Preclinical Development

Nanaomycin A Selectively Inhibits DNMT3B and Reactivates Silenced Tumor Suppressor Genes in Human Cancer Cells

Dirk Kuck¹, Thomas Caulfield², Frank Lyko¹, and Jose L. Medina-Franco²

Abstract

Enzymes involved in the epigenetic regulation of the genome represent promising starting points for therapeutic intervention by small molecules, and DNA methyltransferases (DNMT) are emerging targets for the development of a new class of cancer therapeutics. In this work, we present nanaomycin A, initially identified by a virtual screening for inhibitors against DNMT1, as a compound inducing antiproliferative effects in three different tumor cell lines originating from different tissues. Nanaomycin A treatment reduced the global methylation levels in all three cell lines and reactivated transcription of the RASSF1A tumor suppressor gene. In biochemical assays, nanaomycin A revealed selectivity toward DNMT3B. To the best of our knowledge, this is the first DNMT3B-selective inhibitor identified to induce genomic demethylation. Our study thus establishes the possibility of selectively inhibiting individual DNMT enzymes. Mol Cancer Ther; 9(11); 3015–23. ©2010 AACR.

Introduction

The genetic information of a human cell is packaged into chromatin, which has an important biological function by controlling the accessibility of the DNA to the cellular transcription and replication machinery. Therefore, chromatin is labeled with different marks on the histones and on the DNA itself (1, 2). These marks represent the epigenetic code resulting from modification and regulation systems. The best-characterized mark on DNA consists of a single methyl group at the C5 position of cytidine nucleotides, which occurs nearly always in a CpG sequence context in differentiated cells.

The human genome encodes three active DNA methyltransferases: DNMT1, DNMT3A, and DNMT3B. DNMT1 and DNMT3B are indispensable for embryonic development in mice, whereas DNMT3A knockout mice die shortly after birth (3). DNMT1 is required for the maintenance of DNA methylation patterns during replication in normal and cancer cells and is essential for their proliferation and survival (4). DNMT3A and DNMT3B are de novo methyltransferases involved in embryonic development and in the establishment of genomic imprints (3, 5, 6). The analysis of DNA methylation patterns in normal and tumor cells has revealed that many, if not all, human tumors exhibit an altered methylation signature. This aberrant methylation pattern is often characterized by hypermethylation and inactivation of tumor suppressor genes such as p16 or RASSF1 (7), which can consequently drive tumor formation. However, in contrast to classic genetic mutations, these so-called epimutations are reversible by the inhibition of DNMTs (8). This reversibility encouraged the development of pharmacologic inhibitors of DNA methylation.

Several approaches have been pursued to inhibit DNMT activity, including small interfering RNA-mediated depletion of DNMTs (9) or the use of suicide nucleoside substrates such as azacytidine and decitabine for covalent enzyme trapping (10). Both drugs were approved by the Food and Drug Administration for the treatment of myelodysplastic syndrome in 2004 and 2006, respectively, and represent the first DNMT inhibitors in clinical use. One of the most recent approaches is the rational development of small-molecule nonnucleoside inhibitors such as RG108 (11–13). The family of nonnucleoside candidate DNMT inhibitors is steadily growing and comprises a large variety of different chemical scaffolds [e.g., polyphenolic compounds such as epigallocatechin-3-gallate (14, 15) or compounds with acidic functions such as caffeic acid (16) or methylenedisalicylic acid (17)]. In addition, approved drugs for other indications such as hydralazine (18, 19), procaine (20, 21), procainamide (22), or antibiotics such as mithramycin A (23) have also been reported to inhibit DNA methylation. Additional DNMT inhibitors are reviewed elsewhere (10, 24, 25).

Antibiotics usually target cell wall synthesis, protein translation, or the DNA replication machinery in bacteria.
However, antibiotics of the anthracycline group, such as daunomycin, doxorubicin, or mitomycin, are also known to have powerful antitumor activity. Many related chemical structures from the anthracycline group have been developed as anticancer drugs (26, 27). Nanaomycin A also belongs to this class of quinone antibiotics isolated from a culture of *Streptomyces* (28). The nanaomycin A mode of action is dependent on its reduction by the respiratory chain-linked NADH or flavin dehydrogenase of the organism. The reduced form of nanaomycin A is quickly autooxidized by molecular oxygen producing singlet molecular oxygen (O$_2^*$). The ability to produce O$_2^*$ is related to the antimicrobial activity of nanaomycin A (29, 30). The chemical structure of nanaomycin A is depicted in Fig. 1A.

