested for specific DNMT1 inhibition. *In vitro*, several oligonucleotides fully methylated at the CpG dinucleotides have shown strong inhibitory activity (nanomolar concentrations) by specifically binding DNMT1. However, in cultured cells, these substances alter methylation patterns but do not cause significant genome wide demethylation. The lack of general demethylation could be due to the induced cell growth arrest, which prevents passive demethylation (Bigey *et al.* 1999). The antiproliferative activity of this kind of substances has been proved to be reversible (Knox *et al.* 2000).

3.3.1.2.2 Cytidine and 2'-deoxycytidine analogues

The effects of cytidine and 2'-deoxycytidine analogues on genomic DNA methylation have been very extensively studied. All these compounds only inhibit DNMTs when incorporated into dsDNA (Taylor and Jones 1982). Inside the cell, 2'-deoxycytidine analogues are transformed into the corresponding 2'-deoxynucleotides triphosphate before being incorporated into DNA during replication. Cytidine analogues are converted first into nucleotides diphosphate (NDP), which can undergo

two different pathways. On the one hand, part of this NDP pool is reduced to the corresponding 2'-deoxynucleotides diphosphate. After phosphorylation, the resulting 2'-deoxynucleotides triphosphate are incorporated into DNA during S phase. On the other hand, the rest of the NDP pool is phosphorylated to nucleotides triphosphate and become incorporated into RNA, interfering then with RNA metabolism and protein synthesis. Because of that, cytidine analogues have much less specific effects on cells than 2'-deoxycytidine analogues. Cells lacking any of the enzymes involved in these metabolic reactions are resistant to these drugs. Also cytidine and 2'-deoxycytidine analogues that do not bind any of these enzymes cannot be incorporated into DNA, and so do not affect DNMT activity (reviewed in Haaf 1995).

Once the chemical is incorporated into DNA, the way DNMTs are trapped depends on the characteristics of the cytosine mimic ring. The position 6 of analogues that conserve the cytosine 5,6 insaturation (5-azacytidine, 5-aza-2'-deoxycytidine, 5-fluoro2'-deoxycytidine, 1-β-*D*-arabinofuranosyl-5-azacytosine and pyrimidin-2-one ribofuranoside; compounds **3**, **4**, **5**, **6** and **9** in Fig.9) is attacked by the cystein residue in DNMT motif IV (see 3.1.2.1). As result, DNMTs become covalently bound to DNA. On the other hand, the analogues that do not have the 5,6 insaturation (pseudoisocytidine and 5,6-dihydro-5-azacytidine, compounds **7** and **8** in Fig.9) cannot be attacked by the cystein residue in DNMT catalytic pocket. In this case, the enzyme is not covalently trapped but forms a very stable complex with the analog (reviewed in Christman 2002).

The covalent attachment of DNMTs to DNA not only depletes the nuclear DNMT pool, but also triggers DNA repair mechanisms. The repair takes place while the DNMTs concentration is low, making DNA methylation not efficient and resulting in a decrease of genomic 5-methylcytosine content. Additionally, restoration of DNA integrity is error-prone and mutations are more likely to happen than during normal replication. This increase in the mutation risk constitutes one of the major drawbacks of these drugs.

The compounds containing 5-azacytosine residues (5-azacytydine, 5-aza-2'-deoxycytidine, 1-β-*D*-arabinofuranosyl-5-azacytosine) are particularly mutagenic. The 5-azacytosine rings can be opened by hydrolysis, which is favoured by the covalent attachment of DNMTs to the position 6. The remnant of the base cannot base pair

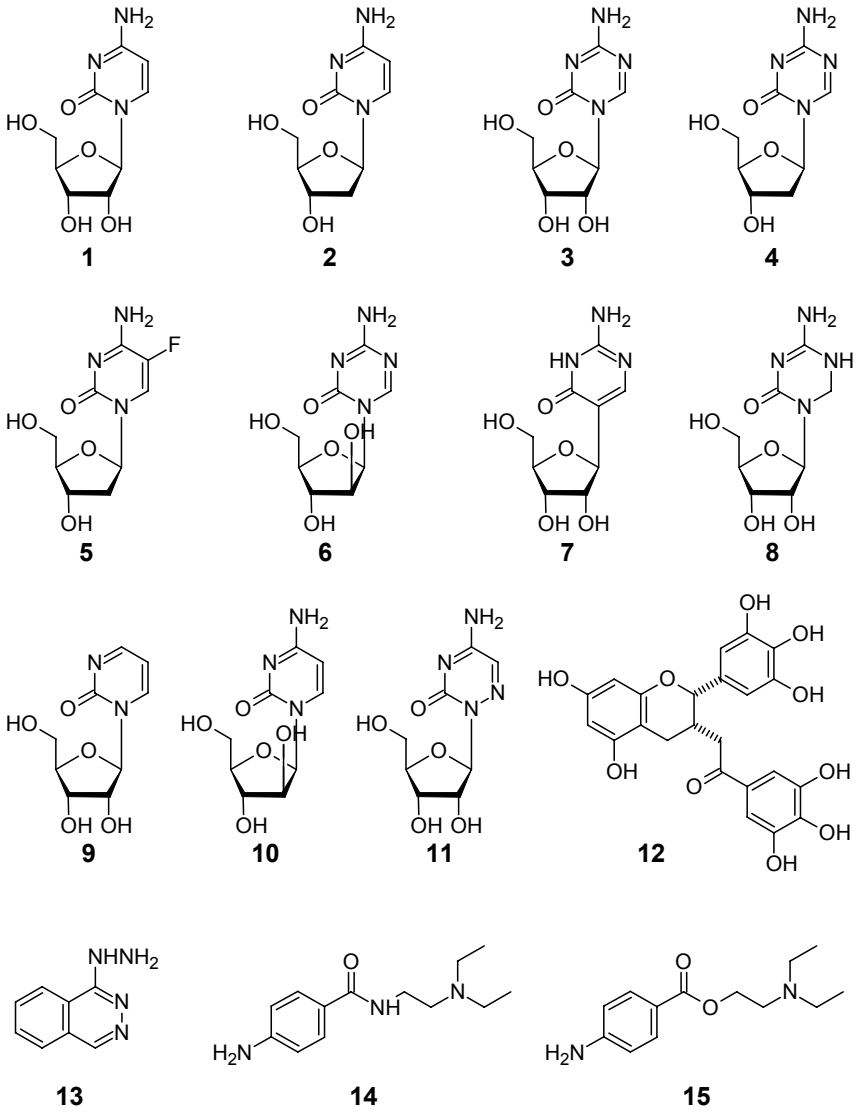


Fig.9: Chemical structure of cytidine (**1**), 2'-deoxycytidine (**2**) and some substances whose possible demethylating effects have been studied (see text): 5-azacytidine (**3**), 5-aza-2'-deoxycytidine (**4**), 5-fluoro-2'-deoxycytidine (**5**), 1-β-*D*-arabinofuranosyl-5-azacytosine (**6,** fazarabine), pseudoisocytidine (**7**), 5,6-dihydro-5-azacytidine (**8**), pyrimidin-2-one ribofuranoside (**9**, zebularine), 1-β-*D*-arabinofuranosylcytosine (**10**, cytarabine), 6-azacytidine (**11**), (-)-epigallocatechin-3-gallate (**12**), 1-hydrazinophthalazine (**13**, hydralazine), 4-aminobenzoic acid 2-diethylaminoethylamide (**14**, procainamide), 4-aminobenzoic acid 2-diethylaminoethyl ester (**15**, procaine).

as cytosine and thus, if it is not repaired, induces mutations during replication (Jackson-Grusby *et al.* 1997).

5-azacytidine (compound **3** in Fig.9) was the first cytidine analogue tested as possible inhibitor of DNA methylation. As already mentioned, when incorporated into DNA, this chemical traps DNMTs by covalent bond formation. Due to its major incorporation into RNA, 5-azacytidine is seldom employed in studies related to DNA methylation. However, it has been proposed that irreversible cell cycle arresting caused by this compound when used at micromolar concentrations is mostly due to its effects on DNMTs (Poot *et al.* 1990). Other inconveniences for the use of this chemical are its mutagenicity and its fast inactivation due to spontaneous hydrolysis and degradation by cytidine deaminase (Jackson-Grusby *et al.* 1997, reviewed in Haaf 1995). In any case, 5-azacytidine treatment is at the moment a potential chemotherapy for myelodysplastic syndrome (Silverman *et al.* 2002, Kornblith *et al.* 2002).

5-aza-2'-deoxycytidine (zdC, also known as *Decitabine*, compound **4** in Fig.9) is one of the most used demethylating drugs for assays in cultured cells. In contrast to 5-azacytidine, zdC is a 2'-deoxynucleoside and it cannot be incorporated into RNA. Because of that, zdC effects on cells are mostly due to the specific inhibition of DNMTs. It is well known that zdC reduces DNA methylation levels, stops cell proliferation and reactivates the expression of genes whose promoters are hypermethylated in cancer cells. However, as 5-azacytidine, zdC is mutagenic, suffers hydrolysis in aqueous solutions and is degraded by cytidine deaminase (Jackson-Grusby *et al.* 1997, Jüttermann *et al.* 1994, reviewed in Haaf 1995). Despite all these problems, the anticancer effects of zdC are under study. Several clinical trials are underway to establish the use of this compound, alone or in combination with others, as chemotherapy for diverse types of cancer, including advanced solid tumors and different leukaemia and lymphoma (for instance, see Issa *et al.* 2004; Aparicio *et al.* 2003).

5-fluoro-2'-deoxycytidine (compound **5** in Fig.9) works in a similar manner as 5-aza-2'-deoxycytidine, but the stabilisation of the complexes between the DNMTs and DNA containing 5-fluoro-2'-deoxycytidine requires the transfer of the methyl group from SAM to the 5 position of the 5-fluorocytosine ring. The methylation

reaction is stopped at this stage because the fluor atom in the 5 position cannot be abstracted. As result, DNA and enzyme are covalently linked and cannot separate. In the cells, 5-fluoro-2'-deoxycytidine is rapidly deaminated to 5-fluoro-2'-deoxyuridine, which is toxic. To avoid this phenomenon, simultaneous administration of high doses of 2'-deoxythymidine is necessary (Sheikhnejad *et al.* 1999, Jones *et al.* 1982, Jones and Taylor 1980).

1-β-*D*-arabinofuranosyl-5-azacytosine, also named fazarabine or Ara-AC (compound **6** in Fig.9), differs from 5-azacytidine in the stereochemistry of the 2' hydroxyl group. Because of this, fazarabine has a double effect: it causes DNA demethylation but also interfers the DNA synthesis, like cytarabine (1-β-*D*-arabinofuranosylcytosine or AraC). In opposition to 5-azacytidine and 5-aza-2'-deoxycytidine, fazarabine deamination is not catalyzed by cytosine deaminase (Barchi *et al.* 1996, Kees and Avramis 1995). This compound has been tested in several clinical trials, the last one reported in 1999 (Wilhelm *et al.* 1999).

Pseudoisocytidine (compound **7** in Fig.9) causes a decrease on genomic DNA methylation levels, but only a few studies analysing this effect have been published (Jones *et al.* 1982, Jones and Taylor 1980). As 5-azacytidine, is incorporated into both DNA and RNA (Zedek 1979).

5,6-dihydro-5-azacytidine (DHAC, compound **8** in Fig.9) incorporated into DNA forms very stable complexes with the DNMTs because the 5,6-dihydro-5-aza-cytosine ring mimics the transition state of the cysteine nucleophilic attack to the position 6 in the cytosine ring (see Fig.4A). The subsequent depletion of DNMTs leads to DNA demethylation (Sheikhnejad *et al.* 1999, Jones *et al.* 1982, Jones and Taylor 1980). DHAC restores the activiy of hypermethylated genes in several cell lines, though the methylation status after treatment has not been studied (Izbicka *et al.* 1999a, Izbicka *et al.* 1999b). In cells, DHAC is preferentially incorporated into RNA because the formation of the corresponding nucleoside triphosphate is favoured respect to the 2'-deoxynucleoside triphosphate (Avramis *et al.* 1989, Kees and Avramis 1995). DHAC has undergone several clinical trials as cancer chemotherapy either alone or in combination with other drugs (for instance, Vogelzang *et al.* 1997, Samuels *et al.* 1998).

Pyrimidin-2-one ribofuranoside (zebularine compound **9** in Fig.9) is a well known inhibitor of cytidine deaminase (Carlow and Wolfenden 1998). Its antitumor activity was already described in 1991 but its effects on DNA methylation were found later. Zebularine reduces global DNA methylation in the cells by trapping covalently the DNMTs with a similar mechanism to that one of 5-azacytidine or zdC but it is not as powerful as these drugs in inducing DNA hypomethylation. However, zebularine has some advantages: it is more stable in aqueous solution and less toxic than 5-azacytidine or zdC (reviewed in Yoo *et al.* 2004). (Driscoll *et al.* 1991, Zhou *et al.* 2002 Cheng *et al.* 2003). Like the other nucleosides, RNA incorporates more zebularine than DNA (Ben-Kasus *et al.* 2005).

The activity *in vivo* of 5-azacytidine, zdC and other cytidine analogues is reduced partially because these chemicals are degraded by cytidine deaminase. The administration of zebularine as inhibitor of cytidine deaminase is also explored as a way of increasing zdC efficience (Laliberté *et al.* 1992, Lemaire *et al.* 2005).

The effects of 1-β-*D*-arabinofuranosylcytosine (cytarabine, compound **10** in Fig.9) and 6-azacyditine (compound **11** in Fig.9) on DNA methylation have also been analysed, but none of these compounds causes DNA hypomethylation in cells (Jones and Taylor 1980, Stopper *et al.* 1995).

3.3.1.2.3 *S*-adenosylmethionine analogues

Different substances that can compete with SAM for DNMT binding, like sinefungin, have been analysed *in vitro* and in cultured cells (for instance Zingg *et al.* 1996, Boehm and Drahovsky 1983). However, SAM is the universal donor of methyl groups in the cell, hence its analogues have pleiotropic effects due to the interference with more metabolic pathways than DNA methylation.

3.3.1.3. Other substances altering DNA methylation

Many other substances alter DNA methylation either globally or at specific promoters but they have not been deeply characterized. In some cases, even the mechanism by which these chemicals exert their effect is not known.

Treatment of cultured cell with inorganic salts of arsenic, cadmium or selenium induces DNA hypomethylation. Several mechanisms have been suggested

to explain this phenomenom, from poisoning DNMTs to interference with SAM metabolism. In any case, the effects of these compounds are pleiotropic. Whereas selenium may have adenoma preventive properties, cadmium and arsenic are considered carcinogens (Jacobs *et al.* 2004, Takiguchi *et al.* 2003, Rossman 2003, reviewed in Davis and Uthus 2004). Nevertheless, arsenic salts are on clinical trials as a potential chemotherapy for leukaemia and other diseases (reviewed in Evens *et al.* 2004).

Hydralazine (1-hydrazinophthalazine, compound **13** in Fig.9) is an antihypertensive drug which induces a lupus-like disease in patients treated with it. The research about this side effect led to the discovery of the alterations that this substance induces in DNA methylation. Hydralazine provokes global DNA hypomethylation, apparently through two mechanisms: by inhibiting directly the enzymatic activity and by inhibiting the expression of DNMT1 and DNMT3a (Ángeles *et al.* 2005, Deng *et al.* 2003). Hydralazine also decreases the presence of 5-methylcytosine at the promoter of genes that are silenced by hypermethylation in cultured cancer cells, reactivating their transcription (Segura-Pacheco *et al.* 2003). In patients, low doses of hydralazine recover the expression of hypermethylated tumor-suppressor genes without major alterations in global DNA methylation (Zambrano *et al.* 2005).

Procainamide (4-aminobenzoic acid 2-diethylaminoethylamide, compound **14** in Fig.9) is employed to treat heart arrhythmia in humans. Like hydralazine, procainamide induces a lupus-like disease in the patients treated with it. Procainamide also reduces the global content of 5-methylcytosine in DNA and at the hypermethylated promoters, restoring the expression of tumor-suppressor genes in cancer cells and in patients (Segura-Pacheco *et al.* 2003). However, the mechanism of demethylation is not known. *In vitro*, procainamide intercalates on naked DNA, inducing changes in its structure (B to Z transitions). This binding may also take place *in vivo*, interfering with protein-DNA interactions, including DNMT binding to its target (Thomas and Messner 1986, Zacharias and Koopman 1990). Other data suggest that procainamide may compete with DNA for the binding of DNMTs (Scheinbart *et al.* 1991, Cornacchia *et al.* 1988, Deng *et al.* 2003).

Different natural compounds, like (-)-epigallocatechin-3-gallate (compound **12** in Fig.9) or genistein, induce changes in the DNA methylation patterns of cultured cells. The mechanism is not clear and, in addition, these compounds also interfere with other metabolic and signalling pathways (reviewed in Davis and Uthus 2004).

3.3.2. Inhibitors of Zn(II)-dependent HDACs

In the last ten years, a myriad of substances able of inhibiting Zn(II)-dependent HDACs have been described. Several of these compounds have been or are currently on clinical trials as possible cancer chemotherapy agents. As mentioned above, the acetylation pattern of histones is maintained by the coordinated action of two types of enzymes: HATs and HDACs. The inhibition of one of this group of enzymes disrupts the equilibrium and results in either histone hypoacetylation, if HATs are inhibited, or hyperacetylation, when HDACs are inhibited. The finding that some compounds that induce cancer cells differentiation are inhibitors of Zn(II)-dependent HDACs prompted the search for more substances with similar antitumor properties but better *in vivo* availability and less toxicity (Richon *et al.* 1998). All these chemicals are supposed to bind the Zn(II) cation present in the bottom of the catalytic pocket of these HDACs. The high number (at least 11) of class I and class II human HDACs together with the homology of their catalytic domains makes difficult the design of selective inhibitors for a certain enzyme of this group. Nevertheless, each given inhibitor has different affinities for the different HDACs. In some cases, these differences are minimal but in others not. For instance, the benzamide MS-275 cannot inhibit HDAC8 (see 3.3.2.3). Additionally, some of the Zn(II)-dependent HDACs have substrates other than histones, so inhibition of these enzymes affects the acetylation of other proteins, like α-tubulin. There is still little knowledge about the different specificities of the HDACs and their inhibitors because their discovery is very recent: the first description of a human HDAC was published in 1996 and the last ones, in 2002 (Furukawa *et al.* 1996, Kao *et al.* 2002, Gao *et al.* 2002, Petrie *et al.* 2003).

Even though inhibition of Zn(II)-dependent HDAC activity provokes massive histone hyperacetylation, it only affects the transcription of 1-10% of the genes in

human and murine cells. Moreover, the proportion of overexpressed genes is comparable to that one of repressed genes (Peart *et al.* 2005, Kultima *et al.* 2004, Chiba *et al.* 2004, Gray *et al.* 2004, Glaser *et al.* 2003). The mechanism by which HDACs inhibitors cause gene activation or repression is not well understood. It seems that histone hyperacetylation is not sufficient to increase the expression of many genes. Also indirect effects, like changes in the expression of transcription factors, can contribute to the expression patterns induced by HDACs inhibitors (reviewed in Marks *et al.* 2003).

Despite all these drugs induce histone hyperacetylation, most of the effects on cell cycle and gene expression depend on the drug concentration and the model system employed to test them (Grant 2004). For instance, butanoic acid and trichostatin A inhibit G1 to S transition in HeLa cells (Finzer *et al.* 2001) but in A549 these chemicals causes predominantly G2 and M arrest, respectively (Blagosklonny *et al.* 2001). Also, the subset of genes whose expression changes upon HDAC inhibition depends on the conditions of the experiment. The reason underlying this behaviour is not known, but it makes impossible the comparison of studies performed independently of each other.

A common feature of these treatments is the induced overexpression of cyclin-dependent kinase inhibitor 1A (*CDKN1A*), a gene involved in cell cycle control and which is repressed in many tumors (Marks *et al.* 2003). In some systems, HDAC inhibition also reactivates the transcription of the growth arrest and DNA-damage-inducible beta gene (*GADD45β*). The phase(s) in which the cell cycle is halted due to the treatment with HDACs inhibitors may be determined by the transcription levels of both *CDKN1A*, whose overexpression is responsible for the arrest in G1, and *GADD45β*, whose overexpression mediates G2/M arrest (Hirose *et al.* 2003).

Three parts can be recognized in the structure of most of the known HDACs inhibitors: the Zn(II) binding moiety, which chelates the cation at the bottom of the HDAC catalytic pocket; a bulky region that acts as a cap, binding the outer part of the catalytic tube; and a hydrophobic linker between the Zn(II) binding group and the cap. The linker can be either aliphatic or aromatic. The inhibitors of class I and class II HDACs can be clasified according to the functional group that interacts with the Zn(II) cation. The biggest set is constituted by the hydroxamic acids, but also

carboxylic acids, benzamides, epoxides and compounds with other functionalities have been described.

3.3.2.1. Hydroxamic acids

Hydroxamic acids cause the reversible inhibition of the Zn(II)-dependent HDACs. The use of combinatorial chemistry allowed the synthesis of thousands hydroxamic acids and the screening of these libraries resulted in the identification of very potent and specific inhibitors. Indeed, in cultured cells, micromolar or submicromolar concentrations of these substances induce global histone acetylation and cell cycle arrest or apoptosis (reviewed in Monneret 2005).

Trichostatin A (TSA, compound **16** in Fig.10) is a chemical produced by *Streptomyces platensis*. It is the first hydroxamic acid whose HDACs inhibitory properties were discovered (Yoshida *et al.* 1990) and it is widely used in cultured cells. Additionally, it has shown promising effects in some murine tumor models (Marks *et al.* 2003) whereas in others is completely inactive (Qiu *et al.* 1999). No trials in humans have been reported.

Suberoylanilide hydroxamic acid (SAHA, compound **17** in Fig.10) was first identified as a substance that induces cell differentiation. It is currently tested on clinical trials for different sort of cancers: phase III studies for T- and B-cell lymphoma and phase I for a variety of solid tumors (Kelly *et al.* 2003).

Pyroxamide (suberoyl-3-aminopyridineamide hydroxamic acid, compound **18** in Fig.10) was found in a screening of chemicals whose design was inspired on SAHA. It has undergone clinical trials as treatment for leukemia, lymphoma, multiple myeloma and eye cancer, but the results have not been yet reported (Butler *et al.* 2001).

Oxamflatin (compound **19** in Fig.10) was found to inhibit cancer cells growth in 1996, but it was identified as a HDAC inhibitor in 1999. It has antitumor properties in mice, but has not been tested in humans (Sonoda *et al.* 1996, Kim *et al.* 1999, reviewed in Monneret 2005).

Cyclic-hydroxamic acid containing peptides (CHAPs) are synthetic cyclic peptides made of 4 amino acids, whose structure is based on that one of trapoxin (see 3.3.2.4). The side-chain of one amino acid ends in a hydroxamic group, in such

a way that the hydroxamic group chelates the Zn(II) cation in the HDAC, the amino-acid side chain is the linker and the rest of the peptide constitutes the cap (see CHAP30, compound **32** in Fig.11) . The peptidic nature of these substances permits the synthesis of large number of analogues by using combinatorial chemistry. The part of the peptide that binds the outer area in the catalytic pocket of the enzymes is thought to be responsible for the selectivity in the binding to the different HDACs. Then, it could be possible to find specific inhibitors for each HDAC by changing the amino acids in the CHAPs. These substances have anticancer properties in murine xenografs, but no studies in humans have been reported (Furumai *et al.* 2001).

PXD101 (compound **20** in Fig.10) and NVP-LAQ824 (compound **21** in Fig.10) are hydroxamic acids developed by Topotarget Prolifix and Novartis, respectively, in the last few years (Remiszewski *et al.* 2003, Plumb *et al.* 2003). At the moment, both are under phase I clinical trials (Monneret 2005).

Tubacin (compound **22** in Fig.10) is a specific inhibitor for HDAC6, found in a screening of several thousand chemicals. Cell treatment with this substance results in accumulation of acetylated α-tubulin and inhibition of cell motility without altering the cell cycle progression (Haggarty *et al.* 2003). No data on animal models or clinical trials are reported.

Probably in the next few years several other hydroxamic acids with HDAC inhibitory activity will enter on clinical trials as cancer chemotherapy because many research groups are still working on the design, synthesis and study of the effects of these compounds.

