Identification of DNMT1 Selective Antagonists Using a Novel Scintillation Proximity Assay*§

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Background: DNA methylation contributes to the heritable regulation of gene expression.

Results: Chemical inhibitors of DNA methyltransferase (DNMT) enzymes were identified.

Conclusion: Several inhibitors nonspecifically inhibited multiple methyltransferases through generation of H2O2, but one compound showed selective inhibition of DNMT1 independent of the production of H2O2.

Significance: Selective DNMT1 inhibitors will allow for more precise elucidation of the role of this enzyme in gene regulation.

A novel scintillation proximity high throughput assay (SPA) to identify inhibitors of DNA methyltransferases was developed and used to screen over 180,000 compounds. The majority of the validated hits shared a quinone core and several were found to generate the reactive oxygen species, H2O2. Inhibition of the production of H2O2 by the addition of catalase blocked the ability of this group of compounds to inhibit DNA methyltransferase (DNMT) activity. However, a related compound, SW155246, was identified that existed in an already reduced form of the quinone. This compound did not generate H2O2, and catalase did not block its ability to inhibit DNA methyltransferase. SW155246 showed a 30-fold preference for inhibition of human DNMT1 versus human or murine DNMT3A or -3B, inhibited global methylation in HeLa cells, and reactivated expression of the tumor suppressor gene RASSF1A in A549 cells. To our knowledge, this work represents the first description of selective chemical inhibitors of the DNMT1 enzyme.

Heritable regulation of gene expression in mammals can be encoded directly within DNA sequences (genetic) or carried in modifications to the DNA or the nucleosomal histones used to package the DNA in the chromatin (epigenetic). The most well-characterized DNA modification is the addition of a methyl group to the C5 position of cytosine, typically within the context of a CpG sequence (reviewed in Ref. 1). In normal mammalian cells, the majority of CpGs are methylated except CpG islands. Because of the increased frequency by which methylcytosines mutate to TpG, CpGs are under-represented in the whole genome (2). However, regions of unmethylated CpGs, defined by a minimum observed/expected CpG ratio of 0.65 and a GC content >55% over a distance of 500 bp, are known as CpG islands (3). Over 37% of these islands are located in the promoters of genes and ~70% of known genes have a CpG island within a region 2 kb upstream and 1 kb downstream of the start site of transcription (4). Methylated cytosines serve as binding sites for the methyl-CpG binding domain proteins, MeCP2, MBD1, MBD2, MBD3, MBD4 (5), and Kaiso (6). These proteins, in turn, interact with other chromatin remodeling enzymes and cause methylated DNA to be compacted into a chromatin environment repressive for transcription (7).

Mammalian genomes encode three active DNA methyltransferases (DNMT): the de novo methyltransferases DNMT3A and DNMT3B and the maintenance methyltransferase DNMT1 (1). The genes encoding all three of these enzymes are essential for development, with the earliest lethality seen in Dnmt1 knock-out embryos (8). DNMT3A and -3B are able to act on unmethylated DNA to establish methylation patterns imposed during embryogenesis and gametogenesis (9). DNMT1 generally shows preference for hemimethylated DNA and is responsible for transmission of lineage-specific DNA methylation patterns during replication, but some evidence suggests the clear separation of target specificity may be an oversimplification (1). DNMT1 may contribute to both de novo and maintenance methylation in certain regions of the genome alone or in collaboration with DNMT3A and DNMT3B (10, 11).

DNA methylation is known to regulate gene imprinting, X-chromosome inactivation, and transcriptional silencing of repetitive elements during normal development (1). However, DNA methylation patterns are frequently distorted in cancer cells leading to aberrant gene expression. For example, hypomethylation of intergenic regions can occur, leading to activation of transposable elements and increased genomic instability. Likewise, hypermethylation of the promoters of many tumor suppressor genes, such as retinoblastoma gene 1, E-cadherin, and RASSF1A often lead to loss of tumor suppressor functions.

The abbreviations used are: DNMT, DNA methyltransferase; SPA, scintillation proximity assay; HTS, high throughput screen; 5-AZA, 5-azacytidine/DACOGEN; AdoMet, S-adenosylmethionine; LC-MS/MS, liquid chromatography tandem mass spectrometry; CAT, catalase; SAR, structure-activity relationship; H2DCFDA, 2′,7′-dichlorodihydrofluorescein diacetate.

