DNA methyltransferase inhibitors in cancer: a chemical and therapeutic patent overview and selected clinical studies

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Introduction: DNA methylation is an epigenetic modification that modulates gene expression without altering the DNA base sequence. It plays a crucial role in cancer by silencing tumor suppressor genes (TSG). The DNA methyltransferases (DNMT) are the enzymes that catalyze DNA methylation and they are interesting therapeutical targets since DNA methylation is reversible such that an aberrant hypermethylation of DNA can be reverted by inhibition of DNMTs. Today, two drugs are on the market for the treatment of myelodysplastic syndrome, azacitidine and decitabine.

Areas covered: Here, we present a review of the patents describing the chemistry and biological activities of novel DNMT inhibitors and discuss select clinical studies.

Expert opinion: DNMT inhibitors have shown efficacy in clinics. However, highly efficient and specific DNMT inhibitors have not yet been identified. Improving methods will certainly lead to the prediction of novel directly binding inhibitors in the future.

Keywords: anticancer agents, DNA methylation, epigenetics, inhibitors

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1. Introduction

Recently it has been demonstrated that by controlling the access to the genetic information it is possible to modulate cellular phenotypes. In eukaryotes, DNA is organized into chromatin: 147 base pairs are wrapped around a nucleosome which consists of an octamer of the core histones H2A, H2B, H3, and H4 [1]. Nucleosomes condense and form higher-order chromatin structure [2]. Numerous modifications of histone proteins and the DNA occur during embryonic development in order for differentiation to take place by repressing or expressing specific arrays of genes [3,4]. The initial definition of epigenetics as “the interactions between genes and their products leading to the realization of the phenotype,” by Waddington, has constantly evolved to become more recently “the changes happening on a chromosome without altering its DNA sequence, leading to a heritable and stable phenotype” [5,6].

Epigenetic information is regulated by chromatin modifications that involve both histones and DNA. Histones undergo numerous post-translational modifications such as acetylation or methylation (reviewed in [7]). By recruiting other factors or preventing their binding, most of these modifications locally alter the chromatin structure, resulting in an open or closed configuration and thereby expressing or repressing gene transcription (reviewed in [2]). Histone modifications are flexible epigenetic marks that can be reversed [8] and the setting and removal of marks has been extensively studied, especially the deacetylation of lysine residues. Many Histone Deacetylase Inhibitors (HDACi) have been characterized, and vorinostat...
and romidepsin have been approved by the USA Food and Drug Administration (FDA) for the treatment of Cutaneous T-Cell Lymphoma (CTCL) [9].

In humans, DNA methylation is the most stable epigenetic mark [10]. It occurs at position 5 of cytosine mainly in a CpG dinucleotide context and it is catalyzed by the C5-DNA methyltransferases (DNMTs) [11]. Briefly, three families of DNMT have been characterized: DNMT1, DNMT2, and DNMT3 (comprising Dnmt 3A, 3B, and 3L). DNMT1 is mainly responsible for DNA methylation maintenance by methylating the newly synthesized DNA strand after DNA replication. DNMT3A and DNMT3B are mainly responsible for de novo DNA methylation. The CpG dinucleotides are mainly located in CpG islands (occurring at circa 60% of all gene promoters), in repeated sequences and in CpG island shores [12-15]. If promoter CpG islands are methylated, the corresponding gene is repressed due to a poor recognition by transcription factors and recruitment of proteins involved in the chromatin remodeling such as methyl-binding proteins MBD [16-18]. DNA methylation is crucial for imprinting, X inactivation, embryonic development, and cell differentiation. It is also required for maintenance of chromosomal stability and protection against mutations by insertion through the repression of transposable and repeated elements [11]. Consequently, failure in maintaining DNA methylation and establishment of aberrant DNA methylation patterns are associated with under- or over-expression of certain proteins, ultimately leading to diverse pathologies, such as cancer [17,19]. Besides, even if the genetic origins of cancer are well established, it appears that epigenetic modifications are early events in tumorigenesis [20]. Interestingly, unlike genetic mutations, epigenetic alterations are reversible, as proved by the re-expression of some tumor suppressor genes (TSG) by two well-characterized inhibitors of DNMTs: azacitidine (trade mark Vidaza®, chemical name 5-azacitidine, by Celgene, Summit, NJ, USA) which has been approved by the FDA in 2004 against Myeloid Dysplasic Syndrome (MDS), Acute Myeloid Leukemia (AML), and Chronic Myelomonocytic Leukemia (CML), and decitabine (Dacogen™, chemical name 5-aza-2′-deoxycytidine, by Astex Pharmaceuticals, Dublin, CA, USA) (Figure 1A) which has been approved by the FDA in 2006 against MDS and AML [21].

