

Original Article

Methoxyacetic acid suppresses prostate cancer cell growth by inducing growth arrest and apoptosis

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Abstract: Methoxyacetic acid (MAA) is a primary metabolite of ester phthalates that are used in production of consumer products and pharmaceutical products. MAA causes embryo malformation and spermatocyte death through inhibition of histone deacetylases (HDACs). Little is known about MAA's effects on cancer cells. In this study, two immortalized human normal prostatic epithelial cell lines (RWPE-1 and pRNS-1-1) and four human prostate cancer cell lines (LNCaP, C4-2B, PC-3, and DU-145) were treated with MAA at different doses and for different time periods. Cell viability, apoptosis, and cell cycle analysis were performed using flow cytometry and chemical assays. Gene expression and binding to DNA were assessed using real-time PCR, Western blot, and chromatin immunoprecipitation analyses. We found that MAA dose-dependently inhibited prostate cancer cell growth through induction of apoptosis and cell cycle arrest at G1 phase. MAA-induced apoptosis was due to down-regulation of the anti-apoptotic gene baculoviral inhibitor of apoptosis protein repeat containing 2 (BIRC2, also named cIAP1), leading to activation of caspases 7 and 3 and turning on the downstream apoptotic events. MAA-induced cell cycle arrest (mainly G1 arrest) was due to up-regulation of p21 expression at the early time and down-regulation of cyclin-dependent kinase 4 (CDK4) and CDK2 expression at the late time. MAA up-regulated p21 expression through inhibition of HDAC activities, independently of p53/p63/p73. These findings demonstrate that MAA suppresses prostate cancer cell growth by inducing growth arrest and apoptosis, which suggests that MAA could be used as a potential therapeutic drug for prostate cancer.

Keywords: Prostate cancer, cell death, cell cycle, apoptosis, p21

Introduction

Methoxyacetic acid (MAA, linear chemical formula: $\text{CH}_3\text{OCH}_2\text{COOH}$) is a primary metabolite of ester phthalates widely used in the manufacture of household products (building materials, plastics, textiles, adhesives, paints, and deodorants), food and personal care products (agricultural adjuvants, pesticides, cosmetics, and perfumes), electronics (coatings, stabilizers, and surfactants), and pharmaceutical products (oral pill coatings, viscosity control agents, surfactants, and stabilizers) [1]. Over 18 billion pounds of ester phthalates are used globally each year. Ingestion, inhalation, intravenous injection, and dermal exposure of ester phthalates may lead to toxicities through their

metabolite MAA [1]. MAA is converted from ethylene glycol monomethyl ether (also called 2-methoxyethanol) by alcohol dehydrogenase. In a workplace with daily 2-methoxyethanol exposure of 4.5 $\mu\text{g}/\text{ml}$ (within the permissible exposure limit), urine MAA concentrations reached up to 0.6 millimoles/liter (mM) [2], which could be accumulated to higher concentrations due to the long elimination half-life of 77 hours [3]. Exposure to 2-methoxyethanol increases risks of spontaneous abortion and subfertility in women [4] and decreases sperm counts in men [5]. In pregnant mice, single i.v. injection of 250-325 mg/kg 2-methoxyethanol created peak plasma concentrations of 5-8 mM MAA, which led to embryo malformation and lethality [6].

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MAA causes toxicities through multiple mechanisms. In normal human fibroblasts, MAA treatment induces production of radical oxygen species, resulting in DNA damage and loss of mitochondrial membrane potential [1]. MAA treatment down-regulates expression of estrogen receptor α (ER α) and estradiol-induced gene expression in human breast cancer cell line MCF-7 and mouse uterus [7]. In contrast, it has been reported that MAA exposure increases ER β expression in pachytene spermatocytes, which may be associated with MAA-induced apoptosis of pachytene spermatocytes in rats [8]. In rat seminiferous tubules, MAA treatment alters the expression of androgen receptor (AR) and androgen-binding protein (ABP) in a stage-specific manner. On one hand, MAA treatment up-regulates AR expression in the early and late stages, but down-regulates AR expression in the middle stage [9]; on the other hand, this same treatment down-regulates ABP expression in the late stage, but up-regulates ABP expression in the middle stage [9]. Spermatogenesis requires normal functions of AR [10, 11], ER α [12], and ER β [13] and their disruption leads to testicular degeneration after MAA exposure. In addition, MAA has been found to activate the tyrosine kinase – PI3K pathway and other pathways to enhance or antagonize androgen-induced gene expression [14-16]. Similarly, MAA can enhance the transcriptional activities of ER α and ER β by activating MAPK and inhibiting histone deacetylases (HDACs) [17]. MAA can inhibit HDAC1, HDAC2, and HDAC3, thus increasing the levels of acetylated histone H4, like the other well-known HDAC inhibitors such as trichostatin, valproic acid, and butyric acid [17]. In fact, it has been reported that MAA-induced hyperacetylation of histones H3 and H4 is associated with rapid spermatocyte death following MAA exposure [18].

These previous studies on MAA largely focused on its toxic effects on the reproductive system. Some HDAC inhibitors such as suberanilohydroxamic acid (SAHA) and romidepsin have been approved for the treatment of cutaneous T cell lymphoma, and panobinostat and valproic acid are being tested in the treatment of prostate cancer, breast cancer, cervical cancer, ovarian cancer, and lymphomas [19]. We speculated that MAA might also possess anti-cancer activity. In the present study, we tested this idea and found that MAA can indeed induce apoptosis and growth arrest of prostate cancer cells. MAA-induced apoptosis was highly asso-

ciated with decreased protein expression of baculoviral inhibitor of apoptosis protein repeat containing 2 (BIRC2, also named cIAP1), whereas MAA-caused G1 arrest was closely associated with induction of p21 level and reduction of cyclin-dependent kinase 4 (CDK4) and CDK2 levels. The MAA-induced p21 level was likely due to the inhibition of HDAC activities by this compound, leading to increased association of acetylated histone H3 and H4 with the specificity protein 1 (Sp1) binding sites-rich DNA element on the p21 promoter, independently of p53/p63/p73 proteins. Thus, these results suggest that MAA might possess a potential anti-cancer activity by inhibiting anti-apoptotic protein and inducing apoptosis as well as inducing cell growth arrest via induction of p21.

