Discovery of Two Novel, Small-Molecule Inhibitors of DNA Methylation

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DNA methyltransferases are promising targets for cancer therapy. In many cancer cells promoters of tumor suppressor genes are hypermethylated, which results in gene inactivation. It has been shown that DNA methyltransferase inhibitors can suppress tumor growth and have significant therapeutic value. However, the established inhibitors are limited in their application due to their substantial cytotoxicity. To discover novel compounds for the inhibition of human DNA methyltransferases, we have screened a set of small molecules available from the NCI database. Using a 3-dimensional model of the human DNA methyltransferase 1 and a modified docking and scoring procedure, we have identified a small list of molecules with high affinities for the active site of the enzyme. The two highest scoring structures were found to inhibit DNA methyltransferase activity in vitro and in vivo. The newly discovered inhibitors validate our screening procedure and also provide a useful basis for further rational drug development.

Introduction

DNA methylation plays an essential role in mammalian cell regulation. Alterations in DNA methylation patterns can promote tumorigenesis and predispose genes to mutational events.[1] It has been shown that promoter hypermethylation is associated with gene inactivation and that tumor suppressor genes can be epigenetically silenced in many cancer types.[1,2] Unlike mutagenic events, however, epigenetic mutations can be reverted. Demethylation of aberrantly silenced genes can restore gene expression and function, an effect which has been observed via demethylation of specific genes[3] and global genomic demethylation.[4]

In humans, methylation takes place at cytosines located 5′ to guanine (CpG). These CpG dinucleotides are unevenly distributed within the genome, and are primarily clustered in CpG islands that have important regulatory functions for epigenetic control of gene expression.[5] DNA methylation patterns are established and maintained by several methyltransferase enzymes. During mammalian embryonic development, methylation patterns are established by de novo methyltransferases DNMT3A and DNMT3B,[6] and in differentiated cells, methylation patterns are maintained by DNMT1. DNMT1 is closely associated with the DNA replication machinery and presumably copies methylation patterns from the parental strand to the daughter strand.[7] It has been shown that reduction of DNMT1 activity causes a significant decrease in the global methylation level of mice[8] and that DNMT1 is necessary to maintain aberrant CpG island methylation in human cancer cells.[9] For this reason, DNMT1 has become an attractive target for drug development and experimental cancer therapy.

The most widely known methylation inhibitors are cytidine analogues: 5-azacytidine and its derivative 5-aza-2′-deoxycytidine. Both compounds have been used in the majority of methylation inhibition experiments and also in a large number of clinical trials.[10] Cells incorporate azanucleotides into DNA during replication and thereby substitute genomic cytosine with 5-azacytosine. However, the modified pyrimidine ring of azanucleotides blocks the completion of the methyl group transfer reaction, and the DNA methyltransferase remains bound to DNA.[11,12] These covalent protein−DNA adducts are believed to be responsible for the toxicity of azanucleosides on mammalian cells[12,13] and represent a major drawback for their clinical application.

In this paper, we identify small-molecule compounds that can block the active site of DNA methyltransferases. Using a previously established three-dimensional model of the catalytic domain of the human DNMT1 enzyme,[14] we screened a database of compounds for chemicals with high affinity to the DNMT1 catalytic pocket. Two of the best scoring compounds identified in our screen, NSC303530 and NSC401077, were chosen as candidate inhibitors and tested experimentally. Both compounds were able to inhibit DNA methyltransferases in vitro and in vivo, presumably via blockage of the enzyme's active site. Our results suggest that these novel compounds are potent inhibitors with significant potential for further drug development.