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\[†\] This article contains supplemental "Materials," Figs. S1–S5, and Table S1.
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Significance:
Selective DNMT1 inhibitors will allow for more precise elucidation of the role of this enzyme in gene regulation.
function in certain cancer cells and tumors (12). Because DNMT1 and DNMT3B are overexpressed and amplified in numerous cancers, there has been considerable interest in the development of selective inhibitors. At present there are two approved compounds known to affect DNA methylation, 5-azacytidine/VIDAZA (5-AZA) and 5-aza-2'-deoxycytidine/DACOGEN (5-DAC) (13). Both are cytidine analogs that, upon cellular import, are converted to the activated triphosphate 5-aza-dCTP and subsequently incorporated into DNA. The nitrogen at the 5-position of 5-AZA causes an irreversible covalent complex to be formed between the carbon at position 6 in the incorporated altered nucleobase and the DNMT enzyme and triggers its proteosome-mediated degradation (14). In recent years both compounds have shown promise in acute myeloid leukemia and myelodysplastic syndrome as investigators have shifted from using high amounts of drug based on maximum tolerated doses to lower doses that are more effective at decreasing DNA methylation and show less direct cytotoxicity (15, 16).

Despite the clinical utility of 5-AZA and 5-DAC there is a need for inhibitors that do not rely on DNA incorporation for activity, and that show improved stability and selectivity. Selective DNMT inhibitors may allow targeting of diseases driven by a particular DNMT enzyme with reduced off-target effects. Such chemicals might also serve as useful tool compounds to identify DNMTs. Knockdown of DNMT3B, for example, has been shown to selectively induce apoptosis of tumor versus normal cells and selectively reactivates methylation-silenced gene expression without causing global or juxtacentromeric satellite demethylation (17). Conversely, it has been reported that partial deficiency of DNMT1 prevented intestinal polyp formation in Apcmin mice (18), whereas cre-mediated deletion of the Dnmt1 gene in fibroblasts from mice expressing a conditional Dnmt1 allele caused global demethylation and uniform p53-dependent apoptosis (19).

The present report describes the identification of several DNA methyltransferase inhibitors using a novel scintillation proximity (SPA)-based high throughput assay. Whereas the majority of the identified compounds showed activity against both the maintenance methyltransferase, DNMT1, and the de novo methyltransferases, DNMT3A, at least one chemical showed activity against the majority of the identified compounds showed activity against both the maintenance methyltransferase, DNMT1, and the de novo methyltransferases, DNMT3A, at least one chemical showed activity against both the maintenance methyltransferase, DNMT1, and the de novo methyltransferases, DNMT3A, at least one chemical showed activity against both the maintenance methyltransferase, DNMT1, and the de novo methyltransferases, DNMT3A, at least one chemical showed activity against both the maintenance methyltransferase, DNMT1, and the de novo methyltransferases, DNMT3A, at least one chemical showed activity against both the maintenance methyltransferase, DNMT1, and the de novo methyltransferases, DNMT3A, at least one chemical showed activity against both the maintenance methyltransferase, 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processed as before and put in vials with 10 ml of scintillation fluid and read on a traditional scintillation counter. Data were plotted in GraphPad Prism 6 and a nonlinear sigmoidal dose-response curve fitted to the data to determine IC50 values.

Secondary screening consisted of the following assays: a biochemical mDNMT3A SPA assay performed in triplicate on active compounds cherry-picked from the library (1472 of ~180,000 compounds, Z-score ≤ −3.86, row-averaged normalized activity ≤0.7); an assay designed to remove nonspecifically active colored compounds (Color Assay), which was performed in triplicate in the same manner as the biochemical SPA, except the compound was added after the 3.5-h incubation and before the addition of SPA beads; a biochemical SPA assay measuring activity of the RNA methyltransferase TrmA catalytically active colored compounds (Color Assay), which was performed twice at varying concentrations of hydrogen peroxide and/or 50 units of catalase where appropriate. Results were visualized on a 1% agarose, 1× TPE, 0.2 μg/ml of chloroquine gel.

Redox Assays—Hydrogen peroxide generation was measured using a colorimetric assay based on the ability of horseradish peroxidase to catalyze the oxidation of phenol red and produce a change in absorbance at 610 nm after reaction termination as previously described (25). To determine the redox effects in the biochemical DNMT assay using hDNMT1 Δ581 (0.188 μg/reaction), the filter method was utilized with the addition of varying concentrations of hydrogen peroxide and/or 50 units of catalase where appropriate.

