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Chapter 17

Inhibitors of DNA methylation

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Abstract. DNA methylation is involved in numerous biological processes and is deregulated in human diseases. The modulation of the activity of the enzymes and proteins in charge of DNA methylation, as for example the DNA methyltransferases (DNMTs), can represent a powerful strategy to alter DNA methylation patterns and restore biological processes that are aberrant in diseases. In this chapter, we will present examples of inhibitors of DNMTs (DNMTi). We will review their fields of application either as therapeutic molecules, for example in cancers, cardiovascular, neurological, and infectious diseases or as bioengineering tools. Finally, novel strategies to target DNA methylation and overcome the limits of single DNMT inhibitors will be described. These strategies consist in either targeting the methyl group reader proteins rather than targeting directly DNMTs or to combine within the same molecule a DNMT inhibitor with an additional active moiety, *e.g.* HDAC inhibitor, to improve efficacy and lower secondary effect of such drug.

17.1 How to inhibit DNA methyltransferases

The DNA methylation mechanism involves three main actors: DNA, DNA methyltransferase (DNMT) and the co-factor *S*-adenosyl-L-methionine (SAM). DNMTs scan DNA, recognize CpG sites and flip out 2'-deoxycytidine into the catalytic pocket of the enzyme. A cysteine residue (C1226 in human DNMT1, C711 in human DNMT3A and C652 in human DNMT3B) binds to position 6 of the cytosine (Figure 17.1 top), then transfer of the methyl group from the SAM occurs on the C5 position of the cytosine (Figure 17.1, grey arrow), the cysteine is released by β -elimination and the enzyme is ready to start a new catalytic cycle (Figure 17.1 top).

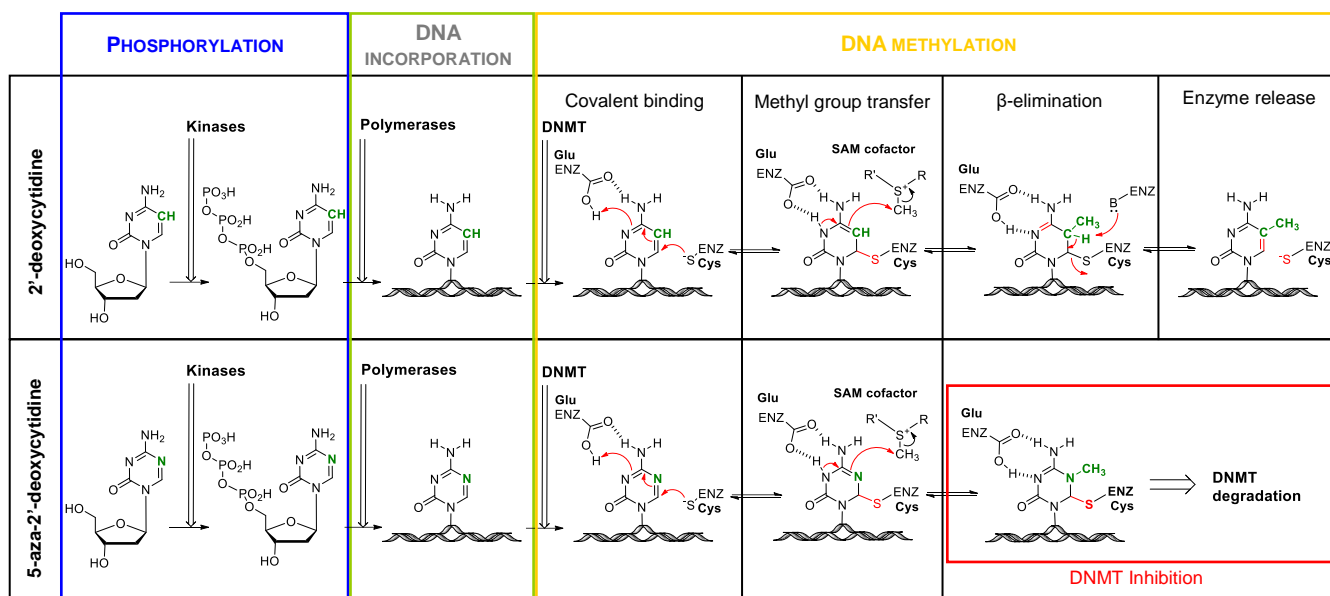


Figure 17.1. Schematic representation of DNMT catalytic cycle mechanism of inhibition by 5azadC.

DNMTs can be inhibited by targeting different parts of the catalytic pocket (the deoxycytidine binding site, the SAM binding site or both),-by targeting allosteric sites or by interfering with their binding to DNA. When the DNA is the target, for example by DNA binders, it is important to design specific compounds that do not inhibit other enzymes acting on DNA. A possibility is to use DNA ligands specific of CpGs sites. An interesting alternative are compounds that recognize the cytidine binding site into the DNMT catalytic pocket and, in addition, bear a chemical moiety able to react with the catalytic cysteine. Cytidine analogs, leading to a suicidal covalent complex targeting, are the best example and are described in 17.2. Another strategy is to target the co-factor binding pocket by SAM analogs for example. Since SAM is the most widely used enzyme cofactor after ATP (Struck, 2012), a challenge lies in the design of compounds specific for the DNMT SAM-binding site and not binding to other methyltransferases. The design of SAM analogs that also bind to the 2'-deoxycytidine binding site, such as bisubstrate inhibitors (Bon, 2021), could confer this specificity and in addition it could potentially give higher affinity compounds and more potent inhibitors. Below, we listed the principal inhibitors representing the different possible mechanisms of inhibition of DNMTs.

17.2 Chemistry and structure of DNMT inhibitors

DNMT inhibitors (DNMTi) are grouped in two families: the nucleoside inhibitors and the non-nucleoside ones (Figure 17.2). To date, two compounds, **5-azacytidine (5aza, azacytidine, Vidaza®)** and **5-aza-2'-deoxycytidine (5azadC, decitabine, Dacogen™ and in combination Inqovi®)** (Figure 17.2), are approved by the FDA for the treatment of hematological cancers. 5Aza and 5azadC were the first DNMTi and their story began with their synthesis in 1964 (Sorm, 1964). They were initially

tested as anti-metabolite agents against acute myelogenous leukemia (AML) (Cihak, 1974, Sorm, 1964), showing anticancer properties (Evans, 1968). However, it was in the late 70's - early 80's that a big turn was taken in their use when Jones and Taylor showed that non-toxic doses of 5aza and 5azadC induced cell differentiation by DNMT inhibition (Jones, 1980, Taylor, 1979).

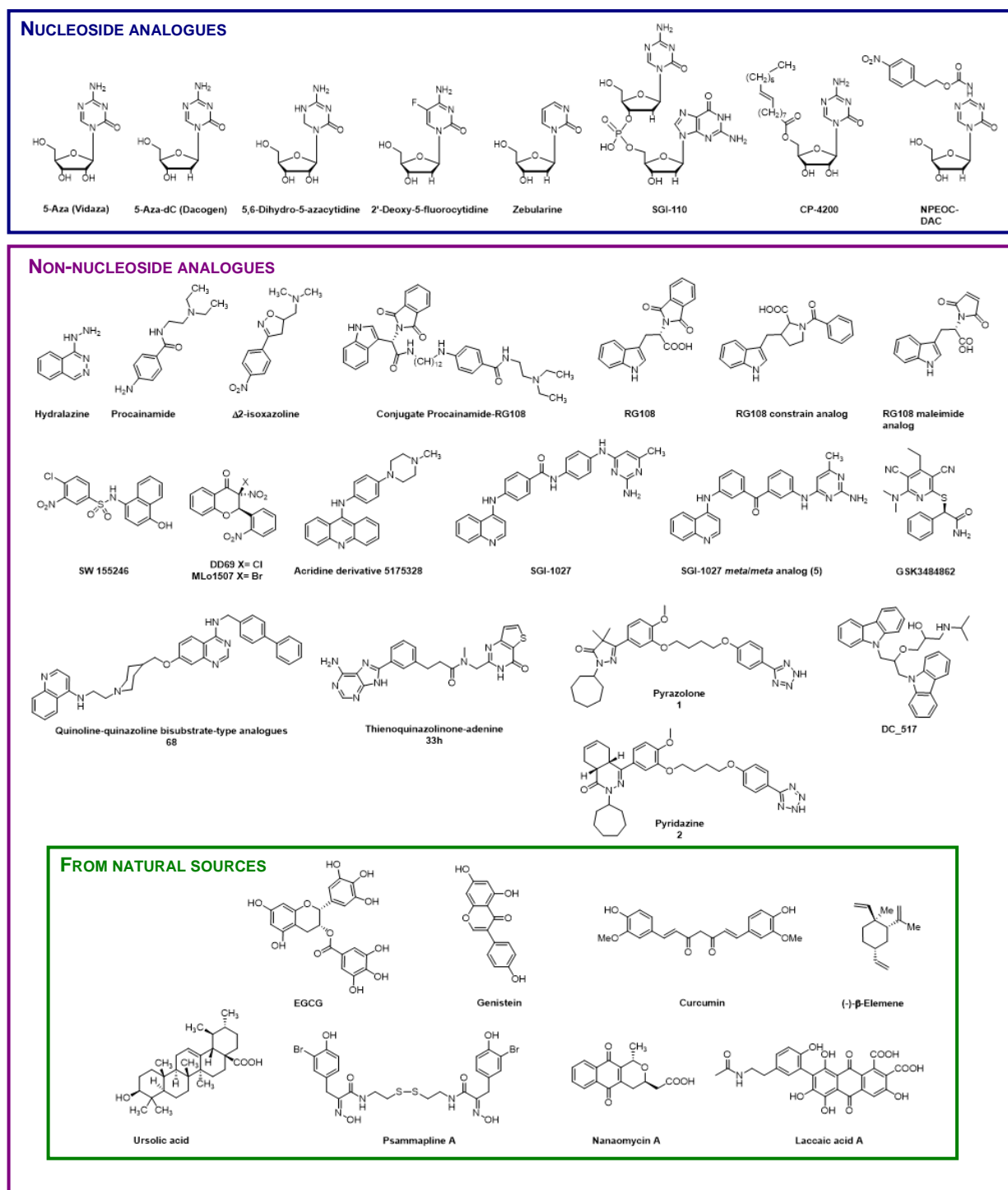


Figure 17.2. Selection of compounds described as DNMT inhibitors

A new paradigm was found, as these compounds were shown to be able to reprogram cells, *i.e.* induce silenced-genes expression and thus restore normal cell functions. The *aza*-nucleosides, once phosphorylated in cells and incorporated into DNA, replace the cytosine in the catalytic pocket of the enzyme and act as suicide substrates of DNMTs (cf. Figure 17.1). In DNA, at the CpG sites, 5azadC is flipped out from the double-helix into the catalytic pocket, the catalytic cysteine binds covalently to C6 position, but the β -elimination, essential to release the enzyme, cannot occur after the methyl group transfer because of the presence of a nitrogen atom at the C5 position. The enzyme is thus irreversibly trapped on the DNA and further degraded by the proteasome (Santi, 1984), inducing DNA demethylation.

Based on these findings, 5aza (Vidaza®) and 5azadC (Dacogen™) were approved in 2004 and 2006, respectively, by the FDA for the treatment of acute myelodysplastic syndrome (MDS), and chronic myelomonocytic leukemia (CMML). However, these drugs are chemically and metabolically unstable, present a low bioavailability (Chan, 1979, Notari, 1975), and, as they are incorporated at every deoxycytidine, are not selective (Karahoca, 2013). Nevertheless, the proof of concept for DNMTi as a therapeutic target was established and since then many efforts have been dedicated to identify novel DNMT inhibitors (DNMTi).

First, the chemical instability of 5aza and 5azadC was addressed. Indeed, The 5-azacytosine moiety was shown to be sensitive to rapid hydrolysis under mild condition over two steps; a reversible ring opening to the N-formylguanylylribosylurea followed by an irreversible formation of guanylylribosylurea specie (Chan, 1979). More stable analogs were designed, like **5,6-dihydro-5-azacytidine**, **2'-deoxy-5,6-dihydro-5-azacytidine**, **2'-deoxy-5-fluorocytidine** and **zebularine** (cf. Figure 17.2). **5,6-Dihydro-5-azacytidine** showed weak inhibition of DNA methylation and was withdrawn from clinical trials (Yogelzang, 1997). Its analog, **2'-deoxy-5,6-dihydro-5-azacytidine**, was shown not to bind covalently to DNMTs, but simply to occupy the cytidine pocket (Sheikhnejad, 1999), resulting in methylation inhibition in several cell lines (CRF-CEM and HL60) with very low cell toxicity (Matoušová, 2011). **2'-Deoxy-5-fluorocytidine** was described as more stable in aqueous media and as potent *in vitro*, but it is currently rather recognized as a pro-drug of a thymidylate synthase inhibitor, because it is metabolized into 5-fluorouridine (Boothman, 1989). **Zebularine** is also a stable compound with a weaker inhibition activity and cytotoxicity than azacytosine analogs (Flotho, 2009). Its mode of action is different from 5aza and 5azadC, since it forms a very stable, but reversible complex with DNMTs that showed a slow dissociation kinetic (Champion, 2010, van Bemmelen, 2009).

