Mithramycin A inhibits DNA methyltransferase and metastasis potential of lung cancer cells

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Abnormal CpG island hypermethylation of multiple tumor-suppressor genes (TSGs) can lead to the initiation and progression of human cancer. The cytosine of the CpG island on the promoter region is methylated by 5′-cytosine-methyltransferases (DNMTs). Pharmacologic inhibitors of CpG island methylation provide a rational approach to reactivate the TSGs in tumor cells and to restore the critical cellular pathways in cancer cells. Mithramycin A (MMA) is known to be a GC- and CG-rich DNA-binding agent. We sought to determine whether MMA could inhibit CpG island methylation and DNMT expression in lung cancer cells. We found that MMA reduced the CpG island methylation of antimetastasis TSGs, including *SLIT2* and *TIMP-3* genes, and was associated with the prevention of metastasis. When highly metastatic CL1-5 lung cancer cells were treated with low doses (10 nmol/l) of MMA for 14 days, they reexpressed mRNA levels for these genes. MMA also inhibited the invasion phenotypes of CL1-5 cells as indicated by its inhibition of cancer cell migration using wound-healing and transwell assays. Molecular docking of MMA onto the DNMT1 catalytic domain revealed that MMA might interact with the catalytic pocket of DNMT1. Western blots showed that DNMT1 protein levels were depleted after MMA. These data support the idea that MMA has demethylation and antimetastasis effects on lung cancer cells. This mechanism might be mediated by the interaction of MMA and DNMT1, leading to the depletion of the DNMT1 protein and the reversal of the metastasis phenotype in lung cancer cells. *Anti-Cancer Drugs* **18:1157–1164**

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Introduction

CpG island methylation is a common feature of many human cancers, and is thought to play an important role in cancer initiation and progression [1–3]. Abnormal hypermethylation of CpG islands on tumor-suppressor genes (TSGs) can lead to transcriptional silencing, including reduced gene expression of $p14^{ARF}$, $p16^{INK4a}$, *RARβ*, *RASSF1A*, *DAP-kinase*, *SLIT2*, *tissue inhibitor of metalloproteinase 3* (*TIMP-3*) and *hMLH1*, and tumorigenesis [1–6]. Cytosines in the CpG islands of the promoter regions of these genes are methylated by 5′-cytosine-methyltransferases (DNMTs), which have been identified and are associated with TSG hypermethylation [7,8]. Overexpression of DNMTs has been reported for various malignancies, including hepatomas, and prostate, breast and lung tumors [8–11]. Studies show that increased DNMTs in human cancers are associated with metastasis and poor prognosis [8–10]. Overexpression of DNMTs in cancers also creates novel therapeutic targets and encourages the search for inhibitors of DNMTs as anticancer treatments. Moreover, these epigenetic changes are potentially reversible, in contrast to genetic alterations, which generally are not. Pharmacologic inhibitors of DNA methylation thus provide an attractive and rational approach to the reversal of the epigenetic silencing of TSGs, with the hope that they will reactivate those genes in the tumor cells and restore activity in critical cellular pathways [12].

The first extensively studied DNMT inhibitors were 5-azacytidine (Vidaza) and 5-aza-2′-deoxycytidine (Decitabine). These nucleoside analogs were incorporated into DNA in place of the natural base cytosine during DNA replication. They covalently bound to the active sites of DNMTs, inhibiting the enzymatic activity of DNMTs [13]. Unfortunately, in clinical trials they showed side effects such as hematopoietic toxicity and neutropenia [1,14,15]. They were also unstable in aqueous solution, making them difficult to apply both experimentally and clinically [16]. Antisense oligonucleotides such as MG98 have also been investigated in phase II trials in patients with metastatic renal carcinoma. They, however, showed no clear evidence of antitumor activity, which can be explained by a lack of ability to interact with the target [17]. Therefore, there is an urgent need now for the development of effectual and low-toxicity inhibitors of DNMTs.

