ANTICANCER RESEARCH *37*: 3553-3561 (2017)
doi:10.21873/anticanres.11725

Inhibitory Effects of ATP and Adenosine on Cholangiocarcinoma Cell Proliferation and Motility

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Abstract. *Background/Aim: Inhibitory effects of extracellular nucleotides have been investigated in many types of cancers. Herein, we aimed to determine the effects of ATP and adenosine and their receptor profile on cholangiocarcinoma (CCA) cells. Materials and Methods: Two CCA and one immortalized cholangiocyte cell line were used. The effects of ATP and adenosine on cell proliferation and motility were examined by MTT and wound-healing/trans-well invasion assays, respectively. Purinergic receptor profiling was carried out by reverse transcription-polymerase chain reaction (RT-PCR). Results: ATP and adenosine induced proliferation-inhibitory and motility-inhibitory effects in all cell lines tested. However, immortalized cholangiocytes showed resistance in proliferation inhibition. Several P2 receptors were commonly expressed in all cells, whereas no adenosine receptor was expressed. Furthermore, no synergistic effects of ATP and adenosine were observed in CCA cells. Conclusion: ATP and adenosine had anti-proliferative and anti-motility effects in CCA cells, while there was a smaller effect on normal cholangiocytes. These data indicate the potential use of ATP and odenosine as a novel therapy for CCA.*

Cholangiocarcinoma (CCA) is a silent and lethal disease. The incidence of CCA is increasing worldwide (1-3). The highest incidence rate is in Southeast Asia (4), particularly in the northeastern provinces of Thailand, where the incidence has been reported to be as high as 115 per 100,000 males (5). Patients are usually diagnosed with locally advanced or metastatic disease. Unfortunately, current chemotherapies are not effective against CCA and no randomized trial has demonstrated a curative benefit of a systemic therapy yet (6). Surgery is still the only curable treatment option (7).

New therapeutic strategies and novel compounds for CCA treatment are being sought. One of the emerging therapeutic agents for cancer is ATP. ATP signals through a subfamily of purinergic receptors known as P2Y and P2X receptors. Also, ATP could be hydrolyzed into adenosine and signal through adenosine receptors. Effects of ATP and adenosine on cancers vary depending on the cancer type, potentially due to the differential expression of purinergic receptors on each cancer type, and the dosage of extracellular ATP or adenosine used (8-10). Short- and long-term (trophic) purinergic signaling in humans has been studied in both normal and pathogenic conditions, including cancer (11-15). Some researchers have suggested the variable responses of cancer cells to extracellular nucleotides. Extracellular ATP inhibited growth and motility of nasopharyngeal carcinoma cells by inducing apoptosis through P2Y2 receptor (16). P2X7, as one of the ATP receptors, was shown to mediate pyroptosis from cytosolic LPS and a catalytic activity of caspase-11 (17). In addition, P2X7 receptor was shown to be a critical player in antitumor immune responses in mice. Tumor growth and metastatic spread were accelerated strongly in mice lacking the P2X7 receptor (18). On the other hand, some studies have shown an induction in the proliferation of human hepatocellular carcinoma cells *in vitro* and *in vivo* (19) and an increase of cell invasion in prostate cancer cells (20, 21) *via* an activation of the P2Y2 receptor. ATP at a low dose (1 μM) also induced cell-cycle progression in MCF-7 cells (22), and increased breast cancer MDA-MB-231 cell proliferation *via* the P2Y2 receptor (23). Nevertheless, high dose of ATP induced cell death through P2X7 and AMPK/mTOR signaling in colon cancer cells (24). In addition, P2X7 receptor is required to mediate cell death in lung cancer cells (25). Herein, the inhibitory effects of ATP and adenosine on CCA cell lines were revealed.

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Key Words: ATP, adenosine, purinergic receptor, cholangiocarcinoma, growth inhibition, motility inhibition.

Materials and Methods

Cell culture. All cell lines were maintained as described previously (26-28). HuCCA-1 and RMCCA-1 were maintained in HAM's F-12 medium (Hyclone, Pittsburgh, PA. USA) supplemented with 10% fetal bovine serum (FBS) (Gibco, Grand Island, NY, USA), while MMNK-1 were maintained in Dulbecco's modified Eagle's medium (DMEM) (Hyclone, Pittsburgh, PA. USA) supplemented with 10% FBS. 1% Penicillin/Streptomycin (Gibco, Grand Island, NY, USA) was added in all media. All cell lines were maintained at 37˚C with 5% CO_2.

Cell proliferation assay. Cells were plated with suitable medium in tissue culture treated 48-well plates. After overnight adhesion to the plate, the treatment group was incubated with the indicated concentrations of agonists ranging from log [agonist]=–5.5 (3.16 μM) to log [agonist]=–2.5 (1000 μM) in a half log increment. Ten and 100 μM of ATPγS (Abcam, Cambridge, MA, USA) and 2 units of apyrase (Sigma-Aldrich, St. Louis, MO, USA) was added to assess the effects of non-hydrolysable ATP analog and degradation products. Media were changed and new treatments were added every other day for 4 days. MTT reagent (Life Technologies, Grand Island, NY, USA) was added to the final concentration of 0.5 mg/mL and incubated for 2.5 h at 37˚C, and then a half volume of stop solution (10% SDS in 50% dimethylformamide in dH_2O) was added and mixed thoroughly before reading the absorbance at 560 nm and 650 nm on a plate reader.