Materials and methods

Cell culture

The sources and cell culture conditions of two immortalized human normal prostatic epithelial cell lines (RWPE-1 and pRNS-1-1) and four human prostate cancer cell lines (LNCaP, C4-2B, PC-3, and DU-145) were described previously [20]. Cells were cultured in a 5% CO₂ humidified incubator at 37°C.

Cell viability assay

The number of live cells was determined using the CellTiter-Glo[®] Luminescent Cell Viability Assay (Promega Corp, Fitchburg, WI, USA) as described previously [20]. Cell viability was calculated as (luminescence of the treatment group – background luminescence) ÷ (luminescence of the control group – background luminescence) × 100%. The data are presented as the mean and standard error of the mean (SEM) of three independent experiments.

Detection of apoptotic nucleosomes

Cells were seeded on 12-well plates with 1×10^5 cells/well in triplicate per group in the complete culture medium with FBS. After overnight incubation, cells were treated with 5 mM MAA for 24 hours (h); a control group was treated with PBS. Apoptotic nucleosomes were detected using Cell Death Detection ELISA kit (Roche Diagnostics Corporation, Indianapolis, IN, USA) according to the manufacturer's instructions [21]. Absorbance was measured at 405 nm (A405) with a reference wavelength at 490 nm (A490) using a plate reader (Bio-Tek U.S.,

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Table 1. PCR primers

Primer		Sequence
p21	Forward	5'-ACCCATGCGGCAGCAA-3'
	Reverse	5'-CGCCATTAGCGCATACA-3'
GAPDH	Forward	5'-TAAAAGCAGCCCTGGTGACC-3'
	Reverse	5'-CCACATCGCTCAGACACCAT-3'
Sp1-rich region	Forward	5'-CAGCGCACCAACGCAGGCG-3'
	Reverse	5'-CAGCTCCGGCTCCACAAGGA-3'
Adjacent region	Forward	5'-GGTGTCTAGGTGCTCCAGGT-3'
	Reverse	5'-GCACTCTCCAGGAGACACA-3'

Winooski, VT, USA). The amount of apoptotic nucleosomes was represented by A405-A490.

Cell cycle analysis

Cells were treated without or with 5 mM or 20 mM MAA for 24 h. The percentage of cells at G1/G0, S, and G2/M phases was determined by flow cytometry analysis as described previously [20].

Western blot analysis

Cells were treated without or with MAA at concentrations of 5 mM or 20 mM for 0, 12, 24, 48, and 72 h. Proteins were extracted for Western blot analysis as described previously [20]. Rabbit anti-caspase 7, rabbit anti-caspase 6, and rabbit anti-caspase 9 antibodies were purchased from Cell Signaling Technology (Danvers, MA, USA). Mouse anti-p53, mouse anti-p63, rabbit anti-p73, rabbit anti-caspase 10, mouse anti-caspase 8, rabbit anti-BIRC2, and rabbit anti-BIRC3 antibodies were obtained from Santa Cruz Biotechnology (Dallas, TX, USA). Mouse anti-GAPDH, mouse anti-caspase 3, and rabbit anti-cleaved poly (ADP-ribose) polymerase (PARP) antibodies were purchased from EMD Millipore Corp (Billerica, MA, USA). Rabbit anti-CDK2 and mouse anti-CDK4 antibodies were purchased from GeneTex, Inc., Irvine, CA, USA. Rabbit anti-cyclin D1 antibodies were bought from Abcam, Cambridge, MA, USA.

Reverse transcription (RT) and quantitative (q) PCR analysis

Cells were treated with 20 mM MAA for 0, 12, 24, 48, and 72 h. Total RNAs were extracted for RT and qPCR analysis as described previously [22]. Results were normalized against GAPDH levels using the formula ΔCt (Cycle threshold) = Ct of target gene - Ct of GAPDH. The mRNA

level of a control group was used as the baseline; therefore, $\Delta\Delta\text{Ct}$ was calculated using the formula $\Delta\Delta\text{Ct} = \Delta\text{Ct}$ of target gene - ΔCt of the baseline. The fold change of mRNA level was calculated as fold = $2^{-\Delta\Delta\text{Ct}}$. PCR primers used are shown in **Table 1**.

Chromatin immunoprecipitation (ChIP) assay

Cells were plated at a density of 2×10^6 cells per dish in four 100-mm dishes in each group and incubated overnight. Cells were treated without or with 20 mM MAA for 24 h and fixed in 1% formaldehyde for 10 minutes. ChIP assays were performed using Magna ChIP™ G Chromatin Immunoprecipitation Kit (EMD Millipore Corp, Billerica, MA, USA) according to the manufacturer's instructions. Briefly, 1 mL of 10x glycine was added to the cells to quench formaldehyde; after washing with ice-cold PBS containing protease inhibitor cocktail II, the cells were resuspended in 0.5 mL of SDS lysis buffer with protease inhibitor cocktail II; after centrifugation, cell pellets were resuspended in 0.5 mL nuclear lysis buffer; the lysates were sonicated for about 10-15 seconds on ice; after centrifugation, an aliquot of 5 microliter (μL) of the supernatant chromatin preparation was set aside as the Input fraction; then, 50 μL chromatin preparations were diluted with 450 μL ChIP dilution buffer and added with 5 μg of rabbit anti-acetyl-histone H3, rabbit anti-acetyl-histone H4 (EMD Millipore Corp), or rabbit IgG (Santa Cruz Biotechnology) for overnight incubation at 4°C; protein G magnetic beads (20 μL) were added for 1 h incubation; next, protein G magnetic bead complexes were separated with magnetic separator and washed with washing buffers; immune complex samples and inputs were eluted with 100 μL of ChIP elution buffer and digested with proteinase K at 62°C for 2 h; immunoprecipitated DNA samples and Inputs were purified with DNA purification spin column and analyzed by PCR with the products analyzed by 2% agarose/ethidium bromide gel electrophoresis. PCR primers used for ChIP assays are shown in **Table 1**.