3.3.2.2. Carboxylic acids

The compounds in this group are short-chain carboxylic acids: butanoic acid, phenylbutanoic acid and valproic acid (2-propylpentanoic acid). These substances are reversible HDACs inhibitors, but much less potent than hydroxamic acids. First, the carboxylic group, which is supposed to chelate the metal cation in the catalytic pocket of the HDACs, has less affinity for Zn(II) than the hydroxamic acid has. Second, the molecules are smaller than the hydroxamic acid with inhibitory capacity, so the surface for the interaction with the enzyme is smaller. As result, the

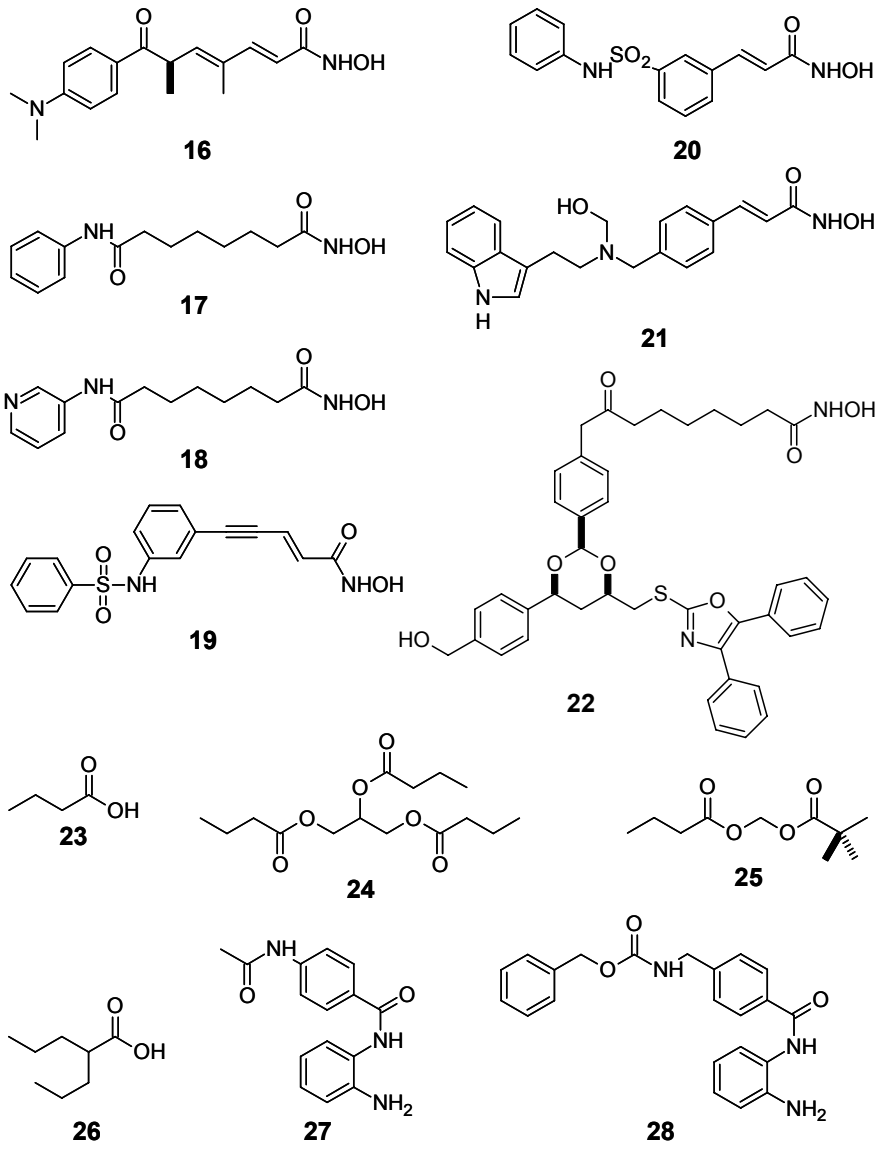


Fig.10: Structure of some Zn(II)-dependent HDACs inhibitors. Trichostatin A (**16**), suberoylanilide hydroxamic acid (SAHA, **17**), suberoyl-3-aminopyridineamide hydroxamic acid (pyroxamide, **18**), oxamflatin (**19**), PXD101 (**20**), NVP-LAQ824 (**21**) and tubacin (**22**) are hydroxamic acids. Butanoic acid (**23**), its prodrug froms tributanoylglycerol (**24**) and pivaloyloymethyl butanoate (**25**) are carboxylic acids, as well as 2-propylpentanoic acid (valproic acid, **26**). N-acetyldinaline (also named CI-994, compound **27**) and MS-275 (**28**) are benzamides.

interactions enzyme-inhibitor are not so strong and, to inhibit the HDACs *in vitro*, the required concentrations of these chemicals are about 3 orders of magnitude higher than the required concentrations of hydroxamic acids described in 3.3.2.1.

These compounds have pleiotropic effects because of their interaction in the cell with macromolecules other than HDACs. For instance, valproic acid is an ion channel blocker used to treat epilepsy and butanoic acid stimulates Ca(II) release (Göttlicher *et al.* 2001, Hamodeh *et al.* 2004). Despite being much less specific and less potent HDACs inhibitors than hydroxamic acids, butanoic, phenylbutanoic and valproic acids are currently at different stages of clinical trials for their use as anticancer agents, either alone or in combination with other substances (reviewed in Monneret 2005).

Butanoic acid (compound **23** in Fig.10) is known to induce histone hyperacetylation by inhibiting the HDACs since 1978 (Vidali *et al.* 1978; Candido *et al.* 1978). This aliphatic acid has a very low solubility in water at neutral pH, is rapidly degraded *in vivo* and it has to be administered in multigram doses for efficiency. To overcome these disadvantages, different prodrugs with better bioavailability, like tributyrin (tributanoylglycerol, compound **24** in Fig.10) and *Pivanex®* (AN-9, pivaloyloxymethyl butanoate, compound **25** in Fig.10), have been developed. All of them are undergoing clinical trials as cancer chemotherapy (reviewed in Monneret 2005).

Valproic acid (compound **26** in Fig.10) is an anticonvulsant drug with undesirable consequences, like teratogenicity. The research on these side effects provoked the discovery of the HDAC inhibitory capacity of the chemical (Göttlicher *et al.* 2001, Phiel *et al.* 2001). Additionally, in some cell lines (murine teratocarcinoma F9, human erythroleukemia K562, human embrionary kidney HEK293T and others), valproic acid increases the rate of HDAC2 protein degradation trough the ubiquitin proteasome pathway. This fact contributes to increase histone acetylation (Krämer *et al.* 2003) and suggests that valproic acid can be a good treatment for the cancers in which HDAC2 is overexpressed (Zhu *et al.* 2004). This compound is currently on clinical trials against some malignancies (reviewed in Monneret 2005).

3.3.2.3. Benzamides

This family of compounds complexes Zn(II) in the catalytic pocket of the HDACs through their *N*-(2'-aminophenyl) benzamide moiety. This functional group is bulkier than the hydroxamic or the carboxylic acids, and, as result, benzamides do not inhibit HDACs whose catalytic pocket is narrow, for instance, HDAC8 (Vannini *et al.* 2004). Nevertheless, these chemicals inhibit in a reversible way some Zn(II)-dependent HDACs and induce histone hyperacetylation in cultured cells (Kraker *et al.* 2003, Saito *et al.* 1999).

N-acetyldinaline (also known as CI-994, compound **27** in Fig.10) was identified already in 1993 as a substance with cytostatic and antileukemic effects on rat models, but its HDAC inhibitory properties were not identified until 2003 (el-Beltagi *et al.* 1993, Kraker *et al.* 2003).

MS-275 (also known as MS-27-275, compound **28** in Fig.10), found in a secreening for compounds with HDAC inhibitory properties, also induces accumulation of hyperacetylated histones *in vivo* (Saito *et al.* 1999).

Both *N*-acetyldinaline and MS-275 are on clinical trials as treatments for different tumors, either alone or in combination with other drugs. Due to their poor solubility in water, both substances require oral administration (reviewed in Monneret 2005).

Other compounds with similar structure to MS-275 and *N*-acetyldinaline have been synthesized. Some of them have succeeded in inhibiting HDACs *in vitro*, in cultured cells and in murine models, also stopping the growth of cancer cells, but no data about clinical trials have been reported (Fournel *et al.* 2002).

3.3.2.4. Epoxides

In contrast to the HDACs inhibitors mentioned before, epoxides inhibit HDACs in an irreversible way, probably because the epoxide functionality reacts with an amino-acid residue of the enzyme yielding a covalent bond which links the inhibitor to the HDAC. Epoxides are very powerful inhibitors *in vitro*, but they lack any activity in cells and *in vivo*, very likely due to the lability of the epoxide group. Therefore, none of them has undergone clinical trials (reviewed in Monneret 2005).

Trapoxin A (*cyclo*-(*L*-phenylalanyl-*L*-phenylalanyl-*D*-pipecolinyl-*L*-2-amino-8-

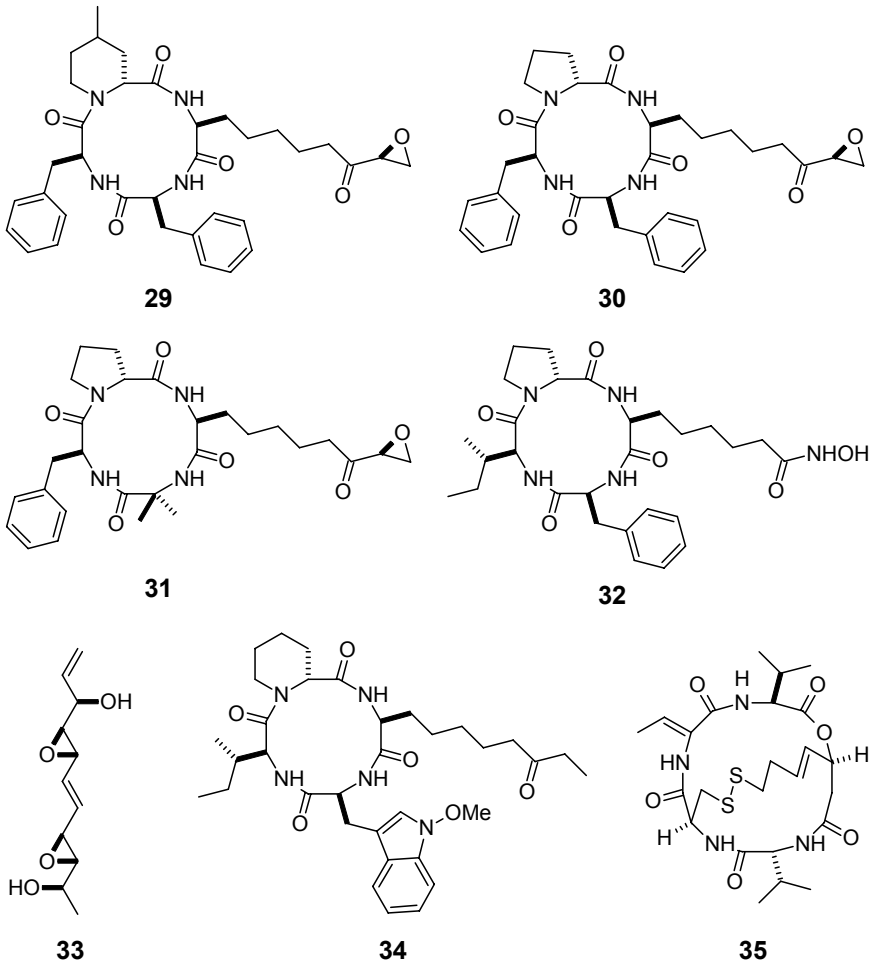


Fig.11: Structure of some Zn(II)-dependent HDACs inhibitors. Trapoxin A (**29**), trapoxin B (**30**) and chlamydocin (**31**) are natural cyclic peptides containing an epoxide functionality. The design of the cyclic-hydroxamic acid containing peptide CHAP30 (**32**) is based on the structure of trapoxins. Depudecin (**33**) is a natural compound with two epoxide rings. Apicidin (**34**) is a cyclic peptide containing an ethylketone. Depsipeptide FK228 (**35**) is another natural product.

oxo-9,10-epoxy-decanoyl), compound **29** in Fig. 11), trapoxin B (compound **30** in Fig. 11), HC-toxin and chlamidocyn (compound **31** in Fig. 11) are natural products with a cyclic peptide structure. The HDAC inhibitory properties of trapoxin A *in vitro* and in cultured cells were already described in 1993 (Kijima *et al.* 1993). The structure of these compounds has been taken as model for the design of novel HDACs inhibitors, the CHAPs (see 3.3.2.1).

Depudecin (compound **33** in Fig.11) is a substance produced by the fungus *Alternaria brassicicola*. Its effects on the phenotype of transformed cells were already described in 1992, but its HDACs inhibitory capacity was not discovered until 1998 (Sugita *et al.* 1992, Kwon *et al.* 1998).

3.3.2.5. Other inhibitors of Zn(II)-dependent histone deacetylases

Other compounds that bind Zn(II) through functional groups different than hydroxamic acid, carboxylic acid and epoxide also inhibit HDACs. For instance, apicidin (compound **34** in Fig.11), apicidin A, apicidin C and apicidin D_1 are cyclic peptides that chelate Zn(II) through an ethylketone functionality. All the four substances inhibit HDACs *in vitro* and in cultured cells (Singh *et al.* 2002).

Depsipeptide FK228 (also known as FR901228, compound **35** in Fig.11) is a cyclic antibiotic produced by *Chromobacterium violaceum*, found in a screening for antitumor compounds (Ueda *et al.* 1994). This chemical is a potent HDAC inhibitor (Nakajima *et al.* 1998). Once in the cell, the disulfide bridge in the molecule is reduced and one of the resulting thiol groups is able to interact with the cation Zn(II) in the HDAC active site (Furumai *et al.* 2002). The anticancer properties of this depsipeptide are currently evaluated on several phase I and II clinical trials.

3.3.3 Inhibitors of NAD^+-dependent histone deacetylases

The screening for inhibitors of NAD^+-dependent HDACs led to the discovery of splitomicin (1,2-dihydro-3H-naphtho[2,1-b]pyran-3-one, compound **36** in Fig.12). Based on the structure of that compound, other inhibitors of sirtuins have been designed but the effects of these substances have been analysed only *in vitro* and in yeast, not in higher eukaryotic cells (Bedalov *et al.* 2001, Posakony *et al.* 2004).

3.3.4. Histone acetyltransferase inhibitors

It is known that CBP and p300 act as transcriptional activators of some oncogenes (reviewed in Lund and van Louhizen 2004). Additionally, in some types of leukemia, chimera proteins produced by translocations cause an abnormal

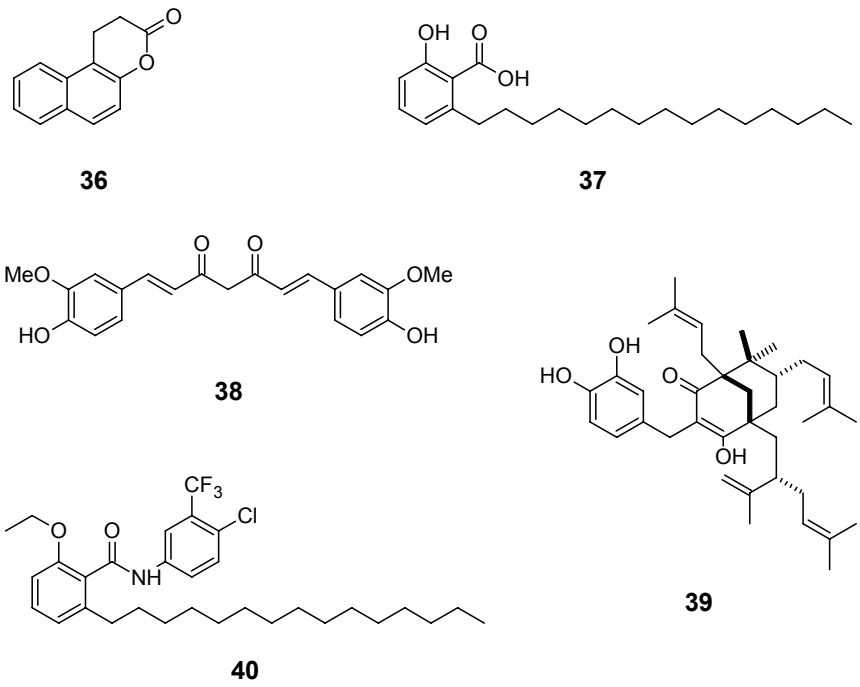


Fig.12: Other substances that alter the activity of epigenetics-related enzymes: splitomicin (**36**) is an inhibitor of sirtuins; anacardic acid (**37**), curcumin (**38**) and garcinol (**39**) inhibit HATs and *N*-(4-chloro-3-trifluoromethylphenyl)-2-ethoxy-6-pentadecyl-benzamide (**40**) activates selectively p300.

recruitment of the HATs which may contribute to tumor development (Marmorstein 2002). So the inhibition of HATs may have anticancer properties. There are a few chemicals with known HAT inhibitory activity and the effects of some of them have been analysed only *in vitro*.

Complexes and salts of Cu(II) induce apoptosis in hepatoma cells (Kang *et al.* 2005). This effect is thought to be a direct consequence of the inhibition of the HAT activity (Kang *et al.* 2004).

Anacardic acid (2-hydroxy-6-pentadecylbenzoic acid, compound **37** in Fig.12) is obtained by hydrogenation of a mixture of insaturated compounds extracted from cashew nut shell. *In vitro*, this substance inhibits both p300 and

PCAF, but no studies in cultured cells or *in vivo* have been reported (Balasubramanyam *et al.* 2003).

Curcumin ((*E,E*)-1,7-bis(4-hydroxy-3-methoxyphenyl)-1,6-heptadiene-3,5-dione, compound **38** in Fig.12) is a natural compound that inhibits p300 and CBP, but also other enzymes, like HIV-1 integrase or the thioredoxin reductase (Balasubramanyam *et al.* 2004, Fang *et al.* 2005). In cultured cells, it stops cell proliferation and causes apoptosis. It is thought that curcumin has also chemopreventive properties (reviewed in Dorai and Aggarwal 2004). Indeed, it is undergoing clinical trials for colon cancer prevention and advanced pancreas tumors treatment. However, if the benefitial effects of this chemical are due to HAT inhibition or to other mechanisms is not known.

Garcinol (compound **39** in Fig.12) is a chemical found in the fruit of *Garcinia Indica*. It inhibits *in vitro* p300 and CBP acetylation of histones in a competitive way, and, in cultured cells, it also represses histone acetylation, causing apoptosis (Balasubramanyam *et al.* 2004b). Garcinol has cancer chemopreventive effects in murine cancer models (Yoshida *et al.* 2005).

Finally, mimics of acetyl CoA have been designed to selectively inhibit HATs. The first of these compounds, named Lys-CoA, is not absorbed by the cells, so it has no activity *in vivo*. The second generation has been tested only *in vitro* (Cebrat *et al.* 2003).

3.3.5. Histone acetyltransferase activators

Activation of HATs may lead also to increased histone acetylation and hypothetically, it can rescue the transcription of tumor-suppressor genes silenced by histone hypoacetylation.

The compound *N*-(4-chloro-3-trifluoromethylphenyl)-2-ethoxy-6-pentadecyl-benzamide (CTBP, compound **40** in Fig.12), a derivative of anacardic acid, enhances p300 but no PCAF activity *in vitro*. No results of experiments in cultured cells or mice have been reported (Balasubramanyam *et al.* 2003).

3.3.6. Other substances altering epigenetic mechanisms

The increase in the knowledge about the epigenetic mechanisms and their deregulation in cancer cells will surely provide in the near future new targets for the design of antitumor drugs. Chemicals interfering with mechanisms that silence tumor-suppressor genes may cause their reactivation. On the other hand, compounds that inhibit the transcription of oncogenes and retrotransposones may also inhibit cancer cell proliferation. Alternatively, the alteration of the functioning of epigenetic machineries can lead to cell growth arrest and/or dead by avoiding normal chromatin condensation and decondensation along the cell cycle.

3.3.7. Synergy of different drugs

Sometimes, the combination of chemicals interfering with different cellular functions results in the enhancement of their antitumor effect. The epigenetic drugs collaborate with different substances to trigger the growth arrest, differentiation and/or apoptosis of cancer cells.

The synergy of DNMTs inhibitors and HDACs inhibitors has been widely studied. The treatment with both types of substances allows the reduction of the dose of both, decreases undesirable side effects and increases cancer cells death and growth arrest. Because of that, simultaneous treatment of a diverse number of malignancies with DNMTs and HDACs inhibitors is currently under evaluation on clinical trials (reviewed in Feinberg and Tycko 2004, Laird 2005). In some cases, inhibition of DNMTs activity is not enough to recover the expression of some genes with hypermethylated promoters. In such a situation, simultaneous treatment with both demethylating agents and substances that induce histone hyperacetylation restores the expression of the genes (Cameron *et al.* 1999). It is also reported that the combination of DNMTs inhibitors and HDACs inhibitors increases histone hyperacetylation respect to the treatment with only HDACs inhihibitors (Zhu *et al.* 2001).

The combination of HDACs inhibitors and topoisomerase II inhibitors is also currently studied on clinical trials. In cultured cells, the pre-treatment with TSA enhances the toxicity of etoposide, doxorubicin, cisplatin and ellipticine. It is thought

that the opening of the chromatin structure upon HDAC inhibition facilitates the access of topoisomerase inhibitors to their targets, favoring their action (Kim *et al.* 2003b). Also combinations of DNMTs inhibitors and topoisomerase-II inhibitors are explored, for instance DHAC and cisplatin (Samuels *et al.* 1998).

Another example is the synergy of all-trans retinoic acid (ATRA) and HDACs inhibitors for the treatment of leukemias with translocations of the *RAR*α gene. In these cases, the resulting fusion proteins abnormally recruit HDACs to certain promoters. Both ATRA and the HDAC inhibitor are supposed to collaborate to restore the transcription of those genes (Côté *et al.* 2002).

4. Objectives

The regulation of epigenetic mechanisms is a promising target for development of cancer chemotherapy. Large effort of researchers and pharmaceutical companies has been dedicated to the design of antitumor drugs which interfere with the aberrant epigenetic patterns. However, the chemicals tested to now have also undesired side effects that limit their use. Mutagenicity, induction of chromosomal instability in normal cells and toxicity for liver and other organs, among other effects reduce or abolish the therapeutic application of these substances. Because of that, we were interested on searching for novel compounds with fewer side effects. These chemicals should specifically activate the expression of tumor-suppressor genes silenced by aberrant epigenetic mechanisms. Our aim was to explore the effects of two types of drugs, compounds that reduce DNA methylation and HDACs inhibitors, in the same model system, the breast cancer cell line MCF7.

Study of procaine as a DNA-demethylating agent

Procaine (4-aminobenzoic acid 2-diethylaminoethyl ester, compound **15** in Fig.9) is a local anesthetic used in humans. The structural similarity of procaine and procainamide (4-aminobenzoic acid 2-diethylaminoethylamide, compound **14** in Fig.9) led us to hypothesize that procaine could also influence DNA methylation. To prove this hypothesis, procaine treatments were performed on MCF7 breast cancer cells. Except otherwise indicated, treatments were 3 days long to permit the cells go through at least one replication round after the drug uptake, and so allowing the passive demethylation to occur (see 3.1.2.2). The monitored parameters were:

1. Procaine-induced changes on global DNA methylation, including dependence of the effects on drug concentration and treatment duration.
2. Procaine-induced changes on DNA methylation at the hypermethylated promoter of a tumor-suppressor gene and if these changes correspond with reexpression of the gene.
3. Procaine-induced alterations in cell proliferation, since anticancer drugs should have cytostatic and/or cytotoxic effects on tumor cells.

Comparative study of seven Zn(II)- dependent HDACs inhibitors

The hydroxamic acids CY and CX were developed at pharmaceutical companies by combining molecular modelling and high-throughput screening of chemical libraries. When we started this project, no data about the cellular response to these compounds was published. Thus, the comparison of the different effects of these compounds with the effects of well characterized HDACs inhibitors (TSA, SAHA, MS-275 butanoic acid and valproic acid) was addressed by analysing:

1. *In vitro* inhibition of HDAC activity.
2. Effects in cell proliferation after 24 h treatments.
3. HDACs inhibition in living cells, evaluated indirectly by determining the increase of histone hyperacetylation after 24 h-treatmets.
4. Alterations in the cellular concentration of HDAC1 and HDAC2 enzymes after 24 h of drug treatment, since it may contribute to changes in histone acetylation.
5. Analysis of transcriptional regulation of different genes after 24 h HDACs inhibitor treatment, including the possible correlation with alterations in histone post-translational modifications and HDACs recruitment at their promoters.

5. Materials and Methods

5.1. Solutions

Except otherwise indicated, all the solutions were made with Milli-Q water and kept at room temperature. Commercial reagents and solutions were stored according the manufacturer's instructions. Whenever employed, protease inhibitors (*Complete® Protease Inhibitor Cocktail EDTA Free,* Roche) and DTT (Sigma) were freshly added.

Below there is a list of the solutions and buffers home-prepared, ranked by alphabetical order:

0.1 M acetic acid	glacial acetic acid (Merck) diluted in water.
1% acetic acid	1% (v/v) glacial acetic acid in water.
10 M ammonium acetate	ammonium acetate (Sigma) dissolved in water.
4.0 M ammonium sulfate	$(NH_4)_2SO_4$ (Sigma) dissolved in water and pH adjusted to 7.9 with NaOH solution.
10% APS	10% (w/v) ammonium persulfate (Bio-Rad) in water, stored at 4ºC.
10 mg/mL BSA	bovine serum albumine (Sigma) dissolved in water, aliquoted and stored at –20ºC.
Cell fixation solution	formaldehyde saturated solution in water (Sigma, concentration c.a. 37% (w/v)) was ten-fold diluted in PBS. Final concentration formaldehyde c.a. 4% (w/v).
Cell permeabilization solution	0.1% (v/v) *Triton® X-100* (Sigma) in PBS.
ChIP dilution buffer	0.01% (w/v) SDS (Merck) , 1.1% (v/v) *Triton® X-100*, 1.2 mM EDTA (Sigma), 16.7 mM Tris (Merck) pH=8.1, 167 mM NaCl (Merck), 1 tablet

	Complete® Protease Inhibitor Cocktail EDTA Free in each 50 mL.
ChIP elution buffer	1% (w/v) SDS, 0.1 M $NaHCO_3$ (Sigma).
ChIP high salt immune complex wash buffer	0.1% (w/v) SDS, 1% (v/v) *Triton® X-100*, 2.0 mM EDTA, 20 mM Tris pH=8.1, 0.50 M NaCl.
ChIP LiCl immune complex wash buffer	1% (v/v) *Igepal® CA-630* (indistinguishable from *NonidetP-40®*, Sigma), 1% (w/v) sodium deoxycolate (Sigma), 1.0 mM EDTA, 10 mM Tris pH=8.1.
ChIP low salt immune complex wash buffer	0.1% (w/v) SDS, 1% (v/v) *Triton® X-100*, 2.0 mM EDTA, 20 mM Tris pH=8.1, 0.15 M NaCl.
ChIP SDS lysis buffer	1% (w/v) SDS, 10 mM EDTA, 50 mM Tris pH=8.1, 1 tablet *Complete® Protease Inhibitor Cocktail EDTA Free* in each 50 mL.
1 μg/mL DAPI	DAPI (Sigma) was dissolved in methanol (Merck) at 1 mg/mL concentration. The resulting solution was 1000-fold diluted with water and stored at 4ºC in the darkness.
6 x DNA loading buffer	30 % (v/v) glycerol (Sigma) in water containing 0.05% (w/v) bromophenol blue (Bio-Rad).
DNA lysis-cell buffer	0.65 M sucrose (Sigma), 20 mM Tris pH=7.8, 10 mM $MgCl_2$ (Merck), 2% (v/v) *Triton® X-100*. Storage at 4ºC.
DNA proteinase K buffer	50 mM Tris pH=8.5, 10 mM EDTA pH=8, 100 mM NaCl, 1% SDS.
2 mM dNTP mixture	prepared by mixing equimolar amounts of 100 mM dATP, 100 mM dTTP, 100 mM dGTP, 100 mM dCTP (Promega) and diluting the resulting solution 12.5-fold (final concentration, 2 mM each dNTP) with water. The mixture was aliquoted and stored at –20ºC.
0.5 M EDTA	EDTA (Sigma) dissolved in water, NaOH solution was added to adjust pH to 8.0.
70% ethanol	70% (v/v) absolute ethanol (Merck) in water.
75% ethanol	75% (v/v) absolute ethanol in DEPC-treated water.
2.5 M glycine	glycine (2-aminoetanoic acid, Fluka) dissolved in water.