Results and Discussion

Protein structures derived from computational approaches represent valuable tools for structure-based research. However, the application potential of such models is usually limited by the sequence similarities between the template structures and the target protein.[36] As a general rule, homology models based on more than 25% of sequence similarity to experimentally resolved structures can be used to address basic structural questions and to plan mutagenesis experiments. Models based on more than 50% of sequence similarity are usually very accurate and can be used in drug discovery processes, including small molecule screens. Indeed, many recent approaches to rational drug design have used homology models as valuable research tools for identification, validation, and optimization of compounds.[37] The homology model used in this study is based on very high conservation of catalytic motifs between individual DNA methyltransferases and an extensive (>50%) sequence conservation of the amino acids constituting the active site cleft. In addition, the atomic coordinates of the model have been experimentally validated in previous experiments.[14]

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To identify small molecules with favorable binding characteristics to an established three-dimensional model of the human DNMT1 catalytic domain,[14] we searched a database of compounds (the Diversity Set) available from the National Cancer Institute. The Diversity Set consists of 1990 compounds that are representative of the chemical diversity of more than 140 000 chemicals. In addition, all of the compounds are easily obtainable for laboratory testing. To refine the database for docking calculations, we have eliminated compounds not suitable for the DNMT1 active site considering their size, hydrophobicity, and uncommon atom types.

We created a screening procedure for the analysis of a large database of compounds that was designed with a sufficient degree of flexibility to allow for structurally diverse molecules to fit into the binding position of the DNMT1 active site. The screening procedure was also designed to be capable of recreating the conformation of known substrates and rank their interaction with the protein on a high to low affinity scoring list. To establish our screening procedure, we compiled a calibration set, which consisted of 10 selected molecules (see Materials and Methods for details). We divided the calibration set into three subsets: "positive", "neutral", and "negative", representing the different binding affinities to the DNMT1 active site. Molecules from the first two subsets could be manually docked into the binding pocket and were distinguished by a possible binding conformation (negative van der Waals values). However, stabilizing electrostatic interactions were obtained only with three positive substrates that ranked higher than the neutral compounds. The two "negative" compounds were too large to be placed inside the binding pocket. These "negative" compounds were used as a low threshold for the screening procedure. All compounds below this threshold were discarded.

By using the calibration set we were able to overcome one of the main drawbacks of a screening methodology based on a fast but not fully flexible docking algorithm. In a rigid protein docking algorithm,[15] inflexible side chains can prevent the establishment of certain conformations due to steric hindrance. The DOCK5 program with default settings was able to predict the correct conformations for DNMT1 substrates (cytidine and its derivatives) but had problems with the remaining calibration set structures, docking them outside of the active site. The docking results were strongly influenced by the conformation of the catalytic cysteine C1226, which favored cytosine and its analogues. This was an inherent characteristic of our model,[14] which is based on experimental structures with catalytic cystein positioned very close to cytosine-like substrate. Because DOCK5 uses a rigid receptor-docking algorithm, the side chain torsion angle of C1226 was manually corrected to a more "open" conformation. The modified conformation is very similar to the conformation of the catalytic cysteine in the crystal structure of the HhaI methyltransferase (6MHT) and is also in agreement with the Ramachandran plot. We also tested various high values of the bump parameter, allowing more overlaps between the ligand and the cleft residues. By increasing the number of allowable surface overlaps and disabling the internal DOCK minimizer options (minimize_final_pose_rigid and minimize_final_pose_torsion set to "off"), we allowed the program to dock and establish conformations for these molecules inside the binding pocket. It also enabled the docking program to sample the active site space more extensively and find more potential starting conformations.

The calibration set was added to the main database for reference purposes, resulting in 1558 structures in the final filtered database. These structures were docked into the DNMT1 active site by the DOCK v. 5.1.0 program, with optimized parameters (i.e. allowing more overlapping surfaces and with internal minimizer disabled). Every docked ligand was saved in various conformations in the active site. These conformations were clustered and scored, and after a quick minimization step, the most probable binding mode was selected. To converge to an energy minimum, the best binding mode was subjected to a full minimization procedure, which allowed for active site and ligand flexibility. Finally, consensus scoring and force field energy values, along with ligand placement and active site distortion (rms deviation), were taken into account to distinguish the best binding compounds. We found that the best results were obtained by using energy values from the minimization procedure (vdw and electrostatics values) as the primary score and consensus scoring as additional information. This methodology resulted in the identification of several hits with high affinity to the DNMT1 active site that were capable of binding in a similar position as the native substrate, cytosine. Two structures, NSC303530 and NSC401077 (Figure 2, Table 1) scored significantly higher than the remaining hits and were therefore chosen as potential inhibitor candidates.