The relevance of aberrant methylation for cancer is also illustrated by the fact that the methylation status of the promoter of TSG is used as a biomarker for diagnosis (such as Septin9 in blood plasma for colon cancer commercialized by Quest Diagnostics and GSTPi in urine by LabCorp) or prognosis (the use of temozolomide depends on the methylation status of MGMT in glioblastoma, test of MDxHealth).

For all these reasons, DNA methylation appears to be a particularly interesting target from a therapeutic point of view. However, the two clinically approved DNMT inhibitors (DNMTi) are not selective toward the different DNMTs, are chemically instable, and have strong secondary effects, for example, renal toxicity or myelotoxicity [9]. In addition, whether their efficiency relies only to their demethylation activity is not clear. Therefore, there is a need to identify novel, more specific and selective inhibitors. Here we review the different patents describing new DNMTi.

2. DNMTs inhibitors

There are two families of DNMTi: the nucleoside analogues, mainly derivatives of cytidine, such as decitabine and azacitidine, that need to be incorporated into the DNA to be active and then form a suicidal covalent complex with the DNMTs; and the non-nucleoside compounds that differ by chemical structure but in general bind to the DNMTs and exhibit their action by variable mechanisms.

2.1 Nucleoside analogues

2.1.1 First generation compounds

The first molecules that had been characterized as DNMTi were initially used as antimetabolites and cytotoxic agents in leukemia chemotherapies. In 1977 and 1978, Constantinides et al. highlighted their hypomethylating properties, leading to the differentiation of 10T1/2 cells (clonal mouse embryonic cell line) into functional striated muscle cells [22,23].

These compounds are 5-azacytidine (azacitidine) and 5-aza-2′-deoxycytidine (decitabine): 2 cytosine analogues in which the carbon atom in position 5 is replaced by a nitrogen atom (colored in red in Figure 1A) and linked to a ribose or a deoxyribose, respectively. Following this discovery, several other analogues had been synthesized and tested for DNMT inhibition, among them are 5,6-dihydro-5-azacytidine and 5-fluoro-2′-deoxycytidine (Figure 1B).

When these analogues are transported into cells by the nucleoside transporter hCNT1 [24], they undergo phosphorylation followed by conversion into their tri-phosphorylated active forms. The ribose analogues are incorporated both into RNA and DNA (after deoxy-conversion), whereas the deoxyribose analogues are incorporated only into DNA. To be active, these compounds need to be integrated into the genome during DNA replication in the S phase of the cell cycle by the DNA polymerase, which provides certain specificity toward rapid proliferating cancer cells. Once into the DNA, the cytosine analogues within DNMT target sites are recognized as target cytosine by the enzyme and undergo the
same reaction as normal cytosines with the formation of the covalent intermediate between the catalytic cysteine of the enzyme and 6-position of cytosine analogues (Figure 1C). However, unlike with cytosine, the β-elimination reaction cannot occur, because of the lack of a proton at the 5-position, resulting in the irreversible formation of a covalent complex. The enzyme is thereby trapped on the DNA by the suicide inhibitor, triggering DNA repair and degradation of the DNMT. The same mechanism holds true for 5-fluoro-2¢-deoxycytidine. Besides the DNMT inhibition, the ribose analogues are also incorporated into RNA, decreasing the incorporation into DNA and also affecting various RNA-cytosine C5-methyltransferases. This might explain why decitabine is more active than azacitidine, and also why it shows less secondary effects.

Zebularine (Figure 1B) is another nucleoside analogue, which was synthesized in the early 60s as an antimetabolite and later described as a DNMT1 inhibitor [25]. Then, a patent was filed by the American National Institute of Health for this specific application [26] followed by a patent covering its prodrugs [27]. Although zebularine is now widely used as a reference compound in addition to azacitidine and decitabine in many laboratories, no pharmaceutical development or clinical trials have been reported in the literature.