10 mg/mL glycogen	commercial solution 20 mg/mL glycogen (Roche) was diluted 1:1 with water, aliquoted and stored at –20ºC.
HDAC activity buffer	25 mM Tris pH 7.5, 10% (v/v) glycerol, 1 mM EDTA, 50 mM NaCl.
HDAC quenching solution	1.0 M HCl and 0.4 M acetic acid in water.
0.25 M hydrochloric acid	37% (w/v) aqueous HCl (Merck) solution (saturated solution) 40-fold diluted in water.
16 mM hydroquinone	hydroquinone (Sigma) dissolved in water. Prepared inmediatly before use and protected from light.
IP buffer	20 mM HEPES (Sigma) pH=7.5, 100 mM KCl (Merck), 1.0 mM $MgCl_2$, 0.05% *Igepal® CA-630*.
80% isopropanol	80% (v/v) 2-propanol (Merck) in water.
4 x Laemmli buffer	0.20 M Tris pH=6.8, 8% (w/v) SDS, 40% (v/v) glycerol, 0.4 M DTT, 0.001% (w/v) bromophenol blue (BioRad).
10% NP-40	10% (v/v) *Igepal® CA-630* (Sigma, indistinguishable from *Nonidet-P40®*) in water.
Nuclear extract buffer A	10 mM Tris pH=7.5, 15 mM KCl, 2.0 mM $MgCl_2$, 0.1 mM EDTA, 1.0 mM DTT, 1 tablet *Complete® Protease Inhibitor Cocktail EDTA Free* in each 50 mL buffer.
Nuclear extract buffer B	50 mM Tris pH=7.5, 1.0 M KCl, 30 mM $MgCl_2$, 0.1 m EDTA, 1.0 mM DTT, 1 tablet *Complete® Protease Inhibitor Cocktail EDTA Free* in each 50 mL buffer.
Nuclear extract buffer C	20 mM Tris pH=7.5, 10% (v/v) glycerol, 2.5 mM $MgCl_2$, 1.0 mM EGTA disodium salt (Sigma), 0.5 mM DTT, 1 tablet *Complete® Protease Inhibitor Cocktail EDTA Free* in each 50 mL buffer.
200 U/mL nuclease P1	lyophilised nuclease P1 (Sigma) dissolved in water, aliquoted and stored at –20ºC.
0.1 mM oligonucleotides	lyophilised primers (Sigma-Genosys) were dissolved in the adequate volume of water (aliquots stored at –20ºC). Whenever necessary, dilutions 1:10 in water were prepared. For sequences, see Tables 2-5.
PBS	137 mM NaCl, 2.7 mM KCl, 10 mM Na_2HPO_4 (Sigma), 2.0 mM KH_2PO_4 (Sigma), pH=7.4.

0.1% Ponceau S	0.1% (w/v) Ponceau S (Sigma) in 5% (v/v) aqueous acetic acid.
2.5 M potassium chloride	KCl (Merck) dissolved in water.
1 mg/mL propidium iodide	propidium iodide (Sigma) solution in water, aliquoted and stored at 4ºC in the darkness.
Protein extract buffer	50 mM Tris pH=7.5, 120 mM NaCl, 0.5 mM EDTA, 0.5 % (v/v) *Igepal® CA-630* and 1 tablet *Complete® Protease Inhibitor Cocktail EDTA Free* EDTA-Free in each 50 mL buffer.
10 mg/mL proteinase K	proteinase K (Sigma) solution in water, aliquoted and stored at –20ºC.
10 mg/mL RNase A	RNase A (Sigma) solution in water, aliquoted and stored at –20ºC.
RSB buffer	10 mM Tris pH=7.5, 10 mM NaCl, 3 mM $MgCl_2$, 1 tablet *Complete® Protease Inhibitor Cocktail EDTA Free* in each 50 mL buffer.
10% SDS	10% (w/v) sodium dodecylsulfate (Merck) in water.
SDS-PAGE resolving gel	acrilamide/bisacrilamide from commercial solution 40% acrilamide/bisacrilamide 37.5:1 in water (Bio-Rad), 0.38 M Tris pH=8.8, 0.1% (w/v) SDS, 0.04% (v/v) TMEDA (Sigma), 0.1% (w/v) APS. The amount of acrilamide/bisacrilamide solution was adjusted to get a final concentration ranging from 7.5% to 15% (w/v) acrilamide in the gel, depending on the aim of the separation.
SDS-PAGE running buffer	26 mM Tris, 0.19 M glycine, 0.1% (w/v) SDS in distilled water, pH=8.6 (approximately).
SDS-PAGE stacking gel	5% acrilamide/bisacrilamide 37.5:1 (from the same commercial solution as in SDS-PAGE resolving gel), 0.125 M Tris pH=6.8, 0.1% (w/v) SDS, 0.1% (v/v) TMEDA and 0.1% (v/v) APS.
Sodium bisulfite solution	sodium metabisulfite ($Na_2S_2O_5$, Sigma) was dissolved in water, pH was adjusted at 5.0 with concentrated aqueous NaOH and finally water was added to get a solution 4.0 M sodium bisulfite ($NaHSO_3$). Solution was prepared inmediatly before use.
5.0 M sodium chloride	NaCl (Merk) dissolved in water.
3 M sodium hydroxide	NaOH (Merck) dissolved in water.

3.0 M sodium acetate	sodium acetate (Sigma) solution in water.
0.5 x TBE	0.044 M Tris, 0.044 M boric acid, 1.0 mM EDTA in distilled water, pH=8.3 (approximately).
TE	10 mM Tris, 1.0 mM EDTA, pH=8.0.
Transfer buffer	0.19 M glycine, 0.025 M Tris, 15% methanol in distilled water. For histones, also 0.02% (w/v) SDS.
1.5 M Tris pH=8.8	Tris base (Merck) dissolved in water, pH adjusted with hydrochloric acid.
1.0 M Tris pH=7.5	Tris base dissolved in water, pH adjusted with hydrochloric acid.
1.0 M Tris pH=6.8	Tris base dissolved in water, pH adjusted with hydrochloric acid.
20 mM Tris pH=6.8	1.0 M Tris pH=6.8, 50-fold diluted in water.
WB blocking buffer 1	5% non-fat powder milk (Nestlé) in WB wash buffer 1.
WB blocking buffer 2	5% non-fat powder milk in WB wash buffer 2.
WB wash buffer 1	0.1% (v/v) *Tween® 20* (Sigma) in PBS.
WB wash buffer 2 (NET buffer)	50 mM Tris pH=7.5, 5.0 mM EDTA, 150 mM NaCl, 0.25% (w/v) gelatin (Merck), 0.05% (v/v) *Igepal® CA-630*; pH adjusted to 7.4.
10 mM zinc (II) sulfate	$ZnSO_4$ (Sigma) solution in water.

5.2. Cell culture reagents and basic methods

The human breast cancer cell line MCF7 (American Type Culture Cell Collection) was grown in DMEM 4.5 g/L glucose (Invitrogen) supplemented with 10% fetal bovine serum (Invitrogen), 0.25 μg/mL amphotericine B (Invitrogen), 50 U/mL penicillin and 50 μg/mL streptomycin (Invitrogen) under an atmosphere saturated of water and containing 5% CO_2. For detachment, medium was aspirated from the dish, cells were washed once with PBS and then incubated with trypsin solution containing EDTA (Sigma) for 3 min. Then, cells were pipetted to disgregate clumps

and diluted with fresh medium.Splitting was done every 3 days and each time cells were 6 to 9 fold diluted.

5.3. Drug treatments

All the dishes needed for each experiment were plated at the same density and in parallel. Twenty-four hours after seeding, cells were washed with PBS and fresh medium containing the desired final concentration of drug was added. All the drug stock solutions (see Table 2) were filtered through 0.2 μm pore for sterilization, aliquoted and kept at –20ºC. Stock solutions were ten-fold diluted with water if necessary. Controls were done by adding only the corresponding solvent to the cells (vehicle).

When serum starving was necessary as control, a dish of cells was maintained in medium with no FBS supplement in parallel with the drug-treated dishes.

Table 2: Drugs stock solutions

Drug	**Supplier**	**Solvent**	**C/mM[(1)]**
procaine	Sigma	water	270
procainamide	Sigma	water	270
5-aza-2'-deoxycytidine	Sigma	water	1.0
butanoic acid	Merck	PBS	1000
valproic acid	Sigma	PBS	1000
MS-275	Calbiochem	DMSO	1.0
trichostatin A	Sigma	ethanol/water 1:9	1.0
SAHA	Galchimia S.A.	DMSO	5.0
CX	Kindly provided by xxxxxxxxxxx[(2)]	DMSO/water 1:19	3.8
CY	Kindly provided by yyyyyyyyyyy[(2)]	water	1.0

(1) Concentration of the stock solution.
(2) Compounds CX and CY were gifts from pharmaceutical companies.

5.4. Cell growth assay

For this assay, ice-cold PBS and cell fixation solution are necessary.

After treatment, cells cultured in 6-well dishes ($0.5\text{-}1.0 \cdot 10^6$ cells) were washed with PBS, detached with trypsin and harvested in 1.5 mL tubes by centrifugation ($16 \cdot 10^3$ RCF for 10 s). Pellets were washed once with cold PBS, then resuspended in cell fixation solution and stored at 4ºC until the moment of the analysis. In each experiment, number of cells per millilitre was counted for two different aliquots per sample by using a Neubauer chamber. The result was the calculated average. When necessary, cell suspensions were diluted with PBS.

5.5. Flow cytometry

The solutions required for this analysis were: ice-cold PBS, ice-cold absolute ethanol, 10 mg/mL RNase and 1 mg/mL propidium iodide.

After treatment, cells cultured in 6-well dishes ($0.5\text{-}1.0 \cdot 10^6$ cells) were washed once with PBS, detached with trypsin, harvested in 1.5 mL tubes by centrifugation and washed twice with cold PBS. For fixation, pellets were resuspended in 0.33 mL ice-cold PBS and, under gentle shaking, 0.66 mL cold ethanol were added dropwise. Cells were stored at 4ºC for a time between 0.5 h and one week. One or two hours before the analysis, samples were washed once with PBS and resuspended in 0.5 mL PBS containing 5 μL RNase A solution. Immediately before introducing the cells in the cytometer, DNA was stained by adding 15 μL of propidium iodide solution to the cell suspension. All the centrifugations were done at $16 \cdot 10^3$ RCF for 10 s.

Distribution of cell population was analysed in a *FACScalibur® Flow Cytometry System* (Beckton Dickinson) at low cell flow. For each histogram, 10000 events were counted. The resulting data were processed with *Modfit® LT MAC* (Beckton Dickinson) software.

5.6. TUNEL assay

Analysis of fluorescence-labelled samples was done by Dr. Jesús Espada. Mounting was done according to the published procedure (Espada *et al.* 2005). The following solutions and reagents were employed: PBS, cell fixation solution, cell permeabilization solution, TUNEL label mix (Roche), TUNEL enzyme (Roche), 1 μg/mL DAPI, 70% ethanol, absolute ethanol, *p*-xylene (1,4-dimethylbenzene, Merck) and DePeX (Servan).

Briefly, after treatment, cells grown on 22 mm^2 microscope coverslips were washed 3 times with PBS. Coverslips were submerged in cell fixation solution at 37ºC during 30 min. After one wash with PBS, samples were placed under cell permeabilization solution for 15 min at room temperature and then washed 3 times with PBS. Coverslips were placed upside down on 20 μL of mixture 1:10 TUNEL enzyme/TUNEL label mix solutions and incubated for 1 h at 37ºC. Then, glasses were rinsed twice with PBS, incubated during 10 min at room temperature with DAPI solution and washed 3 times with water. Finally, cells were dehydrated with the following sequence: one wash with 70% ethanol, one wash with absolute ethanol and a last wash with *p*-xylene. Coverslips were carefully drained and mounted onto microscope slides by usin a drop of DePeX.

Preparations were examined in a fluorescence microscope. For the detection of fluorescein (TUNEL), excitation at 450-500 nm and detection at 515-565 nm were employed and for DAPI, excitation at 340-380 nm and detection with a 430 nm low-pass filter (max DAPI emission: 452 nm).

5.7. Polymerase chain reaction (PCR)

Depending on the sequence to be amplified, either *FastStart® Taq* (Roche) or *EcoTaq®* (Ecogen) was used as DNA-polymerase. In general, a master mix was prepared containing the indicated amount per sample of the following reagents:

10 x DNA-polymerase reaction buffer (see below) 2.5 μL,

CG-RICH® solution (see below) 5.0 μL,

50 mM $MgCl_2$ (only if 10 x polymerase buffer does not contain Mg(II))	0.75 μL,
2 mM dNTP mixture	2.5 μL,
0.1 mM oligonucleotide (sense and antisene)	equimolar amounts (1 to 50 pmole),
Taq DNA-polymerase	0.5 U,
water	the necessary to have in each reaction tube 25 μL final volume, including the template solution.

The 10 x DNA-polymerase reaction buffers were supplied by the DNA-polymerase's manufacturers. The buffer corresponding to *FastStart® Taq* contains the adequate concentration of Mg(II) for the activity of the enzyme, so reactions with this polymerase were not supplemented with $MgCl_2$ solution.

The addition of 5 x *CG-RICH®* solution (Roche) to the reaction mixture was required to get the expected product when amplifying certain sequences (indicated in Tables 4-6).

The master mix was dispensed in thin-walled 0.2 mL tubes and the adequate amount of each DNA sample (template) was added to the corresponding tube. A blank reaction with no template was also run in parallel, as control for possible contaminations.

Except other indication, PCR reactions had a first step of denaturation (5 min at 95ºC), 30 to 40 cycles (30 s at 95ºC, 30 s at the annealing temperature, 30 s at 72 ºC) and a final extension step (7 min at 72ºC).

In Tables 3-6, PCR reaction conditions can be found for each pair of primers (concentration, number of cycles, annealing temperature and presence of *CG-RICH®* solution). Other details are given in the corresponding paragraphs of this section (methylation-specific PCR, bisulfite genome sequencing, RT-PCR, etc.).

5.8. DNA agarose gels

The following reagents were used: agarose *LM-sieve* (Pronadisa), 0.5 x TBE, 10 g/L ethidium bromide solution in water (Sigma), 6 x DNA loading buffer.

Agarose was dissolved in hot TBE, ethidium bromide was added to 0.1 μg/mL final concentration and the solution was poured on the casting tank. Usually, 2% gels (2.0 g agarose in each 100 mL TBE) were prepared.

Samples were mixed with the corresponding volume of 6x DNA loading buffer, loaded and gels were run in 0.5x TBE at 6 V/cm. Bands were visualized under 254 nm light. Molecular weight markers encompassing the size of the expected product (PCR or restriction-enzyme digestion) were used.

5.9. DNA extraction

The reagents used to isolate DNA from cultured cells were: DNA lysis-cell buffer, DNA proteinase K buffer, 10 mg/mL proteinase K, 10 mg/mL RNase A, *Light Phase Lock Gel®* (PLG) tubes (Eppendorf), phenol solution saturated with TE (Sigma), phenol/chloroform/isoamyl alcohol 25:24:1, saturated with TE (Sigma), 3.0 M sodium acetate, absolute ethanol and 70% ethanol.

Cells ($2 \cdot 10^6$ to $6 \cdot 10^6$) were washed once with PBS, detached and collected by centrifugation (387 RCF, 5 min, 4ºC). Next, cytoplasms were lysated: cell pellets were resuspended in 2 mL PBS, each suspension was mixed with 2 mL DNA lysis-cell buffer and incubated on ice during 10 min. After centrifugation (387 RCF, 5 min, 4ºC), the extracts rich in nuclei were washed once with PBS and resuspended in 0.5 mL DNA proteinase K buffer. RNase A solution (10 μL) and proteinase K solution (2.0 μL) were added and samples were incubated at 37ºC o/n. The resulting viscous crudes were emulsionated with 0.5 mL saturated phenol in PLG tubes, then rotated at room temperature for 15 min, centrifuged ($16 \cdot 10^3$ RCF, 5 min, room temperature) and the aqueous layers were carefully transferred to clean PLG tubes. The same washing procedure was repeated with 0.5 mL phenol/chloroform solution. DNA was precipitated from the last aqueous solution by addition of 50 μL sodium acetate solution and 1.5 mL ethanol. The tubes were inverted several times, centrifuged ($16 \cdot 10^3$ RCF, 2 min, room temperature) and the pellets were washed twice with 70% ethanol, air-dried and redissolved in water (10 to 20 μL).

DNA concentrations in the final solution (typically 1.0 to 4.0 μg/μL) were determined by measuring their absorbance at 260 nm (ε=20 (cm·μg/μL)$^{-1}$). The presence of contaminants (protein and/or phenol residues) was estimated by measuring the ratios A_{260}/A_{220} (usual values 2.0-2.2) and A_{260}/A_{280} (usual values 1.6-1.8).

5.10. Quantitation of the genomic 5-methylcytosine DNA content by HPCE

5.10.1. DNA hydrolysis

The next solutions and reagents were necessary: 200 U/mL nuclease P1, 10 mM zinc (II) sulfate, 50 U/mL alkaline phosphatase suspension (Sigma) and 1.5 M Tris pH=8.8.

According to the method described in Fraga *et al.* 2002, aliquots of DNA solutions (5 μL) containing 1-5 μg of highly pure DNA were heated for 3 min in boiling water bath and immediately cooled in ice. Then samples were digested o/n at 37ºC with 1.25 μL nuclease P1 solution in the presence of zinc (II) sulfate (0.75 μL stock solution). Finally, 1.0 μL commercial suspension of alkaline phosphatase and 1.0 μL Tris were added, reaction mixtures were incubated at 37ºC for 2 h, and stored at 4ºC.

5.10.2. Analysis of the nucleoside mixture by HPCE

The analysis was performed by Dr. Mario F. Fraga according to the method described in Fraga *et al.* 2002. Briefly, an uncoated fused-silica capillary (Beckman-Coulter; 60.2 cm x 75 μm; effective length 50 cm) was used in a capillary electrophoresis system (*P/ACE® MDQ*, Beckman-Coulter) connected to a data-processing station (32 Karat software). The running buffer was 14 mM $NaHCO_3$ pH=9.6 containing 20 mM SDS. Running conditions were 25 ºC with an operating

voltage of 17 kV and on-column absorbance was monitored at 254 nm. Before each run, the capillary system was conditioned by washing with 0.1 M sodium hydroxide for 3 min and was equilibrated with the running buffer for 3 min. Hydrolyzed DNA samples were injected under pressure (0.3 psi) for 3 s. Buffers and samples were filtered through 0.45 μm pore filters. All the samples were analysed in duplicate and three analytical measurements were made per replicate. The relative methylation of each DNA sample was taken as the percentage of 5-methyl-2'-deoxycytidine in total cytydine: mdC peak area x 100/(mdC peak area + dC peak area).

5.11. Analysis of the genomic 5-methylcytosine DNA content by DNA digestion with methylation-sensitive restriction enzymes.

Restriction enzymes MspI (20 U/μL), HpaII (10 U/μL) and McrBC (10 U/μL); the corresponding concentrated reaction buffers 10 x, 100 mM GTP solution and 10 mg/mL BSA solution were purchased from New England Biolabs and stored at –20ºC. 6 x DNA loading buffer and a gel 1% (w/v) agarose *NuSieve 3.1* (BioWhittaker) in TBE 0.5 x containing 0.1 μg/mL ethidium bromide were prepared as described in section 5.8.

For each reaction, 1 μg DNA mixed with 50 units of enzyme and 5 μL of the corresponding concentrated buffer (*buffer 2* for MspI and McrBC, *buffer 1* for HpaII) in 50 μL final volume was incubated at 37ºC during 3 h. The reaction of McrBC was supplemented wit 50 nmole GTP (0.5 μL stock solution) and 5 μg BSA (0.5 μL stock solution). Controls were performed incubating in parallel 1 μg DNA with 5 μL *buffer 2* in 50 μL final volume. After digestion, enzymes were inactivated by heating (65ºC, 20 min), crudes were mixed with 6x loading buffer and 5 μL were run in a 1% agarose *NuSieve* gel.

5.12. Analysis of DNA methylation at specific promoters

To analyse the changes in cytosine methylation at specific sequences, genomic DNA samples are modified with bisulfite. When DNA is incubated in the presence of bisulfite at acid pH, cytosine residues are deaminated to uracil, but, if the cytosine ring is methylated in the 5' position, the reaction is so slow that it is possible to stop it after conversion of the non-methylated cytosine moieties and before the transformation of the methylated ones (Wang *et al.* 1980). This selective modification allows the analysis of cytosine methylation at specific DNA sequences by different techniques, some of them based on PCR, since uracil residues will be amplified as thymines whereas the methylated cytosines will remain as cytosine in the PCR product (see Fig.14).

Two of these PCR-base techniques were employed in this work: methylation-specific PCR (MSP) and genomic bisulfite sequencing. Since the strands of dsDNA are not fully complementary anymore after cytosine deamination, primers must be designed to amplify selectively one of both strands (see scheme in Fig.14). MSP provides information about the methylation status of the cytosines encompassed by the oligonucleotides but bisulfite genomic sequence describes the methylation of cytosines in the sequence between the primers (Fig.14).

For MSP analysis of a given DNA sequence, two PCRs per sample are necessary: one with primers specific to amplify the methylated allele and the other one with primers specific to amplify the unmethylated one. Each oligonucleotide of theses two pairs encompasses at least one CpG dinucleotide. The pair of primers designed to amplify the methylated allele only match with the target sequence if all the cytosines in the encompassed CpG dinucleotides are methylated and, because of that have not been deaminated in the bisulfite reaction. The pair of primers designed to amplify the unmethylated allele only match with the target sequence if all the CpG dinuclotides are non methylated and so have become uracil after the bisulfite treatment (see Fig.14). PCR product is usually a very short fragment (typically 50 to 150 bp). MSP is a highly sensitivite technique for the detection of small proportion (as low as 0.1%) of methylated (or unmethylated) alleles in the sample (Herman *et al*. 1996).

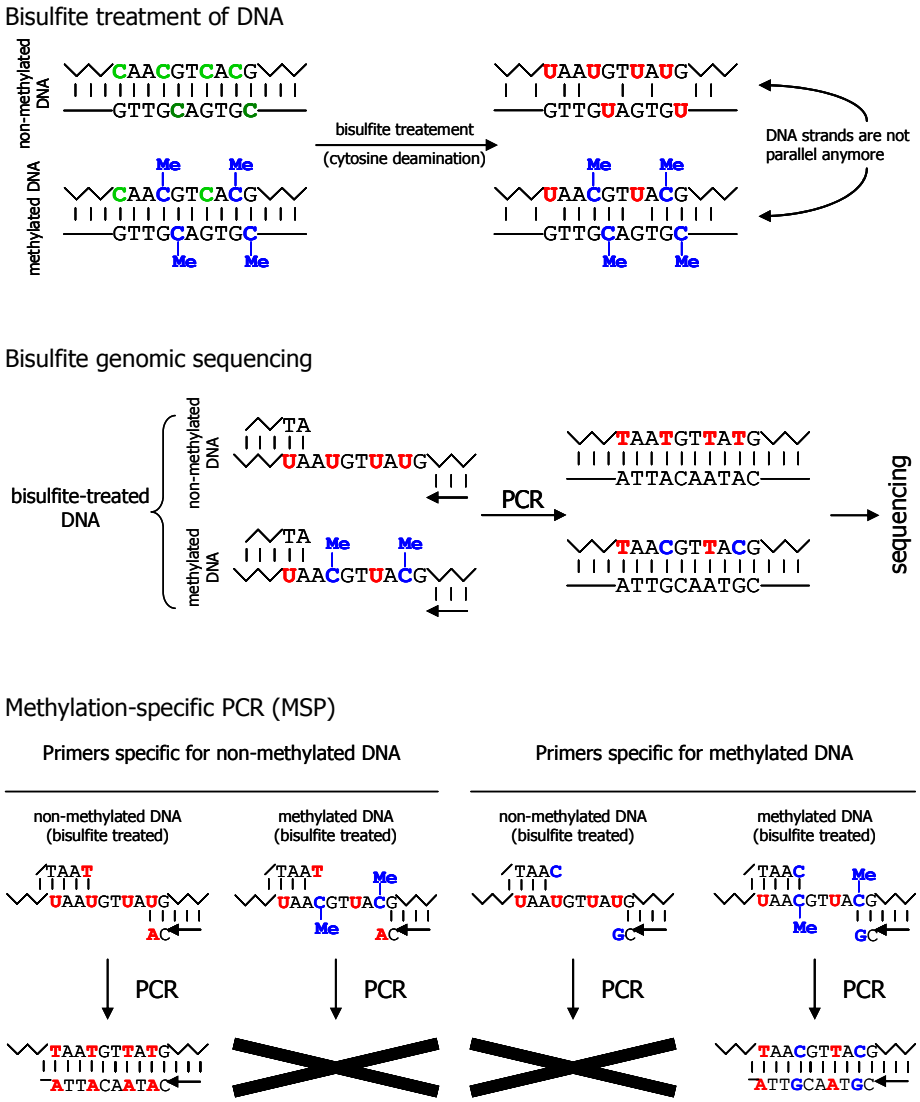


Fig.14: Scheme showing the steps necessary for MSP and bisulfite genomic sequencing. Upper panel: bisulfite treatment of DNA is employed to deaminate selectively non-methylated cytosine rings. After the reaction, DNA strands are not fully complementary anymore. Middle panel: bisulfite genomic sequencing. Primers are designed to amplify the target sequence on one of both DNA strands independently of the methylation status. PCR products are sequenced. Lower panel: MSP. Oligos are designed to discriminate between the methylated and the non-methylated forms of the target sequence.

