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Natural products and chemical biology tools: alternatives to target epigenetic mechanisms in cancers

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Abstract

DNA methylation and histone acetylation are widely studied epigenetic modifications. They are involved in numerous pathologies such as cancer, neurological disease, inflammation, obesity, etc. Since the discovery of the epigenome, numerous compounds have been developed to reverse DNA methylation and histone acetylation aberrant profile in diseases. Among them several were inspired by Nature and have a great interest as therapeutic molecules. In the quest of finding new ways to target epigenetic mechanisms, the use of chemical tools is a powerful strategy to better understand epigenetic mechanisms in biological systems. In this review we will present natural products reported as DNMT or HDAC inhibitors for anticancer treatments. We will then discuss the use of chemical tools that have been used in order to explore the epigenome.

1/ Introduction

Epigenetic modifications, including DNA methylation and histone acetylation, have attracted plenty of attention for several years in the field of anticancer therapies. These modifications are deregulated in cancer leading to silencing of tumour suppressor genes and therefore participating to tumour formation and proliferation.[1, 2] Efforts have been put into the discovery of inhibitors of DNA methyltransferase (DNMT) and histone deacetylase (HDAC), the enzymes responsible for these modifications. Although four HDAC inhibitors (HDACi) are today approved by the FDA (vorinostat, panabinostat, belinostat,

romidepsin) and one by the Chinese CFDA (chidamide), the discovery of isoform-selective inhibitors is necessary in order to target more efficiently and selectively the HDACs. Regarding DNMT inhibitors (DNMTi), two compounds are FDA approved for treatment of leukaemia, 5-azacytidine (5azaC) and 5-aza-2-deoxycytidine (5azadC).[3, 4] They are both nucleoside analogues that need to incorporate into DNA/RNA to be active. This incorporation is neither site- nor cell-specific, which can lead to important secondary effects. Additionally, their instability limits their use. Research efforts aimed at identifying non-nucleoside DNMTi,[5] but to date none of them has showed enough potency to be considered as drug candidate.

In this context, natural products constitute a vital source to find novel chemical scaffolds as HDACi or DNMTi. Because living beings are constantly battling for survival, often against living being of different species or even different life kingdoms altogether, it is hypothesized that there is an evolutionary pressure for the generation of secondary metabolites that can act as antibiotics, antifungals, phytoalexins, etc.,[6] or have no evident utility altogether.[7] Because of this demanding task, Nature produces an overwhelming number of structures, with beautiful complexity and diversity, which interact with a variety of enzymes and proteins in different types of organisms. Researchers have successfully taken advantage of the chemical scaffolds optimized by Nature to design new drugs.[8, 9] One of the main challenges of the use of natural products is the identification of “hits”, as natural products are most of the time found in complex mixtures and in minute amounts. However new techniques for high-throughput screening are continuously being reported and there is good hope that they will allow facilitate drug discovery.

Additionally, the use of chemical tools scanning the epigenetic space directly in the relevant biological system can constitute a powerful strategy. These chemical biology strategies can be highly valuable to either better understand the mode of action of natural products in cells or to identify new epigenetic pathways specific of a pathological context and lead to the discovery of new epi-targets.

In this review, we will first report the natural products that were identified as HDACi and DNMTi and more briefly for other epi-targets. We restricted the scope of this review to isolated single compounds that could be used as new drug or new hit for drug discovery without considering natural extracts.

Then we will describe how chemical tools were used in Epigenetics to explore the epi-drug mechanism of action and to identify new therapeutic targets.

2/ Histone deacetylase-modulating natural products

HDACs are divided in four different classes. Class I, II and IV are zinc-dependent enzymes with a highly conserved deacetylase domain and major residues differences at the entry of the active site.[10] Class I HDACs are comprised of nuclear enzymes HDAC1-3 and more distantly related HDAC8, and possess an additional internal cavity, which is believed to be used for water entry and acetic acid removal after the deacetylation reaction.[10] Class II HDACs are able to shuttle in and out of the nucleus and comprises HDAC4-7, 9 and 10, while HDAC 11 is the sole member of class IV. Class III consists in seven NAD^+-dependent enzymes, called sirtuins (SIRT1-7).

The ubiquity of HDACs and their crucial role in physiological processes and the large diversity of their substrates [11] – in addition to histones – lead therefore pan-inhibitors of HDACs to often show numerous undesirable side effects [12] making isoform selectivity a highly desirable property.

2.1 For HDAC inhibition (Class I and II)

The discovery, in the late 1970s,[13, 14] of the effect of sodium butyrate on histone acetylation is among the first evidence [15] pointing towards the involvement of small molecules of natural origin in the modulation of the acetylation of histones. Sodium butyrate was later shown to be a non-competitive inhibitor of HDAC,[16] and was followed by Trichostatin A in 1990,[17] Trapoxin in 1993,[18] and many others in the following years. Trapoxin, additionally, played a central role in the understanding of HDAC enzymes, as the irreversible nature of its binding to HDAC was used to design an affinity tag for the first isolation of a mammalian HDAC enzyme.[19, 20]

The large variety of natural products inhibiting HDACs can be roughly classified into different structural families and whether they bind the Zn atom found in the HDAC cavity or not.

Zinc-binding inhibitors

General structure

HDACi with a zinc-binding mode of action follow an almost ubiquitous structure, summarized graphically in Fig. 1. The main feature is the zinc-binding group (or ZBG), which can be a mono- or

bi-dentate ligand, binding the metal atom in a reversible or irreversible manner. Among the known ZBG are hydroxamic acids – found in the archetypal HDACi suberoylanilide hydroxamic acid (SAHA, vorinostat, Fig. 1) – thiols, epoxyketones, α-hydroxyketones, carboxylic acids, etc.. Because this pharmacophore mimics the substrate of HDAC enzymes,[21] namely acetyllysines, the ZBG is followed by a hydrocarbon linker, reminiscent of the aliphatic side-chain of lysine, fitting the internal channel found in HDAC composing the catalytic pocket, and topped by a cap. This cap can be simple, such as the aromatic ring of SAHA, or more complex surface-binding groups (*vide infra*). Because HDAC isoforms show significant differences in the surface grooves neighbouring the catalytic pocket, surface-binding groups are often used to achieve isoform selectivity. However, other interesting results demonstrating the influence of the linker,[22, 23]) the ZBG [24] and other new features in the pharmacophore [12] might be leading towards a paradigm shift.[25]

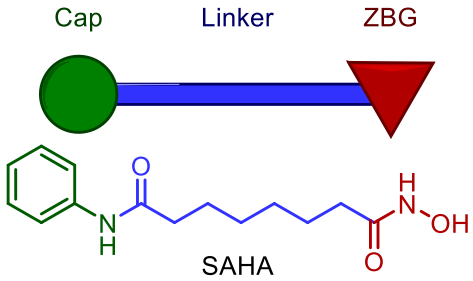


Figure 1. Common pharmacophore for zinc-binding HDACi.

Cyclic depsipeptides with hidden thiol (Fig. 2)

The reported cyclic depsipetides have complex cap group allowing for a more extensive recognition of the external parts of HDACs and thus a potentially better discrimination between isoforms. All are "prodrugs", where the active thiol is "hidden", either as disulfides that get reduced in the cell (usually by glutathione) or as a thioester in the case of largazole that gets hydrolysed.

Romidepsin

Romidepsin was isolated, under the name of FR901228, from *Chromobacterium violaceum* in 1994 by a team from Fujisawa Pharmaceutical Company [26] for its antitumor activity. Sometimes simply referred to as depsipeptide or FK228, romidepsin is one of the four HDACi, and the only one from natural sources, approved by the FDA for treatment of cutaneous [27] and peripheral T-cell

lymphoma.[28] At the time of the publication of this review, it is in clinical trials, alone or in combination with other drugs, in 15 other cases. (Table 1)

Table 1. Clinical trials involving romidepsin for cancer treatment

Treated disease	In combination with	Trial phase	Clinicaltrials.gov ID
Relapsed or refractory T-cell lymphoma	Duvelisib	I	NCT02783625
T-cell non-Hodgkin lymphoma	Chemotherapy, stem cell transplant	II	NCT01908777
Locally recurrent or metastatic triple negative breast cancer	Cisplatin, Nivolumab	I/II	NCT02393794
Lymphoma, chronic lymphocytic leukemia, or solid tumors with liver dysfunction	-	I	NCT01638533
Previously untreated peripheral T-cell lymphoma	Lenalidomide	II	NCT02232516
Relapsed or refractory aggressive lymphoma	Gemcitabine hydrochloride, oxaliplatin, dexamethasone	I	NCT02181218
Relapsed or refractory lymphoma or multiple myeloma	Pralatrexate	I/IIa	NCT01947140
Stage IB-IVB relapsed or refractory T-cell lymphomas	Pegylated liposomal doxorubicin hydrochloride	I	NCT01902225
Recurrent or refractory peripheral T-cell lymphoma	Pembrolizumab	I/II	NCT03278782
Peripheral T-cell lymphoma	Durvalumab, pralatrexate, oral azacitidine	I/IIa	NCT03161223
Relapsed or refractory peripheral T-cell lymphoma or stage IIB-IV cutaneous T-cell lymphoma	Volasertib	I	NCT02757248
Cutaneous T-cell lymphoma	Brentuximab vedotin	I	NCT02616965
T-cell leukemia or lymphoma undergoing donor stem cell transplant	-	I/II	NCT02512497
Microsatellite Stable Metastatic Colorectal Cancer	Azacitidine, pembrolizumab	I	NCT02512172
Stage IA-IVB cutaneous T-cell lymphoma	Carfilzomib	I	NCT01738594

Data obtained from https://www.cancer.gov/about-cancer/treatment/clinical-trials/intervention/romidepsin

Spiruchostatins

Spiruchostatins A and B were isolated in 2001 from *Pseudomonas sp*. by Masuoka *et al*., followed by spiruchostatin C in 2001 and D in 2004.[29] Spiruchostatins A-D all show low nanomolar IC_{50} values for HDAC1 and values in the hundreds for HDAC6, demonstrating high selectivity for class I HDAC.[29, 30] These values were obtained in a reducing environment (100 mM dithiothreitol) in order

to obtain the open dithiol, as unreduced spiruchostatin A lost almost all its inhibitory potency.[31] Crabb *et al*. reported in 2008 potent growth inhibition against four human cancer cell lines (MCF7 and BT474, breast; A2780, ovarian; HT29, colon), with IC_{50} values ranging from 1.2 for HT29 to 6.7 nM for BT474, while Narita *et al*. reported similar results on almost all of thirty-nine human cancer cell lines tested with spiruchostatin A and B in 2009,[30] and C and D in 2013.[29] The mean GI_{50} value over all the lines tested allows to give an order of potency: D (3.8 nM) > B (5.6 nM) > A (15 nM) > C (28 nM).[29]

Rehman *et al*. studied in 2014 the activity of spiruchostatins A and B in human lymphoma U937 cells and reported that both compounds induced apoptosis in the treated cells, resulting in an increase of the population of cells in sub-G_1 cell phase, as well as an increased acetylation of histones H3 and H4.[32] The authors determined that induced apoptosis was heavily dependent on the generation of reactive oxygen species (ROS) and that the inhibitors caused an increase in the expression of caspase-3 and 8, which play a key role in apoptosis signalling pathways.