Second, a pro-drug approach was chosen aiming at releasing the active molecule in the organism. This approach resulted in the generation of **NPEOC-DAC** (Byun, 2008), **CP-4200** (Brueckner, 2010) and **SGI-110** (Chuang, 2010, Yoo, 2007). **SGI-110** or **guadecitabine**, composed of a 5azad-CpG dinucleotide, is a pro-drug of 5azadC, it entered clinical trials and is the most promising pro-drug with a comparable *in vitro* and *in vivo* DNMT inhibition activity. The advantage of SGI-110 is that it is less sensitive to cytidine deaminase than 5azadC, which improves its metabolic stability.

Third, non-nucleoside compounds were investigated. However, most of them lack specificity, show weak activity against the enzyme or do not induce a strong DNA methylation inhibition in cellular models. Several natural products were reported as DNMTi (Figure 17.2), for example, (-)-**epigallocatechin-3-gallate (EGCG)** (Fang, 2003) and **laccaic acid** (Fagan, 2013). These natural products lack specificity and their mode of action was afterwards questioned, as the one of **genistein** (Fang, 2005), **psammaphin A** (Baud, 2012, Pereira, 2012) and **curcumin** (Liu, 2009). Indeed, indirect or off-target effects seem to be responsible for the observed inhibition of DNA methylation. All these compounds are known as multi-target compounds (Lopez, 2015, Pechalrieu, 2017). Similarly, Hann's team highlighted an indirect decrease in DNMT1 expression in cancer cells (lung cancer and hepatocarcinoma) *via* ERK1/2- and AMPKa-mediated inhibition of the transcription factor Sp1 after treatment with two natural products, **β -elemene** (Zhao, 2015) and **ursolic acid** (Yie, 2015). **Nanaomycin A** is a more specific compound, because it was found to target specifically DNMT3B, and shown to induce genomic DNA methylation inhibition in colon cancer cell line (Kuck, 2010a). Nevertheless, in a recent study, Penter *et al.* did not observe any DNA methylation inhibition by nanaomycin A in four neuroblastoma cell lines, although they observed an interesting synergistic activity when used in combination with doxorubicin (Penter, 2015). To conclude, none of the natural products described to date clearly targets the DNMTs directly in cells and their mechanism of action is controversial (Li, 2010, Medina-Franco, 2011, Suh, 2012).

The same difficulty to identify potent and specific non-nucleoside DNMTi is found among the synthetic compounds. For example, drug repurposing showed that **hydralazine** and **procainamide** are weak DNMTi (Candelaria, 2012, Chuang, 2005). These two FDA approved drugs are vasodilator and anti-arrhythmic compounds, respectively, that were described as DNMTi because patients developed auto-immune disease (*lupus erythematosus*) correlated to an abnormal DNA methylation pattern of T-cells (Cornacchia, 1988). Despite the weak DNMT inhibition activity of hydralazine, a molecular modeling study (Singh, 2009a) showed that hydralazine could be involved in a complex network of hydrogen bonds into the cytidine pocket. Today, hydralazine is still extensively studied, especially in combination with valproic acid (VPA), an HDAC inhibitor (Dueñas-Gonzalez, 2014). Procainamide was also described to potentially interact within the catalytic side by molecular modeling (Singh, 2009a), while biophysical studies previously demonstrated that it acted as DNA ligand and decreased the processivity of DNMTs (Lee, 2005). Procainamide and procaine were indeed described as DNA ligands with a certain specificity for CG-rich regions, potentially targeting the DNA binding of the DNMT as described above in paragraph 17.1 (Villar-Garea, 2003). Procainamide was an interesting starting point for drug design and several constrained derivatives were synthesized and tested (Castellano, 2008, Castellano, 2011). In parallel, procainamide conjugated to RG108, an *in silico* identified pharmacophore (see paragraph below), induced a synergy in the inhibition of the enzymatic activity of DNMT3A and DNMT1 (Halby, 2012). Initially, it was hypothesized that the procainamide

moiety of these compounds would bind to DNA and RG108 moiety would bind to DNMT catalytic pocket. Later, a molecular modeling study suggested that the conjugates could occupy both SAM and cytidine pockets (Yoo, 2013) explaining the increased activity (cf. paragraph 17.1).

In parallel, virtual screening-based studies led to the identification of **RG108** (Brueckner, 2005, Siedlecki, 2006, Stresemann, 2006), **DC_517** (Chen, 2014), **thienoquinazolinone-adenine 33h** (Newton, 2020) and **pyrazolone/pyridazine** derivatives (Huang, 2021). Recent experimental studies revealed that RG108 has only a weak activity against human DNMTs (Halby, 2012, Suzuki, 2010) and more potent derivatives were since synthesized such as **maleimide** (Suzuki, 2010), **constraint analogs** (Asgatay, 2014) and other derivatives (Rondelet, 2017, Rotili, 2014). Nevertheless, their ability to inhibit genomic DNA methylation has still to be established for all these inhibitors. New generation compounds seem to be more promising, as they showed DNA methylation inhibition and/or gene re-expression in cells. **DC_517** and **Cpd 33h** showed micromolar inhibition activity against DNMT1 ($IC_{50} = 1.7 \mu M$) (Chen, 2014) and DNMT3B ($IC_{50} = 8 \mu M$) (Newton, 2020), respectively. DC_517 reduced proliferation and induced apoptosis in HCT116 colorectal cancer cell line (Chen, 2014). Regarding pyrazolone/pyridazine derivatives, enzymology studies interestingly suggested that they act as allosteric inhibitors with high-micromolar range activity on purified DNMTs (Huang, 2021) but their efficacy in cells needs to be confirmed. Virtual screening-followed by structure optimization also led to the discovery of **GSK3484862** (Pappalardi, 2021), a highly potent DNMT1-selective inhibitor with $IC_{50} = 0.23 \mu M$ vs $IC_{50} > 50 \mu M$ for DNMT3A/B. In cells, GSK3484862 decreased promoter methylation and reinduced expression of VIM gene, and it also induced global decrease of DNA methylation levels in murine embryonic stem cells with limited toxicity (Azevedo Portilho, 2021).

Several High Throughput Screening (HTS) campaigns were performed and allowed to identify new families of DNMTi. **3-Chloro-3-nitroflavanones** were identified (Ceccaldi, 2011) with sub-micromolar activity against Dnmt3a/3L complex and exhibiting a phenotype in zebrafish embryos similar to the one observed with the 5aza. This scaffold was further optimized and **3-bromo-3-nitroflavanones** showed improved stability and cellular activity in several cancer models *in vitro* and *ex vivo* (Lobo, 2021, Marques-Magalhães, 2021, Pechalrieu, 2020). A naphthoquinone, diclone, and other flavonoids were identified in another screening campaigns, opening the path to study the impact on the epigenome of plants, animals and humans when addressing the toxicology of pesticides (Ceccaldi, 2013). Molecular modeling studies suggested that the most potent compounds occupy both the cytosine and SAM pockets (Ceccaldi, 2011). **SW155246**, an aromatic sulfonamide, was also identified by HTS (Kilgore, 2013). Interestingly, this compound was able to induce a weak methylation inhibition and reactivation of tumor suppressor genes (TSGs) in human lung carcinoma. Acridine derivatives, known to intercalate into DNA, were described to modulate DNA methylation (Hossain, 2013). Among them, derivative **517328** was the most potent analog inhibiting DNMT1 *in vitro* (~30% inhibition at $10 \mu M$) and DNA methylation of TSGs in colon and pancreatic cancer cell lines. This compound demonstrated that DNA binders inhibit DNMTs, however the specificity of such

inhibitors remains an important limitation to their use. **SGI-1027** is another weak DNA ligand that was identified to be a DNMT1 inhibitor (Datta, 2009). Molecular modeling studies suggested that SGI-1027 could inhibit DNMTs by occupying both cytidine and SAM pockets (Yoo, 2013) and Gros *et al.* (Gros, 2015) confirmed, by biophysical studies, that it interacts with DNA and functions as a SAM non-competitive but DNA-competitive inhibitor. SGI-1027 was described to induce DNMT1 degradation and TSG re-expression in colon cancer cell lines and was quickly considered as the most promising starting point for DNMTi design. Several derivatives (Gamage, 2013, Rilova, 2014, Valente, 2014) succeeded in increasing significantly DNMT inhibition potency with the *meta/meta* analog, but no potent inhibition of genomic DNA methylation or TSG re-expression has so far been shown. Further investigations to characterize the mode of action of this compound family indicated that the *meta/meta* analog strongly interacted with DNA and inhibited DNMT by DNA interaction and destabilization of the DNMT/DNA/SAM complex (Gros, 2015).

Finally, Halby *et al.* carried out a rational design based on bisubstrate analogues and identified **quinoline-quinazoline** DNMTi with compound **68** as their lead compound. This DNMTi strongly inhibits DNMT3A ($EC_{50} = 1.1 \mu\text{M}$) with a good selectivity over DNMT1 ($EC_{50} = 100 \mu\text{M}$). Besides it potently reduced promoter methylation of *CDKN2A* together with an increase of its expression level (Halby, 2017).

To conclude, to date only nucleoside DNMTi were FDA-approved and SGI-110 is a promising pro-drug of 5azadC, evaluated in clinical trials both in hematological and solid cancers. An increasing number of new non-nucleoside DNMTi was published in the last few years, but most lack selectivity or demonstrated impact on genomic DNA methylation and TSG re-expression, except for GSK3484862, an allosteric inhibitor, that is to date the most promising chemical probe for DNMT. Several strategies are therefore implemented to discover more potent DNMTi.

19.3 Potential applications of DNMT inhibitors

DNA methylation is crucial for the control of gene expression and cell proliferation. Indeed, DNA methylation on the CpG islands of gene promoters leads to the silencing of the corresponding gene. This participates in the dynamics of the gene expression regulation in cells and is crucial for normal cell functions. However, many factors, including diet, stress, environmental conditions, etc. can lead to abnormal DNA methylation patterns. These deregulations are often responsible for dysfunctions and development of diseases such as cancers, neurological and cardiovascular diseases, and abnormal plant growth (Figure 17.3). As all epigenetic modifications, DNA methylation is reversible and therefore can be chemically reversed. The main application of DNMTi is by far their use as anticancer agents. However, the importance of DNA methylation in various biological contexts has led to increasing research in the context of other pathologies, such as neurodegenerative and cardiovascular diseases and in other fields, such as plant growth optimization.

19.3.1 DNMTi application in cancers

In cancer, hypermethylation of TSG promoter regions is observed together with a global hypomethylation (Esteller, 2008). The hypermethylation of promoters of genes, such as *P53*, *P16*, *P15*, *RARβ2*, *HIC1* and *RASSF1A*, results in their silencing and participates in tumor formation, maintenance and proliferation.

a. Nucleoside analogues

As single agent

As described above, 5aza and 5azadC are the most extensively used DNMTi. These nucleoside analogues are able to decrease TSG-promoter methylation levels in cancer cells and induce TSG re-expression, resulting in cell reprogramming and, eventually, cell cycle arrest and apoptosis (Fahy, 2012). A historical overview of their development and application is described in (Issa, 2009). As single agents, 5aza or 5azadC are FDA- and EMA-approved to treat certain forms of leukemia. To date there is no approval of 5aza or 5azadC monotherapies for solid tumors and only phase I and II clinical trials are ongoing for example for liver, colorectal, breast prostate, pancreatic and non-small cell lung cancers and head neck carcinoma, (<https://clinicaltrials.gov/ct2/home>). Table 17.1 reports some examples of current clinical trials.

Table 1. Examples of DNMTi FDA-approved and in phase II and III clinical trials as a single drug or in combination Phase of clinical trials are mentioned in brackets.

	Status	Drugs	Type of cancer targeted (phase of study)
as a single agent	FDA-approved	5aza (Vidaza®)	AML, MDS
		5azadC (Dacogen™)	AML, MDS, CMML
	in clinical trial	Guadecitabine	AML (III), MDS (III), CMML (III), Gastrointestinal Stromal Tumors (ii)
		5Aza	T-cell lymphoma (III), DLBCL (III)
		Disulfiram (DSF)	Glyoblastoma (II), Non-small Cell Lung Cancer (III)
in combination	with other epi-drug	5AzadC + SAHA	haematological cancers (II)
		5AzadC + VPA	AML (II), MDS (II)
		5Aza + entinostat	Colon and rectal cancer (II), non-small lung cancer (II)
	with chemotherapies	5AzadC + cedazuridine (Inqovi®)	MDS (FDA-approved)
		5Aza or 5AzadC + ATRA	AML (II), MDS (II), prostate cancer (II)
		5Aza or 5AzadC + Venetoclax	AML (III), MDS (III), NHL (II)
		5Aza + Glasdegib	AML (III), MDS (III), CMML (III)
		5AzadC + Ivosidenib	AML (III)
	with immunotherapies	5Aza + Magrolimab (anti CD-47)	AML (III), MDS (III)
		5AzadC + Talacotuzumab (anti CD-123)	AML (III), MSD (II)
		5AzadC + Camrelizumab (anti PD-1)	Hodgkin Lymphoma (III)

As described above, to overcome 5aza and 5azadC drawbacks (*i.e.* chemical instability, poor delivery and side-effects), other nucleoside analogs were developed (Figure 17.2), but they need to be used at higher doses due to their lower efficacy and up to date no clinical trial were undertaken with these compounds.

Despite their promise for non-solid tumor treatment, the direct use of nucleoside analogs as a single therapeutic agent is quite limited considering their instability, their side effects and their lower therapeutic index in solid tumors.

