Bioorg. Med. Chem. Lett. 40 (2021) 127908

Contents lists available at ScienceDirect

Bioorganic & Medicinal Chemistry Letters

journal homepage: www.elsevier.com/locate/bmcl

A novel class of selective non-nucleoside inhibitors of human DNA methyltransferase 3A

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ARTICLE INFO

Keywords:
Non-nucleoside DNA methyltransferase inhibitors
DNMT1 inhibitors
DNMT3A inhibitors
Acute myeloid leukemia target

ABSTRACT

Screening of a small chemical library (*Medicines for Malaria Venture* Pathogen Box) identified two structurally related pyrazolone (inhibitor 1) and pyridazine (inhibitor 2) DNMT3A inhibitors with low micromolar inhibition constants. The uncompetitive and mixed type inhibition patterns with DNA and AdoMet suggest these molecules act through an allosteric mechanism, and thus are unlikely to bind to the enzyme's active site. Unlike the clinically used mechanism based DNMT inhibitors such as decitabine or azacitidine that act via the enzyme active site, the inhibitors described here could lead to the development of more selective drugs. Both inhibitors show promising selectivity for DNMT3A in comparison to DNMT1 and bacterial DNA cytosine methyltransferases. With further study, this could form the basis of preferential targeting of *de novo* DNA methylation over maintenance DNA methylation.

Epigenetic modifications of proteins and nucleic acids are crucial for normal development.[1–3] Human DNA undergoes methylation largely at CpG dinucleotides, and the patterns are developmentally regulated and tissue-specific; these patterns contribute to the epigenetic code, which is essential for viability.[3–6] Aberrant methylation patterns can result in hypermethylation of gene promoters, leading to the silencing of critical tumor suppressor genes, resulting in tumorigenesis.[5,6] DNA methylation is carried out by a family of enzymes (DNMTs, Fig. 1) while demethylation is carried out by the ten-eleven translocation (TET) enzyme family.[7–10] DNMTs rely on the methyl donor S-adenosyl-l-methionine (AdoMet) and display both random and ordered kinetic mechanisms.[11–15] The housekeeping protein DNMT1 primarily acts on hemimethylated DNA, and the two *de novo* methyltransferases DNMT3A and DNMT3B, act predominately on unmethylated DNA.[16–18] The DNMT3s, which also include a catalytically inactive regulatory protein DNMT3L, are mostly expressed during the early development phase of mammalian germ cells.[1,4,16,19] DNMT1, meanwhile, is expressed throughout the lifetime of mammalian somatic cells and is localized near replication forks.[17,20]

All DNMTs share the same domain architecture. The less conserved of their two domains is the *N*-terminal domain, which contains regulatory segments including the replication fork binding RFD sequence in DNMT1 and the H3 binding ADD sequence in DNMT3s.[1,4,20,21] The C-terminal, or catalytic domain has the highly conserved methyltransferase motifs (I-X) that are found in both prokaryotic and eukaryotic methyltransferases (see Fig. 1).[22] These motifs are responsible for cofactor binding and catalysis.[4,21] DNMT3A forms tetramers with DNA binding occurring along the seam of the dimerization domain (see Fig. 2).[21] Mutations that disrupt the oligomeric state of DNMT3A occur in a number of cancers, and in particular, acute myeloid leukemia (AML).[5,23–26] Both catalytically active DNMTs, and in particular, DNMT3A, interact with diverse partners and disruptions to these interactions alter the function of DNMT3A and contribute to tumor-specific changes in methylation patterns.[5,23,27,28]

In the last twenty years, interest in developing drugs that target epigenetic pathways has increased, particularly for histone and DNA modifying enzymes.[29–31] An obvious feature of these pathways is their inherent reversibility, unlike mutational changes which frequently demand therapeutic strategies leading to cytotoxic interventions. Interestingly, the FDA approved DNMT nucleoside inhibitors, azacytidine and decitabine are highly cytotoxic. These prodrugs are converted to the triphosphates, incorporated into DNA and inhibit DNMTs through the