Cell Culture—A549 and HeLa cells (ATCC, Manassas, VA) were cultured in RPMI 1640 (Invitrogen) supplemented with 10% FBS (Gemini Bio Products, West Sacramento, CA), 100 units of penicillin, 100 μg of streptomycin, 2 mM l-glutamine, 0.1 mM minimal essential medium-non-essential amino acids, and 0.1 mM minimal essential medium-sodium pyruvate (all from Invitrogen) at 37 °C and 5% CO2. HBE330KT cells, a kind gift of Dr. John Minna, were cultured in ACL4 media supplemented with 2% FBS at 37 °C and 5% CO2. Cell viability was determined by counting viable cells after trypan blue staining. Cells were seeded in 96-well plates in triplicate at a density of 4,000 cells/well and treated with varying doses of different compounds for 72 h. The compound was added at time 0 and again at 48 h with a media change for cell based assays measuring toxicity and in vivo methylation. Cell viability was evaluated by measuring ATP levels with CellTiter-Glo from Promega (Madison, WI) according to the manufacturer’s protocol. Treated/control values were plotted in GraphPad Prism and IC50 values were determined from a nonlinear log(inhibitor) versus response-variable slope (four parameter) fit of the data.

Intracellular Reactive Oxygen Species Assay—Reactive oxygen species was measured by a fluorimetric assay using carboxy-H2DCFDA as the probe. A549 cells were plated at a density of 5,000 cells per well into a black 384-well plate (Corning, Tewksbury, MA) and incubated overnight. Cells were then washed once with PBS and treated with 50 μM carboxy-H2DCFDA for 1 h. After washing three times with PBS, cells were treated with increasing concentrations of compound or 100 μM H2O2 as a positive control. After 4 h incubation, fluorescence was measured using excitation wavelength of 480 nm and emission of 520 nm on the EnVision Multilabel Reader.

Global DNA Methylation—Genomic DNA from HeLa cells treated with compound for 72 h was isolated and purified using the Promega Wizard SV genomic DNA purification system. 5-AZA (6 μM) was used as a positive control. Global methylation levels were determined, following DNA hydrolysis, by an LC-MS/MS method as described previously (26).

RASSFIA Expression—RNA from A549 cells treated with compound for 72 h was isolated and purified using a Qiagen RNeasy Mini Kit. 5-AZA (6 μM) was used as a positive control. cDNA was generated with 1 μg of total RNA with SuperScript III First-strand Synthesis (Invitrogen). The expression of RASSFIA was analyzed by quantitative real-time RT-PCR, as described previously with RNASE P used as an internal reference gene to normalize expression of RASSFIA (27). The expression data are shown as a ratio of 1/ΔCt mean because untreated A549 cells did not show a quantifiable amount of RASSFIA expression. To further confirm the lack of RASSFIA expression in A549 and its induction by various compounds, a set of reaction products were visualized on a 2% agarose gel stained with EtBr (supplemental “Materials”).

RASSFIA Promoter Methylation—Genomic DNA from A549 cells treated with compound for 72 h was isolated and purified using Promega Wizard SV genomic DNA purification system. 5-AZA (6 μM) was used as a positive control. The DNA was deaminated as previously described (28). After bisulfite treatment, the RASSFIA promoter sequence was amplified from the deaminated DNA samples using forward primer 5’-GGGTTTTTAGGTTTGTATTTAGGGTTT’T-3’ and reverse primer 5’-CAACCTCATAAATACAATACCTCCCTCCC-3’ with M13 sites and a hot start DNA Taq Polymerase. After amplification, the promoter sequence was purified and transformed into TOP10 cells using the TOPO TA Cloning Kit for Sequencing from Invitrogen. Colonies were tested for the RASSFIA promoter insert using colony PCR. DNA was isolated from positive clones using the PureLink Quick Plasma DNA Miniprep Kit from Invitrogen. Plasmid DNA was then submitted for Big Dye Terminator sequencing at the Mc Dermott DNA Sanger Sequencing Core Facility at the University of Texas Southwestern Medical Center using vector primer T3. Sequence readouts were then aligned and compared using Sequencer 5.0 software. The analyzed CpG sites are named
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did not hallucinate.

According to RASSF1A (Homo sapiens) GenBank™ accession NM_001782 with the start codon notated.

Synthesis and Structure-Activity Relationship of SW155246 Analogs—A limited number of SW155246 analogs were generated to evaluate the role of the hydroxy group on the 1-position of the naphthyl ring (see supplemental “Materials”). SW155246-1 lacks the hydroxyl on the 1-position of the naphthyl ring, whereas SW155246-2 possesses an O-methyl group at the same position. These compounds were subjected to the filter method for the biochemical DNMT assay using hDNMT1 Δ581 (0.153 µg/reaction).

