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A novel adenosine analog, thio-Cl-IB-MECA, induces G_0/G_1 cell cycle arrest and apoptosis in human promyelocytic leukemia HL-60 cells

Eun-Jin Lee, Hye-Young Min, Hwa-Jin Chung, Eun-Jung Park, Dae-Hong Shin, Lak Shin Jeong [1], Sang Kook Lee *

[a] College of Pharmacy, Ewha Womans University, 11-1 Daehyun-dong, Seodaemun-ku, Seoul 120 750, Republic of Korea

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Abstract

Human A_3 adenosine receptor (A_3AR) agonists have been shown to play important roles in several physiological and pathological processes, including growth inhibition of human cancer cells. On this line, we recently found that a novel adenosine analog, 2-chloro-N^6-(3-iodobenzyl)-4′-thioadenosine-5′-*N*-methyluronamide (thio-Cl-IB-MECA) was a potent human A_3AR agonist, and is superior to a known agonist Cl-IB-MECA [Jeong LS, Jin DZ, Kim HO, Shin DH, Moon HR, Gunaga P, et al. J Med Chem 2003;46:3775]. Here, we report that a novel A_3AR agonist, thio-Cl-IB-MECA inhibited the growth of human promyelocytic leukemia HL-60 cells by arresting cell cycle and induction of apoptosis. Thio-Cl-IB-MECA induced the cell cycle arrest of G_0/G_1 in the early time and at lower concentration (up to 25 μM). At higher concentration (50 μM), the apoptotic cell deaths were manifested by observation of the increase of sub-G_0 phase of cell cycle distribution, DNA fragmentation and poly(ADP-ribose) polymerase (PARP) cleavage. In addition, the down-regulation of checkpoint protein cyclin D1 and c-myc by thio-Cl-IB-MECA was well correlated with the arrest of cell cycle transition of G_1 to S phase. Further study revealed that the growth inhibitory activity of thio-Cl-IB-MECA is also related with the modulation of Wnt signaling pathway. The levels of β-catenin, phosphorylated forms of GSK-β and Akt were down-regulated by the treatment of thio-Cl-IB-MECA (10 nM) in a time-dependent manner, providing one of plausible mechanistic evidence for the involvement of the Wnt signaling pathway in the HL-60 cell growth inhibitory effects by thio-Cl-IB-MECA. These results suggest that a novel A_3AR agonist, thio-Cl-IB-MECA can down-regulate Wnt signaling, inhibit proliferation and induce apoptosis in HL-60 leukemia cells, and thus provide the possibility of this compound in the potential therapeutic value of the treatment of leukemia.

Keywords: A_3 adenosine receptor agonist; Thio-Cl-IB-MECA; HL-60 cells; Cell cycle arrest; Apoptosis; Wnt signaling

1. Introduction

A natural purine metabolite adenosine plays important roles in a variety of physiological and pathophysiological processes, including modulation of cell proliferation [1]. Recent studies also reported that most of physiological functions of adenosine are mediated by the activation of specific cell surface adenosine receptors. At least four adenosine receptors, A_1, A_{2A}, A_{2B}, and A_3, have been cloned and identified in different tissues. In particular, the A_3 adenosine receptor (A_3AR) was recently identified and cloned from brain tissues and cardiac muscles [2,3]. The relative high expression of A_3AR was also found in several tumor cell lines [4–7]. A_3AR belongs to the G_i protein-coupled receptor, and its activation subsequently leads to the inhibition of adenylate cyclase activity, cAMP formation, and PKA expression, resulting in the initiation of various cellular signaling pathways. Since A_3AR is closely related to several diseases, including cardiac ischemia, cerebral ischemia, inflammation, and tumorigenesis, A_3AR agonists are considered as one of promising therapeutic agents for the treatment of ischemia and cancer [8–11]. Indeed, several reports demonstrate that A_3AR agonists inhibit cell proliferation and induce apoptosis against several human cancer cell lines. Especially, adenosine analogues, IB-MECA and Cl-IB-MECA were developed as potent and selective A_3AR agonists, and also suppressed cell proliferation and tumor growth in in vitro cell cultures and in vivo animal model, respectively [12–15]. On this

Abbreviations: A_3AR, A_3 adenosine receptor; thio-Cl-IB-MECA, 2-chloro-N^6-(3-iodobenzyl)-4′-thioadenosine-5′-*N*-methyluronamide

* Corresponding author. Tel.: +82 2 3277 3023; fax: +82 2 3277 2851.
E-mail address: sklee@ewha.ac.kr (S.K. Lee).