2.1.2 Second generation compounds
2.1.2.1 NPEOC-DAC
Researchers at the University of Southern California synthesized a series of 5-azacytidine derivatives in which the N4 amino function is protected by a 2-((p-nitrophenyl)
ethoxycarbonyl (NPEOC) group that is removed by the cellular carboxylesterases. The main compound NPEOC-DAC (Figure 2) was shown to be converted into decitabine in cells that expressed carboxylesterase 1 [28]. A full characterization of the pharmacological properties of NPEOC-DAC was subsequently published in the literature [29], but no development as a potential drug has yet been reported.

2.1.2.2 CP-4200
Researchers at Mount Sinai School of Medicine in a collaboration with Clavis Pharma (Oslo, Norway) registered two patents on lipophilic esters of azacitidine with the aim of improving the cellular uptake of the compound [30,31]. It was demonstrated subsequently that CP-4200, an elaidic acid ester of azacitidine (Figure 2), acts as a pro-drug of azacitidine with the advantage of delayed delivery of azacitidine.

DNMT1 depletion was observed upon treatment of three cell lines with CP-4200 similarly as with azacitidine. The in vivo antitumor activity of the compound has been characterized in an orthotopic acute lymphoblastic leukemia (ALL) mouse model and CP-4200 demonstrated a better therapeutic efficacy compared to azacitidine [32]. Phase I conducted by Clavis Pharma in MDS was expected to start in 2011 but the clinical trial has been delayed due to priority given to the development of another compound by the company [33].

2.1.2.3 RX-3117
RX-3117 is a nucleoside analogue (Figure 2) designed on the basis of the previously known antitumor properties of cyclopentenyl-cytosine derivatives and developed by Rexahn (Rockville, MD, USA) [34]. RX-3117 was selected among a series of uridine and cytidine analogues bearing a fluorinated cyclopentenyl ring including hydroxy-groups corresponding to the 3’ and/or 2’ alcohol functions of the nucleoside [35]. In vitro, cytotoxicity was analyzed on a panel of 16 human cancer cell lines: RX-3117 induced cell growth inhibition in a sub-micromolar range (IC50 varying from 0.18 to 2.67 µM). In vivo, antitumor efficacy was evaluated against the human colon carcinoma HCT116 xenograft model (subcutaneous) in nude mice: RX-3117 caused significant inhibition of tumor growth at the doses of 2 and 10 mg/kg (i.p.) three times per week for five weeks. However, no experimental data corresponding to epigenetic effects and demethylation targets were disclosed.

The possible inhibitory effect of RX-3117 on DNMT1 was published subsequently. Treatment of MDA-MB-231 cells with RX-3117 caused a reduction of the cellular amount of DNMT1 in a dose-dependent manner (1 to 5 µM for 24 h) as determined by Western blot analysis [36]. The mechanism of action of RX-3117 remains not fully elucidated. The compound is incorporated into RNA and DNA and inhibits DNA synthesis. The authors assume a possible direct inhibition of DNMT [37]. Further experiments are needed to clearly establish its potential role in DNMT1 modulation.

Phase I clinical studies have started in 2012 in Europe but without any specific indication related to an epigenetic mechanism of action [38].

2.1.2.4 Cytosine analogues
Substituted cytosines, mainly at the N4-position (Figure 2, examples C and G), were developed by researchers in Poland [39]. Some of these derivatives were previously patented by the same group for their anti-aging properties [40]. Thirteen compounds were just tested in vitro for inhibition of the bacterial M.SssI in the methylation of plasmid DNA. The authors claimed a competitive inhibition of SAM binding but no data was reported for the inhibition of human DNMT1. It is difficult from a chemical point of view to conclude on the mechanism of action of these molecules. Further pharmacological investigations are therefore necessary to validate these compounds.