DNMT1 Western Blot—HeLa and A549 cells were treated with 10 μM SW155246 or 4 μM 5-AZA for 3 days, with the compound replaced on day 2. The cells were trypsinized and washed twice with 5 ml of PBS. The cell pellet was lysed on ice with 200 μl of RIPA buffer with 10 mM PMSF and 2 μl of 100× proteinase inhibitor mixture (Calbiochem, San Diego, CA). The cell mixture was pipetted several times to ensure cells were mixed well with buffer, then drawn into a 1-ml syringe with a 25-gauge needle 10 times to shear DNA. The BCA method was used to calculate the total protein concentration, and BSA was used as the standard. The cell lysates were mixed 1:1 with 2× Laemmli loading buffer and stored at −20 °C prior to use in the Western blot. Samples (25 μg/lane) were run on an SDS-polyacrylamide gel (4% stacking and 8% separation gel) and transferred to nitrocellulose membrane (Bio-Rad) at 80 V for 1.5 h. The blot was blocked in 5% nonfat milk and DNMT1 was probed with a 1:300 dilution of an anti-DNMT1 antibody (sc-207011, Santa Cruz Biotechnology), and β-actin with a 1:200 dilution of an anti-actin antibody (sc-47778, Santa Cruz) overnight at 4 °C. A 1:2500 dilution of a rabbit IgG-HRP secondary antibody, (GTX213110—01 GeneTex, Irvine, CA) was used for DNMT1, and a 1:2500 dilution of a mouse IgG-HRP (Santa Cruz) was used for β-actin. After washing, the blot was incubated with ECL solution (Thermo Pierce Scientific) for 1 min and exposed to x-ray film.

RESULTS

High Throughput assay—A SPA was used to conduct a high throughput screen for inhibitors of DNA methyltransferases. Although the screening was conducted using mDNMT3A, a hemimethylated DNA template consisting of a biotinylated 58-mer with 12 methylated CpG sites annealed to an antisense 58-mer without biotinylation or methylation was utilized for the substrate so that subsequent secondary assays could evaluate compound specificity for the maintenance methyltransferase DNMT1 versus the de novo methyltransferases DNMT3A and -3B (Fig. 1A). Briefly, DNA template and enzyme were mixed together in 384-well plates in a buffer containing DTT to keep the enzymatic site of the methyltransferase reduced. Compounds were added and the reaction initiated by addition of tritiated AdoMet. In the absence of inhibitors, mDNMT3A methylated the available CpG sites with tritiated AdoMet. After a 3.5-h incubation, streptavidin-labeled PVT scintillation proximity beads were added. Oligos containing labeled CpG sites were brought into sufficiently close proximity to the SPA bead via the biotin-streptavidin interaction and light was produced. Wells containing putative inhibitors showed reduced light generation. Sinefungin, a AdoMet analog originally isolated from S. griseolus (29), was utilized as a control and routinely resulted in >90% inhibition at 10 µM.

Approximately 180,000 compounds were screened in singlet at a concentration of 10 µM in this assay (Fig. 1b). Using a Z-score of <-3.86 and a normalization value of <0.7 (30% inhibition), 1472 compounds were selected and subsequently cherry picked for further analysis. These compounds were re-screened in the SPA assay in triplicate. Using a more stringent normalized cutoff value of <0.534 (50% inhibition), 640 positive compounds remained (43% validation rate). Compounds were subsequently screened in secondary assays designed to eliminate DNA intercalators, colored compounds that nonspecifically inhibited light transmission from the SPA beads, and compounds that showed greater than 40% inhibition of the TrmA RNA methyltransferase. Approximately 320 compounds were discarded using this screening strategy. The remaining 320 compounds were screened in a final filter-binding assay to validate the results obtained by SPA. The filter assay was identical to the SPA assay except that, instead of adding SPA beads, the completed reaction was blotted onto a DE81 filter mat that was washed prior to reading in a scintillation counter. This evaluation yielded 56 compounds showing greater than 50% inhibition of mDNMT3A in the filter-binding assay (27% validation rate). The explanation for this relatively low validation rate is not completely understood, although a subsequent direct comparison of IC₅₀ values obtained for validated hits from both screens has indicated that the SPA assay is approximately 2–4-fold more sensitive in identifying inhibitors (data not shown). Of the validated hits, 26 compounds were available for immediate re-supply and re-screened in the filter-binding assay. Eighteen of 26 hits were confirmed (69% validation rate) and subjected to a final gel-based intercalation assay to remove any intercalators that were not previously removed by the fluorescent HTS intercalation assay. In addition, hits were checked for a clean screening history in assays run to that date in the University of Texas Southwestern HTS core. A final hit list of 12 compounds was generated and is shown in Fig. 2, along with the percent inhibition of mDNMT3A activity noted in the final filter-binding assay. As is evident from the structures, four major chemotypes are represented. The first chemotype consisted of naphthoquinones. The second group represented a specific subset of naphthoquinone imines. The third chemotype consisted of compounds with an oxadiazole core, and the final group contained nihydroxyn derivatives. A representative compound from each chemotype was screened against hDNMT1 and mDNMT3a in the filter-binding assay at two concentrations (supplemental Fig. S1). All four chemotypes demonstrated similar activity against both DNMT enzymes, although chemotypes 1 and 3 demonstrated a slight preference for hDNMT1.

