ORIGINAL PAPER

Trimethylaurintricarboxylic acid inhibits human DNA methyltransferase 1: insights from enzymatic and molecular modeling studies

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Abstract DNA methyltransferase 1 (DNMT1) is an emerging target for the treatment of cancer, brain disorders, and other diseases. Currently, there are only a few DNMT1 inhibitors with potential application as therapeutic agents or research tools. 5,5-Methylenedisalicylic acid is a novel scaffold previously identified by virtual screening with detectable although weak inhibitory activity of DNMT1 in biochemical assays. Herein, we report enzyme inhibition of a structurally related compound, trimethylaurintricarboxylic acid (NSC97317) that showed a low micromolar inhibition of DNMT1 (IC_{50}=4.79 μM). Docking studies of the new inhibitor with the catalytic domain of DNMT1 suggest that NSC97317 can bind into the catalytic site. Interactions with amino acid residues that participate in the mechanism of DNA methylation contribute to the binding recognition. In addition, NSC97317 had a good match with a structure-based pharmacophore model recently developed for inhibitors of DNMT1. Trimethylaurintricarboxylic acid can be a valuable biochemical tool to study DNMT1 inhibition in cancer and other diseases related to DNA methylation.

Keywords Cancer · DNMT · Docking · Enzyme inhibition · Epigenetics · Methyltransferase

Introduction

DNA methylation is an epigenetic change that results in the addition of a methyl group at the carbon-5 position of

cytosine residues. DNA methyltransferase enzymes (DNMTs) mediate this process. To date, three types of DNMTs have been identified in the human genome, including the two de novo methyltransferases (DNMT3A and DNMT3B) and the maintenance methyltransferase (DNMT1), which is the most abundant among the three [1–3]. DNMT1 is responsible for duplicating the pattern of DNA methylation during replication, it is essential for proper mammalian development, and it has been proposed as the more interesting target for experimental cancer therapies [4]. DNA methylation represents a central mechanism for mediating epigenetic gene regulation. Thus, the development of DNMT inhibitors provides novel opportunities for cancer therapy [1, 5–7]. It is worth nothing that epigenetic alterations are not only associated with cancer but also with psychiatric and other diseases [8–10].

Human DNMT1 is a protein with 1616 amino acids whose structure can be divided into an N-terminal regulatory domain and a C-terminal catalytic domain [11–13]. The mechanism of DNA cytosine-C5 methylation is schematically depicted in Fig. 1 [4, 14, 15]. DNMT forms a complex with DNA and the cytosine which will be methylated flips out from the DNA. The thiol of the catalytic cysteine acts as a nucleophile that attacks the 6-position of the target cytosine to generate a covalent intermediate. The 5-position of the cytosine is activated and conducts a nucleophilic attack on the cofactor S-adenosyl-L-methionine (SAM) to form the 5-methyl covalent adduct and S-adenosyl-L-homocysteine (SAH). The attack on the 6-position is assisted by a transient protonation of the cytosine ring at the endocyclic nitrogen atom N3, which is stabilized by a glutamate residue. An arginine residue may participate in the stabilization of the intermediate making a hydrogen bonding interaction with the carbonyl oxygen of cytosine. The covalent complex

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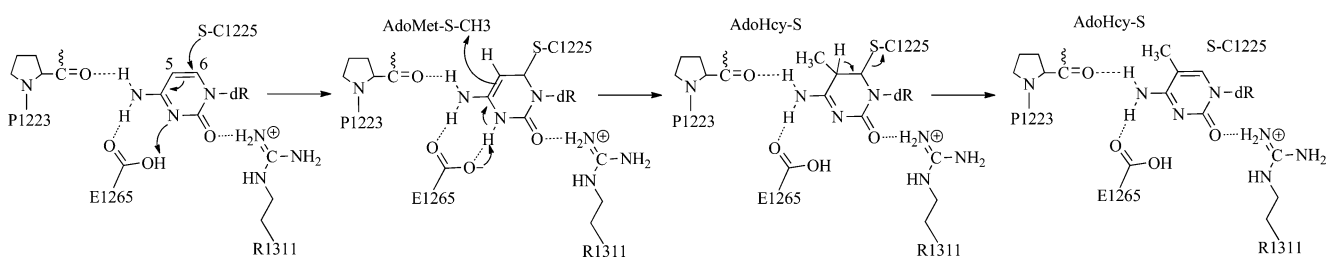
Fig. 1 Proposed mechanism of DNA cytosine-C5 methylation. Amino acid residue numbers are based on the homology model

between the methylated base and the DNA is resolved by deprotonation at the 5-position to generate the methylated cytosine and the free enzyme.