Statistical analysis

Results from this study were presented as the mean \pm SEM. Statistical analysis was performed using two-tailed Student's *t* test. A *p*-value < 0.05 was considered statistically significant.

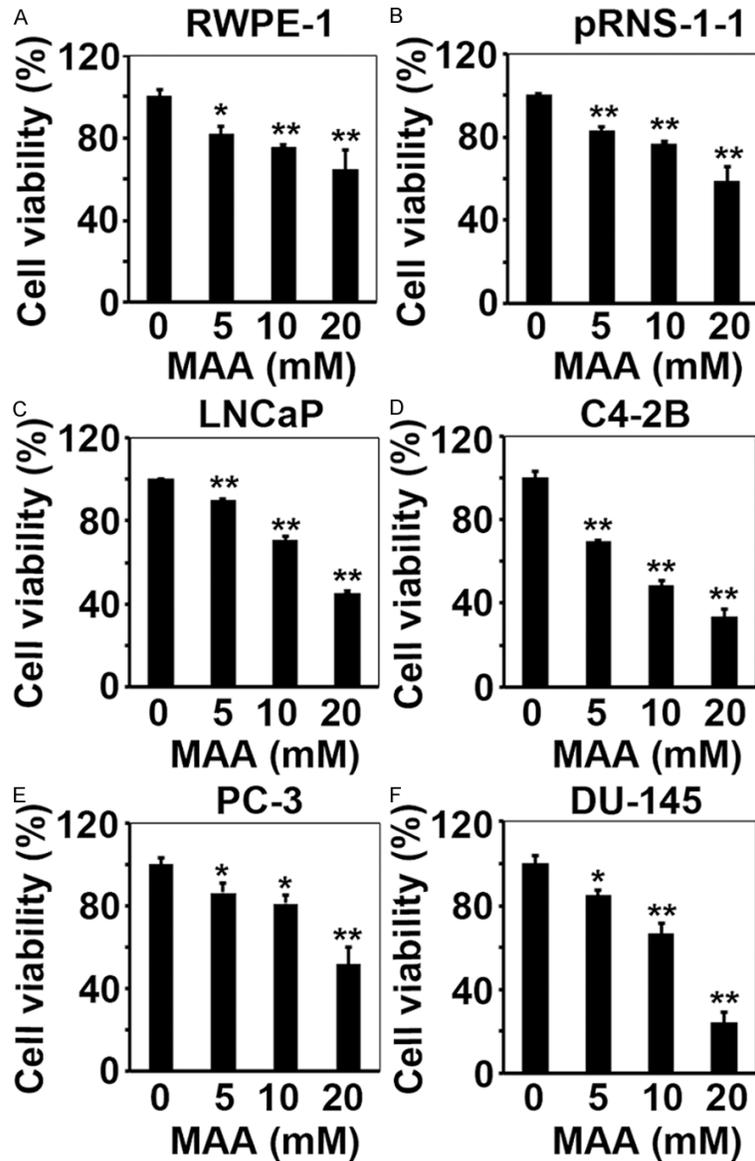


Figure 1. MAA inhibits prostate cancer cell growth. A-F: Normal prostatic epithelial cells and prostate cancer cells were plated in 96-well plates in triplicate per group and were treated with 0, 5, 10, and 20 mM MAA for 72 h. The live cell numbers were determined using the CellTiter-Glo® Luminescent Cell Viability Assay. The data are presented as the mean ± SEM of three independent experiments. **p* < 0.05; ***p* < 0.01.

Results

MAA inhibits prostate cancer cell growth

To study the effects of MAA on prostate cancer cell growth, we treated two immortalized human normal prostatic epithelial cell lines RWPE-1 and pRNS-1-1 and four prostate cancer cell lines LNCaP, C4-2B, PC-3, and DU-145, with 5, 10, and 20 mM of MAA. We chose to start with 5 mM MAA because a previous study

showed that the IC₅₀ was 5.6 mM for MAA to inhibit cell growth of human leukemia cell line HL60 [23]. We found that MAA inhibited cell growth in all of the six cell lines in a dose dependent fashion (Figure 1A-F). Interestingly, four prostate cancer cell lines (LNCaP, C4-2B, PC-3, and DU-145) were more sensitive to MAA than were two normal prostatic epithelial cell lines (RWPE-1 and pRNS-1-1), as the number of viable cells was decreased by approximately 50% to 75% in the four prostate cancer cell lines (Figure 1C-F), whereas it was only reduced by 40% in RWPE-1 and pRNS-1-1 cells, when these cell lines were individually treated with 20 mM MAA (Figure 1A, 1B).

MAA induces apoptosis of prostate cancer cells

To test if MAA induces apoptosis of prostate cancer cells, we measured apoptotic nucleosomes in untreated and MAA-treated cells. We found that 5mM MAA treatment for 24 h significantly increased the amounts of apoptotic nucleosomes in LNCaP, C4-2B, PC-3, and DU-145 cells, compared to the untreated control groups (Figure 2A-D, *p* < 0.05 or 0.01). Consistently, PARP cleavage in all four prostate cancer cell lines was induced by MAA in a dose- and time-

dependent manner (Figure 2E, 2F). Since PARP cleavage has been widely used as an indicator of apoptosis [24, 25], these results indicate that MAA induces apoptosis of four prostate cancer cell lines.