In contrast, for bisulfite sequencing both methylated and unmethylated alleles are amplified in the same PCR reaction. Thus, only one pair of oligonucleotides is required and the primers should not encompass any CpG. When this is not possible, degenerated oligonucleotides considering both the presence and absence of methylation must be used to avoid a possible bias in the experiment (Frommer *et al.* 1992). This is the case of the primers BS-RARβ2-S3 and BS-RARβ2-AS3 in Table 4. PCR products are sequenced. If these PCR products are cloned before sequencing, several colonies must be analysed in order to have an overview of the population. Then, each sequencing gel or electropherogram shows that there is only cytosine or tymine in each cytosine position of the original DNA, depending on the methylation status for that specific residue. If PCR products are not cloned and the sample is not from a homogeneous population of cells, the sequencing gel or the electropherogram shows that each cytosine position is occupied by a mixture of cytosine and tymine, in a proportion according to the percentage of methylation for each particular residue in the original sample.

5.12.1. Bisulfite treatment

DNA was modified with sodium bisulfite as described by Herman *et al.* in 1996, by using the next solutions: sodium bisulfite solution, 3 M sodium hydroxide and 16 mM hydroquinone. To recover DNA after the reaction, *Wizard® DNA Clean-Up System* (Promega), 80% isopropanol, 10 mg/mL glycogen solution, 10 M ammonium acetate, ice-cold absolute ethanol and ice-cold 70% ethanol were necessary.

For each sample, 1 μg genomic DNA was diluted up to 50 μL and was incubated with sodium hydroxide (5.7 μL of 3 M solution) at 37ºC for 10 min. Then hydroquinone (33 μL of 16 mM solution) and sodium bisulfite (0.53 mL of 4.0 M solution) were added and samples were heated o/n at 50ºC.

DNA was desalted and concentrated by using the kit *Wizard® DNA Clean-Up System*: each crude was mixed with 1 mL of DNA-binding resin and the suspension was transferred to a column. A vacuum manifold was used to drain supernatants and washing solutions. After removal of supernatant, resin was washed twice with 1.0 mL

80% isopropanol and the residual solvent was eliminated by centrifuging ($16 \cdot 10^3$ RCF, 2 min, room temperature) the column placed onto a 1.5 mL tube. For DNA recovery, the column was transferred to a clean tube, 50 μL of preheated (50ºC) water were applied on top of the resin and, after 2 min, DNA was eluted by centrifugation ($16 \cdot 10^3$ RCF, 1 min, room temperature).

Once desalted an concentrated, DNA was desulfonated with sodium hydroxide (5.7 mL of 3.0 M solution) for 20 min at 37ºC. Immediately after, DNA was precipitated by addition of 1 μL glycogen solution, 17 μL ammonium acetate (10 M) and 0.22 mL cold absolute ethanol and keeping the resultant mixture at –80ºC for 30 min-2 h. Tubes were centrifuged ($4.5 \cdot 10^3$ g, 20 min, 4ºC), pellets were washed once with cold 70% ethanol and air dried. DNA was redissolved in 25 μL of water (final concentration 40 ng/μL approximately).

5.12.2. Methylation-specific PCR

For each sample, two parallel PCR reactions were carried: one with specific primers for the methylated promoter (labelled as *RARβ2*-M-S and *RARβ2*-M-AS in Table 3) and another one with primers for the corresponding non-methylated allele (*RARβ2*-U-S and *RARβ2*-U-AS in Table 3). In both cases, 40 ng of bisulfite-treated DNA were amplified with *FastStart® Taq* DNA-polymerase system as described in section 5.7, with no addition of *CG-RICH®* solution. Details are shown in Table 3. Products were separated in a standard agarose gel and visualized with ethidium bromide staining.

5.12.3. Bisulfite genomic sequencing

In this analysis, 80 ng of bisulfite-treated DNA were amplified with *FastStart® Taq* DNA-polymerase system in 50 μL final volum, with the primers and conditions described in Table 4.

After PCR, reaction crudes were run in a 2% (w/v) agarose gel (prepared as described in 5.8) and bands with the products were excised under 254 nm light. DNA was extracted from the bands either with *QIAquick® Gel Extraction Kit* (Qiagen) or by freezing the band, centrifuging ($16 \cdot 10^3$ RCF, 10 min, room temperature) it and

purifying the DNA from the supernatant employing *E.Z.N.A.® Cycle-Pure Kit* (Omega Bio-Tek). Both kits were used according to the manufacturer's protocols. Pure PCR products were sequenced at the Unidad de Análisis Genómico (C.N.I.O., Madrid, Spain), by using as primers the same oligonucleotides that were employed for the PCR reaction.

5.13. RNA isolation

Several reagents and solutions were required for RNA isolation: PBS, *TRIzol®* (Invitrogen), chloroform (Merck), isopropanol (Merck), 75% ethanol in DEPC-treated water, DEPC treated water, RNase-Free DNase Set (Quiagen) and *RNeasy® Mini Kit* (Qiagen).

Cell dishes (containing $0.5 \cdot 10^6$ to $2 \cdot 10^6$ cells) were washed twice with PBS, then *TRIzol®* was added (40 μL per 1 cm^2 of dish surface), cells were detached with a scrapper, transferred to sterilised 1.5 mL tubes and incubated at room temperature for 5 min. Next, chloroform (0.2 mL per 1 mL *TRIzol®*) was emulsionated with the resulting lysates, shaking (vortex) during 5 min at room temperature. After centrifugation ($13 \cdot 10^3$ RCF, 15 min, 4ºC), the aqueous layers (0.5 mL per 1 mL *TRIzol®*) were placed in clean 1.5 mL tubes. RNA was precipitated by adding one volume isopropanol to the aqueous solution, mixing by inversion and incubating at room temperature during 10 min. Finally, tubes were centrifuged ($13 \cdot 10^3$ RCF, 20 min, 4ºC), pellets were washed once with 75% ethanol (centrifugation at $5.2 \cdot 10^3$ RCF, 5 min, 4ºC) and allowed to air-dry. To dissolve RNA, 50 μL DEPC-treated water were added to each pellet and, after that, tubes were heated at 65ºC for 5 min. Aliquots of 10 μL were taken for concentration and quality analysis and the remaning 40 μL were immediately stored at –80ºC.

RNA concentrations in the final solution (typically 1.0 to 3.0 μg/μL) were determined by measuring the absorbance of the solutions at 260 nm (ε=25 $(cm \cdot \mu g/\mu L)^{-1}$). The presence of contaminants (protein and/or phenol residues) was estimated by measuring the ratios A_{260} / A_{220} (usual values 2.0 - 2.2) and A_{260} / A_{280}

Table 3: Primers employed for MSP

Gene	Primer name	Sequence	C/μM (1)	Cycles	T_a/°C (2)
RARβ2,methylated +105 to +256	Rarβ-M-S	TGTCGAGAACGCGAGCGATTC	2.0	35	58
	Rarβ-M-AS	CGACCAATCCAACCGAAACGA			
RARβ2,unmethylted +99 to +262	Rarβ-U-S	TTGGGATGTTGAGAATGTGAGTGA$(T)_3$			
	Rarβ-U-AS	CTTACTCAACCAATCCAACC$(A)_4$CAA			

(1) Final concentration in PCR reaction; (2) annealing temperature. Oligos taken from Esteller *et al.*. 2002.

Table 4: Primers employed for bisulfite genomic sequencing

Gene (1)	Primer name	Sequence (2)	C/μM (3)	Cycles	T_a/°C (4)	CG-rich (5)
RARβ2 (6) -282 to +64	BS-RARβ2-S2	GTGAGTTAGGAGTAGYGTTTYGG$(T)_9$	1.0	40	48	no
	BS-RARβ2-AS2	CTTTAAC$(A)_6$TAAACCCTCCTAA				
RARβ2 (6) -197 to +249	BS-RARβ2-S3	AAAAYGTYGGTTTGTGYGTTYGTTG	1.0	40	54	no
	BS-RARβ2-AS3	ATCCAACCRAAACR$(A)_5$TTCCC$(A)_6$				
CDKN1A -222 to +94	BS-p21-S	ATTGGGGGAGGAGGGAAGT	0.8	40	55	yes
	BS-p21-AS	CTCACCTCCTCTAAATACCTC				
GADD45β -303 to -31	BS-gadd45-S	GTTGTYGGGAAATTAGGAGAAA	1.0	40	50	yes
	BS-gadd45-AS	CTAAACTAAAAACCTCCRACAC				
IGFBP3 (7) -282 to +191	BS-igfbp3-S	TGTTGAGGTGGTTTGGAGTGT	1.0	40	55	yes
	BS-igfbp3-AS	CRAAACAACACCAACAAAATCA				
JUND -296 to +27	BS-junD-S	GGGYGGGYGTTTGGTGGT	0.8	40	55	yes
	BS-junD-AS	CACTTATACRCCCTCTTATAACC				
MT1X -322 to -102	BS-mt1L-S	GGGGGTTGGGGGTGTGTAGAT	1.0	40	60	yes
	BS-mt1L-AS	TCCTTACACCCRCCCCTACTAAAT				
MT2A -457 to -196	BS-mt2a-S	AGTTTTGGTTGTTATAGAGTTGTAA	1.0	40	50	yes
	BS-mt2a-AS	ATCAATTCCCTAAAAACRACC				

(1) Gene symbol and region encompassed by the oligonucleotides relative to the transcription start; (2) some of the oligos are degenerated. In that case, Y represents C or T and R represents A or G; (3) final concentration in PCR reaction; (4) annealing temperature; (5) presence of CG-rich solution in the reaction; (6) primers taken from Arapshian *et al.* (2000); (7) primers from Fraga *et al.* 2004.

(usual values 1.6-1.8). Also 4 μL per sample were run for 20 min in 1% (w/v) agarose gel to visualize the integrity of RNA.

For analysis of *JunD* expression (*JunD* is a monoexonic gene), 20-30 μg RNA per sample were brought up to 100 μL, treated with RNase-free DNase and recovered from the digestion with *RNeasy® Mini Kit*. With this procedure, near 30% of the starting material was lost. Both digestion and purification with *RNeasy® Mini Kit* were done according to manufacturer's instructions.

5.14. Reverse transcriptase PCR

Semiquantitative RT-PCR was performed to study the alterations in the transcription of certain genes after drug treatment. Two different protocols were employed: the "one-step protocol", to analyse the transcription of a single gene (*RARβ2*) for all the samples, and the "two-step protocol", to detect changes in the expression of several genes in the same set of samples.

5.14.1. One step protocol

In this case, 0.01 mM oligonucleotides sense and antisense for *RARβ2* and *β-actin* mRNA were used, as well as *Enhanced Avian RT-PCR kit* (Sigma) containing the following solutions: 10x PCR buffer, 25 mM $MgCl_2$, dNTP mix (10 mM each dNTP), 20 U/μL RNase inhibitor, 20 U/μL enhanced avian myeloblastosis virus reverse transcriptase and 5 U/μL *AccuTaq® LA* DNA-polymerase.

In a similar way as for simple PCR, a master mix was prepared containing (per reaction): 2.5 μL 10 x PCR buffer, 1.5 μL 25 mM $MgCl_2$, 0.5 μL dNTP mix, 0.5 μL RNase inhibitor, 1.0 μL each primer solution, 0.5 μL enhanced avian myeloblastosis virus reverse transcriptase, 0.25 μL *AccuTaq® LA* DNA-polymerase. DEPC-treated water was added to adjust the final volume of the reaction to 25 μL. The mix was dispensed in the 0.2 mL thin-wall reaction tubes containing the RNA samples (12 ng each). Tubes were placed in the thermocycler. First, tubes were incubated at 50ºC during 45 min for cDNA synthesis. Then, PCR cycling was performed to amplify the

cDNA: denaturation (3 min at 95ºC), followed by 5 cycles with annealing temperature 58ºC (30 s at 95ºC, 30 s at 58ºC, 1 min at 72ºC), 30 cycles with annealing temperature 51ºC (30 s at 95ºC, 30 s at 51ºC, 1 min at 72ºC) and a final extension at 72ºC during 5 min. As for genomic DNA amplification, a tube with master mix but no template was the negative control.

Products were visualized in 2% (w/v) agarose gel with ethidium bromide staining, as usually.

5.14.2. Two-step protocol

For the first step, consisting in the cDNA synthesis, *ThermoScript® RT-PCR System for First Strand cDNA Synthesis* (Invitrogen) was employed. The kit contains 50 μM oligo(dT)$_{20}$, 10 mM dNTP mix, 5 x buffer, 0.1 M DTT, 40 U/μL *RNaseOUT®*, 15 U/μL *ThermoScript®* reverse transcriptase, 2 U/μL RNase H, DEPC-treated water. For each sample/blank, in a clean thin-walled 0.2 mL tube, 2.0 μL oligo(dT)$_{20}$ solution, 4.0 μL dNTP mix, 4 μg total RNA and DEPC-treated water up to 24 μL were mixed. Then RNA was denatured by heating at 65ºC for 5 min followed by quick cooling on ice bath. A master mix containing the indicated amounts of reagents per sample was made: 8.0 μL 5 x buffer, 2.0 μL DTT, 2.0 μL *RNaseOUT®*, 2.0 μL DEPC-treated water and 2.0 μL reverse transcriptase. After dispensing 16 μL master mix in each 0.2 mL tube, reaction coktails were incubated in a termocycler for 1 h at 50ºC and then for 5 min at 85ºC to inactivate the enzymes. Once tubes were at room temperature, RNA was digested with RNase H (1.0 μL commercial solution per sample) for 20 min at 37ºC.

The second step, the amplification of the resulting cDNAs, was carried as standard PCR with *EcoTaq®* DNA-polymerase in presence of 1.5 mM $MgCl_2$. Reaction conditions (including the amount of cDNA used as template) for each pair of oligonucleotides were searched by amplifying different quantities of cDNA from a positive control and chosing the ones that better reflected changes in template concentration. Primers and other details are specified in Table 5. After amplification, 10 μL each crude were mixed with 2 μL 6 x loading buffer and run in 2% (w/v) agarose gel.

Table 5: Primers for RT-PCR

Gene and product size (1)	Primer name	Sequence	C/μM (2)	Cycles	T_a/ºC (3)	CG-rich (4)
Rarβ2 (5) 288 bp (33 kbp)	RT-RARb-S RT-RARb-AS	AGAGTTTGATGGAGTTGGGTGGAC GACGAGTTCCTCAGAGCTGGTG	0.4	SEE TEXT (One step RT-PCR protocol)		
β-actin (6) 400 bp (1.0 kbp)	RT-actin-S RT-actin-AS	ACCATGGATGATATCG ACATGGCTGGGGTGTTGAAG	0.4			
CDKN1A 70 bp (1.2 kbp)	RT-p21-S RT-p21-AS	GGCAGACCAGCATGACAGATT GGATTAGGGCTTCCTCTTGGAG	0.4	33	50	yes
GADD45β 320 bp (0.8 kbp)	RT-gadd45-S RT-gadd45-AS	TCGGATTTTGCAATTTCTCC TGTCACAGCAGAAGGACTGG	0.08	30	60	no
IGFBP3 (7) 196 bp (3.5 kbp)	RT-igfbp3-S RT-igfbp3-AS	GTCAACGCTAGTGCCGTCAG CATGCCCTTTCTTGATGAT	0.4	31	60	no
JunD 342 bp (0.3 kbp)	RT-junD-S RT-junD-AS	GTGCCCAGGAACTCAGAGAG CGTTTGTTCCCCGAGTAGAA	0.08	35	60	no
MT1X (8) 45 bp (0.6 kbp)	RT-mt1L-S RT-mt1L-AS	GCTCGCCTGTTGGCTCC TCTGACGTCCCTTTGCAGATG	0.08	30	60	no
MT2A 314 bp (1.4 kbp)	RT-mt2a-S RT-mt2a-AS	CAACCTGTCCCGACTCTAGC ATAGCAAACGGTCACGGTCA	0.08	30	60	no
GAPDH 372 bp (2.6 kbp)	RT-gadph-S RT-gadph-AS	TCTTCTTTTGCGTCGCCAG AGCCCCAGCCTTCTCCA	0.04	30	57	no

(1) The expected size for the amplification of contaminant DNA is shown in brackets; (2) final concentration in PCR reaction; (3) annealing temperature; (4) presence of CG-rich solution in the reaction; (5) primers taken from Bovenzi *et al.*. 2001; (6) primers taken from Sirchia *et al.*. 2000; (7) primers from Fraga *et al.*. 2004 (8) primers taken from Glaser *et al.*. 2003.

5.15. Chromatin immunoprecipitation

To investigate the modification status of histones at the promoter of candidate genes, standard ChIP assays were performed as previously described (Gregory *et al.*, 2002) in cells with or without CY (1 μM) treatment. To crosslink the macromolecules, 37% formaldehyde in water (Sigma), 2.5 M glycine and ice-cold PBS containing 1 tablet *Complete® Protease Inhibitor Cocktail EDTA Free* (Roche) in each 50 mL were used. Chromatin was released with ChIP SDS lysis buffer. For the immunoprecipitation, several reagents were required: ChIP dilution buffer, salmon

sperm DNA/protein A agarose slurry 50% (Upstate), antibodies (see Table 7), ChIP low salt immune complex wash buffer, ChIP high salt immune complex wash buffer, ChIP LiCl immune complex wash buffer, TE and ChIP elution buffer. Finally, DNA was isolated from the immunoprecipitated by using 5.0 M NaCl, 0.5 M EDTA, 1.0 M Tris pH=6.8, 10 mg/mL proteinase K, phenol/chloroform/isoamyl alcohol 25:24:1 solution, 3.0 M sodium acetate, 70% (v/v) ethanol, 10 mg/mL glycogen and isopropanol. The scheme of the procedure is shown in Fig.15.

First, chromatin was crosslinked by adding formaldehyde directly to the medium of cell dishes containing $3\text{-}6\cdot10^6$ cells. Final concentration of formaldehyde was 1% in the case of using anti-histone antibodies and 2% if anti-HDAC antibodies were employed. After incubating for 15 min at room temperature in a rocking platform, excess of aldehyde was quenched by adding glycine to a final concentration 0.125 M. The medium was removed after 5 min and dishes were washed twice with cold PBS containing *Complete® Protease Inhibitor Cocktail EDTA Free*. Cells were scrapped, collected in conical tubes by centrifugation (690 RCF, 5 min, 4ºC) and decanted.

For chromatin release, cells were resuspended in cold ChIP SDS lysis buffer (0.2 mL per million cells) and incubated on ice for 10 min. Next, lysates were divided in 0.3 mL aliquots (in tubes 1.5 mL) and sonicated during 10 s (output 3, 90% duty cycler in a *Sonifier 250*, Branson) to shear DNA in fragments of size about 0.5 kbp. Aliquots were pooled.

In order to balance the concentration of material in the samples, two aliquots of 4 μL were taken from each pool, diluted 100-fold with water and absorbances at 260 nm were measured. Then pools were diluted approximately 10-fold with cold ChIP dilution buffer, in such a way that the concentration of starting material (and A_{260}) was the same for all the samples. From each one of these dilutions, a 2.0 mL aliquot was stored at –20ºC to use as "input" control. The rest of the fractions were incubated (rotating wheel, 30 min, 4ºC) with protein A agarose beads blocked with salmon sperm (80 μL slurry for each 2.0 mL diluted lysated), to eliminate the material that can bind inespecifically the resin. Agarose beads were pelleted (690 RCF, 4 min, 4ºC) and each solution was divided in several 2.0 mL aliquots, one for each antibody and one to use as "no antibody" control (this one will be processed in parallel with the rest). Antibodies (5 μg each) were added to the

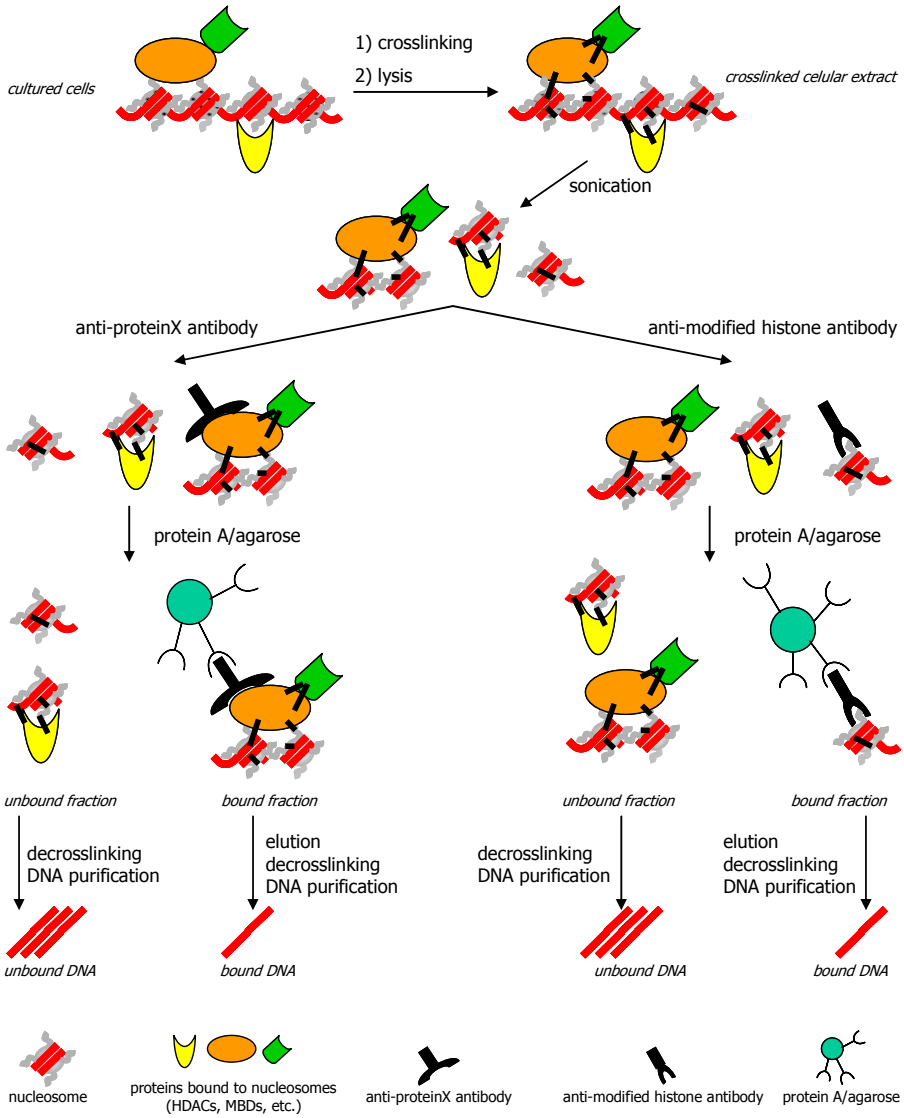


Fig.15: Scheme of the ChIP technique. Cultured cells (whose chromatin is representd in the upper left corner) are first treated with formaldehyde and then lysated to release the crosslnked chromatin (black thick lines represent the covalent bonds arising from the crosslinking). Then, DNA is fragmented by sonication and the resulting mixture is aliquoted. Each aliquot is incubated with a different antibody recognizing either proteins bound to chromatin (left side) or histone modifications (right side) and the fraction bound to the antibody is immunoprecipitated with protein A/agarose beads. Supernatant ("unbound fraction") is separated and kept. Chromatin is eluted from the beads ("bound fraction") and DNA is purified from both bound and unbound fractions after decrosslinking. The presence or absence of a certain sequence in each fraction is determinated by PCR using adequate pimers.

corresponding aliquots and all the tubes (except for the "input") were rotated at 4ºC o/n. Then 60 μL salmon sperm DNA/ protein A agarose beads were added to each tube and incubated with rotation at 4ºC during 1 h. The resin (now containing the immunoprecipitate) was collected by centrifugation (90 RCF, 1 min, 4ºC) and supernatants, which constitute the "unbound" fractions, were saved.Beads were washed sequentially with the following solutions (1.0 mL each) in a rotating platform:

one 5 min wash with 1.0 mL low salt immune complex wash buffer,

one 5 min wash with 1.0 mL high salt immune complex wash buffer,

one 5 min wash with 1.0 mL LiCl immune complex wash buffer,

two 5 min wash with 1.0 mL TE buffer.

To remove supernatants, resin was pelleted as indicated above (90 RCF, 1 min, 4ºC).

Antibody-chromatin complexes were eluted with two washes (15 min each) with 0.25 mL ChIP elution buffer. Both eluates were combined in the called "bound" fractions.

DNA was released by heating at 65ºC for 4 h 0.5 mL aliquots of each sample (including "input", "unbound" and "bound" fractions) mixed 20 μL 5 M NaCl. After addition of 10 μL 0.5 M EDTA and 20 μL 1.0 M Tris pH=6.8 to each aliquot, proteins were digested with 2 μL proteinase K solution for 1 h at 45ºC. The resulting solutions were washed twice by emulsionating with 1 vol phenol/chloroform/isoamyl alcohol and separating phases by centrifuging ($16 \cdot 10^3$ RCF, 5 min, room temperature). DNA was precipitated from the aqueous layer with 0.1 vol sodium acetate, 1.0 μL glycogen solution and 0.7 vol isopropanol, incubating at –20ºC o/n. Finally, tubes were centrifuged ($13 \cdot 10^3$ RCF, 15 min, 4ºC), pellets were washed once with 70% ethanol and, after evaporation of the residual ethanol, DNA was dissolved in 25 μL water.

Average size of the fragments was estimated by running 5 μL input DNA in a 2% agarose gel. DNA concentration in each fraction was determined by absorbance at 260 nm (dilutions 10- or 100-fold in water) and DNA purity was estimated with the ratios A_{260}/A_{220} (usual values 2.0-2.5) and A_{260}/A_{280} (usual values 1.6-1.8).

PCR amplifications were performed as indicated in section 5.7 with specific primers for each of the analysed promoters (see Table 6) and either *EcoTaq®* or *FastStart® Taq* as DNA-polymerase. The sensitivity of amplification was evaluated on PCR amplifications were performed as indicated above with specific primers serial dilutions of input DNA. The amount of template for reaction depended on the amplified sequence (5 to 20 ng).