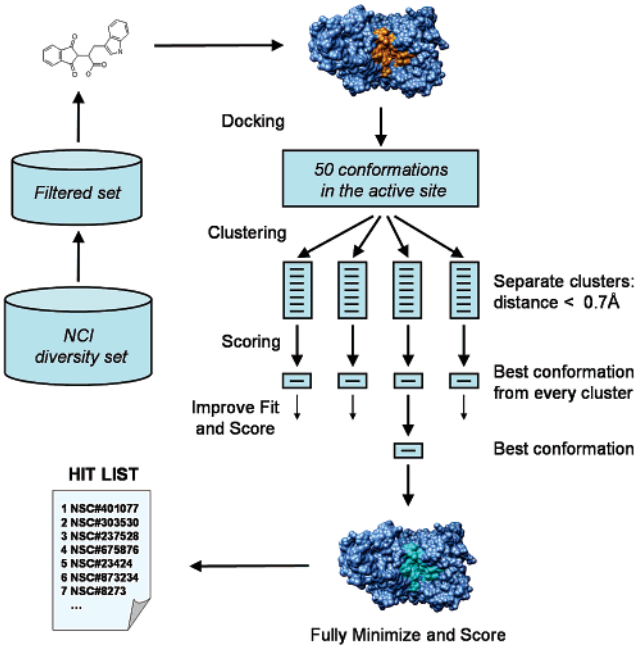

Figure 1. Schematic representation of the docking and scoring procedure. Filtering removes molecules not suitable for docking from the NCI Diversity Set. Remaining ligands are docked into the DNMT1 active site and a multistep fitting and scoring procedure is used to determine the most likely conformation. After determining the best conformation, the whole protein−ligand complex is minimized and consensus scoring is applied. The resulting "hit list" ranks ligands according to scoring functions and energy values.

To experimentally validate in silico predictions, we obtained test samples for NSC303530 and NSC401077 and assayed them for their ability to inhibit purified recombinant CpG methylase M.SssI in a cell-free in vitro assay. Increasing concentrations resulted in a detectable decrease in DNA methyltransferase activity, as visualized by the disappearance of a methylated restriction fragment in this assay (Figure 3A). These results provided experimental confirmation of inhibitory activity and suggested IC_{50} values of 400 and 600 nM, respectively, after normalization to 1 nM enzyme concentration (Figure 3A). Last, we also analyzed the effect of NSC303530 and NSC401077 on the genomic DNA methylation level of a human tumor cell line. We incubated NALM-6 leukemia cells for 72 h with 0, 1, 10,

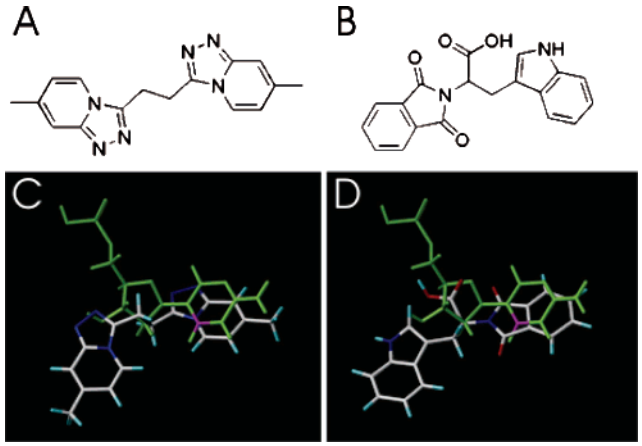

Figure 2. Identification of two novel candidate DNA methyltransferase inhibitors by computational screening. (A, B) Chemical structures of NSC303530 and NSC401077. (C, D) Superimposed conformations of cytidine (green) and NSC303530 (C) or NSC401077 (D) docked into the active site of DNMT1. The covalent bond between cytosine and the catalytic cysteine of DNMT1 is formed at the cytosine-6 position (magenta).