[1] Equally contributed as a corresponding author.

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line, we recently designed and synthesized a new ligand, thio-Cl-IB-MECA, which possessed three times higher affinity to human A_3AR than Cl-IB-MECA and relatively high selectivity to human A_3AR versus human A_1 and A_{2A} adenosine receptors [16].

In the present study, we examined the effect of thio-Cl-IB-MECA on the growth of human promyelocytic leukemia HL-60 cells. We found that thio-Cl-IB-MECA, a novel A_3AR agonist, can inhibit cell proliferation and induce apoptosis, and one plausible mechanism on the growth inhibition might be the regulation of β-catenin and GSK-3β, key signaling components of Wnt signaling pathway.

2. Materials and methods

2.1. Chemicals

3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), RNase A, propidium iodide, bisbenzimide H 33258 (Hoechst 33258), and mouse monoclonal anti-β-actin primary antibody were purchased from Sigma (St. Louis, MO, USA). RPMI medium 1640, fetal bovine serum (FBS), non-essential amino acid solution (10 mM, 100×), trypsin–EDTA solution (1×), and antibiotic–antimycotics solution (PSF) were from Invitrogen Co. (Grand Island, NY, USA). Mouse monoclonal anti-p53, anti-c-myc, rabbit polyclonal anti-Bcl-2, anti-cyclin D1 primary antibody, and horseradish peroxidase (HRP)-conjugated anti-mouse and anti-rabbit IgG were from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Mouse monoclonal anti-PARP, anti-β-catenin, and anti-GSK-3β were from BD Biosciences (San Diego, CA, USA). Rabbit polyclonal anti-phospho–Akt, anti-phospho–GSK-3β, and anti-Akt antibodies were from Cell Signaling Technology (Beverly, MA, USA).

Thio-Cl-IB-MECA (LJ-529) was synthesized as described by Jeong et al. [16] (Fig. 1).

2.2. Cell culture

Human promyelocytic leukemia HL-60 cells, obtained from the American Type Culture Collection (ATCC), were cultured in RPMI medium 1640 supplemented with 10% heat-inactivated fetal bovine serum, 100 units/ml penicillin, 100 μg/ml streptomycin, and 250 ng/ml amphotericin B. Cells were maintained at 37 °C in humidified atmosphere with 5% CO_2.

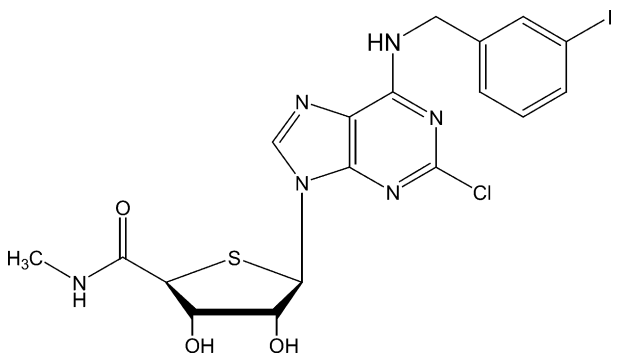

Fig. 1. Chemical structure of thio-Cl-IB-MECA.

2.3. Evaluation of growth inhibitory potential

HL-60 cells (1×10^5 cells/ml in 96-well plates) were treated with various concentrations of test compounds for 3 days. After treatment, MTT (5 mg/ml in PBS) was added to media (final 500 μg/ml) and further incubated for 4 h. Media were discarded, and 200 μl of DMSO was added to each well to dissolve formazan. The absorbance was measured at 570 nm. The result was expressed as a percentage, relative to solvent-treated control incubations, and the IC_{50} values were calculated using non-linear regression analysis (percent survival versus concentration).