Thailandepsins and burkholdacs

The first members of the thailandepsin family were isolated in 2011 from bacterium *Burkholderia thailandensis* by Cheng *et al*. as thailandepsins A and B,[33] and Biggins *et al*., as thailandepsins A and C (named burkholdac B and A respectively).[34] Thailandepsins D-F were later discovered by Wang *et al*. in 2012,[35] while Klausmeyer and coworkers isolated a compound they named spiruchostatin C in 2011.[36] However, considering the biological source of the latter, reason mandates to include it in the thailandepsin/burkholdac family. In this review we will use the nomenclature suggested by Narita *et al*., *i.e.* burkholdac C.[29]

Thailandepsins A-F strongly inhibit recombinant HDAC1 with IC_{50} values ranging from 0.2 to 2.6 nM (for forms C and F respectively), while being much weaker inhibitors for HDAC6 (0.67-1.92 μM) and HDAC4 (8.67-132 μM).[35] Burkholdac C showed similar trends against HDAC1, 4 and 8.[36] Wang *et al*. reported that thailandepsins A and B inhibit HDAC1-3 (class I) in the low nanomolar range, the distantly related HDAC8 (class 1) being only inhibited at low micromolar levels. HDAC6 (class IIB) showed better response, but thailandepsin A and B were not as active against HDAC4, 7, 9 (class IIA).

Additionally, the reduced dithiol form was more active compared to the non-reduced one, with a more than 10,000-fold increase for HDAC2.[33] Additionally, thailandepsins A-C were screened against an extensive panel of human cancer cell lines, with GI_{50} values at low nanomolar concentrations for over 90% of the tested lines.[33, 37]

Thailandepsin A was used against ovarian cancer cells by Wilson *et al*. in 2012,[38] anaplastic thyroid cancer (ATC) cells by Weinlander *et al*. in 2014,[39] and neuroendocrine (NE) cancer cell lines by Jang *et al*. in 2017.[40] In ATC cells, treatment with the compound resulted in a clear inhibition of cell growth, accompanied by an increased acetylation of histone H4, and a dose-dependent increase in $P21^{WAF1}$ and $P27^{Kip1}$, and a decrease of survivin and *BCL-2* expressions.[39] In NE cancer cells, thailandepsin A strongly inhibited the growth through the activation of the Notch pathway, without cytotoxicity to lung fibroblasts.[40]

Interestingly, the groups of Xiao and Gong have produced systems in which thailandepsin A is incorporated in micelles for delivery in cancer cells, showing improved targeting of tumours over the free form.[41-43]

Largazole [44]

Isolated in 2008 from a cyanobacterium *Symploca sp.*, in Key Largo,[45] largazole, unlike the other members of this family, "hides" its thiol as a hydrolysable thioester. Largazole and its thiol have been tested *in vitro* for HDAC inhibition activity, both with HeLa nuclear extracts,[46, 47] and with recombinant [46, 48] or purified [47, 49] HDAC enzymes. The IC_{50} values differ widely, ranging from low nanomolar to low micromolar. A general trend seems however to suggest a low nanomolar affinity for HDAC1 and lower potency for class II HDACs. Largazole also inhibited cell growth in a number of human cancer cell lines: HCT-116 (colon, GI_{50} 44 nM),[46] MCF7 (breast, IC_{50} 5 nM),[47] MM1.S (multiple myeloma, IC_{50} 6.2 µM),[49] and HCT-116, A549, HEK293, HLF (colon, lung, kidney, lung fibroblast, with GI_{50} of 0.08, 0.32, 1.36 and 0.98 µM respectively).[50] Souto *et al*. observed that treatment with largazole induced the accumulation of leukaemia NB4 cells in the pre-G_1 cell phase at 1 µM and massive cell death at 5 µM, as well as increasing the acetylation level of histone H3 and the expression of $P21^{WAF1/CIP1}$.[48]

Liu *et al*. reported that largazole induces cell cycle arrest at the G_1 phase at low concentrations (1-3.2 nM) and G_2/M arrest at higher concentrations (≥ 10 nM) in human colon cancer HCT116 cells in 2010.[51] This was associated to increased acetylation of histone H3, with an increase in *P21, P19, P15* and *P57* and a decrease in *CDK6* and *cyclin D1* levels. A more general analysis of the genes affected by largazole revealed that overrepresented functional classes include those involved in chromatin assembly, negative regulation of cell cycle, transcription (up-regulated), positive regulation of transcription, intracellular protein cascade, positive regulation of nucleic acid metabolism, RNA biosynthesis and metabolism (down-regulated). No inhibition of HDAC6 was observed, as shown by the absence of change in the tubulin acetylation status. When tested on *nu/nu* mice, largazole was well tolerated up to the highest tested concentration (50 mg/kg), with good bioavailability and HDAC inhibition already at 5 mg/kg.[51] More recently, Wu *et al*. reported that treatment of lung cancer A549 cells had similar effects, with up-regulation of caspase-3 and 9, *P21* and *P27* The authors observed hyperacetylation of histone H3 and an enhanced recruitment of the *P21* promoter to acetyl H3.[52]

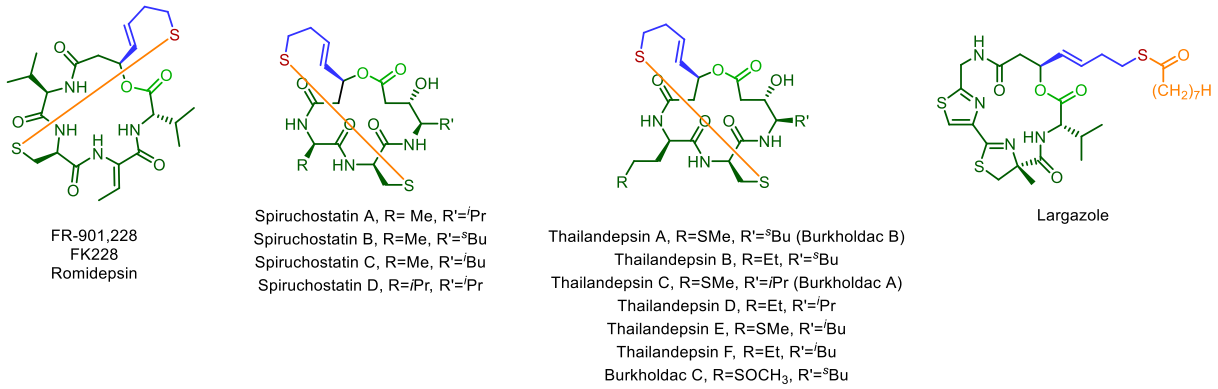


Figure 2. Structure of the discussed cyclic depsipeptides. The colours represent the different parts highlighted in fig. 1 (the lighter green highlight the ester of the depsipetides), the orange colour represents the prodrug moiety.

Cyclic tetrapeptides (Fig. 3)

(1-Alanine)-chlamydocin

Chlamydocin was first isolated from the fungus *Diheterospora chlamydospora* in 1974 by Closse *et al*. [53] and was shown to exhibit cytostatic activity on P-815 mouse mastocytoma cells [54, 55] – with

poor results in animals,[54] and without any hypothesized mode of action. More recently, chlamydocin was identified as HDACi,[56, 57] and was shown to inhibit the proliferation in seven human cancer cell lines.[58] The HDAC inhibition activity in A2780 ovarian cancer total cell lysates was concentration-dependent with an IC_{50} value of 1.3 ± 0.5 nM. This translated, in intact cells, in a hyperacetylation of histones H3 and H4, with maximal levels at a concentration of 10 nM, already detectable 2 h after treatment. Chlamydocin induced growth inhibition of A2780 cells at 1 µM after 24 h; with arrest in G_2/M and apoptosis. The latter was shown to involve caspase-dependent cleavage of $P21^{cip1/waf1}$ and to be associated with proteasome-mediated degradation of survivin.

The closely related 1-alaninechlamydocin was isolated from a Great Lakes-derived fungal isolate, identified as *Tolypocladium* sp. in 2014,[59] and tested in two human pancreatic carcinoma cell lines, MIA PaCa-2 and Panc-1, as well as an immortalized human pancreatic ductal cell line, hTERT-HPNE. It showed potent antiproliferative and cytotoxic activities against MIA PaCa-2 with GI_{50} and LC_{50} values of 5.3 and 22 nM respectively, and inhibited the proliferation of Panc-1 and hTERT-HPNE at low nanomolar concentrations. For the two latter, however, 1-alaninechlamydocin did not exhibit cytotoxicity up to 10 µM. Exposition of MIA PaCa-2 cells to the HDACi, led to a decrease of cells in the G_1 phase and an accumulation of cells in G_2/M, while showing signs of early stage apoptosis. HDAC inhibition activity with an IC_{50} of 6.4 nM is close to the potency observed in live cells, suggesting that it is the major mode of action responsible for the *in vitro* biological effects.

Trapoxin

Trapoxin A (also known simply as Trapoxin) and Trapoxin B were isolated from a fermentation broth of the fungus *Helicoma ambiens* in 1990 by Itazaki *et al*. and were reported to induce the morphological reversion from transformed to normal in *sis* oncogene-transformed NIH3T3 cells.[60] In 1993, Kijima *et al*. showed that Trapoxin is an inhibitor of mammalian HDAC, observing an increase of histone acetylation in cells and the inhibition of partially purified mouse HDAC from FM3A cells.[18] The inhibition was found to be irreversible, as the enzyme treated with trapoxin did not recover its activity after 18 h of dialysis. Additionally, it was found that the HDACi induced specific arrest of the cell cycle of rat 3Y1 fibroblasts in both G_1 and G_2 phases.

Although epoxiketones are commonly thought to be irreversible HDAC inhibitors, as demonstrated by its use for the first isolation of HDAC 1,[19] trapoxin has been found to reversibly inhibit HDAC6.[57] The tight binding was thought to result from the reaction of the epoxide moiety, however Porter *et al.* showed that it is the ketone moiety that undergoes a nucleophilic attack to form a zinc-bound tetrahedral gem-diolate, leaving the epoxide moiety intact.[61]

The closely related FR225497, described in 2000,[62] showed nanomolar range inhibition of partially purified human HDAC and antitumor activity in human T-cell leukemia Jurkat cells and human colon adenocarcinoma HT-29 cells, with IC_{50} of 152 and 158 ng/mL respectively.

HC-Toxin

Isolated in 1971 from the fungus *Helminthosporium carbonum*,[63] HC-Toxin was shown to be an inhibitor of yeast, plant and mammal HDAC [64] and has been used successfully against breast cancer cells,[65, 66] neuroblastoma (NB) cells,[67, 68] and intrahepatic cholangiocarcinoma cells (ICC).[69]

Deubzer *et al*. observed that "nanomolar doses of HC-Toxin (IC_{50}<20 nM) induce G_0/G_1-cell cycle arrest and apoptosis in both established NB cell lines and primary cell cultures but not in primary human fibroblasts".[67] Additionally, the authors determined that treatment with HC-Toxin induces neuronal and neuroendocrine differentiation, and suppresses the invasive growth of NB cells, overall demonstrating an activation of the retinoblastoma tumour suppressor network in NB cells.

