A_3 Adenosine Receptor Antagonist, Truncated Thio-Cl-IB-MECA, Induces Apoptosis in T24 Human Bladder Cancer Cells

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Abstract. *Background: Human A_3 adenosine receptor (A_3AR) plays an essential role in several physiopathological processes. Thus far, A_3AR-selective ligands have been evaluated as anti-inflammation and anticancer therapeutic agents. Among these ligands, truncated thio-Cl-IB-MECA is a newly reported antagonist, and its function has not been studied. Materials and Methods: Cell viability was measured by MTS assay. Cell cycle progression was analysed by propidium iodide (PI) flow cytometric assay. The apoptotic effects were investigated by Hoechst staining and annexin V-FITC/PI staining. The signal-transduction mechanism was explored by Western blot. Results: Truncated thio-Cl-IB-MECA induced the growth arrest of T24 cells at sub-G_1 phase and provoked apoptosis but not necrosis. Apoptotic death was mediated by the activation of extracellular signal-regulated kinase (ERK) and c-Jun N-terminal kinase (JNK). Conclusion: Since truncated thio-Cl-IB-MECA induces anti-proliferation and apoptotic effects via ERK and JNK activation, it may function as an anticancer agent in human bladder cancer cells.*

Bladder cancer is a common malignancy occurring worldwide (1). It is a heterogeneous disease that can be classified into two categories based on the progression and occurrence of tumours in patients: namely, superficial and muscle-invasive tumours. In 70% of patients presenting with superficial bladder cancer, the tumours tend to recur but are mostly not life-threatening (2). In the remaining bladder cancer patients, the tumours present as being muscle-invasive, associated with a high risk of death from distant metastasis (3). Despite aggressive therapeutic approaches, such as radiotherapy or chemotherapy, there are more than 17 million deaths from this disease annually worldwide (4). Thus, bladder cancer is rightly regarded as a significant global public health issue which necessitates the development of more effective anticancer drugs.

A natural purine metabolite, adenosine is a critical signaling molecule that induces various pathological and physiological functions through adenosine receptors (ARs) (5). The ARs are distributed throughout a broad range of tissues and subdivided into A_1, A_{2A}, A_{2B} and A_3, which are all transmembrane spanning receptors coupled to G proteins (6). These four subtypes can be distinguished by their indirect effects on adenylate cyclase activity. The subtype groups of A_1, A_3 and A_{2A}, A_{2B} have effects on the inhibition and stimulation of adenylate cyclase activity and cAMP production, respectively (7). For these reasons, ARs lead to intricate intracellular signals in the human body. In particular, the A_3 subtype has been considered as a good therapeutic target for drug development. Agonists and antagonists for A_3AR have anti-inflammatory effects and anticancer effects (8). Selective A_3AR agonists such as Cl-IB-MECA, IB-MECA and thio-Cl-IB-MECA have been reported to suppress cell proliferation in several cancer cell types, including thyroid, breast, colon, prostate, melanoma, lung and leukemia (9-13). IB-MECA also has anti-inflammatory effects in rheumatoid arthritis (14). The triazole derivatives, which are known to function as potent A_3AR antagonists, appear to induce anti-inflammatory

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effects, anti-asthmatic effects and the anti-glaucoma effect (15, 16). Although A_3AR antagonists are generally associated with anti-inflammatory action, they have also been suggested to function as anticancer agents (17).

In this study, the effect of truncated thio-Cl-IB-MECA on the growth of T24 human bladder cancer cells was examined. It was demonstrated that truncated thio-Cl-IB-MECA inhibited T24 cell proliferation, thereby inducing sub-G_1 cell cycle arrest, and also induced apoptotic cell death *via* ERK and JNK activation in T24 human bladder cancer cells.

Materials and Methods

Reagents. (2*R*,3*R*,4*S*)-2-[2-Chloro-6-(3-iodobenzylamino)-9H-purin-9-yl] tetrahydrothiophene-3, 4-diol was acquired from the Department of Bioinspired Science and College of Pharmacy, Ewha Women's University, South Korea. CellTiter 96 AQ_{ueous} One Solution Cell Proliferation Assay (MTS) reagent was purchased from Promega (Madison, WI, USA). Phenazine methosulfate (PMS), Hoechst stain solution and propidium iodide were purchased from Sigma (St. Louis, MO, USA). FITC Annexin V Apoptosis Detection Kit I, antibodies specific to cyclin A, cyclin D1, cyclin E, and ppRb were purchased from BD Biosciences (San Diego, CA, USA). The ERK inhibitor PD98059 and JNK inhibitor SP600125 were purchased from Calbiochem (France Biochem, Meudon, France). Antibodies specific to PARP, caspase-3, caspase-9, Bax, Bcl-2, cytochrome *c*, phospho-ERK1/2, phospho-SAPK/JNK, and anti-mouse IgG-horseradish peroxidases were purchased from Cell Signaling Technology (Beverly, MA, USA). Antibodies specific to p21, p27, ERK1, JNK, phospho-Rb, GAPDH, and anti-goat IgG-HRP were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Anti-p53 was from Oncogene (Cambridge, MA, USA) and anti-rabbit IgG-HRP was from Assay Designs (Ann Arbor, MI, USA).