One potential inhibitor is mithramycin A (MMA, plicamycin), an anticancer antibiotic. MMA is a member of a group of aureolic acid-type polyketides that are

produced by the soil bacterium *Streptomyces argillaceus* [18]. MMA binds to GC-rich or CG-rich DNA sequences [19,20]. In clinical studies, MMA has been used for the treatment of Paget's disease and tumor-related hypercalcemia [21,22]. It has also been used to treat various types of cancer, including chronic myeloid leukemia and testicular carcinoma [23,24]. MMA enhanced tumor necrosis factor-induced apoptosis in human erythroleukemic TF-1 cells and prevented the development of resistance to the chemotherapeutic agent adriamycin, by downregulation of the mutidrug resistance gene 1 and depletion of P-glycoprotein [25,26]. Recent studies suggest a more specific mechanism for MMA effects based on its ability to bind preferentially to GC-rich or CG-rich regions of DNA. It was found that MMA interferes with expression of genes bearing GC-rich DNA motifs in their promoters for the consensus sequences of the transcription factor, Sp1 [27]. For this study, we hypothesized that MMA binds to the CpG promoter regions of TSGs and blocks the methylating effects of DNMT enzymes.

Materials and methods

Cell culture and drug treatment

The cell line CL1-5, which has a high potential for metastasis, was generated from CL1-0 by progressive selections through the invasion chamber [28], and was cultured in RPMI (Invitrogen, Carlsbad, California, USA). The cell line was provided by Dr. Pan-Chyr Yang (Department of Internal Medicine, National Taiwan University Hospital, Taipei, Taiwan). The A549 and H647 cell lines we used were derived from lung tumors cultured in Dulbecco's modified Eagle's medium and RPMI medium, respectively (Invitrogen). MRC-5 cells are a human lung normal fibroblast cell line and were cultured in Dulbecco's modified Eagle's medium (Invitrogen). The cell lines including A549, H647 and MRC-5 were purchased from American Type Culture Collection (Manassas, Virginia, USA). All media were supplemented with 10% fetal bovine serum (Invitrogen) and 1% penicillin/streptomycin (Invitrogen).

Cell cytotoxicity assay

Cells (1×10^5) were plated in six-well culture dishes and incubated at 37°C in a 5% CO_2 atmosphere. On the following day, cells were treated with MMA (Sigma, St Louis, Missouri, USA) at concentrations of 10, 20 or 50 nmol/l for 48 h at 37°C. Cytotoxicity was measured as a decrease in the number of viable cells counted. The cell number was determined by direct counting using Trypan blue dye exclusion to identify the viable cells. Dimethyl sulfoxide (0.1%) rather than MMA was added to control cultures.

Methylation-specific PCR assay for the *SLIT2* and *TIMP-3* genes

The promoter methylation status of the *SLIT2* and *TIMP-3* genes was determined by chemical treatment with sodium bisulfite and subsequent methylation-specific PCR analysis and sequencing analyse, as described previously [29–31]. All PCRs were performed with positive controls for both unmethylated and methylated alleles, and with no DNA control.

Wound-healing assay for migration analysis

CL1-5 cells were treated with 10 nmol/l MMA for 14 days. Treated and untreated cells were then plated at a density of 1×10^6 cells in 10-cm culture dishes and incubated at 37°C. The following day, cells were cultured in fresh medium with 10% serum and 1% penicillin/streptomycin. A scratch in the form of a lane was made through the confluent monolayers with a plastic pipette tip. Several wounded areas were observed and then photographed through a microscope 24 h after the scratch.

Transwell assay for invasion analysis

CL1-5 cells were treated with 10 nmol/l MMA for 14 days and then plated in 12-well plates (1×10^6 cells/well). Each well had an upper chamber containing a suspension of cells separated by an 8-μm membrane (Falcon, Franklin Lake, New Jersey, USA) from a lower chamber containing medium with attractant (10% serum). We seeded the cells to an upper chamber and observed the cells invading the lower chambers. After allowing the cells to migrate for 16 h, they were removed from the upper side of the membrane with a cotton swab. Cells on the lower side of the membranes were fixed in 1% formaldehyde and stained with 0.1% crystal violet. After mounting and photographing, treated and untreated CL1-5 cells that migrated across the membrane were counted in five independent visual fields under microscopy.