2.1.2.5 Thio-cytidine derivatives
Two patents on thio-cytidine derivatives, which exhibit lower toxicities than the existing drugs decitabine and azacitidine, have been disclosed by the Southern Research Institute (Birmingham, AL, USA) [41]. Pharmacological data of two derivatives, T-dCyd and 5-aza-T-dCyd (Figure 2), including induction of DNMT1 depletion, insertion into replicating DNA, stability versus deamination, and in vivo efficacy on human xenograft models were provided. Decitabine, 5-azacytidine, zebularine, and ara-C were used as reference compounds. The most potent compound 5-aza-T-dCyd induced a complete depletion of DNMT1 in human myeloid leukemia KG1 cells treated with 0.1 µM compound for 72 h. To reach the same efficiency, 3 µM for T-dCyd was needed under these experimental conditions. Also T-dCyd was more rapidly incorporated into DNA to higher levels in comparison to decitabine but no data are given for 5-aza-T-dCyd. The presence of the thio-deoxyribose appears to increase the chemical stability of 5-aza-T-dCyd when incubated in PBS buffer (pH = 7.4) as compared to decitabine. T-dCyd and 5-aza-F-dCyd have been evaluated for their in vivo antitumor activities in the human lung NCI-H23 model. Significant efficacy in terms of tumor growth inhibition has been observed particularly for 5-aza-F-dCyd. Since recent findings indicate that lower doses of azacitidine and decitabine need to be used to have epigenetic effects, the authors suggest exploring treatments with cycles of lower doses of 5-aza-T-dCyd to validate the effect on cellular DNMT1. One more time, further investigations are awaited to confirm the pharmacological activities through the inhibition of DNA methylation.

2.1.2.6 Decitabine-p-deoxyguanosine – S110
SuperGen (now Astex Pharmaceuticals) has synthesized a series of oligonucleotides (di-, tri-, and tetranucleotides) as pro-drugs of decitabine [42]. Among them the dinucleotide “decitabine-p-deoxyguanosine” exhibited demethylating potency comparable to decitabine. Now known as SGI-110.
and formerly S110 (Figure 2), this derivative showed improved pharmaceutical properties in terms of pharmaco-kinetics and metabolic stability compared to the parent drug, decitabine [43,44].

SGI-110 is currently in Phase II clinical for the treatment of MDS and AML [45].

2.1.2.7 SAM- or SAH-analogues
Methylgene (Montreal, Canada) has synthesized a series of SAH-analogues including mainly modifications of the adenine nucleus or various substitutions at the adenine N4-position (Figure 3) [46].

More than 100 compounds have been evaluated for their ability to inhibit DNMT1 and DNMT3b2 in vitro. The most potent derivatives exerted an inhibitory effect of DNMT3b2 with an IC50 close to 0.25 and 0.7 µM for DNMT1 (Table 1).

Some compounds resulted in a selective inhibition of DNMT3b2 or DNMT1 but no clear structure-activity relationship could be obtained. Furthermore, experimental results of cellular effects related to an inhibition of DNA methylation were not provided.

2.2 Non-nucleoside compounds
2.2.1 SGI-1027
SuperGen has also developed a series of 4-anilinoquinoline derivatives substituted with various aromatic and heterocyclic rings that induce DNMT depletion, as shown by induction...
of DNMT1 degradation and demethylating activity in a cellular assay (Figure 4) [47]. These compounds were derived from former DNA-interacting agents bearing the N-methylquinolinium moiety [48]. Subsequently, SGI-1027 (compound E in the patent text) was selected among the quinoline compounds and its biochemical and cellular hypomethylating activities were fully characterized [49].

SGI-1027 inhibited DNMT1, DNMT3a, and DNMT3b as well as M.SssI in a similar IC$_{50}$ range (6 to 13 µM) with a SAM-competitive mechanism. In cellular assays, selective degradation of DNMT1 through a proteasomal pathway was observed, although the underlying mechanism remained unclear. Furthermore, treatment of human colon cancer RKO cells led to the reactivation of silenced TSG. However, since this publication no further studies nor pharmaceutical development of SGI-1027 have been reported.

2.2.2 Alcyne derivatives
Researchers at the University of Louisville, KY, have filed a patent on alcyne derivatives [50]. Such compounds are claimed to exert “useful activity” in the classical MTT cytotoxicity assay. By using the COMPARE program, a strong correlation between Compound 1 and halomon was found, suggesting a common mechanism of action (Figure 4). Halomon, a polyhalogenated monoterpene isolated from a marine red algae, has been described as a potential DNMTi [51].

Figure 3. Examples of SAH and SAM analogues from WO2006078752.
Pharmacological data are provided only for Compound 1. The inhibitory activity of DNMT1 induced by Compound 1 was measured by determining the content of 5-methylcytosine in nuclear extracts from tumor cell lines (A549, lung and HeLa, cervix) incubated with 1 and 0.5 µM Compound 1 after two hours. *In vivo* antitumor effects in the A549 model were not statistically significant but the lower dose evaluated (25 mg/kg) seemed to exert better activity than the higher dose (50 mg/kg), leading the authors to suggest a similar mechanism of action as decitabine. No further studies on Compound 1 have been published in the literature.