Cell culture. T24 human bladder cancer cells were obtained from the American Type Culture Collection (Rockville, MD, USA). T24 cells were cultured in DMEM supplemented with 10% fetal bovine serum (FBS) (Hyclone Laboratories, Logan, UT, USA) and incubated at 37˚C with 5% CO_2.

Reverse transcription-polymerase chain reaction (RT-PCR) analysis. To perform RT-PCR, total RNA was isolated using Easy-BLUE™ total RNA extraction kit (iNtRon Biotechnology, Seoul, Korea). Reverse transcription was conducted by using ProSTAR™ (Stratagene, LaJolla, CA, USA). After the cDNA was synthesised using Oligo (dT), it was amplified by PCR. The GAPDH and A_3AR primers have been previously described (18, 19). The thermal cycling conditions were as follows: 5 min at 92˚C, followed by 40 cycles of 30 s at 92˚C, 1 min at 58.6˚C and 30 s at 72˚C.

Cell viability assay. Cell viability was determined by the MTS assay. Approximately 0.5×10^4 cells per well were seeded in 96-well plates and grown overnight. The cells were treated with different concentrations of truncated thio-Cl-IB-MECA and incubated for 24 and 48 h, respectively. MTS solution was applied to cells for 1-2 h at 37˚C. Optical absorbance was measured at 492 nm using an ELISA reader (Apollo LB 9110; Bertholol, Technologies GmbH, Germany).

Hoechst staining. Cells plated on coverslips were treated with the compound and incubated for 48 h. The coverslips were washed with phosphate-buffered saline (PBS) and fixed with 4% paraformaldehyde for 1 h at room temperature. After washing with PBS, the fixed cells were stained for 20 min with Hoechst stain solution at 37˚C. After washing with PBS, the coverslips were completely dried and mounted on microscope slides. The slides were then observed under fluorescent microscopy.

Cell cycle analysis by flow cytometry. Approximately 1×10^5 cells per well were plated onto 6-well plates and incubated to adhere for overnight. The cells were then treated with the test compound. After 48 h, the cells were harvested and fixed with 70% ethanol at –20˚C. The fixed cells were then washed with PBS prior to staining. The cells were stained for 30 min with PBS containing 50 μmg/ml of propidium iodide and 100 μg/ml of RNase A. The proportion of apoptotic cells were analysed by FACScalibur and CellQuest software (BD Bioscience, San Jose, CA, USA).

Apoptosis assay by annexin V-FITC and propidium iodide staining. Approximately 1×10^5 cells per well were plated onto 6-well plates, incubated to adhere overnight, treated for 48 h with the compound, harvested, and washed with PBS. Staining was conducted with FITC Annexin V Apoptosis Detection Kit I, in accordance with the manufacturer's recommendations. The percentage of early apoptotic cells was calculated by annexin V-positivity and PI-negativity, whereas the percentage of the late apoptotic cells plus necrosis was calculated by annexin V-positivity and PI-positivity.

Western blot analysis. The cells were lysed in lysis buffer containing 20 mM Tris pH 7.4, 0.5% NP-40, 200 mM sodium chloride, 1 mM ethylenediaminetetraacetic acid (EDTA), 10 mM β-glycerophosphate, 10 mM sodium pyrophosphate, 0.05% sodium deoxycholic acid and protease inhibitor cocktail. In the event of cytosolic fraction, the cells were lysed with cytosolic lysis buffer containing 10 mM HEPES-KOH pH 7.9, 10 mM KCl, 2 mM $MaCl_2$, 0.1 mM EDTA, 0.2 mM NaF, 0.4 mM PMSF, 0.1 mM Na_3VO_4, 1 mM DTT. Protein concentrations were determined by Bio-Rad protein assay (Bio-Rad Laboratories, Hercules, CA, USA), and equal amounts of protein were subjected to 12% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), and then transferred to polyvinylidene difluoride membranes (Millipore, Billerica, MA, USA). The membranes were blocked with 5% non-fat dry milk dissolved in TBST containing 25 mM Tris-HCl pH 7.4, 140 mM sodium chloride, 3 mM potassium chloride, and 0.1% Tween-20. Primary antibodies specific to cyclin A, cyclin D1, cyclin E, ppRb, PARP, caspase-3, caspase-9, Bax, Bcl-2, cytochrome *c*, phospho-ERK1/2, phospho-SAPK/JNK, p21, p27, p53, ERK1, JNK, phospho-Rb and GAPDH were applied to the membranes. Horseradish peroxidase-conjugated secondary antibodies were utilised to visualise the bound primary antibodies with Westzol® plus Western Blot detection system (iNtRON Biotechnology).