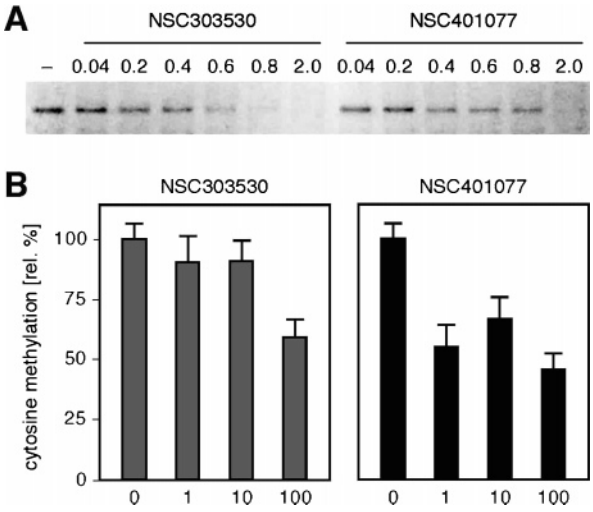

Figure 3. NSC303530 and NSC401077 inhibit DNA methyltransferase activity in vitro and in vivo. (A) In vitro methylation assay. A 798 bp DNA fragment was incubated in the presence of M.SssI methylase with increasing concentrations of inhibitor, as indicated. Subsequent digestion of the DNA fragment with the methylation-sensitive restriction enzyme *Bst*U I is indicative of decreasing DNA methyltransferase activity. (B) Cellular methylation assay. NALM-6 leukemia cells were incubated with the compounds dissolved in the culture medium, concentrations (in μM) are indicated below the bars. Genomic cytosine methylation levels were determined by capillary electrophoretic analysis of isolated genomic DNA. All measurements were performed at least in triplicate, and standard deviations are indicated by error bars. Inhibitor concentrations are indicated (in μM).

or 100 μM of NSC303530 and NSC401077 and quantitatively determined genomic cytosine methylation levels by capillary electrophoresis. This revealed a significant reduction in DNA methylation after incubation with 100 μM NSC303530, while NSC401077 caused reduced levels of DNA methylation at all test concentrations (Figure 3B). The lower methylation level of cells treated with 1 μM NSC401077 as compared to 10 μM test substance concentration (Figure 3B) is presumably due to experimental variations. On the basis of these results, we estimated the cellular IC_{50} value for DNA methylation in the range of 100−200 μM for NSC303530 and 50−100 μM for NSC401077. Together, these experiments strongly suggest that NSC303530 and NSC401077 are able to inhibit DNA methyltransferases, in vitro and in human tumor cells.

The chemical structures of NSC303530 and NSC401077 (Figure 2A,B) are similar to each other. Both compounds contain two indene-based heterocycles linked by a three-carbon chain. Furthermore, their pharmacophore alignment shows high similarities in the arrangement of hydrogen-bond donors and acceptors and hydrophobic features (Figure 4). These similarities would suggest analogous binding positions. Indeed, our calculations show that both compounds bind in a similar manner deep inside the active site cleft (Figure 5), occupying the same part of the binding pocket as the native cytosine (Figure 2C,D). A comparison of functional group placement between these new compounds and cytosine clearly shows that NSC303530 and NSC401077 do not have a ring structure similar to cytosine. Thus, they are highly unlikely to function as mechanism-dependent inhibitors. Although NSC303530 has a ring with double bonded atoms at the 5 and 6 positions that are placed in the cleft similarly to the cytosine ring (Figure 2C), there is no nitrogen atom at the C5 position that could block the methyl transfer reaction. The situation is even more obvious for NSC401077: the compound has a carbonyl group in the region of the cytosine-6 position (Figure 2D), which would prevent the formation of a covalent bond between the ligand and the SH group of the catalytic cysteine. This suggests that both compounds are mechanism-independent inhibitors.