2.4. Observation of morphological changes by treatment with thio-Cl-IB-MECA

HL-60 cells were treated with 25 and 50 μM thio-Cl-IB-MECA for 24 h. Before harvesting, morphological changes were observed under a phase-contrast microscope. Cells were collected by centrifugation at $2000 \times g$ for 5 min. Cells were washed with ice-cold PBS and then fixed with 4% paraformaldehyde. The fixed cells were placed on slides and stained with Hoechst 33258 for 30 min. After staining, cells were observed under confocal microscope (Zeiss, LSM 510).

2.5. DNA fragmentation

HL-60 cells were plated in 100 mm culture dish at a density of 4×10^6 cells/dish. Twenty-four hours later, the cells were treated with various concentrations of test agent for 24 h. After treatment, cells were collected by centrifugation, washed with PBS and lysed with lysis buffer containing 50 mM Tris–HCl, pH 7.5, 20 mM EDTA, and 1% NP-40. After centrifugation, 1% SDS and RNase A (0.5 μg/μl) were added to the supernatant and then incubated at 55 °C for 2 h. Subsequently, proteinase K (5 μg/μl) was added and then incubated at 37 °C for 2 h. Cellular DNA was extracted by addition of equal volume of phenol:chloroform:isoamyl alcohol (25:24:1), and DNA was precipitated with 0.5 vol of 10 M ammonium acetate and 2.5 vol of cold ethanol at −20 °C overnight. Precipitated DNA was dissolved in 50 μl of 10 mM Tris buffer (pH 8.0) containing 1 mM EDTA. DNA samples (4 μg) were resolved by electrophoresis in 1.8% agarose gel, and then stained with SYBR Gold (Molecular Probes, Eugene, OR, USA) and visualized under the UV transilluminator.

2.6. Analysis of cell cycle dynamics by flow cytometry

Cell cycle analysis by flow cytometry was performed as previously described [17]. Briefly, HL-60 cells were plated at a density of 2×10^6 cells per 100 mm culture dish and incubated for 24 h. Fresh media containing test samples were added to culture dishes. After additional incubation for 24 h, cells were collected by centrifugation, and resuspended with PBS. Cells were centrifuged at $2000 \times g$ for 5 min, washed again with PBS, and then fixed with 90% ethanol. Fixed cells were washed with phosphate–citrate buffer (pH 7.2), and incubated with a staining solution containing 0.2% NP-40, RNase A (30 μg/ml), and propidium iodide (50 μg/ml) in phosphate–citrate buffer (pH 7.2) for 30 min. Cellular DNA content was analyzed by flow cytometry using a Becton Dickinson laser-based flow cytometer. At least 20,000 cells were used for each analysis, and results were displayed as histograms. Cell cycle distribution was analyzed using ModFit LT 2.0 program.

2.7. Western blot analysis

Cells were treated with various concentrations of thio-Cl-IB-MECA for the indicated times. For evaluation of effect on the activation of Wnt signaling pathway, cells were deprived of serum for 16 h, and then incubated with complete medium containing 10 nM thio-Cl-IB-MECA for the indicated time interval. After incubation, cells were lysed with boiling 2× sample loading buffer (250 mM Tris–HCl (pH 6.8), 4% SDS, 10% glycerol, 0.006% bromophenol blue, 2 mM sodium orthovanadate, and 2% β-mercaptoethanol), and protein concentration of each sample was determined by BCA method. Each protein (30–50 μg) was subjected to 8 or 10% SDS–PAGE. Proteins were transferred onto PVDF membranes by electroblotting, and membranes were incubated for 1 h with blocking buffer (5% non-fat dry milk in phosphate-buffered saline, 0.1% Tween 20 (PBST)). Membranes were then incubated with indicated antibodies overnight at 4 °C, and then washed three times (each for 5 min) with PBST. After washing, membranes were incubated with corresponding HRP-conjugated IgG diluted 1:2000 in PBS for 1 h at room temperature. Membranes were washed three times with PBST, and blots were detected using ECL reagent (Amersham Corp., Arlington Heights, IL, USA) [17]. The expression of β-actin was used as an internal standard.

2.8. Statistical analysis

Data were presented as means ± S.E. for the indicated number of independently performed experiments. Statistical significance ($P < 0.05$) was assessed by one-way analysis of variance (ANOVA) coupled with Dunnett's *t*-tests.