Statistical analysis. All the presented data and results are confirmed in at least three independent experiments. The data are expressed as the means±SD. Statistical comparisons were conducted by Student's *t*-test. $P<0.005$ was considered statistically significant.

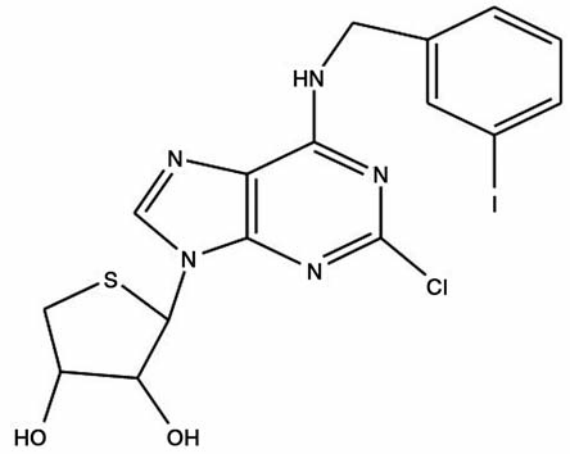

Figure 1. *The chemical structure of (2R,3R,4S)-2-[2-chloro-6-(3-iodobenzylamino)-9H-purin-9-yl] tetrahydrothiophene-3,4-diol (truncated thio-Cl-IB-MECA).*

Results

Anti-proliferative and apoptotic effects of an A3AR antagonist, truncated thio-Cl-IB-MECA, in T24 human bladder cancer cells. Truncated thio-Cl-IB-MECA is a newly synthesised antagonist with high selectivity and affinity to A_3AR as previously described (20, 21) (Figure 1). Prior to the application of truncated thio-Cl-IB-MECA to T24 human bladder cancer cells, RT-PCR was performed to investigate whether A_3ARs are expressed in T24 cells, and the A_3 subtype transcript was detected (Figure 2A). First, the anti-proliferative effects of truncated thio-Cl-IB-MECA on T24 human bladder cancer cells were examined. The cells were exposed to different concentrations of truncated thio-Cl-IB-MECA for 24 h and 48 h, and the cell viability was measured by MTS assay. It was observed that cell growth was significantly inhibited in a time- and dose-dependent manner, and morphological changes were apparent under inverted phase-contrast microscopy (Figures 2B and 2C). It was then ascertained whether nuclear morphology would be altered by treatment with truncated thio-Cl-IB-MECA. Nuclear staining with Hoechst revealed morphological changes of T24 cells at a concentration of 50 μM but not at 30 μM, and nuclear modifications such as distinct condensed chromatin were noted at 50 μM (Figure 2D). Accordingly, it was surmised that truncated thio-Cl-IB-MECA might trigger apoptotic death in T24 human bladder cancer cells. Truncated thio-Cl-IB-MECA-induced apoptosis in T24 cells was examined and confirmed by annexin V and PI staining, using FACS analysis. As shown in Figure 2E, the percentage of viable cells was reduced from 90.17% to 58.32%, but the percentages of early and late apoptotic cells were increased from 9.58% to 26.23% and from 0.25% to 15.40% by this treatment, respectively. However, the percentage of necrotic cells remained unchanged. These results indicate that truncated thio-Cl-IB-MECA inhibits cell proliferation and induces the apoptotic process in early and late stages, but does not induce necrosis in T24 human bladder cancer cells.

