A DNA Methyltransferase I Inhibitor Mithramycin A in Cancer Cells: A Pilot Study

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(Received: 12 April 2007, accepted: 9 May 2007)

ABSTRACT

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 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 restoring of 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 CpG island methylation of anti-metastasis TSGs, *SLIT2*, which associated with the prevention of metastasis. When highly metastatic CL1-5 lung cancer cells were treated with low does (10 nM) of MMA for 14 days, they re-expressed mRNA levels for *SLIT2* genes. MMA also inhibited the invasion phenotypes of CL1-5 cells as indicated by its inhibition of cancer cell migration using transwell assays. Western blots showed that DNMT1 protein levels were depleted after MMA. These data support the idea that MMA has demethylation and anti-metastasis effects on lung cancer cells. This inhibitory mechanism of MMA in DNA methylation may be mediated by the depletion of DNMT1 protein.

Key words: mithramycin A, DNMT, metastasis, lung cancer

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 (Esteller, 2005; Momparler, 2003; Nephew *et al.*, 2003). 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 (Dammann *et al.*, 2005; Esteller, 2005; Hsu *et al.*, 2004; Momparler, 2003; Nephew *et al.*, 2003; Wang *et al.*, 2003). 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 (Lin *et al.*, 2007; Robert *et al.*, 2003). Overexpression of DNMTs has been reported for various malignancies, including hepatomas and prostate, breast and lung tumors (Girault *et al.*, 2003; Lin *et al.*, 2007; Patra *et al.*, 2002; Saito *et al.*, 2003). Studies show that increased DNMTs in human cancers are associated with metastasis and poor prognosis (Girault *et al.*, 2003; Lin *et al.*, 2007; Saito *et al.*, 2003). 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 are generally not. Pharmacologic inhibitors of DNA methylation thus provide an attractive and rational approach to reversal of epigenetic silencing of TSGs, with the hope that they will reactivate those genes in tumor cells and restore activity in critical cellular pathways (Hellebrekers *et al.*, 2007).

The first extensively studied DNMT inhibitors were 5-azacytidine (Vidaza) and 5-aza-2'-

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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 (Juttermann *et al.*, 1994). Unfortunately, in clinical trials they showed side effects such as hematopoietic toxicity and neutropenia (Aparicio *et al.*, 2003; Esteller, 2005; Issa *et al.*, 2004). They were also unstable in aqueous solution, making them difficult to apply both experimentally and clinically (Beisler, 1978). Antisense oligonucleotides such as MG98 have also been investigated in Phase II trials in subjects with metastatic renal carcinoma, but showed no clear evidence of antitumor activity, which may be explained by a lack of ability to interact with the target (Winquist *et al.*, 2006). Therefore, there is an urgent need now for the development of effectual and low toxic 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* (Fig. 1) (Ming, 2003). MMA binds to GC or CG-rich DNA sequences (Carpenter *et al.*, 1994; Fox *et al.*, 1985). In clinical studies, MMA has been used for treatment of Paget's disease and tumor-related hypercalcemia (Hadjipavlou *et al.*, 2001; Koh, 2003). It has also been used to treat various types of cancer, including chronic myeloid leukemia and testicular carcinoma (Kennedy *et al.*, 1995; Koller *et al.*, 1986). MMA enhanced tumor necrosis factor-induced apoptosis in human erythroleukemic TF-1 cells, and prevented development of resistance to the chemotherapeutic agent adriamycin, by downregulation of the mutidrug resistance gene 1 and depletion of P-glycoprotein (Duverger *et al.*, 2004; Tagashira *et al.*, 2000). Recent studies suggest a more specific mechanism for MMA effects based on its ability to bind preferentially to GC 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 (Remsing *et al.*, 2003). For the present study, we hypothesized that MMA binds to CpG promoter regions of TSGs and blocks the methylating effects on TSG promoters of DNMT enzymes.

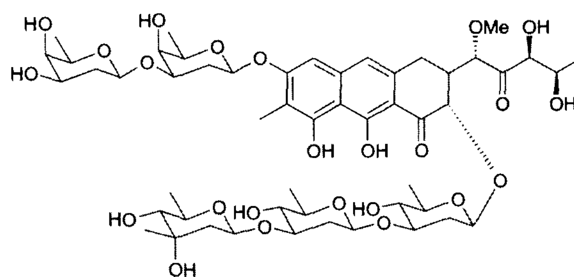

Figure 1. The structure of MMA. The drug contains five sugar rings, an aglycon chromophore, and one unique dihydroxymethoyoxopentyl side chain.