Table 6: Primers for ChIP

Gene (1)	Primer name	Sequence	C/μM (2)	Cycles	T_a/ºC (3)	CG-rich (4)
CDKN1A -468 to -249	ChIP-p21-S ChIP-p21-AS	GCAGAGAGGTGCATCGTTT ACATTTCCCCACGAAGTGAG	0.8	35	60	no
GADD45β -112 to +61	ChIP-gadd45-S ChIP-gadd45-AS	CCAGAAAAGGCGGAAAAATA AATAGGAGGGGCGAATGACT	0.8	35	55	yes
IGFBP3 (5) -89 to +87	ChIP-igfbp3-S ChIP-igfbp3-AS	CTCCCAACCCCCACTCCT GGATGGGGCGACAGTACAC	0.8	35	55	yes
JUND -230 to +25	ChIP-junD-S ChIP-junD-AS	GGGAGATCGGTCGTACACA CTTGTGCGCCCTCTTATAGC	0.8	35	60	yes
MT1X -213 to +5	ChIP-mt1L-S ChIP-mt1L-AS	CACGTACTGCCAGGTTCTCA AGATGAAAAGCGTGGTGGAG	0.8	35	60	yes
MT2A -961 to -784	ChIP-mt2a-S ChIP-mt2a-AS	TTCCAGTGTTCCCGCTAGTT CAAGCTTGGTCCTCAGGAAG	0.8	35	60	yes

(1) Gene symbol and region encompassed by the oligonucleotides relative to the transcription start; (2) final concentration in PCR reaction; (3) annealing temperature; (4) presence of CG-rich solution in the reaction; (5) primers from Fraga *et al.*. 2004.

5.16. *In vitro* HDAC inhibitory activity assay

5.16.1. Preparation of active nuclear extracts

The necessary materials for this preparation are: PBS, nuclear extract buffer A, nuclear extract buffer B, nuclear extract buffer C, 4.0 M ammonium sulfate and

dialysis tube *Servapor ® Dialysis Tubing* 16 mm 10000 MWCO (Servan). All buffers and solutions were cooled at 4ºC before use.

Exponentially growing MCF7 cells (15 to $18 \cdot 10^6$) were collected in a 15 mL tube by centrifugation (110 RCF, 5 min, 4ºC) and washed with PBS. Pellet was resuspended in a mixture of 10 mL nuclear extract buffer A and 0.68 mL nuclear extract buffer B. Cells were centrifuged again (689 RCF, 5 min, 4ºC), washed once with 10 mL nuclear extract buffer A and finally resuspended in 4 mL nuclear extract buffer A. Cytoplasms were broken with a dounce homogenizer (15 strokes), 0.3 mL nuclear extract buffer B were added and suspension was stroken 5 more times. After centrifugation ($7.7 \cdot 10^3$ RCF, 8 min, 4ºC), pellet was suspended in 4 mL nuclear extract buffer A and stroken 5 additional times. Nuclei were transferred to a centrifuge tube, 0.4 mL ammonium sulfate solution were added and the mixture was rotated at 4ºC for 30 min. Solution was cleared by centrifugation ($12 \cdot 10^3$ RCF, 30 min, 4ºC). The resulting supernatant was dialyzed against 250 mL nuclear extract buffer C during 2.5 h at 4ºC. Aliquots of 0.4 mL were made and immediatly frozen at –80ºC.

5.16.2. HDACs immunoprecipitation from active nuclear extracts

The reagents employed to immunoprecipitate active HDACs from the nuclear extracts prepared as described in 5.16.1 were: 2.5 M KCl, IP buffer, antibodies anti-HDAC1 and anti-HDAC5 (Upstate, see Table 7), protein A sepharose 50% suspension (Upstate), protein G sepharose 50% suspension (Upstate), 10 mg/mL BSA and HDAC activity buffer.

For this experiment, the required volume of nuclear extract is 0.28 mL for each planned in vitro reaction (including the negative control), i.e., for 10 reactions, 2.8 mL are necessary.

First, ionic strength in the nuclear extract was increased with KCl (12 μL KCl solution per 0.28 mL extract). The pool was separated in 3 aliquots: the first and the second (for 5 reactions each one) were rotated for 1 h at 4ºC with 40 μg anti-HDAC1 and anti-HDAC5 antibodies, respectively. The third one, the negative control (only

one reaction, i.e., 0.29 mL), was brought up to 0.30 mL with IP buffer and incubated in parallel with the others.

In the meantime, 0.33 mL protein A sepharose slurry was mixed with 0.22 mL protein G sepharose. Beads were centrifuged ($16 \cdot 10^3$ RCF, 1 min, 4ºC) and blocked with 2 mL IP buffer containing 300 μg BSA (30 min at 4ºC in a rotating platform). The pool of beads was divided into 3 aliquots, one containing 0.18 mL suspension and the other two, 0.91 mL each one.

After centrifugation ($16 \cdot 10^3$ RCF, 1 min, 4ºC), supernatant of the beads was removed and the mixtures containing the nuclear extracts were added: each one of the extracts incubated with antibody was pooled with one of the big sepharose aliquots, whereas the non-antibody control was mixed with the small aliquot of sepharose. These suspensions were rotate at 4ºC during 2 h.

Finally, the slurries were centrifuged ($16 \cdot 10^3$ RCF, 0.5 min, 4ºC), washed 4 times with IP buffer (0.3 mL buffer for each 0.3 mL nuclear extract) and a last time with HDAC activity buffer. For each immunoprecipitate (HDAC1 and HDAC5), the content of the tubes was resuspended in 1.5 mL HDAC activity buffer, aliquoted in 5 tubes (0.3 mL per tube), centrifuged ($16 \cdot 10^3$ RCF, 0.5 min, 4ºC) and decanted. In parallel, the control beads were washed once with 0.3 mL HDAC activity buffer. Each pellet was resuspended in 100 μL fresh HDAC activity buffer and used immediately for the activity assay (see 5.16.3).

5.16.3. In vitro HDAC activity assay

In addition to the active nuclear extract (see 5.16.1) or immunoprecipitated HDACs (5.16.2); drugs solutions (Table 2), [^{3}H]acetate-labelled histones (1.8 nCi/μg) dissolved in water (4 μg/mL), HDAC activity buffer, HDAC quenching solution, ethyl acetate (Merck) and *Optiphase HiSafe 3®* (Wallac) scintillation cocktail were necessary for this assay.

For each reaction, either 40 μL MCF7 active nuclear extract diluted with 50 μL HDAC activity buffer or 100 μL beads suspension from the immunoprecipitation of HDAC1 and HDAC5 were taken as source of enzyme and incubated with the drugs for 10 min at room temperature. Dilutions of the chemicals were prepared in such a

way that volumes of 1.0 μL were added to each mixture. Then, 10 μL [^{3}H]acetate-labelled histones were added to each tube and the reaction was allowed to proceed at 30ºC during 60 min (in case of using the beads, reaction tubes were shaken in a *Thermomixer* at 600 rpm to keep the resin in suspension). Deacetylation was stopped with of 37.5 μL HDAC quenching solution and the released [^{3}H]-acetic acid was extracted with 0.7 mL ethyl acetate. Phases were separated by centrifugation ($16 \cdot 10^3$ g, 5 min, room temperature). Finally, 0.45 mL aliquot of each organic layer was counted in 3 mL scintillation cocktail in a *1409 Liquid Scintillation Counter* (Wallac).

5.17. Histone acidic extraction

The following solutions were required for this protocol: PBS, RSB, 10% NP-40, 0.25 M HCl, acetone (Merck), 20 mM Tris pH=6.8.

Cells ($2 \cdot 10^6$ to $6 \cdot 10^6$) were washed once with PBS, detached and collected by centrifugation (200 RCF, 5 min, 4ºC). After one wash with PBS, cells were resuspended in 4 mL RSB with 0.4 mL NP-40 solution and incubated on ice for 10 min. Nuclei were centrifuged (690 RCF, 5 min, 4ºC), washed once with 4 mL cold RSB buffer, resuspended in 0.5 mL 0.25 M HCl and rotated at 4ºC during 4 h. Samples were centrifuged ($16 \cdot 10^3$ RCF, 5 min, 4ºC), supernatants were transferred to clean tubes and histones were precipitated with 8 vol acetone and incubation at 4ºC for 4 h. Finally, samples were centrifuged ($16 \cdot 10^3$ RCF, 5 min, 4ºC), washed twice with acetone and, once the residual solvent evaporated, 0.2-0.4 mL Tris solution were used to dissolve histones. Typically, 0.2 to 0.4 mg protein were obtained.

5.18. Preparation of whole-cell protein extracts

Dishes containing 0.5-2·10^6 cells were washed with cold PBS. Protein extract buffer (40 μL per 1 cm^2) was added and plates were incubated on ice for 15-30 min (until cells detach). Suspensions were pipetted thoroughly, transferred to 1.5 mL tubes and the extracts were cleared by centrifugation (16·10^3 RCF, 30 min, 4ºC). Supernantants were placed in a clean tube and stored at –80ºC.

5.19. Determination of protein concentration

Histone concentration and protein concentration in whole protein extracts were determined with Bio-Rad Protein Assay Kit and *DC* Protein Assay Kit (Bio-Rad), respectively, following the manufacturer's instructions. The first reagent is based on Bradford's method and for that, 0-8 μg of protein (contained in 0-20 μL of protein or standard solution) were mixed with 1 mL diluted Coomassie brilliant blue solution. Absorbances were measured at 595 nm. *DC* Protein Assay Kit is based on Lowry's method and is compatible with the presence of detergent in the sample. For the assay, 0-30 μg of protein sample or standard (contained in 20 μL) were mixed in a final volume of 0.92 mL colorimetric cocktail. After 15 min incubation at room temperature, absorbances at 750 nm were measured.

In both cases, calibration curves were performed with BSA standard solutions. When necessary, protein solutions were diluted to get an absorbance value in the linear range.

5.20. Denaturing polyacrilamide gel electrophoresis of proteins (SDS-PAGE)

First, gel was made. SDS-PAGE resolving gel (7.5 mL) was prepared by mixing the reagents in the order of citation (see 5.1). The resulting solution was

immediately poored into a plastic cassette for *XCell SureLock®* Mini-Cell (Invitrogen) for gels 1.0 mm thick, overlayed with 1.5 mL either ethanol or isopropanol and allowed to polymerize for 30 min at room temperature. Alcohol was drained completely and 3 mL freshly prepared SDS-PAGE stacking gel mixture was added on top of the resolving gel. Combs (10 or 12 wells) were placed and the gel was left 30 min at room temperature for polymerization.

To perform the electrophoresis, one or two cassettes were placed in the *XCell SureLock®* Mini-Cell system, the cell was filled with SDS-PAGE running buffer, the combs in the gels were removed and the samples, previously mixed with 4 x Laemmli buffer and denatured (3 min at 100ºC, then ice), were loaded. For histone analysis, 1 μg per sample of acidic extract (5.17) were separated in a 15% SDS-PAGE gel, for HDACs (HDAC1 and HDAC2) 25 μg of whole-cell protein extract per sample were separated in 7.5% or 10% SDS-PAGE gel. Electrophoresis were run at 25 mA (one single gel) or 50 mA (two gels) until the bromophenol blue present in the Laemmli buffer started leaving the gel. Adequate molecular weight standards were used.

5.21. Detection of proteins by Western blotting

After separation in a SDS-PAGE gel, proteins were transferred to *Immoblilon-P*^{SQ} PVDF membrane (0.22 μm pore size, Millipore) in a *Mini Trans-Blot Cell* (Bio-Rad). One piece of *Immobilon* membrane of the size of the gel was cut and equilibrated for 2 min in methanol (Merck) and then in transfer buffer. Four pieces of *Whatman®* 3MM paper and two fibber pads with surface slightly bigger than the gel were also soaked in transfer buffer. The transfer sandwich was assembled (see Fig.), placed in the tank full with transfer buffer and transference was performed at 60 V during 2.5 h at room temperature. Then, membrane was soaked for 1 min in 1% acetic acid and stained with Ponceau S solution to visualize the proteins and verify that the transfer was properly done.

Table 7: Antibodies

Antigen	Epitope	Host	Manufacturer	Dilution(1)	Buffer (1)
HDAC1	Amino acids 53-482 of mouse HDAC1	rabbit	Upstate	1/1000	System 2
HDAC5	KLH conjugated peptides corresponding to amino acids 536-545 and 194-206 of human HDAC5	rabbit	Upstate		Only used in IP
Acetyl H4	AGGKGGKGMGKVGAKRHSC with all K acetylated	rabbit	Upstate	1/2000	System 1
Acetyl H3	ARTKQTARKSTGGKAPRKQLC K9 and K14 acetylated	rabbit	Upstate	1/5000	System 2
H3	Synthetic peptide corresponding to amino acids 124-135 of human H3	rabbit	Abcam	1/1000	System 1
H4	Calf thymus histone H4	rabbit	Upstate	1/1000	System 2
HDAC1	Synthetic peptide corresponding to amino acids 467 to 482 of human HDAC1	rabbit	ABR		Only used in ChIP
HDAC2	Synthetic peptide corresponding to amino acids 475 to 488 of human HDAC2	rabbit	ABR		Only used in ChIP
LAP2	Amino acids 34-156 of rat Lap2	mouse	Transduction laboratories	1/1000	System 2
HDAC2	KLH conjugated synthetic peptide corresponding to amino acids 471 to 488 of human HDAC2	rabbit	Abcam	1/1000	System 2

(1) Dilution and buffer system employed for Western Blotting. System 1 is the PBS-based buffer and System 2, the NET-based one.

Membrane was washed twice with PBS and then blocked with either WB blocking buffer 1 or 2 (see below) for 1 h at room temperature. After that, the *Immobilon*® piece was rinsed with the corresponding wash buffer (WB wash buffer 1 or 2, see below), hybridized with the primary antibody (see Table 7 for antibodies and conditions) during 2 h at room temperature or o/n at 4ºC, washed 3 times with WB wash buffer, incubated with the secondary antibody for 45 min and rinsed 4 times with WB wash buffer. For developing, *Ortho CP-G Plus* films (Agfa) and *ECL® Western Blotting Detection Reagent* (Amersham) were used according to manufacturers' instructions.

For blocking, washing and antibodies dilutions, two systems of buffers were employed, one based on PBS/*Tween® 20* (WB wash buffer 1 and WB blocking buffer 1) and the other one consisnting on a more complex solution, the NET buffer (WB wash buffer 2 and WB blocking buffer 2). The first one was used for routine except in the cases were antibodies gave strong background signal. Then, NET-based buffers were employed instead of PBS/*Tween® 20*.

5.22. Quantitation of global histone modifications by high-performance capillary electrophoresis

This analysis was performed as in Fraga *et al.* 2005, a method which is a variation of that one reported by Lindner *et al.* 1992.

5.22.1. Histone fractionation by reverse phase high-perfomance liquid chromatography

Histones isolated from $2 \cdot 10^6$ to $6 \cdot 10^6$ cells were dissolved in 0.2 mL and separated in a *Jupiter® 5 μm C18* column (Phenomenex), protected by a *SecurityGuard® C18-widepore* guardacolumn cartridge (Phenomenex), in a Beckman HPLC gradient system (Beckman-Coulter). Flow was set at 1.0 mL/min, detection at 220 nm and the mobile phase was:

0-5 min 20% eluent B,
5-65 min linear gradient from 20% to 80% eluent B,
65-70 min linear gradient from 80% to 100% eluent B,
70-75 min 100% eluent B,
75-80 min linear gradient from 100% to 20% eluent B;

where eluent A is 3% (v/v) HPLC-grade TFA (Merck) in water and eluent B 3% TFA in HPLC-grade acetonitrile (Merck). Between two consecutive injections, the system was allowed to equilibrate for 5 min with 20% eluent B.

Eluate was collected in 0.5 mL fractions (*FC 203B* fraction collector, Gilson) and the ones containing H3 or H4 were freeze dried. Since we found H3 usually in 3 consecutive fractions, these were pooled before lyophilization.

5.22.2. High-perfomance capillary electrophoresis analysis of isolated histone fractions

Capillary electrophoresis analysis of HPLC-fractionated histones was done by Dr. Mario F. Fraga according to the reported procedure (Fraga *et al.* 2005, Lindner *et al.* 1992) The non-, mono, di-, tri- and tetra-acetylated derivatives of H3 or H4 were resolved by high performance capillary electrophoresis (HPCE). A non-coated fused-silica capillary (Beckman-Coulter) (60.2 cm x 75 mm, effective length 50 cm) was used in a CE system (*P/ACE® MDQ*, Beckman-Coulter) connected to a data-processing station (32 KaratTM Software). The running buffer was 110 mM phosphate buffer, pH 2.0 containing 0.03% (w/v) (hydroxypropyl)methyl cellulose (Sigma). Running conditions were 25º C and operating voltage of 12 kV. On-column absorbance was monitored at 214 nm. Before each run, the capillary system was conditioned by washing with 0.1 M NaOH for 3 min, washing with 0.5 M H_2SO_4 for 2 min and equilibrated with the running buffer for 3 min. Samples were injected under pressure (0.3 psi) for 3 s.

6. Results

6.1. Procaine: a novel DNA demethylating agent

6.1.1. Procaine decreases global DNA methylation

6.1.1.1. Concentration dependence of procaine induced DNA demethylation

In order to investigate procaine-induced changes of 5-methylcytosine levels in genomic DNA, MCF7 cells were grown during 3 days in the presence of different concentrations of the drug (from 0.005 mM to 0.5 mM). Positive and negative control treatments were performed in parallel using either the well characterized DNA demethylating agent zdC (at 2.5 and 5.0 μM concentrations) or the solvent, respectively. DNA was extracted, hydrolysed and the resulting 2'-deoxynucleosides were separated by HPCE and detected by measuring the absorbance at 254 nm. This technique allows the quantitation of the proportion of the different bases present in DNA. Examples of electropherograms from different 2'-deoxynucleoside mixtures are shown in Fig. 15A (see Figure legend for details).

The effect of procaine on the global content of 5-methylcytosine is shown in the next plot Fig. 15B. When procaine concentration increases, the area of the peak for mdC decreases respect to the area of the dC peak, however the effect is much less stronger than that one of zdC: 0.5 mM procaine is not enough to reduce DNA-methylation as much as 2.5 μM zdC (Fig. 15B and C).

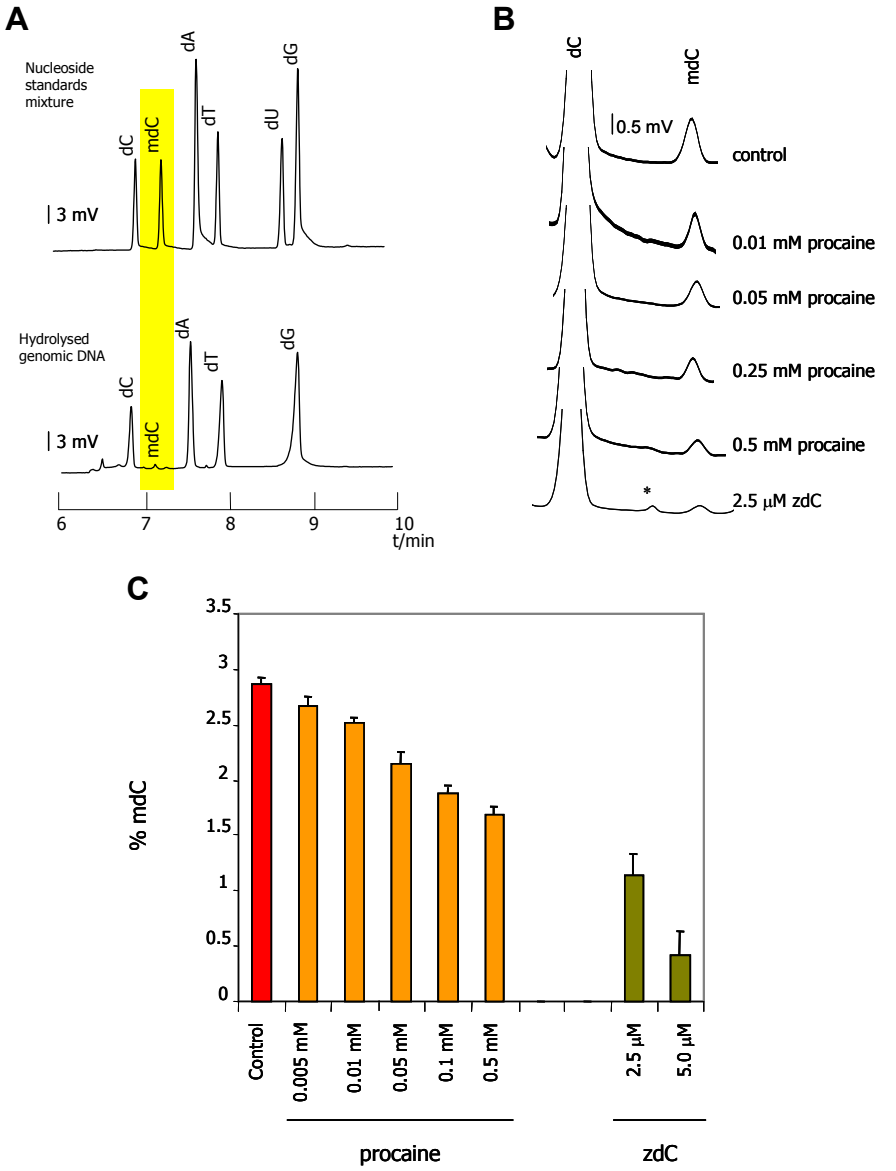


Fig. 15: HPLC analysis of global DNA methylation. **A**) Two examples of electropherograms from different 2'-deoxynucleosides mixtures are shown. The upper graph corresponds to a standard solution containing equimolar amounts of each 2'-deoxynucleoside (dC, mdC, dA, dT, dU and dG). The lower plot is the electropherogram for hydrolysed genomic DNA from MCF7 cells. The yellow rectangle marks the peak for mdC. **B**) Enlargement of mdC peaks of DNA samples from MCF7 cells treated with different concentrations of procaine or zdC. Intensities are normalized taking as reference the peak for 2'-deoxycytidine. **C**) The diagram shows the percentage of mdC in genomic DNA relative to global dC content for different doses of procaine and zdC after 3 days treatment.

Further analysis of global DNA methylation was performed using methylation-sensitive restriction enzymes. Aliquots of genomic DNA from cells cultured with vehicle, 5.0 μM zdC, 0.25 mM or 1.0 mM procaine were digested with MspI or HpaII or McrBC. MspI and HpaII are isoschizomers: both recognize the sequence CCGG but, whereas MspI is insensitive to the methylation status of the second cytosine residue, HpaII only cuts if that residue is unmethylated. On the other hand, McrBC recognizes sites in the form $(G/A)^{m}C(N)_{40\text{-}3000}\ ^{m}C(G/A)$, where ^{m}C represents a 5-methylcytosine residue and $(N)_{40\text{-}3000}$ any DNA sequence from 40 to 3000 bp long (but the optimal length is 55 to 103 bp). McrBC cuts if one or both DNA strands are methylated.

Digestion products were run in an agarose gel and visualized with ethidium bromide staining (Fig. 16). The strongest effects on DNA methylation were found with 5 μM zdC, since for this sample the smear with HpaII is very similar to that one got with MspI, but McrBC digestion was not as extensive as in the other cases. Both

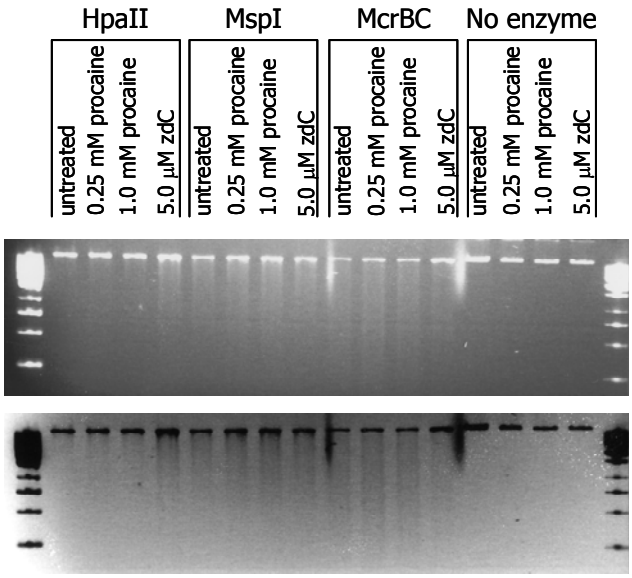


Fig. 16: Methylation-sensitive restriction enzyme analysis of genomic DNA isolated from procaine or zdC treated cells. HpaII does not cut if the recognition sequence is methylated, MspI is its methylation-insensitive isoschizomer and McrBC only cuts if the recognition site is methylated. DNA was visualized by ethidium-bromide staining after gel electrophoresis.The same picture is displayed in positive (upper panel) and negative (lower panel) colours.

DNA samples from procaine treated cells seem to be slightly more digested by HpaII than the control DNA, but no effect could be observed in the McrBC reaction. These results reveal the same tendency as the ones obtained by HPCE: procaine induces some loss of DNA methylation but not as much as zdC. However, restriction enzyme digestion is not as quantitative and sensitive to variations in global methylation as high-performance capillary eletrophoresis.

6.1.1.2. Time dependence of procaine induced DNA demethylation

Next, the time dependence of DNA demethylation induced by procaine was studied and compared to the effects of procainamide and zdC. The same concentration of procaine and procainamide was chosen in order to make easier the comparison, given the structural similitude of both chemicals. MCF7 cells were cultured in parallel with 0.5 mM procaine, 0.5 mM procainamide or 5.0 μM zdC. After 24 h, 48 h and 72 h treatments, DNA was extracted to measure the percentage of methylcytosine by HPCE. The result of the quantitations is displayed in Fig. 17.

