Chapter 9

Chemical compounds targeting DNA methylation and hydroxymethylation

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Abstract

DNA methylation and its oxidised forms participate in the interpretation and regulation of the human genome. Many questions arise around the enzymes responsible of these chemical modifications of DNA and their roles in regulating these modifications, which are very dynamic but are specific in the location and context (tissues, diseases, etc.). We reviewed here the major enzymes involved in DNA methylation and oxidation, with a focus on the DNA methyltransferases and TET enzymes. The principal compounds that inhibit these enzymes are presented since they will help address these questions.

Keywords: DNA methylation, DNA hydroxymethylation, inhibitors, DNMT, TET.

Abbreviations:

1-mA = 1-methyl-Adenine
2OG = 2-oxo-glutarate
3-mC = 3-methyl-Cytosine
3-mT = 3-methyl-Thymine
5-aza-C = 5-aza-Cytosine
5-azadC = 5-aza-2'-deoxyCytosine
5-caC = 5-carboxy-Cytosine
5-fC = 5-formyl-Cytosine
5-hmC = 5-hydroxymethyl-Cytosine
5-mC = 5-methyl-Cytosine
5-xC = 5-modified Cytosine
6-mA = 6-methyl-Adenine
AM-PD = Active modification – Passive Dilution
AML = Acute Myeloid Leukemia
BAH1 and BAH2 = Bromo-Adjacent Homology domains 1 and 2;
BER = Base Excision Repair
CFP1 = CpG-binding protein, CXXC finger protein 1
CIP
CMML = Chronic MyeloidMonocytic Leukemia
CpA = Cytidine pairing Adenosine
CpC = Cytidine pairing Cytidine
CpG = Cytidine pairing Guanosine
CpT = Cytidine pairing Thymidine
CXXC = CXXC domain;
DMAP domain = DNA methyltransferase-associated protein 1-interacting domain;
DNMT = C5 DNA methyltransferase
DSBH = Double Stranded β Helix
EGCG = EpiGalloCatechin Gallate
ELISA = Enzyme Linked ImmunoSorbent Assay
EMA = European Medicines Agency
FDA = Food and Drug Administration
FH = Fumerate Hydratase
FTO = Fatt mass obesity associated protein

HDAC = Histone deacetylase
IDAX = Inhibition of the Dvl and Axin complex)
IDH = Isocytrate dehydrogenase
LC-MS = Liquid Chromatrography – Mass Spectrometry
LCI = Low Complexity Insert
MALDI-TOF = Matrix assisted laser desorption/ionization – Time Of Flight
MBP = Methyl Binding Protein
MDS = MyeloDysplastic Syndrome
MLL = Mixed lineage leukemia
mTet1 = murine TET
NgTet1 = Naegleria Grubi TET
NLS = nuclear localization signal
NOG = N-Oxalyl Glycine
PBD = PCNA-binding domain
PHD = Plant Homeo Domain
PRMT = Protein arginine methyltransferase
PWWP = proline-tryptophan-tryptophan-proline domain
R/S-2HG = R/S-2-hydroxyglutarate
RFTD = Replication Foci Targeting sequence (RFTS) domain;
ROS1 = Repressor of Silencing 1
SAH/AdoHys = *S*-adenosyl- L -homocysteine
SAM/AdoMet = *S*-adenosyl-L-methionine
SDH = Succinate de hydrogenase
SPR = Surface Plasmon Resonance
TCA = Tricarboxylic acid
TDG = Thymidine DNA glycosylase
TET = Ten Eleven Translocation
TLC = Thin Layer Chromatography
TRDMT1 = tRNA aspartic acid methyltransferase

Table of Contents

9.1. Introduction

DNA methylation and its oxidised forms participate in the interpretation and regulation of the genome. Chemical modifications of cytosine in chromosome DNA are (highly) dynamic and are one of the regulatory elements that, together with histone modifications and chromatin remodeling, allow the DNA to interact with the protein machineries that interpret, repair, splice the genetic information. The cytosine modifications identified to date are: 5-methylcytosine (5mC), 5-hydroxymethylcytosine (5hmC), 5-formylcytosine (5fC) and 5-carboxycytosine (5caC).
In humans, DNA methylation is the most stable epigenetic mark [1] and it occurs at C5 position of cytosine (5mC), mainly in a CpG dinucleotide context. The CpG dinucleotides are mainly located in the CpG islands (occurring at *ca* 60% of all gene promoters), in repeated sequences and in CpG island shores [2]. If promoter CpG islands are methylated, the corresponding gene is repressed due to a poor recognition by transcription factors and recruitment of proteins involved in chromatin remodeling such as methyl DNA-binding proteins (MBPs) [3].

Failure in maintaining DNA methylation and establishment of new DNA methylation patterns are associated with under- or over-expression of the affected genes, ultimately leading to inflammation, cancer and other diseases. DNA methylation is catalysed by the DNA methyltransferases (DNMT) that mediates the transfer of a methyl group from the *S*-adenosyl-L-methionine (SAM or AdoMet, **1**) to position 5 of cytosine in DNA [4] (**Fig. 9.1, Fig. 9.2A**).

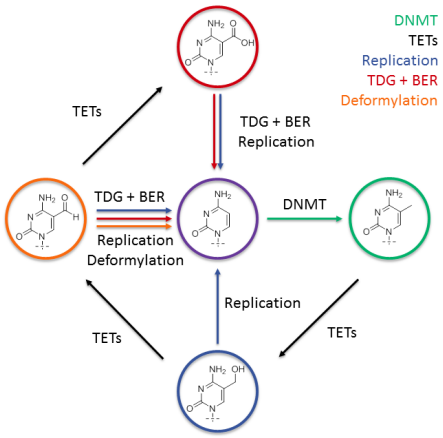

Figure 9.1: Methylation of cytosine in DNA and successive oxidation of 5-methylcytosine catalysed by TETs. *Cytosine (C), mainly at CpG sites, is methylated by the DNA methyltransferases (DNMT) at position 5 using S-adenosyl-L-methionine (SAM or AdoMet) as methyl donner to give 5-methylcytosine (5mC). Methylated cytosine (5mC) can undergo iterative oxidation by TET enzymes followed providing 5-hydroxycytosine (5hmC), 5-formylcytosine (5fC) and 5 carboxycytosine (5caC), respectively. Each base has a passive and/or active demethylation pathway leading to cytosine (C). Possible 5mC demethylation pathways include: 1) passive dilution, 2) active modification followed by passive dilution, 3) TDG + BER, thymine DNA glycosylase-mediated base excision repair and 4) deformylation.*

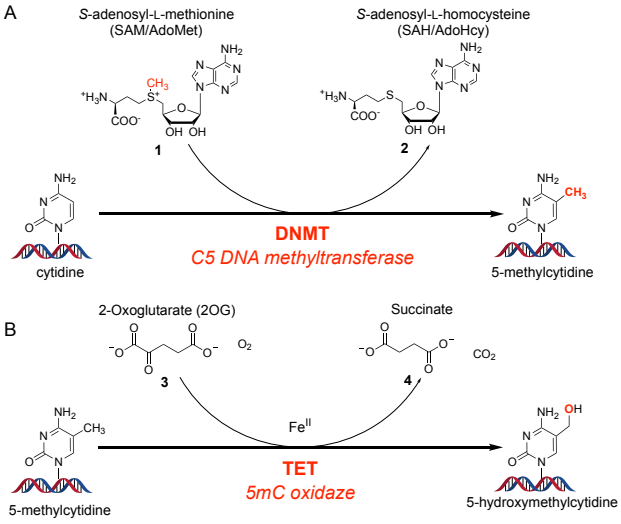

Figure 9.2. DNMT- and TET-catalysed cytosine methylation and oxidations*. The structures of substrates, co-factors and products are indicated.*

The Ten Eleven Translocators (TET) enzymes catalyse the 2-oxoglutarate (2OG, **3**) dependent oxidation of 5mC in a cascade of iterative steps to give 5-hydroxymethyl cytosine (5hmC), 5-formylcytosine (5fC) and 5-carboxycytosine (5caC) (**Fig. 9.1, Fig. 9.2**). The discovery of TET oxidation of 5mC to 5hmC in 2009 garnered interest as an important epigenetic regulator [5, 6]. Since the identification of 5hmC in human cells, other DNA modifications (5fC, 5caC, as well as 5-hydroxymethyluracil (5hmU)) have been identified and found to be catalysed by TET1-3 [7–11].