Induction of cell cycle arrest at sub-G_1 phase and regulations of cell cycle-related proteins by truncated thio-Cl-IB-MECA. Cell cycle progression in truncated thio-Cl-IB-MECA-treated cells was analysed by flow cytometry. The cells that accumulated at sub-G_1 phase were increased from 14.83% to 61.56%, but the population of cells in G_0/G_1 phase decreased from 52.98% to 10.46% upon compound treatment for 48 h (Figure 3A). These findings led to the postulation that truncated thio-Cl-IB-MECA induces cell cycle arrest at sub-G_1 phase, indicating early stages of the cell cycle progression. The regulation of checkpoint-related proteins corresponding with cell cycle dynamics was then analysed. As shown in Figure 3B, the expression levels of cyclin A, cyclin D1 and cyclin E were reduced in truncated thio-Cl-IB-MECA-treated cells. The expression level of p-pRb protein, which is regulated by G_1 cyclins, cyclin-dependent kinases (CDKs), was also reduced. Considering the upstream effecter of cell cycle regulating factors, the effects of truncated thio-Cl-IB-MECA on expression of p21 and p27 that are involved in the regulation of G_1/S cyclins and p53 tumour suppressor were examined. The expression levels of p21 and p53 were clearly elevated in the compound-treated cells, whereas there was almost no change in the level of p27 expression. These results suggest that truncated thio-Cl-IB-MECA mediates cell cycle arrest in the sub-G_1 phase *via* the down-regulation of G_1/S cyclins and the up-regulation of p21, which is involved in the p53-dependent pathway.

Truncated thio-Cl-IB-MECA induces apoptosis via the processing of caspases and PARP, and regulation of Bax, Bcl-2 and cytochrome c. Caspase-8, caspase-9 and caspase-3 are critical factors in the process of apoptosis including proteolytic cleavage of PARP. As shown in Figure 4A, cleaved PARP (89 kDa) was detected in the compound-treated cells. The levels of cleaved caspase-9 (35 kDa and 37 kDa) and caspase-3 (17 kDa and 19 kDa) increased in a dose-dependent manner. However, cleaved forms of caspase-8 (43 kDa) were not detected in truncated thio-Cl-IB-MECA-induced apoptosis (data not shown). Because apoptosis has been known to be regulated by mitochondrial-related proteins, composed of anti-apoptotic and pro-apoptotic members (22), the modulation of Bax/Bcl-2 family proteins and cytochrome *c* release was evaluated in truncated thio-Cl-IB-MECA-induced apoptosis by Western blotting. As shown in Figure 4B, the level of pro-apoptotic Bax was elevated in 30 μM and 50 μM truncated