Pro-drugs of nucleoside analogues

Pro-drugs of 5aza and 5azadC, which delay the release of the active molecule in the organism and lower its degradation, showed a real improvement of the drugs. CP-4200 is a lipophilic ester of 5aza patented for its better cellular uptake and activity *in vivo* in an orthotropic acute lymphoblastic leukemia (ALL) mouse model (Silverman, 2009), but no clinical trial was undertaken up to date. SGI-110 (guadecitabine) is a 5azadC pro-drug, which avoids its decomposition by deaminase (Chuang, 2010). In the last few years many clinical trials were started with SGI-110 alone (for example in phase III clinical trials for AML (Acute Myeloid Leukemia), MDS (Myelodysplastic Syndrome) and CMML (Chronic Muelomonocytic Leukemia), in phase II for gastrointestinal cancer and in phase I for testicular cancer) and in combination with immunotherapies (for example in phase II for kidney, ovarian, melanoma and (non-) small cell lung cancers).

Nucleoside DNMTi in combination with other drugs

With other epidrugs

Nucleoside DNMTi were studied in combinations with other epidrugs, as for example 5azadC was combined with trichostatin A (TSA), an HDACi, in colorectal carcinoma cell line. Whereas TSA alone was not able to cause re-expression of *MLH1*, *TIMP3*, *P15* and *P16* genes, a pre-treatment with a low dose of 5azadC led to their re-expression (Cameron, 1999). The combination of 5azadC and valproic acid (VPA), another HDACi, was evaluated in AML and MDS (Yang, 2005) and tested in phase II clinical trials (NCT00414310). However, no improvement in outcome was reported. The association of 5aza or 5azadC with the FDA-approved HDACi vorinostat (SAHA) was proven to be of interest in hematological cancers (Silverman, 2008), in colorectal cancer cells (Najem, 2019) and *in vivo* in a colon carcinoma CT26 experimental lung metastasis mouse model (Yang, 2012). In addition, 5aza was reported to be active in combination with entinostat and mocetinostat, benzamide inhibitors of HDACi (Fandy, 2009) and these combinations are tested in phase I and II clinical trials in hematological cancers. 5aza/entinostat combination is also studied in solid cancers, such as advanced non-small cell lung cancer (NCT01886573) (Juergens, 2011), advanced breast cancer (NCT01349959) and metastatic colorectal cancer (NCT01105377) (see Table 17.1 for some examples). Combination of

5aza/pracinostat, an hydroxamic acid based HDACi, is in phase III for the treatment of AML (NCT03151408).

With “classical” chemo- and immunotherapies

Great promises arise from the use of nucleoside DNMTi in combination with chemotherapies or immunotherapies in tumors (Table 17.1). The rationale of these combination approaches relies on the fact that, due to their capacity to globally affect cells and restore cell functions, DNMTi can increase the sensitivity to other anticancer agents, resulting in higher efficacy (Ahuja, 2014, Ahuja, 2016, Azad, 2013, Cameron, 1999). A plethora of clinical trials, involving multi-anti-cancer agents in combination with both nucleoside DNMTi (5aza and 5azadC), are currently ongoing, mainly in hematologic cancers with 39 phase III/IV active clinical trials. Many phase III/IV trials have been undertaken in the last few years. 5aza and 5azadC are in phase III in combination with “classical” chemotherapies such as doxorubicin, all-*trans* retinoic acid (ATRA) or cytarabine. Both nucleosides are also in phase III and IV in combination with Venetoclax, a Bcl2 inhibitor, Glasdegib, a Hedgehog pathway inhibitor, Ivosidenib, an IDH1 inhibitor, Eprentapopt, a P53 reactivator, or the kinase inhibitors Midostaurin, Gilteritinib, Clofarabine, exclusively against hematological cancers, *i.e.* AML, MDS or CMML. Besides this, several combinations of nucleoside DNMTi with monoclonal antibodies, such as anti-CD47 (Magrolimab), anti-CD3 (Visilizumab), anti-CD123 (Talacotuzumab), anti-TIM-3 (Sabatolimab) or anti-PD-1 (Camrelizumab), have reached phase III and IV. Finally, a combination of 5azadC and Cedazuridine, a cytidine deaminase inhibitor, was approved in 2020 for the treatment of MDS (Inqovi®).

In parallel, several studies described an enhancement of radiosensitivity by 5azadC treatment in lung cancer A549 and glioblastoma U373MG cells (Kim, 2012), SaOS2, HOS, and U2OS osteosarcoma cells (Li, 2014) and MDA-MB-231 and MDA-MB-435 breast cancer cell lines (Wang, 2013a). In all cases, the increase in radiosensitivity was correlated with cell cycle-, apoptosis- and radiosensitivity-related gene up-regulation and G2/M cell arrest.

In summary, nucleoside DNMTi are frequently used for hematological cancers. New clinical trials are ongoing to optimize the treatment schedule, using a low-dose strategy, and evaluate DNMTi efficacy in solid tumors. SGI-110 could eventually overcome the compound stability limitation. In parallel, their use in combination with other anticancer agents is giving promising results increasing the efficacy of the treatments and decreasing the side effects, since lower doses of each drug are used.

b. Non-nucleoside DNMTi

As described above, several non-nucleoside DNMTi have been reported, but none obtained the same potency and validation as 5aza and 5azadC. Still ongoing studies and clinical trials are required to better understand the therapeutic potential of DNA methylation (Erdmann, 2015). Hydralazine (de la Cruz-Hernandez, 2011, Graça, 2014b) failed as single therapeutic agent in phase II for breast and

rectal cancer (Wang, 2009). Its combination with VPA (Duenas-Gonzalez, 2008) was completed in phase II against cervical cancer (NCT00404326) and refractory solid tumors (NCT00404508) more than 10 years ago and no further studies had been started since.

Indirect effects on DNA methylation are also of therapeutic interest. In the presence of disulfiram, *in vitro* DNMT1 activity was shown to be reduced, and global hypomethylation was observed in prostate cancer cell lines, with decrease in *APC* and *RAR-β* promoter methylation accompanied by their re-expression. Xenograft tumor volumes were shown to be reduced under disulfiram treatment independently of the dose (from 10 to 40 mg.kg⁻¹) (Lin, 2011). However, a completed Disulfiram phase II study prostate cancer showed a limited impact (NCT01118741). Combination of disulfiram with copper improved its potency in refractory cancers (Li, 2020a). This combination is currently in phase II against metastatic breast (NCT03323346) and pancreatic (NCT03714555) cancers and in phase III against glioblastoma (NCT02678975).

RG108, found by virtual screening, was shown to inhibit methylation in NALM6 (leukemia cell line) and in HCT116 (colorectal cancer cell line), inducing re-expression of *P16* and *TIMP3* (Stresemann, 2006). In prostate cancer cell lines (LNCaP and 22Rv1), it decreased DNMT activity together with cell growth inhibition and apoptosis (Graça, 2014a). However, the mechanism by which RG108 treatment resulted in a decrease of the methylation level in cells remains to be understood. Nevertheless, since it is commercially available, it was used as a tool to inhibit DNA methylation in several cellular models (see below).

19.3.2 DNMTi application in neurological and psychiatric disorders

DNA methylation, together with other epigenetic modifications, is also deregulated in neurological diseases and psychiatric disorders, such as schizophrenia, epilepsy or bipolar disorder (for review, (Mohd Murshid, 2022)). However, to date, no DNMTi has been approved for these diseases, the main hurdle being the poor capability of the existing DNMTi to cross the blood-brain barrier (BBB). Nevertheless, interesting results, listed here below, highlight the great interest of targeting DNA methylation in neurological and psychiatric disorders.

a. Memory formation

Memory loss is involved in many neurodegenerative diseases, such as Alzheimer's and Parkinson's diseases, schizophrenia or post-traumatic stress disorders. DNA methylation is implicated in memory formation, as it regulates gene transcription in central nervous system (CNS) and is required in long-term memory formation (Day, 2010). Additionally, DNA methylation was demonstrated to be involved in synaptic plasticity as *Dnmt1/Dnmt3a* double knockout in mice exhibit long-term potentiation (LTP) deficiencies (Feng, 2010). To better understand the implication of DNA methylation in various brain regions, rats were treated with RG108 by using the object-in-place paradigm, a test based on the memory of the positioning of objects in an enclosed space, which

requires both hippocampus and perirhinal cortex brain regions. Using this model, Michnick *et al.* demonstrated that DNA methylation was required for long-term but not short-term memory (Michnick, 2015). In parallel, expression of *Pp1*, a memory suppressor gene, and *Reln*, coding for reelin, a positive memory regulator, was shown to be controlled by promoter methylation. Following fear conditioning, an increase in *Pp1* promoter methylation and a decrease in *Reln* promoter methylation levels was observed in adult rat hippocampus. Infusion of 5aza in adult mouse brain inverted these effects, decreased *Pp1* methylation and enhanced the low methylation level of *Reln*, which inversely correlated with the expression of both genes (Miller, 2007). In rats trained for contextual fear memory, the authors observed that intra-anterior cingulate cortex infusions of 5aza or zebularine, 30 days post-training, disrupted remote memory (Miller, 2010). Reelin is also implicated in synaptic plasticity that is involved in storage memory. Levenson *et al.* demonstrated that zebularine induced a significant methylation decrease in one of the two CpG islands of the *Reln* promoter, whereas the other CpG methylation level remained steady (Levenson, 2006). The impact of DNMTi in synaptic plasticity was studied by Nelson *et al.* in hippocampus slices, where 5azadC was reported to decrease the genomic DNA methylation level, concomitantly with a reduction of miniature excitatory post-synaptic current frequencies in neurons, impacting neuronal activity (Nelson, 2008). Tetrodotoxin, known to decrease neuronal activity, also induced a loss of methylation and the same effects were observed both by using RG108 or by knocking down *Dnmt1* and *Dnmt3a* (Meadows, 2015). Treatment of honeybees with RG108 also induced relearning and bees were able to discriminate between a known and a new odor (Biergans, 2016).

Altogether, these studies show that DNA methylation is implicated in memory regulation, long-term memory and synaptic plasticity. DNMTi were shown to interfere with the long-term memory and to enhance synapse receptiveness in neurons (synaptic upscaling). Consequently, DNMTi were shown to impair memory, suggesting a positive effect on post-traumatic stress disorders (PTSD) caused by long-lasting traumatic memory (see below). Animal studies using 5azadC were, in most cases, carried out by direct injection in brain regions or on slice cultures. However, 5azadC injected intraperitoneally in mice led also to an increase of *Bdnf* expression and a lower global DNA methylation in the hippocampus (Sales, 2011). These effects on memory functions were hypothesized as limiting factor for the use of decitabine in MDS patients (Aydin, 2012).

b. Schizophrenia

Schizophrenia is a cognition disorder often characterized by hallucination, paranoia and failure to adopt a “normal” behavior in social situations. It is now quite clearly established that a deregulation of the GABA ((γ -aminobutyric acid)ergic/glutamatergic) network in the hippocampus and cortex is characteristic of psychotic disorders including schizophrenia (Lewis, 2005). More specifically, a down-regulation of GABAergic genes, such as *glutamic acid decarboxylase₆₇* (*GAD₆₇*) and *RELN*, was measured in post-mortem samples of schizophrenia patients (Guidotti, 2000). This down-regulation

was correlated with an hypermethylation of their CpG island promoter regions (Chen, 2002, Grayson, 2005) and with an increase in DNMT1 (Veldic, 2004) and DNMT3A (Zhubi, 2009) mRNA expression in schizophrenia post-mortem cortical GABAergic neurons. In a model of prenatal-stress mice exhibiting a schizophrenia-like behavior, high levels of Dnmt1 and Dnmt3a expression occurred in GABAergic neurons (Matriciano, 2013). Additionally, a knock-down of *Dnmt1* in mouse primary cortical cultures showed that *Reln* expression is controlled by Dnmt1 (Noh, 2005).

In this context, DNMTi were tested to decrease the methylation level and restore a normal expression of *GAD₆₇* and *RELN*. 5aza, zebularine and procainamide were tested in cell cultures and an increase in the expression of *GAD₆₇* and *RELN* and a decrease in *DNMT1* expression were observed (Kundakovic, 2007). However, 5aza and zebularine, the most active molecules in the cellular context, present limiting potential to cross the blood-brain barrier (BBB).

c. Bipolar Disorders

Bipolar disorders are maniac-depressive disorders characterized by a succession of elevated mood and depression periods. Aberrant DNA methylation patterns are implicated in the etiology of bipolar disorders and, very similarly to schizophrenia, patients with bipolar disorders show a down-regulation of *GAD₆₇* and *RELN* (Guidotti, 2000). Because of the lack of relevant animal models to study bipolar disorders, very few studies involving DNMTi have been carried out. Mainly, VPA, an HDACi, was showed to decrease DNA methylation without decrease in DNMT expression (Aizawa, 2015). VPA is FDA approved for bipolar disorders and in clinical studies in combination with antipsychotics.

d. Epilepsy

Epilepsy is a group of neurological diseases for which causes are unknown in most cases. Epileptic patients are subject to recurrent unprovoked seizures, which are brief to quite long periods of intense shaking.