Methylation at position 5 of cytosine is a reversible modification, and there are four different demethylation pathways that could lead to the reformation of cytosine identified to date (**Fig 9.1**). First, the 5mC mark is not maintained during DNA replication, thus 5mC is diluted by subsequent rounds of cell division, a process known as passive DNA demethylation. Second, 5mC can be iteratively modified to provide 5hmC, 5fC and 5caC, which then can be diluted in cell division, a combination of active modification followed by passive dilution (AM-PD). The third process is an active pathway via a combination of Thymidine DNA Glycosylase (TDG) and Base Excision Repair (BER) enzymes. As part of DNA repair mechanism, TDG recognizes a thymidine guanosine pair mismatch and deaminates the thymidine base leaving an a-basic sugar. TDG likewise performs this excision efficiently on 5fC and 5caC [12] followed by DNA repair using the Base Excision Repair (BER) mechanism that introduces C. Finally, recent studies by Iwan *et al.* demonstrated, by labelling the 5fC sugar and base independently, that 5fC can be directly converted by the human cell to C without the change in base or sugar, suggesting direct deformylation [13]. The protein(s) associated with this process of 5fC however, have not been identified to date. In plants, a direct 5mC to C pathway is present, following a similar TDG-BER mechanism, where Repressor Of Silencing 1 (ROS1) recognises 5mC [14].

Many questions arise around these enzymes and their roles in regulating cytosine modifications, which are very dynamic but are specific in the location and context (tissues, diseases, etc.). How are they precisely regulated in a concerted manner? Why was this mechanism of DNA demethylation selected in mammals? Their role in diseases, such as cancer, neuronal diseases, inflammation, infection, is

well established, but the mechanisms involved are still to be fully understood. Compounds that inhibit the enzymes responsible for DNA methylation and oxidation will help address these questions.

9.2. DNMT enzymes

DNA methylation was first described by Holliday & Pugh [15] and Riggs [16], who hypothesised its role in gene regulation during development. Since then it was shown that DNA methylation can induce gene silencing when it occurs on promoters of genes (e.g. imprinted genes, transposons silencing, X-inactivation, tumour-suppressor genes in cancer). DNA methyltransferases activities were isolated and observed in several eukaryotic cells [17]. The first mammalian DNMT to be identified was Dnmt1 in mouse cells [18]. Interestingly all C5 DNA methyltransferases share 10 motifs that are relatively conserved (the Motif IV contains the Pro-Cys dipeptide, the Cys being involved in the catalytic reaction making a covalent bond with the dC). Dnmt1 has a preference for hemi-methylated DNA and it is thus called maintenance enzyme. The Dnmt3A and 3B were discovered later [19] and characterised to be active also on non-methylated DNA; and are thus considered *de novo* DNMTs. Dnmt3L was shown to lack the catalytic motif [20], resulting in a catalytically inactive form. However Dnmt3L plays an important role, especially in development and in imprinting [21]. It enhances the activity of Dnmt3A and 3B [22] and interacts with several proteins involved in chromatin regulation. See [23, 24] for recent reviews on the biology of DNMTs. The highly conserved DNMT2 has been shown to methylated tRNA and has been renamed tRNA aspartic acid (D) methyltransferase 1 (*TRDM1*) [23, 25].

9.2.1. Structures and mechanism of DNMTs

DNMTs belong to the C5 DNA methyltransfrases, which have a three-dimensionally conserved catalytic pocket. The C-terminal contains the motifs of DNA binding motifs and catalysis of methyl transfer. After base flipping out of the DNA double helix and binding of the cytosine in the catalytic pocket, the catalytic cysteine in the Pro-Cys motif (PCQ or PCN) binds to position 6 of cytosine (**Fig. 9.1**). Then the methyl group from the SAM is transferred to position 5 of

the cytosine, creating a steric clash that releases the 5mC by β-elimination and resolves the DNA-DNMT complex.
In addition to the catalytic domain at the C-terminus, the DNMTs possess regulatory N-terminal domains that includes a nuclear localisation signal and domains for protein-protein interactions to chromatin, transcriptional and replication regulators [26]. Several structures of the mammalian DNMTs have been resolved showing the particular features of the different isoforms and some protein partners. In particular, the three DNMT3s have a PHD (plant homeodomain)-like domain, ADD (ATRX-DNMT3-DNMT3L domain) for the interaction with the tail of histone H3 [26] and DNMT3A and 3B a PWWP domain found in DNA-binding proteins and involved in nucleosome recognition [27], interacting with H3K36me3 [26].The N-terminus of DNMT1 is rich in protein-interaction domains that localize it to the nucleous (NLS), to replication forks (PBD, PCNA-binding domain, DMAP domain, RFTS domain, BAH1 and 2 [28] and to un-methylated and hemi-methylated DNA (CXXC zinc finger domain; BAH1 and BAH2). These domains are also involved in the enzymatic control of the protein, for example its inhibition when bound to non-methylated DNA and its switch to catalytically active form when bound to hemi-methylated DNA [26, 29, 30].

9.2.2. DNMTs in diseases

It is clearly established that aberrant DNA methylation profile is associated with cancers [31–35]. In parallel to a global hypomethylation, hypermethylation at promoters of specific genes are observed in cancer cells. In particular, genes, such as tumour suppressor genes, are commonly silenced by promoter hypermethylation. These features have been exploited for the development of biomarkers for the detection of many cancers [36, 37], including for colon cancer (ColoVantage® and Epi proColon®), lung cancer (Epi proLung). Moreover, DNA methylation is an anticancer therapeutic target: 5aza and 5azadC have been approved for the treatment of certain hematological cancers. These drugs are also in clinical trials, mainly in combination, for several solid tumors [38, 39]. However, these aberrant patterns are not limited to cancers [40]. Alteration in DNA methylation, caused by genetic mutations in the DNMTs or DNMTs deregulation, is involved

in psychiatric, cognition, neuronal, ageing disorders, in cardiovascular diseases, bacterial and viral infections, genetic diseases such as cystic fibrosis [41, 42]. Finally, epigenomics studies are revealing the role of DNA methylation in obesity, allergy, autoimmune diseases, addiction and inflammation [42, 43].

Nevertheless, despite the established roles of DNA methylation and DNMT1 and DNMT3s in diseases-[42, 44], which DNMT isoform is best to therapeutically target and in which pathology remains to be determined. Chemical tools can thus be useful to address these important questions. Another unanswered question is in regards to Dnmt2. Mammalian Dnmt2 was discovered by its homology with the most conserved C5-DNA methyltransferases [45] but it was shown not to methylate DNA but rather RNA cytosines, the $tRNA^{Asp}$ [46, 47] and was subsequently renamed TRDMT1 (tRNA aspartic acid methyltransferase 1). It contains only the catalytic domain and interestingly is present in species that do not have DNMT1 or DNMT3 (such as *Drosophila* and *Schizosaccharoyces pombe*) [25]. Today it is still debated whether in certain species, Dnmt2 is able to methylate DNA or if its action is limited to RNA methylation [48, 49].

Thus, while DNMT enzymes are well-studied, many questions remain to be answered. Chemical tools that specifically inhibit the DNMTs can contribute towards answering these biological questions.

9.3. Inhibitors of DNA methylation

Two families of inhibitors have been identified for DNMTs: the *nucleoside analogues*, of which 5-azacytidine (5azaC (**5**)) and 5-aza-deoxycytidine (5azadC(**6**)) are approved anti-leukaemia drugs (known as azacitidine and decitabine), and the *non-nucleoside analogues*, which are composed of very different scaffolds (**Fig. 9.3**).