3. Results

3.1. Growth-inhibitory effect of thio-Cl-IB-MECA in HL-60 cells

HL-60 cells were treated with various concentrations of thio-Cl-IB-MECA for 3 days, and viable cells were measured by MTT method. As shown in Fig. 2A, thio-Cl-IB-MECA inhibited the growth of HL-60 cells in a dose-dependent manner. This anti-proliferative effect was apparent at the concentration of 50 μM. In addition, we examined morphological changes by treatment with thio-Cl-IB-MECA using a phase-contrast microscope. As illustrated in Fig. 2B, even though the number of cells was gradually

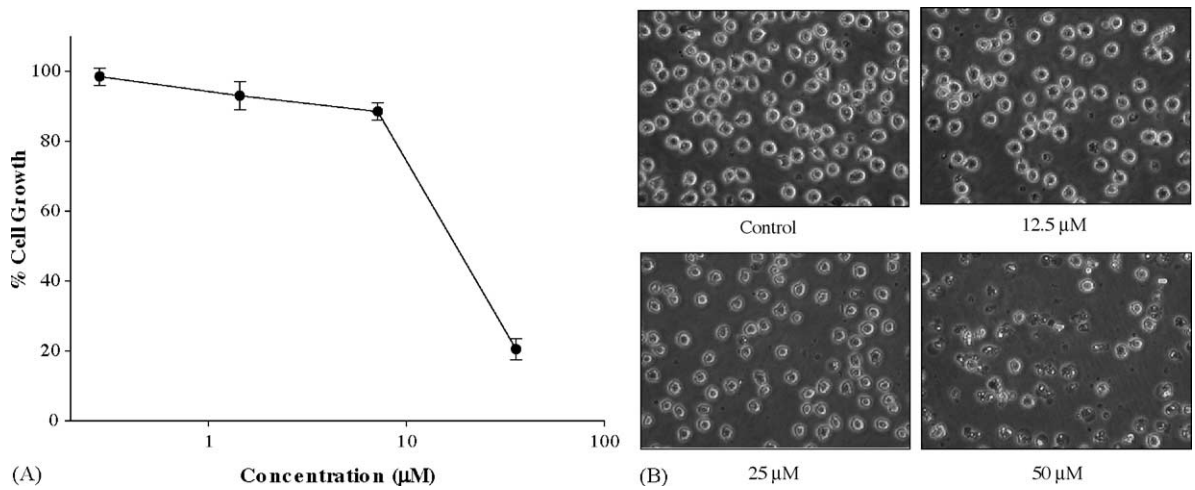

Fig. 2. (A) Growth inhibitory effect of thio-Cl-IB-MECA in cultured HL-60 cells. Cells were treated with the indicated concentrations of thio-Cl-IB-MECA for 3 days and determined the cell growth with MTT method. Values were averaged expressed as a percentage relative to the untreated control. Values indicate the mean ± S.D. in triplicate tests. (B) Morphological change in HL-60 cells treated with thio-Cl-IB-MECA. Morphological change of HL-60 cells treated with DMSO (control) alone or various concentrations of thio-Cl-IB-MECA for 24 h were observed under the phase-contrast microscope and photographed (magnification, 100×).