MAA blocks G1/S transition of prostate cancer cell cycle

To assess if MAA induces cell cycle arrest, we analyzed the percentages of cells in the G1

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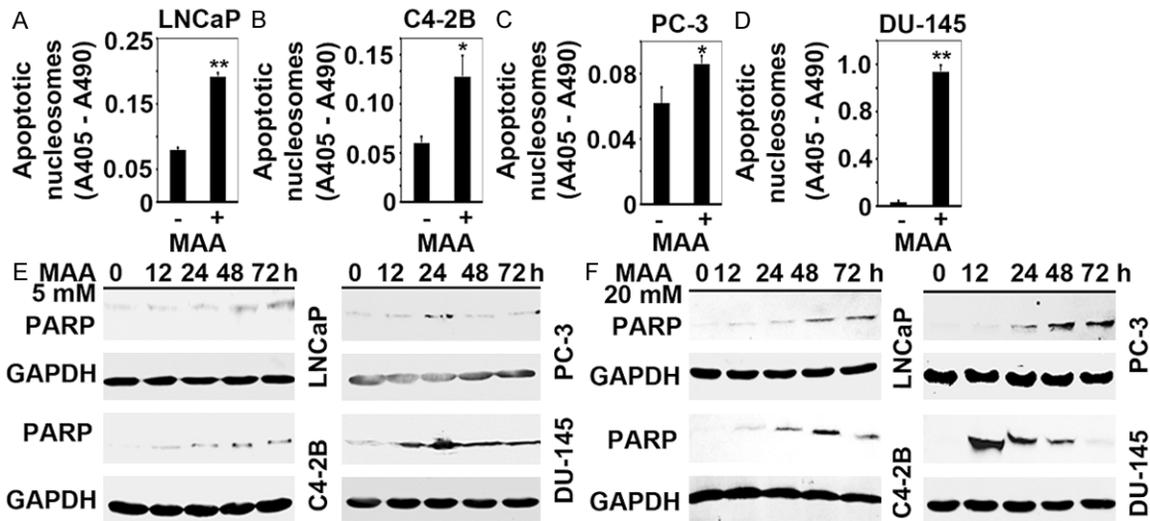


Figure 2. MAA induces apoptosis of prostate cancer cells. (A-D) Prostate cancer cells were plated in 12-well plates in triplicate per group and treated with 5 mM MAA for 24 h; the control group was treated with PBS. Apoptotic nucleosomes were detected using Cell Death Detection ELISA kit, which were calculated as absorbance at 405 nm (A405) – absorbance at 490 nm (A490). The data are presented as the mean \pm SEM of three independent experiments. * $p < 0.05$; ** $p < 0.01$. (E, F) Prostate cancer cells were treated with 5 mM (E) or 20 mM (F) MAA for up to 72 h. Protein extracts were used for Western blot analysis of cleaved PARP. For the loading control, the blots were probed for GAPDH.

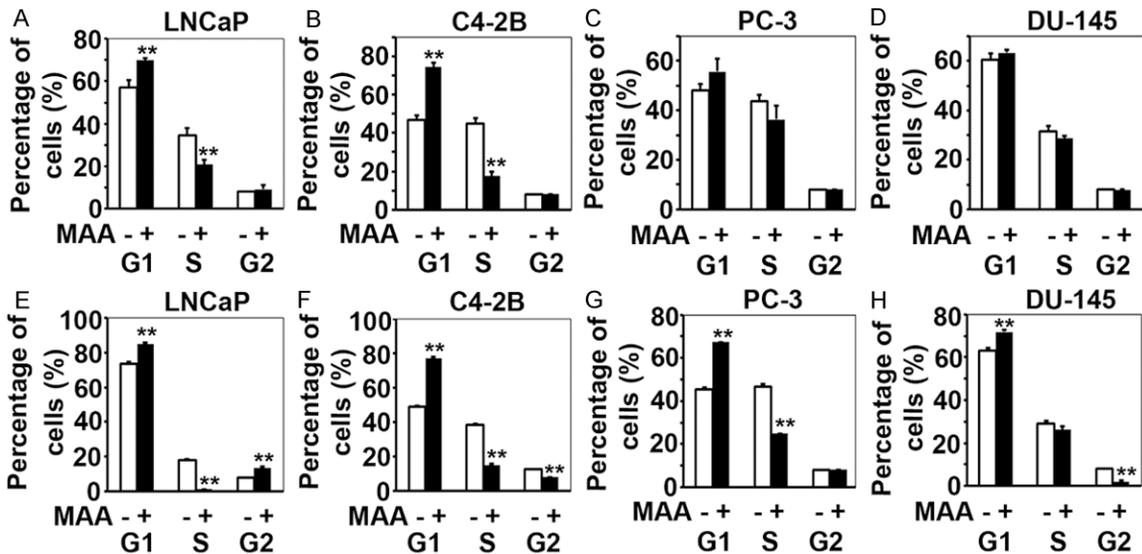


Figure 3. MAA blocks G1/S transition of prostate cancer cell cycle. (A-H) Prostate cancer cells were plated in 60-mm dishes in triplicate per group and treated with 5 mM (A-D) or 20 mM (E-H) MAA for 24 h; the control group was treated with PBS. The percentages of cells at G1 (and G0), S, and G2 (and M) phases were determined by flow cytometry analysis. The data are presented as the mean \pm SEM, $n = 3$. ** $p < 0.01$.

(and G0), S, and G2 (and M) phases of the cell cycle using flow cytometry analysis. We found that 5 mM MAA treatment significantly increased the percentage of LNCaP and C4-2B cells at the G1/G0 phase, but significantly decreased the percentage of cells at the S

phase (Figure 3A, 3B, $p < 0.01$). However, although some effects were found in PC-3 and DU-145 cells, the differences were not statistically significant at the low dosage of MAA (Figure 3C, 3D, $p > 0.05$). At a high dose such as 20 mM, MAA treatment significantly