9.3.1. Cytosine analogues

5azaC (**5**) was synthetized as an anti-metabolite and was described for its anti-leukaemia properties in 1964-65 by Sorm and Vesely [50, 51]. However, it was its impact on DNA methylation and on the reprogramming of cells by Jones and Taylor [52, 53] that allowed the understanding of its mechanism of action [54–56]. 5azaC and 5azadC incorporate into DNA instead of dC. Once the DNMT is bound to the

position 6 of the 5azadC, the β-elimination and restauration of the 5-6 double bond cannot occur and the DNMT is irreversibly trapped on the DNA (suicide complex), inducing its degradation by the proteasome [57]. The trapping of the DNMT1 was elegantly visualized by using fluorescent DNMT1 fusions [56].

5azaC (**5**) was approved by the FDA and then the EMA, together with the deoxy analogue 5azadC (**6**) for the treatment of acute myeloid leukaemia (AML), myelodysplastic syndrome (MDS) and chronic myelomoncytic leukaemia (CMML).

Both 5azaC and 5azadC are chemically unstable (storage and handling is sensitive) and have a very short half-life in patients [58]. Several efforts were made to generate stable analogues (such as 5-fluorocytidine (**8**) and zebularine (**7**)) and prodrugs (reviewed in [59, 60][61]). The most promising prodrug is the dinucleotide version of 5azadC (**6**), SGI-110 (**9**) or guadecitabine, which is in clinical trials against several haematological and solid cancers. Very interesting results are also being obtained in the clinic by combination studies with other epigenetic inhibitors, conventional chemotherapies or immunotherapies [38, 62].

Nevertheless, despite being the most potent compounds to demethylate DNA identified to date, they induce other effects due to their chemical instability, their incorporation into RNA and DNA, and also by the formation of suicide complexes. Indeed, the nucleoside analogues are incorporated into DNA (and 5azaC also in RNA) instead of C, but not only at CpG sites. They then induce the formation of a suicide-substrate with DNMT that is processed by the proteasome and the DNA repair machinery. Because of their mode of action, they are not selective for an isoform of DNMTs and induce effects other than just DNA demethylation. To overcome this, several studies have focused on the identification of DNMT inhibitors that are not nucleoside-analogues.

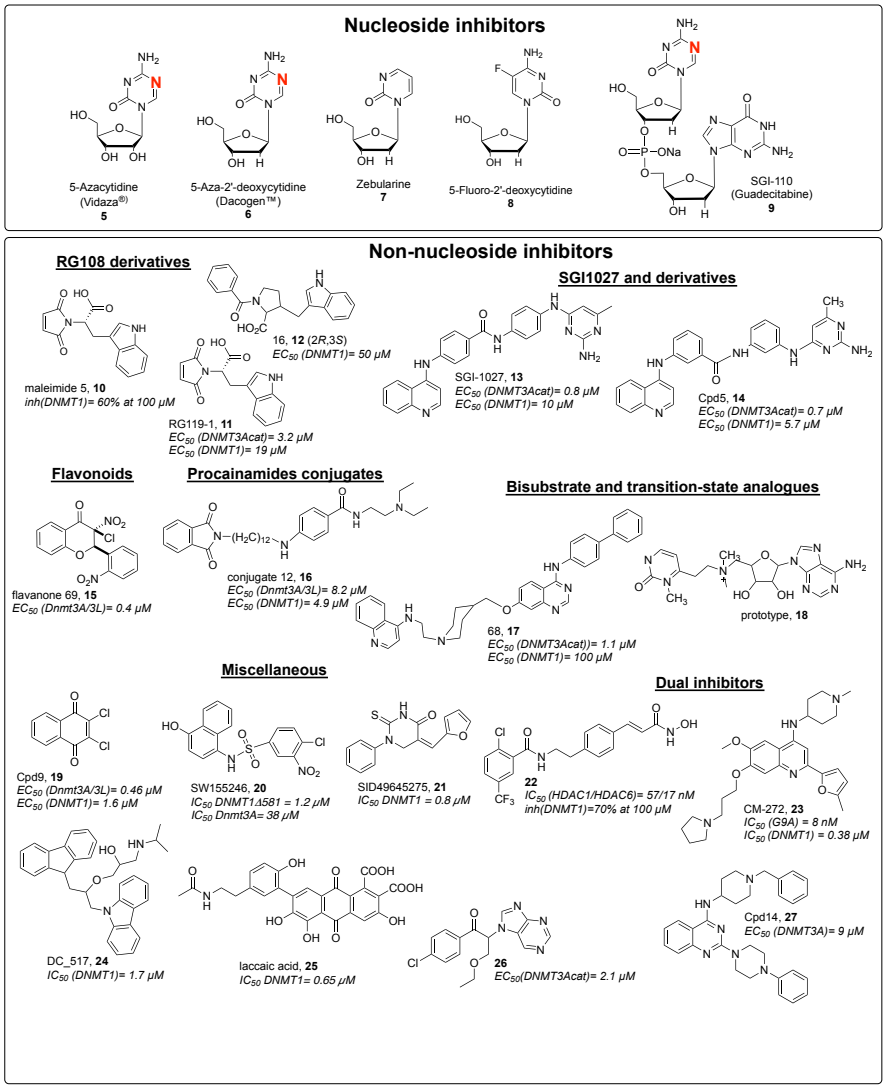

Figure 9.3: Selection of inhibitors of DNMTs. *Top panel: the nucleoside inhibitors. Bottom panel: the non-nucleoside inhibitors. The concentration at which 50% of the maximal activity of the enzyme is inhibited is indicated for DNMT1, DNMT3A and/or the DNMT3A/3L complex.*

9.3.2. Non-nucleoside analogues

The family of non-nucleoside inhibitors has been largely reviewed [24, 42, 59], so this section will focus on a selection of inhibitors that have shown effects on cellular phenotypes, on gene expression or have provided new concepts for the discovery of new inhibitors (**Fig. 9.3**). We have chosen to omit most of the compounds that act by binding to DNA, such as intercalators (*i.e.*, acridine derivatives, [63] or minor groove binders (*i.e.,* bisbenzimidazoles [64]) that lack specificity for CpG sites. Natural products have also not been included since they have been reviewed recently and show little specificity for the DNMT enzymes [65]. Importantly direct comparisons of inhibition properties of these compounds cannot be made, because the assays and conditions used for each study are very different, as well as the choice of the enzyme used (reviewed in [66]), which can have an important impact on the inhibition [67].

The first synthetic non-nucleoside inhibitor, *N*-phthaloyl-l-tryptophan RG108, was identified by *in silico* screening against a model of DNMT1 and showed reactivation of tumour suppressor genes in different cancer models [68, 69]. It is worth noting that, the compound is not active in enzymatic studies against mammalian DNMT1 and DNMT3A [70–72]. However, it is of interest from the perspective of inhibiting DNA methylation since several groups have shown that it leads to demethylation of genes in cellular models [73–77] and *in vivo* [75, 78]. Modifications on RG108 resulted in compounds with improved activity against the mammalian DNMTs (maleimide 5**,** (**10**), (**12**) and RG119-1 (**11**)[70, 71, 79] (**Fig. 9.3**)**)** and highlighted the interactions of the compound within the catalytic pocket. These studies provide new potential for improving this chemotype.

By modulation of DNA minor groove binders, Datta *et al*. identified SGI-1027 (**13**) [80] which interacts weakly with DNA and inhibits DNMT3A and DNMT1 in the micromolar range [81]. Further modifications by Valente *et al.* resulted in a more active meta-meta analogue Cpd5 (**14**) ([82] **Fig. 9.3**), which shows a stronger interaction with DNA at CG-rich regions [81]. Chemical modifications to better characterize the structure-activity relationships and increase the cellular potency are on-going.

The screening of a library of flavonoids identified a family of

3-chloro-3-nitroflavanones which was able to inhibit DNMT3A and DNA methylation in zebrafish embryos (Cpd 69 (**15**) **Fig. 9.3**; [83]). This family of compounds showed selectivity for the C5 DNA methyltransferases, compared to other non-specific flavones such as epigallocatechin gallate (EGCG).