To date, only the DNMT inhibitors 5-azacytidine and 5-aza-2′-deoxycytidine have been developed clinically. These two drugs are nucleoside analogues, which, after incorporation into DNA, cause covalent trapping and subsequent depletion of DNMTs [5, 15]. Aza nucleosides are FDA approved for the treatment of myelodysplastic syndrome, where they demonstrate significant, although usually transient improvement in patient survival and are currently being tested in many solid cancers [16–18]. 5-Aza-2′-deoxycytidine however, has relatively low specificity and is characterized by substantial cellular and clinical toxicity [5].

There are an increasing number of substances reported to inhibit DNMTs [19–21]. Representative DNMT inhibitors and other candidate demethylating agents are depicted in Fig. 2. Computational screening of chemical databases has contributed to the discovery of lead compounds with distinct scaffolds such as RG108 [22]. Similarly, molecular modeling and docking are playing a key role in the

understanding of the mechanism of action of a number of DNMT inhibitors at the molecular level [23, 24].

We recently conducted a virtual screening of the National Cancer Institute (NCI) database identifying 5,5-methylenedisalicylic acid (NSC14778) (Fig. 2) as a DNMT1 inhibitor with an $IC_{50}=92$ μM [25]. Molecular docking of 5,5-methylenedisalicylic acid with an homology model of the catalytic domain of DNMT1 indicated the key role of the carboxylic acids in the binding recognition including hydrogen bonds with amino acid residues that are involved in the catalytic mechanism of methylation [25]. Recently, researchers have shown an increased interest in the biological activity of 5,5-methylenedisalicylic acid and related compounds. For example, 5,5-methylenedisalicylic acid also emerged as an experimentally validated hit of a virtual screening with the viral NS5 RNA methyltransferase, a promising drug target against flaviviruses which are the causative agents of severe diseases such as Dengue or Yellow fever [26]. Moreover, in an independent virtual screening, the aurintricarboxylic acid (which is a structural analogue of 5,5-methylenedisalicylic acid) was identified as a highly potent inhibitor of the methyltransferase activities on flaviviral methyltransferases [27]. Also, a recent study shows that aurintricarboxylic acid inhibit two AdoMet-dependant RNA methyltransferases of the severe acute respiratory syndrome (SARS) coronavirus [28].

We hypothesize that trimethylaurintricarboxylic acid (NSC97317; Fig. 2) is an inhibitor of DNMT1. To note, this compound is a structural analogue of aurintricarboxylic acid and 5,5-methylenedisalicylic acid. Moreover, NSC97317 is *freely available* at the NCI Drug Synthesis and Chemistry Branch [29]; this fact may increase the impact and applicability of the insights of this work. In order to test this hypothesis and identify a novel DNMT inhibitor, herein we report enzyme inhibition and molecular modeling studies that confirm this hypothesis.
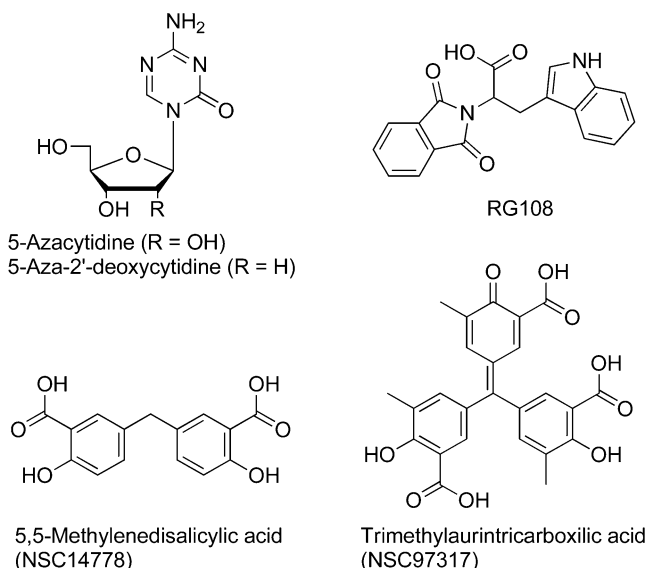
Fig. 2 Chemical structures of representative inhibitors of DNA methyltransferases. NSC97317 is described in this work