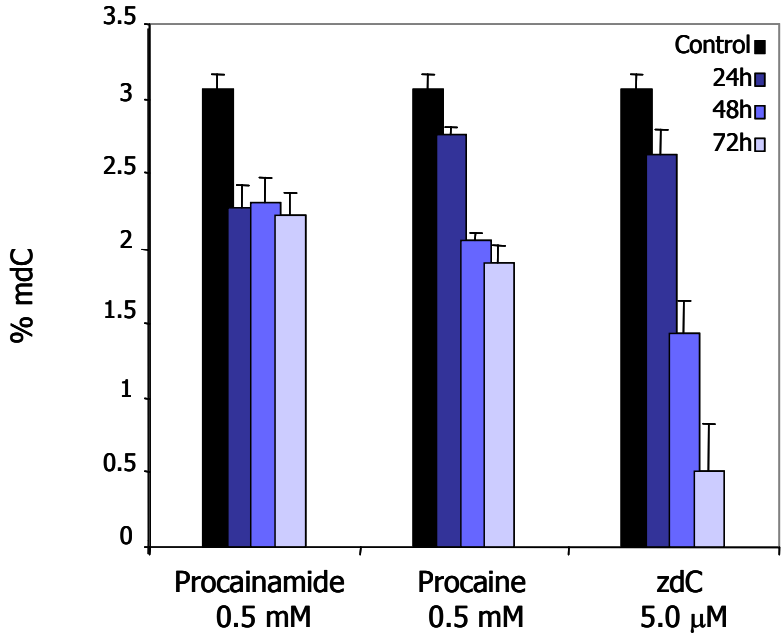


Fig. 17: Time-dependence of DNA demethylation caused by procainamide, procaine and zdC at the indicated concentrations. The proportion of mdC was determined by HPCE.

Procainamide and procaine reach their maximum effect about 24-48 h after drug addition. In contrast, the loss of methylation induced by zdC decreases continuously along the three days. Also the final mdC content in DNA is much lower in samples treated with zdC than in samples treated with procainamide or procaine.

6.1.2. Procaine induces the expression of tumor-suppressor genes silenced by DNA hypermethylation

6.1.2.1. Procaine reduces methylation at *RARβ2* promoter.

Once demonstrated that procaine is able to induce demethylation in a global genomic scale, we wondered whether the reduction of 5-methylcytosine content also occurs in aberrantly hypermethylated gene promoters. We selected the isoform 2 of retinoic acid receptor beta (*RARβ2*) gene, a well-known tumor suppressor gene silenced in MCF7 cells by hypermethylation of its promoter (Arapshian *et al.* 2000, Sirchia *et al.* 2000, Bovenzi *et al.* 2001, Esteller *et al.* 2002, Paz *et al.* 2003).

In order to study procaine effects on *RARβ2* promoter, cells were cultured for 3 days in medium containing different concentrations of procaine or zdC, DNA was extracted and modified with bisulfite. By using MSP, the presence of a population of non-methylated *RARβ2* promoter alleles could be detected when 10 μM or more procaine was added to the cells as well as when 2.5 μM or 5.0 μM zdC were employed (Fig. 18A).

To monitor the loss of methylation at single CpG resolution, bisulfite genomic sequencing of *RARβ2* promoter was performed on DNA from non-treated cells, cells treated with 5.0 μM zdC and cells treated with 0.5 mM procaine. Amplification with the adequate primers (one pair was BS-RARβ2-S3 and BS-RARβ2-AS3 and the other, BS-RARβ2-S2 and BS-RARβ2-AS2) was performed on bisulfite-deaminated DNA, PCR products were isolated and sequenced. The analysis showed that zdC had the strongest effects on methylation but the loss of 5-methylcytosine is not as marked as one would expect from the effects of the same chemical on global DNA methylation. Procaine also reduces the level of methylation in the promoter (Fig. 18B).

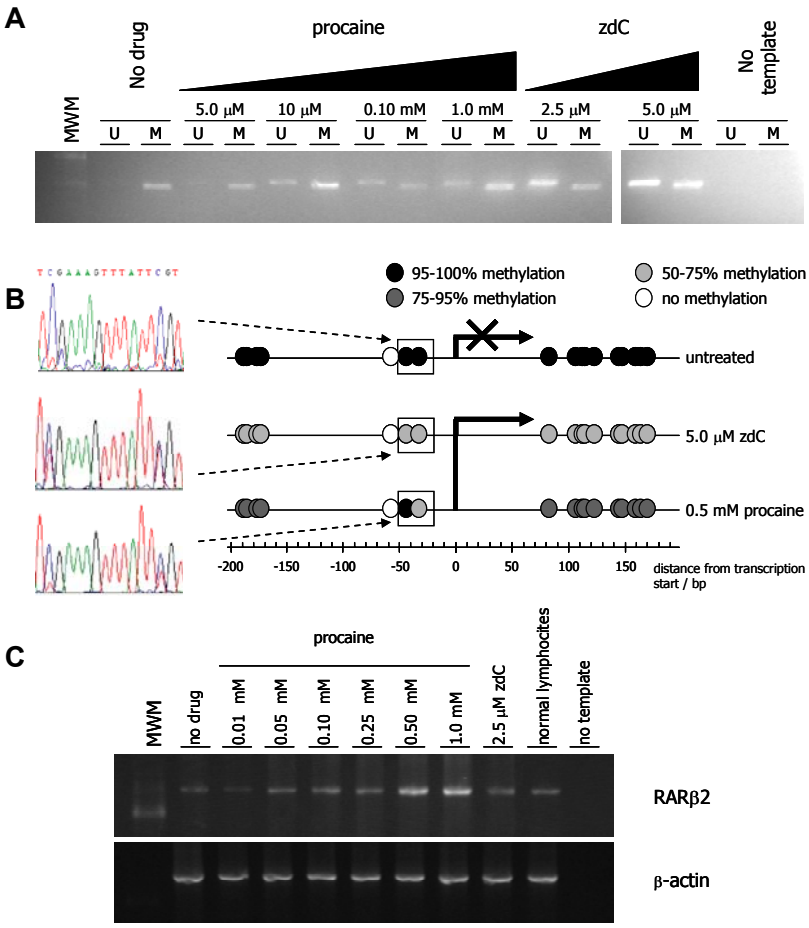


Fig. 18: Analysis of *RARβ2* promoter methylation and gene expression. **A**) MSP analysis of *RARβ2* promoter after treatment with procaine and zdC. Lines labelled with "U" correspond to the reaction with primers specific for the non-methylated sequence, whereas "M" labels the reactions with primers specific for the methylated sequence. Concentrations range from 0 (no drug) to 1.0 mM procaine and from 0 to 5.0 μM zdC. **B**) Genomic bisulfite sequencing of the *RARβ2* promoter region by using the pair of primers BS-RARβ2-S2 and BS-RARβ2-AS2 for the region upstream to the transcription start site and the pair BS-RARβ2-S3 and BS-RARβ2-AS3 for the region downstream to it. Left: upper panel, part of the electropherogram for the untreated cells; middle panel, part of the electropherogram for the cells treated with 5.0 μM zdC and lower pannel, part of the electropherogram for the cells treated with 0.5 mM procaine. Right: Scheme displaying the percentage of methylation for each CpG in the promoter. Each circle represents a CpG dinucleotide and the colour code is given in the upper part of the figure. The boxes mark the CpG dinucleotides shown in the left panels. **C**) Semiquantitative RT-PCR measurement of changes in *RARβ2* (upper panel) and β-actin (lower panel) expressions upon drug treatment. The different concentrations of drugs are indicated. RNA extracted from normal lymphocytes was used as positive control.

6.1.2.2. Procaine reactivates *RARβ2* transcription

In order to see if that decrease of 5-methylcytosine was enough to recover gene expression, the presence of RARβ transcript was monitored by semiquantitative RT-PCR after 3 days of treatment. RNA extracted from normal lymphocytes was used as a positive control. As expected, concentrations of procaine over 50 μM increase the amount of *RARβ2* transcript (Fig 18C).

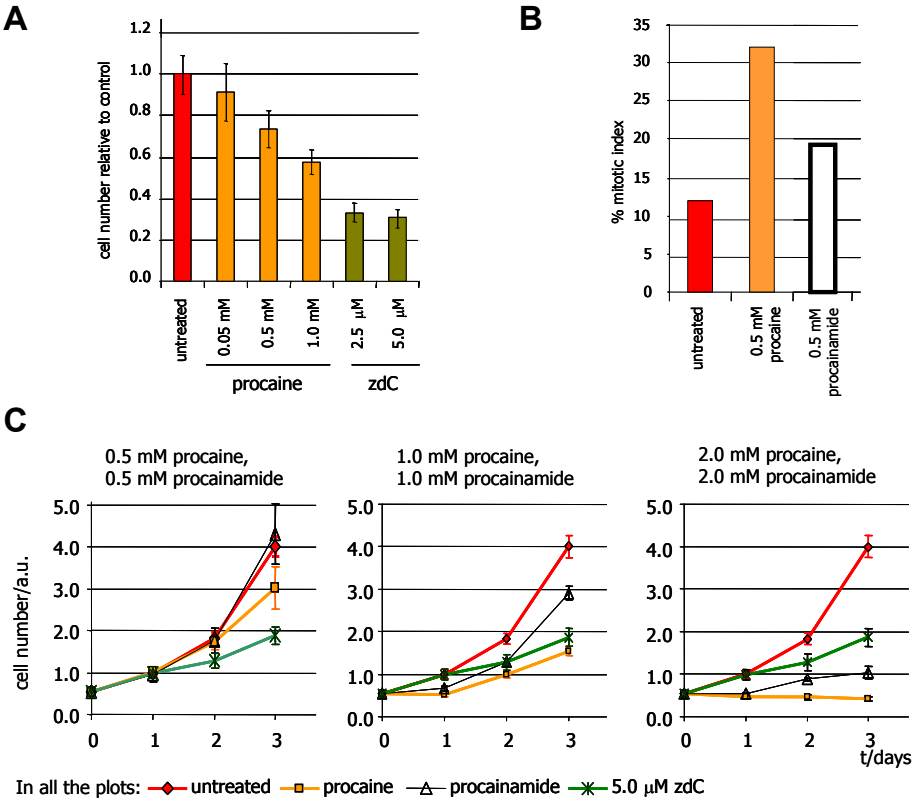


Fig. 19: Cell growth effects of procaine. **A**) Effect of different procaine and zdC concentrations on cell growth after 3 days treatment. **B**) Mitotic index expressed as % found in each of the indicated immunostained samples after 3 days of drug treatment. **C**) Time course of the effect of procaine and procainamide on cell growth. Left: 0.5 mM procaine and 0.5 mM procainamide; middle: 1.0 mM procaine and 1.0 mM procainamide and right: 2.0 mM procaine and 2.0 mM procainamide. In all the plots, untreated cells and 5.0 μM zdC were used as controls.

6.1.3. Procaine reduces cell proliferation

Finally, the influence of drug treatment in cell proliferation was examined. Firstly, cells were treated during 3 days with different concentrations of procaine (from 0.05 mM to 1.0 mM) or zdC (either 2.5 or 5 μM). Attached cells were harvested and counted in a Neubauer chamber. The number of procaine treated cells decreases in a dose-dependent manner. However, for all the tested concentrations zdC has a stronger effect than procaine (Fig. 19A).

In order to investigate time-dependence of drug effects on cell proliferation, non-treated cells and cells grown with 5.0 μM zdC, 0.5, 1.0 or 2.0 mM procaine and 0.5 1.0 or 2.0 mM procainamide for 24, 48 and 72 were counted. The results are plotted in Fig. 19C. For all the analysed concentrations, procaine slows cell proliferation more than procainamide. No alteration in the number of cells (relative to the controls) is observed with 0.5 mM procainamide at any time point whereas the same concentration of procaine retarded cell growth after the second day. When both chemicals were added at 1.0 or 2.0 mM, the number of cells per dish did not change during the first 24 h. If the concentration of the substances was 1.0 mM, the amount of cells increased in the second and third days, more in the plates treated with procainamide. The highest dose of procaine (2.0 mM) completely inhibited cell proliferation.

Cytological changes upon 0.5 mM procaine and 0.5 mM procainamide treatments were studied in more detail using microscopical techniques. After 3 days with the corresponding drug, cells were fixed and stained with DAPI, to visualize the nuclei. We observed that both procaine and procainamide induced an increase in the number of mitotic figures, which was higher in the case of procaine treatment (Fig. 19B and Fig. 20). In order to explore the extent of the apoptosis induced by these drugs, TUNEL assay was also performed. Serum starved cells were taken as positive control. This immunofluorescence technique is based on the enzymatic labelling of the free terminal 3' hydroxyl groups in the DNA nicks that are generated during apoptosis. Fluorescein-dUTP was selected as the nick label in this work because it can be directly observed under a fluorescence microscope. As shown in Fig20,

neither 0.5 mM procaine nor 0.5 mM procainamide triggered the apoptotic response in MCF7 cells.

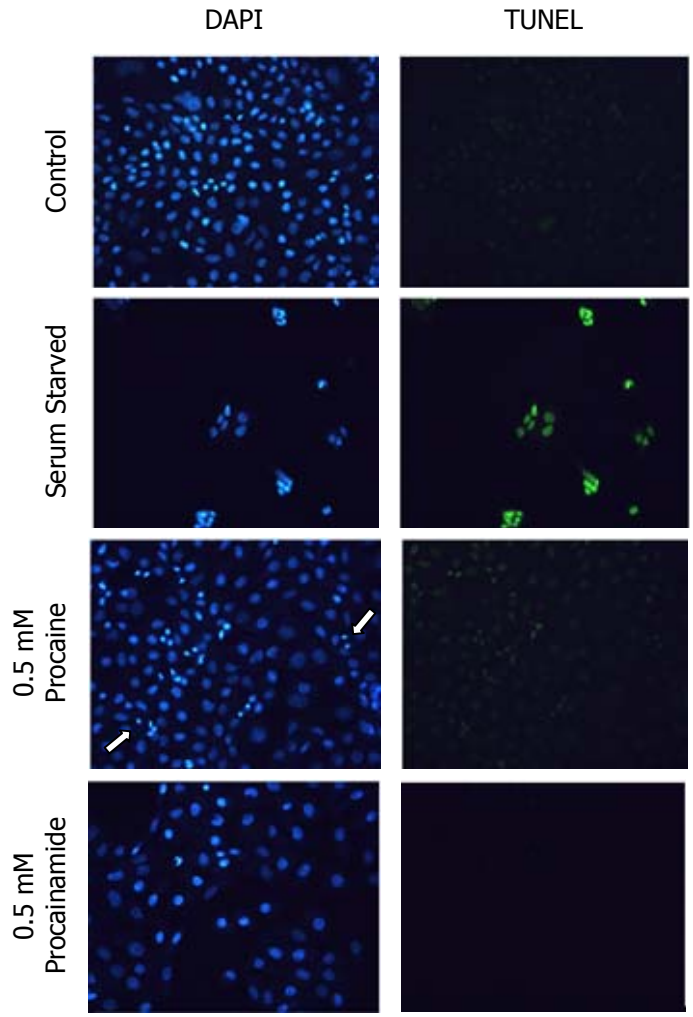


Fig. 20: TUNEL assay for analysis of apoptosis. DAPI (left panels) and TUNEL (right panels) staining of MCF7 cells after 3 days treatment. From left to right: untreated cells, cells after 3 days serum starvation (TUNEL positive control), cells treated with 0.5 mM procaine, cells treated with 0.5 mM procainamide. Some mitotic figures are pointed by an arrow.

6.2. Comparative study of seven HDACs inhibitors

6.2.1. In vitro comparison of the HDAC inhibitory properties.

6.2.1.1. Inhibition of the HDAC activity present in nuclear extracts

In order to compare the ability of valproic acid, butanoic acid, MS-275, TSA, SAHA, CX and CY to inhibit Zn(II)-dependent HDACs contained in nuclei, *in vitro* HDAC activity assays were performed. To this end, MCF7 nuclear extracts were incubated in the presence of the drugs or vehicle, with hyperacetylated histones containing tritium-labelled acetyl groups. The acetic acid resulting from the hydrolysis was extracted with ethyl acetate and the liberated counts were measured. The released radioactivity is proportional to the acetate groups hydrolysed from histones.

The results displayed in Fig. 21A are the average of two independent experiments. All the substances inhibit to some extent the HDAC activity contained in MCF7 nuclear extract. Hydroxamic acids are the most potent chemicals, showing significant effect already at micromolar concentrations: 60% of the activity is abolished by 0.1 μM TSA and 10 μM CX, near 70% is inhibited by 1.0 μM SAHA and 1.0 μM CY abolishes about 50% of the HDAC acitvity. All these four compounds at 50 μM concentration block almost completely the HDAC activity in the extracts. On the other hand, to reach a similar degree of inhibition, millimolar concentrations of carboxylic acids (butanoic and valproic acid) are necessary.

In the same extract, benzamide MS-275 has very little effect on HDAC activity at concentrations up to 50 μM. The increase in the concentration of this compound caused the appearance of a precipitate in the reaction mixture, thus the dose of this chemical which is necessary for the inhibition of more than 50% of the activity present in the extract could not be determined.

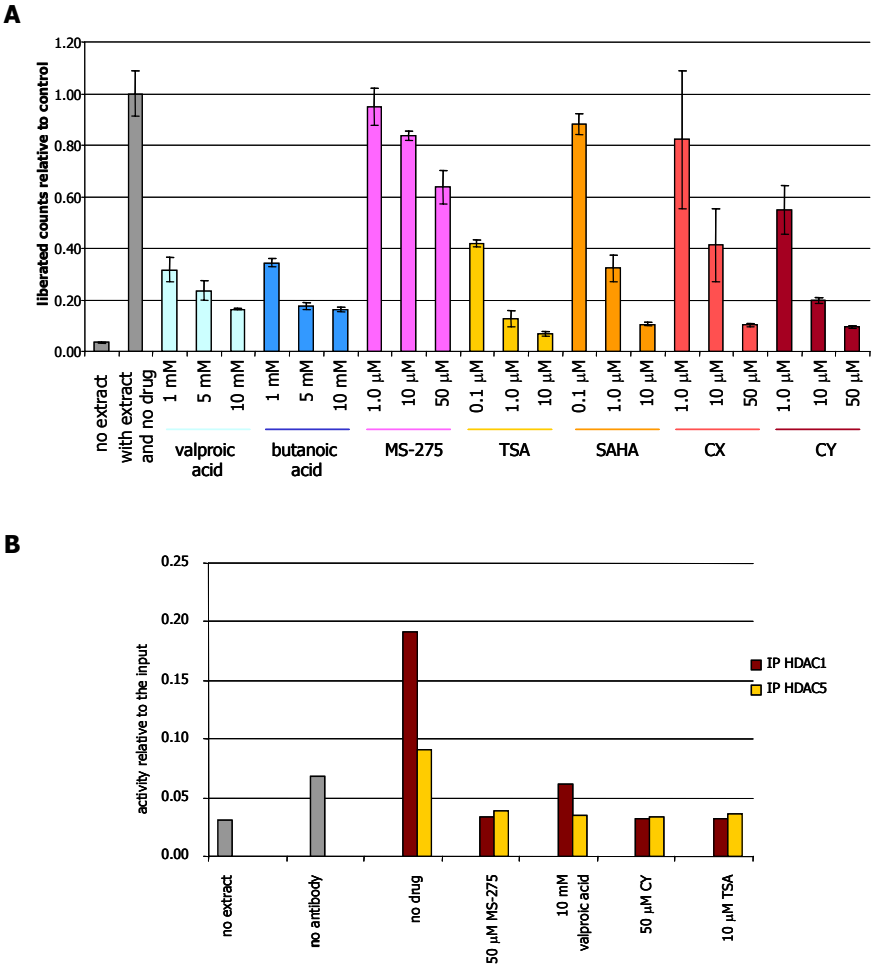


Fig. 21: *In vitro* HDAC assays **A**) *In vitro* inhibitory activity assay. Aliquots of MCF7 nuclear extract were incubated for 10 min at r.t. with the indicated concentration of chemicals (butanoic acid, valproic acid, MS-274, TSA, SAHA, CX, CY), then radiolabelled histones were added and reaction was allowed to proceed for 2 h at 37ºC. After quenching, acetic acid was extracted with ethyl acetate and counts of the organic phase were measured. In the graphic, the resulting counts for each reaction are normalised to the reaction in absence of HDAC inhibitor. **B**) *In vitro* inhibitory activity assay with immunoprecipitated HDAC1 and HDAC5. Values are relative to the activity of the equivalent volume of crude nuclear extract. "No extract" is the control reaction with no addition of any enzyme, "no antibody" is the control immunoprecipitation with no primary antibody and "no drug" is the reaction of the immunoprecipitated extracts with histones in the absence of inhibitor. Both immunoprecipitations were performed in parallel. The results for HDAC1 are shown in brown and, for HDAC5, in yellow.

6.2.1.2. Inhibition of immunoprecipitated HDAC1 and HDAC5

In order to explore a potential specific inhibition for particular HDACs, HDAC1 and HDAC5 were immunoprecipitated from nuclear extracts. Only the anti-HDAC1 antibody immunoprecipitated a significant amount of activity (Fig. 21B). At the employed concentrations, all the substances tested with these preparations (MS-275, valproic acid, CY and TSA) reduced HDAC1 activity to the background level.

6.2.2. HDACs inhibitors stop MCF7 cell proliferation

6.2.2.1. Determination of the IC50 for 24 h treatments

An important characteristic of anticancer drugs is the ability to inhibit cancer cell proliferation and/or cause apoptosis of cancer cells. In a first approach to compare the antiproliferative effects of the seven HDACs inhibitors on MCF7 cells, the IC_{50} for 24 h-treatments was determined. The IC_{50} is defined as the concentration of substance necessary to get, at the end of the treatment, half as many cells as if there would not be any drug. Additionally, the use of the IC_{50} for the experiments on cells provides a good reference to compare the effects of compounds with diverse chemical nature.

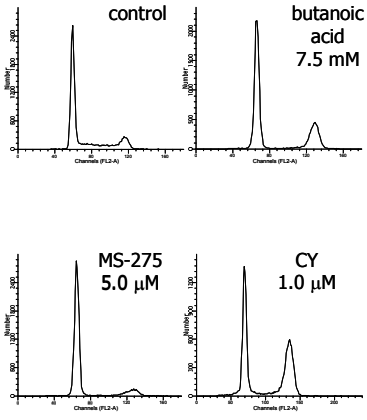
It is widely reported that 24 h incubations with HDAC inhibitors are long enough to result in extensive histone hyperacetylation in cultured cells. These reports include the drugs employed in this study (see for instance, Göttlicher *et al.* 2001, Plumb *et al.* 2003, Munster *et al.* 2001, Catley *et al.* 2003, Saito *et al.* 1999). Because of this reason, all the treatments we performed on MCF7 cells with the HDACs inhibitors were 24 h long.

Cells were cultured for 24 h with different concentrations of each compound, harvested and counted. The resulting IC_{50} values are shown in Fig. 22A. Millimolar concentrations of valproic and butanoic acid are required to alter cell proliferation, whereas hydroxamic acids and MS-275 slowed proliferation at micromolar concentrations.

A

Drug	IC_{50}
MS-275	5.0 μM
valproic acid	7.5 mM
butanoic acid	7.5 mM
TSA	0.25 μM
SAHA	5.0 μM
CX	3.5 μM
CY	1.0 μM

B



C

Treatment	G1/G0	S	G2/M	Sub-G0
control	65	25	10	0
MS-275	87	2	11	0
valproic acid	65	8	27	0
butanoic acid	70	4	26	1
TSA	47	11	40	2
SAHA	49	9	42	1
CX	52	8	39	2
CY	48	9	43	1

Fig. 22: Effects of the seven HDACs inhibitors on cell cycle. **A**) IC_{50} concentrations of each drug for MCF7 cell growth after 24 h treatment. **B** and **C**) Cell cycle analysis of cells treated for 24 h with different HDACs inhibitors at their IC_{50} concentration. **B**) Examples of histograms which were used to calculate the data shown in C. **C**) Table with the percentage of cells in each phase (values are the average of two independent experiments).

6.2.2.2. Structure-activity relationship between the chemicals and the induced cell cycle arrest

Cells treated during 24 h with each inhibitor at the corresponding IC_{50} concentration were harvested, fixed and submitted to flow cytometry analysis with propidium iodide staining of the nuclei. Examples of the resulting histograms are shown in Fig. 22B and the results of the integration, in Fig. 22C. All the chemicals reduced the proportion of cells in S phase. Hydroxamic and carboxylic acids induced arrest in G2/M, but the second ones also produced the block of the G1 to S transition. MS-275 stopped the cycle only in G1/G0. With all the hydroxamic acids and with butanoic acid a small amount of cells in the sub-G0 region (related to cell death) was observed.

6.2.3. HDACs inhibitors induce global H3 and H4 hyperacetylation in MCF7 cells

6.2.3.1. All the seven inhibitors induce similar changes in acetylation of H3 and H4

Next, it was addressed if, the inhibition of certain HDACs causes the accumulation of hyperacetylated histones in cells. Thus, we compared the ability of valproic acid, butanoic acid, MS-275, TSA, SAHA, CX and CY to induce an increase of histone acetylation in MCF7 cells. Histone extracts from cells treated with the chemicals or vehicle were prepared. After HPLC isolation of H3 and H4 from the extracts, both histones were analysed sequentially by HPCE. Under the selected running conditions, the electrophoretic mobility of histones is determined by the degree of acetylation, allowing the separation of non-, mono-, di-, tri- and tetraacetylated H3 or H4. If absorbance at 220 nm is employed for detection, the integration of the peaks in the electropherogram can be used to calculate the proportion of the different species. The scheme of the analysis is depicted in Fig. 23.