Role of Reactive Oxygen Species in Compound Activity—As reported by Johnston and colleagues (25) a number of HTS assays that have utilized a reducing agent in the assay and/or in storage buffers have resulted in identification of several false positive hits because the compounds generated H₂O₂ by redox cycling in reducing agents that could indirectly modulate the activity of susceptible targets. In fact, addition of H₂O₂, albeit at
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**A**

- Enzyme
- Compound
- 37°C
- 5′-AdoMet

**B**

**mDNMT3A 180K SPA Screen**

SPA x 1, z-score ≤ -3.86, norm value ≤ 0.7

**mDNMT3A SPA on 1472 Cherry Picked Compounds**

SPA x 3 (norm value ≤ 0.534), Color Assay x 1

**Intercalation and hDNMT1, M.Sssl, TrmA SPA Assays for 640 Compounds**

All assays x 3, TrmA norm value ≥ 0.6, compound omitted if mDNMT3A SPA & Color Assay norm values within 0.25 of each other

**mDNMT3A Filter Binding Assay for 320 Compounds**

mDNMT3A Filter Binding Assay x 1, norm value ≤ 0.5, immediate resupply from vendors

**26 out of 56 Compounds Resupplied**

mDNMT3A Filter Binding Assay x 2

**18 Confirmed Hits**

Clean screening history, Gel Based Intercalation Assay x 2

**12 Hits**
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FIGURE 1. SPA used for HTS of DNMT inhibitors. A, schematic of assay used to screen for DNMT3A inhibitors. Two custom oligos were utilized as the substrate. One was a 58-mer oligo, biotinylated (Biotin) on the 5' end and methylated at 12 CpG sites (black circles). The other oligo was an antisense 58-mer without biotinylation or methylation. The two oligos were annealed to produce a biotinylated, hemi-methylated double-stranded oligo template. Assay components were mixed and the reaction initiated by the addition of tritiated S-adenosylmethionine (\[^{3}\text{H}\]AdoMet). If the compound did not inhibit the reaction, the double-stranded oligo would be methylated and AdoMet incorporated. Addition of streptavidin-coated PVT (dark gray SPA beads) allows for light production when bound to double-stranded oligos with \[^{3}\text{H}\]AdoMet incorporated. B, screening strategy for DNMT inhibitors. See text describing the HTS assay under “Experimental Procedures” and “Results” for more details. Norm, normalized.

FIGURE 2. Screening hits grouped in chemotypes. The % mDNMT3A inhibition when treated with 10 \(\mu\text{M}\) compound for 3.5 h and processed using the high throughput filter-binding method is in parentheses. An asterisk indicates the representative compound from each chemotype chosen for further analysis.

relatively high concentrations, to the biochemical filter-based methyltransferase assay, inhibited methyltransferase activity (Fig. 3A). The quinone moiety present in chemotypes 1, 2, and 3 has the potential to generate such reactive oxygen species. Production of \(\text{H}_2\text{O}_2\) was therefore monitored upon addition of 10 \(\mu\text{M}\) of a representative compound from each of the chemotypes using a colorimetric assay. As shown in Fig. 3B, compounds SW025890 (chemotype 1), SW045263 (chemotype 2), and SW000026 (chemotype 3) generated a change in absorbance equivalent to the addition of 100 \(\mu\text{M}\) \(\text{H}_2\text{O}_2\). The shift in absorbance by the quinone-containing compounds could be blocked by the addition of catalase to the assay buffer (Fig. 3C). Significantly, the ability of compounds representing chemotypes 1, 2, and 3 to inhibit hDNMT1 activity \textit{in vitro} could be blocked by the addition of catalase (Fig. 3D), suggesting the mechanism of action of these classes of compounds was through generation of the reactive oxygen species, namely \(\text{H}_2\text{O}_2\), which may be oxidizing the catalytic cysteine in the DNMT enzyme. Compounds with an oxidized quinone core were therefore not considered further here due to the lack of specificity in their mode of action. Although the representative chemotype 4 compound (SW152843) did not appear capable of generating reactive oxygen in these assays, subsequent testing of the chemical stability of compounds representing this structural type by LC-MS/MS revealed significant instability in media only (data not shown). This was manifest by the poor repeatability of its ability to inhibit DNMT1 activity (e.g. Fig. 3D). Thus, continued evaluation of this structural class was also not warranted.