Procainamide and procaine were described to weakly bind to DNA and inhibit DNMTs [84, 85] and were chemically modified to improve the inhibition [86, 87]. The conjugation to diverse moieties resulted in micromolar inhibitors of DNMT1 and Dnmt3A/3L (conjugate 12 (**16**), **Fig. 9.3** [88]).

High-throughput screening campaigns against different DNMTs and use of diverse biochemical assays resulted in the identification of miscellaneous compounds, the most potent of which are shown in **Fig. 9.3**. Among these, the antraquinone laccaic acid (**25**) was described as a DNA-competitive inhibitor of DNMT1 with a weak activity in breast MCF-7 cancer cells (at 200 μM) [89]. A naphthoquinone, diclone (**19**), a pesticide and fungicide, was specific of DNMT1 and Dnmt3A and inactive against the histone methyltransferase G9A. This observation opened the path to studying the impact on the epigenome of plants, animals and human when addressing the toxicology of pesticides [90]. SW155246 (**20**), an aromatic sulphonamide, showed a weak selectivity against DNMT1 *vs* DNMT3A and DNMT3B and induced a weak inhibition of methylation and reactivation of TSGs in human lung carcinoma [91]. DC_501, DC_517 (**24**)[92] and SID49645275 (**21**) [93] were identified to inhibit DNMT1 in the low micromolar level and to inhibit cell proliferation. The mechanism of action and the selectivity still need to be explored.

9.3.3. Transition state and bisubstrate analogues

Recently, progress has been made in the design of transition state and bisubstrate analogues. We have shown that seven cytosine-adenosine compounds, designed as transition-state analogues of the methylation reaction of position 5 of cytosine by DNMT1 and DNMT3, did not result in inhibitors of DNMTs but rather of histone arginine methyltransferases PRMT4 [94].

Interestingly, based on the design of mechanism-based transition analogues, Miletic *et al.* have described and evaluated a set of adenosyl-1-methyl-pyrimidin-2-one derivatives *in silico* as leads for

the synthesis of mechanism-based suicide-inhibitors of DNMT1 ([95], prototype (**18) Fig. 9.3**).

Another chemical approach we explored is the bisubstrate analogues that have successfully been applied to inhibit adenine DNA methyltransferases. Using this approach we have identified compounds (**17)** as potent inhibitor of DNMT3A able to demethylate promoters of tumour suppressor genes and to reactivate gene expression in cancer cells ([96], **Fig. 9.3**).

9.3.4. New approaches to DNMT inhibitor design

Finally, a very promising approach is the design of dual inhibitors [97]. By modifying BIX-01294, inhibitor of histone H3 lysine 9 methyltransferase G9A, Rotili *et al.* identified new quinazoline derivatives as inhibitors of DNMT3A with activity in cancer cells (cpd 14 (**27**) **Fig. 9.3** [98]). José-Enériz *et al.* successfully modulated this family of G9A and DNMT inhibitors to obtain a dual G9A-DNMT1 inhibitor, CMC-272 (**23**) (**Fig. 9.3**), with anti-leukaemia effect in an *in vivo* model of mice engrafted with ALL-derived CEMO-1 cells [99]. This is the first example of DNMT dual inhibitors active *in vivo*. Other dual inhibitors are currently being explored, such as (**22**) (**Fig. 9.3**), resulting from chemical optimisations based on HDAC inhibitors [100].

Taken together, the transition-state and bisubstrate analogues, as well as dual inhibitors, are interesting chemical approaches that need to be further explored to obtain new, potent and selective inhibitors of DNMT. Another strategy worth exploring is to develop protein-protein interaction inhibitors (PPI) for DNMTs. PPIs have been successful for other epigenetic targets, and it is worthwhile since the DNMTs are involved in protein complexes that direct DNA methylation [101, 102]. For example, the inhibition of the DNMT1/CFP1 interface with peptides was shown to affect methylation level of cancer cells and to synergize with temozolomide [103]. Most recently, Ye et al. determined the crystal structure of DNMT1 in two different states and suggest the possibility to design inhibitors of the conformational transition necessary for DNMT1 activity [30].

9.4. TET enzymes

Methylation at the 5 position of cytosine (5mC) is recognized by the TET enzymes that catalyse the oxidation of 5mC in a cascade of iterative steps (**Fig. 9.1**). TETs are part of the oxygenase superfamily that uses Fe(II) for catalysis, using O_2 and 2-oxoglutarate (2OG, **3**) as co-factors, to generate the oxidized substrate, CO_2 and succinate (**4**) [6]. Recent developments reveal that the 5-methyl oxidations at cytosines play an extensive role in epigenetic regulation and key steps in the DNA demethylation pathways. Studies have shown that TETs are fundamental in mammal development and mutations or overexpression of the protein are linked to various diseases.

9.4.2. Discovery and biological roles of TETs

DNA methylation is a well-established modification and it has long been known that passive dilution alone cannot fully account for the rapid rate of genome methyl remodeling after mouse fertilization, indicating the possibility of an additional mechanism that would remove 5mC [104]. The TET proteins were originally linked as being fusion partner of mixed lineage leukaemia (MLL) [105] and later identified as enzymes related to JPB1, that facilitate the oxidation of thymidine (T) in the nucleus to 5-hydroxyuracil (5hmU) [106]. The first discovery of 5hmC modification was made in 2009, were 5hmC was detected in brain tissue at high abundance [5]. At the same time, it was revealed that TET enzymes are capable of oxidizing 5mC to 5hmC [6]. Shortly after, it was discovered that TET enzymes can iteratively oxidize 5mC to 5caC [107].

TET enzymes are widely conserved through evolution [111], including in *Naegleria grubi* TET (NgTET). Herein, we focus on studies involving human TETs (hTETs), with some references to other model organisms, such as murine TETs (mTET) [110] and NgTETs [112].

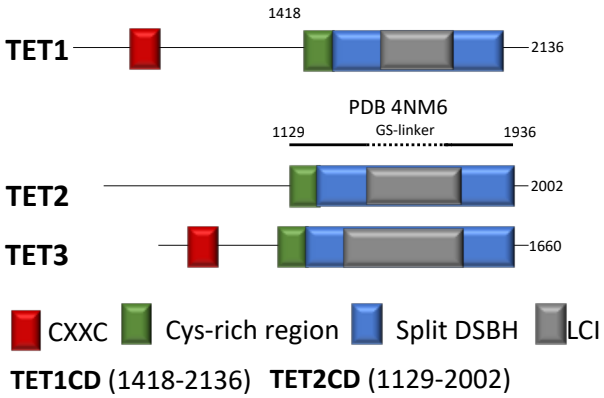

Figure 9.4: Domain architecture of the human TET proteins. *The 'canonical' sequences for the three human TETs are shown. The minimum regions required for efficient catalysis (often referred to as the "catalytic domain", CD) have been reported as follows: TET1 (1418-2136), TET2 (1129-2002), TET3 (689-1596))* [6, 108–110]. *The CD includes the Cys-rich region (green), the DSBH domain (blue) and a low-complexity insert (LCI, grey). The DSBH catalytic region is 'split', with the first two active site Fe(II) coordinating residues of the HXD…H motif in the first segment, and the second His and the 2OG binding Arg in the second segment, flanking the LCI. Additionally, TET1 and TET3 have an N-terminal CXXC Zinc-finger domain that can bind DNA and act to recruit to target genomic sites.*