Materials and Methods

Cell Culture and Drug Treatment

The cell line CL1-5, which has a high potential for metastasis, was kindly provided by Dr. Pan-Chyr Yang (Department of Internal Medicine, National Taiwan University Hospital, Taipei, Taiwan) and cultured in RPMI (Invitrogen). The A549 and H647 cell lines we used are derived from lung tumors cultured in DMEM and RPMI medium, respectively (Invitrogen, Carlsbad, CA, USA). MRC-5 cells are a human lung normal fibroblast cell line and were cultured in DMEM medium (Invitrogen). The cell lines including A549, H647 and MRC-5 were purchased from American Type Culture Collection. 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 6-well culture dishes and incubated at 37°C in a 5% CO_2 atmosphere. The following day cells were treated with MMA (Sigma Chemical Co., St. Louis, MO, USA) at concentrations of 10 nM, 20 nM or 50 nM 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 viable cells. DMSO (0.1%) rather than MMA was added to control cultures.

Methylation-specific PCR Assay for the SLIT2 Genes

The promoter methylation status of the *SLIT2* gene was determined by methylation-specific PCR (MSP) analysis as previously described (Dallol *et al.*, 2002; Herman *et al.*, 1996). The PCR were

performed with positive controls for both unmethylated and methylated alleles and with a no DNA control.

Transwell Assay for Invasion Analysis

CL1-5 cells were treated with 10 nM MMA for 14 days and then plated in 12-well plates (1 x 10^6 cells per well). Each well had an upper chamber containing a suspension of cells is separated by an 8.0 µm membrane (Falcon, Franklin lake, USA) from a lower chamber containing medium with attractant (10% serum). We seeded the cells to an upper chamber and observed the cells invading into lower chambers. After allowing cells to migrate for 16 h, cells 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, the number of treated and untreated CL1-5 cells that migrated across the membrane was counted in five independent visual fields under microscopy.

RNA Extraction and Semiquantitative Reverse Transcriptase-Polymerase Chain Reaction (RT-PCR) Assay

Total RNA was extracted using Trizol reagent (Invitrogen). cDNA was synthesized using SuperScriptTM reverse transcriptase (Invitrogen) with the protocols provided by the manufacturer. Expression levels of *DNMT1*, *SLIT2* were detected using the *ß-actin* gene as an internal control. The primer nucleotide sequences of *DNMT1* and *SLIT2* genes and their PCR conditions were as described previously (Dallol *et al.*, 2002; Mizuno *et al.*, 2001).

To quantify the relative levels of mRNA expression in the RT-PCR assay, the value of the internal standard (*ß-actin*) in each reaction was used to quantify baseline gene expression of that sample and relative values were calculated for *DNMT1* and *SLIT2* genes for untreated and treated samples. The number of cycles and the amount of primers and the cDNA used were determined to provide quantitative amplification during multiplex RT-PCR.

Cell Lysis and Western Blot

Cells were lysed on ice using RIPA buffer (0.05M Tris-HCl, pH 7.4, 0.15M NaCl, 0.25% deoxycholic acid, 1% NP-40, 1mM EDTA, 0.5 mM DTT, 1 mM phenylmethylsulfonyl fluoride, 5 µg/ml leupeptin, 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 SDS gel loading buffer (60 mM Tris base, 2% SDS, 10% glycerol, and 5% ß-mercaptoethanol). Samples containing equal amounts of protein (50 µg) were separated on an 8% SDS-PAGE and electroblotted onto Immobilon-P membranes (Millipore Co., Bedford, MA, USA) in a transfer buffer. Immunoblotting was performed using antibodies against DNMT1 (1:2000, Asia Hepato Gene Co., Kaohsiung, Taiwan) and ß-actin (1:1500, Abcam Ltd., Cambridge, UK) as an internal control. Each western blot analysis was repeated three times.

Results

Growth inhibition of cancer cells, but not normal lung cells, by low does of MMA

Clinical toxicity is a major concern for clinical applications of anticancer agents. Therefore, we treated normal and cancer cells with a range of low concentrations of MMA (10 *n*M, 20 *n*M and 50 *n*M) for 48 hours. The IC_{50} values were 79.5 *n*M, 55.3 *n*M, and 34.7 *n*M for normal MRC-5 lung cells, CL1-5 lung cancer cells, and H647 lung cancer cells, respectively. Interestingly, treatment with 10 *n*M of MMA inhibited the growth of human cancer cell lines but not the growth of normal human fibroblasts (Fig. 2). Because low doses of MMA showed low toxicity to normal cells, we used 10 *n*M to treat cancer cells in all subsequent studies.