Compound SAR and Identification of a Novel DNMT1 Selective Inhibitor—In reviewing the original screening data, it was observed that a number of additional hits belonging to chemotype 2 were not selected for further evaluation because they failed to inhibit mDNMT3A by >50% in the filter-binding assay. In an effort to identify hits with a more specific mode of action, these compounds were further screened against hDNMT1. Interestingly, several of these compounds showed quite potent activity for hDNMT1 \(\Delta S81\), at least in the SPA, and several represented already reduced forms of the quinone. One of these compounds, SW155246, was tested in a dose-response curve for the ability to inhibit hDNMT1 and mDNMT3A in the filter-based biochemical methyltransferase assay (Fig. 4A). Significantly, as shown in Fig. 4B, SW155246 demonstrated a 30-fold preference for hDNMT1 \(\Delta S81\) (IC\(_{50} = 1.2 \mu\text{M}\)) versus mDNMT3A (IC\(_{50} = 38 \mu\text{M}\)). The AdoMet analog singefungin was used as a positive control. Activity against hDNMT3B + 3L was similar to that observed against mDNMT3a (data not shown). This compound, unlike the original hits from chemotype 2, failed to generate \(\text{H}_2\text{O}_2\) as measured using the colorimetric assay (Fig. 4C) and unlike SW045263 and SW025890, the ability of SW155246 to inhibit DNA methyltransferase activity in the biochemical assay was not inhibited by the addi-
tion of catalase (Fig. 4D), suggesting a more specific mode of action for this compound.

**Compound Effects on Cell Viability**—Prior to ascertaining whether SW155246 was effective at inhibiting the DNMT enzymes in a cell-based assay, it was first necessary to determine its effects on cellular viability because knockdown of DNMT enzymes by genetic approaches has been shown to affect cell growth and health (17, 19, 30, 31). SW155246 was thus evaluated for activity against two tumor cell lines, HeLa and A549, and one normal immortalized human bronchial epithelial line, HBEC30KT (supplemental Fig. S2) in comparison to the known pan-DNMT inhibitor, 5-AZA. Cell viability was monitored using Cell Titer Glo, which measures the amount of ATP produced. SW155246 showed a variable degree of cell-growth inhibition, with IC50 values ranging from 8 to 20 μM. 5-AZA was a more potent cytotoxin with IC50 values ranging from 3 to 6 μM.

**Compound Effects on in Vivo Methylation**—Using the cell viability data as a guide, effects of SW155246 in comparison to 5-AZA on global genomic methylation in HeLa cells was evaluated at concentrations that would not be expected to result in significant cell death. An LC-MS/MS-based genomic methylation assay was employed (26). This assay relies on acid hydrolysis of DNA to yield free cytosine and 5-methylcytosine followed by separation on a C18 reversed phase column using the ion-pairing agent nonafluoropentanoic acid and quantification using stable isotope dilution. Using this assay, the basal level of methylcytosine in HeLa cells was found to be 2.2% of all cytosines. Previous studies utilizing genetic means of blocking either DNMT1 or DNMT3A have demonstrated changes in global DNA methylation upon inhibition of either enzyme (19, 32). Several concentrations of compounds were evaluated with the top concentration chosen to be at or below the cell viability IC50. SW155246 was able to inhibit genomic methylation in HeLa cells at concentrations of 10 μM and higher (Fig. 5A) below its IC50 of 18.6 μM for cell viability. By comparison, treatment with 5-AZA resulted in much more significant effects on global methylation. It is hypothesized that the ability of 5-AZA to inhibit both DNMT1 and DNMT3A/3B and thus both maintenance and de novo methyltransferase activity accounts for its more potent effects on global DNA methylation.

To carry the analysis of SW155246 further, the ability of this compound to specifically affect promoter methylation and subsequent gene expression for a tumor suppressor gene known to be regulated by methylation was tested. *RASSF1A* is a known tumor suppressor gene that is not expressed in A549 cells due to promoter methylation. Addition of 10 μM SW155246 resulted in re-expression of this gene as measured both by real-time PCR (Fig. 5b) and a gel-based analysis (supplemental Fig. S3), albeit at lower levels than those induced by 5-AZA. Analyzing...
sis of the RASSFIA promoter showed a loss of methylation at low frequency at several CpG sites after treatment with 10 μM SW155246 (Fig. 6). Notably, two of these sites are the same as those altered after treatment of A549 cells with 6 μM 5-AZA and are fully methylated in untreated A549 cells.