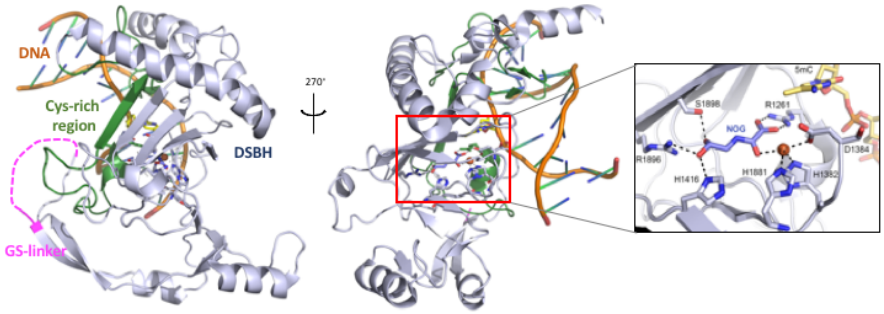

Figure 9.5. Crystal structure of human TET2 in complex with DNA. *(LHS) Two different views of the human TET2 catalytic domain complexed with dsDNA-5mC and NOG. Each domain is colored (Cys-rich region – green; DSBH – light blue; GS-linker (15 GS-repeat replacing LCI, delΔ1099-1936) – magenta; dsDNA-5hmC – orange). (RHS) NOG is bound at the active site. PDB:4NM6.*

To date, three TET enzymes have been found in humans (hTET). The hTET proteins are approximately 180 to 230 kDa in length and all human TETs (hTET1-3) can carry out iterative oxidation of 5mC [6]. The catalytic domain (CD) of TETs contains the cysteine rich domain and a split double stranded ß-helix (DSBH) connected via a flexible region known as the Low Complexity Insert (LCI) (**Fig 9.4**). The DSBH domain, which contains a jelly-roll / cupin fold, is highly conserved among the TETs, and a signature motif of the 2OG oxygenase superfamily (see [113] for review). DSBH forms a catalytic core where it positions the 5mC DNA substrate at the active-site adjacent to Fe(II) enabling catalytic oxidation, while the cysteine rich region structurally stabilizes the DSBH and DNA interactions. TET1 and TET3 have an additional DNA-binding CXXC domain at the N-terminus, which preferentially binds CpG rich regions. TET2 lacks the CXXC Zinc-finger domain but instead has been shown to interact with IDAX (CXXC4) which then interacts with DNA [6, 114].

While the cysteine rich domain is essential for the catalytic activity [114], truncations in LCI retains the activity in all hTET1-3, albeit at reduced catalytic efficiency [108, 114]. The first structural insight came in 2013 from the crystal structure of hTET2 in complex with 5mC-containing double-stranded DNA (dsDNA) (**Fig. 9.5**) [114]. To enable crystallization of hTET2, Hu *et al.* replaced the 837 amino acid (aa) flexible loop LCI by a GS (15 aa) linker. The structure revealed that the phosphate backbone of DNA interacts with multiple arginine and lysine residues of hTET2. The bound helical DNA structure is distorted, where the G-5mC hydrogen bond interaction is disrupted and 5mC flips into the catalytic core of the DSBH scaffold. The methyl group of 5mC is placed in proximity to the catalytic Fe(II) at the active site, which is held in place by H1382, D1384 and H1881 (**Fig 9.5**). 2OG coordinates to the metal and interacts with R1261, H1416, S1898 and R1896 of hTET2.

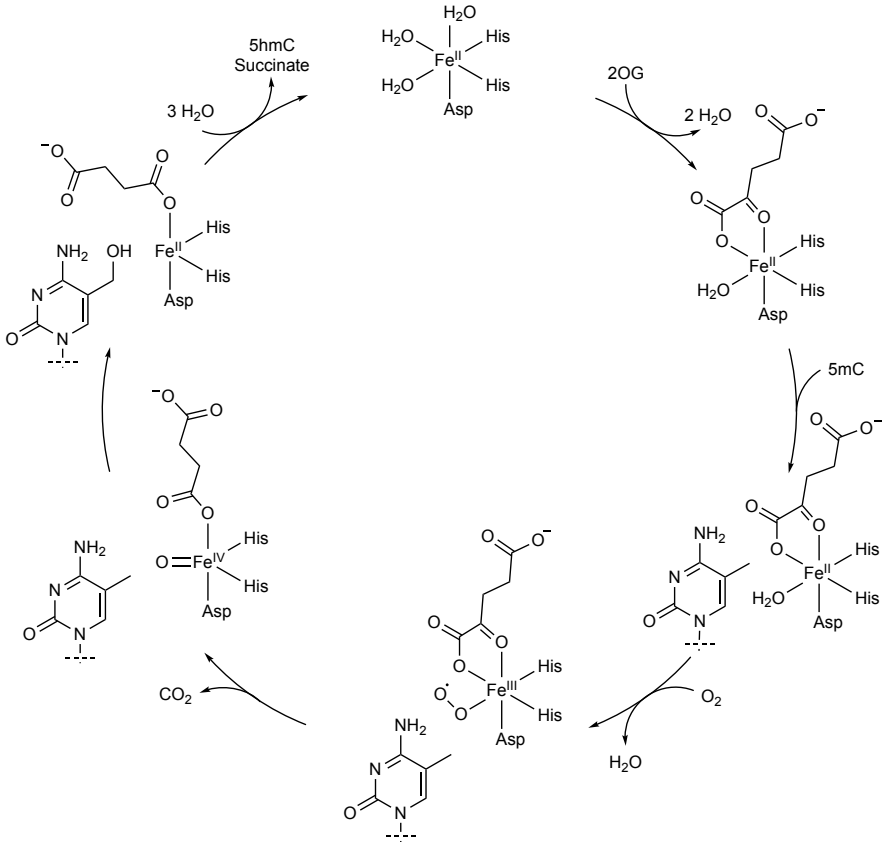

Figure 9.6: Proposed mechanism of the JmjC domain of TET enzymes. *TET protein binds to 2OG, molecular oxygen and the 5mC substrate. After generation of highly reactive Fe(IV) species, 5-methyl on cytosine is oxidized. The catalytic cycle is completed by the release of CO_2, succinate, substrate and the coordination of water to Fe(II).*

9.4.3. Mechanism of enzyme catalysis

The human TET enzymes contain a DSBH domain which catalyses the oxidization of 5mC, using a similar mechanism as other 2OG oxygenases [115–117] (also see Chapter 8). In the first step of the proposed mechanism, 2OG enters the active site and binds to Fe(II) releasing water molecules (**Fig. 9.6**). The DNA substrate binds with the 5mC methyl directed towards Fe(II). Molecular oxygen (O_2) then binds and reacts with the Fe(II) to form Fe(III) species. The radical oxygen molecule reacts further with 2OG giving a Fe(IV) intermediate and carbon dioxide (CO_2). The highly reactive Fe(IV) metal reacts with a proton on the methyl of 5mC substrate to provide the 5hmC

product in two steps, and Fe(II) is regenerated. The newly 5hmC substrate and succinate can then be replaced by water molecules completing the catalytic cycle. Oxidative steps of 5hmC to 5fC and 5fC to 5caC catalysed by TET are thought to follow a similar pathway.

The rate of oxidation by TETs is highly context dependent. The catalytic domain by itself is not believed to bind to specific DNA sequence but instead the catalytic domain 'slides' along the DNA strand until the active site binds the modified cytosine (5xC, 5-position modified cytosine, x= m, hm or f, (**Fig 9.7**. highlighted in red)). Enzyme kinetic analysis of hTET2 reveal selectivity for oxidation of 5mC over 5hmC or 5fC on dsDNA substrate, with lower (Kcat: ~3.4-4.6 fold) and higher K_M (~1.4-2.7 fold) than for 5mC [118]. The C-H proton extraction and inter – intra molecular proton stabilization are understood to be important in determining the differential rates of catalysis. Furthermore, hydroxyl group at the 2' position on the sugar (i.e. RNA) is tolerated (**Fig 9.7**. Highlighted in green) [119].