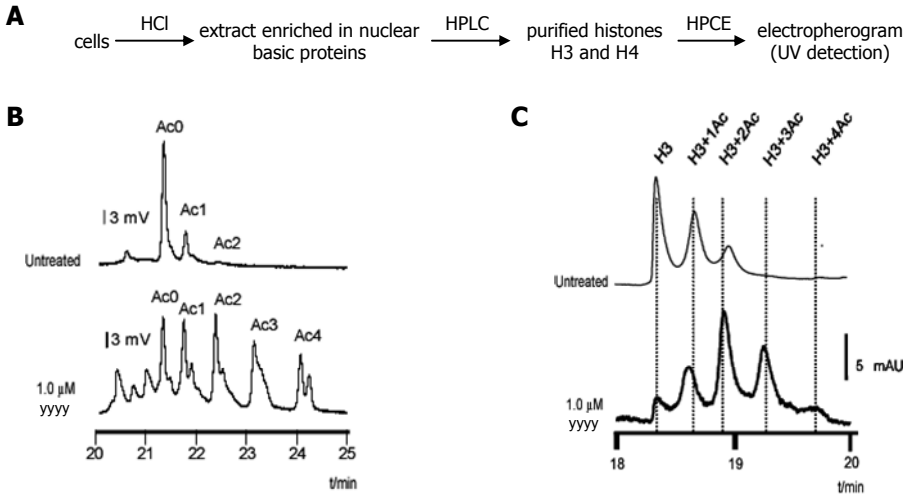


Fig. 23: H3 and H4 acetylation analysis by HPCE. **A**) Scheme of the general procedure. **B** and **C**) Typical electropherograms of H4 and H3 (respectively) in MCF7 cells: upper plots, untreated cells; lower plot, cells treated for 24 h with 1.0 μM CY.

Firstly, the response of the cells to the IC_{50} concentration of the drugs was analysed. All the compounds induced very similar changes in H3 acetylation (Fig. 24A and C): the proportion of species with at least one acetyl group is doubled respect to the untreated cells and with all the treatments, the diacetylated form is the predominant, the amount of monoacetylated is slightly less and the tetraacetylated species constitutes a very small proportion.

However, the influence of the inhibitors on H4 is more heterogeneous (Fig. 24B and D). In general, the proportion of monoacetylated H4 doubles upon treatment, but some substances, like MS-275, increase this value more than others like CX or TSA. Also the distribution of the acetylated species is not uniform, ranging from the changes induced by CX (in this case the monoacetylated H4 is the predominant and the tetraacetylated represents a very small proportion) to the ones caused by MS-275 (the major form is the triacetylated but the di- and the tetraacetylated species are in comparable amounts).

We also analysed the effects of the drugs on H4 at lower concentrations. To simplify the experiment, we selected 3 substances, one from each chemical class: butanoic acid, MS-275 and CY. All these three drugs were added to the

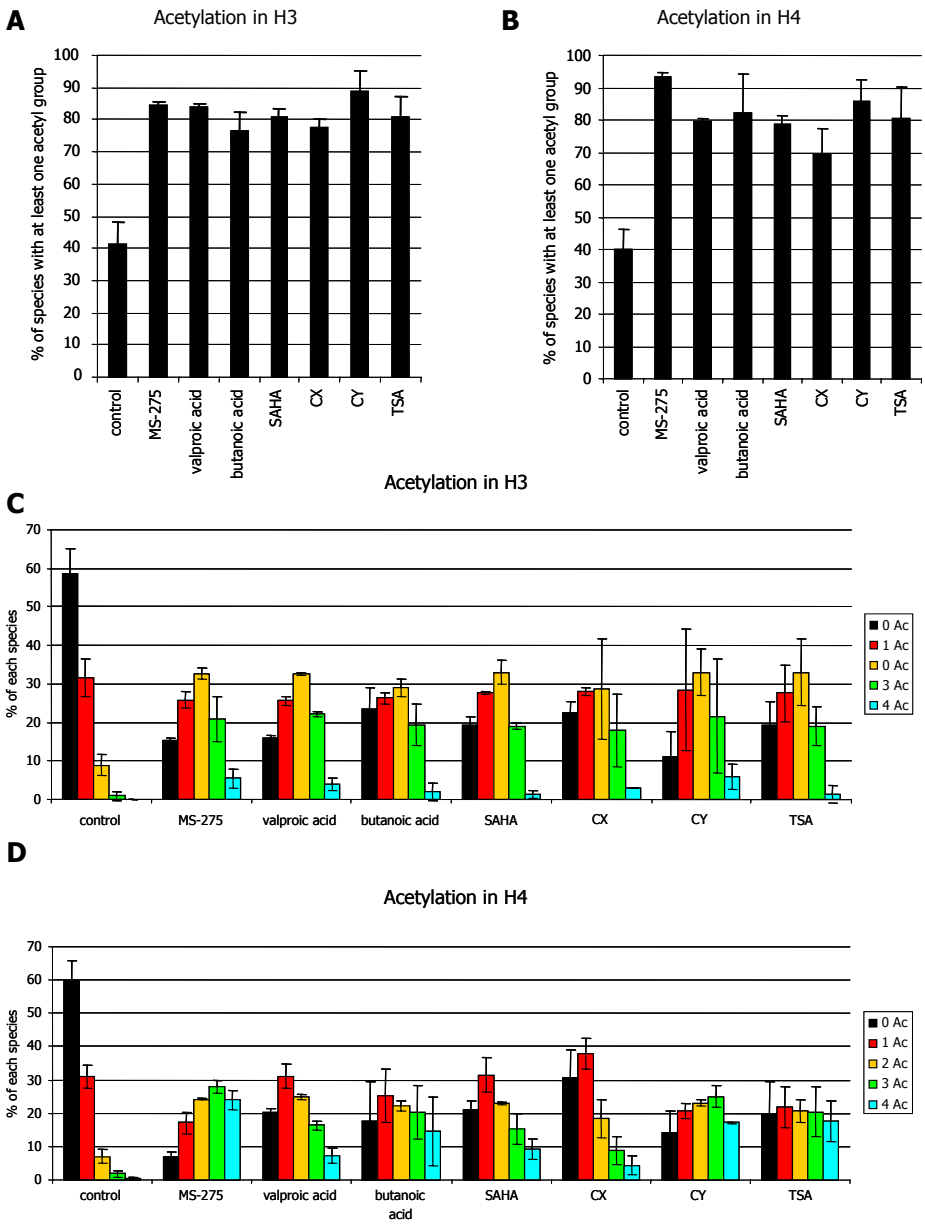


Fig. 24: Acetylation changes induced by the drugs when added to the cells at the IC_{50} concentration during 24 h. Proportion of the different species was measured by HPCE. A, B) Acetylation of H4 and H3 (respectively) expressed as percentage of species with at least one acetyl group. C, D) Proportion of non-acetylated (black), mono- (red), di- (yellow), tri- (green) and tetraacetylated (blue) H4 or H3 (respectively).

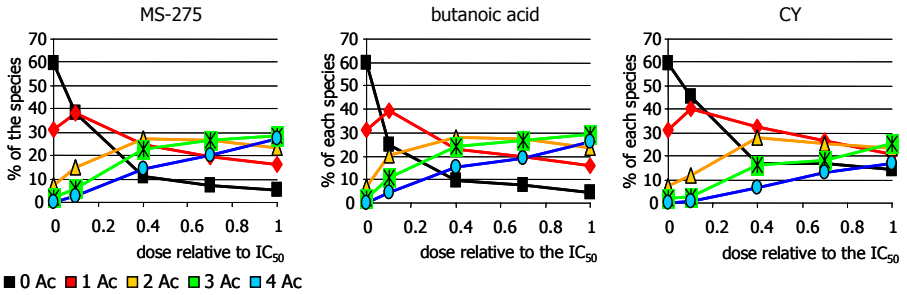


Fig. 25: Analysis of changes in histone H4 acetylation. Distribution of histone H4 with different degree of acetylation after 24 h treatment with MS-275 (left), butanoic acid (middle) and CY (right) at the following concentrations: 0.1-fold, 0.4-fold, 0.7-fold and 1.0-fold IC_{50}. Colour code as in Fig. 24: black means no acetyl groups; red, monoacetylation; yellow, diacetylation; green, triacetylation and blue, tetraacetylation.

cells at 0.1-fold, 0.4-fold, 0.7-fold and 1.0-fold IC_{50} concentration, histones were extracted and analysed as described above. The results are displayed in Fig. 25. With all the three compounds, monoacetyl H4 is the major species at 0.1-fold IC_{50} and no other of the tested concentrations induced as much monoacetylation. The proportion of diacetylated species seems to reach its maximum from 0.4-fold to 0.7-fold IC_{50} while the fractions corresponding to tri- and tetraacetyl H4 increase continuously with the concentration of drug. Almost no difference is seen when comparing the plot for butanoic and the plot for MS-275, while the response to CY seems to be less concentration-dependent: when the amount of drug varies from 0.1-fold to 1.0-fold IC_{50}, the drop in the proportion of the low-acetylated species (non- and monoacetylated) and the augment in the percentage of the high-acetylated forms (tri- and tetraacetylated) is not as pronounced as in the other two cases.

6.2.3.2. Changes in global histone acetylation analysed by Western blotting

An alternative way of investigating changes in histone acetylation is the use of Western blotting with antibodies that recognize the acetylated N-termini of histones. We have chosen one for H4 raised against the fully acetylated histone tail and another which is specific for H3 tail with both K9 and K14 acetylated (see Table 7 for details). The load control was performed with antibodies that bind the C-termini

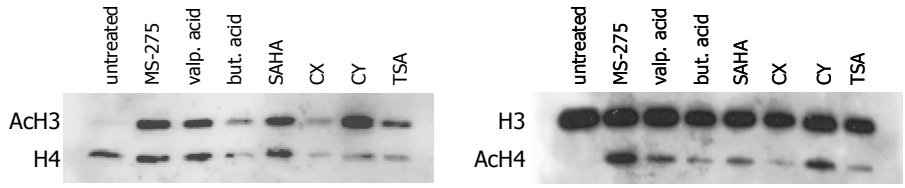


Fig. 26: Effects of drug treatments on H3 and H4 acetylation. Western blotting against diacetylated H3 (AcH3, left panel) and tetraacetylated H4 (AcH4, right panel). Cells were treated with each drug for 24 h at the IC_{50} concentration, histones were extracted and Western blotting was performed. Antibodies against C-terminus of H4 and H3 were used as load control. Membranes were probed sequentially with the anti-acetylated histone and the corresponding control antibodies.

of H3 and H4 respectively.

Both Western blotting and HPCE have their advantages and inconveniences in the study of acetylation. Capillary electrophoresis is a quantitative technique but requires higher amount of sample than Western blotting. On the other hand, Western blotting and other antibodies-based techniques can give some information about the positions whose acetylation is changing.

In Fig. 26 Western blottings against acetyl H4 and acetyl H3 are shown. For that experiment, histones were extracted from cells maintained for 24 h with the drugs at their corresponding IC_{50} concentration. Neither acetylation in H3 nor in H4 could be detected in the untreated cells with the employed antibodies but all the treated samples gave some signal. In the case of H4, the intensities of the bands correlate with the percentage of tetraacetylated species calculated by HPCE: the strongest bands are for MS-275 and CY and the weakest one for CX. In agreement with the HPCE results, no major differences could be observed in acetylation of H3 induced by the different chemicals.

6.2.4. None of the seven HDACs inhibitors alters HDAC1 or HDAC2 protein levels in MCF7 cells

Some HDACs inhibitors are known to alter the expression and the degradation of diverse HDACs and these phenomena can also affect global histone acetylation (Ajamian *et al.* 2003, Krämer *et al.* 2003). For instance, valproic acid

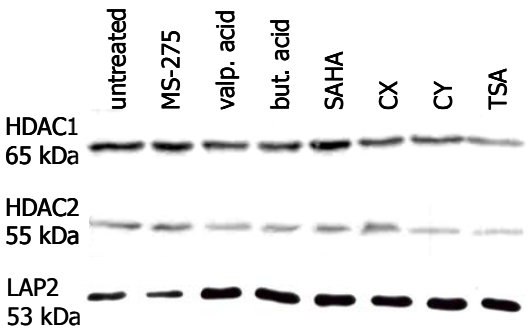


Fig. 27: Effects of drug treatments on HDACs expression. Western blotting against HDAC2 and HDAC1. The nuclear protein LAP2 was used as loading control. Cells were treated for 24 h with the indicated drug at the IC_{50} concentration, whole cell extract were prepared and samples employed for Western blotting.

induces degradation of HDAC2 (Krämer *et al.* 2003). Because of that, we analysed the protein levels of HDAC1 and HDAC2 after drug treatment.

Whole cell protein extracts were prepared after treating the cells with the chemicals at the IC_{50} concentration. No changes in HDAC1 or HDAC2 protein amounts were observed in Western blots (Fig. 27).

6.2.5. Histone acetylation restores the expression of tumor-suppressor genes

6.2.5.1. Tumor-suppressor genes silenced by histone hypoacetylation.

Once analysed the HDAC inhibitory properties of these chemicals *in vitro* and their ability of inducing global histone hypoacetylation in cultured cells, the possible changes in histone modifications at the promoters of certain genes were addressed. The six target genes were selected among those whose expression has been reported to increase upon treatment with HDACs inhibitors. The details and the references are listed in Table 8.

Table 8: Genes overexpressed upon HDACs inhibition

Gene symbol	Acc. nº	description	CpG island	Reexpression found with	References
CDKN1A	AF497972	cyclin-dependent kinase inhibitor 1A (*p21,Cip1, WAF1*)	yes	All HDAC inhibitors	Blagosklonny et al. 2002 Marks et al. 2001
GADD45	AF533019	growth arrest and DNA-damage-inducible,	yes	TSA, butanoic acid	Chen et al. 2002 Hirose et al. 2003
JunD	NT_011295.10	jun D transcription factor (AP-1 family)	yes	TSA, oxamflatin	Kim et al. 2003 Gobl et al. 1999 Kim et al. 1999
IGFBP3	M35878.1	insulin-like growth factor binding protein 3	yes	TSA, butanoic acid, valproic acid	Thelen et al. 2004 Gray et al. 2004 Choi et al. 2002 Walker et al. 2001
MT1X	NT_010498.14	metallothionein 1X	yes	Butanoic acid, SAHA, TSA, MS-275, valproic acid, FK228 (1)	Ghoshal et al. 2002 Dressel et al. 2000 (3) Glaser et al. 2003 Kultima et al. 2004
MT2A	NT_010498.14	metallothionein 2A	yes	Butanoic acid, TSA, valproic acid (2)	Joseph et al. 2004 Dressel et al. 2000 (3) Kultima et al. 2004

(1) Except for TSA, SAHA and MS-275, who are reported to induce overexpression in Glaser et al. 2003, the rest of the drugs are found to increase "MT1" expression, but in the corresponding references (Ghoshal et al. 2002, Dressel et al. 2000, Kultima et al. 2004) is not specified which of the MT1 genes are overexpressed.
(2) Kultima et al. do not specify which of the MT2 genes is overexpressed.
(3) Dressel et al. only mention overexpression of "metallothionein" in general.

CDKN1A is one of the proteins that control cell transition from G1 to S phase. Its expression is considered to be reactivated by all the HDAC inhibitors in all the systems where it is silenced through promoter deacetylation. GADD45β is thought to be responsible for the cell cycle arrest in G2/M that HDACs inhibitors induce under certain circumstances. JUND is a transcription factor belonging to the AP-1 family. Its ability to slow cell growth has been reported. *IGFBP3* codes for a protein not only involved in modulation of IGF signalling but also in growth arrest or apoptosis in different cells, such as our model MCF7. *Metallothionein IX* and *2A* are part of the metallothionein cluster (chromosome 16), constituted by genes coding for proteins that are involved in the metabolism of transition metal ions either essential (zinc, copper) or toxic (cadmium, mercury). Depending on the type of tumor, different profiles of overexpression or downregulation of metallothioneins are found. These proteins could also be responsible for the resistance to some antineoplastic treatments like cisplatin and radiation.

6.2.5.2. Selective response of gene expression to HDACs inhibitors

The changes in transcription were analysed for all the 6 genes by semiquantitative RT-PCR after culturing the cells for 24 h with the different chemicals at the IC_{50} dose. The house-keeping gene *GAPDH* was used as load control. The results are shown in Fig. 28A. Under the employed PCR conditions, *CDKN1A* mRNA is not detected in the untreated cells, but all of the inhibitors induce its expression in similar extents. Transcription of *JUND* is similarly increased, but in this case CY is much less efficient than the other drugs. Except for MS-275, all the chemicals enhance the expression of *GADD45β* and *IGFBP3*, however the response of the latter gene seems to be more drug-dependent. The transcription of *MT1X* and *MT2A* seem to be unaltered by the addition of the chemicals to the cells.

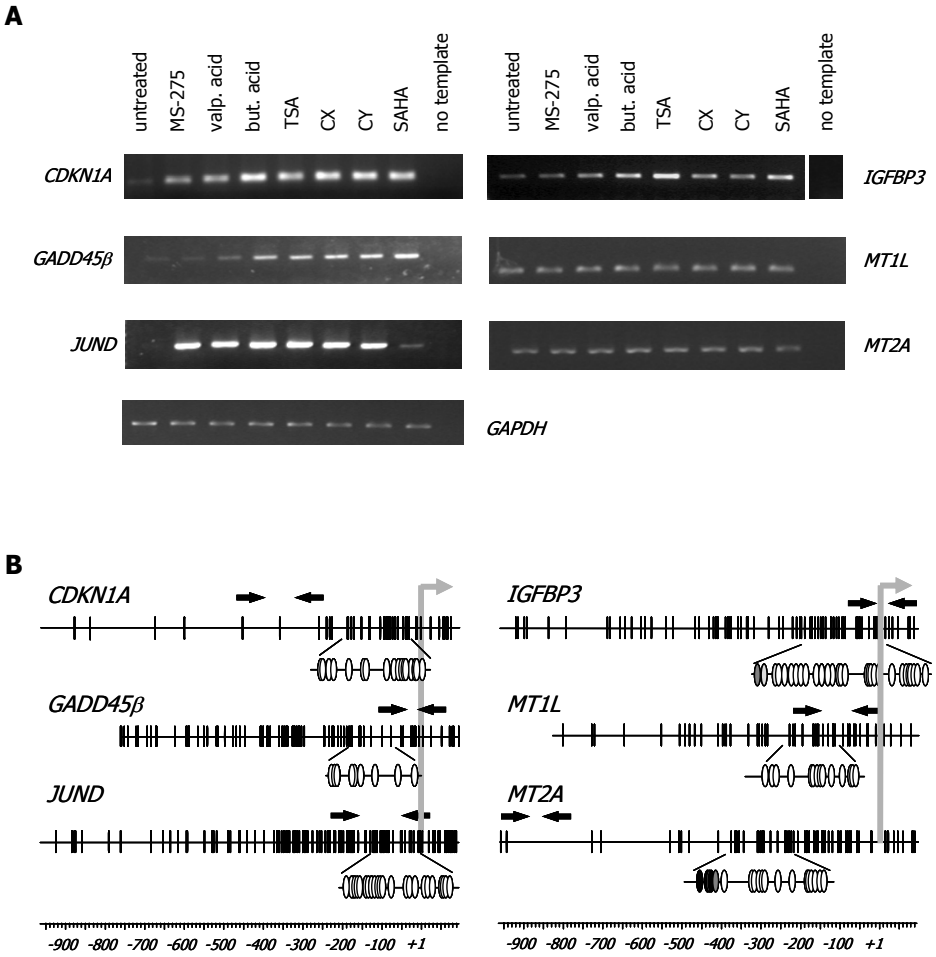


Fig. 28: Analysis of the expression and promoter methylation of *CDKN1A*, *GADD45β*, *JUND*, *IGFBP3*, *MT1X* and *MT2A*. **A**) Results of the semiquantitative RT-PCR. MCF7 cells were treated for 24 h with the seven compounds at the IC50 concentration. GAPDH was used as load control. **B**) Maps showing the promoter areas of the six analysed genes. Transcription start is indicated by the grey arrow and the position of the oligos employed for ChIP, by the black arrows. The result of the bisulfite genomic sequencing analysis for each gene is summarized in the graphics with circles: each circle represents a CpG dinucleotide, white if methylation is not detected and black if the cytosine residues are fully methylated.

6.2.5.3 None of the studied genes is silenced by DNA hypermethylation

The promoters of *CDKN1A*, *GADD45β*, *JUND*, *IGFBP3*, *MT1X* and *MT2A* are CpG islands. As indicated in the Introduction, the hypermethylation of CpG islands associated to promoters causes gene silencing. To know whether this phenomenom contributes in MCF7 cells to the silencing of the six studied genes, the corresponding promoters were analysed by bisulfite genomic sequencing. The result shows clearly that all of them are devoid of 5-methylcytosine. Maps of the sequenced regions are displayed in Fig. 28B.

6.2.5.4. HDACs inhibition alters HDAC2 recruitment and histone modifications at gene promoters

Finally, chromatin immunoprecipitation assays were carried out to evaluate the possible alterations at the promoters of these genes caused by the drugs. In order to reduce the number of samples to handle, only untreated cells ("control" cells) and cells grown for 24 h in the presence of 1.0 μM CY were compared.

It has been discussed in section 3.1.6 that the epigenetic mechanisms interact with each other. Thus, the presence of histone modifications different than acetylation could also be affected by HDACs inhibition. Therefore, we not only analysed the association of tetraacetylated H4 to the promoters, but also the presence of dimethylated K4H3 and K9H3 before and after CY treatment. The locations of the primers used for each gene are shown in the maps in Fig. 28B. For all the promoters studied, treatment with CY results in an increase of the two modifications associated with transcriptionally active chromatin: acetylated H4 and dimethylated K4H3. The signals indicating the presence of dimethylated K9H3 in the promoters of both *MT1X* and *MT2A* disappear upon CY treatment (Fig. 29).

The binding of HDAC1 and HDAC2 to the promoters was also studied, in order to see if there was any release of these enzymes due to the inhibition. The result showed that HDAC2 disappears from most of the promoters (*CDKN1A*, *JUND*,

IGFBP3, *MT1X* and *MT2A*) in the cells treated with CY. The presence of HDAC1 is only visible at the MT2A promoter (Fig. 29).

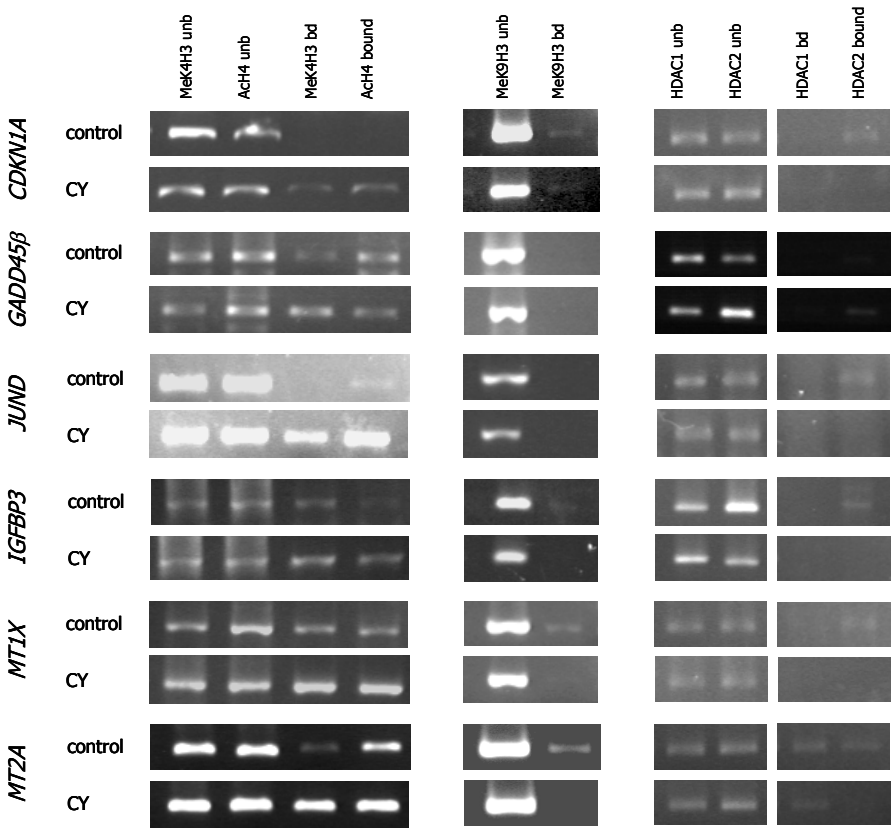


Fig. 29: ChIP analysis of the changes in histone modifications and HDACs recruitment at the promoters of *CDKN1A*, *GADD45β*, *JUND*, *IGFBP3*, *MT1X* and *MT2A* upon CY treatment. Immunoprecipitations were performed with anti-dimethylated K4H3 (MeK4H3), anti-tetraacetylated H4 (AcH4), anti-dimethylated K9H3 (MeK9H3), anti-HDAC1 and anti-HDAC2 antibodies. "control" are the untreated cells, "CY" are the samples treated with 1 μM CY, "bd" means bound fraction and "unb", unbound fraction.

7. Discussion

7.1. Procaine: a novel DNA demethylating agent

7.1.1. Possible mechanisms of procaine-induced DNA demethylation

Procaine is a substance that reduces the content of 5-methylcytosine in genomic DNA of MCF7 cells. However, the behaviour of this chemical is different than that of zdC. Firstly, after 3 days of treatment, doses of procaine as high as 0.5 mM are not as potent in decreasing DNA methylation as micromolar concentrations of zdC (Fig. 15 and 16). Second, the evolution of 5-methylcytosine proportion in DNA along the 3 days of treatment is very different from one drug to the other one: whereas zdC treatment results in a continuous strong reduction in DNA methylation, procaine (also procainamide) reaches its maximum effect between the first and the second day and,after that,the amount of 5-methylcytosine remains constant (Fig.17).

The different behaviour can be explained by considering that zdC and procaine induce DNA demethylation through different mechanisms. As mentioned in the introduction, zdC is a deoxynucleoside that becomes phosphorylated in the cell and the resulting dNTP is incorporated into the DNA during the S phase of the cell cycle (Taylor and Jones 1982). Once incorporated into DNA, zdC inhibits the DNMTs by trapping covalently these enzymes (reviewed in Christman 2002). Although the mechanism of procaine action is not clear, since this chemical is not a nucleoside or derivative, it seems obvious that its activity does not come from its incorporation into

DNA. Thus, contrary to zdC, procaine does not need any DNA replication round to be able of inhibiting the DNMTs. This fact could explain why procaine reaches its maximum effect faster than zdC.

Procainamide, structurally related with procaine (see Fig. 9), inhibits DNA methylation *in vitro*, probably due to DNA-competitive inhibition of the DNMT activity (Scheinbart *et al.* 1991, Cornacchia *et al.* 1988, Deng *et al.* 2003). Procainamide and also hydralazine bind naked DNA (either linear or circular) and induce B to Z transitions in DNA structure. The alteration of DNA structure could interfere with protein-DNA interactions, including DNMTs activity (Thomas and Messner 1986, Zacharias and Koopman 1990). Since both procaine and procainamide are substances with a very similar chemical structure, it can be suggested that both act through the same mechanism.