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https://doi.org/10.1016/j.bmcl.2021.127908
Received 2 November 2020; Received in revised form 15 January 2021; Accepted 18 February 2021
Available online 8 March 2021
0960-894X/

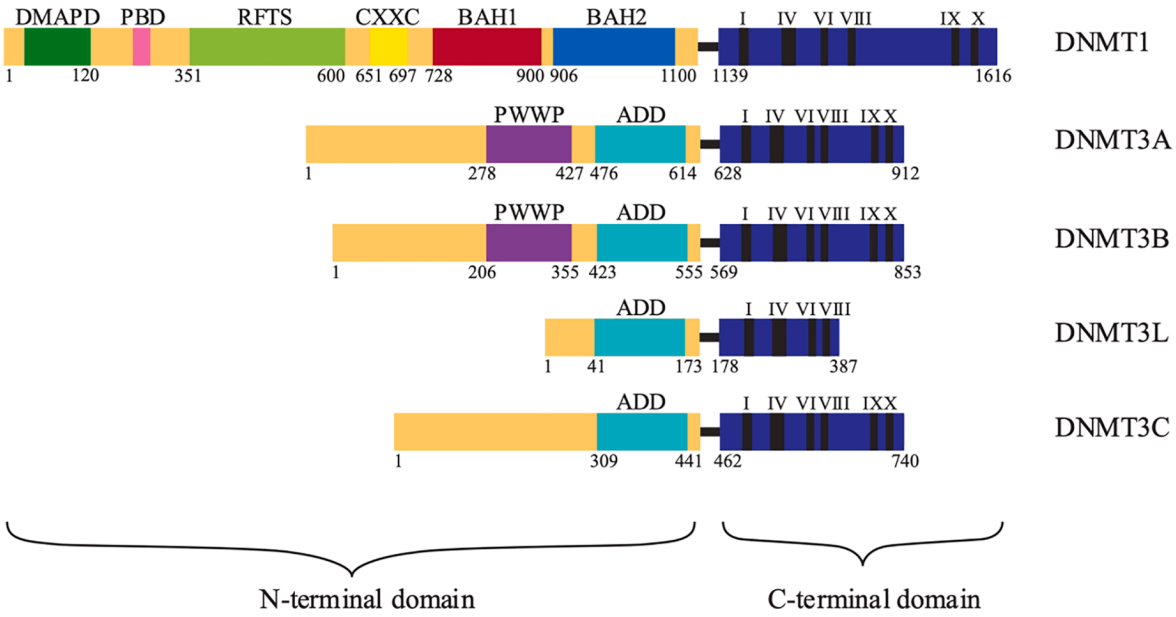

Fig. 1. Comparison of the primary structures of human DNMTs. The C-terminal domain contains conserved motifs (I-X) and is active in the absence of the *N*-terminal domain. The N terminal domain has several conserved segments known to interact with regulatory proteins and histones. The abbreviations used are: DMAPD – DNA methyltransferase associated protein 1 interacting domain, PDB – PCNA binding domain, RFTS – Replication foci targeting domain, BAH – bromo-adjacent homology domain, ADD – ATRX-DNMT3-DNMT3L domain.