Table 1. Binding Energies and Score Calculations for the Calibration Set and the Two Highest-Ranked Hits[a]

compound	*E*(vdw)	*E*(elec)	*E*(total)	G_score	D_score	F_score	PMF	Chem Score	cons. score
cytidine	−20.1	−27.0	−48.1	−156.8	−79.0	−17.4	−16.6	−1.8	2
5-azacytidine	−20.4	−26.6	−47.0	−153.3	−74.7	−17.0	−11.7	−0.5	2
zebularine	−16.7	−25.5	−42.2	−112.0	−72.6	−9.9	1.1	−0.8	0
adenosine	−14.7	−16.8	−31.5	−186.6	−96.7	−8.9	−12.8	−3.2	3
guanosine	−10.0	−15.6	−25.6	−85.6	−47.8	−3.2	−33.3	−4.0	1
5-methyluridine	−17.0	−10.4	−27.4	−121.6	−86.8	3.0	−0.9	−3.9	2
NSC4092	−23.9	−1.1	−25.0	−192.6	−105.4	−9.8	32.4	−12.9	3
NSC21970	−13.2	−1.1	−14.4	−135.0	−62.7	−9.6	−0.6	−11.8	2
NSC57278	−16.8	0.2	−16.6	−154.3	−88.2	−4.1	22.7	−10.0	3
NSC19555*	−11.4	−4.7	−16.1	−80.6	−50.4	−1.9	−7.0	−7.9	2
NSC27292*	−7.2	−0.9	−8.1	−163.4	−82.5	−3.4	17.2	−12.0	3
NSC303530	−28.2	−35.1	−63.3	−181.6	−57.7	−8.4	−0.5	−10.0	3
NSC401077	−34.4	−37.6	−71.9	−188.6	−79.7	−13.4	−0.6	−11.1	4

[a] Columns 2−4 show the calculated binding energies (kcal/mol) after a full minimization procedure. Total energy, *E*(total), is the sum of van der Waals interactions, *E*(vdw), and electrostatic interactions, *E*(elec). NSC401077 and NSC303530 both have a lower *E*(total) value than 5-azacytidine, which suggests a strong interaction between those compounds and the DNMT1 active site. Columns 5−10 show scores from several scoring functions. The consensus score also ranks the two compounds higher than the original inhibitor. Molecules marked with an asterisk are not docked inside the active site pocket because of steric hindrance. Molecular structures of the compounds listed in the table can be found at http://129.43,27.140/ncidb2.

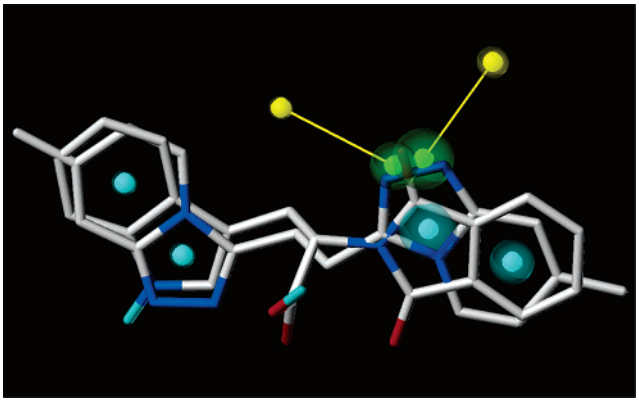

Figure 4. Pharmacophore alignment of NSC303530 and NSC401077 by GASP.[16] Both compounds consist of two indene-based heterocycles linked by a three-carbon chain. Spheres show similarities in feature positions: lipophilic (cyan) and hydrogen-bond acceptors (green) and donors (yellow). The smaller the sphere radius, the more similar the feature position is in both compounds.