RNA extraction and semiquantitative reverse transcriptase-PCR assay

Total RNA was extracted using Trizol reagent (Invitrogen). cDNA was synthesized using SuperScriptTM reverse transcriptase (Invitrogen) with the protocols being provided by the manufacturer. Expression levels of *DNMT1*, *SLIT2*, *TIMP-3*, $p21^{WAF}$, *CDH1* (*E-cadherin*) and *Caspase-3* were detected using the *β-actin* gene as an internal control. The primer nucleotide sequences of *DNMT1*, *SLIT2 and TIMP-3* and their PCR conditions were as described previously [30,32,33]. The primers used for mRNA analysis were TCACCGAGACACC ACTGGAG (forward) and TGGAGTGGTAGAAATCTG TC (reverse) for *p21*; CGGGAATGCAGTTGAGGATC (forward) and AGGATGGTGTAAGCGATGGC (reverse) for *CDH1;* and TTTTTCAGAGGGGATCGTTG (forward) and CGGTTAACCCGGGTAAGAAT (reverse) for *caspase-3*.

To quantify the relative levels of mRNA expression in the reverse transcription (RT)-PCR assay, the value of the internal standard (*β-actin*) in each reaction was used to

quantify the baseline gene expression of that sample. Relative values were calculated for *DNMT1*, *SLIT2*, *TIMP-3*, *p21^{WAF}*, *CDH1* and *Caspase-3* genes for untreated and treated samples. The number of cycles and the amount of primers and cDNA used were determined, to provide a quantitative amplification during multiplex RT-PCR.

Cell lysis and Western blot

Cells were lysed on ice using radioimmunoprecipitation buffer (0.05 mol/l Tris–HCl, pH 7.4, 0.15 mol/l NaCl, 0.25% deoxycholic acid, 1% NP-40, 1 mmol/l ethylenediaminetetraacetic acid, 0.5 mmol/l dithiothreitol, 1 mmol/l phenylmethylsulfonyl fluoride, 5 μg/ml leupeptin and 10 μg/ml aprotinin). Lysates were then centrifuged at 13 000 r.p.m. at 4°C for 10 min. Protein extracts were solubilized in sodium dodecyl sulfate (SDS) gel-loading buffer (60 mmol/l Tris base, 2% SDS, 10% glycerol and 5% β-mercaptoethanol). Samples containing equal amounts of protein (50 μg) were separated on an 8% SDS–polyacrylamide gel electrophoresis and electroblotted onto Immobilon-P membranes (Millipore, Bedford, Massachusetts, USA) in a transfer buffer. Immunoblotting was performed using antibodies against DNMT1 (1 : 2000; Asia Hepato Gene, Kaohsiung, Taiwan) and β-actin (1 : 1500; Abcam, Cambridge, UK) as an internal control. Each Western blot analysis was repeated three times.

Molecular modeling and docking of DNMT1 with mithramycin A

The protein sequence of DNMT1 (NP_001370) was retrieved from the National Center of Biotechnology Information (NCBI) and used to search for templates by phi-BLAST [34] against the Protein DataBank (PDB). Multiple alignments of the sequences within the DNMT1 catalytic domain and structural templates including DNMT2 (1G55) [35], M. HaeIII (1DCT) [36] and M. HhaI (6MHT) [37] were carried out using CLUSTALW [38]. The structural model of the DNMT1 catalytic domain was built using the HOMOLOGY module of the INSIGHT2005 (Accelrys, San Diego, California, USA), based on multiple alignments. The model was minimized in 200 steps using the DISCOVER3 module with a consistent valence force field. Molecular mechanics minimization was then performed on the model soaked with water until the maximum derivative was less than 0.001 kcal/mol Å. Finally, the model was qualified by PROCHECK [39] and used for docking experiments. The pocket of the binding site was defined by the residues within 5.0 Å of the cytosine nucleotide of the hemimethylated DNA. Molecular docking of the DNMT1 catalytic domain with MMA was performed and evaluated using the LigandFit program of the Discover Studio Modeling environment (Accelrys).