Methods

Experimental

Trimethylaurintricarboxylic acid (NSC97317; Fig. 2) was obtained from the NCI Drug Synthesis and Chemistry

Branch [29]. The inhibition of the enzymatic activity of DNMT1 was tested using the HotSpot[SM] platform for methyltransferase assays available at Reaction Biology Corporation [30]. HotSpot[SM] is a low volume radioisotope-based assay which uses tritium-labeled AdoMet (^{3}H-SAM) as a methyl donor. NSC97317 diluted in DMSO was added by using acoustic technology (Echo550, Labcyte) into enzyme/substrate mixture in nano-liter range. The reaction was initiated by the addition of ^{3}H-SAM, and incubated at 30°C. Total final methylations on the substrate (Poly(dI-dC)) were detected by a filter binding approach. Data analysis was performed using Graphed Prism software (La Jolla, CA) for curve fits. Reactions were carried out at 1 μM of S-adenosyl-L-methionine (SAM). S-adenosyl-L-homocysteine (SAH) is used as standard positive control in these assays. NSC97317 was tested in 10-dose IC_{50} (effective concentration to inhibit DNMT1 activity by 50%) with three-fold serial dilution starting at 100 μM.

Computational

Homology model

We employed a homology model of the catalytic domain of human DNMT1 recently developed by our group; the homology model has been used to rationalize, at the molecular level, the binding mode of established DNMT1 inhibitors [31]. Despite the fact that a recent crystallo-graphic structure for human DNMT1 has been recently published (*vide infra*), the structure corresponds to the enzyme bound to *unmethylated DNA* and this structure it is not suitable to model small-molecule inhibitors of DNMT1. This is because in the crystallographic structure the catalytic loop has an open conformation and the catalytic cysteine is far from the binding site (more than 9 Å) [32]. Therefore, the geometry of the catalytic site does not represent the catalytic mechanism of DNA methylation. Briefly, to build the homology model, the catalytic domain of the human DNMT1 was taken from the UniProt (UniProt ID: P26358) [33]. The DNMT1 sequence was aligned based on the sequence of DNA methyltransferases M.HhaI (PDB ID: 6MHT), M.HaeIII (PDB ID: 1DCT) and DNMT2 (PDB ID: 1G55) and built based on the template 3D structures using Prime (PRIME, version 2.2, Schrödinger, LLC, New York, NY, 2010). The co-factor was included in this model and the DNA double helix was constructed from the structure of M.HhaI. The variable small loops and gaps were filled by knowledge-based, homology or ab initio approach of ORCHESTRAR, and then missing long loop was modeled using Loop Search module implemented in Sybyl 8.0. The loops showing the highest homology and the lowest root mean square deviations were selected. The side chains and hydrogen atoms were added and the