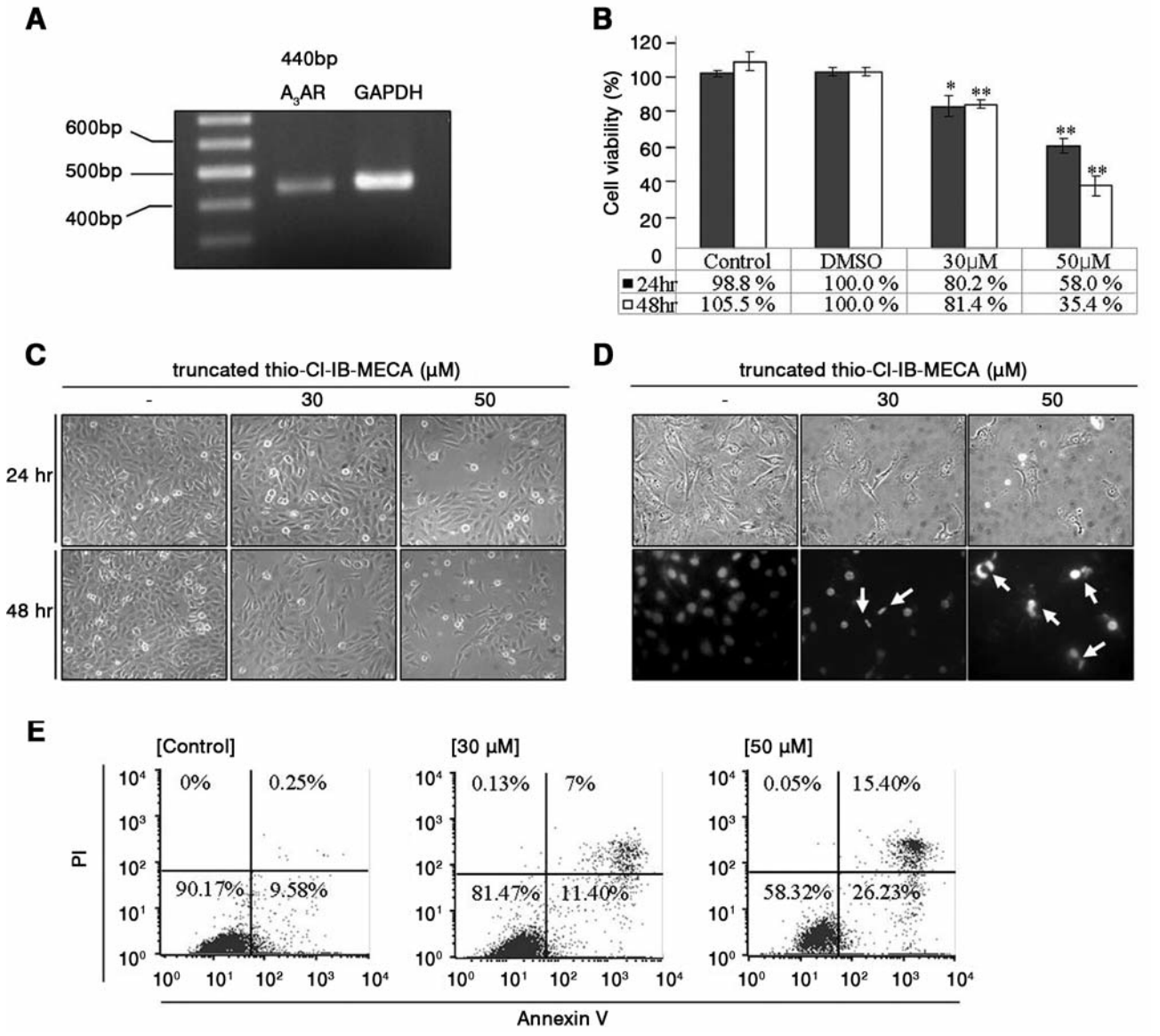

Figure 2. *Anti-proliferative and apoptotic effects of A_3AR antagonist, truncated thio-Cl-IB-MECA, in human bladder cancer T24 cells. A: A_3AR mRNA was analyzed by RT-PCR, and compared with GAPDH as the expression of housekeeping gene in T24 cells. B: The cells were treated with the indicated concentrations for 24 h and 48 h, and the cell viability was measured by MTS assay. Values are shown as mean±SD of three independent experiments conducted in triplicate for each treatment. *p<0.005 and **p<0.0005 vs. untreated control cells. C: Cells treated with indicated concentrations of truncated thio-Cl-IB-MECA for 24 h and 48 h were observed under phase-contrast microscopy (×20) and photographed. D: The cells were treated with the indicated concentrations of the compound for 48 h, and the nuclear morphological changes were observed under fluorescent microscopy by Hoechst staining (×40). E: The cells were stained with Annexin V/PI, and analyzed by FACS.*

thio-Cl-IB-MECA-treated sample groups, whereas the level of pro-survival Bcl-2 was reduced, and then the release of cytochrome *c* into the cytosol was increased by truncated thio-Cl-IB-MECA treatment. These results suggest that truncated thio-Cl-IB-MECA induces apoptosis *via* the processing of caspase-9, caspase-3 and PARP, and the activation of the mitochondrial pathway may be involved in the apoptotic process mediated by truncated thio-Cl-IB-MECA.

Activation of ERK and JNK are required in truncated thio-Cl-IB-MECA-induced apoptosis. A_3AR is strongly associated with mitogen-activated protein kinase (MAPK) pathways, which are known to be involved in cell proliferation and survival. Therefore, it was determined whether MAPK pathways are involved in truncated thio-Cl-IB-MECA-induced apoptosis in T24 human bladder cancer cells. Western blot analysis revealed that the level of phosphorylated ERK1/2 and JNK was markedly