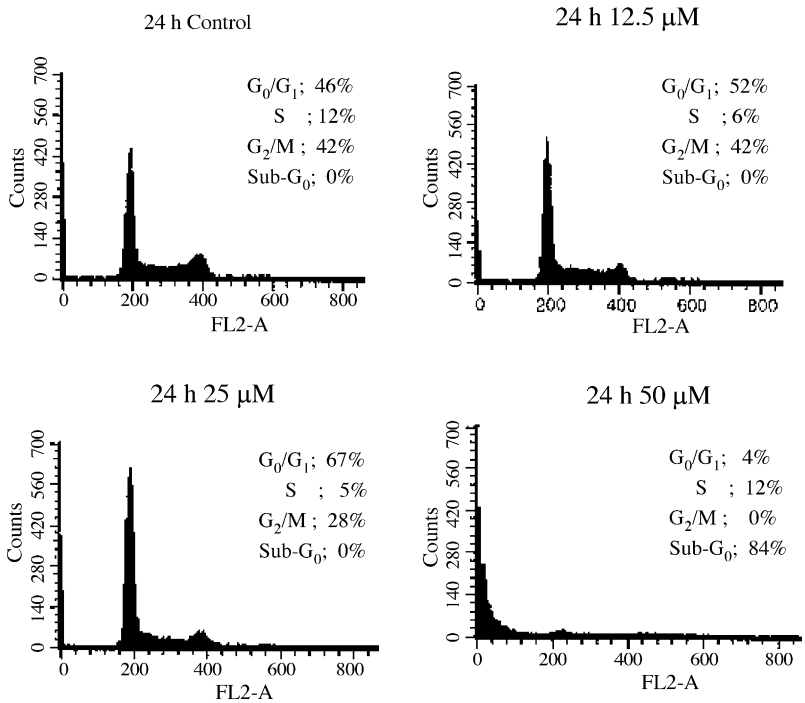

Fig. 3. The effect of thio-Cl-IB-MECA on cell cycle progression in HL-60 cells. Cells were treated with the indicated concentrations of thio-Cl-IB-MECA for 24 h and then the distribution of cell cycle was analyzed by flow cytometry.

decreased, apparent morphological changes were not observed in cells treated with thio-Cl-IB-MECA up to 25 μM. However, at 50 μM, cells with distinct morphology (irregular shaped, gray-colored cells) were markedly increased, indicating induction of cell death.

3.2. Cell cycle distribution by thio-Cl-IB-MECA

In order to examine whether the growth inhibitory effect of thio-Cl-IB-MECA is related to induction of cell cycle arrest or apoptotic process, HL-60 cells were treated with various concentrations (12.5, 25, or 50 μM) of thio-Cl-IB-MECA for 24 h, and the distribution of cells in various compartments of the cell cycle was analyzed by flow cytometry. When treated with the compound up to 25 μM, cells significantly accumulated in the G_1 phase of the cell cycle in a concentration-dependent manner. However, the treatment with 50 μM of the compound drastically induced the increase of cell population in the sub-G_1 phase, indicative of apoptotic cell death (Fig. 3).

3.3. Effects on DNA fragmentation by thio-Cl-IB-MECA

To further clarify whether the cytotoxic effect of 50 μM thio-Cl-IB-MECA was associated with apoptosis, DNA was extracted from HL-60 cells after exposure to various concentrations of the compound (12.5, 25, or 50 μM) for 24 h and subjected to agarose gel electrophoresis. As illustrated in Fig. 4A, DNA fragmentation, which is one of hallmarks of apoptotic phenomena, was clearly detected by treatment with 50 μM thio-Cl-IB-MECA. The nuclear morphological changes were also detected by nuclear staining with the Hoechst 33258 dye under the confocal microscope. The exposure of 25 μM of the compound did not show any distinct changes compared to control cells. However, the treatment with 50 μM thio-Cl-IB-MECA induced the increase of chromatin condensation and fragmentation (Fig. 4B). These results suggest that high concentration (50 μM) of thio-Cl-IB-MECA induces apoptosis.

3.4. Effects of thio-Cl-IB-MECA on the expression of regulatory proteins

To investigate whether the induction of G_1 cell cycle and apoptosis mediated by thio-Cl-IB-MECA was related to the expression of regulatory proteins, cyclin D1, c-myc, Bcl-2, and PARP levels were determined by Western blot analysis. As shown in Fig. 5, when the HL-60 cells were treated with various concentrations of the compound for 24 h, the levels of cyclin D1 and c-myc protein expression were gradually decreased in a concentration-dependent manner. In contrast, the treatment with 50 μM thio-Cl-IB-MECA, the concentration which induced apoptotic cell death, was clearly induced PARP cleavage. In addition, time-dependent down-regulation of c-myc and cyclin D1 expression was also evoked by treatment with 25 μM thio-Cl-IB-MECA. However, thio-Cl-IB-MECA did not affect on the expression level of Bcl-2 protein, indicating that Bcl-2 might not be involved in thio-Cl-IB-MECA-induced apoptosis.