In the case of ICC, the screening of 34 HDACi showed that HC-Toxin exhibited the highest antitumor activity, inhibiting the cell proliferation and clone formation ability of the targeted cells, which was associated with an increase in the acetylation of H4.[69] The authors also observed an arrest of the cell cycle at the G_0/G_1 stage and an increase in apoptotic cell, as well as morphological changes, potentially indicating cell differentiation due to HC-Toxin.

FR235222

FR235222 was first isolated from a fungus, *Acremonium* sp. No. 27082, in 2003 by Mori *et al*.[70] After determining and measuring its inhibitory activity on mammalian HDAC, the authors showed the activity of the HDACi as immunosuppressant, both *in vitro* [70] and *in vivo*,[71] which was further studied by Matsuoka *et al* in 2007.[72] Petrella *et al*. showed in 2008 [73] that, at 50 nM, FR235222

induces the accumulation of acetylated histones H4 and inhibits cell proliferation of human promyelocytic leukaemia U937 cells, human chronic myelogenous leukaemia K562 cells and human T cell leukaemia Jurkat cells, while having no effect on apoptosis. The antiproliferative activity of the inhibitor was linked to its ability to cause growth inhibition at the G_0/G_1 phase transition of cell cycle, and was postulated to be, at least partially, linked to an increase in the expression of Annexin A1 (ANXA 1). At higher concentration (0.5 µM), FR23522 induced apoptosis in all three cell lines.

Later work of the group in 2010 with human prostate adenocarcinoma LNCaP cells,[74] showed an increase in the acetylation of histone H4 as well as apoptosis through a caspase-dependent mechanism at a concentration of 0.5 µM, and an increase in the expression of ANXA1. It is worth noting that after siRNA-mediated inhibition of the expression of the latter, the apoptotic potency of the HDACi was only partially reduced.

Microsporin A

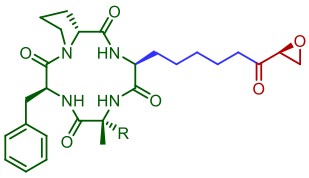
Isolated from the cultured marine-derived fungus identified as *Microsporum* cf. *gypseum* (along with Microsporin B), Microsporin A showed interesting *in vitro* cytotoxicity against human colon adenocarcinoma HCT-116 (IC_{50} 0.6 µg/mL) and a mean IC_{50} value of 2.7 µM on 60 cell lines.[75] *In vitro*, on HeLa cell nuclear extract, Microsporin A inhibited a mixture of HDACs and HDAC8 with an IC_{50} of 0.14 µM and 0.55 µM respectively.

Apicidins

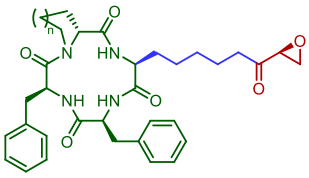
Apicidin and Apicidin A were first isolated by Darkin-Rattray *et al*. in 1996 from fermentations of fungal *Fusarium spp*. (ATCC 74289, ATCC 74322) and shown to have antiprotozoal activity, through their low nanomolar inhibitory effect on protozoal HDAC.[76] More apicidins were isolated further on: B and C by in 2001,[77] D_{1-3} in 2002,[78] E in 2010,[79] and F in 2013.[80] Among them, apicidin, apicidins A and D1 were reported to inhibit HDACs in HeLa cell extracts with IC_{50} values of 1, 2 and 0.3 nM respectively.[78] While apicidin was reported to selectively inhibit HDAC 2, 3 and 8, (IC_{50} of 120, 43, and 575 nM respectively),[81] it also reduced the expression of HDAC3 and 4 in endometrial cancer cells,[82] and inhibited the expression of HDAC4 and 7 in ovarian cancer cells,[83] and in salivary mucoepidermoid carcinoma cells.[84] Apicidin inhibited proliferation of various cancer cell

lines, through the increase in the expression of *P21*$^{WAF1/Cip1}$ and gelsolin,[85] and, in human acute promyeolcytic leukemia cells HL60, through the selective induction of Fas/Fas ligand, resulting in the activation of caspase-3 and 9.[86] Cheong *et al*. in 2003 studied the effect of apicidin in human Bcr-Abl-positive leukemia K562 cells and found similar activation of caspase-3 and 9 without the involvement of Fas/Fas ligand death receptor pathway.[87] In endometrial and ovarian cancer cells, Ueda *et al*. observed strong anti-growth activity of apicidin, with the induction of apoptosis occurring in concert with the increase in levels of P21^{WAF1}, P27^{KIP1} and the decrease of cyclin A and E-cadherin.[88] Ahn *et al*. also reported the inhibition of the migration of human ovarian cancer cells via HDAC4 silencing,[83] and the anti-tumour effect of apicidin on Ishikawa human endometrial cancer cells, *in vitro* and *in vivo*,[82] through an increase in the acetylation of histones H3 and H4, leading to the up-regulation of *P21* and the decrease of cyclins A, B1, D1 and E, and CDK2 and 4.[89]

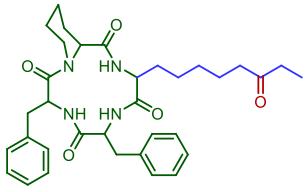
In 2011, Ahn *et al.* reported that apicidin induced apoptosis, through up-regulation of *P21*^{WAF1}, and autophagy in human oral squamous carcinoma cells,[90] while cell growth inhibition of salivary mucoepidermoid carcinoma cells was hypothesized to be mediated by the inhibition of IGF-1R, an upstream regulator of MAPK and AKT/mTOR, signaling pathways involved in cell survival and proliferation events.[91]



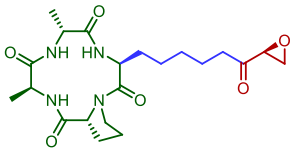
Chlamydocin, R=Me
1-Alaninechlamydocin, R=H



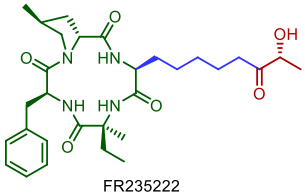
Trapoxin A, n=2
Trapoxin B, n=1



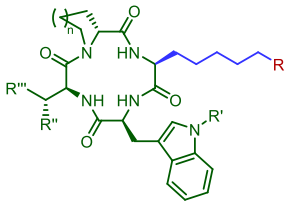
FR225497



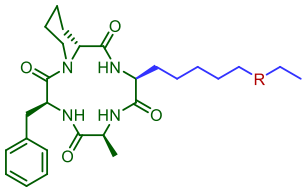
HC toxin



FR235222



Apicidin, n=2, R=COEt, R'=OMe, R''=Et, R'''=Me
Apicidin A, n=2, R=COEt, R'=H, R''=Et, R'''=Me
Apicidin B, n=1, R=COEt, R'=OMe, R''=Et, R'''=Me
Apicidin C, n=2, R=COEt, R'=OMe, R''=Me, R'''=Me
Apicidin D_1, n=2, R=COCH(OH)Me, R'=OMe, R''=Et, R'''=Me
Apicidin D_2, n=2, R=CH(SOH)Et, R'=OMe, R''=Et, R'''=Me
Apicidin D_3, n=2, R=CH_2CH(OH)Me, R'=OMe, R''=Et, R'''=Me
Apicidin E, n=2, R=*n*Pr, R'=OMe, R''=Et, R'''=Me
Apicidin F, n=2, R=COOH, R'=OMe, R''=Ph, R'''=H



Microsporin A, R=(C=O)
Microsporin B, R=(CHOH)

Figure 3- - Structure of the reported cyclic peptides. The colours represent the different parts highlighted in fig. 1

Table 2. Selected examples of targeted genes by cyclic HDACi.

Compound	Cell line	Targeted genes
Spiruchostatin A,B	U937 (lymphoma)	*Fas, caspase-3, 8, Bid*
Thailandepsin A	8505C (anaplastic thyroid cancer)	*$P21^{WAF1}$, $P27^{Kip1}$, survivin, BCL-2*
Largazole	NB4 (leukemia)	*$P21^{WAF1/CIP1}$*
	HCT116 (colorectal carcinoma)	*P21, P19, P15, P57, CDK6, cyclin D*
	A549 (adenocarcinoma)	*caspase-3, 9, P21, P27*
Chlamydocin	A2780 (ovarian carcinoma)	*caspase-3, $P21^{cip1/waf1}$*
HC-Toxin	BE(2)-C (neuroblastoma)	*N-myc, Skp2, Mad2, survivin*
FR235222	LNCaP (adenocarcinoma)	*caspase-3, ANXA1*
Apicidin	Ishikawa (endometrial adenocarcinoma)	*$P21^{WAF1}$, $P27^{KIP1}$, cyclin A, E-cadherin*

Linear HDACi (Fig. 4)

Trichostatin A

Isolated from the fungus *Streptomyces hygroscopicus* in 1976 by Tsuji et al.,[92] is a classic linear hydroxamic acid derivative and one of the first natural product determined to have HDAC inhibitory activity.[17] Because it is one of the most potent known HDAC inhibitors,[93] with nanomolar inhibition of recombinant HDAC 1 and 6,[46] trichostatin A (TSA) is routinely used as a positive control and as a reference to compare other HDACi.[46, 67] TSA has been reported to inhibit cell growth in, among others, lung adenocarcinoma, hepatoma, cervix carcinoma, and small cell lung cancer, through regulation *$P21^{waf/cip1}$*, *P27*, *Bcl-Xl*, cyclin A and E, etc.[94, 95] It is worth noting that the hydroxamate moiety has been shown to display mutagenicity, potentially limiting its clinical use.[96]

Depudecin

Discovered in the culture broth of the fungus *Alternaria brassiciola* in 1992, depudecin was determined by Kwon *et al*. in 1998 to have an inhibitory effect on HDAC *in vitro*, with an IC_{50} of 4.7 µM on purified FLAG epitope-tagged HDAC1.[97] Depudecin was also shown to induce morphological changes in NIH3T3 fibroblasts transformed with v-*ras* and v-*src* oncogenes and to induce a hyperacetylation of histones *in vivo*.

Linear sulfides, disulfides, thiols

Psammaplins

Psammaplin A was first isolated in 1987 from an unidentified marine sponge [98] and later on from other sources such as *Thorectopsamma xana* [99]. It consists of a symmetrical bromotyrosine-derivative. The dimeric form, bisaprasin, as well as a large variety of related psammaplins (B-J),[99, 100] were reported to exhibit high inhibitory potency against HDACs, with IC_{50} values ranging from 2.1 to 327 nM, with psammaplins A and F and bisaprasin being the most potent at 2.1, 4.2, and 9 nM. The trend is mirrored in the activation of *P21* promoter regions in cancer cells.[100] Psammaplin A inhibited cell growth in monolayers of lung tumour A549 cells and breast tumour MDA-MB-435 cells, with IC_{50} values of 1.35 and 1.15 µM, and *in vivo*, with tumour inhibition in the A549 lung xenograph mouse model, with low toxicity.