Results

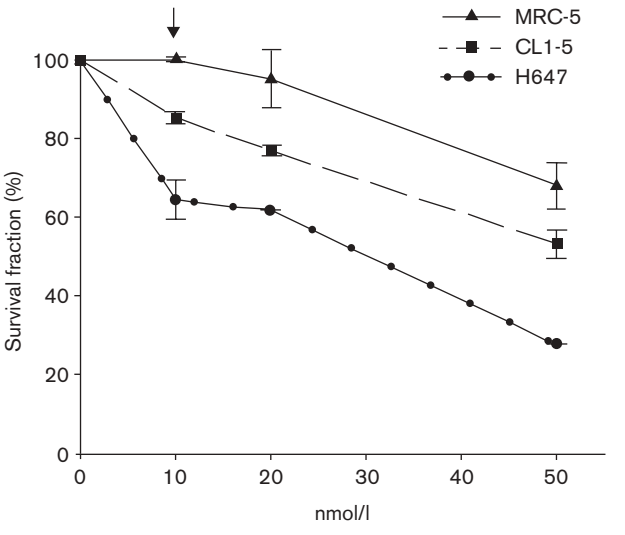
Growth inhibition of cancer cells, but not normal lung cells, by low doses of mithramycin A

Clinical toxicity is a major concern in the clinical applications of anticancer agents. Therefore, we treated normal and cancer cells with a range of low concentrations of MMA (10, 20 and 50 nmol/l) for 48 h. The IC_{50} values were 79.5, 55.3 and 34.7 nmol/l for normal MRC-5 lung cells, CL1-5 lung cancer cells and H647 lung cancer cells, respectively. Interestingly, treatment with 10 nmol/l of MMA inhibited the growth of human cancer cell lines, but not of normal human fibroblasts (Fig. 1). As MMA showed low toxicity to normal cells, we used the low dose to treat cancer cells in all subsequent studies. The results indicated that the cell viability after treating with 10 nmol/l MMA for 14 days was around 90% compared with that of untreated cells (data not shown).

Reversal of hypermethylation on *SLIT2* and *TIMP-3* promoters, and the reactivation of their mRNA expression by mithramycin A

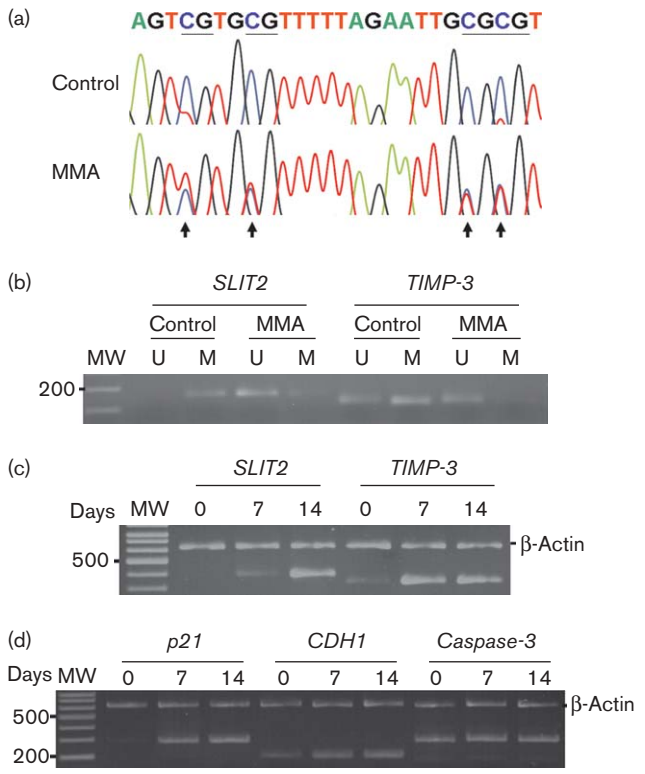
As MMA is a GC-rich DNA-binding agent, we speculated that it might bind to CpG regions and block DNMT methylation activity. We treated CL1-5 cells, whose antimetastatic TSGs such as *SLIT2* and *TIMP-3* are downregulated by promoter hypermethylation, with MMA in a low dose (10 nmol/l) for several doubling times (14 days). The methylation-specific analysis and bisulfite sequencing showed that MMA decreased the methylation of the *SLIT2* and *TIMP-3* promoters (Fig. 2a and b), and concomitantly increased the expression of mRNA for *SLIT2* and *TIMP-3* genes (Fig. 2c).