SW155246 Mode of Action Studies and SAR—To confirm that the ability of SW155246 to inhibit hDNMT1 activity in vivo was not through generation of reactive oxygen species, compound was added to A549 cells at 10 μM. As controls, two of the original chemotype 1 and 2 hits that were found to generate H₂O₂ in vitro were also evaluated. Both SW045263 and SW025890 when used at 10 μM generated H₂O₂ in A549 cells as measured by addition of 50 μM of the sensor dye, carboxy-H₂DCFDA, whereas SW155246 failed to show a shift in absorbance indicative of the production of H₂O₂ (Fig. 7). Similarly, catalase did not affect cell viability of SW155246-treated cells (supplemental Fig. S4), suggesting a redox-insensitive mechanism is responsible for the loss in cell viability.

Because 5-AZA is known to affect stability of the DNMT proteins, the effects of SW155246 on stability of hDNMT1 was evaluated (Fig. 8). Although 4 μM 5-AZA clearly resulted in degradation of the DNMT1 protein, as would be expected, up to 10 μM SW155246 had no effect. Thus, SW155246 inhibits the activity of the DNMT1 enzyme without affecting protein levels or generating reactive oxygen species.

A limited SAR analysis was conducted and demonstrated that loss of the hydroxyl group (SW155246-1) or addition of a methylated oxygen on the 1-position of the naphthyl ring (SW155246-2) completely abolished the ability of this compound to inhibit hDNMT1 activity in vitro and reduced cell-based cytotoxicity (Fig. 9 and supplemental Fig. S5). Furthermore, mining of the original mDNMT3A HTS data revealed that: 1) compounds with a bulky substituent at the 2-position on the napthyl ring, such as that observed in SW045263, failed to show specificity for DNMT1 versus 3A and 2) equally potent activity was observed for compounds like SW155246 that were already reduced and compounds that were quinones (supplemental Table S1.).

DISCUSSION

The most widely used DNA methyltransferase inhibitors are the cytidine analogs, 5-AZA and 5-DAC. These compounds are
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A covalent complex is formed between the DNMT enzyme and the carbon at position 6 in the altered nucleobase due to the nitrogen at position 5. This triggers proteosome-mediated DNMT1 degradation (13, 14). At high doses these compounds lose their potent hypomethylation activity due to the appearance of cytotoxicity unrelated to DNA methylation (33). More recent use of these compounds employing low dose strategies have demonstrated clinical efficacy in myelodysplastic syndrome and acute myeloid leukemia. Despite their advantages, significant challenges arise from the instability of 5-AZA and 5-DAC in aqueous solutions as well as in vivo deamination. Current studies are focused on improving the stability and delivery of these agents. A number of additional compounds that function as reversible inhibitors of methyltransferase activity have been identified over the years (reviewed in Ref. 14) but there has been difficulty in many cases in validating their role as methyltransferase inhibitors. Thus, there is a need for potent, selective DNA methyltransferase inhibitors, both as tool compounds and as potential therapeutics.

The data presented herein represent the first identification of a semi-selective DNMT1 inhibitor with low micromolar efficacy in a biochemical assay measuring DNA methyltransferase activity. SW155246 shows a 30-fold selectivity for inhibition of hDNMT1 versus mDNMT3A. Although 2-fold more mDNMT3A enzyme (~147 nM) was used relative to hDNMT1 (~60 nM) in the biochemical assays, the amount of compound is far in excess of this; thus, the differential specificity cannot be explained by differences in enzyme abundance. The SAR represented by the screening data indicates that bulky groups at the 2-position of the naphthyl ring diminish interaction of the compounds with DNMT1, whereas small groups or no substitution at this position promote inhibition of DNMT1; however, this remains to be more fully explored. It may be that compounds with the bulky substituent form only weak interactions with both DNMT3A and DNMT1 and their ability to produce H2O2 allows for modest inhibition of both enzymes. Smaller compounds lacking this bulky substituent, such as SW155246, however, may form a tighter interaction with DNMT1 that allows for potent and specific inhibition of this enzyme not dependent on generation of H2O2. SW155246 also demonstrated the ability to demethylate cytosines in vivo in a cell-based assay and induced re-expression of the tumor suppressor gene, RASSFIA. The change in expression was correlated with an increased frequency of demethylation of specific CpG sites within the RASSFIA promoter. This activity was not as pronounced as that observed using the known DNA methyltransferase inhibitor 5-AZA, possibly due to the broader activity of 5-AZA for both maintenance and de novo methyltransferases.

In addition, SW155246 showed only a 4-h half-life in the presence of the HeLa cells used in this assay (data not shown) as measured by LC-MS/MS. As compound was added only at the beginning of the 3-day incubation and again on day 2, the amount of active compound available to affect in vivo methylation was likely lower than represented by the amount added. Thus, there is potential for more potent in vivo activity if issues with compound stability can be resolved.