In temporal lobe epilepsy, the most common epilepsy syndrome in human, low levels of reelin were observed in brain specimens (Heinrich, 2006) in correlation with a hypermethylation of its promoter region (Kobow, 2009). A genome-wide study revealed a global DNA hypermethylation in chronic epileptic rat correlated to a decrease in reelin gene expression at the mRNA level (Kobow, 2013). Moreover, *Gria2*, a Glu2A subunit of ionotropic glutamate receptor, has been identified to play a role in epilepsy, and its hypermethylation and corresponding decrease in mRNA expression was correlated to the intensity and frequency of seizure in rats (Machnes, 2013). In an equivalent rat model of kainic acid-induced epilepsy, bisulfite sequencing showed a hypermethylation of *Grin2b/Nr2b*, resulting in a lower level of the GRIN2B protein, another glutamate receptor subunit, together with a hypomethylation of the *Bdnf* gene in epileptic hippocampus (Parrish, 2013). RG108 treatment on kainite-treated hippocampal slice cultures showed an inhibition of the *Gria2* hypermethylation and a beneficial increase in *Gria2* activity (Machnes, 2013). Zebularine also decreased the methylation level

of *Grin2b/Nr2b*, concomitantly with an increase in GRIN2B protein level (Parrish, 2013). All these findings are in favor of applying DNMTi to epilepsy treatment. It was also demonstrated that treatment with RG108 led to a lower methylation level of *RASgrf1* gene and increase its expression, which was associated with the suppression of epileptic seizure in epileptic mice model (Chen, 2017).

e. Post-traumatic stress disorder

Post-traumatic stress disorders (PTSD) appear in patients who experienced a psychologically traumatic event, such as violent death witnessing, domestic violence, child abuse, etc. and result in severe anxiety associated with memory avoidance, flashbacks, nightmares and emotional arousal. For example, war veterans commonly suffer from PTSD.

In PTSD patients, altered DNA methylation was observed in the peripheral blood immune cells. Together with other genes (Uddin, 2010), *BDNF* was reported to be hypermethylated (Smith, 2011). In rat models, epigenetic modifications were identified to play a role in fear memory and Miller *et al.* demonstrated that *Dnmt3a* and *3b* are up-regulated, whereas level of *Dnmt1* remains steady in rat hippocampus, in response to contextual fear conditioning (Miller, 2007). Models of maltreated rats compared to normal animals also showed an increase in DNA methylation levels of *Bdnf*, associated with a decrease of *Bdnf* expression. This modification is a lasting effect, which was observed in adulthood and passed to the next generation (Roth, 2009). DNA methylation of *BDNF* contributes to learned fear and BDNF is important for the persistence of the pathological fear. Therefore, DNMTi could be envisaged to erase the memory of the trauma in PTSD patients (Zovkic, 2013). Administration of DNMTi (5aza or zebularine) induces *Bdnf* expression even in adult animals. In PTSD, an over-consolidation of the fear memory is observed and nucleoside DNMTi and RG108 were able to block the contextual fear memory formation. RG108 was also tested directly in rat brains and shown to abolish long-term fear memory (Miller, 2010).

In rodent, neonatal maternal separation (NMS) attenuates neuron differentiation and can lead to stress-induced behavioral disorders. It results in a decrease in *RAR α* expression concomitant with an increase in the *RAR α* promoter methylation. *Dnmt1* was shown to be up-regulated, whereas no change was measured in *Dnmt3a* and *Dnmt3b* expression in adult dentate gyrus-derived neural precursor cells (ADP). 5-AzaadC was capable of increasing the neuronal differentiation and decreasing methylation of *RAR α* (Boku, 2015).

f. Depression

Sadness, low mood, loss of motivation, and low self-esteem characterize pathological depression, which, in the most severe cases, can lead to suicide. For example, 38.000 cases of suicide were reported in 2010 in the USA, which represents more than the number of human beings killed by car accident (34.000) or twice more than victims of homicide (16.000) (Nature Editorials, 2014). About 60% of the subjects who committed suicide had depression or related mood disorders.

Until recently, the only epidrugs used to treat depression were HDACi (Sun, 2013). However, a couple of years ago, DNA methylation was proven to be involved in this disorder, and the environment was shown to play a major role, particularly early-life environmental stress (Booij, 2013). Dnmt3a was identified in a mouse model as regulating emotional behavior (LaPlant, 2010). In patients with severe depression and previous suicidal attempts, *BDNF* hypermethylation was proposed as a biological marker of suicidal behavior (Kang, 2013). Additionally, the gene encoding for P11 protein, a modulator of neuronal function involved in depression, was demonstrated to be hypermethylated in rodent and human depression. Interestingly, after treatment with anti-depressant, such as escitalopram (a serotonin reuptake inhibitor), the methylation of *P11* was shown to return to normal (Melas, 2012). Hypermethylation of the promoter region of *TRKB*, encoding for tropomyosin receptor kinase B (TrkB), a BDNF receptor, was also reported in suicide completers accompanied by a decrease of mRNA expression (Ernst, 2009).

Sales *et al* observed that 5aza, 5azadC and RG108 possessed anti-depressant-like effects in rats, as increasing the mobility in the tail suspension test and decreasing the immobility time in the forced swimming test (Sales, 2011). This was correlated to an increase of *BDNF* expression level. In a model of chronic ultra-middle stress exposure in mice, representative of a depression-like behavior, zebularine and RG108 reverted the depression-like behavior with an increase in *Gdnf* expression (Uchida, 2011).

g. X-chromosome related diseases and autism disorders

Patients with Rett syndrome, predominantly occurring in women, show a normal development for the first 6-18 months, then progressively loose speech and hand use, associated with seizure and autism. Rett syndrome is an X-chromosome-related neurological disease caused by a mutation in the MeCP2 (methyl-CpG-binding protein 2) protein (Amir, 1999, Guy, 2007). This protein is known to bind methylated DNA, repress transcription, and to be involved in gene regulation *via* the recruitment of the co-activator CREB1 (Lyst, 2015). Although DNA methylation pattern is described as crucial for MeCP2 recognition, no DNMTi was used to treat Rett syndrome. However, treatment of hippocampal cells of MeCP2 knock-out mice with methyl donor SAM was able to rescue neurotransmission event frequencies and thus to partially compensate MeCP2 loss of function (Nelson, 2008).

Additionally, in X-fragile syndrome, another related autism disorder, an hypermethylation in the promoter region of the *FMR1* gene is observed (Sutcliffe, 1992). The treatment of X-fragile cell lines with 5azadC was reported to slightly re-induce *FRM1* expression (Tabolacci, 2005).

h. Parkinson's and Alzheimer's diseases

Parkinson's disease (PD) is a CNS disease due to the degeneration of dopamine-producing neurons, and it affects more than 4 million people worldwide. The movement-related symptoms include shaking and dementia and can occur in a later stage of the disease. Hypomethylation is observed in PD

patients in the CpG rich island of *SNCA*, gene coding for α -synuclein (Jowaed, 2010). α -Synuclein is a protein involved in aberrant soluble oligomers that lead to neurons death. The reduced levels of *SNCA* methylation were shown to result from a “sequestration” of DNMT1 in the cytosol in an α -synuclein-transgenic mice and post-mortem PD brains (Desplats, 2011). 5-azadC was tested on dopaminergic neurons and resulted in viability decrease and increase of apoptosis associated with an up-regulation of α -synuclein (Wang, 2013b). DNMTi can thus have deleterious effects for PD patients.

Alzheimer’s disease (AD) is a neurodegenerative disorder resulting in severe dementia. The methylation profile of AD disease patients is controversial as both hypo- and hypermethylation were reported (Coppieters, 2011). Nuclear immunostaining of DNMT1 and other component of the methylation complex such as MBD2/3 was significantly diminished in neurons (Mastroeni, 2010), where a loss of methylation of the amyloid precursor proteins was observed. A decrease in *BACE* and *PSEN1* (encoding for presenilin 1) methylation was also shown in AD patients and was associated with a potential over-expression of amyloid β -peptides (Mastroeni, 2010, Scarpa, 2003). A cocktail of DNA methylation enhancers, such as folate, is currently in phase III clinical trial (NCT00056225). Moreover, a dramatic global hypermethylation in the gray matter of post-mortem human brain tissues was also observed in AD patients (Coppieters, 2014) together with the increase of methylation in certain regions such as *MCF2L* and *ANK1* genes (De Jager, 2014). Additional data are crucial to confirm the hypermethylation profile of AD patient and to potentially envisage the use of DNMTi for AD treatment.

i. Aging-related senescence and amyotrophic lateral sclerosis

In amyotrophic lateral sclerosis (ALS), motor neurons death leads to muscle weakness and evolves to breathing difficulties. This neurodegenerative disease, often associated with aging, has a 3-5-year survival prognostic (Cleveland, 2001). In motor cortex tissues and spinal cord motor neurons of ALS patients, levels of DNMT1 and DNMT3A were shown to be increased (Chestnut, 2011, Cho, 2010). Oh *et al.* reported an increase in DNMT1 and DNMT3A expression in ALS mesenchymal stromal cells (ALS-MSCs) compared to normal MSCs. RG108 treatment of ALS-MSCs isolated from ALS patients’ bone marrow induced anti-senescence factors (*TERT*, *VEGF*, *ANG*), together with the down-regulation of senescence factors (*ATM*, *P21*). Additionally, a significant improvement in ALS-MSC migration and their differentiation into neurons was observed (Oh, 2015b). Thus, RG108 treatment is promising for a more efficient ALS treatment by autologous cells therapies.

In addition, Oh *et al.* evaluated the effect of RG108 on human bone marrow mesenchymal stromal cells (hBM-MSCs), which are used in cell therapies. They confirmed the anti-senescence effect of RG108 in *in vitro* cultures with an increase in the expression of anti-senescence factors (*TERT*, *VEGF*, *bFGF* and *ANG*) and a decrease of senescence-related factors (*ATM*, *P21* and *P53*) (Oh, 2015a). Thus, an optimized dose of RG108 (5 μ M), for which cell viability is maintained, could greatly improve

hBM-MSC potency, which could constitute a real progress in the improvement of the stem cell therapies.

j. Neuronal stem cell

Neuronal stem cell (NSC) cultures are potential sources of transplantable cells to treat neurodegenerative diseases. DNA methylation was proven to be essential for NSC differentiation and proliferation. Folic acid was shown to stimulate neonatal rat NSC proliferation *in vitro* (Li, 2013), whereas zebularine treatment resulted in the attenuation of their proliferation (Lin, 2014, Luo, 2013) and a reduction of their migration (Singh, 2009b). Hence DNMTi are deleterious for NSC growth, but the use of DNMT activators can provide an interesting way to optimize NSC cultures.

DNA methylation was shown to be implicated in many neurological diseases and psychiatric disorders. The consequences of DNA methylation depend on the disease and the use of DNMTi can be, in a few cases, unfavorable, like in Alzheimer's disease, but it can also have a positive outcome in pathologies like schizophrenia, depression and post-traumatic stress disorders. Despite the high interest in controlling DNMT activity, the path to use DNMTi as drugs to treat neurological diseases and psychiatric disorders is still long. First, it is made difficult by the lack of animal models to study these pathologies. Second, active molecules must cross the blood-brain barrier, and, for example, 5aza or zebularine are not able to do so. Additionally, toxicity is not acceptable for psychiatric diseases, which often require chronic and life-long treatment. Finally, the specificity of action is also an issue as DNA methylation is involved in biological processes in all tissues and organs. Despite these limitations, the better comprehension of DNA methylation in neurological diseases will lead to new therapeutic strategies and new small molecule DNMTi are urgently needed for neurodegenerative diseases and psychiatric disorders (Szyf, 2015).

19.3.3 DNMTi application in cardiovascular diseases

Cardiovascular diseases (CVDs) are the leading cause of death in most of the developed countries and environmental factors such as diet, smoking habits or impaired metabolism are critical risk factors. Therefore, it is not surprising that epigenetic modifications, including DNA methylation, are involved in these pathologies.

DNA methylation patterns were reported to be altered in several CVDs. In hypertension, the hypermethylation of the *HSD11B2* promoter, a gene affecting blood pressure, was observed (Friso, 2008). Hypermethylation was also reported for the ATP-binding cassette transporter A1 gene (*ABCA1*) in coronary heart disease (Guay, 2014) and, in an atherosclerosis *Apoe*^{-/-} mouse model, the hypermethylation of specific vascular homeostasis genes was described (Zaina, 2014). A study on about 300 Singapore Chinese subjects also proposed DNA methylation as CVD risk biomarker, specifically the *Alu/STAT2* methylation level (Kim, 2010). In parallel, in the early-stage

atherosclerosis Apoe^{-/-} mouse model a global hypomethylation was reported in peripheral blood mononuclear cells (Lund, 2004). Concerning the use of DNMTi in CVDs, in the context of norepinephrine-induced heart hypertrophy, a 6-days treatment with 5azadC restored a normal protein expression profile of the whole cardiac proteome and rescued the phenotype of norepinephrine-treated rats (Xiao, 2014). 5azadC was also shown to facilitate inorganic phosphorus mineralization of human aortic smooth muscle cells (HASMCs) via upregulation of the alkaline phosphatase (ALP), a risk factor for cardiovascular disease in patients with chronic kidney disease (CKD) (Azechi, 2014).