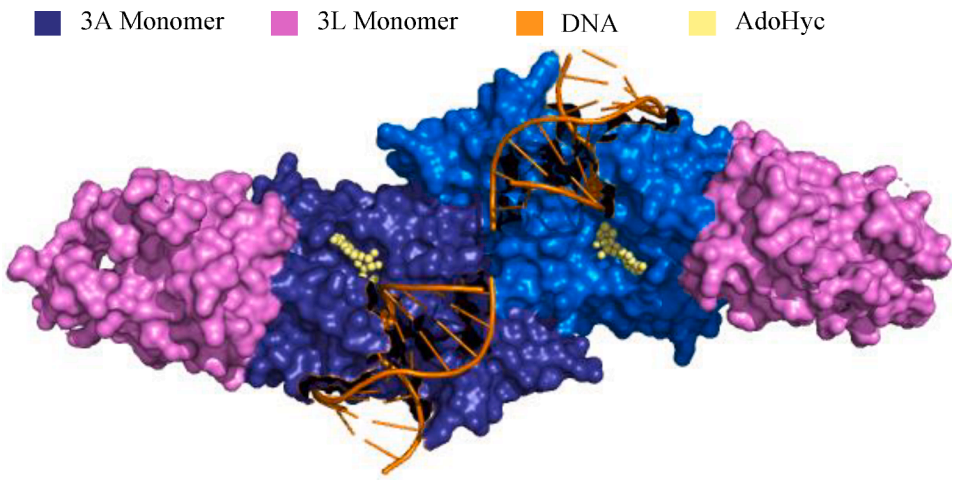

Fig. 2. Crystal structure of DNMT3A-3L heterotetrameric complex; the DNA is modeled from the M.*Hha*I-DNA cocrystal structure (PDB code 6f57).

formation of an irreversible suicide complex.[30,32,33] The dose-limiting toxicity manifested by these drugs has led to the search for non-nucleoside inhibitors; interestingly, many of these act by binding the enzyme active site or act by unknown mechanisms[29,30].

Our interest is to determine if new mechanistic classes of DNMT inhibitors can be identified. The long term goal is to develop therapeutic approaches not hampered by the toxicity and related issues associated with currently used and recently described DNMT inhibitors.[29,30] For example, there are over 60 known proteins which interact with DNMT3A,[34] some of which are implicated in directing DNMT3A to inappropriately methylate and regulate tumor suppressor genes.[26,27] Moreover, the clinically identified DNMT3A mutations in diverse cancers are known to alter the stability and functional outcomes of the complexes formed between DNMT3A and its partner proteins.[26,27,35] This network of interactions could be the basis of tumor-specific protein–protein inhibitors (PPIs).[30,36] Certainly, the recent progress in developing PPIs for diverse therapeutic targets, including leukemia, forms a strong basis for such a strategy.[37,38] Finally, allosteric enzyme modulators can provide a basis for enhanced selectivity and, potentially, decreased toxicity.[39–41]

Here we describe our initial compound screening effort, relying on open source chemical library constructed from the *Medicines for Malaria Venture* (MMV) Pathogen Box. The library consists of 400 drug-like molecules with known activities against targets for neglected tropical diseases. The relative merits of using a library of well-established molecules that show good bio-activity versus other approaches have been well described.[42] Using 50 compounds of the library, we first determined that a compound concentration of 60 μM resulted in 5% of the molecules showing 90% or more inhibition. We then relied on a modified version of our standard radiochemical assay using tritiated AdoMet,[26] which measures DNA methylation (see Methods, Supplementary). The assay uses poly dI-dC which is an excellent DNMT3A substrate, due to the presence of multiple sites for DNMT3A-mediated methylation. The conditions allow for multiple catalytic turnovers with an excess of DNA.[26,27] Importantly, many literature reports describing DNMT screens are actually done under conditions