### 2.2.3 Cyclopenta- and cyclohexathiophene derivatives

SuperGen filed a patent on 33 new cyclopenta- and cyclohexathiophene compounds as inhibitors of DNMTs, particularly DNMT3b (Figure 4) [52]. A protocol for the DNMT3b enzymatic assay is briefly described in the example part of the patent. Nevertheless, experimental values permitting to estimate the activity of the claimed derivatives are not given.

### 2.2.4 Tryptophane derivatives (RG108)

Researchers in Heidelberg, Germany, registered a patent covering a large family of small organic compounds including substituted phthalimide derivatives as DNA methylation inhibitors [53]. Most of the compounds were already known in the literature but their use as DNMTi was not described. Among the nine preferred products of the invention, the phthaloyl tryptophane derivative named RG108 was the most investigated (Figure 5A). RG108 is the L-enantiomer of the corresponding racemic NSC401077 as referred in the NCI database. The RG108 compound was identified during a virtual screening of 1553 molecules extracted from the NCI database using a three-dimensional model of the catalytic domain of the human DNMT1 [54]. Based on this structural homology modeling of DNMT1 and docking of the compound into the modeled binding pocket of the enzyme, binding energies of the compound were calculated and compared with cytidine and azacitidine. *In vitro* enzymatic experiments were performed with the bacterial methyltransferase M.SssI, RG108 being used at concentrations up to 500 µM. In contrast to azacitidine, RG108 did not induce DNMT1 depletion in HCT116 cells. These results suggested that RG108 does not act as a covalent inhibitor of DNMT.

RG108 also induced demethylation of genomic DNA in HCT116 and NALM-6 cell lines without affecting the viability under the experimental conditions mentioned in the text. The compound lacking the carboxylic function was found inactive in the DNA methylation assay. Simultaneously, a detailed characterization of the effect of RG108 and its ability to reactivate epigenetically silenced TSG was published [55]. Then, a second patent focusing on the RG108 structure was presented by the Cancer Research Technology group (London, UK). Sixty-six phthalimide and succinimide derivatives of tryptophan together with various benzamides were exemplified, from which 22 exerted an inhibition of DNA methylation with an IC₅₀ of 50 µM or less [56]. Furthermore, based on the RG108 structure, the Curis American group (Curis, Inc., Cambridge, MA, USA) synthesized a series of tryptophan derivatives containing a zinc-binding moiety, consisting of an hydroxamic acid, to provide synergistic effects by inhibiting the DNMT and HDAC enzymes with a dual pharmacophore [57]. This strategy was also applied by Curis to several antitumoral targets. However, only HDAC inhibition values were given for a selection of compounds. Consequently, the ability of such derivatives to inhibit DNMTs remains to be proven.

RG108 has been mentioned as a reference compound in the literature, but its potency and selectivity toward the DNMT enzymes is dependent on the biological assays. Two independent research groups reported a weak activity of RG108 on DNMT1 and DNMT3A/3L at unusually high concentrations (500 µM to 1 mM) [58,59]. The compound is not further developed.

### 2.2.5 Procainamide derivatives

Procaine and procainamide have been used for years as anesthetic and antiarrythmic agents, respectively (Figure 5B). Inhibition of DNA methylation induced by procainamide was first described in 1988 as a secondary effect [60]. It entered a clinical trial to reactivate TSG in cancers [61]. It has been shown later that these two drugs have an affinity for CpG-rich DNA regions, providing an explanation to their effect on DNA methylation [62]. Recently, the procainamide moiety was linked to a DNMTi such as RG108 derivatives or flavonoid derivatives with the aim of targeting the CpG-rich region (Figure 5B) [63]. Both hDNMT1 and mDNMT3A/3L inhibition was evaluated for 12 products, the most potent exerting IC₅₀ values in the micromolar range. Also specificity for DNMT3A/3L versus other methyltransferase enzymes (EcoDam and G9a) was demonstrated for three compounds [59]. Antiparasitic activity against *Trypanosoma brucei* was also studied. Further studies are awaited to confirm the therapeutic interest of these compounds.