Atherosclerosis is characterized by an inflammation of arterial wall and it is the major cause of stroke and heart attack. Gene expression in endothelial cells changes dramatically when submitted to blood flow variations and disturbed flow is pro-arterogenic. In mice, disturbed blood flow induces *Dnmt* expression, moreover several down-regulated mechano-sensitive genes were identified and their expression was shown to be under their promoter methylation control and reversed by 5azadC (Dunn, 2014). Besides, estrogen receptors (ER α , ER β) are arteroprotective and hypermethylation of *ESR1* (ER α) and *ESR2* (ER β) promoters, associated with their silencing, were shown in atherosclerotic tissues and in senescing cells. Treatment with 5azadC was carried out on smooth muscle cells (SMCs) and endothelial cells (ECs), resulting in the decrease of estrogen receptor gene promoter methylation accompanied by an increase of ER levels in both vascular cell lines. Additionally, the combination 5azadC/TSA showed some synergetic effect, while TSA alone had no effect (Kim, 2007).

Ischemia is a decrease in blood supply to tissue, which results in hypoxia and leads to cardiac fibrosis. In hypoxia-induced pro-fibrotic state of human cardiac fibroblast, an increase in *DNMT1* and *DNMT3B* expression and a global hypermethylation were reported. The expression of pro-fibrotic genes, such as *alpha-smooth muscle actin* (*ASMA*) or *collagen 1*, increased and this was enhanced by treatment with TGF β , a pro-fibrotic cytokine. Interestingly, treatment with 5azadC significantly reduced TGF β effects and levels of ASMA and collagen 1, decreasing pro-fibrotic effects and positioning DNMT as potentially valuable therapeutic target in ischemic heart disease (Watson, 2014). Finally, exploring the influence of DNMTi on mouse embryonic stem cells (mESCs), zebularine was shown to re-express cardiac-specific genes, such as *Nkx2.5* and *Gata4* (Horrillo, 2013).

DNA methylation is also implicated in heart failure. Indeed, a higher level of homeobox gene *PITX2c* promoter methylation accompanied with increased DNMT1 and decreased PITX2 protein levels was identified in heart disease atrium. In the HL-1 cell line, 5azadC reversed *PITX2c* promoter methylation and increased the PITX2 and KIR2.1 protein level (Kao, 2013), which could have a positive outcome in case of heart failure.

Recently, DNA methylation and expression levels of COX2 in human heart mesenchymal stem cells (HMSCs) were shown to be involved in the senescence and ageing of HMSCs. Treatment with 5azadC lowered COX2 methylation level, restored its expression level, promoting HMSCs proliferation and delating ageing (Sun, 2021b). This highlighted the potential role of COX2 as a new therapeutic target

against cardiovascular diseases. Despite the evidence of the role of DNA methylation in CVDs (Chaturvedi, 2014), no epidrug is currently in clinical trial for CVDs.

19.3.4 DNMTi application in other human pathologies

a. Obesity

Obesity has a high prevalence in industrialized countries where high caloric diet is common. This major public health problem can lead to diabetes or cardiovascular diseases. Following a 5 days high fat diet, DNA methylation changes of the transcriptional co-activator PGC-1 α involved in oxidative energy metabolism were observed (Brøns, 2010). Variations of DNA methylation were also reported by Wang *et al.*, who analyzed DNA methylation in peripheral blood leukocytes and determined that *UBASH3A* was hypermethylated, whereas *TRIM3* was hypomethylated in obesity cases (Wang, 2010b). Variability of DNA methylation in individual adipose tissues was shown to influence their response to caloric restriction in terms of weight loss (Bouchard, 2010). A comparison of methylation levels in abdominal adipose tissues before and after gastric bypass and weight loss showed a decrease of DNA methylation in both tissues after gastric bypass (Benton, 2015). Expression of adiponectin, a protein regulating glucose and lipid metabolism (Yamauchi, 2002), was inversely correlated with insulin resistance, type 2 diabetes and cardiovascular diseases (Kadowaki, 2006). Kim *et al.* established that adiponectin expression was under epigenetic control; by hypermethylation of the R2 promoter region; observed in high-fat diet obese mice compared to normal-diet lean mice. RG108 was tested in an obese mice model (*db/db* mice) and increased adiponectin expression levels were shown to lead to an improvement of glucose intolerance and insulin resistance (Kim, 2015). These recent results are promising for obesity-related disease therapeutics.

b. Alcohol addiction

Alcohol abuse with its associated diseases and behaviors is responsible for about 6% of the death worldwide (WHO 2014 Global status report on alcohol and health). In the United Kingdom, alcohol was classified as the most harmful drug for oneself and others (Nutt, 2010). The influence of alcohol consumption on DNA methylation is controversial and seems to be highly dependent on the studied tissues. For example, DNA in post-mortem human brains was reported to be hypomethylated in alcoholics (Ponomarev, 2012). This hypomethylation was shown to result in the increased expression of long-term repeat-containing human endogenous retrovirus. A decrease in DNMT3A and DNMT3B levels was also shown in post-mortem alcoholic patients brains compared to healthy individuals. DNMT3B expression was reported to be inversely proportional to blood alcohol concentration, whereas no variation was reported for DNMT1 expression level (Bönsch, 2006). Genome-wide DNA hypermethylation in lymphoblasts of 165 female subjects showed a correlation of the-increase in DNA methylation with the drinking frequency (Philibert, 2012).

DNMTi were studied for the treatment of alcohol addiction and encouraging outcomes were described. In an alcohol-exposed rat model submitted to the 2-bottle choice test and treated or not with 5aza, Warnault *et al.* found that for 5aza-treated animals the rate of alcohol intake was significantly lower compared to non-treated alcohol-dependent rats (Warnault, 2013). These results also showed that inhibition of DNA methylation enabled to specifically reduce alcohol consumption and preference, but did not influence other rewarding substance intake. DNA methylation was suggested to increase the expression of endogenous factors interfering with alcohol drinking behavior, such as BDNF (Logrip, 2009) and GDNF (Carnicella, 2008) that prevent the escalation from moderate to excessive consumption. Barbier *et al.* studied long-term behavior in alcohol-conditioned rats after 3 weeks of abstinence and established a causal relationship between DNA methylation, alcohol intake and seeking behavior. They measured an increase in Dnmt1 and no change in Dnmt3A and Dnmt3B levels. Using intracerebroventricular infusion of RG108, they lowered the DNA methylation level in mPFC (medial prefrontal cortex) of alcohol-conditioned rats ~~and~~, while no change was observed in controls. DNMTi also prevented escalation of alcohol consumption. Seven genes involved in neurotransmission and coding for synaptic proteins were down-regulated in alcohol-dependent rats. Among them, synaptotagmin 2 (*Syt2*) presented a higher methylation level in alcohol post-dependent rats and a synaptic transmission deregulation was reported in post-dependent mPFC neurons. A *Syt2* knock-down resulted in compulsive-like drinking behavior. Interestingly, *Syt2* normal methylation level and expression were restored by RG108 treatment (Barbier, 2015).

Although variations of DNA methylation levels in the brain of alcoholic patients seem to be heterogeneous, the results of DNMTi on preclinical animal models are an interesting starting point for the use of DNMTi to limit alcohol dependence.

c. Inflammation and allergy

Inflammation is a reaction of the immune system to harmful stimuli. In allergy, also known as hypersensitivity (*e.g.* hay fever, allergic asthma, etc.), inflammation is triggered by an unsuitable immune response to harmless environmental cues. Similarly, in auto-immune diseases, an abnormal immune response is directed towards tissues normally present in the organism. Among the CD4⁺ T-cells (T-helper (Th) cells), T-helper 1 (Th1) express specifically interferon- γ (INF γ) (Brand, 2012), whereas T-helper 2 (Th2) express interleukin 4 (IL4), IL5 and IL13. The INF γ (Winders, 2004) and IL4 (Kwon, 2008) cytokine expression is under DNA methylation control and differentiation of naïve T-cell into Th1 or Th2 cells results in a modification of the methylation levels of CpG sites in *INFG* coding for INF γ (White, 2006). The level of *INFG* promoter methylation in CD4⁺ lymphocytes of adults and children with asthma was shown to depend on age, sex and tissues (Lovinsky-Desir, 2014). However, in this study, the authors hypothesized that the increase of *INFG* promoter methylation might not be the only reason of its lower expression and further investigation are required. In-mouse models, *Dnmt3a* and *Dnmt3b* were more expressed in Th2 cells compared to Th1 cells, whereas no

difference in *Dnmt1* expression was observed (Yu, 2012). Moreover, in the same study, *INFG* and *IL4* expression was lower in mutant mice lacking *Dnmt3a* or *Dnmt3a/3b* but not in mutants lacking only *Dnmt3b*. Altogether these data highlight the importance of DNA methylation in the regulation of cytokine expression in CD4⁺ T-cells and in inflammation reactions.

In inflammation, the balance of T-cell sub-type is disturbed with a shift from Th1 towards Th2 response. As a result, the ratio INF γ vs IL4 production is modified, which can be considered as an indicator of inflammation risk (Shahid, 2002). In patients with bronchial asthma, T-cell differentiation into Th2 sub-type and IL4 associated cytokine are much higher than in controls (Kwon, 2008). Although genetics predispositions are involved in asthma (Lloyd, 2009), environment such as pollution, cigarette smoke, etc. is also well-known to have a crucial influence, which rises the importance of epi-mutations in asthmatic patients. A comparison of 21 monozygotic adult twins discordant for asthma showed a decrease in *INFG* and *FOXP3* expression in the case of asthma with a greater decrease for cases with second-hand smoke (SHS) during childhood, which was shown to significantly affect *INFG* and *FOXP3* methylation levels (Kohli, 2012, Runyon, 2012). A similar study with monozygotic twins concluded the importance of DNA methylation of genes involved in immune response associated with psoriasis, like *TNFSF11*, in CD4⁺ T-cells (Gervin, 2012). It is important to underline the significance and increased interest in epidemiology studies on the epigenome of monozygotic twins, starting from the seminal paper of Fraga *et al.* in 2005 (Fraga, 2005). Concerning seasonal allergic rhinitis (SAR), peripheral blood mononuclear cells from SAR patients and healthy controls were challenged with allergen. Interestingly, the DNA methylation profile of SAR patients varied during and outside pollen season (Nestor, 2014). DNMTi were tested *in vitro* and *in vivo* for their ability to control inflammation. In asthma, upon allergen sensitization/challenge by ovalbumine treatment a comparison of sensitized and non-sensitized mice showed an increase in *INFG* promoter methylation correlating with a decrease in INF γ level. This increase was reversed by 5azadC treatment (Brand, 2012).

In acute lung injury (ALI), an inflammatory lung disease mainly caused by sepsis, the combination of 5azadC and TSA reduced mortality level in LPS (lipopolysaccharide)-induced ALI mouse model with a 80% survival rate (Thangavel, 2014). Primary bone marrow-derived macrophages of LPS-treated mice expressed higher levels of pro-inflammatory chemokines and cytokines, which were decreased upon 5azadC/TSA treatment, resulting in cell survival increase (Thangavel, 2015).

Rheumatoid arthritis is a chronic inflammatory auto-immune disease involving synovial inflammation and causing joint pains. In fibroblast-like synoviocytes (FLS) of arthritic rats, the methyl-CpG-binding protein MeCP2 was selectively over-expressed, whereas secreted frizzled-related protein (SFRP4) was down-regulated. A knock-down of MeCP2 resulted in an enhancement of the SFRP4 level. SFRP4 is known to activate the Wnt pathway, which is involved in FLS abnormal proliferation. Treatment of FLS with 5azadC resulted in an increased *SFRP4* expression and a decreased cell proliferation (Miao, 2013). Finally, a strong increase in promoter methylation of Th2 genes (*IL4* and *IL5*) with an opposite

effect for Th1 genes (*INFG* and *IL10*) was observed in PMBC of children allergic to cow's milk compared to healthy ones (Berni Canani, 2015).

These recent studies described above suggest the potential of DNMTi in the development of new anti-inflammatory treatment in asthma, acute lung injury and rheumatoid arthritis.

d. Infectious diseases

Viral infections

In 2014, 35 million people were affected by HIV (human immunodeficiency virus)-1 worldwide. Most infected people have a normal quality of life when treated with anti-retrovirus therapy, but an interruption in the treatment can reactivate latent viruses (Van Lint, 2013). Efforts have been made to improve the anti-HIV therapies. It was shown that methylation of proviral DNA of HIV (Kumar, 2015) or human T-lymphotropic virus (HTLV) (Saggiaro, 1991), is involved in latency establishment and in their escape from the immune system. In HIV-infected cells, an increase of DNMT1 (Mikovits, 1998) and DNMT3A/3B levels (Chandel, 2015) has been observed. However, the influence of DNA methylation is quite controversial and a hypermethylation of HIV-1 5' long terminal regions (LTR) was reported in latent HIV reservoirs of patients who did not present viremia compared to viremic patients (Blazkova, 2009). A strategic option could be to activate HIV in their reservoir to kill infected cells, while anti-retrovirals are used to block new infections. In this context, Fernandez *et al.* demonstrated that, in association with TNF α known to activate HIV replication, the use of 5azadC activated HIV twice more than the single use of TNF α in J-Lat cells. However, this seems to be cell-line specific, as in other cell lines, such as J1.1 and U1, 5azadC inhibited HIV activation (Fernandez, 2010). This variability highlights the complexity in the dynamic of DNA methylation and the need of novel chemical inhibitors to probe the role of DNA methylation in different biological contexts.

HIV is also known to be associated with certain cancers such as non-Hodgkin's lymphomas (NHL). In HIV-positive NHL patients, a clear up-regulation of DNMTs was observed. In HIV-positive aggressive B-cell lymphomas, the HIV-TAT protein was shown to be secreted by infected cells and to result in cell proliferation. In this context, an up-regulation of DNMTs was observed and 5azadC treatment was able to re-express *P16* (Luzzi, 2014).