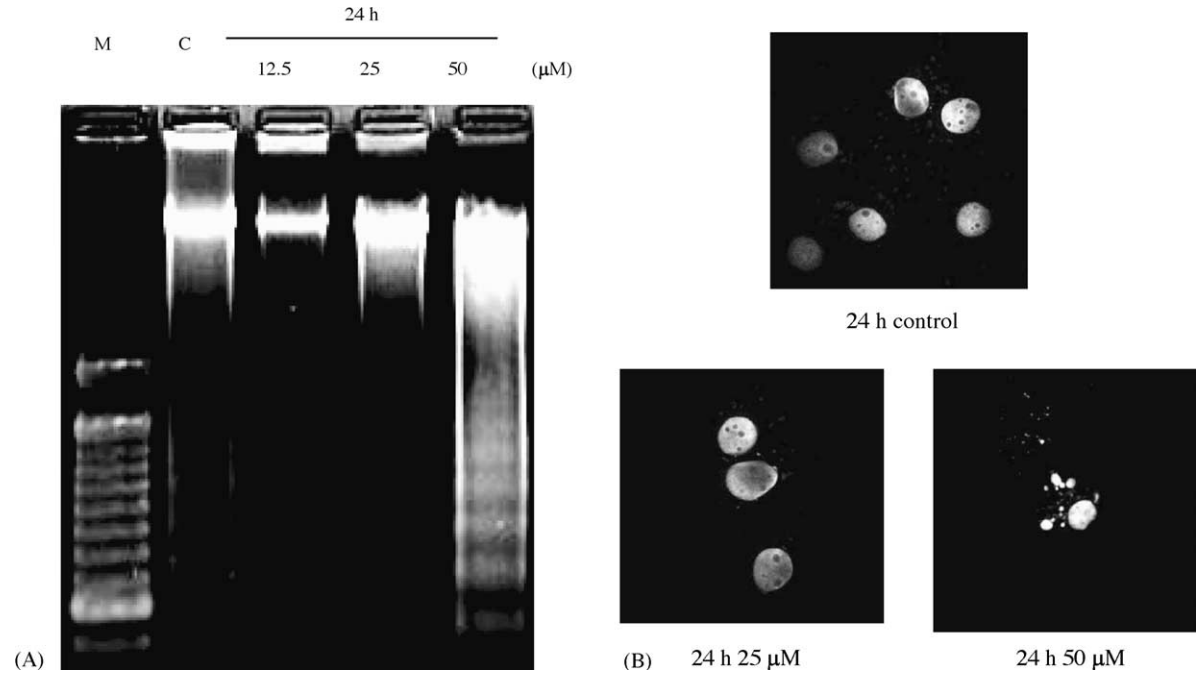

Fig. 4. Effects of thio-Cl-IB-MECA on DNA fragmentation in HL-60 cells. Cells were treated with various concentrations of thio-Cl-IB-MECA for 24 h, and then DNA fragmentation was determined by agarose gel electrophoresis (A) or nuclear staining examination with the Hoecht 33258 fluorescent dye (B).

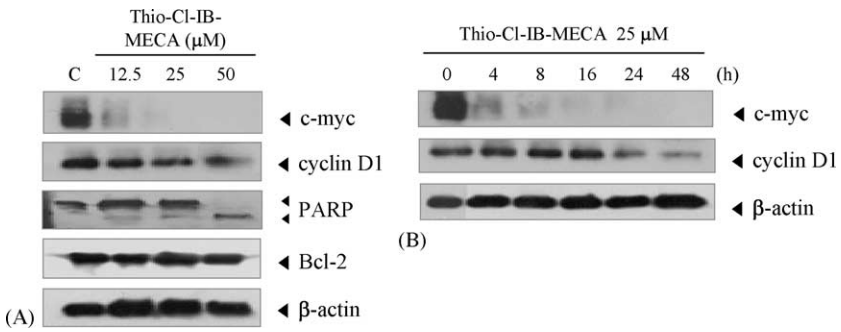

Fig. 5. The effects of thio-Cl-IB-MECA on the expression of c-myc, cyclin D1, and PARP cleavage in HL-60 cells. (A) Cells were treated with the indicated concentrations of thio-Cl-IB-MECA for 24 h and then analyzed the expression of proteins by Western blotting. (B) Time-dependent expressions of regulatory proteins were analyzed with thio-Cl-IB-MECA (25 μM) in HL-60 cells.