Fig. 1



The cytotoxicity of MRC-5, CL1-5 and H647 cells treated with 0, 10, 20 and 50 nmol/l of MMA for 48 h. Note that no cytotoxicity (see arrow) was found when normal MRC-5 lung fibroblast cells were treated with 10 nmol/l MMA. MMA, mithramycin A.

Fig. 2



Demethylation and reexpression effects of MMA on the *SLIT2* and *TIMP-3* genes of CL1-5 cells. (a) The chromatograms of the bisulfite sequencing of the CpG island in the *SLIT2* promoter showed that MMA treatment increased unmethylated pick (in red) compared with methylated pick (in blue) indicated by arrow. (b) Demethylation was assayed by a methylation-specific PCR of *SLIT2* and *TIMP-3* in a lung cancer cell line after 14 days of 10 nmol/l MMA. The increase of the unmethylated product (U) and decrease of the methylated product (M) after MMA treatment indicates demethylation of the promoter. (c) Reverse transcription-PCR analysis of mRNA expression of the *SLIT2* and *TIMP-3* genes in CL1-5 cells. (d) mRNA expression of Sp1 downstream genes including *p21*, *CDH1* and *Caspase-3* after MMA treatment at 10 nmol/l for 14 days. MMA, mithramycin A; MW, molecular weight.

Inhibition of motility of CL1-5 cells after mithramycin A-induced reactivation of *SLIT2* and *TIMP-3* genes

SLIT2 and *TIMP-3* genes are candidate antimetastasis TSGs [4,30,31,40]. To examine whether the reactivation of *SLIT2* and *TIMP-3* could lower the motility of CL1-5 lung cancer cells, we carried out wound-healing and transwell assays after treating CL1-5 cells with 10 nmol/l MMA for 14 days. In wound-healing assays, MMA markedly decreased the migration of CL1-5 cells. Untreated CL1-5 cells migrated rapidly and filled in the wound significantly faster than the treated cells (Fig. 3a). The anti-invasion effect of MMA was also observed in transwell assays. A greater number of untreated cells moved through the pores of the membranes compared with MMA-treated cells (Fig. 3b). Quantitative data from the photographs of five different visual fields indicated that MMA significantly lowered the ability of CL1-5 cells to be invasive (Fig. 3c, $P < 0.001$). This implies that MMA can inhibit cellular migration and that this inhibition might be mediated by a reactivation of multiple antimetastatic TSGs.