Similarly, human papillomavirus (HPV)-positive lung cancers exhibited high levels of DNMT3B (Lin, 2005) and treatment with 5azadC was able to restore normal levels of *E-cadherin* expression in the presence of HPV (D'Costa, 2012). These findings could be exploited to improve the treatment of virus-induced cancers like HIV-positive NHL, papilloma virus-positive cancers or Epstein-Barr virus-positive Burkitt's lymphoma (Paschos, 2009).

Hepatitis C virus (HCV) was also shown to lower E-cadherin levels and induce an hypermethylation of the *E-cadherin* promoter, which is known to induce morphological changes, alter cell-cell adhesion and induce epithelial-mesenchymal transition, critical in tumorigenesis (Park, 2014). DNA

methylation was reported to be deregulated in HCV. DNMT activity was shown to be essential for HCV cell infection and the use of 5aza or 5azadC induced a significant decrease in HCV infection (Chen, 2013). Therefore, DNMTs can represent potential targets for HCV treatment.

Bacterial infections

Epigenetic changes regulate the bacteria-specific innate immune response, as described for inflammation (cf. paragraph 17.3.4.c). Alterations of DNMT expression levels were measured in mice provided with *E. coli* or *E. coli*-LPS contaminated water compared to control mice. This highlighted the role of bacterial LPS in the alteration of the epigenetic response (Kovalchuk, 2013). In *E. coli*-infected human uroepithelial cells, DNMT activity was demonstrated to be more than 10-fold enhanced compared to non-infected cells (Tolg, 2011). DNMT1 expression was also altered following *Porphyromonas gingivalis* or *Fusobacterium nucleatum* infection (Yin, 2011). In parallel, pre-treatment of gingival epithelial cells with 5aza significantly reduced the up-regulation of cytokine genes *IL6* and *CXCL1*, observed upon *P. gingivalis* or *F. nucleatum* exposure. However, no reduction in bacterial invasion of gingival epithelial cells was observed (Drury, 2015). Hence, although DNA methylation is involved in bacterial infection, the use of DNMTi is still to be studied.

Parasite infections

DNA methylation was shown to be involved in several parasite infections. Quinazoline-based DNMTi, show oral efficacy in *P. berghei* infected mice (Bouchut, 2019) and quinoline–quinazoline DNMTi induced parasite death including artemisinin-resistant pathogens together with a decrease in methylation level (Nardella, 2020). DNA methylation inhibition was also studied in *Biomphalaria glabrata*, a mollusk, host of the pathogen *Schistosoma mansoni*. In this invertebrate model, 3-bromo-3-nitroflavanones were shown to reduce DNA methylation in *B. glabrata* and its offspring together with phenotype modification but with no noticeable toxicity. Interestingly, the identified hypomethylated loci were associated with reduced gene expression (Luviano, 2021).

The importance of the epigenetic modulation of the host in case of infective disease starts to be established and this could open the door to the use of epidrugs as anti-infectious agents. This would certainly be facilitated by the discovery of more potent and selective small molecule DNMTi.

e. Embryo growth

The establishment and maintenance of DNA methylation patterns are crucial in the early development of the human embryo (Guo, 2014). Altered DNMT activity in the embryo can lead to early pregnancy loss (Yin, 2012). Early DNA methylation pattern establishment is also important in the *in vitro* fertilization (IVF) process and development of pre-implanted embryos was shown to be dependent on correct methylation (Dobbs, 2013). To better understand and improve the success rate in IVF process, studies were carried out on DNA methylation pattern in animal model embryos. *S*-Adenosyl-L-

homocysteine (SAH), a common inhibitor of methyltransferase, was used to study *in vitro* mouse embryo development. Administered at a period of time preceding *de novo* methylation, SAH results in a decrease of DNA methylation and improvement of development competency of *in vitro* cloned embryos compared to non-treated ones (Jafari, 2011). Treatment of buffalo skin fibroblast donor cells with the combination 5azadC/TSA decreased global DNA methylation, apoptosis and improved the development of cloned embryos (Saini, 2014). RG108 in combination with an HDAC inhibitor showed synergetic effects on somatic cell nuclear transfer, a technique that is known to have limited efficiency in mammalian cloning and in which the DNA methylation profile was shown to be incompletely reprogrammed (Peat, 2012). RG108 at high doses (500 μ M) increased embryoblasts compared to non-treated controls, which could be promising for the improvement in cloning efficiency (Li, 2011, Watanabe, 2013).

19.3.5 DNMTi application in metabolite production

To date, no direct therapeutic studies targeting DNA methylation in fungal infection were carried out. However, DNA methylation was found to be involved in fungal biosynthetic genes repression. When cultured in the presence of 50 μ M of 5aza, atlantic-forest-soil-derived *Penicillium citreonigrum* produced fungal exudates, formally known as guttates, secondary metabolites strongly enriched in several specific components, as the azaphilone family, and exhibited new secondary metabolites from the atlantinone family (Wang, 2010a). Likewise, supplementation of the *Alternaria sp.* fungus culture medium by 5aza induced the production of toxic metabolites, such as alternariol, altenusin, alternariol-5-*O*-methyl ether, 3'-hydroxyalternariol-5-*O*-methyl ether, which are known as plant-disease inducers and that are dormant in normal culture conditions (Sun, 2012).

Additionally, DNMTi and HDACi can be added to bacterial cultures to diversify the metabolite production. For example, 5aza was added to marine fungus *Leucostoma perisoonii* culture medium to optimize the production of cytosporones; two unknown cytosporones were obtained, one of which exhibiting some activity against *Plasmodium falciparum* (Beau, 2012). This modulation of the biosynthesis pathways through DNA methylation tailoring could lead to the production and identification of novel molecules that can be therapeutically active compounds

19.3.6 DNMTi in plants

Variations in plant growth conditions, such as temperature, induce epimutations, which can lead to significant modifications in the phenotype, as for example the variation of petal number of the *Rosa hybrid*. This modification was related to DNA hypermethylation of the *RhAG* promoter at low temperature (Cortijo, 2014, Ma, 2015). In contrast to humans, cytosine methylation in plants does not occur only in a CpG context and 30% of the cytosines are methylated compared to 5-8% in humans (Finnegan, 1998). In *Japonica* rice (*Oryza sativa L.*), 5aza or 5azadC (0.3 mM for 16 h to 3 days) were shown to decrease the DNA methylation level and to result in dwarfism. The effect of 5azadC (70% dwarfism effect) was more pronounced than with 5aza (30% dwarfism effect). Unlike in mammals, the

DNA methylation profile is not erased during gametogenesis (Kinoshita, 2004, Kinoshita, 2007), which results in heritable DNA methylation changes in progenies, thus 5aza induced dwarfism in rice was shown to be maintained up to the third generation (Sano, 1990). Upon treatment with 5azadC, a lower methylation at the *Xa21G* promoter was measured. *Xa21G* is a resistance gene to the pathogenic bacterium, *X. oryzae pv. Oryzae* (Ronald, 1997), and, indeed, the new rice line obtained upon 5aza treatment was proven to possess a resistance trait towards bacterial infection (Akimoto, 2007).

Thus, DNMTi can program new features in plants and cultures, like the disease-resistance trait described above or flowering (Kondo, 2007). However, as long as no plant specific DNMTi is identified, attention must be taken that these treatments do not affect the methylation profile of animals and human beings.

19.4 Innovative indirect and combined approaches to better target DNA methylation

Since direct targeting of DNMTs is highly challenging other approaches can be envisaged. Rather than targeting DNA methylation writers, DNA methylation readers responsible for sensing the presence of methylated DNA and triggering signaling cascades can also be considered. Besides, DNMTi can also be coupled with other active moiety to lead to bifunctional inhibitors (Figure 17.4).

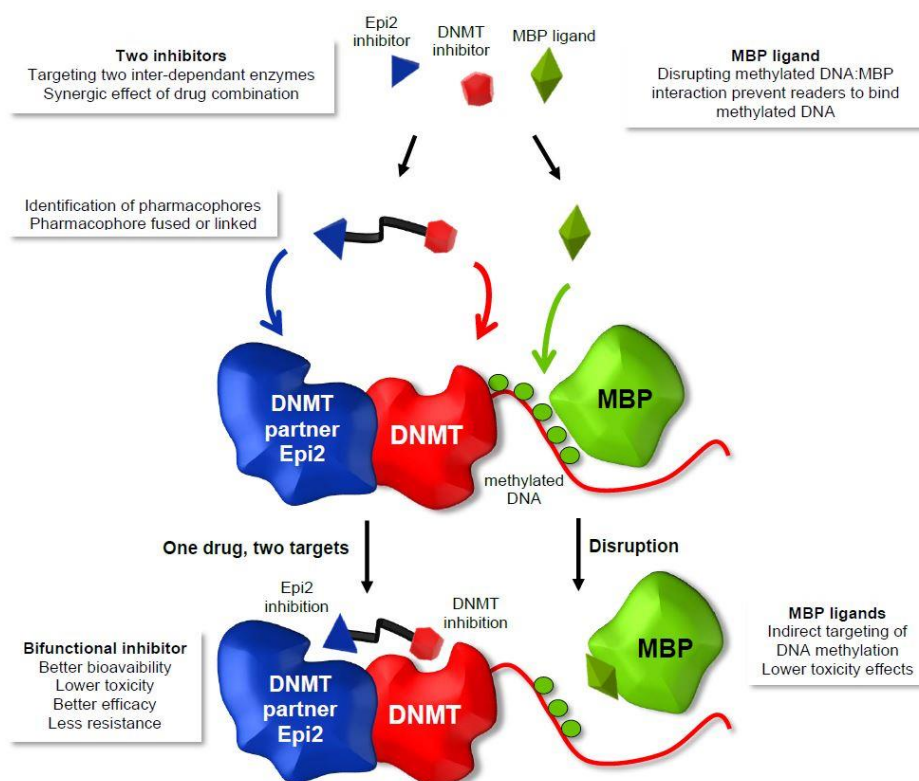


Figure 17.4. Alternative strategies to target DNA methylation by either 1) designing bifunctional inhibitor including a DNMTi and another active moiety and 2) targeting DNA methylation reader proteins, *i.e.* MBPs.

19.4.1 Methyl-CpG binding proteins: nature and probes

In addition to the writers of DNA methylation, the DNMT enzymes, and the erasers, the TET enzymes, the readers proteins, known as Methyl-CpG Binding Proteins (MBPs), recognize and bind to methylated cytosine and transform this modification into a biological signal (Ludwig, 2016). As DNA methylation is involved in numerous biological processes and in diseases, the MBPs play a global role as well (Sidhu, 2017, Stirzaker, 2017, van Roy, 2005). Indeed, abnormal MBPs expressions and structures have been reported in psychiatric disorders (Gigek, 2016), in different cancer types (Gigek, 2016, Li, 2020b, Prokhortchouk, 2006) and in inflammatory diseases (Robinson, 2019), for example. Three different subfamilies of MBPs are known at the date: Methyl-CpG Binding Domain Proteins (MBDs), the SET and RING Associated (SRA) proteins and the Kaiso proteins (Sidhu, 2017).

a. Methyl-CpG binding domain proteins (MBD)

The Methyl-CpG Binding Domain Proteins (MBD) subfamily is the largest group of MBPs with seven members in total: MeCP2 (Lewis, 1992), MBD1 (Fujita, 1999), MBD2 (Hendrich, 1998), MBD3 (Hendrich, 1998), MBD4 (Hendrich, 1998), MBD5 (Baymaz, 2014), MBD6 (Baymaz, 2014). All of them are characterized by an MBD domain comprising approximately 80 amino acids, which mediates their binding to methylated DNA (except for MBD3). MBD4 has in addition an enzymatic activity through its glycosylase domain (Hendrich, 1999). Furthermore, four other proteins possess an MBD binding domain in their structure (Du, 2015): SETDB1 and SETDB2, which also have a SET domain bearing an histone methyltransferase activity (Falandry, 2010, Schultz, 2002), BAZ2A (also known as TIPS5) and BAZ2B, which have a bromodomain, explaining their affinity for acetylated histones (Jones, 2000).

The MBD proteins are involved in several protein regulatory complexes, such as the NuRD complex, which mainly suppresses genes transcription (Xue, 1998). Because of their role in mediating the DNA methylation signal, they constitute an interesting target for chemical modulation, as, for example, the disruption of the binding of the MBD domain to methylated DNA could-potentially reactivate silenced genes.

Currently, only three binders of the MBD domain have been identified in the literature, all—are targeting MBD2: NF449, aurintricarboxylic acid (Wyhs, 2014) and KCC-07 (Zhu, 2018) (cf. Figure 17.5). The first two first compounds were identified in an time-resolved (TR)-FRET screening assay for compounds that disrupt the interaction between the MBD domain of hMBD2 and methylated DNA (IC₅₀ of 290 nM for NF449 and 2.7 nM for aurintricarboxylic acid) (Tang, 2021). Previously, these compounds were known as a P2X1 receptor antagonist and a well-known inhibitor of multiple DNA-protein interactions, respectively. However, both compounds present features that are not favourable for chemical probes or drugs as they interfere with multiple process, lack complete selectivity and bear reactive moieties (El-Ajouz, 2012, Roos, 2017, Sun, 2014). Compound KCC-07 was previously described to interfere with the binding of MBD2-MBD domain to a methylated 45mer DNA with an

IC₅₀ of 1.55 μM (determined by ELISA), while inhibition of MeCP2-DNA binding was undetectable (Reichert 2010). Interestingly, the compound showed *in vivo* activity extending the lifespan of medulloblastoma xenografted mice by 6 days and restoring Brain-specific angiogenesis inhibitor (BAI1) expression (Zhu, 2018). These results are promising for the use of chemical compounds that target MBDs.