Ahn *et al.* found that psammaplin A inhibited proliferation of on human endometrial cancer cells with an IC_{50} value of 5 µg/mL.[101] At this concentration, it dramatically increased the levels of acetylated H3 and H4 histones, while 1 µg/mL caused the accumulation of cells at the G_1 phase. This was correlated with the increased expression of $P21^{WAF1}$ and a decrease in the *P53* levels. Additionally, treatment with the inhibitor caused an increase in apoptosis, with values of 50% after 48 h at 5 µg/mL.

Psammaplins have been shown to inhibit cell growth and possess cytotoxic properties in the low micromolar range in a broad range of human cell lines, such as lung, ovarian, skin, colon, and breast cancer.[99]

Allium organosulfur compounds

The vegetables of the *Allium* genus, such as onions, garlic, scallion, leek and chives, are known for their medical benefits, which are attributed primarily to organosulfur compounds (OSC) released upon processing.[102] These OSC include allicin, alliin, allyl mercaptan (AM), diallyl disulfide (DADS), and *S*-allylmercapto-L-cystein (SAMC), among others. It is hypothesized that *Allium* OSCs might be "funnelled" by metabolization to generate the actual HDAC inhibitors, such as AM, although it seems more than likely that the resulting thiols also react with other proteins.[103]

Amongst the most studied of the OSCs, DADS has been for example shown to increase the acetylation of histone H3 in human colon tumour cell lines Caco-2 and HT-29,[104] and H4 in the former, accompanied by the up-regulation of $P21^{WAF1/CIP1}$,[105] and CDKN1A.[106] The authors also found that

AM inhibited HDAC activity stronger than its dimeric counterpart DADS (92% against 29%).[90] In breast cancer cell line MCF-7, DADS induced the hyperacetylation of histone H4, the activation of caspase-3, and the modulation of BAX, BCL-2, BCL-xL, and BXL-w, suggesting the involvement of the BCL-2 family proteins in the DADS-induced apoptosis.[107] Zhao and coworkers reported in 2006 that DADS led to hyperacetylation of histones H3 and H4 and the up-regulation of $P21^{WAF1}$ in human acute myeloid leukaemia HL-60 cells *in vitro* and *in vivo*.[108]

Allyl mercaptan (AM) is believed to be the metabolite of most OSCs,[109] and has often been studied alongside DADS and reported as a HDACi.[105] For example, DADS and AM induced histone H4 acetylation in HTC in rat hepatoma cells, T47D human breast cancer cells and DS19 mouse erythroleukemic cells,[110, 111] Nian *et al*. reported in 2008 that AM functioned as a competitive inhibitor of HDAC8 (K_i = 24 μM) and caused hyperacetylation of histones H3 and H4 in human colon cancer cells.[112] The authors observed the presence of hyperacetylated histone H3 on the $P21^{WAF1}$ gene promoter and increased binding of the transcription factor SP3. The induction of $P21^{WAF1}$ protein expression coincided with growth arrest in the G_1 phase of the cell cycle.

S-allylmercapto-L-cystein (SAMC) was reported to inhibit cell proliferation of DS19 mouse erythroleukemia cells with significant inhibition already at 0.5 μM [113] and an increase of the acetylation of histone H4.

Bis(4-hydroxybenzyl)sulfide

Isolated from an extract of *Pleuropterus ciliinervis* by Son et al in 2007,[114] bis(4-hydroxybenzyl)sulfide has been shown to have an *in vitro* inhibitory HDAC activity on HeLa nuclear extracts, with an IC_{50} value of 1.43 μM, as well as growth inhibitory activities on six different human tumour cell lines, namely ACHN (kidney), NCI-H23 (lung), PC-3 (prostate), MDA-MB-231 (breast), LOX-IMVI (melanoma), and HCT-15 (colon), with GI_{50} values ranging from 1.45 (for MDA-MB-231) to > 10 μM for NCI-H23.[114]

Sulforaphane and other isothiocyanates

Abundantly found in cruciferous vegetables, such as cauliflower, cabbage, broccoli or Brussel sprouts, isothiocyanates (ITC) result from the hydrolysis of glucosinates by the plant enzyme mirosinase.[115]

Among the most studied ITC are found sulforaphane (SFN), allyl isothiocyanate (allyl-ITC), benzyl isothiocyanate (BITC) and phenethyl isothiocyanate (PEITC). *In silico* and *in vitro* experiments suggest that the active form of SFN, and potentially other ITC, is the metabolite sulforaphane-cysteine.[116, 117] Surprisingly, although SFN has been extensively studied, its HDAC inhibitory activity had never been studied in a cell-free system until 2018, when Choi *et al*. determined that it inhibited HDAC 2 and 9, with IC_{50} values of 36 and 0.6 µM respectively, while exhibiting much lower potency for HDAC 4, 5 and 7.[118]

SFN has been reported to inhibit cell growth, induce G_2/M cell cycle block and increase expression of cyclin B1 in four different human breast cancer cells.[119] In one of these cell lines (MDA-MB-231) apoptosis seemed to be initiated through induction of Fas ligand, resulting in the activation of caspase-3 and 8, while in the others, apoptosis was initiated by decreased Bcl-2 expression, release of cytochrome *c* and activation of caspase-3 and 9 but not 8. Interestingly SFN inhibited HDAC activity and reduced the expression of estrogen receptor-α, among others, although no change in the acetylation of histones H3 or H4 was observed.

Myzak *et al*. found that, when consumed in the diet at an average daily dose of 7.5 µmol per animal for 21 days, SFN suppressed the growth of xenografts of human PC-3 prostate cancer cells by 40% in mice, accompanied by significant decrease in HDAC activity in the xenografts.[120]

PEITC was found to act as a de-repressor of the *P21* promoter in LNCaP androgen-dependent prostate cancer cells in 2008 by Wang and coworkers.[121] Treatment of the cells resulted in cell cycle arrerst and *P53*-independent up-regulation of, among others, $P21^{WAF1}$ and *P27*. PEITC caused histone hyperacetylation, which was associated with the *P21* gene, as found by immunoprecipitation. Interestingly, PEITC also significantly down-regulated c-MYC, a repressor of *P21*.

Interestingly BITC causes G_2/M cell cycle arrest and apoptosis in human pancreatic cancer cells. The authors observed a 60% inhibition in cell growth at concentrations of 5-10 µM, whereas higher concentrations did not significantly increase this value. Treatment with BITC resulted in increased levels of caspase-3 and $P21^{WAF1/CIP1}$, and down-regulation of G_2/M regulatory proteins, such as cyclin B1, CDC2 and CDC25C.[122]

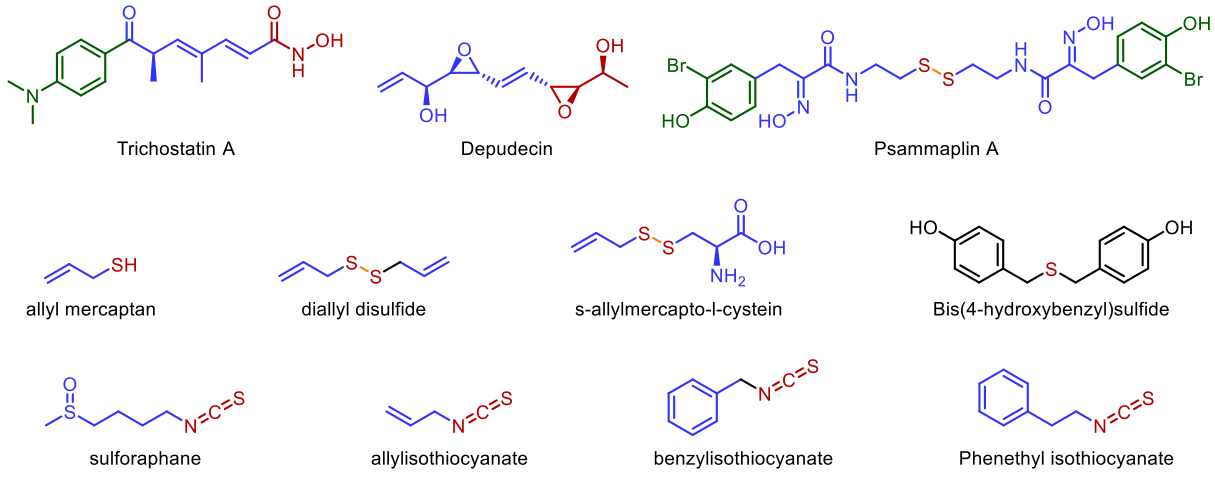


Figure 4. Structure of the discussed linear inhibitors. The colours represent the different parts highlighted in fig. 1.

Non-Zinc Binding Inhibitors (Fig. 5)

Epicocconigrone A and epicoccolide B

Isolated from the endophytic fungus *Epicoccum nigrum* in 2014, epicocconigrone A and epicoccolide B were tested against total protein extracts of K-562 cells and against recombinant proteins and displayed a micromolar HDAC inhibitory activity, with IC_{50} values ranging from 1.6 to 12.9 µM.[123] Both compounds were tested for proliferation and viability of Burkitt's lymphoma RAJI and histiocytic lymophome U-937 cell lines. Epicocconigrone A inhibited 50% of the proliferation of RAJI cells at 5 µM and induced about 30% cell death at 25 µM, and had no effect on U-937 cell viability while inducing growth inhibition of 32% at 5 µM. Epicoccolide B had no effect on cell viability in any of the two cell lines, but inhibited the growth in RAJI and U-937 by 55% and 30% respectively at 25 µM, showing that both HDACi mostly act as cytostatic agents. Notably, neither had any effect on cell viability of healthy peripheral blood mononuclear cells (PBMCs).

Zerumbone

Zerumbone and 6-methoxy-2*E*,9*E*-humuladien-8-one are two sesquiterpenoids isolated from *Zingiber zerumbet*, a wild ginger from Southeast Asia, which display HDAC inhibitory activity.[124] The compounds were tested against partially purified HDAC enzymes (IC_{50} of 8.35 and 1.25 µM respectively) and their antiproliferative effects using human prostate cancer PC-3 cells (GI_{50} of 14.24 and 4.63 µM respectively). Further investigations on six human tumour cell lines and one type of

human primary cells (hepatocytes) showed that both compounds display an inhibitory effect on the tested cancer cells, although with a two- to ten-fold lower activity than SAHA and similar or increased cytotoxicity against hepatocytes.

Ursolic acid

Ursolic acid can be found in a wide variety of fruit and herbs used in daily life, and in large amounts in apple peels. This triterpenoid has been shown, by Chen *et al*. in 2009,[125] to be cytotoxic to a selection of cancer cell lines, in particular to human gingival cancer Ca9-22 cells and human leukaemia HL60 cells, with IC_{50} values of 5.9 and 8.7 µg/mL. The authors showed in a qualitative way that the activity of HDAC 1, 3, 4, 5 and 6 decreased upon treatment with increasing amounts of ursolic acid, and similarly, observed an increase in the acetylation of histone H3.