Molecular modeling of the interaction between mithramycin A and DNMT1

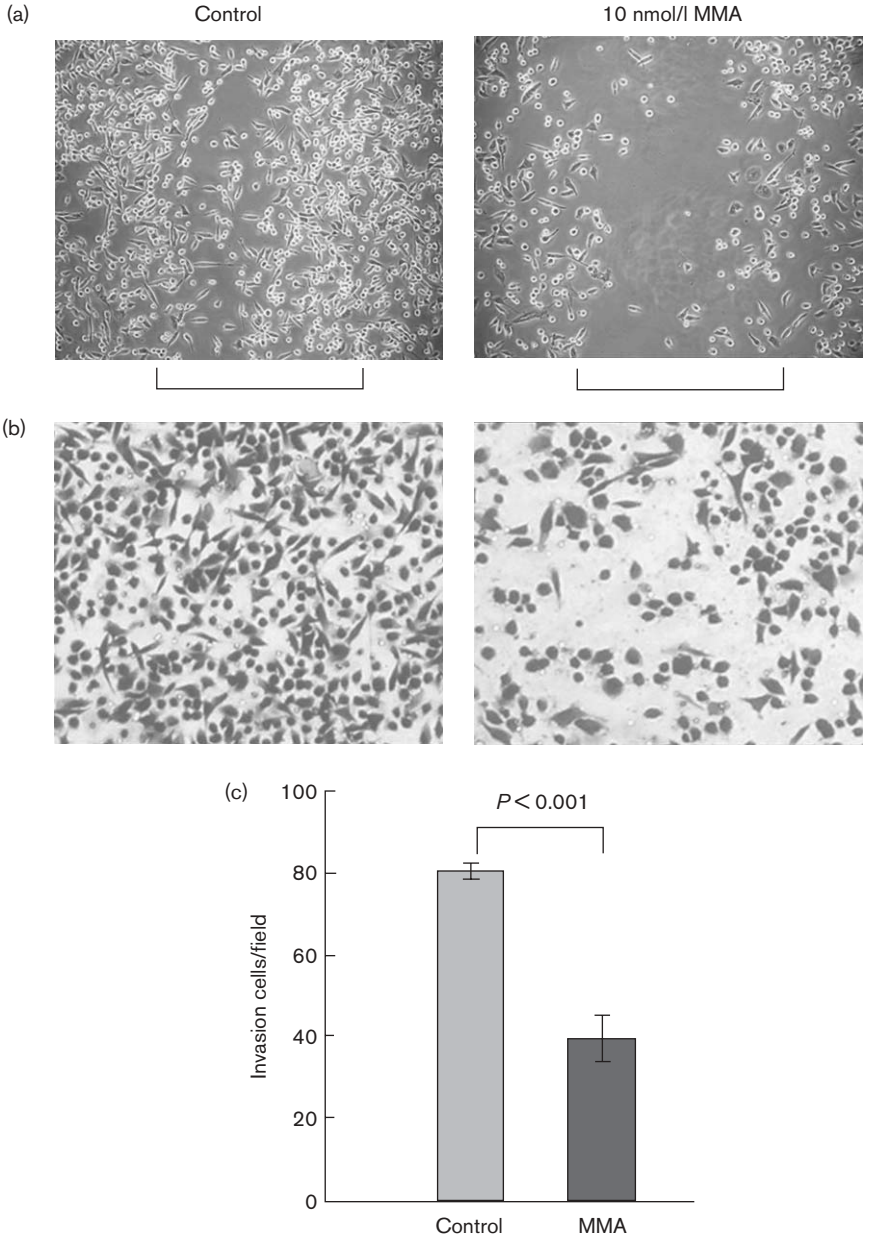
As both DNMTs and MMA can bind to CG-rich DNA sequences, we postulated that there could be an interaction between DNMTs and MMA. The solution structures of MMA bound to DNA have been reported [41]. To investigate this putative inhibition mechanism, the three-dimensional structure of the DNMT1 catalytic domain was modeled on the basis of the various available crystal structures of DNMTs [35–37]. Then we illustrated the possible binding mode of the MMA/DNMT1 complex using the in-silico docking method, which has been applied to other DNMT1 inhibitors [42] A feasible model, which had been folded by evolutionarily conserved motifs identified from multiple sequence alignments, revealed a substantial binding region with hemimethylated DNA and the flipped cytosine. Docking of MMA onto the DNMT1 catalytic domain indicated that the trisaccharide of MMA (Fig. 4a) could fit into the putative cytosine pocket and the aglycon chromophore lay on the space between the two arms that fasten up the hemimethylated DNA (Fig. 4b; left). Interestingly, the methyl group of the second sugar ring in the MMA trisaccharide seems to be oriented to the same position as that of the methylated cytosine in hemimethylated DNA (Fig. 4b; right).

Depletion of DNMT1 protein levels by mithramycin A

To analyze further whether the demethylating effect of MMA is mediated by the inhibition of DNMT1, we performed Western blot assays to detect protein levels of DNMT1 during MMA treatment. Interestingly, the levels of the DNMT1 protein were decreased more by treatment with 10 nmol/l MMA for 14 days than by treatment with dimethyl sulfoxide only or with 50 nmol/l MMA for 48 h in CL1-5 and A549 lung cancer cells (Fig. 5a). Note that a light supershift band of DNMT1 was observed in the Western blot in the CL1-5 cells (Fig. 5a).