3.5. Involvement of Wnt signaling in thio-Cl-IB-MECA-induced cell growth regulation

It is documented that the activation of A_3AR is associated with the down-regulation of Wnt signaling [13]. To investigate the involvement of Wnt signaling in thio-Cl-IB-MECA-mediated anti-proliferative effects, HL-60 cells were treated with thio-Cl-IB-MECA (10 nM) for the indicated times. The activation of Akt (p-Akt) by serum stimulation was significantly alleviated in the thio-Cl-IB-MECA-treated cells (Fig. 6). In addition, the phosphorylated form of GSK-3α/β (p-GSK-3α/β) in the control cells was gradually increased during the incubation time up to 60 min, but a noticeable decrease of phosphorylated GSK-3α/β was detected after 30 min in thio-Cl-IB-MECA-treated cells. Consequently, the level of β-catenin was gradually decreased by treat-

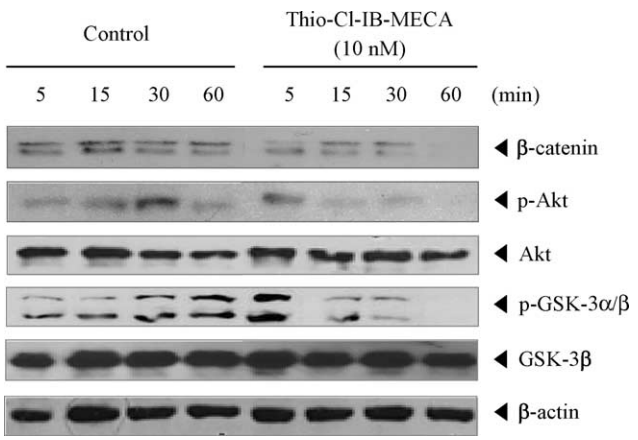

Fig. 6. Down-regulation of Wnt signaling by treatment with thio-Cl-IB-MECA. Cells were treated with thio-Cl-IB-MECA (10 nM) for the indicated times as described in Section 2. The expression of relevant proteins was analyzed by Western blotting.

ment with thio-Cl-IB-MECA in a time-dependent manner.

4. Discussion

Recent studies revealed that A_3AR plays a key role in a variety of physiological functions including the modulation of cell proliferation. On this line, A_3AR agonists have been found to be active as antitumor agents in in vitro and in vivo. In particular, IB-MECA and Cl-IB-MECA were considered as potential antitumor agents [13,14]. In the course of development of potential A_3AR agonists from synthetic nucleosides, we recently found that a novel nucleoside thio-Cl-IB-MECA is superior to Cl-IB-MECA in terms of selectivity and affinity to A_3AR [16]. Based on the antitumor activity of Cl-IB-MECA, we explored to determine the anti-proliferative activity of thio-Cl-IB-MECA against human cancer cells and investigated the possible mechanism of action. Thio-Cl-IB-MECA inhibited the proliferation of human promyelocytic leukemia cells in vitro through arresting cell cycle progression and inducing apoptosis. Significantly, the lower concentrations of thio-Cl-IB-MECA (up to 25 μM) were found to be effective in the suppression of cell proliferation with cytostatic effect, but the higher concentration (50 μM) of thio-Cl-IB-MECA induced cytotoxic effect (Fig. 2). The cytostatic mechanism of thio-Cl-IB-MECA in HL-60 cells appeared to be related to the induction of cell cycle arrest at G_1 phase (Fig. 3). Cyclin D1 and c-myc expression were also down-regulated by treatment with thio-Cl-IB-MECA in a time- and dose-dependent manner, which was well correlated with G_1 phase cell cycle arrest by the compound (Fig. 5). In addition, the cytotoxic effect and induction of apoptosis by treatment with the high concentration (50 μM) of thio-Cl-IB-MECA were to be explained by increase of DNA fragmentation and PARP cleavage (Figs. 4 and 5). Further study might be needed to explore the pro-apoptotic mechanism of thio-Cl-IB-MECA in relation to regulation of relevant proteins, such as activation of caspases.