In this study, we characterized the epigenetic effects of nanaomycin A, which we had identified by an in silico screening approach aiming for novel inhibitors of DNMT1 (17). Nanaomycin A showed no activity against DNMT1, despite sharing structural similarities to other active compounds, such as methylendisalicylic acid derivatives, yet we did observe a potent antiproliferative effect in different tumor cell lines. When we analyzed the genomic methylation levels in different tumor cell lines, we found a distinct nanaomycin A–dependent reduction, as well as transcriptional reactivation and expression of the RASSF1A tumor suppressor gene. To identify the cellular target of nanaomycin A, we applied a biochemical in vitro methylation assay using human DNMT1 or DNMT3B and detected a selective inhibition of DNMT3B. To better rationalize the biochemical activity at the molecular level, we conducted molecular docking studies of nanaomycin A with a homology model of DNMT3B. The docking model suggests that nanaomycin A can bind in the catalytic site of the enzyme. Currently, this is the first report of a DNMT3B-selective small-molecule inhibitor with cellular activity, highlighting the feasibility of discovering compounds that selectively inhibit individual DNMT enzymes.

**Materials and Methods**

**Compounds**

Nanaomycin A (NSC267461) was obtained from the National Cancer Institute (NCI)/Developmental Therapeutics Program Open Chemical Repository (http://dtp.cancer.gov), dissolved in DMSO to 50 mmol/L, and stored at −80°C. Chemical structure of nanaomycin A is shown in Fig. 1A. 5-Azacytidine, RG108, and procainamide were purchased by Sigma-Aldrich. 5-Azacytidine, RG108, and procainamide were dissolved in water; RG108 was dissolved in DMSO.

**Cell culture**

A549, HL60, and HCT116 cells were obtained directly from the American Type Culture Collection and passaged in our laboratory for <6 months after resuscitation. A549, HCT116, and HeLa were cultured in DMEM/Ham’s F12 (Biochrom) supplemented with 10% FCS (Invitrogen). HL60 cells were cultured in RPMI 1640 supplemented with 5% L-glutamine and 10% FCS (Invitrogen) at 37°C and 5% CO$_2$. Determination of cell viability was done by counting the viable cells after trypan blue staining. Therefore, cells were seeded in triplicates in six-well plates at a density of 250,000 per well. Cells were treated with increasing doses of nanaomycin A for 72 hours. Caspase-3/7 activation was measured by the Caspase-Glo 3/7 assay from Promega according to the manufacturer’s protocol. Cells were seeded in 96-well plates in triplicates at a density of 10,000 per well and incubated for 72 hours. Assays were measured by a Tecan Infinite 200 microplate reader.

**DNA methylation analysis**

Genomic DNA was purified using the DNeasy Blood and Tissue kit (Qiagen). Global methylation levels were determined by capillary electrophoresis as described previously (31). Briefly, 5 μg of genomic DNA were enzymatically hydrolyzed to single nucleotides, and the nucleotides were derivatized with the fluorescent marker BODIPY (Molecular Probes). Derivatized nucleotides were separated by capillary electrophoresis and analyzed in a Beckman P/ACE MDQ Molecular Characterization System. Statistical significance was calculated by the Student’s t test.

**Methylation analysis of the RASSF1A promoter region**

For gene-specific methylation analysis, genomic DNA was deaminated using the EpiTect Bisulfite kit (Qiagen). For 454 sequencing, bisulfite-treated genomic DNA was amplified using sequence-specific primers containing treatment-specific barcodes and 454 linker sequences: Pair1, GCCTCCCTCGCGCCATCAGTCAGGA-CAGTGGGGATTTTTTTTTTTA (For_RASSF1A_1) and GCTTTGCGACGCCCTAGTCAGGCAAACACT-CAATAAACTCAAC (Rev_RASSF1A_1); Pair2, GCCCTCCCTCGCGCCATCAGTCACGAGTGGG- GATTTTTTTTTTTTTA (For_RASSF1A_2) and GCTTTGCGACGCCCTAGTCAGGCAAACACT-CAATAAACTCAAC (Rev_RASSF1A_2).