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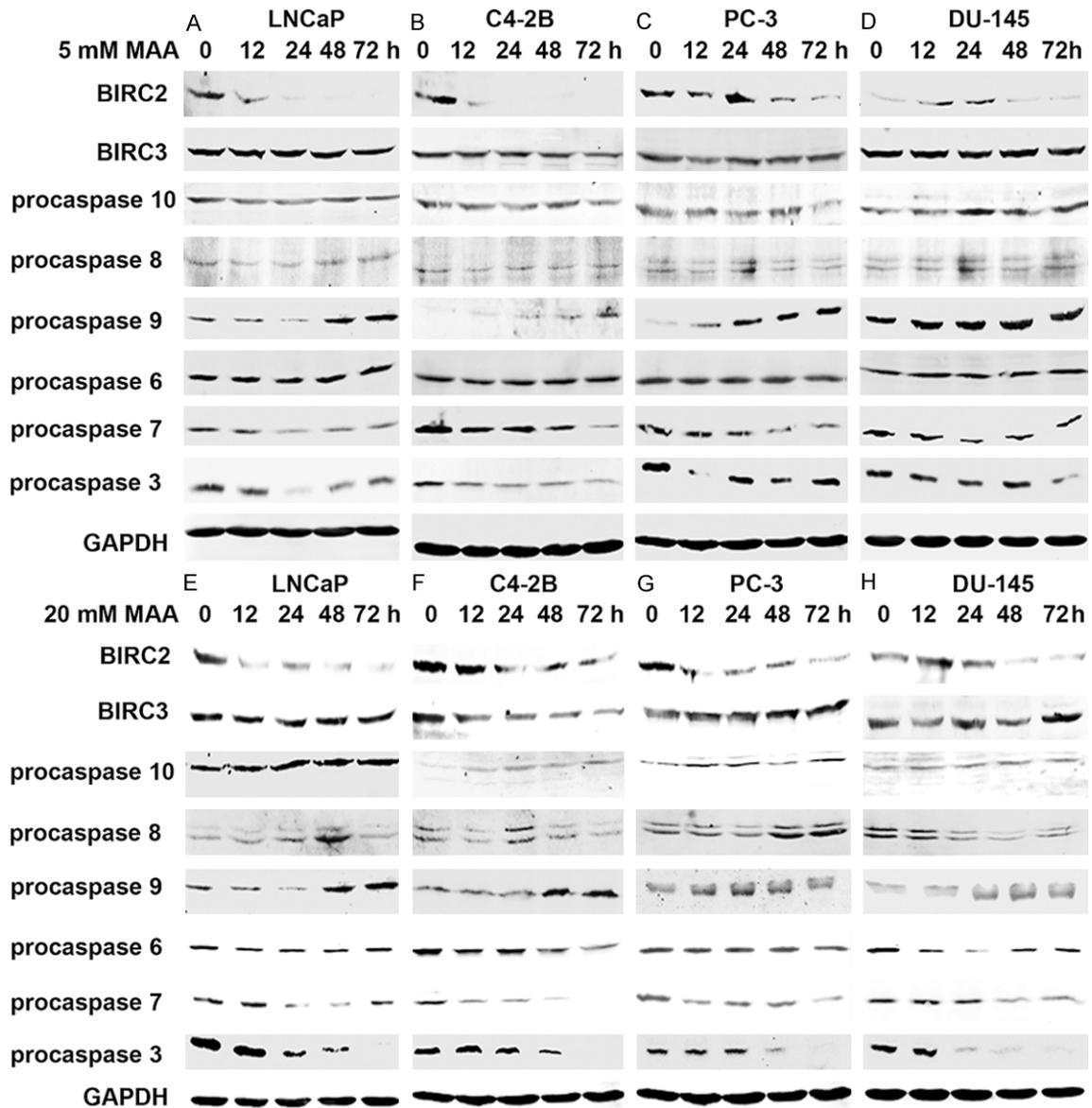


Figure 4. MAA decreases protein expression of BIRC2 and activates caspases 7 and 3. (A-H) Prostate cancer cells were treated with 5 mM (A-D) or 20 mM (E-H) MAA for up to 72 h. Protein extracts were used for Western blot analysis of the indicated proteins. For the loading control, the blots were probed for GAPDH.

increased the percentage of cells at the G1/G0 phase with the corresponding decrease of cells at the S phase in all four prostate cancer cell lines (Figure 3E-H). These results imply that MAA treatment blocks the G1/S transition, and thus inhibits cell proliferation.

MAA decreases protein expression of BIRC2 and activates caspases 7 and 3

To illustrate the mechanisms underlying MAA-induced apoptosis of prostate cancer cells, we examined the expression of a panel of anti-

apoptotic and pro-apoptotic genes, using Western blot analysis. Although there was not any detectable expression or any change upon MAA treatment for B-cell CLL/lymphoma 2 (BCL2), BCL2-associated X protein (BAX), BCL2-like 1 (BCL2L1), BCL2-associated agonist of cell death (BAD), BH3 interacting domain death agonist (BID), myeloid cell leukemia 1 (MCL1), and CASP8 and FADD-like apoptosis regulator (CFLAR) (data not shown), we found that MAA treatment decreased the protein level of BIRC2 in all four prostate cancer cell lines (Figure

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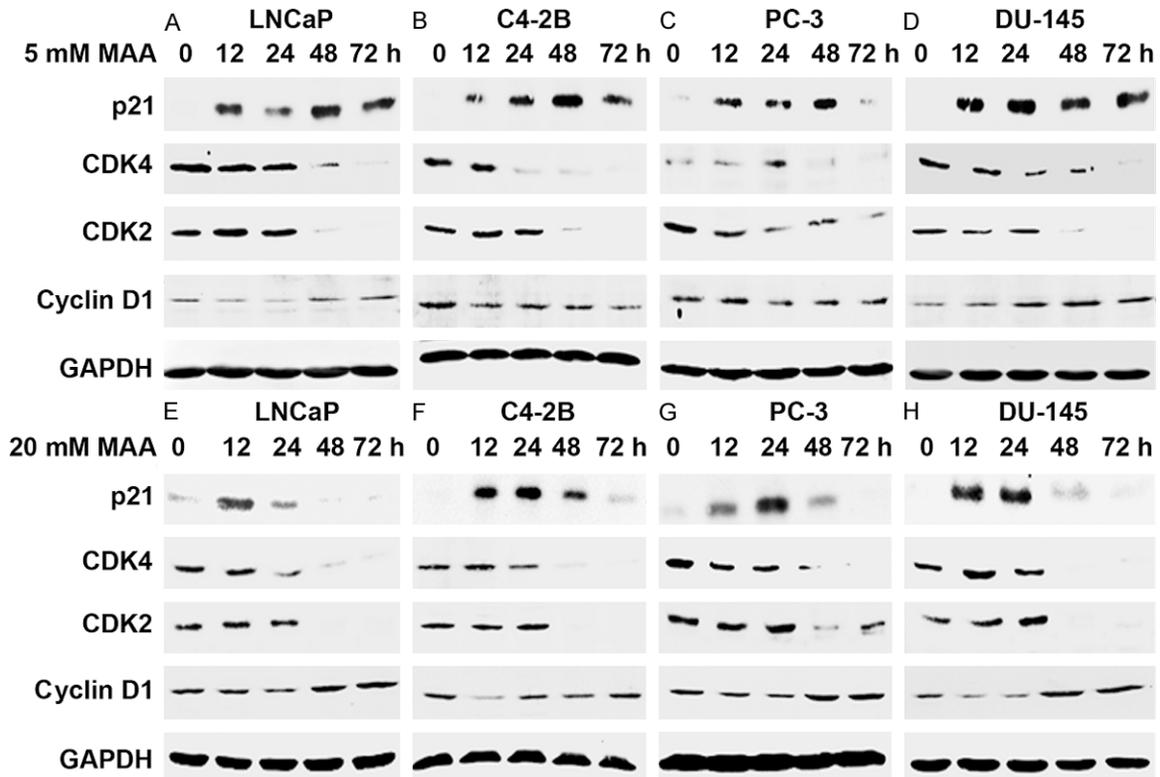