stability of the homology model was validated by checking the geometry using PROCHECK. The homology model coordinates were then energy minimized with Macromodel (MACROMODEL, version 9.8, Schrödinger, LLC, New York, NY, 2010) using MMFF94s force field in a water environment (until converging at a termination gradient of 0.05 kJ mol^{-1}-Å) and the H-bonds were fixed using the SHAKE algorithm during molecular dynamics.
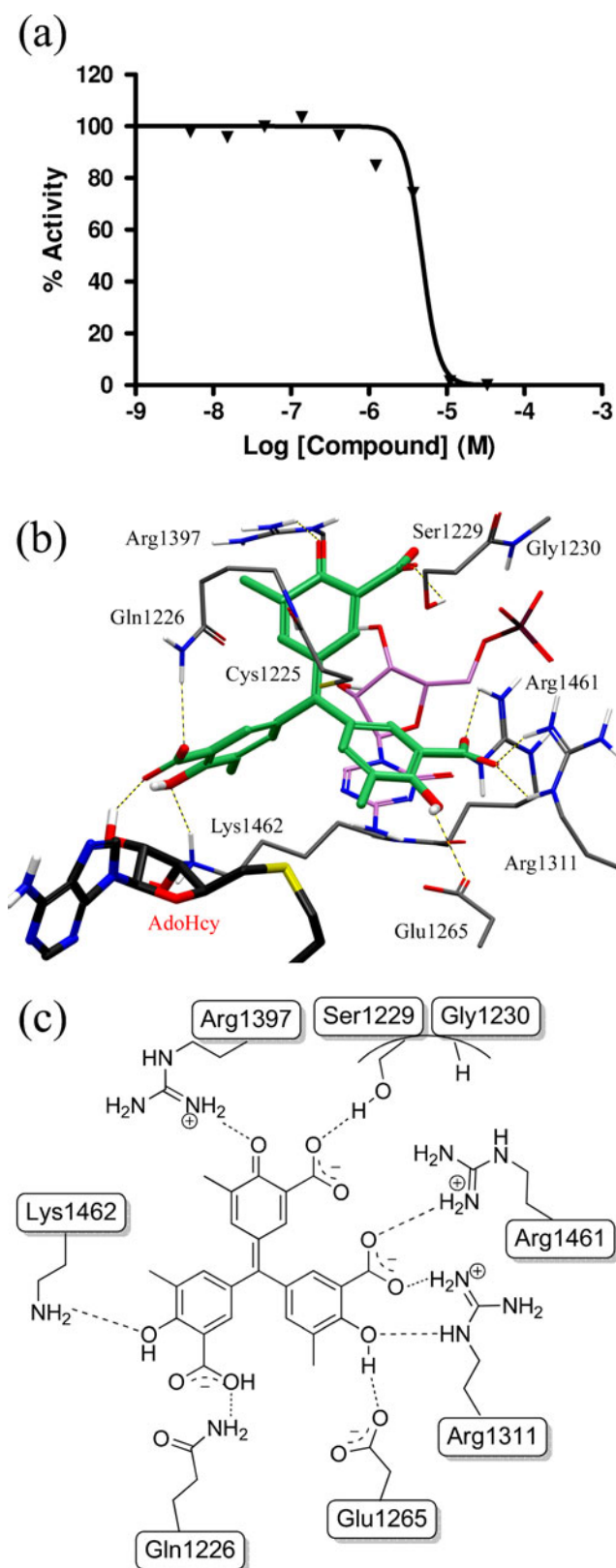
Molecular docking

The starting conformation of NSC97317 was obtained by the conformational search in MacroModel and possible tautomers were explored using LigPrep (LigPrep, version 2.4, Schrödinger, LLC, New York, NY, 2010). The conformational analysis was carried out with Monte Carlo Multiple Minimum and Low-Mode conformational search method, employing the OPLS force field using GB/SA water solvation model. The lowest energy conformation of NSC97317 was docked into the catalytic site of the DNMT1 homology model using Glide extra precision (XP) (GLIDE, version 5.6, Schrödinger, LLC, New York, NY, 2010). We also performed flexible docking of other low-energy conformers of NSC97317 generated during the conformational analysis. Other known DNMT1 inhibitors were used as a reference (*vide infra*). To generate the grids for docking, the complexed DNA structure was removed from the homology model of human DNMT1, excluding the target cytosine. The shape and properties of the catalytic site were mapped onto the grids with default dimensions centered on the target cytosine. The best docking poses were selected and compared to the structure-based pharma-cophore we recently reported for inhibitors of DNMT1 [31]. The same docking protocol was used recently to develop binding models for other DNMT inhibitors [31].

Results and discussion

In order to experimentally test the hypothesis that NSC97317 is an inhibitor of DNMT1, the enzymatic inhibitory activity of this compound was measured using the HotSpot[SM] platform for methyltransferase assay described in Methods. NSC97317 showed a low micromolar inhibition of DNMT1 with IC_{50} value of 4.79 μM. The corresponding dose-response plot is shown in Fig. 3a. To note, the enzymatic inhibitory activity of NSC97317 was better than the activity previously reported for 5,5-methylenedisalicylic acid (IC_{50}=92 μM) [25].