A group from the Ohio State University filed a patent on three structurally different general formula extracted from a library of 169 products including mainly procainamide derivatives [64]. The demethylating properties have been identified by using a two-component reporter gene system (EGFP, enhanced green fluorescent protein) providing simultaneously...
an evaluation of DNA demethylation and cytotoxicity in human breast cancer MCF7 cells. Inhibition of EGFP expression was obtained with 46 derivatives of which 36 did not cause significant cytotoxicity.

The structure of the most potent compounds of the patent, IM25, CI-4-1, and 4e are shown in Figure 5C. ELISA assay and Western blot analysis confirmed that IM25 exhibited demethylating properties in terms of EGFP expression inhibition. Also, IM25 induced global DNA demethylation in MCF7 treated cells. A more detailed characterization of the pharmacological properties of IM25 appeared in the literature [65]. Its efficacy demonstrated on MCF7 cells remains to be studied on other cell lines, then in vivo antitumoral experiments are awaited to assure the therapeutic potential of such compounds.

2.2.6 Flavonoid derivatives

Genistein, the main isoflavone component of soybean, is known for its numerous pharmacological activities, including inhibiting properties of various enzymes such as kinases, topoisomerase, and more recently, DNMTs [66].

Starting from the genistein skeleton, a library of 114 synthetic flavone and flavanone derivatives has been evaluated for their DNMT3A/3L inhibiting properties (Figure 6A) [67]. Some of the compounds have been previously tested for cytotoxic properties [68,69] and antiangiogenic activities [70]. In the same work a novel high throughput screening assay for DNMTi was described [67]. Interestingly the most active compounds on DNMT3A are inactive in inhibiting amino-peptidase N (APN) [70]. Among the series evaluated on DNMT3A/3L, the most active compounds possess a chloro-nitro motif at the C3 position of the flavanone ring and the most potent compound had IC50 of 370 nM in the enzymatic assay (Compound 69, Figure 6A). A methylation radioactive assay measuring the incorporation of 3H-SAM into DNA was also performed on a subset of derivatives. Result confirmed the data obtained with the HT assay, the best compounds in the DNMT assay were the best in the radioactive assay as well. Genistein and RG108 were used as reference compounds in both assays but exerted no or marginal activity under these experimental conditions. Similar results were observed on DNMT1 and M.SssI, indicating no apparent selectivity among the DNA cytosine-C5-methyltransferases (data not shown). However, a relative selectivity for DNMT3A compared to the adenine methyltransferase EcoDam and the protein lysine methyltransferase G9a was found. Docking studies suggested that the chloro-nitroflavanones are located into the catalytic site with interactions with the SAM and the DNA pockets. The most active derivatives were also tested on a zebrafish development model. For the tested ones, the same phenotype as azacitidine was observed. Further characterization is needed to establish if these compounds have an antitumoral potential and then the specificity of such compounds for the DNMTs.

2.2.7 Curcumin

Among numerous reported pharmacological properties, curcumin has been also identified as DNMTi in a virtual screening based on a homology model of DNMT1 (Figure 6B). Confirmation has been obtained by evaluating the inhibiting properties of curcumin on M.SssI with an IC50 of 30 nM [71]. The same group at the Ohio State University filed a patent on a pharmaceutical composition of curcumin allowing to increase its plasma concentration [72]. Western blot analysis showing a downregulation of DNMT1, DNMT3a, and DNMT3b induced by curcumin
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A) RG-108 and derivatives

[Diagram showing RG108 structures from WO2007007054 and WO2008033744]

B) Procaine and derivatives

[Diagram showing Procaine and Procainamide from WO2012038417]

C) Compounds from WO2012087889

[Diagram showing IM25, CI-4-1, and 4e from WO2012087889]

Figure 5. Non-nucleoside compounds. A) RG108 derivatives. B) Procaine, procainamide derivatives. C) Molecules from WO2012087889.

in leukemia MV4-11 cells is also provided and experimental data of TSG reactivation are given, although the mechanism of DNMT downregulation by a direct-binding enzyme inhibitor is unclear. A 70% decrease in tumor growth in a xenografted MV4-11 model had been achieved when the mice were treated at 100 mg/kg without apparent toxicity. The pharmaceutical composition consisting of a combination of PEG and cremophor to improve the bioavailability of curcumin represents one of the key points of this patent. Taking into account the multiple activities attributed to curcumin, further research is needed to confirm its potential effect on the epigenetic regulation.