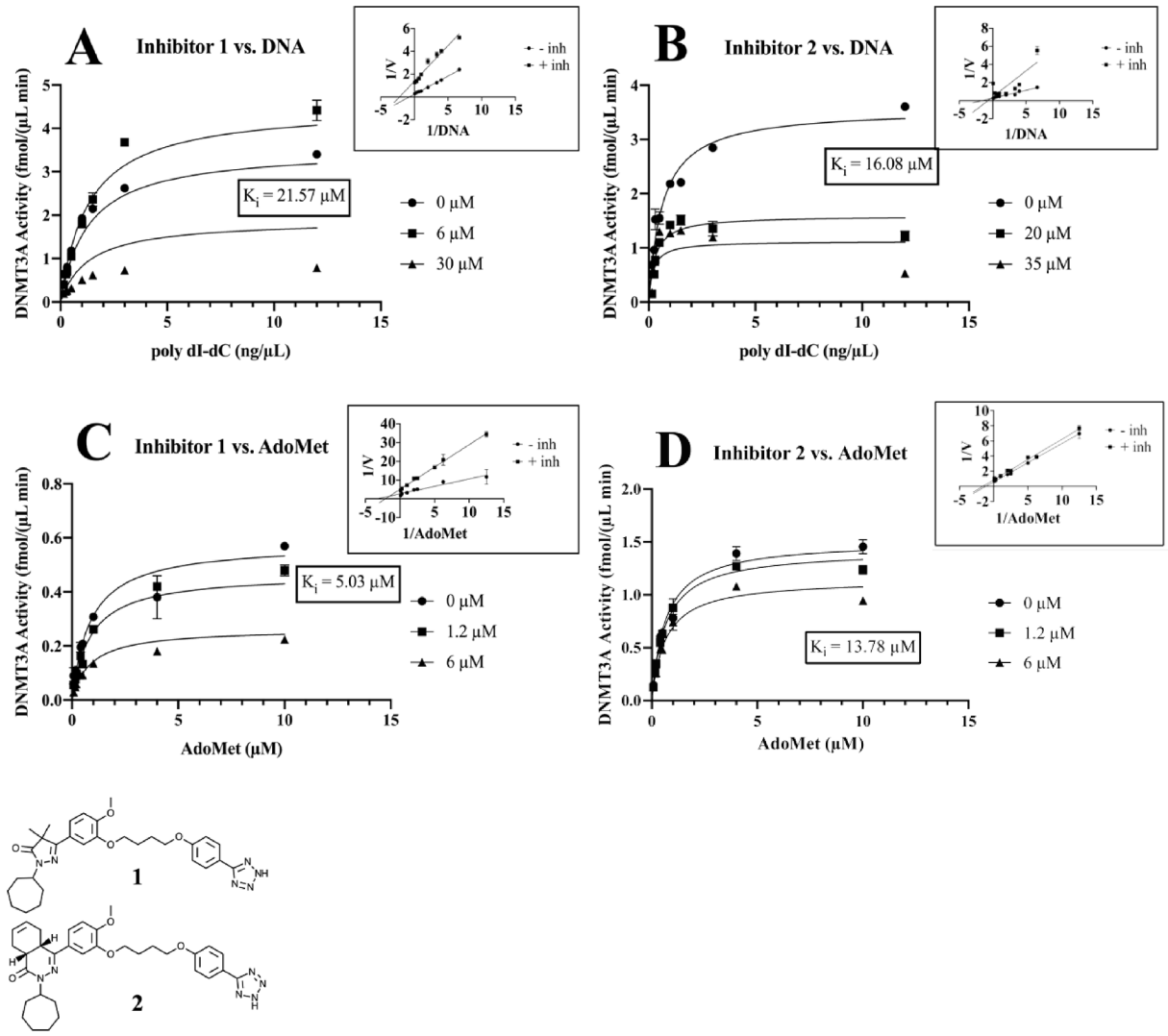

Fig. 3. Best fit plots of the inhibition with respect to both substrates, poly dI-dC (A, B) and AdoMet (C, D). Assays were performed with 150 nM DNMT3A with an excess of the substrate being held constant. Radiolabeled ^{3}H-AdoMet was used to determine product formation. All reactions were assayed for 30 min, then quenched with 0.1% SDS and spotted onto charged nylon membranes for detection. Data was collected with two replicates (n = 2). Fitting was performed with standard inhibition equations being applied to the whole model. Extracted K_i values are boxed, while corresponding reciprocal plot with best-fit lines are shown in top right. Structures of inhibitors are shown (left).

Table 1
Values for the various fits of inhibitors with respect to both substrates. Fits were determined using the *Noncompetitive* and *Uncompetitive* nonlinear regression models in *Prism 8.4.3*. The reported bounds define the 95% confidence interval of the K_i value.