In order to rationalize the putative binding mode of NSC97317 with DNMT1 at the molecular level, we conducted molecular docking simulations. Despite the fact that a recent crystallographic structure for human DNMT1

(vide supra). Therefore, we employed a homology model we recently developed and used to model several established DNMT1 inhibitors (cf. Methods). The geometry of the catalytic site, including the position of the target cytosine modeled in the complex, is in agreement with the catalytic mechanism of DNA methylation. Further details are provided elsewhere [31]. Of note, homology models of human DNMT1 have been used previously to identify novel inhibitors [22] and study the mechanism of action of small-molecule inhibitors [31].
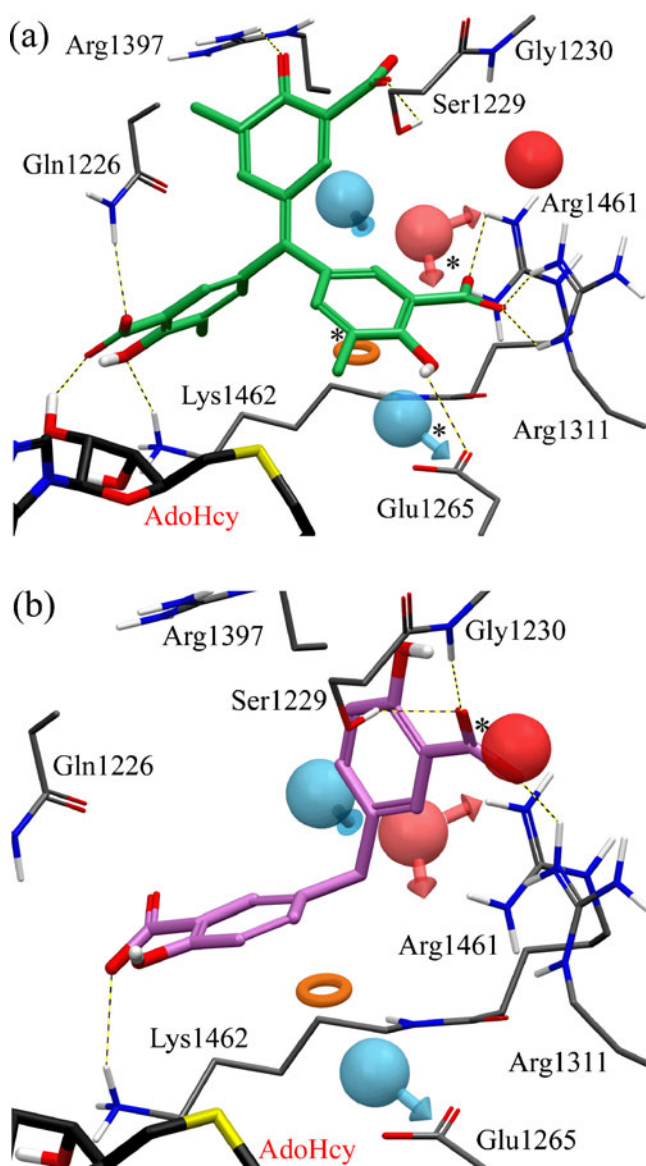
Flexible docking studies of NSC97317 with DNMT1 were performed with Glide XP following the protocol described in Methods. The efficacy of the docking protocol was recently demonstrated in a recent published docking and structure-based pharmacophore study of several known DNMT1 inhibitors [31]. Figure 3 shows three- and two-dimensional (3D and 2D) representations of the optimized docked model of NSC97317 with DNMT1. Selected residues are shown. Of note, the deprotonated form of NSC97317 (expected in aqueous solution) is shown in the binding models. The best docking conformation of NSC97317 was generated by flexible docking. According to this binding model, NSC97317 makes hydrogen bond interactions with Ser1229 and Arg1397. The phosphate group of nucleoside analogues as well as the carboxylate anion of 5,5-methylenedisalicylic acid and RG108 also make hydrogen bonds with Ser1229 [31]. One of the salicylic acid moieties of NSC97317 forms a hydrogen bond network with key residues Glu1265, Arg1311, and Arg1461 (Fig. 3b). It is worth pointing out that Glu1265 and Arg1311 seem to play a crucial role in catalytic mechanism (Fig. 1). Furthermore, hydrogen bonds with Glu1265, Arg1311, and Arg1461 have also been predicted for other DNMT1 inhibitors and could be pharmacophoric. The second methylbenzoic acid moiety of NSC97317 makes hydrogen bonds with Gln1226, Lys1462, and 2′-OH of the co-factor (Fig. 3b). A similar hydrogen bond interaction with the co-factor has been reported for aurintricarboxylic acid, which is a very similar compound to NSC97317, with other methyltransferase. These hydrogen bond interactions are in agreement with the interactions found in the docking models of nucleoside analogues which are the most potent DNMT1 inhibitors. A comparison of the optimized binding mode of