PCR conditions were as follows: initial denaturation at 95°C for 2 minutes, 35 cycles at 95°C for 30 seconds, annealing at 40°C for 30 seconds, elongation at 74°C for 30 seconds, and a final elongation step at 74°C for 2 minutes. 454 deep sequencing was done by the German Cancer Research Center Genomics and Proteomics Core Facility.

**RNA isolation and quantitative real-time PCR**

RNA was extracted using the RNeasy RNA Isolation kit (Qiagen), and on-column DNA digestion was done using the RNeasy-Free DNase Set (Qiagen). For quantitative reverse transcription-PCR (RT-PCR), 1 μg of RNA was reverse transcribed using SuperScript III reverse transcriptase (Invitrogen) according to the manufacturer’s protocol. Each cDNA sample was analyzed in
triplicate using the QuantiFast SYBR Green PCR kit (Qiagen) according to the manufacturer’s instructions. QuantiTect Primer Assays (Qiagen) were used for detection of RASSF1A, DNMT1, and DNMT3B. GAPDH served as internal standard. RT-PCR was done by LightCycler 480 from Roche.

Western blot analysis
Antibodies for detection were used according to the manufacturer’s protocol. The antibodies against β-actin, DNMT1, DNMT3B, and RASSF1A were purchased by Santa Cruz Biotechnology or by Millipore for γH2AX (clone JBW301).

Cloning and purification of recombinant DNMTs
DNMT1 was produced and purified as described before (21). DNMT3B (isoform 2) was produced and purified as described elsewhere (17). Briefly, proteins were expressed in insect cells and purified by affinity chromatography and gel filtration. The protein concentration of purified DNMT was determined by Bradford assay and verified by using Coomassie blue-stained SDS-polyacrylamide gels and suitable molecular mass markers of known concentration.

Biochemical DNMT assay
DNA methylation assays were carried out in total reaction volume of 25 μL containing 0.4 μmol/L hemimethylated or unmethylated oligonucleotide substrate purchased from MWG (upper strand: 5′-GATCGCXGATCGCGXGATXGATCGCGAT-3′, X = 5mC for hemimethylated or X = C for unmethylated substrate, and lower strand: 5′-ATCGCATCGATCGC-GATTCGCGATCGCGATC-3′), purified DNMT in
reaction buffer [100 mmol/L KCl, 10 mmol/L Tris-Cl (pH 7.5), 1 mmol/L EDTA], and bovine serum albumin (1 mg/mL). All reactions were carried out at 37°C in the presence of 0.7 μmol/L [methyl-3H]AdoMet (2.6 TBq/mmol; Perkin-Elmer). After 3 hours, the reaction was stopped by adding 10 μL of 20% SDS and spotting of the whole volume onto DE81 cellulose paper. Filters were baked at 80°C for 2 hours and washed three times with cold 0.2 mol/L NH4HCO3, three times with distilled water, and once with 100% ethanol. After drying, filters were transferred into Mini-Poly Q vial from Perkin-Elmer, and 5 mL of Ultima Gold LSC Cocktail were added per vial. Analysis was done in a scintillation counter, and each measurement was repeated once.

**Molecular docking**

The structure of nanaomycin A was prepared with the program Molecular Operating Environment (MOE; version 2008.10; Chemical Computing Group, Inc.). Docking studies were conducted with Glide (version 5.5; Schrödinger, LLC), and visualizations were carried out with Maestro (version 9.0; Schrödinger, LLC). Docking was done using previously generated homology models of DNMT3B (17) and DNMT1 (32). The scoring grids were centered on the binding mode predicted for 2′-deoxycytidine as we reported previously (17). We used the bounding box size 14Å × 6Å × 10Å, which covers the catalytic pocket and part of the cofactor binding site. We used flexible docking with the extra precision mode in Glide and default parameters. To explore the putative interactions of nanaomycin A with the binding site, the top-ranked binding mode found by Glide in complex with the binding pocket of the enzyme was subjected to full-energy minimization using the MMFF94x force field implemented in MOE until a gradient of 0.001 was reached. The default parameters implemented into the LigX application of MOE were used.

