Nanaomycin A selectively inhibits DNMT3B and reactivates silenced tumor suppressor genes in human cancer cells

Dirk Kuck\textsuperscript{1,2,4}, Thomas Caulfield\textsuperscript{3}, Frank Lyko\textsuperscript{1}, Jose L. Medina-Franco\textsuperscript{3}

\textsuperscript{1}Division of Epigenetics, Deutsches Krebsforschungszentrum, Im Neuenheimer Feld 580, 69120 Heidelberg, Germany, \textsuperscript{2}Torrey Pines Institute for Molecular Studies, 11350 SW Village Parkway, Port St. Lucie, Florida 34987, USA

\textsuperscript{2}Corresponding author phone: +49 6221 42 4731; fax: +49 6221 42 3802; e-mail: d.kuck@dkfz-heidelberg.de.

\textsuperscript{1} Division of Epigenetics, Deutsches Krebsforschungszentrum. 

\textsuperscript{3} Torrey Pines Institute for Molecular Studies.

Running title: Nanaomycin A inhibits DNMT3B and reactivates silenced genes

Key words: DNA methyltransferases; DNA methylation; epigenetics; inhibitors, drug discovery

Abbreviations: DNMT, DNA methyltransferase; SAM, S-adenosylmethionine

Footnotes: J.L.M.-F. is funded by the Menopause & Women’s Health Research Center

Author manuscripts have been peer reviewed and accepted for publication but have not yet been edited.

Copyright © 2010 American Association for Cancer Research
Abstract

Enzymes involved in the epigenetic regulation of the genome represent promising starting points for therapeutic intervention by small molecules and DNA methyltransferases (DNMTs) 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 towards 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.
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 labelled 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, while DNMT3A knock-out 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 known to be essential for their proliferation and survival (4). DNMT3A and 3B 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 like p16 or RASSF1 (7) which can consequently drive tumor formation. However, in contrast to classical 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 siRNA-mediated depletion of DNA methyltransferases (9) or the use of suicide nucleoside substrates like
azacytidine and decitabine for covalent enzyme trapping (10). Both drugs were approved by
the FDA for the treatment of myelodysplastic syndrome (MDS) 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 non-nucleoside inhibitors like
RG108 (11-13). The family of non-nucleoside candidate DNMT inhibitors is steadily growing
and comprises a large variety of different chemical scaffolds, e.g. polyphenolic compounds
like EGCG (14, 15) or compounds with acidic functions like caffeic acid (16) or
methylenedisalicylic acid (17). In addition, approved drugs for other indications like
hydralazine (18, 19), procaine (20, 21), procainamide (22) or antibiotics like 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, like 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 (O₂⁻). The ability to produce O₂⁻ is related to the antimicrobial activity of
nanaomycin A (29, 30). The chemical structure of nanaomycin A is depicted in Figure 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 (31). Nanaomycin A
showed no activity against DNMT1, despite sharing structural similarities to other active compounds, like methylenedisalicylic 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.

In order 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. In order 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 NCI/DTP Open Chemical Repository (http://dtp.cancer.gov), dissolved in DMSO to 50 mM and stored at -80°C. Chemical structure of nanaomycin A is shown in Figure 1A. 5-azacytidine, RG108 and procainamide were purchased by Sigma-Aldrich. 5-azacytidine and procainamide were dissolved in water; RG108 was dissolved in DMSO.

Cell culture

A549, HL60 and HCT116 cells were obtained directly from the ATCC and passaged in our laboratory for fewer than 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% CO₂. Determination of cell viability was performed by counting the viable cells after trypan blue staining. Therefore, cells were seeded in triplicates in 6-well plates at a density of 250,000 cells 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 cells per well and incubated for 72 hrs. 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 (32). Briefly, five micrograms of genomic DNA was 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: For_RASSF1A_1

GCCTCCCTCGCGCCATCAG TCAG GACA GTGGGGATTTTTTTTTTTTTTA
Rev_RASSF1A_1
GCCTTGCCAGCCCCGCTCAG TCAG GACA ACAACTCAATAAACAAC

Pair2:
For_RASSF1A_2
GCCTCCCTCGCGCCATCAG TCAG CACA GTGGGGATTTTTTTTTTTTTTA
Rev_RASSF1A_2
GCCTTGCCAGCCCCGCTCAG TCAG CACA ACAACTCAATAAACAAC

PCR conditions were as follows: initial denaturation at 95°C for 2 min, 35 cycles at 95°C for 30 sec, annealing at 40°C for 30 sec, elongation at 74°C for 74°C, and a final elongation step
at 74°C at 2 min. 454 deep sequencing was performed by the DKFZ 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 performed using the RNase-Free DNase Set (Qiagen). For quantitative RT-PCR, 1 µg of RNA were 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 performed by LightCycler 480 from Roche.