has been recently published [34], the structure corresponds to the enzyme bound to *unmethylated DNA* and it is *not suitable to model small-molecule inhibitors of DNMT1*
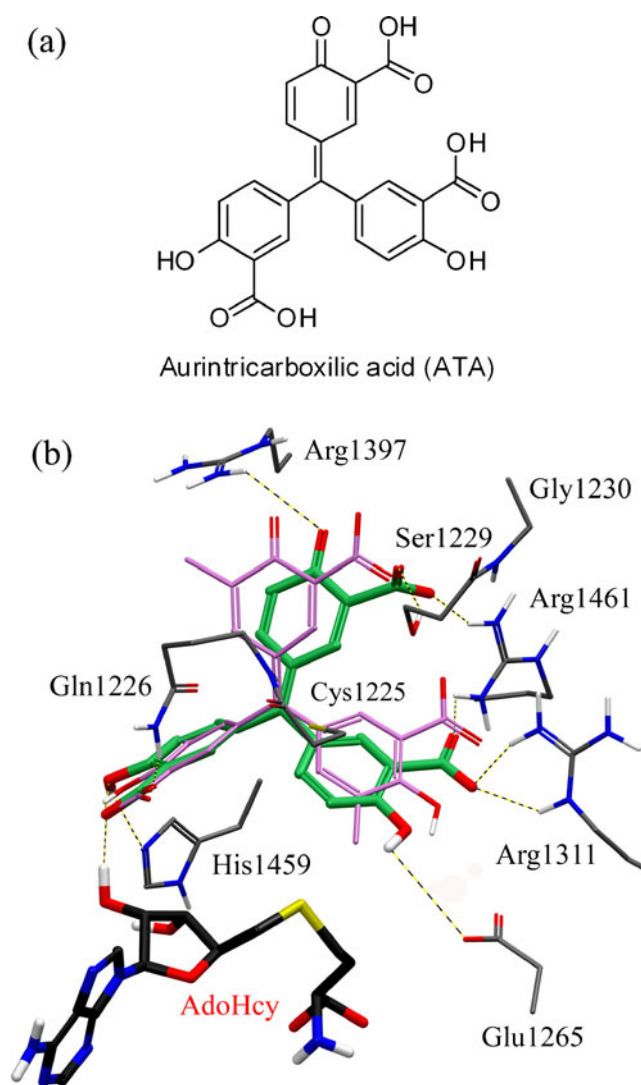
NSC97317 with 5,5-methylenedisalicylic acid revealed that both compounds adopt a different docking position in the binding pocket (*vide infra*). Of note, the docking score of NSC97317 with DNMT1 (-9.45 kcal mol^{-1}) was better than the docking score of 5,5-methylenedisalicylic acid (-7.73 kcal mol^{-1}) and similar with that of nucleoside analogues (data not shown).