2.2.8 Psammaplin

Psammaplin A is a marine natural compound extracted in 1987 from the sponge *Psammaplinapysilla* (Figure 6C).
In 2003, psammaplin and natural analogues were found to inhibit HDAC and DNMT [73]. Based on these findings, a French group patented new synthetic psammaplin derivatives for their inhibition properties of both HDAC and DNMT [74]. Finally the preferred compound UVI5008 was found less active than RG108 (used as a reference product) in terms of DNMT inhibition, and rather found as a potent HDACi comparable to psammaplin A or MS275. Most recently, it was further confirmed that histone deacetylases are the main target of psammaplin and not DNMT [75].

2.3 Antisense oligonucleotides

An antisense oligonucleotide, MG98, directed against DNMT1, was validated as a demethylating agent in cells and tumor models. It entered Phase I and Phase II (in combination) clinical trials [76] but was stopped in 2006 by Methylgene to focus on other product developments [77].

3. Development of a DNMTi screening cell line

To improve screening methods, a patent has been filed detailing the development and application of a DNMTi detection cell line. In these cells, the green-fluorescent protein (GFP) gene has been inserted into the genome after methylation of its promoter. The methylation was shown to be stable, maintained in the cells, such that no GFP was expressed. Upon addition of DNMTi like azacitidine to the cells, DNA methylation dropped leading to expression of GFP.
4. Clinical studies

To further complete this overview we report here a selection of clinical trials showing the most recent advances for demethylation agents and the therapeutic strategies that are explored.

4.1 Hydralazine

Although the demethylating activity of hydralazine is not completely understood [79], it is considered as a DNMTi. Its use as reactivator of TSG expression in vitro, in vivo, and in patients was patented by researchers in Mexico [61]. A sustained release form of hydralazine permitting to deliver a demethylating effective dose and avoiding hypotensive effects has been also protected [80]. Several Phases I and Phases II clinical trials with hydralazine are ongoing mainly against solid tumors, some in combination with other anticancer drugs [see “hydralazine” at ClinicalTrials.gov for more informations].

4.2 Disulfiram

A clinical study is conducted by the Johns Hopkins University, using disulfiram identified as “a potent DNMTi inhibitor and recently found as one of the most potent inhibitors for prostate cancer growth in vitro” [81]. The purpose of the study is to explore the potential benefits of disulfiram treatment as a DNMTi. The study appears to be still active, but not recruiting and no recent informations have been added in ClinicalTrials.gov [82].

4.3 5-Fluoro-2'-deoxycytidine (FdCyd)

5-Fluoro-2'-deoxycytidine (FdCyd) is developed by the National Cancer Institute (USA). Two Phase I and one Phase II clinical trials are ongoing with FdCyd in solid tumors, in combination with an inhibitor of cytidine deaminase, tetrahydrouridine (THU). One of the issues is to evaluate whether treatment with FdCyd and THU alters DNA methylation patterns in tumor biopsies [83-85].

4.4 Azacitidine

Mainly the clinical trials with azacitidine concern its use in combination. For example, a Phase II trial aimed at determining the effect of azacitidine alone and in combination with histone deacetylase inhibitor benzamide MGCD0103 (mocetinostat) on AML or MDS patients over 60 years of age [86].

Several clinical trials deal with the use of azacitidine in solid tumors. Until recently little success was observed probably because the appropriate schedule and dose needs to be found. Today, a randomized Phase II trial is carried out by the Sidney Kimmel Comprehensive Cancer Center, USA and MethylGene, Inc., on Non-small Cell Lung Cancer patients with the combined usage of azacitidine and entinostat. A first study on extensively pretreated patients showed very encouraging results including a complete response [87]. In addition, a correlation with the methylation status of 4 genes (APC, RASSF1a, CDH13, and CDKN2a) was observed [88].

Another strategy that is explored is to apply demethylating agents to sensitize cells to other anti-tumor agents. Celgene Corporation carries out a clinical trial at the Sidney Kimmel Comprehensive Cancer Center, USA to evaluate the safety and to define the Maximal Tolerated Dose (MTD) or the Maximal Administered Dose (MAD) of oral azacitidine as a single agent and in combination with carboplatin (CBDCA) or paclitaxel protein bound particles (ABI-007, ABX) in subjects with relapsed or refractory solid tumors [89].