Western blot analysis

Antibodies for detection were used according to the manufacturer’s protocol. The antibodies against β-actin (sc-130301), DNMT1 (sc-10222), DNMT3B (sc-10235), and RASSF1A (sc-18722) were purchased by Santa Cruz Biotechnology (Germany) or by Millipore for γH2AX (clone JBW301, 05-636).

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 gelfiltration. The protein concentration of
purified DNMT was determined by Bradford assay and verified by using Coomassie bluestained 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 µM hemimethylated or unmethylated oligonucleotide substrate purchased from MWG (upper strand: 5´-GATCGCXGATGCGXGAATXGCGATXGATGCGAT-3´, X= 5mC for hemimethylated or X=C for unmethylated substrate, and lower strand: 5´-ATCGCATCGCATCGCATCGGCATCGGCGATC-3´), purified DNMT in reaction buffer (100 mM KCl, 10 mM TrisCl pH7.5, 1 mM EDTA) and BSA (1 mg/ml). All reactions were carried out at 37°C in the presence of 0.7 µM [methyl-3H] AdoMet (2.6 TBq/mmol, PerkinElmer). After 3 hrs the reaction was stopped by adding 10 µl 20% SDS and spotting of the whole volume onto DE81 cellulose paper. Filters were baked at 80°C for 2 hrs and washed three times with cold 0.2 M NH₄HCO₃, three times with distillated water and once with 100% ethanol. After drying filters were transferred into Mini-Poly Q vial from PerkinElmer and 5 ml Ultima Gold LSC Cocktail was added per vial. Analysis was done in a Scintillation counter, each measurement was repeated once.

Molecular docking

The structure of nanaomycin A was prepared with the program Molecular Operating Environment [Molecular Operating Environment (MOE), version 2008.10, Chemical Computing Group Inc., Montreal, Quebec, Canada]. Docking studies were conducted with
Glide [Glide, version 5.5, Schrödinger, LLC, New York, NY, 2009] and visualizations were carried out with Maestro [Maestro, version 9.0, Schrödinger, LLC, New York, NY, 2009]. Docking was performed using previously generated homology models of DNMT3B (17) and DNMT1 (33). The scoring grids were centered on the binding mode predicted for 2'-deoxycytidine as we reported previously (17). We used the following bounding box size 14Å x 6Å x 10Å, which covers the catalytic pocket and part of the co-factor binding site. We employed flexible docking with the extra precision (XP) mode in Glide and default parameters. In order 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 were 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 MOE’s LigX application were used.
Results

In an earlier study, we conducted a virtual screening of a public compound library from the National Cancer Institute 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 (Figure 1A, chemical structure) was ranked among the top ten percent of all screened compounds from two of the docking programs. However, this quinonionic 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 nM to 10 µM) for 72 hours, we observed in all three cell lines a distinct cytotoxic effect (Figure 1B). We determined the cellular viability by counting viable cells after trypan blue staining and measured IC\textsubscript{50} values of 400 nM for HCT116, 4100 nM for A549, and 800 nM for HL60. These data suggest a broad anti-proliferative 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. In order to elucidate the cellular mechanism of nanaomycin A-dependent cell death, we measured caspase-3 and -7 activities in nanaomycin A-treated cells. Therefore, cells were incubated for 72 hours with the IC\textsubscript{50} concentrations. However, when we compared untreated control cells to cells treated with the calculated IC\textsubscript{50} concentrations, we did not detect activation of caspase-3 and -7 (Figure 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 down regulation 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 (32) (Figure 2). We incubated cells for 72 hours with 500 nM for HCT116, 1000 nM for HL60 and 5000 nM for A549 and isolated genomic DNA. Genomic methylation levels were found to be most significantly \((p<0.001)\) reduced in HCT116 at 500 nM (from 3.8% to 2.2%), in HL60 at 1000 nM (from 3.4% to 2.6%), and in A549 at 5000 nM (from 2.7% to 1.1%). Although we used compounds concentrations in the range of the calculated IC\(_{50}\) value, we measured genomic demethylation also with lower nanaomycin A concentrations e.g. at 100 nM for HL60, or at 3000 nM 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 (5000 nM) A549 cells. We used 454 bisulfite sequencing of the RASSF1A promoter region with coverage rates between 450 and 600 reads per CpG (Figure 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 knock down in A549 cells (34).