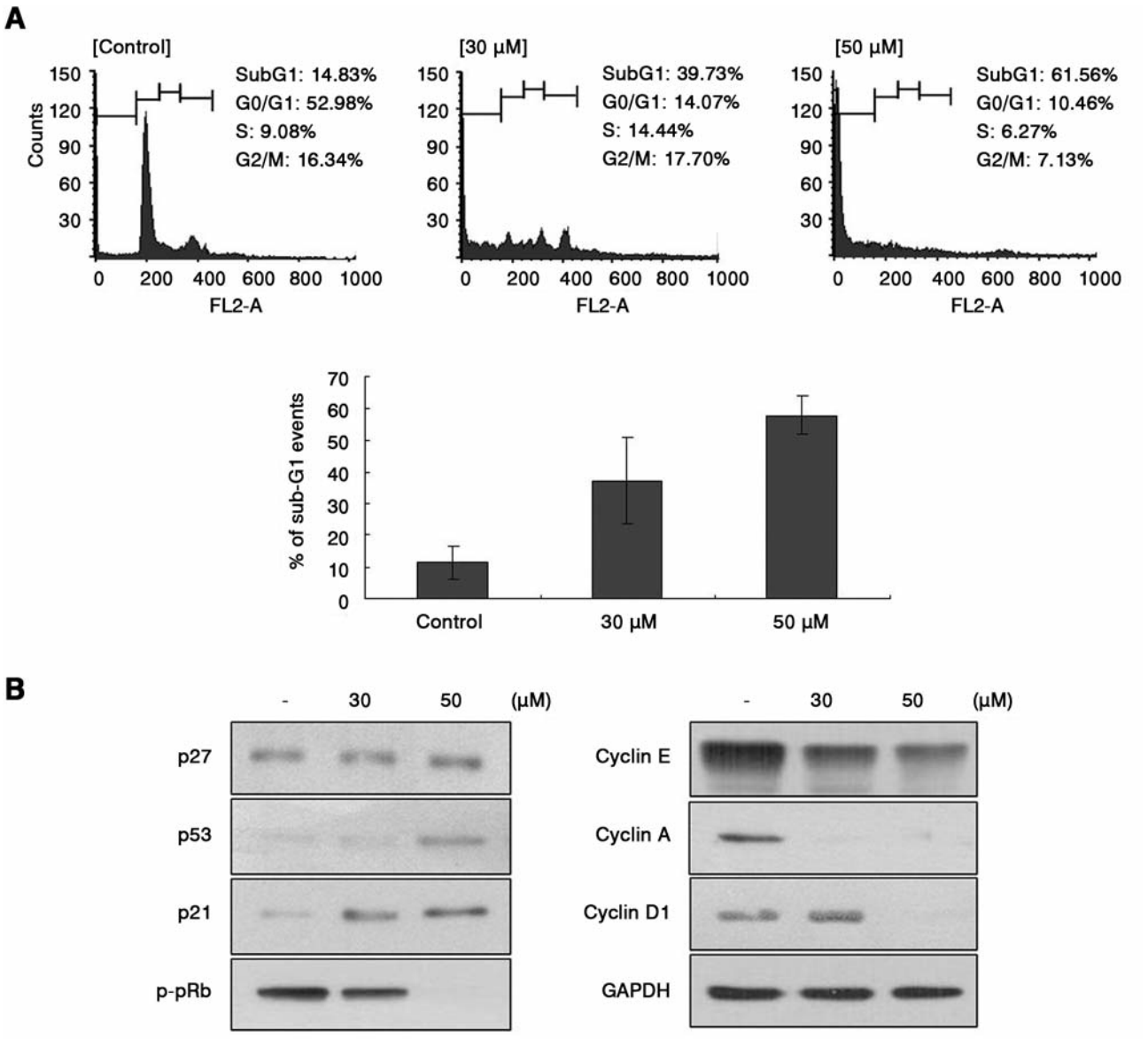

Figure 3. *The effects of truncated thio-Cl-IB-MECA on cell cycle progression in T24 cells. The cells were treated with the indicated concentrations for 48 h. A: The cells were stained with PI staining solution, and 10,000 events were analyzed by flow cytometry. B: The expression levels of cyclin A/D1/E, p21, p27, p53 and p-pRb as cell cycle regulation factors were detected by Western blot analysis.*

elevated in T24 cells treated with truncated thio-Cl-IB-MECA. However, this compound did not affect p38 phosphorylation (Figure 5A). An inhibitory strategy was then applied using the ERK inhibitor PD98059 and the JNK inhibitor SP600125 to truncated thio-Cl-IB-MECA-induced apoptosis. Pretreatment with PD98059 and SP600125 attenuated both ERK and JNK phosphorylations induced by truncated thio-Cl-IB-MECA, respectively. In addition, the processing of PARP and caspase-3/-9 as sub-signal regulators for apoptosis was also inhibited (Figure 5B). These results suggest that the activation of ERK and JNK plays an important role in truncated thio-Cl-IB-MECA-induced apoptosis, and acts as upstream regulators of caspase-3/-9 and PARP, there by inducing apoptosis in truncated thio-Cl-IB-MECA-treated T24 cells.

Discussion

A_3AR plays important roles in several physiopathological processes associated with ischaemia, inflammation, and tumourigenesis (23). A_3AR, which is highly expressed in