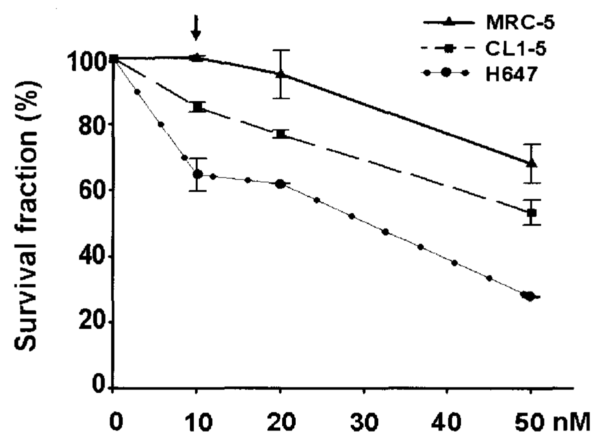

Figure 2. The cytotoxicity of MRC-5, CL1-5 and H647 cells treated with 0, 10 *n*M, 20 *n*M and 50 *n*M of MMA for 48 hours. Note that no cytotoxicity (see arrow) was found when normal MRC-5 lung fibroblast cells were treated with 10 *n*M MMA.

Reversal of hypermethylation on SLIT2 promoter and reactivation of their mRNA expression by MMA

Because MMA is a GC-rich DNA binding agent, we speculated that it may bind to CpG regions and block DNMT methylation activity. We treated CL1-5 cells, whose anti-metastatic TSGs such as *SLIT2* is down-regulated by promoter hypermethylation, with MMA in a low dose (10 *n*M) for several doubling times (14 days). The MSP analysis showed that MMA decreased methylation of *SLIT2* promoters (Fig. 3A) and concomitantly increased expression of mRNA for *SLIT2* gene (Fig. 3B).

Inhibition of motility of CL1-5 cells after MMA-induced reactivation of SLIT2 gene

SLIT2 gene are candidate anti-metastasis TSGs. To examine whether reactivation of *SLIT2* could lower the motility of CL1-5 lung cancer cells, we carried out transwell assays after treating CL1-5 cells with 10 *n*M MMA for 14 days. In transwell assays, MMA markedly decreased the migration of CL1-5 cells. A greater number of untreated cells moved through the pores of the membranes compared with MMA-treated cells (Fig. 4A). Quantitative data from photographics of five different visual fields indicated that MMA significantly lowered the ability of CL1-5 cells to be invasive (Fig. 4B, $P < 0.001$). This implies that MMA can inhibit cellular migration mediated by reactivation of multiple anti-metastasis TSGs.

Depletion of DNMT1 protein levels by MMA

To further analyze whether the demethylating effect of MMA is mediated by inhibition of DNMT1, we performed Western blot assays to detect protein levels of DNMT1 during MMA treatment. Interestingly, levels of the DNMT1 protein were decreased more by treatment with 10 *n*M MMA for 14 days than by treatment with DMSO only or 50 *n*M MMA for 48 hours 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, and Sp1 is a transcription factor for the *DNMT1* gene. To determine whether the depletion of DNMT1 protein by MMA was due to its inhibition of Sp1 transactivation of the *DNMT1* promoter and resulted in a reduction in *DNMT1* mRNA expression, we performed RT-PCR to

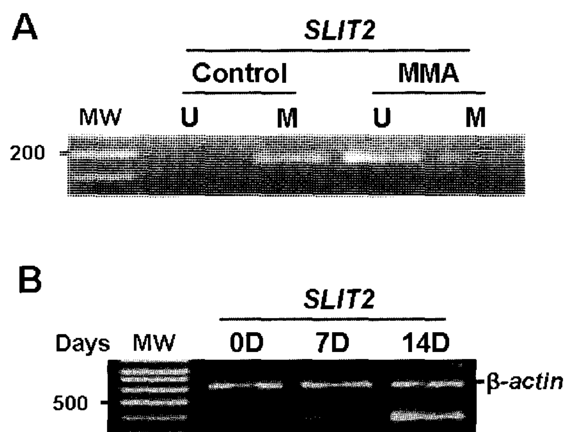

Figure 3. De-methylation and re-expression effects of MMA on *SLIT2* gene of CL1-5 cells. **A.** Demethylation was assayed by an MSP of *SLIT2* in a lung cancer cell lines after 14 days of 10 *n*M MMA. The increasing of the unmethylated product (U) and decreasing of the methylated product (M) after MMA treatment indicates demethylation of the promoter. **B.** RT-PCR analysis of mRNA expression of the *SLIT2* gene in CL1-5 cells.