Previous studies have shown that MMA is an Sp1 inhibitor – Sp1 is a transcription factor for the DNMT1 gene [27,43], and also for the *p21*, *CDH1* and *caspase-3* genes [44–46]. To determine whether the depletion of DNMT1 protein by MMA was due to its inhibition of Sp1 transactivation of the *DNMT1* promoter, in a reduction in *DNMT1* mRNA expression, we performed an RT-PCR to examine the *DNMT1* mRNA expression. A low dose of MMA, however, did not decrease the mRNA expression of the *DNMT1* (Fig. 5b) and other Sp1 target genes such as *p21*, *CDH1* and *caspase-3* (Fig. 2d) between MMA-treated or MMA-untreated cells. This suggests that

Fig. 3



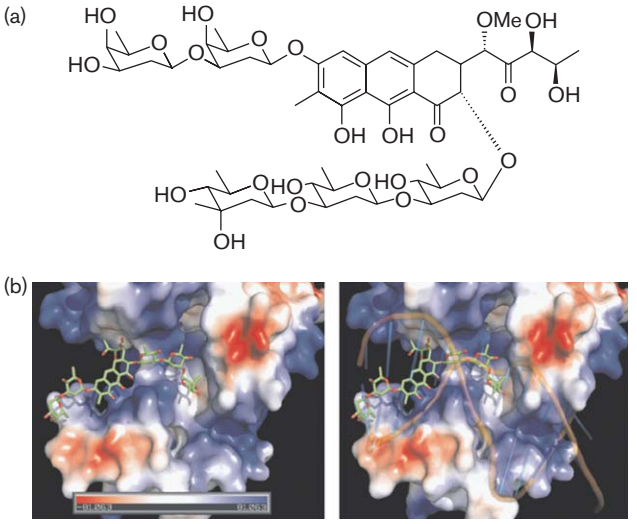
The mobility of CL1-5 cells decreased after treatment with 10 nmol/l MMA for 14 days. (a) Wound-healing assay for migration activity of untreated (left panel) and treated (right panel) CL1-5 cells. Representative images of the migrating cells were captured at 24 h after the scratch (original magnification $\times$ 100). (b) Photo images representing transwell invasion assays in untreated (left panel) and treated (right panel) cells (original magnification $\times$ 200). (c) Quantitative analysis of the inhibition of invasion effects in untreated (gray bar) and treated (black bar) CL1-5 cells. The average number of invasive cells (those that passed through the membrane) was counted in five different fields. MMA significantly decreased the invasion of the CL1-5 cells ($P < 0.001$). MMA, mithramycin A.

DNMT1 depletion by MMA is not due to changes in transcription.

Discussion

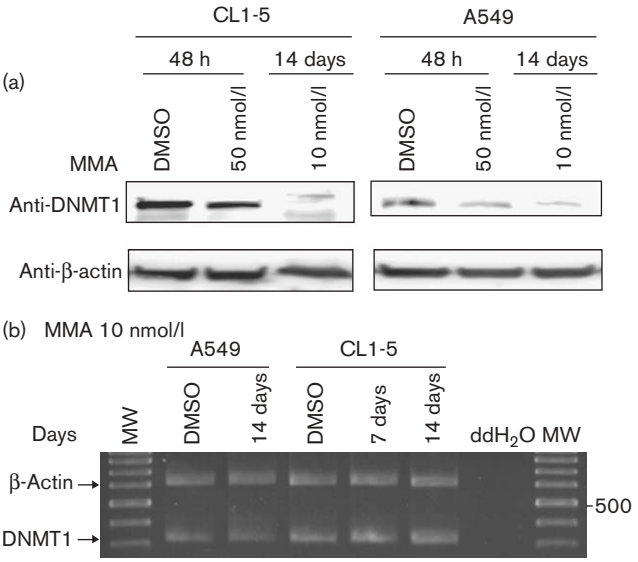
Aberrant DNA hypermethylation on promoter region of TSGs is a key mechanism for tumorigenesis [1,2]. Consequently, researchers are now searching for potential demethylating agents that can reactivate many TSGs in tumor cells, and possibly lead to the suppression of cancer cell growth and invasion. In this study, we discovered the novel effects of MMA including demethylation and reactivation of TSGs. These effects may be mediated via binding with the catalytic domain of DNMT1 and depletion of DNMT1 protein levels. Although MMA is

Fig. 4



Molecular docking model of the DNMT1 catalytic domain bound with MMA or hemimethylated DNA. (a) The structure of MMA contains five sugar rings, an aglycon chromophore and one unique dihydroxymethoyoxopentyl side chain. (b) Left, close-up view of the consensus orientation for MMA in DNMT1 is shown. MMA is represented in stick form and color by atom type with carbon in green, oxygen in red and nitrogen in blue. Right, docking of MMA and hemimethylated DNA (brown line) into the catalytic pocket of DNMT1. The second sugar ring in the trisaccharide of MMA was in the overlapped region with the flipped cytosine of DNA. MMA, mithramycin A.