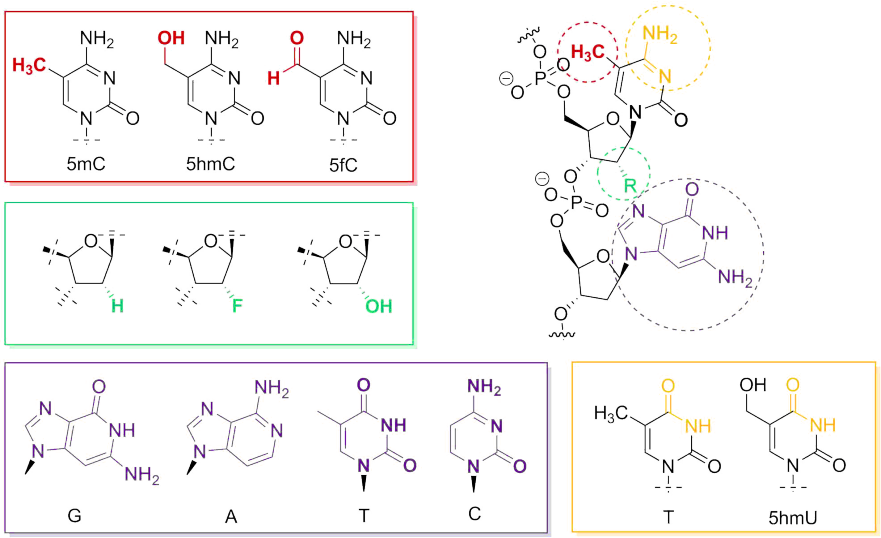

Figure 9.7: Double stranded DNA (dsDNA) representing the variety of different substrates for TET. *Red: 5- modified Cytosine (5mC, 5hmC, 5fC and 5caC); Yellow: Cytosine modified to thymidine; Green: modified 2'-(H (DNA), OH (RNA), F); Purple: phosphate pairing base (CpG, CpA, CpT, CpC); Blue: counter strand (single or double stranded DNA).*

Comparison of identical main strand sequences of double strand DNA (dsDNA), single-strand DNA (ssDNA) and single strand RNA (ssRNA) revealed that both dsDNA and ssDNA can be iteratively oxidised (5mC, 5hmC, 5fC and 5caC) by TETs; however, while 5-methyl RNA cytosine (5mrC) can be oxidised by TETs to form 5hmrC, limited levels of 5frC formation and the absence of 5carC was observed [119]. dsDNA and ssDNA are thus better TET substrates than RNA. It is interesting to note that the 2'-(*R*)-fluorinated derivatives of oxidised 5mC are also substrates of TETs, and although they exhibit decreased catalytic efficiency, they can be used as mechanistic tools [120]. TET activity is thus influenced by the modifications at the 2' position of the cytosine sugar, suggesting that this site contributes towards substrate specificity of TETs (**Fig 9.7.** Highlighted in green). It has been observed that mTET can oxidize thymidine as an alternative substrate (**Fig 9.7** highlighted in yellow), both *in vitro* [121] and *in vivo* [122], to give 5-hydroxymethyl uracil (5hmU) and 5-formyl uracil (5fU), albeit at significantly reduced activity relative to 5mC oxidation.

In the oligonucleotide sequence context, CpG is the preferred substrate over CpC, CpA CpT. In the genome, 60-80% of CpG are methylated [123], where symmetry is frequently observed; this gives rise to 21 possible symmetrical (on both strands) CpG combinations (C, 5mC, 5hmC, 5fC and 5caC) which TET can act on. In NgTET, significant drop in catalytic efficiency is observed by altering CpG site to CpA (~1.75 fold), CpT (~3.80 fold) and CpC (~5.80 fold) [112]. The crystal structure of NgTET1 indicates H-bonding interactions between the guanosine in the CpG pair and the NgTET protein which can account for the preference for CpG [112]. Note that 5-C modifications of the CpG on the reverse strand do not influence the catalytic activity.

The Alkylation repair protein (AlkB) homologs subfamily of 2OG oxygenases are closely related to the TET oxygenases and use Fe(II) catalysis with 2OG (**3**) and O_2 to oxidize DNA or RNA. This subfamily contains ABHs and Fat mass and Obesity-associated (FTO) proteins. In particular, ABH2 and ABH3, demethylases involved in repair processes, are structurally similar to hTET2. The preferred substrates for ABHs or FTO are 1N'-methyl adenine (1mA), 3N'-methyl thymidine (3mT), 3N'-methyl cytosine (3mC), 6N'-methyl adenosine

(6mA), and their oxidation results in unstable hemiaminal intermediate which decomposes to formaldehyde and demethylated base [114].

9.4.4. TETs in development and in disease

5hmC, 5fC and 5caC are found in many cells but their concentrations vary depending on the cell types. Interestingly, while the levels of 5mC remain relatively even across different cell types at approximately ~3.5-4.5% of all cytosine in the genomic DNA, this is not observed for 5hmC, 5fC or 5caC. The levels of 5hmC can range from 0.7% in the central nervous system to 0.03% in the spleen [124]. High levels of 5hmC are commonly found in the brain and neurons. Purkinje neurons for example contain up to 40% of 5hmC abundance relative to the 5mC levels in the cell [5].

While 5fC and 5caC are stable modifications and believed to be part of signaling pathways, 5fC and 5caC are significantly less abundant than 5mC or 5hmC. Levels of 5fC can range between 0.2 parts per million (ppm) in the lungs to 12 ppm in the brain in mice. Interestingly, there is no direct correlation between 5mC/5hmC and 5fC in cells or among the age of the tissue, suggesting that these marks have independent roles and are actively generated over time [125]. Postnatal mice have undetectable levels of 5caC (< 0.1 ppm) but in 12-week-old mice, some tissues, such as liver, can have elevated levels of 5caC (up to 2.0 ppm), while in others such as kidney or brain, 5caC levels remain below the detection limit [125].

Aside from their role as intermediates for demethylation, the function of 5hmC, 5fC 5caC, are not fully understood. Recent studies suggest that each mark has multiple implications. 5hmC formation is detected in active genes and enriched in the promotor regions [126]. While 5fC has been proposed to influence the helical structure of DNA [127], this may be context dependent [128]. Raiber *et al.* demonstrated that additional anchoring of the nucleosome to the DNA can occur *in vitro* and *in vivo*, as a result of histone lysine reacting with the 5fC to form an imine derivative [129]. This would provide enhanced nucleosome organisation within the chromatin remodelling for activation and silencing. Furthermore, 5fC and 5caC can affect the activity of proteins that associate with these marks. For example, with RNA polymerase II, 5fC and 5caC results in a lower rate of incorpo-

ration of pairing G [130]. Additionally, modifications at 5mC are distinctly recognised by an array of reader proteins [131] providing additional level of epigenetic regulation mediated by TET oxidation.

TET1 was first identified as a translocation partner of *MLL* gene in patients with acute myeloid leukemia (AML) [105]. Subsequently, it was demonstrated that MLL fusion protein directly binds to TET1 promoter, causing upregulation of TET1 and global increase in 5hmC levels in MLL-rearranged leukemia [132]. TET1 plays a critical role in the oncogenesis of MLL-rearranged leukemia *in vitro* and *in vivo* through co-activation of the Hoxa9/Meis1/Pbx3 signalling pathway [132]. In glioblastomas, TET1-mediated 5hmC production plays a critical role in tumourgenicity [133]. These studies highlight the therapeutic potential for targeting TET1 in certain cancers. In contrast, TETs have been shown to also have important tumour-suppressor roles in multiple cancers. Mutations in TET2 have been linked to haematopetic malignancies [134]. In myelodysplastic syndrome (MDS), TET2 is the most highly mutated gene [135], and in AML patients, multiple TET2 mutations (including frameshift, nonsense and missense within the protein creating mutated or truncated proteins) are found with reduced or abolished catalytic activity [136]. Furthermore, abnormalities have been observed in lymphoid malignancies, such as hTET2 B/T-Cell lymphoma [137]. Rare occurrences of mutations are found in hTET1/3 in chronic lymphocytic leukaemia (CLL) [138]. TETs are also linked to various solid tumours including gastric, breast, lung, liver and prostate cancer [139]. Downregulation of *TET* gene expression has been observed in multiple solid tumours, with decrease in 5hmC levels and increasing rate of proliferation [140].