Figure 5. MAA induces p21 level but reduces CDK4 and CDK2 levels. (A-H) Prostate cancer cells were treated with 5 mM (A-D) or 20 mM (E-H) MAA for up to 72 h. Protein extracts were used for Western blot analysis of the indicated proteins. For the loading control, the blots were probed for GAPDH.

4A-H). This decrease was specific to BIRC2, as there were not any obvious changes in the protein levels of BIRC3, another member of the inhibitors of apoptosis protein (IAP) family [26]. It has been shown that proteasome-mediated and/or HTRA7 serine protease-mediated degradation of BIRC2 can relieve BIRC2's inhibitory function on caspases, thus activating caspase-mediated apoptosis [27, 28]. Therefore, we examined a panel of key caspases in both extrinsic and intrinsic apoptosis pathways. Caspases are endoproteases that are initially produced as inactive monomeric procaspases, which require dimerization and often cleavage for activation [29]. Among the apoptosis-relevant caspases, the level of procaspase 9 in all four prostate cancer cell lines was induced by MAA treatment at both 5 mM (**Figure 4A-D**) and 20 mM (**Figure 4E-H**), whereas little change of the level of procaspases 10, 8 and 6 was observed with the same treatment (**Figure 4A-H**). By contrast, the level of procaspases 7 and 3 was decreased by MAA treatment at both 5 mM (**Figure 4A-D**) and 20 mM (**Figure 4E-H**). Decrease of the procaspases indicates cleav-

age of the proenzymes and activation of caspases 7 and 3, two key executioner caspases [29].

MAA induces p21 level but reduces CDK4 and CDK2 levels

To determine which protein molecules might be responsible for MAA-induced G1 arrest, we first examined the levels of several cell cycle-regulated proteins during the G1/S transition. It has been shown that in the late G1 phase, the cyclin D-CDK4/6 kinase complex initiates phosphorylation of retinoblastoma protein (pRb), and this phosphorylation dissociates pRb from E2F transcription factors, thus allowing them to be functional and transactivate expression of the genes necessary for G1/S transition. This process is enhanced by the cyclin E-CDK2 complex, but inhibited by p21 [30]. We found that the level of p21 protein was increased as early as 12 h after cells were treated with 5 mM MAA (**Figure 5A-D**). However, surprisingly and interestingly, at 20 mM, MAA-induced p21 level reached a peak at 12 to 24 h, and then started

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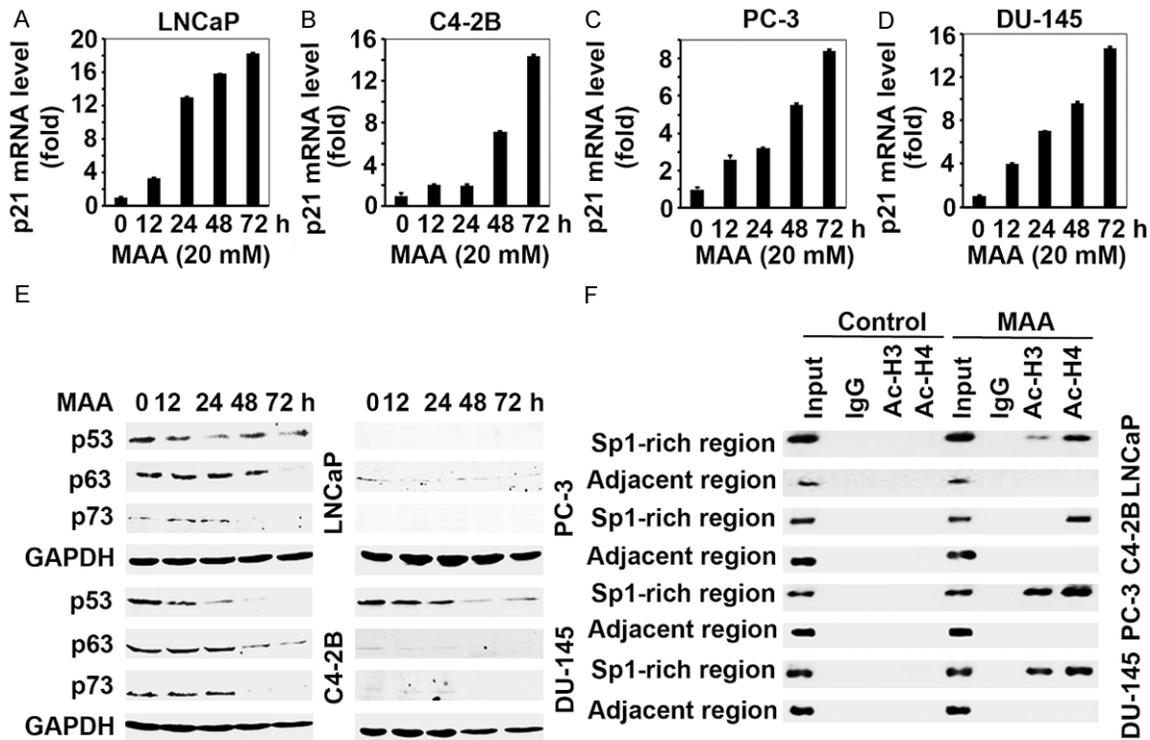