Gymnochrome E

Found in metabolites of the crinoid *Holopus rangii*,[126] Gymnochrome E was shown to inhibit the proliferation of the multi-drug-resistant ovarian cancer cell line NCI/ADR-Res with an IC_{50} value of 3.5 µM, while showing no inhibitory effects on PANC-1 pancreatic carcinoma of DLD-1 human colorectal adenocarcinoma cell lines at a concentration of 6.4 µM. The compound was also found to inhibit HDAC1 with an IC_{50} of 10.9 µM.

(Dehydro)cyclostellettamines

In 2004, Oku *et al*. isolated from the aqueous extract of a marine sponge of the genus *Xestospongia* a family of molecules including cyclostellettamine A and G and dehydrocyclostellettamine D and E. The compounds showed moderate activity against HDAC from K562 cells (human chronic myeloid leukemia cell line), with IC_{50} values ranging from 17 to 80 µM, and good to moderate cytotoxicity against HeLa human cervix carcinoma (IC_{50} 0.6-2.8 µM), P388 mouse leukemia (IC_{50} 1.3-2.7 µM), and 3Y1 rat fibroblastic cells (IC_{50} 3.2-11 µM).[127]

Flavonoids

Apigenin is abundantly present in common fruits and vegetables such as grapefruit, parsley, chamomile and wheat sprouts,[128] and has been shown to be an *in vitro* HDAC inhibitor, with an IC_{50} value of 27 µM for class I HDAC and no observed inhibition of class IIa and IIb.[129] In a 2012 study,

Pandey *et al*. measured the effect of apigenin on human prostate cancer 22Rv1 and PC-3 cells and found a decrease in HDAC activity of 22% and 62% respectively at 40 µM of apigenin.[128] This decrease, however, was linked to a diminished expression of HDAC, at 40 µM of apigenin: in 22Rv1, HDAC 1 and 3 levels were down by 52% and 22% respectively, while in PC-3, these values were of 46% and 48%. Unsurprisingly, both effects together resulted in an overall increase in the acetylation of histones H3 and H4 in both cell lines. Apigenin was found to cause a marked decrease in the percentage of cells in the S-phase of the cell cycle, the treated cells being preferentially arrested in the G_0/G_1 phase, and to induce apoptosis.

Tseng *et al*. have more recently investigated the effects of apigenin in MDA-MB-231 breast cancer cells. It was found that the potential HDACi inhibits cell proliferation in the studied cells, with 40 µM being defined as the "nonapoptotic induction concentration".[130] The authors measured an increase in the acetylation of histone H3, in particular in the $P21^{WAF1/CIP1}$ promoter, while H4 appeared to not be affected by apigenin. In this case, however, the treated cells accumulated in the G_2/M cell phase, unlike what was observed in prostate cancer cells. The reduction upon treatment with apigenin of cyclin A and B and CDK1 levels, which modulate G_2-to-M progression, is given as a possible explanation for this modulation of the cell cycle. Additionally, the authors observed a significant antitumor growth effect *in vivo* in nude mice with MDA-MB-231 cells orthotopically injected, without any sign of toxicity observed in the mice.

Kaempferol is synthesized by a variety of vegetables and fruits, such as tomatoes, hop, red grapes, grapefruit, strawberries and *gingko biloba* and can also be found as an ingredient in honey, and green and black tea.[131] It was found to inhibit HDAC enzymes *in vitro*, with IC_{50} values of 39, 48 and 29 µM for class I HDAC, 2a and 2b respectively.[129] Similar values were found by Berger *et al*. in 2013 with all tested recombinant HDACs of class I, II and IV.[131] The authors further tested kaempferol in human-derived hepatoma cell lines HepG2 and Hep3B, as well as HCT-116 colon cancer cells, and found that it caused a hyperacetylation of histone H3 in all cell lines. This translated in an antiproliferative activity, independently of the p53 status of the tested cell line, shown by the fact that HepG2 has wild-type p53 and Hep3B tumour cells are p53 deficient.

Plants rich in luteolin have been used in Chinese traditional medicine and can be found in celery, broccoli, green pepper, parsley, dandelion, etc.[132] Godoy *et al*. measured IC_{50} values ranging from 17 and 18 µM for inhibition of class I, IIa and IIb HDAC, among the lowest for flavonoid derivatives.[129] In 2011, Attoub *et al*. investigated the effect of luteolin in human epithelioid cancer cells, namely LNM35 (lungs), HT29 (colon), HepG2 (liver), MCF7/6 and MDA-MB231-1833 (breast). The authors observed the inhibitory effect of luteolin *in vitro* on HDAC in HeLa nuclear extracts, with 100 µM doses of luteolin leading to a significant inhibition already visible after 6 h on both histones H3 and H4, with values around 20 and 30% respectively. When incubating 24 h, these values increased to around 80 and 40 %. When using 50 µM doses, a significant activity of about 30 % could be observed on histone H3, but only after 24 h. Luteolin also exhibited an effect on cell viability and apoptosis in all tested cell lines, with IC_{50} values of 60, 65 and 90 µM for HepG2, LNM35 and MCF7/6, respectively, and more than 200 µM for HT29 and MDA-MB231-1833. Further analysis on the LNM35 cell line showed that treatment with 50 µM luteolin induced apoptosis of 20% of the cells, associated with a four-fold increase in caspase3/7 activity. Finally, the authors determined that luteolin could efficiently impair the invasiveness of MCF-7/6 cells in the chick heart invasion model effects at 10 µM concentration, while not exhibiting any detectable toxic effect on the heart tissue.

Chrysin is found in honey, propolis and passion flowers among others,[133] and has been found to weakly inhibit HDAC 2 and 8 *in vitro* (IC_{50} of 129 and 40 µM respectively) out of HDAC 1-11.[134] When treating breast cancer MDA-MB-231 cells with 40 µM of chrysin, Sun *et al*. have observed a significant increased of the expression of $P21^{WAF1/CIP1}$ and an inhibition of cell proliferation, with no signs of cell death, suggesting that the suppression occurred through the induction of differentiation. This translated, *in vivo*, in the inhibition of tumour growth in mice with MDA-MB-231 cell xenograft.[134] Pal-Bhadra *et al*. also observed inhibition of HDAC 2 and 8 in melanoma A375 cells, although concomitant with a decrease in HDAC expression, leading to increase acetylation of histones H3 and H4 and activation of $P21^{WAF1/CIP1}$.[135] As a result, chrysin induced accumulation of cells in the G_1 phase and apoptosis through downregulation of anti-apoptotic Bcl-xL and survivin and

upregulation of caspase-3. In breast cancer cells, Sun *et al*. did not observe significant cell cycle arrest at any phase.[134]

Osajin and pomiferin have been isolated from the ground fruit of *Maclura pomifera* (or the Osage orange) by Son *et al*. in 2007.[136] Their inhibitory HDAC activity was determined on HeLa nuclear extracts, with IC_{50} values of 6.53 and 1.05 µM, respectively. The compounds were tested against a range of cancer cell lines, namely ACHN (kidney), NCI-H23 (lung), PC-3 (prostate), MDA-MB-231 (breast), LOX-IMVI (melanoma), HCT-15 (colon) and hepatocytes as human primary cells, displaying growth inhibitory activities in all of them. Osajin GI_{50} values ranged from 9.65 to 13.32 µM, while pomiferin shower more potency, with values ranging from 1.32 to 5.14 µM. The toxicity on hepatocytes showed the same trend, with a GI_{50} of > 500 µM for osajin and 123 µM for pomiferin.

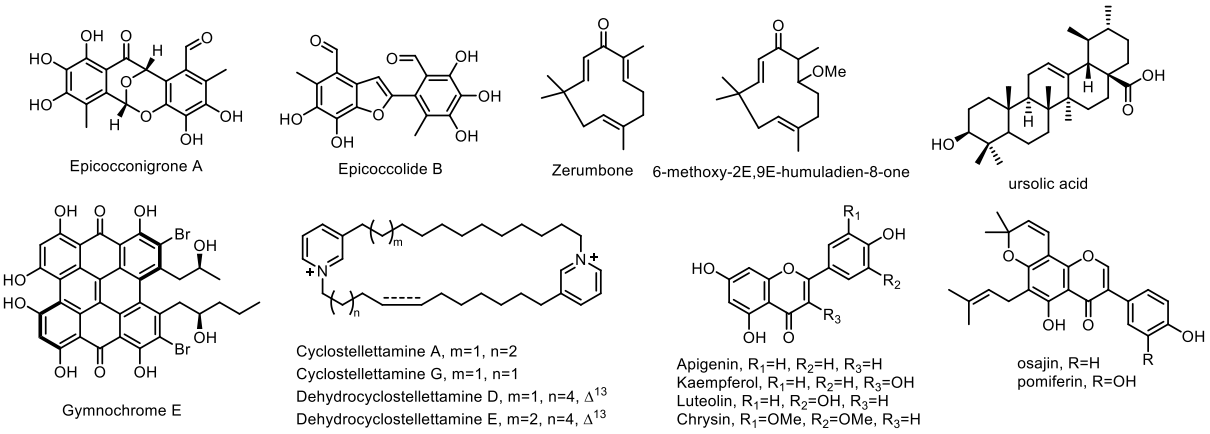


Figure 5- Structure of the reported non-zinc-binding inhibitors.

2.2 For Class III HDAC inhibition

Class III HDACs, sirtuins, are NAD^+-dependant HDACs and do not contain a zinc cation in their active site. Therefore, sirtuins inhibitors are different structurally. SIRT1 and 2 are the most studied for inhibition in anticancer therapies [137] or activation in other pathologies such as type 2 diabetes, inflammatory diseases and neurodegenerative disorders [138]. Few natural products were identified as sirtuin modulators.

Resveratrol was identified as a yeast SIR2 activator [139] increasing yeast lifespan [140] and a human SIRT1 activator inhibiting TNF-alpha induced inflammation in fibroblasts. [141] However, the activator

effects of resveratrol have been contested as no effect was detected in enzymatic assay with SIR2 [142] and the pharmacological effect of resveratrol was shown to be independent of SIRT1 in various models.[143] In cancer resveratrol was shown to induce gastric cancer cell arrest in a SIRT1-dependant manner.[144]

Quercetin was also identified as SIRT1 acetylation activator in yeast but with no effect on lifespan.[140] In HT29 colon carcinoma cells, its metabolite, quercetin 3-*O*-glucuronide, showed a weak inhibition of recombinant SIRT1 enzyme.[145]

Nicotinamide was shown to be a pan-sirtuin inhibitor [146, 147] repressing gastric cancer growth.[148] However, due to the pleiotropic targets of nicotinamide it has probably several in-cell targets.