**Results**

In an earlier study, we conducted a virtual screening of a public compound library from the NCI using a multistep docking approach with a previously validated homology model of the catalytic domain of human DNMT1 (17). Docking was completed using three different programs. From this, nanaomycin A (Fig. 1A, chemical structure) was ranked among the top 10% of all screened compounds from two of the docking programs. However, this quinonic compound did not show any detectable DNMT1 inhibition. When we treated three different human tumor cell lines [HCT116 (colon), A549 (lung), and HL60 (bone marrow)] with increasing concentrations of nanaomycin A (ranging from 10 nmol/L to 10 μmol/L) for 72 hours, we observed in all three cell lines a distinct cytotoxic effect (Fig. 1B). We determined the cellular viability by counting viable cells after trypan blue staining and measured IC50 values of 400 nmol/L for HCT116, 4100 nmol/L for A549, and 800 nmol/L for HL60. These data suggest a broad antiproliferative activity, which does not seem to be restricted to specific cell types.

The balance between cell proliferation and cell death is regulated by apoptotic and necrotic pathways. To elucidate the cellular mechanism of nanaomycin A–dependent cell death, we measured caspase-3 and caspase-7 activities in nanaomycin A–treated cells. Therefore, cells were incubated for 72 hours with the IC50 concentrations. However, when we compared untreated control cells with cells treated with the calculated IC50 concentrations, we did not detect activation of caspase-3 and caspase-7 (Fig. 1C). Thus, we conclude that caspase activation does not play a major role in the measured cell death.

Studies from various cancers have shown that downregulation of genes by DNA methylation as well as maintenance of silencing are important mechanisms through which tumor cells can survive (7). Thus, we decided to analyze a possible interruption of these mechanisms and assessed the ability of nanaomycin A to decrease
the genomic methylation level of HCT116, HL60, and A549 cells by capillary electrophoresis (Fig. 2; ref. 31). We incubated cells for 72 h with nanaomycin A (5,000 nmol/L) for HCT116, 1,000 nmol/L for HL60, and 5,000 nmol/L for A549 and isolated genomic DNA. Genomic methylation levels were found to be most significantly (P < 0.001) reduced in HCT116 at 500 nmol/L (from 3.8% to 2.2%), in HL60 at 1,000 nmol/L (from 3.4% to 2.6%), and in A549 at 5,000 nmol/L (from 2.7% to 1.1%). Although we used compound concentrations in the range of the calculated IC_{50} value, we measured genomic demethylation also with lower nanaomycin A concentrations (e.g., at 100 nmol/L for HL60 or at 3,000 nmol/L for A549; data not shown). This argues against the hypothesis that the observed demethylation might be a toxic side effect of the treatment. From this, we assumed that nanaomycin A hits the same cellular target in all three cell lines.

One of the primary biological outcomes of DNA methylation in cancer cells is transcriptional repression of tumor suppressor genes, thus promoting uncontrolled...
proliferation (7). For this reason, we investigated the promoter methylation of the epigenetically silenced RASSF1A tumor suppressor gene in untreated and nanaomycin A–treated (5,000 nmol/L) A549 cells. We used 454 bisulfite sequencing of the RASSF1A promoter region with coverage rates between 450 and 600 reads per CpG (Fig. 3A). Sequencing confirmed that the RASSF1A promoter was heavily methylated in A549 cells. Nanaomycin A treatment resulted in demethylation that was restricted to a few CpGs, which is in line with the results obtained after DNMT3B knockdown in A549 cells (33).