Taken together, the evidence suggests that TETs can have both oncogenic and tumour suppressor roles, depending on the cellular context. Chemical probes for TETs are thus needed to understand their biological functions in development and in diseases.

9.5. TET enzyme assay and inhibitor development

The availability of robust and quantitative assays is prerequisite for the biochemical and functional studies of enzymes. For the TET enzymes, there are now a wide range of methodologies available to detect and quantitate oxidised 5mC levels, including global genome-wide mapping at base-resolution [141–143]. Analysis techniques of modified nucleosides/oligonucleotides include: (1) antibody-based detection of oxidised 5mC, (2) analytical methods using thin-layer chromatography (TLC) [5, 110, 112, 144] or liquid chromatography coupled with mass-spectrometry (LC-MS, LC-MS/MS) [107, 124, 145], and (3) chemical conversion or enzymatic labelling of modified cytosines (including glucosylation of 5hmC) [146, 147] [148–153]. These methodologies have enabled studies on tissue and genomic distribution and dynamics of oxidized 5mC in biological context. The readers are referred to [154] for an overview of sequencing techniques available for mapping oxidized 5mC on the genome, and [155, 156] for reviews on some approaches to studying the enzyme activities of TETs. In this section, we highlight key methodologies used for kinetic analysis of TETs *in vitro*, and recent TET assays developed for inhibitor discovery.

9.5.1. Kinetic analysis of human TETs.

Availability of reagents, such as modified oligonucleotides, oxidised 5mC antibodies and recombinant TET proteins, in recent years have enabled the development of a variety of biochemical assays for the TETs (**Table 9.1**).

LC-MS/MS is a direct and reliable method for quantification of multiple cytosine modifications simultaneously. While there are a number of variations on the methods and instruments used, the general procedure is that the DNA is purified, digested to nucleosides by nuclease and phosphodiesterase treatment. The digested nucleosides are separated and analysed by LC in tandem with MS (e.g. triple-quadrupole MS, quadrupole-orbitrap MS), and quantitated against labelled nucleoside internal standards. The large dynamic range (from low femtomole range) allows diverse application of this technique, from gDNA to oligonucleotides. LC-MS/MS analysis have been used to determine the substrate preference for TETs for 5mC oxidation over 5hmC/5fC-DNA substrates [107, 118]. Steady state kinetic analysis

of TET2CD(*del*Δ1099-1936) using LC-MS/MS revealed the affinities of modified cytosine to reduce with increasing oxidation (K_M = 0.48, 0.9 and 1.3 μM for 5mC, 5hmC and 5fC respectively) [118].

Detection method	Analytes; Principle	References
Analytical methods		
LC-MS/MS	Nucleosides: Enzymatic digestion of oligonucleotides to nucleosides, followed by dephosphorylation by CIP and LC-MS/MS analysis. Relative quantification of 5mC and oxidised 5mCs.	[114][118]
MALDI-TOF MS	Oligonucleotides: Relative quantification of intact DNA substrate and products using mass-directed detection.	[108][157]
Antibody-based methods		
Dot-blot	Oxidised 5mC (DNA); Detection and quantitation of oxidised 5mC containing intact DNA immobilised on membrane, using specific antibodies (5hmC, 5fC, 5caC) (chemiluminescence / fluorescence)	[158]
ELISA	Oxidised 5mC (DNA); Immobilised DNA substrate on a plate. Upon incubation with TETs, product formation detected using 5hmC antibody (fluorescence)	Epigentek
AlphaScreen	Oxidised 5mC (DNA): Homogenous bead-based assay, with product DNA capture and antibody detection of oxidised 5mC. (luminescence)	[158]
Radiolabeling		
TLC	Nucleosides; Oligonucleotides digested and dephosphorylated with CIP, and labelled with [γ-^{32}P]ATP and T4 polynuclotide kinase. Further treatment with nuclease and analysed on TLC plate. Relative quantification of 5mC and oxidised 5mCs.	[159]
$^{14}CO_2$ assay	By-product formation (CO_2); Measurement of hydroxylation-coupled $^{14}CO_2$ production using 2-oxo[1-^{14}C]glutarate co-factor.	[160]
Other		
Succinate-Glo	Succinate (**5**) by-product of catalysis; Enzyme-coupled assay linking succinate production to ATP production (bioluminescence)	Promega [161]

Table 9.1. Reported assays used for kinetic analysis and inhibitor development for the TET proteins

While LC-MS/MS methodology provides quantitative accuracy and robustness, the multi-step processing of DNA has hampered its use for high-throughput applications. A matrix-assisted laser desorption/ionisation time-of-flight (MALDI-TOF) MS method has provided a middle ground. This assay allows direct measurements of intact DNA without the need for downstream processing steps (e.g. enzymatic and chemical modifications), thus minimising the reagent requirement, processing error and time. Michaelis-Menten kinetic parameters for 2OG (**3**) were determined to be K_M = 15.7 μM (hTET2CD (*del*Δ1099-1936)) and K_M = 24.2 μM (hTET3CD (*del*Δ 689-1596)) using this method. The relatively low 2OG K_M suggests that potency is crucial for 2OG competitive inhibitors.

Assays using antibodies against modified cytosines, such as enzyme-linked immunosobent assay (ELISA) and AlphaScreen, have also helped improve the sensitivity, the volumes and throughput of the assays [158]. Assays using radiolabelling (e.g. 2-oxo[1-^{14}C]glutarate, [γ-^{32}P]ATP) are also utilised for inhibitor screening.

9.5.2. Inhibitors of TET enzyme activity

As for the other 2OG oxygenases, the majority of inhibitors target the catalytic Fe(II), and often mimic or compete with the 2OG binding (see [162] for review of 2OG oxygenase inhibitors (**Fig. 9.8**).

9.5.2.1. 2OG analogues as TET inhibitors

The first TET inhibitor to be identified was *R*-2-hydroxyglutarate (*R*-2HG, **28**), an 'oncometabolite' associated with the gain-of-function mutations in isocitrate dehydrogenases (IDH). IDH is a tricarboxylic acid (TCA) cycle enzyme that catalyse the oxidative decarboxylation of isocitrate to 2OG (**3**). IDH with mutations in the active site (Arg100, Arg132 in IDH1, Arg140, Arg172 in IDH2 found in glioma/leukemia), can further catalyse the reduction of 2OG (**3**) to *R*-2HG (**28**), leading to cellular accumulation of *R*-2HG (**28**) (up to 30-50 mM, compared to < 0.1 mM in normal cells) [163–166]. *R*-2HG inhibits human (and mouse) TETs with IC_{50} at 4-5 mM range [159] [160]. Given the inhibitory effect of TETs by *R*-2HG and significantly reduced global levels of 5hmC in *IDH* mutants, TETs are thought to

play a contributory role in the tumorigenicity of *IDH* mutant cells. Other TCA cycle intermediates, such as succinate (**31**) and fumarate (**32**), can also accumulate as a result of tumour-associated mutations in the TCA cycle enzymes succinate dehydrogenase (*SDH*) and fumarate hydratase (*FH*) [167]. Biochemical assays using recombinant mouse Tet1 and Tet2 have shown that both succinate and fumarate inhibit at 400-600 μM range, and modest changes in global 5hmC levels are observed in human neuroblastoma cells treated with fumarate and succinate esters [160]. Thus, it appears TET activities are sensitive to concentration flux in some TCA cycle intermediates / oncometabolites *in vitro* and in cells, as found in other 2OG (**3**) oxygenases [168, 169][170].

In 2015, a fluorescent polarisation assay based on a fluorophore-linked hydroxamic acid probe (**33**) was reported for Tet1 from NgTET, a model protein with 39% similarity to human TETs [171]. The hydroxyamic acid motif is thought to chelate the active site Fe(II). Indeed, the probe binds to NgTet1 with K_d = 250 nM, and competes with 2OG (NgTET K_d = 250 μM). *N*-oxalylglycine (NOG, **30**), a close isostere and an inactive analogue of 2OG (**3**) and a broad-spectrum inhibitor of 2OG oxygenases [172], was also found to bind to NgTet1 (K_d = 49 μM), demonstrating the utility of FP assay for the identification of 2OG competitive binders of the TET proteins.