However, demethylation can be induced by other mechanisms different than DNMT enzymatic activity inhibition. Hydralazine produces a decrease in DNMT1 and DNMT3A expression by affecting the MAPK pathway. This is thought to be the mechanism by which this chemical induces genomic DNA demethylation, rather than the alteration of DNA structure that hydralazine causes *in vitro* (Deng *et al.* 2003). It has not been studied if procainamide or procaine have a similar effect on DNMTs expression levels.

In any case, the fact that procaine is not incorporated into DNA could be an advantage over zdC. As described in the introduction, zdC incorporated into DNA is mutagenic due to the lability of the 5-azacytosine ring and to the covalent trapping of DNMTs.

7.1.2. Low procaine concentrations cause demethylation of tumor-suppressor genes

Procaine causes demethylation of *RARβ2* promoter and restores the expression of this gene. However, the concentrations of drug required to achieve these two effects are higher for procaine than for zdC (Fig. 19A and C). Bisulfite genomic sequencing shows that 0.5 mM procaine is not able of inducing as much demethylation as 5.0 μM zdC (Fig. 19B).

Similarly, procainamide (the analog of procaine) reduces promoter 5-methylcytosine content in other cancer cell lines and even in murine models. That is the case of the genes encoding for 11β-hydroxysteroid dehydrogenase type 2 (Alikhani-Koopaei *et al.* 2004) and glutathione S-transferase P (*GSTP1*) (Lin *et al.* 2001). Moreover, procainamide causes demethylation of promoters of several tumor suppressor genes in cancer patients (Segura-Pacheco *et al.* 2003).

One of the major drawbacks for the use of DNA demethylating agents as chemotherapy drugs is that global DNA hypomethylation causes chromosomal instability (see 3.2.2.3). The fact that low concentrations (10-50 μM) of procaine induce tumor-suppressor genes expression and promoter demethylation without major changes in global DNA methylation could be an advantage for a possible use of procaine as anticancer agent.

7.1.3. Causes for cell cycle arrest: global DNA-demethylation vs. tumor-suppressor genes reactivation

In 3-days treatments, procaine has a stronger effect on MCF7 cells proliferation than procainamide but weaker than zdC (Fig. 19). The absence of TUNEL-positive cells and the increase in the proportion of mitotic figures after treating the cells with 0.5 mM procaine suggest that this substance triggers mitotic arrest in MCF7 rather than apoptosis (Fig. 19 and 20). Procainamide also arrests MCF7 cell cycle in mitosis (19% mitotic index), but less efficiently than procaine does (32% mitotic index).

The alterations induced by procaine and procainamide in cell cycle may be mediated by the reactivation of tumor suppressor genes silenced through hypermethylation, by the defects in chromatin condensation due to global DNA hypomethylation or by side effects unrelated to its demethylating properties. These three possibilities are not excludent. The two first ones were already discussed in section 3.3. Regarding the third one, both procaine and procainamide are known ion channel blockers, thus these chemicals can interfere with metabolic reactions different than the epigenetic mechanisms.

7.2. Comparative study of seven HDACs inhibitors

7.2.1. Structure-activity relationship for the in vitro inhibition of HDACs

In order to compare the HDAC inhibitory capacity of valproic acid, butanoic acid, MS-275, TSA, SAHA, CX and CY, we performed *in vitro* assays using MCF7 nuclear extracts as source of HDACs and hyperacetylated free histones as substrate. As indicated in the introduction, HDAC1, HDAC2, HDAC3 and HDAC8 are the class I HDACs and all of them are nuclear proteins. Additionally, class II enzymes can be present in the nucleus. Thus, in the MCF7 nuclear extract there are several Zn(II)-dependent HDACs. Although the catalytic domain of these enzymes is highly conserved, its amino acid composition varies slightly among them. As result, the HDACs inhibitors show different degrees of specificity for the different HDACs (see Table 9). For instance, HDAC4 is less senstitive to TSA than HDAC1 is (Furumai *et al.* 2001); HDAC3 is less sensitive to MS-275 than HDAC1, HDAC4 or HDAC6 (Hu *et al.* 2003, Glaser *et al.* 2004); and the concentration of SAHA and TSA necessary to inhibit HDAC8 is higher than the concentration to inhibit HDAC1 or HDAC3 (Vannini *et al.* 2004). Moreover, not in all the enzymes Zn(II) cation is accessible for a bulky functional group like the benzamide. For example, recent molecular modelling and crystallographic data show that the catalytic pocket of HDAC8 is narrower than that one of the other HDACs (Schultz *et al.* 2004,Vannini *et al.* 2004, Somoza *et al.* 2004). This finding explains why MS-275 inhibits HDAC1 and HDAC3, enzymes with a wide catalytic tube, but not HDAC8 (Vannini *et al.* 2004). In summary, not all the HDACs present in MCF7 nuclear extract are inhibited to the same extent by each drug.

In our assay, all the seven substances inhibit the HDAC activity contained in the nuclear extracts, with a clear structure-activity relationship (Fig. 21A). According to the results of the *in vitro* activity assay, the chemicals can be grouped in 3 sets. The first one is constituted by the hydroxamic acids, TSA, SAHA, CX and CY, which

are effective at low micromolar concentrations. The chemicals in the second group, the small carboxylic acids, are active at millimolar concentrations. Finally the benzamide MS-275 has an intermediate inhibitory capacity since 50 μM MS-275 reduces only 40% the activity of the whole nuclear extract. However, 50 μM MS-275 suppresses completely the activity of immunoprecipitated HDAC1 (Fig. 21).

In Table 9 there is a summary of representative *in vitro* data published for these substances. The reported results for valproic acid inhibition of HeLa nuclear extracts are compatible with the values we obtained (Phiel *et al.* 2001). In the case of butanoic acid, the IC_{50} values found in the literature for *in vitro* assays are lower than the ones obtained in our system (see Table 9). Also for the hydroxamic acids (TSA, SAHA, CX and CY) most of the published inhibitory concentrations are one or two orders of magnitude lower than the ones found in this study. We could only observe partial inhibition with 50 μM MS-275, whereas most of the reported inhibitory concentrations for MS-275 range between 0.3 and 6.0 μM (see Table 9 for details). All these discrepancies can be due to the different experimental systems employed: for this work whole nuclear extracts were used and, in the literature, either pure HDACs (recombinant or endogenous) or partially purified nuclear extracts were the source of the HDAC activity. The whole nuclear extracts may contain proteins or factors that influence HDAC activity and which are not present in the reactions with recombinant, immunoprecipitated or purified HDACs. Also the existence of several HDACs in the nuclear extracts may affect the reaction. For instance, HDAC8 may account for the poor effect of 50 μM MS-275 in our nuclear extract (Vannini *et al.* 2004). Moreover, in some reported experiments, the substrates for the enzymes were not histones but synthetic peptides. The variability of the HDAC catalytic domain may also be responsible for different substrate specificity, resulting in different kinetic constants for each HDAC-substrate couple.

Despite the differences, there are some common features between our results and the published data. In all the reported studies the concentration of butanoic acid necessary for the significant HDAC inhibition is in the same order of magnitude than the concentration of valproic acid and about 4 orders of magnitude higher than the concentration of hydroxamic acids, like in our experiments. Also, the required concentration of MS-275 is higher than that one of the hydroxamic acids

and SAHA is slightly less efficient than TSA both in the literature and in our experiments (Remiszewski *et al.* 2003, Vannini *et al.* 2004).

Table 9: Representative data of *in vitro* HDACs inhibition

Drug	Activity (1)	Substrate	$IC_{50}/\mu M$	References
valproic acid	HeLa nuclear extract	histones from HeLa	500-2000	Phiel *et al.* 2001
valproic acid	HeLa endogenous HDAC1	histones from HeLa	400	Phiel *et al.* 2001
butanoic acid	Jurkat endogenous HDAC1	H4-tail peptide	100	Emiliani *et al.* 1998
butanoic acid	Jurkat endogenous HDAC3	H4-tail peptide	100	Emiliani *et al.* 1998
butanoic acid	partially purified FM3A n.e.	histones	120	Kim *et al.* 1999
MS-275	partially purified K562 n.e.	histones from K562	1.5-2.0	Saito *et al.* 1999, Glaser *et al.* 2004
MS-275	rec. Flag-tagged HDAC1	histones	1.2	Fournel *et al.* 2002
MS-275	rec. His-tagged HDAC1	H4-tail peptide	0.3	Hu *et al.* 2003
MS-275	rec. His-tagged HDAC3	H4-tail peptide	8.0	Hu *et al.* 2003
MS-275	rec. His-tagged HDAC8	H4-tail peptide	>100	Hu *et al.* 2003
MS-275	HDAC1/2 from K562	H4-tail peptide	1.5	Glaser *et al.* 2004
MS-275	rec. His-tagged HDAC3	H4-tail peptide	6.1	Glaser *et al.* 2004
MS-275	rec. His-tagged HDAC4 [2)]	H4-tail peptide	0.8	Glaser *et al.* 2004
MS-275	rec. His-tagged HDAC6	H4-tail peptide	0.6	Glaser *et al.* 2004
MS-275	partially purified K562 n.e.	histones from K562	2.4	Li *et al.* 2004
MS-275	rec. His-tagged HDAC1	histones from K562	6.0	Li *et al.* 2004
MS-275	partially purified H1299 n.e.	biot. H4-tail peptide	>10	Remiszewski *et al.* 2003
MS-275	rec. Flag-tagged HDAC1	histones	0.18	Vannini *et al.* 2004
MS-275	rec. Flag-tagged HDAC3	histones	0.20	Vannini *et al.* 2004
MS-275	rec. His-tagged HDAC8	histones	>10	Vannini *et al.* 2004

Table 9 (cont.)

Drug	Activity (1)	Substrate	IC_{50}/μM	References
TSA	Jurkat endogenous HDAC1	H4-tail peptide	0.002	Emiliani *et al.* 1998
TSA	Jurkat endogenous HDAC3	H4-tail peptide	0.002	Emiliani *et al.* 1998
TSA	partially purified FM3A n.e.	histones	0.0014	Kim *et al.* 1999
TSA	rec. His-tagged HDAC1	H4-tail peptide	0.1-0.3	Hu *et al.* 2003
TSA	rec. His-tagged HDAC3	H4-tail peptide	0.1-0.3	Hu *et al.* 2003
TSA	rec. His-tagged HDAC8	H4-tail peptide	0.1-0.3	Hu *et al.* 2003
TSA	partially purified K562 n.e.	histones from K562	0.004	Li *et al.* 2004
TSA	rec. His-tagged HDAC1	histones from K562	0.001	Li *et al.* 2004
TSA	rec. HDAC1	histones from Jurkat	0.005	Woo *et al.* 2002
TSA	rec. Flag-tagged HDAC1	histones	0.006	Furumai *et al.* 2001
TSA	rec. Flag-tagged HDAC4	histones	0.04	Furumai *et al.* 2001
TSA	rec. HA-tagged HDAC6	histones	0.009	Furumai *et al.* 2001
TSA	partially purified H1299 n.e.	biot. H4-tail peptide	0.026	Remiszewski *et al.* 2003
TSA	rec. Flag-tagged HDAC1	histones	0.001	Vannini *et al.* 2004
TSA	rec. Flag-tagged HDAC3	histones	0.0006	Vannini *et al.* 2004
TSA	rec. His-tagged HDAC8	histones	0.49	Vannini *et al.* 2004
SAHA	partially purified K562 n.e.	histones from K562	0.1	Glaser *et al.* 2004
SAHA	HDAC1/2 from K562	H4-tail peptide	0.03	Glaser *et al.* 2004
SAHA	rec. His-tagged HDAC3	H4-tail peptide	0.04	Glaser *et al.* 2004
SAHA	rec. His-tagged HDAC4 (3)	H4-tail peptide	0.04	Glaser *et al.* 2004
SAHA	rec. His-tagged HDAC6	H4-tail peptide	0.06	Glaser *et al.* 2004
SAHA	partially purified K562 n.e.	histones from K562	0.12	Li *et al.* 2004
SAHA	rec. His-tagged HDAC1	histones from K562	0.15	Li *et al.* 2004
SAHA	rec. His-tagged HDAC3	histones from K562	0.12	Li *et al.* 2004
SAHA	partially purified H1299 ne	biot. H4-tail peptide	0.2	Remiszewski *et al.* 2003
SAHA	Rec. Flag-tagged HDAC1	histones	0.12	Vannini *et al.* 2004
SAHA	Rec. Flag-tagged HDAC3	histones	0.11	Vannini *et al.* 2004
SAHA	Rec. His-tagged HDAC8	histones	4.0	Vannini *et al.* 2004
CX	HeLa nuclear extract	H4-tail peptide	0.027	XXXXXXXXXXXXXX
CY	Partially purified H1299 n.e.	biot. H4-tail peptide	0.03	YYYYYYYYYYYYYY

(1) Rec. stands for recombinant, n.e. for nuclear extract, biot. for biotinylated. All the mentioned cell lines are human, except for FM3A, which is derived from mouse. (2) HeLa cells are used to express HDAC4. After the purification, some endogenous HDAC3 remains in the preparation.

7.2.2. Effects on global histone acetylation may depend on the inhibition of only a subset of HDACs

As determined by Western blots with anti-diacetylated H3 and anti-tetraacetylated H4 antibodies as well as by HPCE analysis of the purified histones, all the seven chemicals induced a similar degree of hyperacetylation of both H4 and H3 when employed at the IC_{50} concentration for 24 h treatments in MCF7 cells (Fig. 24 and 26). Surprisingly, H3 and H4 histone hyperacetylation caused by 5 μM MS-275 is comparable to that one caused by the other drugs when used at their respective IC_{50}. Considering that 5 μM MS-275 is not able to inhibit significantly *in vitro* the HDAC activity contained in MCF7 nuclear extracts, the H3 and H4 hyperacetylation induced by 5 μM MS-275 strongly suggests that only a subset of Zn(II)-dependent HDACs is responsible for most of the deacetylation of H3 and H4 in MCF7 cells, and that this function cannot be replaced by other enzymes.

All the drugs seem to have similar efficiency in inducing the acetylation of H3, but the response of H4 varies slightly. Some substances, such as MS-275, CY and TSA, induce a higher proportion of tri- and tetraacetylated species than others, namely SAHA, CX and valproic acid. Many reasons can be argued to explain these differences, from the different affinity of the chemicals for specific HDACs that could play different roles in H4 deacetylation, to different side-effects, the solubility of the drugs, etc.

There are no major differences in the proportion of non-, mono-, di-, tri- and tetraacetylated H4 induced by MS-275, butanoic acid and CY if the concentration of the drugs referred to their respective IC_{50} is the same (Fig. 25). Moreover, the plots for MS-275 and butanoic acid are almost superimposable.

In summary, according to the data obtained by HPCE and Western blotting with antibodies against diacetylated H3 and tetraacetylated H4, there is no clear structure-activity relationship between the chemicals and the effects on global histone H3 and H4 acetylation if the inhibitors are added to the cells at the same concentration (referred to the IC_{50}). Also, the induction of H3 and H4 hyperacetylation may not require the inhibition of all the Zn(II)-dependent HDACs.

7.2.3. Structure-activity relationship for the induced cell cycle arrest: effects on global acetylation vs. effects on gene expression

There are two non-excludent models to explain the mechanism by which HDACs inhibitors cause cell cycle arrest and apoptosis. As described in the introduction, the first one considers that these effects are due to the induced overexpression of tumor suppressor genes. The second one proposes that the disruption of the chromatin structure caused by bulk histone hyperacetylation triggers the mechanisms for cell cycle control and apoptosis.

In our experiments, in general, the concentrations for the inhibition of cell growth after 24 h treatment and that ones necessary for the inhibition of the activity in nuclear extracts were similar (millimolar concentrations for butanoic and valproic acids and micromolar concentrations for the hydroxamic acids), except for MS-275. Cells were more sensitive to MS-275 than the nuclear extracts (Fig. 22A). This fact can be attributed to the presence, in the nuclear extracts, of Zn(II)-dependent HDACs that MS-275 cannot inhibit, like HDAC8. The inhibition of a subset of HDACs seems to be sufficient to alter global H3 and H4 acetylation. Therefore, according to the mentioned models, the induced hyperacetylation could be sufficient to induce cell growth arrest either by disrupting the chromatin structure or by inducing overexpression of tumor-suppressor genes or both. Another possibility could be that MS-275 interferes in the function of other proteins affecting, at least to some extent, cell growth.

Different degrees of cell cycle arrest either in G1/G0 or in G2/M have been reported for these drugs, depending on the studied cell line and the dose of the compound. For instance, butanoic acid and TSA were found to inhibit G1 to S transition in HeLa cells (Finzer *et al.* 2001) but in A549 these chemicals caused predominantly G2 and M arrest, respectively (Blagosklonny *et al.* 2001); CY was reported to stop leukaemia cells in G1/G0 (Weisberg *et al.* 2004), etc. In our experiments, except for MS-275, all the studied drugs stopped the cell cycle in the G2/M phase (Fig. 22B and C). The distribution of the cells in the different steps of the cell cycle was very similar for all the hydroxamic acids (near 50% in G1/G0 and

around 40% in G2/M). The carboxylic acids showed a slightly different effect: in addition to the G2/M arrest, the maintenance of the proportion of cells in G1/G0 (65-70%) together with the reduction in the S phase suggests a G1/G0 arrest. MS-275 produced accumulation of cells mainly in G1/G0. Consequently, there is a relationship between the structure of the chemical and its effects on the cell cycle. As already discussed, there are 11 known human Zn(II) dependent HDACs and some of them have splicing variants. Although small, these differences between the enzymes make possible that the HDAC inhibitors have different specificities for different HDACs. As result, even though the effects of the chemicals on bulk histone H3 and H4 acetylation are identical, subtle differences in the induced acetylation patterns may arise and cause differential gene expression profiles which would account for the diversity of effects on the cell cycle. Additionally, some HDACs have targets different than histones, like p53 or α-tubulin.

As widely reported in the literature, all the seven chemicals compared in this work restore the expression of *CDKN1A* and all except for MS-275 induce *GADD45β*. According to Hirose *et al.* (2003), recovery of *CDKN1A* transcription by HDACs inhibitors is responsible for the arrest in G1/G0, whereas the arrest in G2/M can be due to the induction of *GADD45β*. The data presented here fit with that hypothesis.

7.2.4. Histone hyperacetylation is not sufficient to increase gene expression

As mentioned in sections 6.2.5 and 7.2.3., all these chemicals induce the expression of *CDKN1A* (Fig. 28A). The unique compound which does not cause the increase of *GADD45β* transcription is MS-275 (Fig. 28A). In addition, all the drugs reactivate *JUND* but CY in much less extent than the others (Fig. 28A). *JUND* was found to be repressed through recruitment of mSinA3 complex to its promoter (Kim *et al.* 2003). The expression of *IGFBP3* is increased by all the compounds except for MS-275 (Fig. 28A). In the literature it is found that TSA and butanoic acid cause significant *IGFBP3* overexpression in MCF7 cells (Walker *et al.* 2001).

In the conditions used in this work, no alteration in the mRNA levels of *MT1X* and *MT2A* was appreciated (Fig. 28A). It is reported that in lymphosarcoma

cells *MT1* is not expressed and acetylation of histones caused by HDACs inhibitors is not enough to recover the expression because its promoter is methylated. Demethylation due to zdC treatment is also not sufficient and both HDACs inhibitors and demethylating agents are necessary to restore the expression (Ghoshal *et al.* 2002). In other cases (such as the lung cancer cell line H460 or the breast cancer cells T24, MDA-468 and MDA-435), HDAC inhibition is enough to increase the expression of *MT1X* and *MT2A* (Joseph *et al.* 2004, Glaser *et al.* 2003). The promoters of the six genes we studied contain CpG islands. Genomic bisulfite sequencing revealed that all of them are devoid of 5-methylcytosine in MCF7 cells (Fig. 28B). In consequence, aberrant DNA hypermethylation can not explain the insensitivity of the expression of *MT1X* and *MT2A* towards HDACs inhibition.

Next, the alterations that HDACs inhibition causes on histone modifications and HDAC recruitment at the promoters were analysed by chromatin immunoprecipitation (Fig. 29). Samples treated with 1 μM CY were compared to untreated cells. Firstly, possible changes in the acetylation of H4 were studied by using an antibody raised against the tetraacetylated H4 tail. As expected, there is an increase of acetylation in H4 for all the genes, including *MT1X* and *MT2A*. In some cases (*JUND, IGFBP3*) the differences are much stronger than in others (*CDKN1A*, *MT1X* and *MT2A*).

Methylation in lysine 4 of H3 (K4H3) is considered to be a mark of transcriptionally active chromatin (Jiang *et al.* 2004, Noma *et al.* 2001, Schneider *et al.* 2004), being associated to mRNA elongation by RNA polymerase II in yeast (Ng *et al.* 2003) or induced by the Paf1 complex in Drosophila (Krogan *et al.* 2003b). Recently, it has been described that di- and trimethylation of K4H3 colocalizes with acetylation in K9H3 and K14H3 in the promoter region of the transcriptionally active genes (Liang *et al.* 2004). So, in order to see if methylation of K4H3 at the promoter of the six genes was altered after HDACs inhibition, ChIP using anti-dimethylK4H3 antibody was performed. The enrichment of this modification at the promoter in cells cultured with CY was confirmed for all the six genes. This fact constitutes an additional evidence of the crosstalk existing between different histone modifications: acetylation of lysine residues induces an increase of K4H3 dimethylation. The opposite relationship, methylation of K4H3 influencing histone H3 acetylation, has

been reported. Methylation of K4H3 precludes lysine deacetylation by displacing NuRD (a complex containing HDAC1 and HDAC2) from H3 tail (Nishioka *et al.* 2002, Zegerman *et al.* 2002).

On the other hand, methylation (di- and tri-) of lysine 9 in H3 (K9H3) is related with heterochromatin and transcriptional repression (Stewart *et al.* 2005, Scotta *et al.* 2002, Nakayama *et al.* 2001, Rea *et al.* 2000). Also methylation of K9H3 by SUV39H1 is impaired by methylation in K4H3 (Nishioka *et al.* 2002) and it was suggested that HDACs could recruit SUV39H1 to nucleosomes (Vaute *et al.* 2002). In our case, we only found dimethyl K9H3 in the promoter of a few genes in untreated cells, but after 24 h treatment with CY, that mark disappeared. This might be caused directly by histone acetylation but could also be a consequence of K4H3 methylation or of the loss of HDACs recruited to the promoter. Further studies are necessary to determinate the sequence of these modifications.

It is also remarkable that dimethylated K9H3 is only clearly present in the promoters of *MT1X* and *MT2A*, the only genes in the studied set whose expression is not altered by the treatment with HDACs inhibitors. It could be that this mark is associated with other transcriptional regulators that are not released from the promoters in our experiment but are responsible of keeping the expression of these genes unaltered during the 24 h drug treatment. Another possibility is that the elongation cannot proceed even though the changes in the promoters of these genes upon HDAC inhibition allow the assembly of the transcription machinery. Histone acetylation may not be sufficient for the release of repressor complexes or for the recruitment of elongation factors to the transcribed region.

Finally, it seems that upon CY treatment, HDAC2 leaves the promoters where it was present, as it happens in other cases with other HDACs inhibitors (Duan *et al.* 2005).

8. Conclusions

Procaine: a novel DNA demethylating agent

1. Procaine reduces global DNA methylation, reaching its maximum effect in MCF7 cells between the first and the second day of treatment. However, when compared to 5-aza-2'-deoxycitidine, procaine effects are milder.
2. Procaine reactivates genes that are silenced by DNA hypermethylation. The concentration of drug to restore gene expression only causes a slight reduction in the proportion of 5-methylcytosine on global DNA.
3. Procaine reduces cell cycle proliferation and induces cell cycle arrest at mitosis.
4. All these properties together with the fact that procaine is not incorporated into DNA make procaine a potentially interesting drug for cancer chemotherapy.

Comparative study of seven HDACs inhibitors

5. TSA, SAHA, PXD101, NVP-LAQ824, MS-275, valproic acid and butanoic acid inhibit Zn(II)-dependent HDACs. Their effects on HDAC activity *in vitro* and on cell cycle show a tight structure-activity relationship. The carboxylic acids are the least efficient compounds.
6. All the seven chemicals increase in to a similar extent the global acetylation of H3 and H4 in cultured cells.
7. None of the seven inhibitors alter the protein concentration of HDAC1 or HDAC2 in the cell.
8. The different effects of the HDACs inhibitors in the expression levels of *CDKN1A* and *GADD45β* may be related with the distinctive effects on cell cycle arrest: MS-275 induces arrest in G1/G0 and transcription of *CDKN1A*, but not *GADD45β*;

whereas the rest of drugs induce arrest in G2/M and overexpression of both *CDKN1A* and *GADD45β.*

9. Not all the compounds reactivate the same set of genes.
10. The expression of *MT1X* and *MT2A* is insensitive to any of these treatments, even though the histones associated with their promoters become hyperacetylated and their respective promoters do not exhibit DNA hypermethylation.
11. Inhibition of HDACs causes an increase in acetylated H4 associated at the promoters of the six studied genes.
12. Inhibition of HDACs also results in the accumulation of dimethylated lysine 4 of H3 (a mark associated with gene activation) and the loss of dimethylated lysine 9 of H3 (a mark associated with silent chromatin) in the promoters of the studied genes where this modification was present. This fact supports the existence of a crosstalk between different histone modifications.

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10. Publications

The work of this thesis is reflected in the following publications:

Villar-Garea A, Esteller M. (2004) *Histone deacetylase inhibitors: understanding a new wave of anticancer agents.* **Int J Cancer** 112(2):171-8.

Villar-Garea A, Fraga MF, Espada J, Esteller M. (2003) *Procaine is a DNA demethylating agent with growth-inhibitory effects in human cancer cells.* **Cancer Res** 63(16):4984-9.

Villar-Garea A, Esteller M. (2003) *DNA demethylating agents and chromatin remodelling drugs: which, how and why?* **Curr Drug Metab** 4(1):11-31.