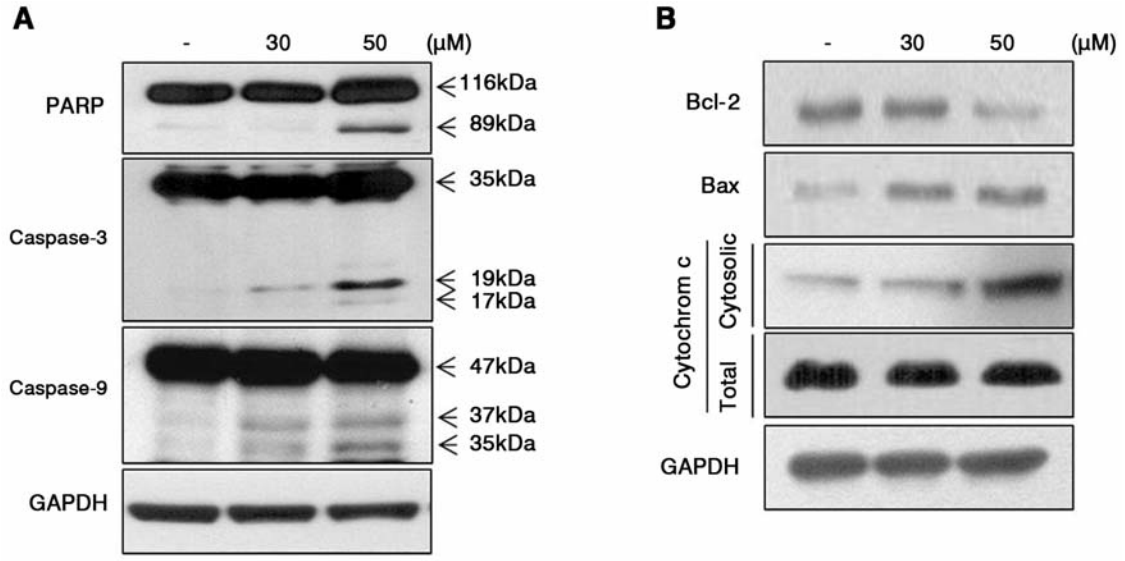

Figure 4. *The effects of truncated thio-Cl-IB-MECA on processing of caspases and PARP and regulations of Bax, Bcl-2 and cytochrome c in T24 cells. The cells were treated with the indicated concentration for 48 h. A: The cleavages of caspase-3, caspase-9 and PARP were detected by Western blot analysis. B: The levels of Bax and Bcl-2 expression and cytochrome c release were detected by Western blot analysis.*

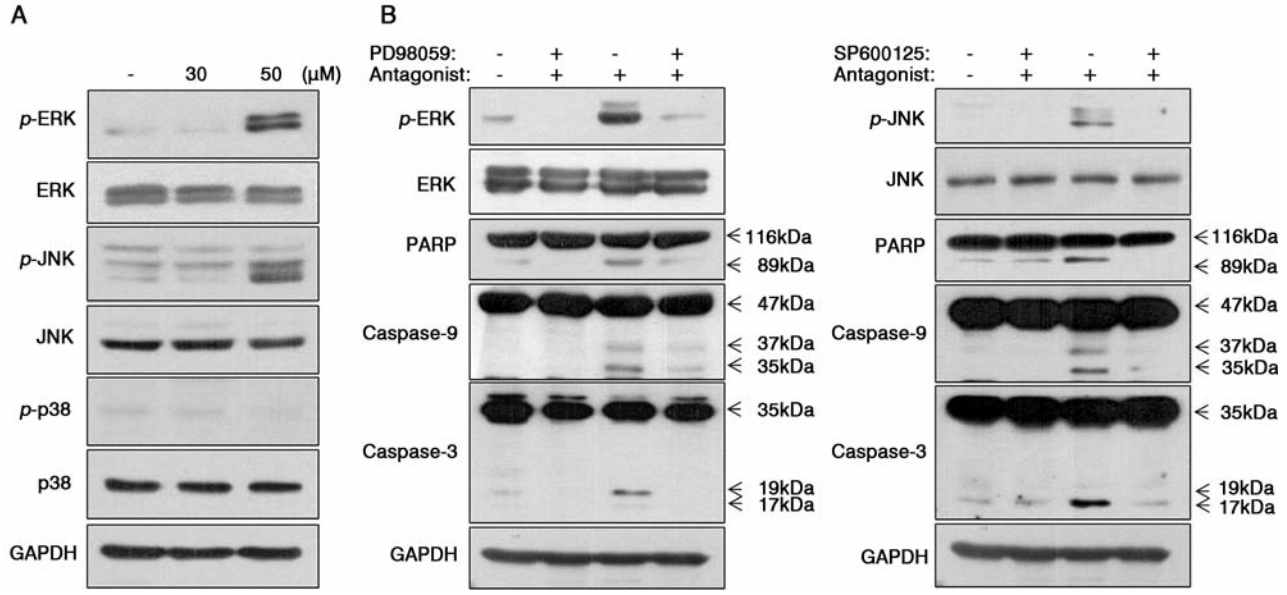

Figure 5. *The effects of truncated thio-Cl-IB-MECA on expression of MAPKs in T24 cells. The cells were treated with the indicated concentrations for 48 h. A: The levels of ERK, JNK and p38 phosphorylation were detected by Western blot analysis. B: The cells were pretreated with 25 μM of ERK inhibitor PD98059 and 10 μM JNK inhibitor SP600125 for 30 min, and then treated with 50 μM of truncated thio-Cl-IB-MECA. The phosphorylation levels of ERK, JNK, and p38, and their sub-signals such as caspases and PARP cleavage were detected by Western blot analysis.*