Figure 6. MAA enhances p21 transcription by inhibiting HDACs, independently of p53/p63/p73. A-D: Prostate cancer cells were treated with 20 mM MAA for up to 72 h. RNAs were analyzed with reverse transcription and quantitative PCR. Fold changes were calculated based on normalization to GAPDH levels and using the untreated control group as base line. The data are presented as the mean \pm SEM, $n = 3$. E: Prostate cancer cells were treated with 20 mM MAA for up to 72 h. Protein extracts were used for Western blot analysis of the indicated proteins. For the loading control, the blots were probed for GAPDH. F: Prostate cancer cells were treated with 20 mM MAA for 24 h and fixed in 1% formaldehyde. The chromatin preparations were immunoprecipitated with anti-acetyl-histone H3 (Ac-H3), anti-acetyl-histone H4 (Ac-H4), or control IgG, plus protein G magnetic beads. The purified DNAs were analyzed by PCR using primers specific for the Sp1 binding sites-rich region (Sp1-rich region) or the adjacent upstream region without Sp1 binding sites (Adjacent region). An aliquot of the chromatin preparations was used as Input control. PCR products were run on 2% agarose/ethidium bromide gel electrophoresis.

to drop down to the basal level from 48 h post treatment (**Figure 5E-H**). By contrast, CDK4 and CDK2 levels started to decrease in most of the four tested cell lines at 48 and 72 h after MAA treatment at 5 and 20 mM, whereas cyclin D1 levels were not dramatically altered after MAA treatment of all of the cell lines (**Figure 5A-H**). These results suggest that MAA may induce G1 arrest by increasing p21 level and decreasing the levels of CDK2 and CDK4.

MAA enhances p21 transcription by inhibiting HDACs

To further illustrate possible molecular mechanisms underlying p21 up-regulation by MAA, we first performed qPCR and found that MAA increased p21 mRNA levels as early as 12 h after its treatment at 20 mM, and p21 mRNA levels continued to rise over the 72 h treatment

period (**Figure 6A-D**). Since p21 transcription is often activated by the p53 family of genes [31, 32], we examined the levels of p53, p63, and p73 proteins. It is known that LNCaP and its derivative C4-2B cells harbor a wild-type TP53 gene, while DU-145 cells have a mutant TP53 gene, but PC-3 cells have a truncation mutation in the TP53 gene hence do not express p53 protein [33]. We found that none of p53, p63, and p73 protein expression was induced by MAA treatment; instead, they were decreased after 48 or 72 h in some cells, such as LNCaP, C4-2B and DU-145 (**Figure 6E**). Also, expression of p53 or p73 proteins was not detected in PC-3 cells, while a low level of p63 protein was present, but decreased during the treatment period (**Figure 6E**). Since it has been reported that p21 expression is repressed by HDAC1 and HDAC4 in a p53-independent mechanism

[34, 35], we examined whether MAA as an HDAC inhibitor could enhance binding of acetylated histone H3 and H4 to the Sp1 binding sites-rich region within the p21 promoter. We adopted the PCR primers spanning the Sp1 binding sites-rich region (abbreviated as Sp1-rich region) and the Sp1 binding sites-deficient adjacent region (abbreviated as adjacent region) used in a previous study [35]. We also used the same anti-acetylated histone H3 and H4 antibodies to perform ChIP analysis as previously described [34]. As shown in **Figure 6F**, MAA treatment increased binding of acetylated histone H3 and/or H4 to the Sp1-rich region of the p21 promoter, which was specific to this region as there was no increase in binding to the adjacent region (**Figure 6F**), suggesting that MAA might induce histone H3 and H4 acetylation at the p21 promoter region and thus open up the Sp1-binding DNA element to Sp1 that in turn activates the expression of p21 at the transcriptional level. Taken together, these results indicate that MAA induces p21 transcription by inhibiting HDAC activity and consequently leading to hyperacetylation of histone H3 and H4 and opening the promoter region of the p21 gene, in a p53 family independent manner.

Discussion

Prostate cancer, particularly castration-resistant prostate cancer, is lethal to the patients, as the currently available treatments can only extend patient's survival by 2.4 to 4.8 months [36]. Thus, new therapeutics are urgently needed for this type of malignancy. HDAC inhibitors can promote growth arrest, differentiation, and apoptosis of cancer cells, with minimal effects on normal tissues, thus HDAC inhibitors are emerging as promising anti-cancer drugs which possess tumor-selective cytotoxicity [37]. MAA has been demonstrated to be an HDAC inhibitor [17, 18], yet its anti-cancer potential has never been assessed. In the present study, we demonstrated that MAA suppressed the survival of four prostate cancer cell lines (LNCaP, C4-2B, PC-3, and DU-145) in a dose-dependent manner by inducing apoptosis and G1 arrest. Although MAA has been shown to cause apoptosis of spermatocytes (5), this cellular toxicity would be acceptable or minimal to most of the prostate cancer patients because the majority of the patients are 60 years or older who have passed their reproductive age [38]. MAA has been shown to be responsible for immunosup-

pression in rats, but it does not suppress humoral immunity in mice [39]. In humans, only a few cases were reported to have mild anemia and leukopenia in individuals exposed to ethylene glycol monomethyl ether [40]. Therefore, MAA is a promising chemical candidate for the treatment of prostate cancer.