While the human TET2-5hmC-DNA complex had been co-crystallised with NOG [114], it was not until recently that NOG was demonstrated to inhibit the catalytic activity of human TET2CD (IC_{50} = 149 μM) using MALDI-TOF MS assay [108]. *R*-2HG was confirmed to weakly inhibit TET2 in a similar range as previously reported, and *S*-2HG (**28**) at IC_{50} > 10 mM using this assay.

9.5.2.2. Non-metal chelating inhibitors of TETs

We have recently reported the development of macrocyclic peptide inhibitors for the human TETs [158]. Using mRNA-display based RaPID technology, macrocyclic peptide binders of human TET1CD *del*Δ1099-1936 were selected from a pool of >10^{12} peptides. Three sequences (TiP1 (**36**), TiP2 (**37**), Tip3M15L (**38**)) were confirmed to bind to TET1 (K_d < 100-220 nM) using surface plasmon resonance (SPR) and inhibit the catalytic activity at approximately IC_{50} = 1 μM

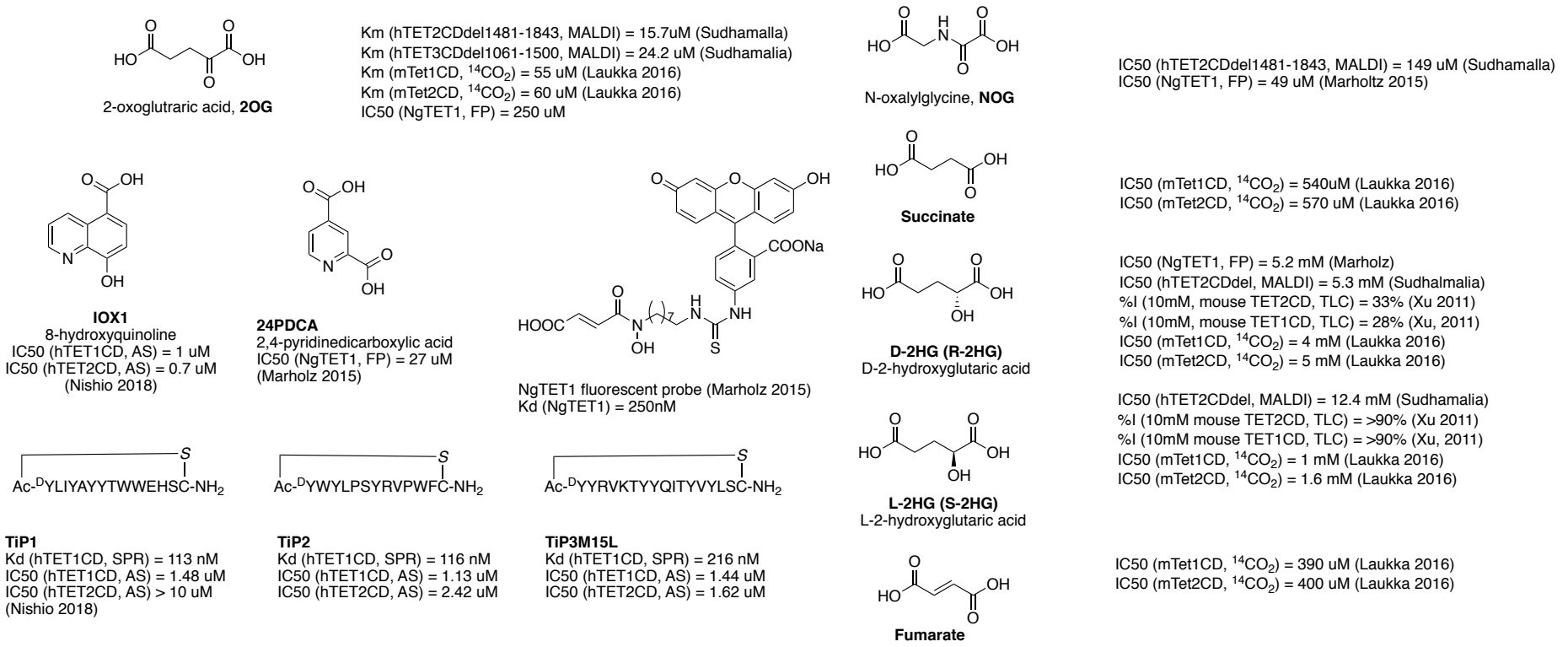

Figure 9.8. Structures of TET inhibitors. *Structures of the co-factor 2OG, TCA cycle intermediates and metabolites, small molecules and cyclic peptides that bind to or inhibit the TET proteins are shown. IC_{50}, K_d or percentage inhibition of activity (%I) are stated at tested concentration. Assay methods used for affinity or inhibition are noted as superscript: 1) MALDI-TOF MS, 2) $^{14}CO_2$ radiolabelling, 3) FP, 4) TLC, 5) AlphaScreen, 6) SPR. A: (Sudhamalla et al. 2017); B: (Laukka et al. 2016), C: (Marholz et al. 2016); D: (Xu et al 2011b) ; E: (Nishio et al. 2018).*

using 5hmC-antibody based AlphaScreen™-based assay. Interestingly, TiP1 selectively inhibited TET1 over TET2, demonstrating that some selectivity between different TET proteins is possible. Unlike IOX1 (**34**), a 2OG competitive, metal-chelating broad-spectrum 2OG oxygenase inhibitor control [172], the macrocyclic peptides does not compete directly with 2OG, demonstrating a novel mode-of-action and a promising approach to developing selective inhibitors.

9.6. Conclusions

DNA methylation is the most conserved epigenetic modification and in mammals it plays an important role in gene regulation. It is involved in normal biological process, but it is also aberrant in several human diseases [35]. In particular it is well studied in cancer, and for example certain FDA-approved cancer diagnostic kits are based on DNA methylation. Nevertheless, several questions remain to be addressed and chemical tools can be of help. The most potent inhibitors of DNA methylation are the nucleoside analogues 5azaC and 5azadC that have their limitations as described above. Non-nucleoside inhibitors have been designed or screened to overcome this. Several have shown to demethylate promoters and reactivate tumour suppressor genes, but none have the potency of the nucleoside inhibitors in cells and *in vivo.* There are on-going efforts to further improve the design of these compounds, and novel strategies, such as the design of dual inhibitors or bisubstrate analogues, are providing promise. Isoform selective inhibitors of DNMTs (Dnmt1, Dnmt2 or Dnmt3) will be of great use for the understanding of the roles of each; as well as compounds that specifically demethylate DNA, which are devoid of off-target effects.

In parallel, it is crucial to understand the downstream chemical fate of DNA methylation. While sequential oxidations of 5mC to 5hmC, 5fC and 5caC are key steps in the passive and active demethylation pathways, each oxidised 5mC mark themselves have important regulatory functions, including active recruitment of chromatin reader modules and protein complexes. TETs are thus involved in

multiple levels of epigenetic regulation, making them an interesting protein family to study. TETs play key roles in development and in disease. In cancer, TETs have been shown to have both oncogenic and tumour suppressor functions. However, the catalytic and non-catalytically dependent TET functions remain unclear in many biological processes.

Recent progresses in structural and biochemical studies have provided great insight into the functions of TET proteins. The substrate specificity, allosteric regulation with respect to other domains (*e.g.* CXXC domain, LCI domain) and the biological impact however, are not fully understood. Selective domain targeting chemical probes for TETs will be valuable to investigate the regulation of cytosine modifications on chromatin and transcription. Efforts have been made towards developing inhibitors targeting the catalytic domain of TETs; however, many of the small molecule inhibitors reported to date are weak inhibitors and are not selective. Potent cyclic peptide inhibitors show promise but cell permeability remains a challenge. It is anticipated that the recent advancements in (high-throughput) assays and structural information will aid in the development of chemical probes for the TET proteins.

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