Inhibitor	1		2	
Substrate	AdoMet	poly dI-dC	AdoMet	poly dI-dC
Best fit K_i (µM)	3.70 – 7.06	12.64 – 40.56	9.16–18.85	11.37 – 23.34
Best fit Mechanism	*Mixed*	*Mixed*	*Uncompetitive*	*Uncompetitive*
Goodness of fit (R^2)	0.957	0.831	0.983	0.891

which compromise interpretation of any inhibition effects, such as excess enzyme over DNA, or less than a single catalytic cycle. [43–45]

The screen of the library generated 12 compounds that showed at least 90% inhibition. The screening assay was repeated on these 12 compounds to verify the inhibitory properties (see Figure 1S), and the results were used to select two structurally similar compounds for further analysis (see compounds 1 and 2, Fig. 3). These two compounds were previously identified as potential inhibitors of TbrPDEs, a class of phosphodiesterases found in *T.* brucei – the parasite responsible for trypanosomiasis (*African sleeping sickness*).[46] Compounds 1 and 2 both show potent and selective inhibition of TbrPDE, good antitrypanosomal effects, and are part of an extensive study of TbrPDE inhibitors involving numerous analogs.[47]

The inhibitory mechanisms of both compounds were examined by varying both substrates. The results were fit to models representing various modes of inhibition (see Methods, Supplemental). The potencies (K_I values) of compounds 1 and 2 against DNMT3A range from 3.7 to 18 µM (AdoMet) and 11 to 41 µM (poly dI-dC), which compare favorably to numerous published efforts.[29,30] The best fits to the inhibition data for both compounds against poly dI-dC and AdoMet are consistent with mixed type or uncompetitive mechanisms (see Fig. 3, Table 1). Importantly, both mechanisms require that compounds 1 and 2 bind allosterically, away from the active site of the enzyme. The mixed type mechanism allows for scenarios in which the inhibitor binds both forms of the enzyme with the pertinent substrate bound, or unbound. In contrast, the uncompetitive mechanisms (Fig. 3, Table 1, Compound 2) implicate a mechanism wherein the inhibitor only binds to the form of