4.5 Decitabine

As in the case of azacitidine many trials are carried out to determine decitabine efficacy in combination with HDACi, other anticancer agents or even stimulators of the immune system. The aim is also to find a therapeutic strategy in solid tumors.

One trial conducted at the Weill Medical College of Cornell University, USA, in collaboration with Genzyme, combines decitabine with “plerixafor,” an inhibitor of stromal cell derived factor - 1α (SDF-1α), as induction and postremission therapy for older patients with AML to improve treatment outcomes through mobilization of leukemia stem cells and alteration of the pharmacodynamics of decitabine. The protocol will establish the safety and feasibility of combining two different doses of plerixafor with a fixed dose and schedule of decitabine [90].

At the University of Utah, USA, in collaboration with Amgen and Eisai, the main objective is “to evaluate the safety and feasibility of the sequential use of decitabine with a targeted biological agent against EGFR (panitumumab) for KRAS wild type tumors in the second or third line treatment of advanced metastatic colorectal cancer.” In the trial, the re-expression or a reduction in promoter methylation of TSG involved in colorectal cancer or in EGFR signaling pathway will be followed. The overall response as progression free survival will be evaluated too [91].

Based on the fact that DNA demethylating agents are able to reprogram cells, the Case Comprehensive Cancer Center, USA, tests whether decitabine can reprogram myelodysplastic cells in more normal-like stem cells [92].

Other interesting tests are going on combining DNMTi to agents that stimulate the immune system. In fact, decitabine may exert its antitumor activity by stopping the cells from dividing by its reprogramming activity. At the Nevada Cancer Institute, NV, USA, in collaboration with the NCI, decitabine is tested in combination with interferon α-2b in patients with unresectable or metastatic solid tumors [93].

A Phase I/II trial carried out at the Emory University, GA, USA, in collaboration with Novartis and Eisai, presents an interesting rational approach based on the reprogramming...
properties of DNMTi. Here patients with triple negative metastatic breast cancer, that do not express estrogen receptor (ER), progesterone receptor (PR), and HER2 and, thus, do not respond to agents such as trastuzumab (Herceptin®) and tamoxifen, are pretreated with decitabine and LBH 589, an HDACi, to restore the ER and then treated with tamoxifen. In fact it has been shown that ER in some triple negative breast cancers is epigenetically silenced. DNMTi (such as decitabine) and HDACi (such as LBH 589) can remove the epigenetic silencing marks (DNA methylation and histone deacetylation) and reactivate ER. This reactivated ER cells are sensible again to agents such as tamoxifen. In the first part of the trial, the reactivation ER will be followed and in a second part the response of tumor growth to tamoxifen will be evaluated [94].

5. Expert opinion

Several DNMTi have been discovered in the past years, but so far a highly efficient and specific compound has not yet been identified. The most potent inhibitors, the nucleoside inhibitors, in particular, azacitidine, display their action mainly after their incorporation into nucleic acids. The resolution of crystal structures of the mDnmt3a/Dnmt3L complex in 2007 [95] and hDNMT1 in 2011 and 2012 [96,97] and ever improving virtual docking routines will certainly lead to the prediction of novel directly binding inhibitors in the future. When combined with continuously improving screening assays on large libraries of tested compounds, the identification of novel, more potent and specific compounds is to be expected.

Furthermore, it is not clear today which DNMT is to be targeted for cancer treatment. DNMT1 is always expressed and is involved in the maintenance of the methylation pattern but for example the de novo DNMT3b is found overexpressed in certain tumor and DNMT3a shows many somatic mutations in tumors. The design of selective inhibitors will help answer this question.

From the clinical trials it appears that the choice of the dose and of the schedule are important elements. The reprogramming properties of DNA demethylation seem to play an important role for the therapeutic response. Again the discovery of potent catalytic inhibitors of the DNMT will help to explore this issue and give new hints for therapy.

It also seems that the determination of methylation status of the tumor tissue of the patient at relevant genes will be a useful marker for the prediction of response even if the level of DNA methylation alone may not be predictive. In addition this methylation status might also be important to determine whether demethylation can induce a sensibilization of cells to other anti-tumor agents given in combination.

Declaration of interest

The authors state no conflict of interest and have received no payment in preparation of this manuscript.
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