We next assessed RASSF1A transcript levels relative to GAPDH transcript levels by quantitative RT-PCR after treatment with several concentrations of nanaomycin A. We used 5-azacytidine (25 μmol/L), RG108 (300 μmol/L), and procainamide (1 mmol/L) as reference compounds (Fig. 3B). We observed a ~6-fold relative induction after 5-azacytidine treatment and an 18-fold relative induction after treatment with nanaomycin A (5,000 nmol/L). Lower concentrations of nanaomycin A yielded lower relative RASSF1A induction (e.g., 3,000 nmol/L induced 2-fold induction; data not shown). Procainamide or RG108 treatments achieved no reactivation.

Parallel assessment of RASSF1A protein expression by Western blotting revealed an initiation of RASSF1A protein expression in A549 cells (Fig. 3C). HeLa extracts were used as positive control for RASSF1A protein expression. In conclusion, we provide evidence for genomic and locus-specific demethylation caused by nanaomycin A that is apparently sufficient to reactivate transcription and expression of a silenced tumor suppressor gene.

Because the known antimicrobial mode of action of nanaomycin A involves free radical generation, we hypothesized that nanaomycin A might directly degrade DNMT1 or DNMT3B in compound-treated cells. To experimentally test this hypothesis, we did Western blotting and quantitative RT-PCR of untreated and nanaomycin A (5,000 nmol/L)–treated A549 cells and failed to detect any evidence for DNMT protein degradation (Fig. 4A) or downregulated DNMT transcription levels (Fig. 4B). We also tested whether DNA might be damaged by nanaomycin A treatment. However, A549 cells treated with nanaomycin A did not show increased levels of the DNA damage marker γH2AX by Western blot analysis (Fig. 4A). These experiments strongly suggest that nanaomycin A does not directly degrade DNMT1 or DNMT3B in compound-treated cells.

After analyzing indirect inhibition of DNMTs, we investigated the possibility of direct inhibition of DNMTs by nanaomycin A. We focused on DNMT1 and DNMT3B, the two most important isoforms in tumorigenesis, using a biochemical in vitro methylation assay consisting of recombinant methyltransferases. Human methyltransferases DNMT1 and DNMT3B were produced via baculovirus-mediated expression in SF9 insect cells and purified by affinity chromatography and gel filtration (21). Strikingly, we found a pronounced selectivity of nanaomycin A toward DNMT3B in the tested compound range with an IC50 value of 500 nmol/L (Fig. 5A). Under these conditions, the enzymatic activity of DNMT1 was not affected by nanaomycin A. To our knowledge, this is the first report of a non-SAH (S-adenosyl-L-homocysteine) analogue acting as a DNMT3B-selective inhibitor (34, 35).

To explore the putative binding mode of nanaomycin A with DNMT3B, we applied molecular docking of the inhibitor with a homology model of the catalytic site of DNMT3B (17). To build our computational model, the homology model of DNMT3B was constructed with Prime (Schrödinger, LLC) using the crystal structure of DNMT3A (PDB code 2QRV) as a template (36). The final
homology model showed a backbone root mean square deviation of only 0.08 Å with the template; further details are published elsewhere (17). Figure 5B shows the optimized binding model of nanaomycin A with DNMT3B. According to this binding model, residues that form the binding pocket of nanaomycin A include Pro650, the catalytic Cys651, Glu697, Arg731, Arg733, Lys828, Gly831, and Arg832. The carboxylic acid group of the ligand is capable of forming hydrogen bonds with the side chain of Arg832. Notably, with nanaomycin A, both the hydroxyl group and adjacent carbonyl oxygen atom are predicted to form an extensive hydrogen bond network with the side chains of Arg731 and Arg733. Additionally, the hydroxyl group of the ligand forms a hydrogen bond with the side chain of Glu697. Interestingly, we do not observe similar hydrogen bonds with the equivalent glutamic acid and arginine residues in docking studies of nanaomycin A with a previously validated homology model of the catalytic site of human DNMT1 (32), which provides a possible structural explanation for the enzyme selectivity of the drug.