The Wnt signaling plays a key role in embryogenesis, regulation of cell proliferation, motility, and cell fate, and aberrant Wnt signaling has been shown to contribute to a variety of human cancers [18–21]. In the absence of Wnt signaling, a complex, which is composed of adenomatous polyposis coli (APC), glycogen synthase kinase (GSK)-3β, and axin, binds β-catenin and sequesters it in the cytoplasm, leading degradation of β-catenin by ubiquitin–proteasome pathway. However, upon activation of Wnt signaling, GSK-3β is phosphorylated and loses its ability on the formation of complex with β-catenin. Stabilized β-catenin in the cytoplasm translocates to the nucleus and complexes with Tcf/Lef transcription factors to mediate the induction of transcription of c-myc and cyclin D1, resulting in activation of cell proliferation [18,19]. Indeed, PKB/Akt is one of upstream kinases that phosphorylates GSK-3β and inactivates its function [22]. Although Wnt signaling has been known to play an important role in colon cancer carcinogenesis [20,21], recent studies demonstrate that Wnt signaling pathway is also constitutively activated in various types of leukemia [23,24]. A_3AR is expressed in human neutrophils and HL-60 cells [12,25]. In addition, an A_3AR agonist IB-MECA interacts with A_3AR, and induces inhibition of Akt phosphorylation, activation of GSK-3β, and subsequent enhancement of β-catenin degradation. These events result in down-regulation of c-myc and cyclin D1 expression, and eventually suppression of cell proliferation in B16–F10 melanoma cells [13]. Our results also indicate that thio-Cl-IB-MECA suppressed cell proliferation by time- and dose-dependent inhibition of c-myc and cyclin D1 expression (Fig. 5). Based on this line, we assumed that one of anti-proliferative mechanism of thio-Cl-IB-MECA might be related to regulation of Wnt signaling pathway via interaction with A_3AR in HL-60 cells. Since thio-Cl-IB-MECA is a potent and selective A_3AR agonist with the K_i value of 0.38 ± 0.07 nM [16], we selected the low concentration (10 nM) of thio-Cl-IB-MECA to examine on the regulation of Wnt signaling pathway for only evaluating an effect via interaction with A_3AR and excluding other effects, such as direct regulation of signaling pathway by this compound. Treatment with thio-Cl-IB-MECA suppressed Akt activation and GSK-3β phosphorylation, and consequently inhibited β-catenin expression, demonstrating down-regulation of Wnt signaling activation by thio-Cl-IB-MECA in HL-60 cells (Fig. 6). Therefore, it is suggested that the regulation of Wnt signaling pathway might be involved in the growth inhibitory effect of thio-Cl-IB-MECA in HL-60 cells, and this result might provide an additional mechanism of A_3AR agonists on regulation of cell proliferation in human leukemia cells. A recent report published by Merighi et al. suggests that the mechanism of Cl-IB-MECA on the suppression of cell proliferation is to induce down-regulation of extracellular signal-regulated kinase (ERK) by activation of phosphatidylinositol-3-kinase (PI3K)/Akt pathway in human melanoma cells [26]. Thus, further studies should be needed to investigate the anti-proliferative mechanism of thio-Cl-IB-MECA in relation to several signal transduction pathways which are affected by A_3AR activation, including protein kinase A and mitogen-activated protein kinases (MAPKs) pathway as demonstrated in other reports [14,26].

In summary, our in vitro studies show that a novel class of A_3AR agonist thio-Cl-IB-MECA inhibits the growth of human leukemia cells by arresting cell cycle progression and inducing apoptosis. In addition, the regulation of Wnt signaling pathway might be involved in one of anti-proliferative mechanism of thio-Cl-IB-MECA in HL-60 cells. Moreover, in our recent study, thio-Cl-IB-MECA suppressed tumor growth in in vivo nude mouse xenograft model without any overt side effects (data not shown). This

report, therefore, may provide a novel chemical class of antitumor agents with thio-sugar nucleosides targeting A_3AR functionality, and also indicate the possible mechanism of this type of compound on the control of cancer cell growth. Further studies are in progress to investigate the additional anti-proliferative and antitumor mechanism of novel thio-sugar nucleosides in in vitro and in vivo experimental systems.

Acknowledgements

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