Computationally expensive methods involving flexible active site side chains are widely considered to be superior for the identification of binding molecules. However, their application for screening large sets of molecules is limited by computational time consumption. Our results show that a rigid docking algorithm with an increased number of bumps and a multistep minimization procedure can be similarly effective in scanning small molecules for potential interactions with a protein. It was possible to sample the active site space efficiently and more independently from the rigid conformation of the side chains by increasing the allowed number of overlaps between the protein and ligand, thus generating a number of different starting conformations. The methodology described in this paper decreases the influence of side chains conformations, which also reduces the problems associated with conformational data derived from crystallographic, NMR, and homology modeling. Our procedure allowed us to establish ligand conformations that would normally be rejected by the rigid docking algorithm due to steric hindrance. In addition, clustering similar ligand conformations decreased the computation time and allowed fully flexible minimization calculations on a protein−ligand complex.

Both compounds identified in this study are novel structures that can provide new insights into the DNA methylation process. NSC401077, in particular, seems to be a good candidate for further analysis and development. The structure of the compound (phthaloyltryptophan) suggests several possibilities for a straightforward synthesis and derivatization. In addition, our data showed a substantial demethylating effect in human tumor cells. This demethylation was recently shown to not affect cellular viability, which further underscores the usefulness of the compound.[33] Furthermore, NSC401077 has good drug-likeness values, including a log *P* value of 2.95, which indicates easy access through the cell barriers. Also, the number of hydrogen bond donors (two) and acceptors (five) and the molecular weight of 334.39 g/mol satisfy Lipinski's "rule of five".[38]

Conclusions

The present study is the first successful example for a rational design of DNA methyltransferase inhibitors. It was accomplished by virtual screening for inhibitors of DNMT1 based on a previously established homology model and a novel docking and scoring methodology. This screen resulted in the discovery of two small molecules that were experimentally confirmed as DNA methyltransferase inhibitors. The experimental data obtained provides a strong indication for the usefulness of our docking and scoring approach. In addition, a recent in-depth analysis of NSC401077[33] has revealed fundamentally novel characteristics of this compound and indicated a substantial developmental potential as an antitumor drug.

Materials and Methods

Receptor Preparation. We have used a model of human DNMT1[14] enzyme to define the area where docking should take