In addition, two compounds have been identified to bind a MBD2 Intrinsic Disordered Domain (IDD) outside the MBD domain: ABA and APC (Kim, 2019) (Figure 17.5). These compounds disrupt the recruitment of P66α by MBD2, which is a key factor of the NuRD complex formation (Gnanapragasam Merlin, 2011) with an IC₅₀ of 1.93 μM for ABA and 1.75 μM for APC (determined by FRET) (Schultz, 2002).

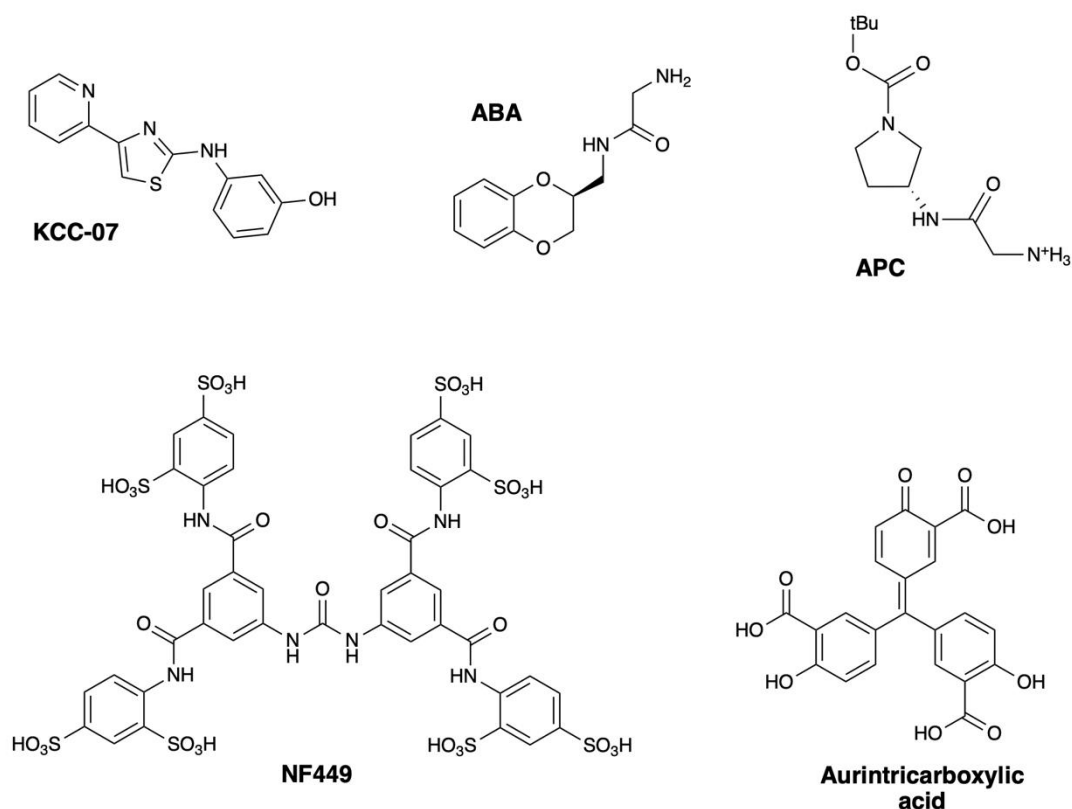


Figure 17.5. MBD2 targeting compounds. KCC-07 interferes with the binding of MBD2-MBD domain to DNA with an IC₅₀ = 1.55 μM (measured by ELISA), NF449 and aurintricarboxylic acid inhibit the interaction of MBD2 with DNA with an IC₅₀ = 290 nM and 2.7 nM, respectively (measured by TR-FRET). ABA and APC bind to IDRs of MBD2 with IC₅₀ = 1.93 and 1.75 μM, respectively (measured by FRET).

b. SET and RING Associated (SRA) domain Proteins

The SRA proteins are also able to bind methylated and hydroxymethylated DNA, in particular hemimethylated DNA, through the SRA domain. UHRF1 (Fujimori, 1998) and UHRF2 (Fujimori, 1998) are the two human members of this family. These proteins can recruit epigenetic silencing factors such

as DNMT1 and HDAC1 and promote gene silencing (Niinuma, 2019). In particular, it has been shown that they play a key role in the recruitment of DNMT1 to hemimethylated DNA (Felle, 2011). These proteins act as “pivots” in the gene silencing machinery since they are able to recruit different types of proteins and enzymes to ensure epigenetic DNA methylation, histone methylation and acetylation and even ubiquitin-related actors (Choudhry, 2018).

So far, no binder of UHRF1 and UHRF2 has been identified that disrupt their interaction with methylated DNA. The described inhibitors for UHRF1 target mainly its Tandem Tudor Domain and Plant Homeodomain (TTD and PHD, respectively), which mediate the binding to silencing-related proteins such as DNMT1 (Achour, 2008) and HDAC1 (Unoki, 2004). The most relevant molecules are NV03 (Senisterra, 2018), NSC232003 (Myrianthopoulos, 2016), BPC (Houliston, 2017) and 2,4-lutidine (Chang, 2021) (Figure 17.6) with an affinity to the TTD domain of $K_d = 2 \mu\text{M}$ for NV03 (determined by ITC and Fluorescence polarisation) (Senisterra, 2018); $50 \mu\text{M}$ for BPC (determined by ITC and DSF) (Houliston, 2017) and $29 \mu\text{M}$ for 1,4-lutidine (measured by TR-FRET) (Chang, 2021). NSC232003, that interacts with UHRF1, decreases the global number of methylated CpGs of a population of U251 glioblastoma cells by 50% (Myrianthopoulos, 2016). However, the specificity of these molecules towards UHRF1 over UHRF2 has not been established.

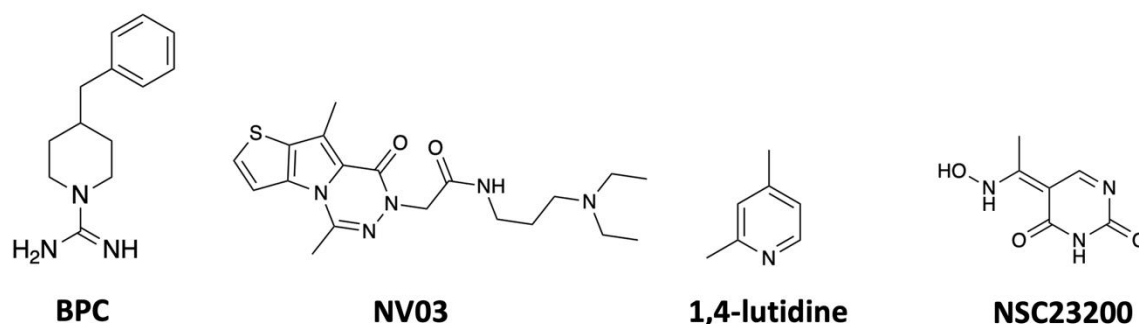


Figure 17.6. UHRF1 binders. BPC, NV03 and 1,4-lutidine bind the UHRF1 Tandem Tudor Domain with $K_d = 2 \mu\text{M}$, $50 \mu\text{M}$ and $29 \mu\text{M}$, respectively. NSC232003 reduced by 50% global numbers of methylated CpGs in U251 glioblastoma cells.

c. *Kaiso Proteins*

The Kaiso protein subfamily is composed of three different members: Kaiso (Daniel Juliet, 1999), ZBTB4 (Filion, 2006) and ZBTB38 (Sasai, 2005). These are Zinc Finger Domains (ZF) proteins that bind methylated and unmethylated DNA in a sequence-specific manner (Donaldson, 2012). They are known to recruit HDAC-dependent transcriptional repressors (for ZBTB4 and ZBTB38) (Sasai, 2005); or the N-CoR repressive complex (for Kaiso) (Yoon, 2003). The implication of these proteins in diseases remarkably differs from one another, going from oncogenesis (Buck-Koehntop Bethany, 2012), to abnormal inflammatory mechanisms (Ocskó, 2018) and even cell cycle dysregulations (Blue, 2018). To this day, no inhibitor or probe has been identified, even if *in silico* screening assays and

computational docking simulations have been realized (Chikan, 2015). This subfamily is interesting since its members do not share similar biological outcomes (Filion, 2006) and their depletion in mice has dramatically opposite consequences between Kaiso (viable and fertile) (Prokhortchouk, 2006) and the two other members (cell damage and genomic instability) (Marchal, 2018, Roussel-Gervais, 2017).

As illustrated, chemical probes for MBPs are still at an early stage with few active molecules identified to date. Mostly they lack specificity and are not potent enough for cellular or *in vivo* assessments. Nevertheless, these proteins remain an interesting chemical target since they could allow studying complex epigenetic mechanisms and widen the spectra of therapeutic approaches for human pathologies.

19.4.2 Bifunctional inhibitors involving DNMTi

Another strategy to address the lack of efficacy and selectivity of DNMTi is the use of bifunctional compounds. This promising strategy consists in designing bifunctional inhibitors formed by the connection of at least two different pharmacophores in a single drug (Tomaselli, 2020). Such compounds have many advantages compared to the use of drug combination. First, selectivity might be improved, pharmacological properties can be modulated and toxicity can be lowered. Besides, both inhibitors are simultaneously present in the same tissue that can help to reach greater efficacy against advanced-stage disease. Bifunctional compounds also limit resistance mechanisms (de Lera, 2016).

Due to the lack of reported potent DNMTi, it is only recently that a few bifunctional inhibitors including a DNMTi moiety have been reported. Synthesis and biological evaluation of four different DNMT/HDAC and DNMT/G9a bifunctional inhibitors have recently been described (Figure 17.7). Because of the co-location of these two targets in the same protein complex (Hervouet, 2018), their involvement in the same diseases (Esteller, 2008), and the synergic effect of drug combinations, targeting these two enzymes, exhibit good potency. The biggest challenge in designing hybrid inhibitors is to maintain or improve activity against both enzymes.

Histone deacetylases (HDAC) are a family of 18 enzymes, divided in 4 classes, which catalyze deacetylation of histone-tail *N*-terminal lysines, leading to chromatin compaction (Ruijter, 2003). In some diseases, particularly in cancer, histone hypoacetylation combined with DNA hypermethylation, cause transcriptional repression (Kondo, 2009). HDAC inhibitors have all the same general structure characterized by a Zinc Binding Group (ZBG) that chelate the zinc ion in the catalytic pocket of the enzyme, a hydrophobic linker and a cap group, which interacts at the entrance of the catalytic pocket. To date, four HDACi were approved: Vorinostat, Belinostat, Panobinostat, and Chidamide, where ZBG is an hydroxamic acid moiety (Ho, 2020).

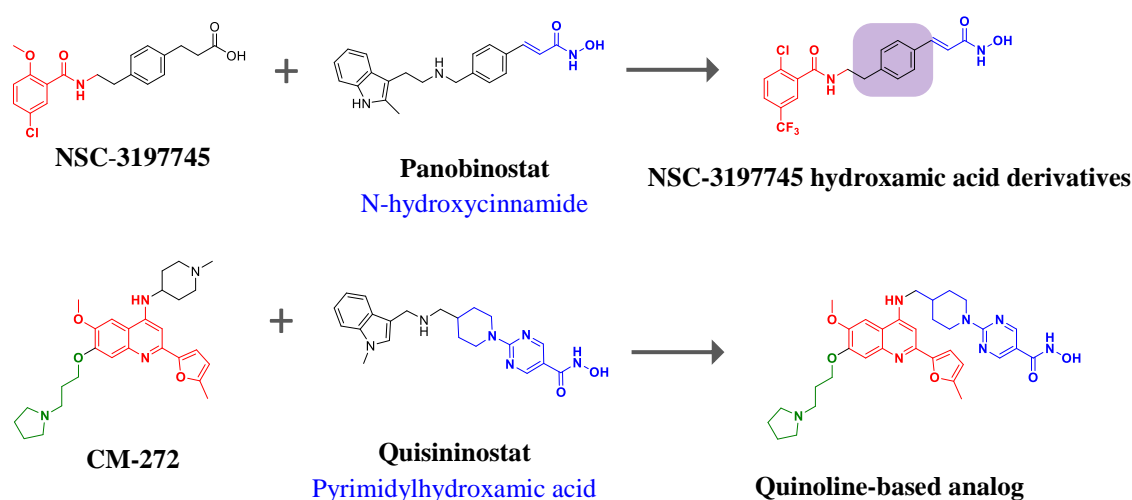
In 2017, the first-in-class bifunctional DNMT/HDAC inhibitor was described (Yuan, 2017). Its design ~~consists in~~ was based on NSC-319745 DNMTi pharmacophore functionalized with an hydroxamic

acid moiety. NSC-319745, had been previously identified by virtual screening as a selective DNMT1 inhibitor with low activity (34% inhibition at 100 μ M on enzymatic assay) (Kuck, 2010b). Interestingly, despite its low efficacy, this DNMTi shares similarities with hydroxamate HDAC inhibitors. Changing carboxylic acid in hydroxamic acid, more precisely with a *N*-hydroxycinnamide moiety, and optimizing its structure allowed to improve the activity against target enzymes. The lead compound finally showed 70%, 23% and 49% inhibition at 100 μ M against DNMT1, 3A et 3B, respectively and IC₅₀ at nanomolar range against two classes of HDAC in enzyme assays. Potent antiproliferative activity was measured in K562 and U937 human cancer cell lines (IC₅₀= 2.9 μ M and 1.1 μ M, respectively) as well as an increased H3K9 and H4K8 acetylation level. This bifunctional inhibitor was shown to initiate *P16* methylation inhibition and its re-expression and to induce apoptosis in leukemia model cancer cells U937.