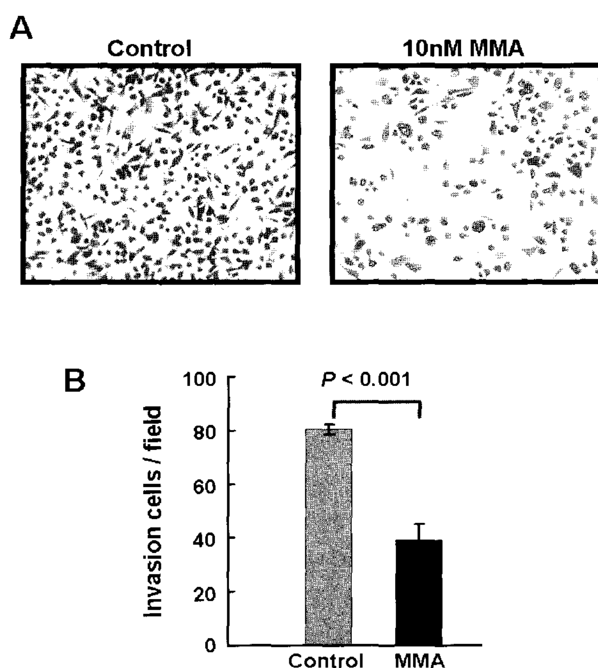

Figure 4. The mobility of CL1-5 cells decreased after treatment with 10 *n*M MMA for 14 days. **A.** Photo images representing transwell invasion assays in untreated (left panel) and treated (right panel) cells (original magnification ×200). **B.** Quantitative analysis of the inhibition of invasion 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 invasion of CL1-5 cells ($P < 0.001$).

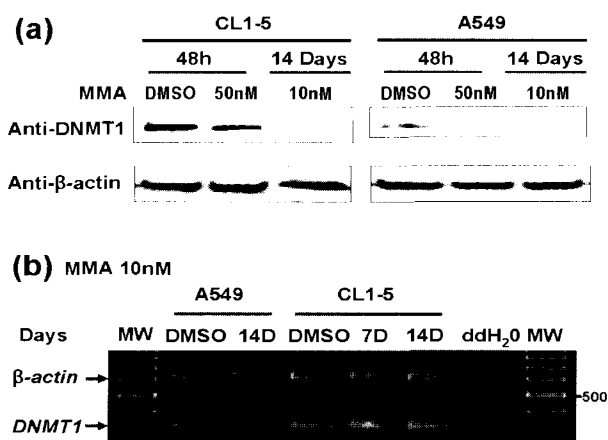

Figure 5. The depleting effect of MMA on DNMT1 protein levels in A549 and CL1-5 lung cancer cell lines. **A.** Western blot analysis of DNMT1 protein levels when maintained in 50 nM MMA for 48h or in 10 nM for 14 days. **B.** RT-PCR analysis of mRNA expression of the *DNMT1* genes in A549 and CL1-5 cell lines at 10 nM at the indicated times.

examine *DNMT1* mRNA expression. However, a low dose of MMA did not decrease the mRNA expression of the *DNMT1* gene (Fig. 5B) between MMA-treated or untreated cells. This suggests that DNMT1 depletion by MMA is not due to changes in its transcription efficiency.

Discussion

Aberrant DNA hypermethylation on promoter region of TSGs is a key mechanism for tumorigenesis (Esteller, 2005; Momparler, 2003). Consequently, researchers are now searching for potential demethylating agents, which can reactivate many TSGs in tumor cells and possible lead to suppression of cancer cell growth and invasion. In the present study we discovered novel effects of MMA including demethylation and reactivation of TSGs in lung cancer cells. These effects appear to be mediated via depletion of DNMT1 protein levels. Although MMA is recognized as an Sp1 inhibitor – it blocked binding of Sp1 to its target genes at concentrations of 100 *n*M to 90 μM (for 48h) in previous studies (Koutsodontis *et al.*, 2004; Remsing *et al.*, 2003; Tagashira *et al.*, 2000) – we found that a low dose (10 *n*M) of MMA did not decrease downstream gene expression of Sp1 target genes such as *DNMT1*. This suggests that such a low concentration of MMA specifically affects methylated TSGs rather than transcription level of Sp1 target genes. The present study is the first to show that low doses of MMA over prolonged periods inhibits DNMT1 protein levels.