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 μM), RG108 (300 μM) and procainamide (1 mM) as reference compounds (Figure 3B). We observed a nearly 6-fold relative induction after 5-azacytidine treatment and a 18-fold relative induction after treatment with nanaomycin A (5000 nM). Lower concentrations of nanaomycin A yielded lower relative RASSF1A induction, e.g. 3000 nM 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 (Figure 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. In order to experimentally test this hypothesis, we performed western blotting and quantitative RT-PCR of untreated and nanaomycin A (5000 nM) treated A549 cells and failed to detect any evidence for DNMT protein degradation (Figure 4A) or down regulated DNMT transcription levels (Figure 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 (Figure 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 gelfiltration (21). Strikingly, we found a pronounced selectivity of nanaomycin A towards DNMT3B in the tested compound range with an IC$_{50}$ value of 500 nM (Figure 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 analogue acting as a DNMT3B-selective inhibitor (35, 36).

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 (37). The final homology model showed a backbone root mean square deviation (RMSD) 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 for the catalytic site of human DNMT1 (33), 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 to nucleoside analogues (38). Treatment of MCF-7 cells with procaine reduced the genomic methylation level approximately 40% and reactivated transcription of the tumor suppressor gene RARß2, although the demethylation around the transcription start site was moderate (20). Procainamide treatment of HCT116 cells also induced global demethylation by approximately 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, 39-41).

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 play 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 knock down, blocking DNMT3B with a small-molecule inhibitor would abolish DNMT3B function without depleting it from the cellular proteomic pool. This will still permit e.g. 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 (42). 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 \(\alpha,\beta\)-unsaturated carbonyl system at the \(\beta\) carbon which proceeds on the less steric side (Fig S1 in Supplementary material). Noteworthy, in the docking model, the sulfur atom of Cys651 is close to the \(\beta\) 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) (Figure 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 isoform of nanaomycin A structure does result; the negative charge located on the oxygen is further stabilized by interaction with positive residues Arg731 and Arg733. This stabilization, plus the covalent addition of the thiol group (Cys651-S\(^{-}\)), 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 towards 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, though, as expected, at a lower level than observed for the non-selective 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 (43). Although the level of demethylation at the RASSF1A promoter region remained lower than that of the genome, it was still sufficient to reactivate transcription and expression of this gene and was comparable to the effects observed with other non-nucleoside inhibitors (20, 22). This result is also in agreement with the data obtained from antisense oligonucleotides-mediated knock down of DNMT3B in A549 cells (34), inducing anti-proliferative 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 (44), nanaomycin A represents the first non-SAH DNM3B selective compound and provides a valuable biochemical tool and benchmark for future studies.

Acknowledgments

Nanaomycin A was kindly supplied by the National Cancer Institute, Drug Synthesis and Chemistry Branch. This work was supported in part by the State of Florida, Executive Office of the Governor’s Office of Tourism, Trade, and Economic Development. J.L.M.-F. wishes to thank the Menopause & Women’s Health Research Center for funding. We thank Andre Leischwitz and Berit Haldemann from the DKFZ core facility for their support.
References


Figure legends

Figure 1. A. Chemical structure of nanaomycin A. B. Cell viability of HCT116, A549 and HL60 cell lines was determined by counting viable cells after trypan blue staining. Cells were treated with increasing doses of nanaomycin A ranging from 10 nM to 10 µM for 72 hours prior analysis. Number of viable cells per milliliter was plotted against nanaomycin A concentrations in nM. Each data point represents the mean ± SD of three wells and the data were analyzed by SigmaPlot Version 10.0. C. Activity of caspase-3 and -7 in HCT116, A549 and HL60 cells was measured by Caspase-Glo 3/7 after 72 hours of incubation with the calculated IC50 concentrations. Results are expressed relative to those obtained in the untreated control cells. Treatment with nanaomycin A showed no induction of caspases under these conditions.