Figure 4 shows the comparison of the optimized binding mode of NSC97317 and 5,5′-methylenedisalicylic acid with

a recently developed pharmacophore hypothesis for known DNMT inhibitors [31]. The pharmacophore model includes five-point features with one negative charge (N), one hydrogen bond acceptor (A), one aromatic ring (R) and two hydrogen bond donors (D). Considering a distance matching tolerance of 2.0 Å, a criteria commonly accepted in pharmacophore modeling [31, 35, 36], only one carboxylic acid group of 5,5′-methylenedisalicylic acid matches with the negative charged feature (Fig. 4b). In contrast, the binding position of NSC97317 matches (considering a distance matching tolerance of 2.0 Å) with the aromatic ring (R), hydrogen bond donor (D) (interaction with Glu1265) and hydrogen bond acceptor (A) (interaction

Fig. 4 Comparison of the binding modes with pharmacophore hypothesis for (a) NSC97317 and (b) 5,5-methylenedisalicylic acid (deprotonated forms). Hydrogen bonds are depicted with dashes. Non-polar hydrogen atoms are omitted for clarity. Selected residues are displayed for reference. *Red sphere:* negative ionizable; *pink sphere with vectors:* hydrogen bond acceptor; *blue sphere with vector:* hydrogen bond donors; and *orange ring:* aromatic ring. Matching features, considering a distance matching tolerance of 2.0 Å, are marked with asterisks

Fig. 5 (a) Chemical structure of aurintricarboxilic acid (ATA). (b) Comparison of the binding model of ATA (carbon atoms in green) and NSC97317 (carbon atoms in pink) with the catalytic domain of human DNMT1. Deprotonated forms of ATA and NSC97317 are shown in the binding models. Selected residues in the binding pocket are shown. Hydrogen bonds are depicted with dashes. Non-polar hydrogen atoms are omitted for clarity

with Arg1311 and Arg1461) of the pharmacophore hypothesis (Fig. 4a). These results further supported the experimental observation that NSC97317 is a better DNMT1 inhibitor than 5,5′-methylenedisalicylic acid.

It seems likely that aurintricarboxylic acid will also inhibit DNMT1 [27]. Preliminary docking studies showed that aurintricarboxylic acid (Fig. 5a) has a very similar binding mode than NSC97317 making almost the same interactions with the catalytic site. Figure 5b shows the predicted binding mode of aurintricarboxylic acid with the catalytic domain of DNMT1. The binding model of the trimethyl analog is shown for comparison. Of note, the deprotonated forms of both ligands (expected in aqueous solution), are shown in the 3D binding model. In addition, the calculated binding score for aurintricarboxylic acid (-8.77 kcal mol^{-1}) is also better than the binding score for 5,5′-methylenedisalicylic acid (-7.62 kcal mol^{-1}). It remains to be determined the experimental enzymatic DNMT1 inhibition of aurintricarboxylic acid.

Collectively, these results support that NSC97317 impair the correct location of DNA in the catalytic site by steric hindrance of this site and through the interaction with residues involved in DNA-DNMT1 recognition. As such, NSC97317, *freely available* at the NCI/DTP, is a valuable biochemical tool to study DNMT1 inhibition in cancer and other diseases related to DNA methylation.

Conclusions and perspectives

We report enzyme inhibition and molecular modeling studies of trimethylaurintricarboxylic acid (NSC97317) with the catalytic site of human DNMT1. NSC97317 is a low micromolar inhibitor of human DNMT1. Enzymatic and molecular modeling studies confirmed our hypothesis that this compound is a more potent inhibitor than 5,5-methylenedisalicylic acid, a structurally related compound. Molecular docking studies with the catalytic domain of DNMT1 indicate that NSC97317 makes hydrogen bond interactions with amino acid residues involved in the mechanism of DNA methylation. The polyanionic structure of NSC97317 and the electropositive binding region of the catalytic site of DNMT1, due to several arginine residues, also seem to play a key role in the binding recognition. The key interactions of the analogue of aurintricarboxylic acid with the binding pocket of DNMT1 can be exploited in the structure-based discovery and optimization of DNMT1 inhibitors. NSC97317 is a promising research tool to study enzymatic inhibition of DNMT1 in the research of cancer and other diseases related to DNA methylation. Follow up studies include the evaluation of NSC97317

as a demethylating agent in cell based assays. Additional work planned is to determine the potential biochemical activity of NSC97317, aurintricarboxylic acid, and other analogues toward different isoforms of DNMT including DNMT3A and DNMT3B.

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