Tanikolide dimer is a dimer of the biologically active *δ*–lactone Tanikolide. It was isolated from *Lyngbya majuscula*, a Madagascar marine cyanobacterium, and shown to inhibit SIRT2. The IC_{50} values range from 176 nM to 2.4 µM depending on the evaluation assay.[149]

More recently, Schnekenburger *et al.* showed that eurochevalierine, a metabolite from the fungus *Neosartorya pseudofischeri*, inhibits sirtuin 1 and 2 with IC_{50} = 10 µM with no effect on sirtuin 3 nor on a panel of class I and II HDACs. Eurochevalierine exhibit a sirtuin inhibitor profile *in vitro* in several cancer cell lines (SK-MEL-28, melanoma; U-373, glyoblastoma and A-549, adenocarcinoma) with increase of acetylation of sirtuin targets and without any toxicity for normal cells. They identified its mode of interaction with SIRT1 and SIRT2 by molecular docking.[150]

2.3 For DNA methylation inhibition

<u>Natural products altering DNA methylation</u>

DNMTs are enzymes responsible for methylation of cytosine in a CpG context, using *S*-adenosyl-L-methionine (SAM) as a methyl donor. In cancers, hypermethylation of the promoter regions of tumour suppressor genes (TSGs) leads to their silencing, which participates to tumour formation and proliferation. As mentioned before the interest of finding non-nucleoside DNMTi remains a crucial challenge. Therefore testing natural products can highlight unexplored DNMTi chemical scaffolds.

For several natural products (Fig. 6), a modification in DNA methylation in cancer cell lines was shown but no direct DNMT inhibition activity has been reported yet.

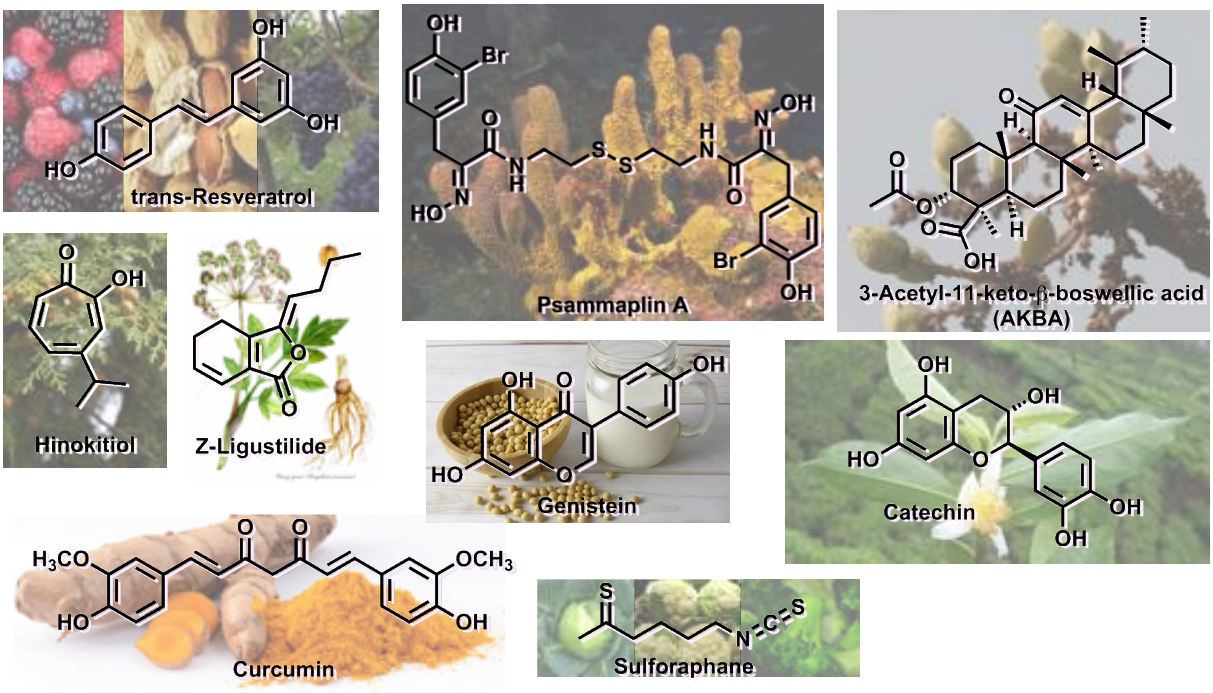


Figure 6: Structure of natural products altering DNA methylation

Resveratrol

Resveratrol (*trans*-3,5,4′-trihydroxystilbene) is a stilbene produced by red grapes, peanuts and berries as part of their defence system in response to an injury. This natural phenolic derivative was shown to decrease methylation of PTEN promoter and induce its reexpression in MCF7 breast cancer cell line.[151] In combination with pterostilbene, another stilbene found in plants, resveratrol resulted in the down-regulation of DNMT3B in HCC1806 triple-negative breast cancer cells without any significant effects on control MCF10A breast epithelial cells.[152] The expression of DNMTs and certain other associated protein, including HDAC1 and MeCP2 was decreased after treatment with resveratrol (10-15 µM) in MDA MB 231 and MCF7 breast cancer cell lines.[153] A decrease in the overall DNA methylation level with 15 µM of resveratrol was observed using nuclear extracts from HCC1806 cells.[152] but no or very weak inhibition of DNMT was shown *in vitro*, which suggests an indirect mechanism of DNA methylation inhibition.

Boswellic acids

Boswellic acids are extracted from the plant *Boswellia serrata*. Among them the acetyl-11-keto-β-boswellic acid (AKBA), a pentacyclic terpenoid, is the major active derivative. It was shown to reduce DNA methylation at several CpG loci in SW48 colorectal cancer cell line and induce the up-regulation of gene expression including TSG such as SAMD14 and SMPD3. A global DNA methylation inhibition was measured in SW48 and SW480 colorectal cancer cell lines following AKBA treatment at 40 µM.[154] Although to date AKBA inhibition activity on purified DNMT was not reported, these results suggest that AKBA induce inhibition of DNA methylation *via* an indirect pathway.

Hinokitiol

Hinokitiol (4-isopropyltropolone) is extracted from the *Chymacyparis obtusa* tree. In colon cancer cell line HCT116, decreased DNMT1 and ubiquitin-like containing PHD and RING finger domains 1 (UHRF1) expressions and increased ten-eleven translocation methylcytosine dioxygenase 1 (TET1) expression were observed after treatment with 10 µM of hinokitiol for 48 to 72 h. TET1 is responsible for the conversion of 5-methylcytosine (5mC) in 5-hydroxymethylcytosine (5hmC) identified as the first step towards DNA demethylation. Following hinokitiol treatment, the global level of 5hmC was increased whereas the global 5mC level remained unchanged. However, looking more closely at specific genes, a decrease of methylation was reported for *MGMT*, *CHST10* and *BTG4* together with an increase of their expression.[155]

Z-Ligustilide

Z-Ligustilide is extracted from the Asian medicinal herb Radix *Angelicae Sinensis* (RAS) with cytotoxic effect against TRAMP1 C1 prostate cancer cell line at concentrations higher than 50 µM. Z-Ligustilide showed an alteration of the DNA methylation level in the *NRF2* promoter and an increase of its expression in a concentration-dependent manner with no changes in DNMT or HDAC1-5 and 8 expression levels. The authors carried out enzymatic assays on M.*SssI* and reported for Z-Ligustilide an IC_{50} = 1055 µM.[156] This can hardly be considered as an inhibitory effect, suggesting an indirect mechanism for DNA methylation inhibition by Z-ligustilide.

Psammaplin A

As mentioned earlier in this review, psammaplin A and derivatives were first isolated in 1987 from unidentified sponge [98] and antibacterial and antitumour activities were reported. These compounds, later isolated from the Papua New Guinea sponge *Pseudoceratina purpurea* showed a nanomolar *in vitro* inhibition of DNMT1 together with HDAC inhibition.[100] However this inhibition value was obtained using a partially purified DNMT, which could be the reason for the lack of activity measured by Baud and coworkers for DNMT1 inhibition.[157]

Sulforaphane

Sulforaphane (SFN) is an isothiocyanate isolated from cruciferous vegetables. After SFN treatment in MCF-7 and MDA-MB-231 cancer cell lines, the methylation status at CpG sites of the telomerase reverse transcriptase *hTERT* gene was altered and its mRNA transcription was down-regulated. This was observed concomitantly with a lower expression of DNMT1 and 3A together with a modification of histone acetylation levels. This suggested that the modification of DNA methylation level occurred *via* an indirect mechanism involving HDAC inhibition (*vide supra*).[158] Another study reported that the treatment with SFN in LnCaP prostate cancer cell line resulted in a lower methylation of the promoter of *cyclin D2*, correlated with prostate cancer progression, which was associated with an increase of *cyclin D2* expression. However a down-regulation of the DNMT1 and DNMT3B expression was also reported, which suggested as well an indirect pathway.[159]

Catechin

Catechin is a flavanol extracted from tea. In MCF7 and MDA-MB-231 breast cancer cell lines, catechin induced inhibition of the *RARβ* gene promoter methylation similar to EGCG. Low cytotoxic effects were observed up to 50 µM.[160] The inhibition activity of catechin against M.*SssI* and DNMT1 was weak since only 40% inhibition was achieved at the highest tested concentration (*i.e.* 20 µM). However in presence of COMT, catechin showed micromolar IC_{50} with 6.5 µM and 4.6 µM against M.*SssI* and DNMT1, respectively.[160] This highlights the indirect mechanism of DNA methylation inhibition of catechin that is COMT-dependant, similarly to quercetin. Although its bioavailability seemed better, catechin was shown to be a good substrate for COMT, which results in an increase of SAH concentration due to their methylation metabolism.[161]

Genistein

Genistein (4',5,7-trihydroxyisoflavone) is the main isoflavonoid from soybean present in significant amounts in Asian diet. In rat, prepubertal genistein exposure resulted in the up-regulation of the TSG *BRCA1*. Although its expression is controlled by epigenetic mechanisms, no evidence was reported in this paper that could confirm genistein as an epi-target(s).[162] Genistein was shown to induce the expression of $P21^{WAF1/CIP1}$ and $P16^{INK4a}$ tumour suppressor genes in prostate cancer cells. This was identified to be the consequence of several chromatin modulations, *i.e.* decrease of DNA methylation in *P21* promoter and increase of histone acetylation *P21* and *P16* transcription start sites.[163] Genistein was also shown to reverse hypermethylation and induce the reexpression of $P16^{INK4a}$, retinoic acid receptor β (*RARβ*) and *MGMT* genes carcinoma KYSE 510 cell line.[164] Genistein treatment induced a decrease of the methylation in promoter regions of *GSTP1* gene in PC-3, DU145 and LNCaP prostate cancer cell lines and of *EPHB2* gene in DU145 cell line associated with an increase of their expression. The same study, however, reported no effect on *BRCA1* methylation or expression.[165] In breast cancer cell lines, genistein was suggested to decrease the *GSTP1* promoter methylation and to increase its expression in MDA-MB-468 cells.[166] Similar results were shown for *BRCA1* and *BRCA2* [167] and *ATM*, *APC*, *PTEN*, *SERPINB5* [168] genes in MCF-7 and MDA-MB-231 cell lines. Molecular modelling shows that Genistein could interact within the DNMT1 active site, cytosine pocket. However, with about 40% inhibition of DNMT activity in nuclear extracts of MCF-7 and MDA-MB-231 cell lines at 100 µM, genistein can not be consider as a potent inhibitor of DNMT. Moreover, genistein causes a decrease of DNMT1 expression that can be the cause of the observed lower DNA methylation level. Noteworthy, following genistein treatment, no significant alteration of DNMT3A and 3B expression was observed MCF-7 and MDA-MB-231 breast cancer cell lines [168] but a decrease of DNMT3B expression was observed in Human neuroblastoma SK-N-SH cell line.[169] Low DNA methylation inhibition activity was confirmed using KYSE 510 cell line nuclear extract [164] and no inhibition activity was observed on Dnmt3A/3L complex [170] indicating an indirect pathway of DNA methylation inhibition, especially since multiple targets were identified for genistein such as the inhibition of HDAC6 recruitment by decreasing Hsp90 activity,[171] tyrosine specific protein kinases,[172] topoisomerase I and II,[173] 5α-reductase [174] and NF-κB.[175]