Figure 2. Relative genomic cytosine methylation levels in percent of HCT116, HL60 and A549 were determined by capillary electrophoretic analysis. Cells were incubated for 72 hours with the indicated nanaomycin A concentration. Error bars represent standard deviation, and asterisks denote a statistically significant (P < 0.001) difference compared to the untreated control cells. Statistical significance was calculated by the Student’s T-Test. Nanaomycin A induced in all three cell lines a significant genomic demethylation compared to control cells.

Figure 3. A. 454 bisulfite sequencing of the RASSF1A promoter region in untreated and nanaomycin A-treated A549 cells. Cells were incubated for 72 hours with nanaomycin A (5000 nM), genomic DNA was isolated, bisulfite treated and the RASSF1A locus was amplified by PCR prior sequencing. Illustrated are the relative demethylation levels in percent.
of treated cells to untreated cells for thirty-one CpG sites within the RASSF1 promoter. Nanaomycin A treatment resulted in demethylation that was restricted to a few CpG sites. B. qRT-PCR analysis of RASSF1A transcription normalized to GAPDH in A549 cells after treatment with 5-azacytidine (AZA) (25 µM), RG108 (300 µM), procainamide (1 mM) and nanaomycin A (5000 nM) for 72 hours. Each data point represents the mean ± SD of three wells. Nanaomycin A and 5-azacytidine were able to reactivate RASSF1A transcription after treatment. C. Western blot of A549 cells treated with nanaomycin A (500 nM, 5000 nM) for 72 hours. Whole-cell lysates were assayed for expression of RASSF1A protein in the upper panel, respectively for β-actin in the lower panel. Fifty micrograms of total protein was loaded for HeLa cells as positive control and 250 µg for A549 samples. Nanaomycin A treatment revealed induction of RASSF1A protein expression in A549 cells.

Figure 4. A. Western blot analysis of A549 cells untreated (control) and treated with nanaomycin A (5000 nM) for 72 hours. Detection was carried out for DNMT1, DNMT3B, γH2AX, and β-actin as loading control. Displayed are representative western blots. Treatment of cells with nanaomycin A caused no significant changes on protein levels. B. Quantitative RT-PCR analysis of DNMT3B or DNMT1 expression normalized to GAPDH in A549 cells after treatment with nanaomycin A (5000 nM) and 5-azacytidine (AZA) (10 µM) as reference compound. Each data point represents the mean ± SD of three wells. Nanaomycin A induced no changes in transcript levels of DNMT3B or DNMT1.

Figure 5. A. Dose-response plots of nanaomycin A against DNMT1 and DNMT3B. The IC50 concentrations were determined by biochemical DNMT assays under identical conditions (500
nM DNMT, 0.7 µM AdoMet, 400 nM hemimethylated oligo). Each data point represents the mean ± SD of three measurements, and the data were analyzed by SigmaPlot Version 10.0.

B. Docking model of nanaomycin A with DNMT3B. Selected amino acid residues of the binding site are shown. Hydrogen bonds are indicated with magenta dashes. Non-polar hydrogen atoms are omitted for clarity.
A

Nanaomycin A

B

IC_{50} 400 nM

HCT116

C

IC_{50} 4100 nM

A549

IC_{50} 800 nM

HL60
A

-220 → +162

B

RASSF1A expression

C

RASSF1A

β-Actin

A549

Control, AZA 25µM, RG108 300µM, Procainamide 1mM, Nanaomycin A 5µM

x-fold induction

CpG sites in RASSF1A promoter

Relative demethylation in %
Molecular Cancer Therapeutics

Nanaomycin A selectively inhibits DNMT3B and reactivates silenced tumor suppressor genes in human cancer cells

Dirk Kuck, Thomas Caulfield, Frank Lyko, et al.

Mol Cancer Ther Published OnlineFirst September 10, 2010.

Updated version
Access the most recent version of this article at:
doi:10.1158/1535-7163.MCT-10-0609

Supplementary Material
Access the most recent supplemental material at:
http://mct.aacrjournals.org/content/suppl/2010/09/10/1535-7163.MCT-10-0609.DC1
http://mct.aacrjournals.org/content/suppl/2010/11/05/1535-7163.MCT-10-0609.DC2

Author Manuscript
Author manuscripts have been peer reviewed and accepted for publication but have not yet been edited.

E-mail alerts
Sign up to receive free email-alerts related to this article or journal.

Reprints and Subscriptions
To order reprints of this article or to subscribe to the journal, contact the AACR Publications Department at pubs@aacr.org.

Permissions
To request permission to re-use all or part of this article, contact the AACR Publications Department at permissions@aacr.org.