Although a definitive mode of action for SW155246 against DNMT1 was not identified, the results described herein demonstrate a number of important features of its activity. First, a gel-based intercalation assay indicated that it does not intercalate DNA (data not shown). Additional screening data using SPA for measuring methyltransferase activity showed poor activity of this compound in inhibiting both the bacterial methyltransferase M.SssI (22% inhibition at 10 μM), the RNA methyltransferase TrmA (37% inhibition at 10 μM), and murine DNMT3A (55% inhibition at 10 μM) but potent activity against human DNMT1 (90% inhibition at 10 μM). Subsequent dose-response studies indicated a 30-fold difference in IC50 values for DNMT3A versus DNMT1, demonstrating the selectivity of this compound for DNMT1. Unlike most of the hits identified here, SW155246 does not act to inhibit DNMT1 through generation of reactive oxygen nor does it act like 5-AZA by causing degradation of the DNMT enzymes. Based on its specificity and potency, further studies are warranted to define its precise mode of action.
Several of the identified compounds, as typified by SW045263 and SW025890, potently inhibited both the maintenance and de novo methyltransferases, but suffered from the nonspecific ability to generate H₂O₂ in both the biochemical methyltransferase assay and in cells. The addition of exogenous catalase blocked the generation of H₂O₂ by these compounds but also blocked their ability to inhibit methyltransferase activity, suggesting that the H₂O₂ generated may have oxidized the catalytic cysteine in the active site of DNA methyltransferases. It is intriguing that a number of recently identified DNMT inhibitors are either quinones like nanaomycin (34) or dichlone (35) or are capable of generating H₂O₂ (epigallocatechin-3-gallate (36)). These results, although raising questions regarding the utility of these types of inhibitors as potential therapeutics due to off target toxicity, do suggest a possible role for the cellular oxidation state in regulating methylation. The role of oxidative stress in modulating generation of glutathionine versus AdoMet is well known (37). Homocysteine, which is generated

![FIGURE 6. RASSF1A promoter methylation sequencing treated A549 cells. A, the hRASSF1A promoter sequence (NM 007182.4) is shown. The top sequence is deaminated DNA with possible methylation sites, highlighted in gray and numbered, aligned with the endogenous sequence on the bottom. ATG start site is highlighted in black with white text. B, A549 cells were treated with 10 μM SW155246 or 6 μM 5-azacytidine for 72 h, with fresh addition of compound at 48 h. The cells were then harvested and genomic DNA was obtained. The genomic DNA was deaminated and the RASSF1A promoter was amplified through PCR. The PCR products were cloned into E. coli and insert DNA was checked using colony PCR. Plasmid DNA was isolated from positive clones and sequenced using Big Dye Terminator chemistry. Sequences were analyzed using Analyst Software. The DNA was confirmed to have complete deamination. Data from 20 clones of each are shown. Conditions and calculations are described under “Experimental Procedures.”](image)

![FIGURE 7. Compound in vivo redox analysis. A 384-well assay to determine whether reactive oxygen species is generated by compounds SW045263 (chemotype 1), SW025890 (chemotype 2), and SW155246 in A549 cells was performed. Compounds were titrated with 5000 cells/well. Carboxy-H₂DCFDA (50 μM) was used as an indicator and fluorescence was read at excitation 480 and emission of 520 4 h after addition of compound. Readings were normalized to no compound samples. Each point represents the average reading of triplicate wells. Conditions for all assays are described under "Experimental Procedures."](image)

![FIGURE 8. Effects of SW155246 and 5-AZA on DNMT1 protein levels. HeLa and A549 cells were treated with 10 μM SW155246 and 4 μM 5-AZA for 72 h, with fresh addition of compound at 48 h and lysed for DNMT1 Western blot analysis. Samples were electrophoresed on a 8% SDS-polyacrylamide gel and probed with DNMT1 antibody. The Western blots were scanned and analyzed with ImageJ. The DNMT1 signal was normalized to β-actin. The normalized expression value is indicated in the bar graph above each blot. NT, non-treated cells; ND, not detectable.](image)
as a by-product of DNA methylation, is at the cross-roads of generation of either methionine or cysteine depending on cellular needs. It may be processed through the transulfation pathway to generate cysteine and GSH or be recycled to methionine and subsequently contribute to AdoMet pools through betaine homocysteine methyltransferase, which is expressed primarily in liver and kidney, or methionine synthase, which is broadly expressed. Studies to date indicate that the modulation of AdoMet pools have broad effects on epigenetic marks (37). Although H₂O₂ has been shown to have a direct effect in modulating the activity of histone deacetylases (38), this is one of the first demonstrations of a direct role of reactive oxygen species in modulating the activity of DNA methyltransferase enzymes.

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