Fig. 5



The depleting effect of MMA on DNMT1 protein levels in A549 and CL1-5 lung cancer cell lines. (a) Western blot analysis of DNMT1 protein levels when maintained in 50 nmol/l MMA for 48 h or in 10 nmol/l for 14 days. (b) Reverse transcription-PCR analysis of mRNA expression of the *DNMT1* genes in A549 and CL1-5 cell lines at 10 nmol/l at the indicated times. DMSO, dimethyl sulfoxide; MMA, mithramycin A; MW, molecular weight.

recognized as an Sp1 inhibitor, it blocked binding of Sp1 to its target genes at concentrations of 100 nmol/l to 90 μmol/l (for 48 h) in previous studies [26,27,44]. We found that a low dose (10 nmol/l) of MMA did not decrease the downstream gene expression of Sp1 target genes such as *DNMT1*, *p21*, *CDH1* and *caspase-3*. This suggests that such a low concentration of MMA specifically affects methylated TSGs rather than the Sp1 target genes. This study is the first to show that low doses of MMA over prolonged periods inhibit DNMT1 protein levels.

CL1-5 cells, which are highly metastatic, are hypermethylated on the promoter regions of *SLIT2* and *TIMP-3* genes. We found that the *SLIT2* and *TIMP-3* genes were demethylated and reexpressed after MMA treatment. This treatment led to several morphological changes such as bigger cell size with more adhesiveness and fewer filopodia. Moreover, MMA also diminishes the motility of the CL1-5 cells. We believe that MMA might affect other antimetastatic TSGs. A genome-wide screening to identify other antimetastasis-associated genes that are affected by MMA is now needed. In addition, the inhibition of DNMT1 by MMA can lead to a global change in the chromatin structure because DNMT1 is known to cooperate with many chromatin modifiers for epigenetic control [1].

An additional benefit of using low-dose MMA is that it showed no cytotoxicity to normal lung cells. At such low concentrations, MMA preferentially targets cancer cells (such as H647 and CL1-5), which show overexpression of DNMT1 proteins (data not shown). Overexpression of DNMTs has previously been reported in various malignancies [8–11]. Therefore, MMA should attack tumor cells more selectively than the more conventional anticancer drugs. If this were true, MMA can be applied to the treatment of many cancer types.

MMA is recognized as a CG-rich DNA-binding agent, implying that it might also have a similar inhibitory effect against DNMT as does the compound procaine. Procaine has been found to possess demethylating ability, possibly by competing with DNMT for the GC sequences [47]. We propose that MMA interferes with DNMT1 binding at the CpG region in TSG promoters through two possible reaction mechanisms. First, MMA might directly bind to the DNMT1 protein. Alternatively, there might be a triplex complex formed with MMA, DNMT1 and double-stranded DNA. We are now conducting more biochemical and biophysical experiments to confirm these possible mechanisms. In addition, both mechanisms will lead to the depletion of the functional DNMT1 enzyme in treated cells, similar to the effect of 5-azacytidine and 5-aza-2′-deoxycytidine [48]. In contrast to 5-azacytidine and 5-aza-2′-deoxycytidine, MMA binds

reversibly to DNA rather than being incorporated into the DNA. Therefore, it is unlikely to have the inherent toxicity caused by the covalent trapping of the enzyme. Of course, these mechanisms will need to be confirmed. We are also currently testing whether the MMA affects DNMT3a and DNMT3b proteins as well. Moreover, there are several aureolic acid analogues of MMA that have been found, such as mithramycin SK [27]. Other MMA analogues can inhibit DNMT1 protein and are worthy investigating further.

Our study found that MMA can reverse the expression of TSGs by reducing their promoter methylation. This is mediated by the binding and depletion of DNMT1 protein. Moreover, MMA can act as a metastasis inhibitor through the reactivation of antimetastasis-associated genes in lung cancer cells.

Acknowledgements

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