Another HDAC/DNMT bifunctional inhibitor was described by Rabal's team (Rabal, 2021). They first worked on a series of quinoline-based analogue as bifunctional DNMT/G9a inhibitor, where G9a is a lysine methyltransferase. Their hit compound exhibited IC₅₀ of 8 nM against G9a and 382 nM against DNMT1 on purified enzymes (San José-Enériz, 2017) and it was shown to act as a substrate competitive inhibitor of G9a and DNMTs (Rabal, 2018). They then designed a multitarget epigenetic inhibitor targeting HDAC, DNMT1 and optionally G9a. Based on structure design, docking and structure activity relationship SAR) analysis, they inserted a ZBG at the solvent exposed area of their inhibitor. They selected the pyrimidylhydroxamic acid moiety of quisininostat since its cap group mimics a quinoline base. Substitution of the quinoline moiety increased activity against DNMT and G9a and finally led to two potent compounds, one acting as a trifunctional G9a/HDAC/DNMT inhibitor and the second one as a bifunctional DNMT/HDAC inhibitor obtained by removing the lysine mimic moiety. Although they lost efficacy against DNMT1, compared with their previous hit compounds, these multifunctional compounds exhibited IC₅₀ in nanomolar range against HDAC1, 2 and 3 and a potent antiproliferative activity on MM.1S multiple myeloma cell line. Due to their poor pharmacological property profiles these compounds should be optimized to enable *in vivo* experiments. By fragment-based rational drug design, Jiang's group worked on a series of hydroxamic acid derivatives of nucleoside bases. This work was inspired by cytosine and hydroxamic acid derivatives, FDA-approved DNMTi and HDACi, respectively. In both studies, both pharmacophores were linked through a triazole with different linker lengths. The first bifunctional inhibitor reported exhibited adenine as DNMT pharmacophore (Ren, 2019), then they completed the series varying the nucleobase (Sun, 2021a). The most potent compound, carrying a cytosine linked to the hydroxamic acid moiety with a linear six-carbon alkyl chain showed potent inhibitory activity against DNMT1 with IC₅₀= 6.39 μ M and nanomolar activities against HDAC1 and HDAC6, comparable to Vorinostat. Western Blot in U937 lymphoma cell lines treated with this DNMT-HDAC inhibitor, showed an increase of H3K9 and H4K8 acetylation levels and a weak decrease of methylation levels in *P16* promoter region that led to an increase of *P16* expression and anti-proliferative activity in a dose-dependent manner. Even though

these bifunctional inhibitors displayed a good potency, ~~it~~ they need further investigations for cancer therapy, especially regarding quantification of DNA methylation inhibition in cells, pharmacological properties as well as comparison with DNMTi and HDACi combinations.

The most potent bifunctional inhibitor known to date was described by Yuan *et al.* (Yuan, 2019). In this study, DC-517, a selective DNMT1 inhibitor described by Luo's group composed of two carbazole groups and a side chain (Chen, 2014), was selected as DNMTi. Previous docking experiments on this molecule suggested the addition of the hydroxamic acid moiety at the end of the side chain, without removing the hydroxyl group, which interacts by hydrogen bonding in the DNMT1 catalytic pocket. Cell viability assays in breast cancer and epithelial cell lines MCF-7, A549, and MDA-MB-231, showed that the (*S*)-enantiomer C02S has the strongest antiproliferative effect, which was higher than compounds SAHA and SGI-1027. C02S achieved DNMT inhibition in the micromolar range against DNMT1. Its activities against DNMT3A and 3B were improved compared to DC-517. However, it induced significant DNMT1 degradation only after 24 h treatment in MCF-7 tumor cells. Moderate HDAC inhibition activity was also reported by hyperacetylation of H3K9 and H4K8 in MCF-7 cell line. C02S also inhibited *P16* and *P21* promoter methylation together with the re-expression of these TSGs. By flow cytometry assay and in PI-FITC/annexin assay it was shown that C02S induced G0/G1 cell cycle arrest that led to apoptosis. It also possessed antiangiogenic activity, confirmed by the inhibition of HUVEC tubule formation in a dose-dependent-manner, and inhibited tumor cells migration and invasion of MDA-MB-231 cells. In *in vivo* model, using mice xenograft 4T1 breast tumor models, C02S significantly reduced tumor volume and mass at 5 and 15 mg/kg/d treatment, that was comparable with the positive control group treated with a combination of SAHA and decitabine. Despite low HDAC inhibition activity (Yuan, 2019), C02S is—the only DNMTi-containing bifunctional inhibitor evaluated *in vivo*, which exhibited a significant effect on solid tumor. To conclude, development of hybrid compounds that can inhibit simultaneously DNMT and HDAC is a very recent strategy which has a real potential in anti-tumor activity.



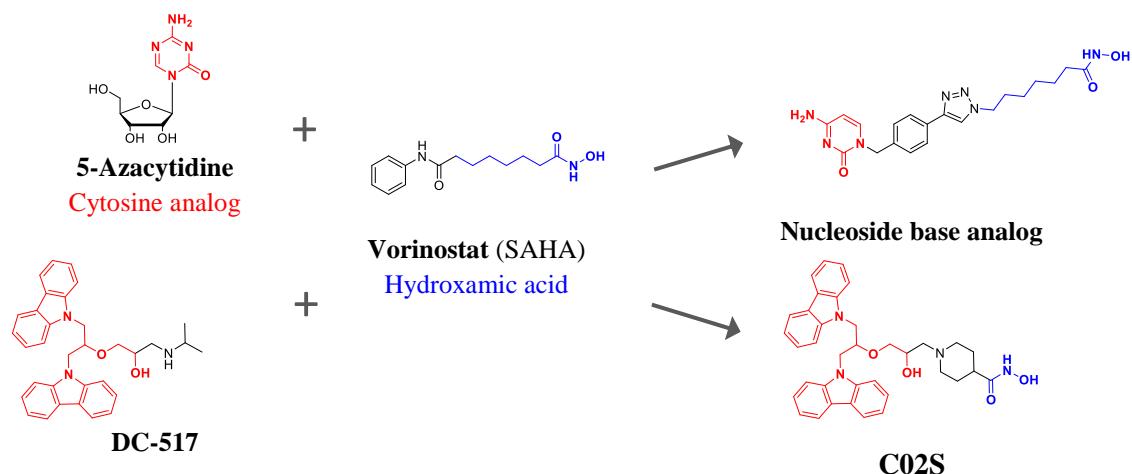


Figure 17.7. Bifunctional epigenetic inhibitors containing DNMTi moieties.

In red the DNMT pharmacophore, in blue the HDAC pharmacophore, in green the G9a pharmacophore, in purple, merged part of pharmacophores

19.5 Limits and hopes of DNMTi applications and new perspectives

The above examples illustrate the major role of DNMTs in the normal and abnormal functioning of cells and how their inhibition by DNMTi can change phenotypes, revealing the high potential of DNMTi; both for therapeutic strategies and for the bioengineering of organisms. It also highlights how, depending on the context, the effects can be inverted. This is related to the dynamics of DNA methylation and its role in controlling gene expression. As illustrated by the above examples, DNMTi are just starting to be applied to diseases other than cancer and these strategies seem very promising. An increasing number of clinical trials are starting to explore-epigenetic reprogramming by DNMTi in combination with other treatments, in particular, in solid tumors. Time will show if these studies can validate the interest of epidrugs beyond hematological cancers, the only diseases they are approved for. Noteworthy, the only FDA-approved DNMTi are 5aza and 5azadC, suicide substrates that are incorporated into DNA and form an irreversible covalent complex with the enzyme. This triggers a subsequent DNMT degradation by the proteasome, inducing a potent inhibition of DNA methylation. It is not clear whether a non-covalent inhibitor can induce this level of demethylation (Erdmann, 2015). To obtain the same potency as covalent-nucleoside inhibitors, non-nucleoside inhibitors could be an alternative, but they would need to be very specific for the DNMTs and today this specificity is lacking. Among non-nucleoside DNMTi, GSK3484862 showed the most promising results with high *in vitro* and in cell potency. Nevertheless, it is urgent to find novel compounds that are very potent inhibitors of DNMTs and bind strongly to the enzyme, in order to induce a strong inhibition in cells and eventually a down-regulation of the DNMTs.

Another feature of 5aza, 5azadC and their analogues is the fact that they are globally incorporated into the DNA instead of deoxycytosines and thus they are not specific for CpGs or certain genomic regions. This can hinder their use by inducing non-desired secondary effects. The use of low doses of the drugs

has diminished these effects, however repeated cycles are necessary for the epigenetic reprogramming, increasing the probability of their appearance. Non-nucleoside inhibitors have the advantage of not needing incorporation into DNA and thus potentially diminishing the side-effects. In conclusions, it is important to pursue the search for new non-nucleoside inhibitors of DNA methylation that are potent, specific for the CpGs and of promoters silencing key genes in pathologies. Several strategies are being explored for the next generation of inhibitors, as allosteric inhibitors, protein-protein ligands and dual inhibitors (Erdmann, 2016).

An interesting alternative is to target the proteins that recognize methylated DNA and trigger the signaling pathways that silence DNA (DNA methylation reader proteins). While, histone reader proteins are a well explored target, the chemical targeting of the MBP is still in its infancy.

Finally, hybrid compounds bearing both a DNMTi moiety and another inhibitor moiety is also promising. Interestingly, targeting not a single enzyme but cancer specific protein complexes can enable cancer cell selectivity. In addition, the merging of two drugs in the same molecules can facilitate the pharmacology compared to drug combinations. Challenges remain in the design of such compounds to maintain both scaffold activity and efficacy.

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ABBREVIATIONS

AD: Alzheimer's disease
ALI: acute lung injury
ALL: acute lymphoblastic leukemia
ALS: amyotrophic lateral sclerosis
AML: acute myeloid leukemia
Ara-C: cytarabine
ASMA: alpha-smooth muscle actin
ATRA: all-*trans* retinoic acid
5aza: 5-azacytidine
5azadC: 5-aza-2'-deoxycytidine
BBB: blood-brain barrier
BDNF: brain-derived neurotrophic factor
BM: bone marrow
CMML: chronic myelomonocytic leukemia
CNS: central nervous system
COMT: catechol-*O*-methyltransferase
CpG: cytosine-guanine dinucleotide
CVD: cardiovascular disease
DNMT: DNA methyltransferase
DNMTi: DNMT inhibitor
EC: endothelial cells
EGCG: (-)-epigallocatechin-3-gallate
EMA: European Medicines Agency
ER: estrogen receptor
ESC: embryonic stem cells
FDA: U. S. Food and Drug Administration
GABA: γ -aminobutyric acid
GAD: glutamic acid decarboxylase
HCV: hepatitis C virus
HDAC: histone deacetylase
HDACi: HDAC inhibitor
HIV: human immunodeficiency virus
HPV: human papilloma virus
HTS: high-throughput screening
INF γ : interferon- γ

IVF: *in vitro* fertilization
LPS: lipopolysaccharide
LTP: long-term potentiation
MDS: myelodysplastic syndrome
MeCP2: methyl-CpG-binding protein 2
mPFC: medial prefrontal cortex
MSC: mesenchymal stromal cells
NHL: non-Hodgkin's lymphomas
NMS: neonatal maternal separation
NSC: neuronal stem cell
PARP: poly-(ADP-ribose) polymerase
PARPi: PARP inhibitor
PD: Parkinson's disease
PTSD: post-traumatic stress disorder
SAHA: suberoylanilide hydroxamic acid, vorinostat (Zolinza®)
SAM: S-adenosyl-L-methionine
SAH: S-adenosyl-L-homocysteine
SAR: seasonal allergic rhinitis
SFRP4: secreted frizzled-related protein
SHS: second-hand smoke
Th: T-helper
TSA: trichostatin A
TSG: tumor suppressor gene
VPA: valproic acid
XRCC1: X-ray repair cross-complementing protein

LEGEND of FIGURES and TABLES

Figure 17.1. Schematic representation of DNMT catalytic cycle mechanism and its inhibition by 5azadC.

Figure 17.2. Selection of compounds described as DNMT inhibitors.

Figure 17.3. Schematic representation of the mode of action of DNMTi as gene re-expressing agents and examples of DNA methylation-induced gene silencing in diverse pathologies.

Figure 17.4. Alternative strategies to target DNA methylation.

Figure 17.5. MBD2 targeting compounds.

Figure 17.6. UHRF1 binders.

Figure 17.7. Bifunctional epigenetic inhibitors containing DNMTi moieties.

Table 17.1. Examples of DNMTi FDA-approved and in phase II and III clinical trials as a single drug or in combination