Discussion

The number of compounds reported to possess DNA demethylating properties without covalent binding is steadily growing. Due to the high conservation of the catalytic domain within the DNMT family, most compounds are not expected to show selectivity to individual DNMT enzymes. Additionally, many of these compounds revealed weaker inhibition potential when compared with nucleoside analogues (37). Treatment of MCF-7 cells with procaine reduced the genomic methylation level ∼40% and reactivated transcription of the tumor suppressor gene RARβ2, although the demethylation around the transcription start site was moderate (20). Procainamide treatment of HCT116 cells also induced global demethylation by ∼15% and a similar degree of locus-specific demethylation at the TIMP3 tumor suppressor gene (22). However, the mode of action of procaine and procainamide probably relies on their intercalation into GC-rich DNA, which seemed to be contradictory to the reported specificity of procainamide (20, 38–40).

Our study establishes nanaomycin A as a novel DNMT inhibitor. The biochemical in vitro methylation assays support the finding that DNMT3B is the major DNMT target of nanaomycin A. Molecular docking studies of nanaomycin A using a homology model of the catalytic domain of DNMT3B suggest that an extensive hydrogen bond network with Glu697, Arg731, and Arg733 plays a key role in the stabilization of the protein-ligand complex. Similar interactions were not observed in docking studies of nanaomycin A with DNMT1, and these results may explain, at least in part, the structural basis of selectivity. It should be noted that, in contrast to a knockdown, blocking DNMT3B with a small-molecule inhibitor would abolish DNMT3B function without depleting it from the cellular proteomic pool. This will still permit, for example, protein-protein interactions, which might weaken the consequences of DNMT3B inhibition. Additionally, DNMT3B is also known to be expressed in multiple splice variants in cancer cells with unknown functions (41). Some of them encode truncated DNMT3B proteins, which might also be able to bind nanaomycin A.

Based on the experimental and theoretical evidence of the reaction between quinones and cysteine-rich proteins, and the docking model developed for nanaomycin A, we hypothesize the following mechanism for inhibition of DNMT3B with nanaomycin A. Cys651.S− initiates
a nucleophilic Michael 1,4 addition to the α,β-unsaturated carbonyl system at the β carbon, which proceeds on the less steric side (Supplementary Fig. S1). Noteworthy, in the docking model, the sulfur atom of Cys651 is close to the β carbon. The diene pushes its electron toward the opposite carbonyl atom. Subsequently, an intermediate enolate forms at the carbonyl atom (C-11) and the oxygen atom (O-10; Supplementary Fig. S1). This occurs as a result of the resonance between the delocalization of the anionic charge of the oxygen and the carbonyl atoms. However, the enol isomer of nanaomycin A structure does result; the negative charge located on the oxygen is further stabilized by interaction with positive residues Arg371 and Arg733. This stabilization, plus the covalent addition of the thiol group (Cys651-S\textsuperscript{-}), blocks the catalytic site DNMT3B. Our binding model of DNMT3B with nanaomycin A is compatible with the described 1,4-addition mechanism. Interestingly, this stabilization is not favored in DNMT1, which has a larger binding pocket. Thus, the selectivity of nanaomycin A toward DNMT3B is probably due to a better stability in the catalytic domain.

The findings of the biochemical assay and the in silico modeling data are supported by the results of the in vivo experiments. Treatment of different cell lines with nanaomycin A showed a significant genomic demethylation, although, as expected, at a lower level than observed for the nonselective DNMT inhibitor 5-azacytidine. Similar findings were reported for mouse embryonic fibroblasts, where constitutive or conditional deletion of Dnmt3b resulted in genomic demethylation of endogenous C-type retroviral DNA. These results have suggested that Dnmt3b, in addition to the major maintenance methyltransferase Dnmt1, is required for maintaining DNA methylation in these cells (42). Although the level of demethylation at the RASSF1A promoter region remained lower than that of the genome, it was still sufficient to re-activate transcription and expression of this gene and was comparable with the effects observed with other nonnucleoside inhibitors (20, 22). This result is also in agreement with the data obtained from antisense oligonucleotide-mediated knockdown of DNMT3B in A549 cells (33), inducing antiproliferative effects as well as a similar limited RASSF1A promoter demethylation associated with reactivated transcription.

Although it is debatable whether the anthracycline group is a good candidate for clinical drug testing due to some lasting cardiotoxicity issues (43), nanaomycin A represents the first non-SAH DNMT3B-selective compound and provides a valuable biochemical tool and benchmark for future studies.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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