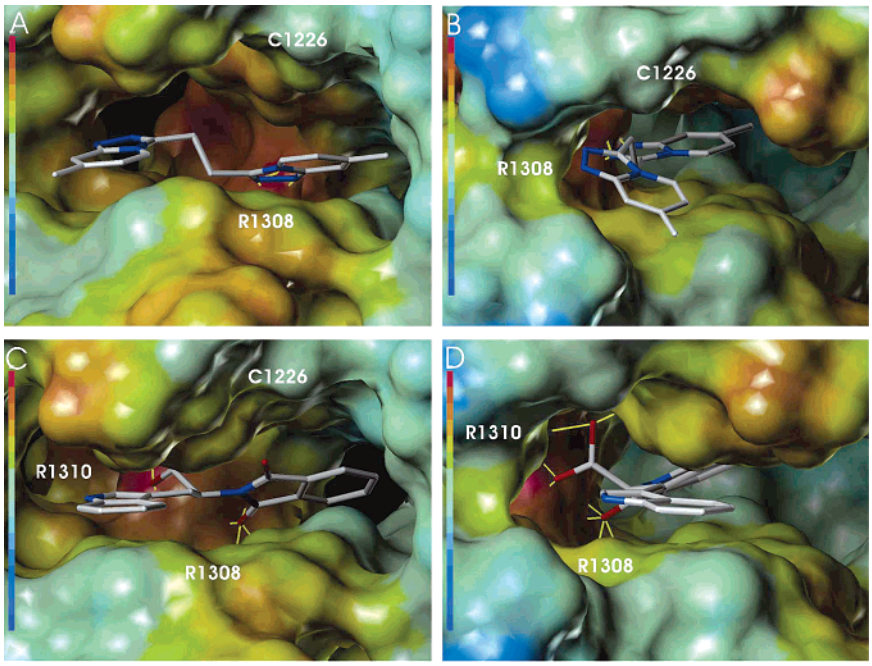

Figure 5. Predicted placement of NSC303530 (A, B) and NSC401077 (C, D) in the active site of DNMT1. Both compounds are docked deep inside the catalytic pocket of DNMT1. For visibility reasons, some of the amino acid residues are not shown. The active site surface is colored according to the electrostatic potential (highest, red; lowest, blue). The red surface inside the cleft is formed by two main residues interacting with the compounds, arginines R1310 and R1308. The surface of the catalytic cysteine C1226 is also shown. Yellow lines indicate predicted hydrogen bonds.

place. The side chain conformation of the catalytic cysteine (C1226) was modified to create more space in the active site and allow for more diverse compounds to be docked. The allowable docking space, enclosed in a box of 31 Å × 16 Å × 26 Å, covered all residues 6 Å away from the center of a docked cytidine molecule and includes the entrance of the catalytic pocket and also part of the space where the Ado-Met cofactor resides. Manual refinement of subsequent steps was done to create a negative image of the DNMT1 active site necessary for the DOCK v. 5.1.0 (DOCK5) docking program.[15] Finally, Kollman charges were assigned using SYBYL,[16] and the GRID program[17] was used to create score grids for docking.

Database Preparation. The Diversity Set[18] of the National Cancer Institute database,[19] containing 1990 molecules, was retrieved and converted from SD into MOL2 format. PERL scripts were used to filter out unwanted types of structures such as compounds bigger than 51 atoms, compounds with no heteroatoms (all-hydrophobic), and molecules with "exotic" atoms (like Ti, Y, Zr, etc.), as AMBER,[20] MMFF94s,[21] and DOCK5 force field parameters for them are unavailable. Less common atom type parameters for DOCK were taken from the UFF force field.[22] SYBYL's SPL scripting language was used to check the resulting set of structures, to fix atom and bond types, to add hydrogens, and to assign Gasteiger-Hückel charges. Finally, to fix bond properties, structures were minimized with SYBYL's implementation of the Merck force field designed for small molecules (MMFF94s). The resulting new data set consisted of 1553 molecules of similar size to cytidine that potentially could fit into the DNMT1 active site, both sterically and electrostatically.

Testing the Docking and Scoring Procedure. To establish a reliable docking and scoring procedure, a calibration set of small molecules was created. The calibration set consisted of three subsets of compounds: "positive substrates" (cytidine, 5-azacytidine, zebularine), "neutral substrates" (adenosine, 5-methyluracyl, guanosine, and three all-hydrophobic compounds, NSC4092, NSC21970, and NSC57278), and "negative substrates" (compounds not fitting sterically into the active site, i.e., NSC27929 and NSC19555). The calibration set was used to test, modify, and calibrate the screening procedure so that it could generate various possible binding modes for each compound and also create a list of molecules in an order representing their binding affinity to the DNMT1 active site. All three "positive substrates" should have similar binding positions and scores placing them at the top of the scoring list. In the case of "neutral substrates" the three nucleotides should bind in a similar place as cytidine but have lower affinity scores compared to "positive substrates", as cytosine represents the natural substrate of DNMT1. The hydrophobic compounds from this set should also fit sterically into the binding pocket, but with much lower affinity scores, since the electrostatic interactions are unsatisfactory. Last, the "negative" compounds should be placed at the bottom of the scoring list, as they are too large to fit in the active site. The ligand position in the active site was compared with the original cytosine docked previously[14] using SYBYL SPL script. We determined the cytosine distance value (CDV), which represents the distance between the centroid of the docked ligand and the centroid of the original cytosine position. Ligand conformations close to the original cytosine were preferred, as we were searching for new compounds binding in a similar position as the original substrate of DNMT1. We tested a variety of docking parameters (such as various minimizer options, different numbers of ligand orientations and conformations), space sampling (number of allowable bumps), and scoring (contact and energy score) to find the optimal combination. The calibration set was then used to test various scoring methods and minimization parameters to create a fast and accurate scoring procedure.