Curcumin

Curcumin (1,7-bis(4-hydroxy-3-methoxy-phenyl)-1,6-heptadiene-3,5-dione; diferuloylmethane) is the major flavonoid component of the rhizome of *Curcuma longa* (spice turmeric) that is used in the traditional Indian medicine.[176] Curcumin has many pharmacological effects in cancers [177] including the modulation of epigenetic mechanisms.[178] Only a low effect of curcumin on the global DNA methylation level in HCT116, HL-60 and HEK293 cell lines were reported.[179] In colorectal cancer cell lines, however, curcumin was reported to alter, in a time dependent-manner, the DNA methylation at a selected subset of loci contrarily to the 5-azadC that showed a global DNA hypomethylation.[180] In MV4-11 leukaemia cell line, curcumin treatment showed a decrease in DNA methylation with reexpression of epigenetically silenced *P15* and a decrease in DNMT1 expression.[181] This suggests an indirect DNA methylation mechanism of curcumin. However, in LNCaP and TRAMP C1 prostate cancer cell lines, a decrease of the DNA methylation inducing the reexpression of Neurog1 and Nrf2 was observed with no effect on the modification of the DNMT1, DNMT3A and DNMT3B expression.[182, 183] Curcumin was identified as a very weak inhibitor of DNMT1 and DNMT3A with respectively 34 and 20% inhibition with 100 μM [184] and of M.*SssI* methyltransferase in a concentration-dependent manner in high micromolar range (50-100 μM).[183] Surprisingly, using a fluorescence assay Liu *et al.* reported an inhibition activity of IC_{50} = 30 nM for curcumin against *M. SssI* methyltransferase. Based on molecular docking, the authors suggested a covalent mode of interaction between curcumin and C1226 of DNMT1. These results are in contradiction with previously reported inhibition activity on DNMT1, as described above. Additionally, the authors observed an in-cell global DNA methylation change with curcumin at concentrations starting from 3 μM. Moreover, although curcumin derivative 5 of this paper was calculated to have the same affinity for DNMT1 by molecular docking, no inhibition was observed in fluorescent enzymatic assay.[185] Globally, similarly to other polyphenolic compounds, curcumin induces DNA methylation inhibition in TSG promoter regions and associated gene re-expression, but, as suggested by the pleiotropic effect of curcumin, this occurs *via* indirect pathways. The interest of curcumin as therapeutic molecule and its potential PAINs behaviour is still intensively debated.[186, 187]

Natural products altering DNA methylation and identified as DNMTi (Fig. 7)

Nanaomycin A

Nanaomycin A is a quinone isolated from *Streptomyces sp.* culture and first used as antibiotic. [188] In three neuroblastoma cell lines, nanaomycin A induced cell growth inhibition, without global DNA methylation inhibition.[189] In three other human cancer cell lines (colorectal HCT116, lung A549 and leukaemia HL60), increasing concentration of nanomycin A inhibited cell growth and reduced the level of global DNA methylation. In A549 cell line, methylation inhibition at a few CpG sites was measured at the promoter of *RASSF1A*, with up-regulation of the gene. Biochemical assays indicated that nanaomycin A does not inhibit DNMT1 but inhibits DNMT3B activity with IC_{50} = 0.5 µM.[190] However more studies are needed to assess this suggested selectivity.

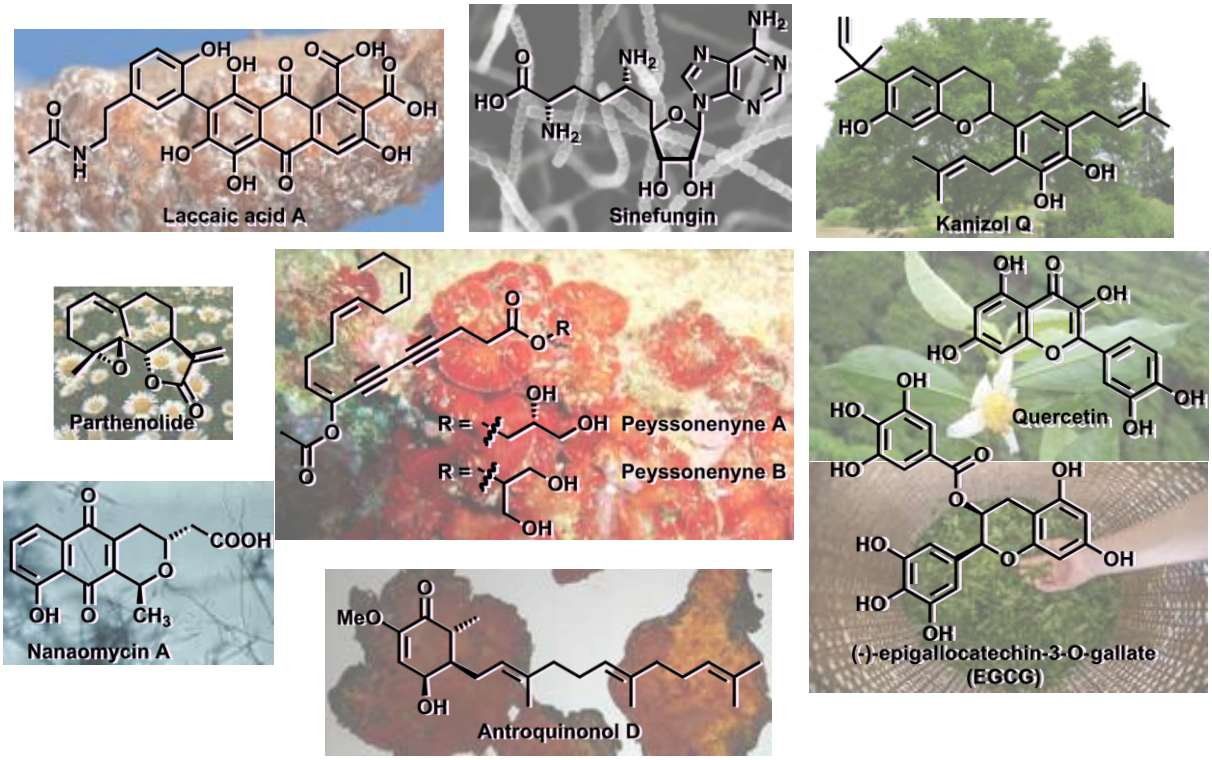


Figure 7: Structure of natural products altering DNA methylation and identified as DNMTi

Laccaic acid

Laccaic acid extracted from insects, *Laccifer lacca* Kerr., is a highly substituted anthraquinone used as a natural food dye in Japan that was first identified as toxic against *Paramecium caudatum* in 1978.[191] This compound was shown to induce the reexpression of *VGF* and *MAL* genes over 10 tested genes in

MCF7 breast cancer cell line and to inhibit an activated, *i.e.* RFTS-domain lacking, DNMT1 with K_i = 310 nM in a DNA-competitive mechanism. Noteworthy, the activated DNMT was reported by the authors to be 640-fold more active than the RFTS domain including enzyme [192] and to date no in-cell DNA methylation inhibition was reported.

Peyssonenynes

Peyssonenynes A and B are two ω3 fatty acids derivatives isolated from Fijian red marine alga *Peyssonnelia caulifera*. In a first study, they were reported to have comparable inhibition activity of DNMT1 with IC_{50} = 16 and 9 µM, respectively, using a methyl transfer radioactive enzymatic assay.[193] However more recently, another study reported a 60% inhibition of DNMT1 with 50 µM of peyssonenynes A and B using a similar radioactive assay. This weak inhibition was shown to be still higher than RG108, commonly described as a DNMTi.[194] To date Peyssonenynes A and B have not been studied to evaluate the in-cell DNA methylation inhibition potency.

Parthenolide

Parthenolide is a sequiterpene lactone identified as NF-κB transcription factor inhibitor present in the plant *Tanacetum parthenium* (feverfew) that is used for its anti-inflammatory properties (Lyss 1998, 33508). A global hypomethylation was shown in K562 and MV4-11 leukaemia cell lines and in MV4-11 xenografted nude mice. The same study reported the hypomethylation of the promoter of the putative TSG HIN-1 associated with the up-regulation of its expression at relatively high dose of parthenolide (*i.e.* 30 µM).[195] Parthenolide was also shown to up-regulate the expression of *p21* in JB6P+ cells with no effect observed on histone methylation and acetylation.[196] A down-regulation of DNMT1 expression was evidenced in MV4-11 and Kasumi-1 leukaemia cell lines treated with 10 µM of partenolide [195] that can lead to an indirect inhibition of the DNA methylation. However, docking experiments suggested a possible covalent direct inhibition of DNMT1 by parthenolide *via* 1,4-conjugated addition between Cys1226 and α,β-unsaturated carbonyl moiety.[195, 197] Fluorescent enzymatic assays showed parthenolide to inhibit M.*SssI* and DNMT1 with IC_{50} = 5 µM and 3.5 µM, respectively[195]

Kazinol Q

Kazinol Q was extracted from the root bark of the Formosan plant *Broussonetia kazinoki* (Ko 1999, 164). In the MDA-MB-231 breast cancer cell line a reexpression of E-cadherin (*CDH1*) gene, silenced through DNA methylation, was observed after 48 h treatment with 5 µM of Kazinol Q whereas no reexpression was observed in this study with EGCG, another natural product identified as DNMTi and described later in this review. Enzymatic assays on DNMT1, lead to the identification of Kazinol Q as the only active inhibitor among 12 structurally diverse compounds isolated from Formosan plants with IC_{50} = 7 µM and compared to EGCG used as positive control (IC_{50} = 3 µM). Noteworthy the pharmacokinetic properties of Kazinol Q are improved compared to EGCG with increased stability and hydrophobicity.[198]

Antroquinonol D

Antroquinonol D (3-demethoxyl antroquinonol) was isolated from *Antrodia camphorate*, a mycelium from Taiwan used by aboriginal people as a medicine. Antroquinonol D was shown to inhibit the growth of three breast cancer cell lines (MCF7, T47D and MDA-MB-231) but not of breast epithelial cells (MCF10A and IMR-90). Interestingly, antroquinonol D was reported to inhibit the migration of MDA-MB-231. The treatment of MDA-MB-231 with 15 µM of antroquinonol D for 6 days resulted in a decrease of methylation at CpG sites and an increase of five TSG expression levels (*i.e*. *FANCC*, *CACNA1A*, *CHD15*, *ASB9* and *COL4A2*). A 75% inhibition of DNMT1 and a 25% inhibition of DNMT3B activity were measured using 22 µM of antroquinonol D. Contrarily to the inhibition of DNMT3B, the DNMT1 inhibition was shown to be SAM-concentration dependent. With additional molecular docking this enables to hypothesise that it could interact in DNMT1 SAH pocket.[199]

Sinefungin

Sinefungin is a SAM analogue originally isolated from the *Streptomyces griseolus* bacteria. As expected, this derivative strongly competes with SAM in bacterial N^6-adenine DNA methyltransferase M•TaqI.[200] Sinefungin was identified as a weak SAM-competitive DNMT1 inhibitor with IC_{50} = 80 µM.[201] As a SAM analogue, this inhibitor can target the full range of SAM-dependent enzymes and as such is not suitable for selective DNMT inhibition. However to date no in cell assays showed evidence of a DNA methylation inhibition of sinefungin in cells.