MAA induces apoptosis of rat germ cells through release of mitochondrial cytochrome c, thus activating caspase 9 and caspase 3 [41]. Cytochrome c release from mitochondria is controlled by the antagonistic actions of pro-apoptotic and anti-apoptotic genes of the BCL2 family [42], which is true in rat germ cells [43]. However, we did not find any MAA-induced changes of BCL2, BAX, BCL2L1, BAD, BID, MCL1, and CFLAR in the four prostate cancer cell lines. Instead, we found that BIRC2 (also called cIAP1) protein expression was consistently decreased by MAA treatment in all four prostate cancer cell lines, which was specific to BIRC2 as BIRC3 (also called cIAP2) expression was not affected. BIRC2, like other IAP family proteins, has ubiquitin protein ligase (E3) activity [28]. BIRC2 binds to tumor necrosis factor receptor associated factor 2 (TRAF2) and becomes activated to initiate ubiquitination of receptor-interacting protein 1 (RIP1), subsequently inhibiting activation of caspase 8 [26]. However, we did not observe any MAA-induced activation of caspase 8 or other initiator caspases such as caspase 10, in the decrease of BIRC2 protein levels. On the other hand, we found activation of caspase 7 and caspase 3. Previously, it was found that although BIRC2 can bind to caspases 7 and 9, it is a weak inhibitor of caspases 9, 7, and 3 [44]. But later on, it was found that BIRC2 potently inhibited activation of procaspase 3 by the cytochrome c-dependent apoptotic protease activating factor 1 (APAF1)-caspase 9 apoptosome complex [45]. This finding explains the observed activation of caspases 3 and 7 when MAA treatment decreased BIRC2 protein levels in our study. Of note, MAA treatment consistently increased the procaspase 9 protein level, though the mechanism of this action is not known. We and other investigators have previously noticed that some apoptosis-inducing chemicals can up-regulate procaspase 9 expression [20, 46]. Our speculation is that the increased procaspase 9 levels might be involved in activation of caspases 7 and 3, which awaits further verification.

Also, we showed that MAA causes G1 arrest in all of the four prostate cancer cell lines regardless of the status of p53. It is known that cyclin D-CDK4/6 complex phosphorylates pRb, leading to separation of pRb from E2F transcription factors, thus transactivating genes needed for the G1/S transition and S phase, including cyclin E. Then, activation of cyclin E-CDK2 complex further phosphorylates and completely releases pRb from interacting with E2Fs. However, association of p21 with cyclin D-CDK4/6 inhibits pRb phosphorylation and induces cell cycle arrest in G1 phase [30]. We found that MAA treatment induced up-regulation of p21 mRNA expression and protein expression. At the earlier time points, such as 12 and 24 h, p21 mRNA transcription could be responsible for the increase of its protein level by MAA. However, the protein level of p21 decreased after 24 h of MAA treatment at 20 mM (**Figure 5E-H**), which was inconsistent with the increase of its mRNA level (**Figure 6A-D**). The mechanism for this difference at later time points remains unclear, which is possibly caused by rapid degradation of p21 protein by caspase 3-mediated cleavage as shown in a previous study [47]. p21 is well known for its role in cell cycle arrest, yet p21 can also inhibit apoptosis by interacting with and inhibiting caspase 3 [48, 49]. On the other hand, active caspase 3 can cleave p21 protein, thus converting the cells from cell cycle arrest to apoptosis [47, 50]. This is consistent to our findings that procaspase 3 was dramatically cleaved hence activated at 48 to 72 h (**Figure 4E-H**), which then cleaved and degraded p21 protein (**Figure 5E-H**). Further, we found that at the high dose of 20 mM, MAA treatment also reduced the percentage of C4-2B and DU-145 cells in G2 phase (**Figure 3F, 3H**). This is possibly due to inhibition of cyclin A-CDK1/2 by p21, as it has been reported that p21 can induce G2 arrest [51], or due to loss of CDK2. On the other hand, we consistently found that 20 mM MAA treatment increased the percentage of LNCaP cells in G2 phase, but there were only few cells left in S phase (**Figure 3E**). We do not have a good explanation for this observation, which is unique only to LNCaP cell line at this 20 mM dose of MAA treatment. It is worthy notice that MAA-induced p21 up-regulation was independent of p53/p63/p73, as we did not find any induction of the p53 family proteins by MAA treatment, rather than seeing a slight decrease

of their expression (**Figure 6E**). Nevertheless, we found that MAA treatment increased binding of acetylated histone H3 and H4 to the Sp1 binding sites-rich region of p21 promoter, suggesting that MAA inhibits HDAC activities that repress p21 expression. Our findings are consistent with two previous studies showing that HDAC inhibitors up-regulate p21 expression through a Sp1-dependent, p53-independent mechanism [34, 35].

In addition, we also found that protein levels of CDK4 and CDK2, but not cyclin D1, were decreased by MAA treatment at 48 to 72 h. It has been demonstrated that CDK4 and CDK2 cooperate to phosphorylate pRb and drive G1/S transition, thus loss of both CDK4 and CDK2 leads to G1 arrest [52]. The timing of loss of CDK4 and CDK2 couples with reduction of p21 protein levels, particularly with 20 mM MAA treatment (**Figure 5E-H**), suggesting that in the absence of p21, loss of CDK4 and CDK2 becomes the main reason for G1 arrest. However, the mechanisms of how MAA treatment leads to loss of CDK4 and CDK2 are not clear, which requires further investigation.

In summary, the results as presented here demonstrate that MAA, as an HDAC inhibitor, can inhibit prostate cancer cell growth through induction of apoptosis and cell cycle arrest. MAA-induced apoptosis is likely due to down-regulation of the anti-apoptotic gene BIRC2, leading to activation of caspases 7 and 3 and turning on the downstream apoptotic events. MAA-induced G1 arrest is due to up-regulation of p21 expression at the early time and down-regulation of CDK4 and CDK2 expression at the late time post its treatment. MAA up-regulates p21 expression through inhibition of HDAC activities, independently of the p53 family members. Thus, our results strongly suggest that MAA could be developed into a potential therapy for prostate cancer.

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Disclosure of conflicts of interest

The authors disclose no conflicts of interest.

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