tumour cells, has been implicated in cell cycle regulation and pro- or anti-apoptotic effects, depending on its activation (24). Based on these relationships between tumours and A_3AR, the A_3 subtype has been considered as a potential target of cancer therapies. Many scientists have tried to synthesise the more selective and potent ligands acting on A_3AR, and have studied their functional analysis. A_3AR agonists such as Cl-IB-MECA, IB-MECA and thio-Cl-IB-MECA have been reported to induce antitumour effects in several cancer cell types (18). Interestingly, a recent study reported that the A_3AR antagonists have anticancer as well as anti-glaucoma and anti-inflammation effects (17). The A_3AR antagonists MRS1191

and L-249313 provoked apoptosis in HL-60 and U937 human leukemia cell lines (25). Additionally, the A_3AR antagonists suppressed cell proliferation under hypoxic conditions, which is typical of solid tumours (23). In this regard, both A_3AR agonists and antagonists represent potential anticancer agents. This study focused on the anticancer effect of truncated thio-Cl-IB-MECA, and applied the effect to T24 human bladder cancer cells expressing A_3AR. Truncated thio-Cl-IB-MECA inhibited the proliferation of T24 cells, thereby inducing nuclear morphological change at 50 μM. However, the aforementioned apoptosis hallmarks are not precise findings in apoptotic death. Accordingly, it was demonstrated that truncated thio-Cl-IB-MECA-induced cell death belongs to apoptotic process of early and late stages but not necrosis. As in the presented anti-proliferation and apoptosis phenomenon by truncated thio-Cl-IB-MECA in T24 cells, cell cycle arrest happened in the sub-G_1 phase. Cell cycle progression is elaborately regulated through a complex network of cell cycle-associated molecules such as CDKs, cyclins, cyclin-dependent kinase inhibitors (CKIs) and pRb proteins (26, 27). This study demonstrated that cell cycle arrest at sub-G_1 correlated to the reduction of cyclin D1/E/A and p-pRb and the up-regulation of p53 and p21.

Apoptosis is mediated *via* two major apoptotic pathways: death receptor-mediated extrinsic pathways and mitochondria-emanated intrinsic pathways (28). Caspase-8 is activated through death receptor while caspase-9 is *via* mitochondrial pathways. These signal transductions following extrinsic or instrinsic pathways result in processing of PARP and caspase-3. This study demonstrated that the activation of caspase-9/-3 and PARP cleavage were induced in truncated thio-Cl-IB-MECA-provoked apoptosis, and the pro- and anti-apoptotic molecules were regulated by mitochondrial pathway associated with the activation of caspase-9.

The MAPKs, are activated by various cellular stresses and growth factors, and are major signaling transduction molecules in apoptosis (29). A_3AR-induced ERK1/2 activation had been suggested to be important for human astrocyte proliferation (30). CHO A3 cells react to adenosine A_3 receptor stimulation with A_3AR agonist, IB-MECA or Cl-IB-MECA, and induce cell cycle arrest and decrease in proliferation *via* ERK1/2 phosphorlyation (31). A recent study showed that Cl-IB-MECA inhibits the proliferation of human thyroid cancer cell independently of A_3 adenosine receptor activation *via* inhibition of phosphorylation of ERK (32). The present data showed that truncated thio-Cl-IB-MECA strongly induces phosphorylation of ERK and JNK but not p38 in T24 human bladder cancer cells. These results were confirmed by the signal recovery effect through pre-treating the PD98059 of ERK inhibitor and SP600125 of JNK inhibitor. Therefore, it can be concluded that the activation of ERK and JNK are major signaling pathways in truncated thio-Cl-IB-MECA-induced apoptosis in T24 cells. Although truncated thio-Cl-IB-MECA pharmacologically does not indicate opposite effects against A_3AR agonists, these can be possible results in that A_3AR is an enigmatic player which presents a double nature in different pathophysiological conditions.

In this present study, the precise mechanism underlying the anticancer effect of a newly reported A_3AR antagonist, truncated thio-Cl-IB-MECA, was elucidated in T24 human bladder cancer cells. Truncated thio-Cl-IB-MECA induced the sub-G_1 cell cycle arrest and ensuing early and late apoptosis. These processes were mediated *via* the activation of ERK and JNK among the MAPK family, and involved the activation of mitochondrial pathway. Taken together, these results imply that truncated thio-Cl-IB-MECA may be a potential therapeutic agent in cancer therapy.

Acknowledgements

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