Quercetin

Quercetin is a flavone extracted from green tea that showed a concentration-dependent growth inhibition of colon cancer RKO cell line. After a 5-days treatment, quercetin inhibited, in a concentration-dependent manner, the methylation of the promoter of $P16^{INK4a}$ gene,[202] key tumour suppressor gene involved in cellular senescence.[203] This was associated with a restored $P16^{INK4a}$ expression.[202] In prostate cancer cell lines PC3 and DU145, two receptor-negative cell lines, quercetin was shown to decrease global methylation level and induce the reexpression of androgen receptor.[204] The direct inhibition of DNA methyltransferases was investigated on M.*SssI* methyltransferase and the inhibition level remained very low even at 20 µM.[160] However as a polyphenol, quercetin is susceptible to be methylated by catechol-*O*-methyltransferase (COMT) and thus deplete the SAM pool. By increasing the concentration of SAH in the medium, quercetin is therefore responsible for the indirect inhibition of SAM-dependent enzyme including DNMTs. The higher inhibition level of quercetin against M.*SssI* (IC_{50} = 1.6 µM) in presence of COMT confirmed this hypothesis.[160]

(-)-Epigallocatechin-3-gallate

(-)-Epigallocatechin-3-gallate (EGCG) is the major polyphenol found in green tea (*Camellia sinensis*). It was first reported to lower promoter methylation and re-induce gene expression of $p16^{INK4a}$, *RARβ*, O^6-methylguanine methyltransferase (*MGMT*) and human *MUTL* homologue 1 (*hMLH1*) in esophageal cancer KYSE 510 cell line.[205] Later on, this was also observed on *RARβ* in MCF7 and MDA-MB-231 breast cancer cell lines [160] and *GAS1*, *TIMP4*, *ICAM1*, *WISP2* in cisplatin-resistant non-small-cell lung cancer (NSCLC) cells.[206] Several groups with very dissimilar results investigated the inhibition activity of EGCG on purified DNMTs. First, using nuclear extract from KYSE 510 cells, EGCG was identified as the most potent, although very weak, DNMT inhibitor among tested family of dietary polyphenols [205] with about 50% inhibition at 20 µM. A competitive mode of inhibition was shown with K_i = 6.9 µM. Another work on purified DNMT1, using radioactive biochemical assay, reported a submicromolar inhibition activity of EGCG with IC_{50} = 0.47 µM.[160] The re-evaluation of EGCG DNMT1 inhibition activity using a similar radioactive assay showed a

dramatically lower inhibition activity with IC_{50} = 70 µM.[179] This disparity in the inhibition activity questioned the real efficacy of EGCG as DNMTi. Moreover, the direct DNMT inhibition of EGCG in cells was questioned and an indirect mechanism was proposed. As for quercetin, the SAM-dependent COMT was shown to methylate catechol structure of EGCG, which results in a decrease of SAM concentration and an increase in SAH concentration. This can indirectly lower DNMT activity.[207] Nevertheless, Lee *et al.* studied the EGCG inhibition activity in presence or in absence of COMT and could not see any variation, which tend to show that EGCG inhibition activity is independent of COMT, contrarily to other green tea polyphenol.[160] In addition, EGCG was also identified as HAT inhibitor and as an activator of HDAC 3 recruitment [208] and its instability in culture medium with half-life time of 130 min, due to auto-oxidation and spontaneous degradation, is also to be considered.[209] Therefore, EGCG is a pleiotropic drug with high potential as anticancer prevention, but it mode of action remains to be elucidated, such as most of the flavonoid epigenetic inhibitors.[210]

2.3 Other epigenetics targets

Besides the most studied epigenetic actors, DNMT and HDAC, several natural products showed activity against other epigenetic targets.

Histone acetyltransferases

In prostate cancer cell line, genistein re-induced *BTG3* expression as a result of decreased DNMT methylation associated with an activation of histone acetyltransferase (HAT).[211] Garcinol, a polyisoprenylated benzophenone derivative extracted from rinds of *Garcinia indica* fruit was also shown to inhibit PCAF and p300 HAT activity *in vitro* with IC_{50} = 5 and 7 µM, respectively and repress acetylation in HeLa cell line.[212] Quercetin was also suggested as a HAT inhibitor in mode-K cells.[213] EGCG was shown to inhibit HAT preventing P300-induced P65 acetylation and NF-κB induction, with no effect on other epigenetic targets such as HDAC, SIRT1 and HMTs.[208]

Curcumin was identified as a specific P300/CREB-binding protein (CBP) HAT activity inhibitor and to decrease P300-induced p53 histone acetylation.[214]

Histone demethylase

Quercetin, resveratrol and curcumin were also identified as inhibitors of the histone demethylase LSD1 using an *in vitro* assay on LSD1 recombinant protein.[215]

Histone methyltransferase

Nahuoic acid A is a polyketide produced by culture of *Streptomyces sp.* that was reported as the first selective SAM-competitive inhibitor of the histone methyltransferase SETD8 with IC_{50} = 6.5 µM over other HMT including G9a, SETD7, DOT1L, EHMT1 and SUV39H2.[216] Nahuoic acids B-E were also reported as SEDTD8 inhibitor.[217] Curcumin was found to downregulate expression of EZH2, responsible for H3K27 methylation in MDA-MB-435.[218] EGCG influences EZH2 expression and H3K27me3 levels but no direct inhibition of EZH2 was demonstrated.[208, 219] Sinefungin is reported as micromolar inhibitor of SEDTD2.[220]

3/Chemical biology tools to better understand epigenetics in pathological context

Natural products are an extremely abundant source of new chemical scaffolds for the discovery of epi-drugs against cancer. However, although this strategy was quite successful for the identification of HDACi, the inhibition of DNMT by non-nucleoside analogues remains a challenge. The complexity of modulating the deregulated epigenetic mechanism is due to the fact that the epigenetic signature is the result of dynamic pathways, that the epigenetic actors are embedded into dynamic complexes and that these actors cross-talk to establish the epigenetic profile of cells. In order to better understand epigenetic mechanism and to identify innovative pathways to target these mechanisms, the use of appropriate chemical tools associated with chemical biology techniques and proteomic analysis can enable the identification of new targets or novel modes of interaction.[221] In this review, we focused on the chemical tools that were used in living cells and lead to the identification of novel targets or interacting proteins.

In the context of epigenetic mechanisms, the use of chemical probes to scan the epigenetic protein complexes in living cells was first applied in 2007 to identify HADCi interacting proteins,[222] Salisbury *et al.* synthesised chemical probes containing a SAHA moiety (a FDA-approved HDACi), a photoactivable crosslinker and an alkyne function to be functionalised by affinity group. SAHA was shown to target several class I and II HDACs, but other proteins including RbAp46 and RbAp48 were

also identified and hypothesised to be part of HDAC complexes. Very interestingly the authors showed that labelling was significantly different in cellular extract and in living cells.

Zhu *et al.* developed chemical tools based on maleimide-based DNMT inhibitors.[223] The authors added to the maleimide inhibitor moiety an alkyne function to be used as a handle. They first identified this chemical probe as a potent DNMT inhibitor with about 60% inhibition at 2 µM on recombinant DNMT1 and with effect on proliferation with GI_{50} = 0.3 µM on MCF7 breast cancer cell line. Thanks to cellular imaging, they showed that the chemical probe co-localised with the nuclei and with anti-DNMT antibody. Finally, carrying out pull-down on cell lysates with and without competing inhibitor, they were able to identify DNMT1 by western blot. Unfortunately no further proteomic analyses were carried out to identify other potential target of these inhibitors.

The bromodomain targeting compound JQ1 was also modified as chemical probe. First Li *et al.* synthesised several affinity based probes containing a diazirine crosslinker moiety and a tag (cyclopropene or alkyne). They show a identical binding potency of the probes compared to JQ1 and co-localisation with nuclei by cell imaging. They performed pull-down experiments in HepG2 carcinoma cell line and in competition with JQ1, and identified, together with BRD4 enrichment, a significant enrichment of nine other proteins that could be either other targets of JQ1 or be part of the protein complex of BDR-4.[224] With the aim of identifying chromatin genomic site of JQ1, Anders *et al.* synthesised biotinylated derivatives of this bromodomain ligand. They performed a pull-down experiment followed by sequencing analysis in MM1.S multiple myeloma cell line. This Chem-Seq technique showed that the genomic sites bound by biotinylated-JQ1 were similar to the one interacting with BRD2, BRD3 and BRD4 including *CCND2*.[225]

In another study involving a bromodomain (BRD4)-interacting compound (GW841819X), Pan *et al.* investigated the influence of a photo-crosslinking group for living cell imaging. The authors reported APEX1 as an off-target of GW841819X and showed the interaction to be responsible of DNMT1 up-regulation.[226] This approach has the potential to be be the starting point of a new indirect targeting of DNA methylation.

A study using chemical probes designed from the histone-lysine *N*-methyltransferase EZH2 inhibitor DzNep was carried out in MCF7 mammalian cells. A large set of proteins was identified including kinases, phosphatases, ATPases, and methyltransferases but without identification of EZH2 or *S*-adenosylhomocysteine hydrolase, SAHH.[227] None of these proteins has been yet validated.

4/ Conclusion – towards new therapeutic targets

In conclusion, natural products are invaluable resources to explore chemical diversity in order to find new chemical scaffold for therapeutic purposes. Natural products and their derivatives represent about 70% of the currently used drugs.[8] In the field of epi-drugs, natural products are often responsible for pleiotropic effect, which *a priori* limit their use. The use of chemical tool and affinity-based proteomic experiments involving natural products have not been undertaken yet, although this could be highly valuable to better understand their pathways. Moreover, looking closely, the multi-target effect of natural products can be a real advantage for therapeutic treatments. Multi-target molecules have been recently highly studied in several pathology such as pain management,[228] diabetic cardiomyopathy,[229] cancer,[230, 231] and mostly Alzheimer's disease.[232-234] Therefore multi-target therapies using natural products can be an alternative for epigenetic treatment of cancers [235] as long as their mechanisms of action remain well controlled and understood.

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