CL1-5 cells, which are highly metastatic, are hypermethylated on the promoter regions of *SLIT2* gene. We found that the *SLIT2* gene was demethylated and re-expressed after MMA treatment. This treatment led to several morphological changes such as bigger cell size with more adhesiveness and fewer filopodia (data not shown). Also, MMA diminishes the motility of CL1-5 cells. We believe that MMA may affect yet other anti-metastasis TSGs. A genome-wide screening to identify other anti-metastasis associated genes that are affected by MMA is now needed. In addition, inhibition of DNMT1 by MMA may lead to a global change in chromatin structure because DNMT1 is known to cooperate with many chromatin modifiers for epigenetic control (Esteller, 2005). Combined treatment with MMA and other DNA damaging reagents such as cisplatin or etoposide may have a synergistic toxicity to cancer cells. This may benefit cancer patients in the future because it may be possible to use lower, nontoxic doses of each agent than would otherwise be used in high dose regimens when either agent is given alone.

There was an additional benefit of using low does of MMA – 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 previous been reported in various malignancies (Girault *et al.*, 2003; Lin *et al.*, 2007; Patra *et al.*, 2002; Saito *et al.*, 2003). Therefore, MMA should more selectively attack tumor cells than do more conventional anti-cancer drugs and if this were true, MMA could be applied to the treatment of many cancer types.

MMA is recognized as a CG-rich DNA binding agent, implying that it may also have a similar inhibitory effect against DNMT as does the compound Procaine. Procaine has been found to possess demethylating ability, possibly through competition with DNMT for the GC sequences (Villar-Garea *et al.*, 2003). We propose that MMA interferes with DNMT1 binding at the CpG region in TSG promoters through two possible reaction mechanisms. First, MMA may directly bind to the

DNMT1 protein. Alternatively, there may be a triplex complex formed with MMA, DNMT1, and double stranded DNA. Both mechanisms will lead to depletion of the functional DNMT1 enzyme in treated cells, similar to the effect of 5-azacytidine and 5-aza-2'-deoxycytidine (Ghoshal *et al.*, 2005). In contrast to 5-azacytidine and 5-aza-2'-deoxycytidine, MMA reversibly binds to DNA rather than being incorporated into 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 in the future. We are also currently testing whether 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 (Remsing *et al.*, 2003). Other MMA analogues may inhibit DNMT1 protein and are worthy of further investigation.

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

Acknowledgements

We thank Dr. Chun-Hua Hsu for assistance on making the picture of MMA structure.

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生物學報（2007）42(2): 55-62

Mithramycin A 抑制肺癌細胞中的甲基轉移酵素及其轉移能力

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(收稿日期：2007.4.12，接受日期：2007.5.9)

摘　要

在抑癌基因啟動子的 CpG 島群上若發生了不正常的過度甲基化時，往往導致人類癌症的發生及進程。負責將 CpG 島群甲基化的是 DNA 甲基轉移酵素 (5'-cytosine-methyltransferase，簡稱 DNMT)。抑制形成 CpG 島群的甲基化的藥物正可以作為恢復抑癌基因的表現及活化癌細胞中抑制癌症的重要路徑的新穎標的。而 Mithramycin A (簡稱 MMA)是一個會與富含 GC 及 CG 序列的 DNA 結合的藥物，因此本研究檢測癌細胞在經過 MMA 的處理之後，是否會抑制 CpG 島群甲基化的情形。我們發現當以低劑量(10 *n*M)的 MMA 處理癌細胞 14 天後，會減少 SLIT2 基因的 CpG 島群過度甲基化的情形，並進而使這個具有抑制癌細胞轉移的 SLIT2 重現基因表達。同時間藉由膜穿透 (transwell) 實驗我們也發現 MMA 可降低具有高轉移能力的癌細胞 CL1-5 的穿越及移動能力。為了瞭解 MMA 的作用途徑，我們使用西方點漬法發現 MMA 會使 DNMT1 蛋白明顯下降，但是 DNMT1 的基因表現不受影響。總結研究結果發現，MMA 具有去 DNA 甲基化及抑制癌細胞轉移的潛力。而抑制的機轉可能藉由降低癌細胞中 DNMT1 的蛋白表達量，進而導致抑制癌細胞轉移的基因啟動子去甲基化且重新恢復表達。

關鍵詞：mithramycin A、甲基轉移酵素、轉移、肺癌