Screening Procedure. The DOCK5 program (with the allowable_bump parameter set to 12, anchor size 5, max_orientations 1000, max_conformations 50) was used to dock selected compounds of the filtered NCI database. To efficiently sample conformational space, every ligand was docked, and its 50 best conformations were saved. By calculating the rmsd between them, we clustered the conformations, distinguishing a set of unique, possible binding modes for every ligand. If the rmsd value was <0.7 Å, two docking positions were clustered. By calculating the cytosine distance value (CDV), we were able to discard clusters of ligands that had been docked outside the active site cleft (CDV > 4 Å). Finally, the top-scoring compound (DOCK5 scoring function) from every cluster was subjected to a quick ligand minimization procedure (100 steps, rigid active site, AMBER force field, calculated with SYBYL) to eliminate most of the energetically unfavorable surface overlaps introduced by the docking program. To choose the most promising ligand binding mode, we used energy values (vdw interactions and electrostatics) as calculated by the SYBYL "dock" module, with regard to the CDV calculated again after the quick minimization. After choosing the optimum binding mode for every compound, we used a full minimization procedure that allowed flexibility of the whole ligand−protein complex to let the system converge into an energy minimum (with AMBER force field, until convergence or 2000 steps, SYBYL) (Figure 1). Subsequently, consensus scoring methodology available from SYBYL was applied; the scoring functions used were G_Score,[28] D_Score,[29] F_Score,[30] PMV,[31] and ChemScore.[32] Twenty of the best compounds from the consensus scoring and, additionally, 10 best scoring compounds from every function alone and from force field parameters computed by SYBYL's "dock" module were chosen. These compounds were closely inspected; their conformation in the active site was manually checked and refined. The level of distortions introduced to the active site structure by a docked ligand was also taken into consideration. The distortion was measured by the rms value of the original DNMT1 active site structure before and after a full minimization with the ligand.

Determination of DNA Methyltransferase Inhibitor Activity. NSC303530 and NSC401077 were obtained from the Drug Synthesis & Chemistry Branch, Developmental Therapeutics Program, Division of Cancer Treatment and Diagnosis, National Cancer Institute. Test samples were dissolved in PBS and their identities were confirmed by ^{1}H and ^{13}C NMR. The in vitro methylation assay was performed as described previously.[33] The methylation reaction contained 500 ng of substrate DNA in reaction buffer (50 mM NaCl, 10 mM Tris-HCl, 10 mM $MgCl_2$, 1 mM dithiothreitol, pH 7.9), 80 μM *S*-adenosylmethionine, and 2 U of M.SssI methylase (0.25 μM, New England Biolabs, Frankfurt, Germany) in a final volume of 50 μL. Reactions were performed at 37 °C for 3 h. After completion, the reaction was inactivated at 65 °C for 15 min, and DNA was purified using the QIAquick PCR Purification Kit (Qiagen, Hilden, Germany). Purified DNA was digested for 3 h at 60 °C with 25 U of BstUI (New England Biolabs, Frankfurt, Germany) and analyzed on 3% TBE agarose gels. The in vivo methylation assay was performed by incubating NALM-6 cells[34] in inhibitor-supplemented RPMI 1640 medium under standard cell culture conditions. After 72 h, cells were harvested for further analysis. Genomic DNA was isolated with the Qiagen DNeasy kit and subsequently analyzed by capillary electrophoresis, as described previously.[35]

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