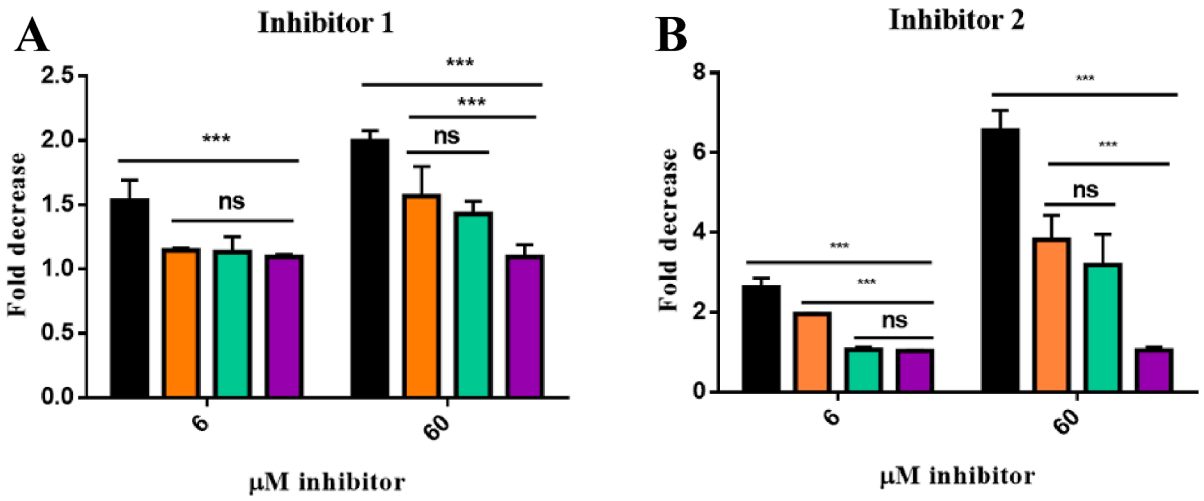

Fig. 4. Inhibitors 1 (A) and 2 (B) modulate the activity of human *de novo* DNMT3A_CD (■), DNMT3A_FL (■) and maintenance DNMT1 (■) DNA methyltransferases but not of the bacterial DNA methyltransferase M.SssI (■). To test the specificity of inhibitor 1 and 2 for these distinct types of CpG DNA methyltransferases, assays were performed with 150 nM DNMT3A_CD (■), DNMT3A_FL (■), 133 U/mL DNMT1 (■) and 266 U/mL M.SssI (A and B). Inhibitors 1 (A) and 2 (B) were tested at 6 and 60 μM with poly dI-dC and AdoMet at a fixed concentration of 1.8 ng/μL and 5 μM, respectively. In all reactions (A and B), enzymes and inhibitor 1 or 2 were pre-incubated for 15 min at 37 °C in methylation reaction buffer (see methods) prior to initiating the reaction by the addition of DNA. Methylation reactions were assayed for 30 min, quenched with 0.1% SDS and spotted onto charged nylon membranes for detection of radiolabeled ^{3}H-AdoMet DNA. Fold decrease was calculated by dividing the product formed in reactions without compound 1 or 2 by the product formed in reactions with compound 1 or 2. Data reflect the mean ± S.D. of 3 independent experiments; one-way analysis of variance (ANOVA) was used to compare the values of all enzymes for each concentration (6 or 60 μM) of inhibitors 1 (A) or 2 (B); ***, $p < 0.01$; ns, $p > 0.05$. mean ± S.D. of 3 independent experiments; one-way analysis of variance (ANOVA) was used to compare the values of all enzymes for each concentration (6 or 60 μM) of inhibitors 1 (A) or 2 (B); ***, $p < 0.01$; ns, $p > 0.05$.

the enzyme already bound by the DNA. The mechanisms of other DNMT inhibitors, when reported, often display competitive mechanisms with DNA, AdoMet, or both.[30,44,48] The simplest interpretation of these mechanisms is that the inhibitor binds the same site as DNA or AdoMet, or, minimally, binds the same form of the enzyme bound by these substrates.[49]

The widespread cellular reliance on AdoMet-dependent methyltransferases suggests that the development of drugs specific for DNA methyltransferases or drugs that distinguish between DNMT1 and DNMT3A will be challenging. This is reflected by the fact that the majority of DNA methyltransferase inhibitors are poorly selective for DNMT3A, likely contributing to the limiting toxicity displayed by these compounds.[30] DNMT1 is critical to cell viability and given the prevalence of DNMT1 throughout the lifetime of somatic cells, the selective inhibition of DNMT3A over DNMT1 is important in the development of cancer treatments.[7] This is especially true of cancers like AML, where prevalence of DNMT3A mutations is particularly high. Further inhibition studies aimed to see if these compounds would affect DNMT1. Additionally, given the implicated allostery, we wanted to investigate if these compounds could inhibit the bacterial cytosine methyltransferase M. SssI (see Methods, Supplemental). This protein has a highly conserved active site with respect to DNMT3A but does not share its allosteric structure.[22] The results with respect to DNMT3A and DNMT1 found both compounds show some selectivity, with inhibitor 2 being the more selective of the two (see Fig. 4). Neither compound shows inhibition of the bacterial DNA cytosine methyltransferase M.SssI, even at 60 μM. Both compounds show little inhibition of DNMT1 at 6 μM, and compound 2 retains this selectivity even at 60 μM. It is intriguing that both inhibitors show greater inhibition of the catalytic domain of DNMT3A (residues 628 to 912, see Fig. 1) than the full length DNMT3A, suggesting that the large *N*-terminal segment interferes with the inhibition. The basis of this difference has diverse molecular explanations, which we are actively investigating. Both compound 1 and 2 are still able to modulate the more biologically relevant full-length form of DNMT3A.

In summary, the screening of a small chemical library of known drugs against human DNMT3A identified two non-nucleoside molecules of low micromolar potency. Both molecules inhibit the enzyme by binding outside the active site, and not only selectively inhibit human over bacterial DNMTs, but also shows some promising preferential targeting of *de novo* over maintenance DNA methyltransferases. This highlights the potential use of these molecules for the treatment of malignancies associated with disruptions to DNMT3A activity. The large number of analogs of these two inhibitors which have been described provides a promising basis for further optimization of this new group of DNMT3A inhibitors, with reasonable prospects of showing improved toxicity over known DNA methyltransferase drugs.[46]

Declaration of Competing Interest

This work was supported by National Science Foundation Grant 1808775 (to N. R.). The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.bmcl.2021.127908.

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