Cell migration assay. Cells were plated in 60 mm tissue culture discs with suitable medium to achieve 100% confluence after overnight incubation. A single scratch was made at the middle of the dish by using 100 μL siliconized pipette tip. The cells were incubated with ATP or adenosine at their IC_{50} values in new media. Pictures were taken every 6 h at the same locations along the gap to observe a progression of cell migration by using Nikon TMS inverted microscope mounted with Nikon D3000 digital SLR camera under 4× objective lens. Distance of migration was analyzed using Adobe Photoshop software.

Cell invasion assay. The insert transwells with 8.0 μm polycarbonate membrane (Corning costar, New York, NY, USA) was coated with 50 μl of a 1:10 mixture of Matrigel™ (BD Biosciences, San Jose, CA, USA) in serum free medium, and then allowed to congeal at 37˚C for 30 min. A total of 5×10^4 cells were added to the top chamber of each well in 200 μl serum free medium. Then, 500 μL of complete medium was added to each bottom well. Treatment was added in both transwell and bottom well. Cells were incubated for 24 h in a 37˚C, humidified incubator with 5% CO_2. After this, the chambers were swabbed with cotton swab to remove cells and Matrigel™ remaining on the top of the membrane. Cells remaining on the underside of the membrane were fixed in methanol for 5 min and stained with 0.5% (w/v) crystal violet in 12% glutaraldehyde in water for 5 min. Following a brief dH_2O wash, cells were counted using phase contrast microscopy.

Reverse transcriptase polymerase chain reaction. Total RNA was extracted using TRIzol reagent (Life Technologies, Grand Island, NY, USA). Following extraction, RNA was treated with RNAse-free DNase I (Roche, Indianapolis, IN, USA). Total RNA was reverse transcribed using Maloney murine leukemia virus (M-MLV) reverse transcriptase (Life Technologies, Grand Island, NY, USA).

Table I. *Primer sequences for purinergic receptors. Rev: Reverse, For: Forward. Splice variations (v) of each gene, if presence, are shown in "expected product size" column.*

Gene	Forward primer	Expected product size (base pair)
P2Y1 For	GTTAGACGCCCCGAAACTGA	254
P2Y1 Rev	CAACGCCGAGCTTACACAAC	
P2Y2 For	TTCCTGTTTCCCGCAGAGTT	328 (v1), 194 (v2,3)
P2Y2 Rev	CACCTGTAGCCCAGCTCATC	
P2Y4 For	CTGCCTGTGAGCTATGCAGT	154
P2Y4 Re	GCAGCGACAGCACATACAAG	
P2Y6 For	ACCCTAAACCTTGCTCTGGC	177 (all)
P2Y6 Rev	CTGGAAGCTGATGCAGGTGA	
P2Y11 For	CTTGAACCTCAGGAGGGTTGTG	282
P2Y11 Rev	CACAGGAAGTCCCCCTGGAA	
P2Y12 For	GCCACTCTGCAGGTTGCAATA	224 (all)
P2Y12 Rev	TCGCCAGGCCATTTGTGAT	
P2Y13 For	TGCCGCCATAAGAAGACAGAG	173
P2Y13 Rev	GGATGCCGGTCAAGAAAACC	
P2Y14 For	CAAACGCTCACTGGGCAAAA	376
P2Y14 Rev	TGCACACAAACACGTTCAGC	
P2X1 For	TCTACGTCATCGGGTGGGT	276
P2X1 Rev	GGGTACAGCCACTGTCTTCC	
P2X2 For	CCACTTCTCCAAGGGCAACA	374
P2X2 Rev	CCTGTCCATGCACAATGACG	
P2X3 For	CTGAGAAAAGCAGCGTGTCC	210
P2X3 Rev	AGAGAACAGTTCCCACTCCCA	
P2X4 For	GGTCAGCTCCGTTACGACC	187
P2X4 Rev	CGCATCTGGAATCTCGGGG	
P2X5 For	TGGAAACGGAGTGAAGACCG	169 (445 in positive control due to an intron span between primers)
P2X5 Rev	GGGGAAACGGATGTGGTTCT	
P2X6 For	AGGGGTTTCCGTCACTCAGA	238
P2X6 Rev	GCCTGTTTTTACACCGTGGC	
P2X7 For	AGCGGAAAGAGCCTGTCATC	242
P2X7 Rev	GTCAGAGGAACAGAGCGTCC	
A1 For	CAACATTGGGCCACAGACCT	222
A1 Rev	ATAGGGGTCAGTCCCACCAC	
A2A For	ATCGCCATTGACCGCTACAT	493
A2A Rev	AGTCGGGGCAGAAGAAAGTG	
A2b For	GGGCTTCTGCACTGACTTCT	175
A2b Rev	AGCAATGACCCCTCTTGCTC	
A3 For	CACCTGTGATGAGCCCTTTCT	577
A3 Rev	GTGAGTGGTGACCCTCTTGT	
GAPDH For	CACCGTCAAGGCTGAGAACG	223
GAPDH Rev	GACGAACATGGGGGCATCAG	

Polymerase chain reaction (PCR) was carried-out using helixamp Taq (Nanohelix, Yuseong-Gu, Daejeon, Korea) for 32 cycles. PCR reaction was carried out with 2 mM $MgCl_2$, 0.2 mM dNTP, 1 μM of forward and reverse primers (Integrated DNA technology, Coralville, Iowa, USA), 0.025 U/μl of Taq polymerase in 10 μl total volume. Primer sequences are shown in Table I. Five microliters of each reaction mixture was mixed with NovelJuice (Gendirex) and

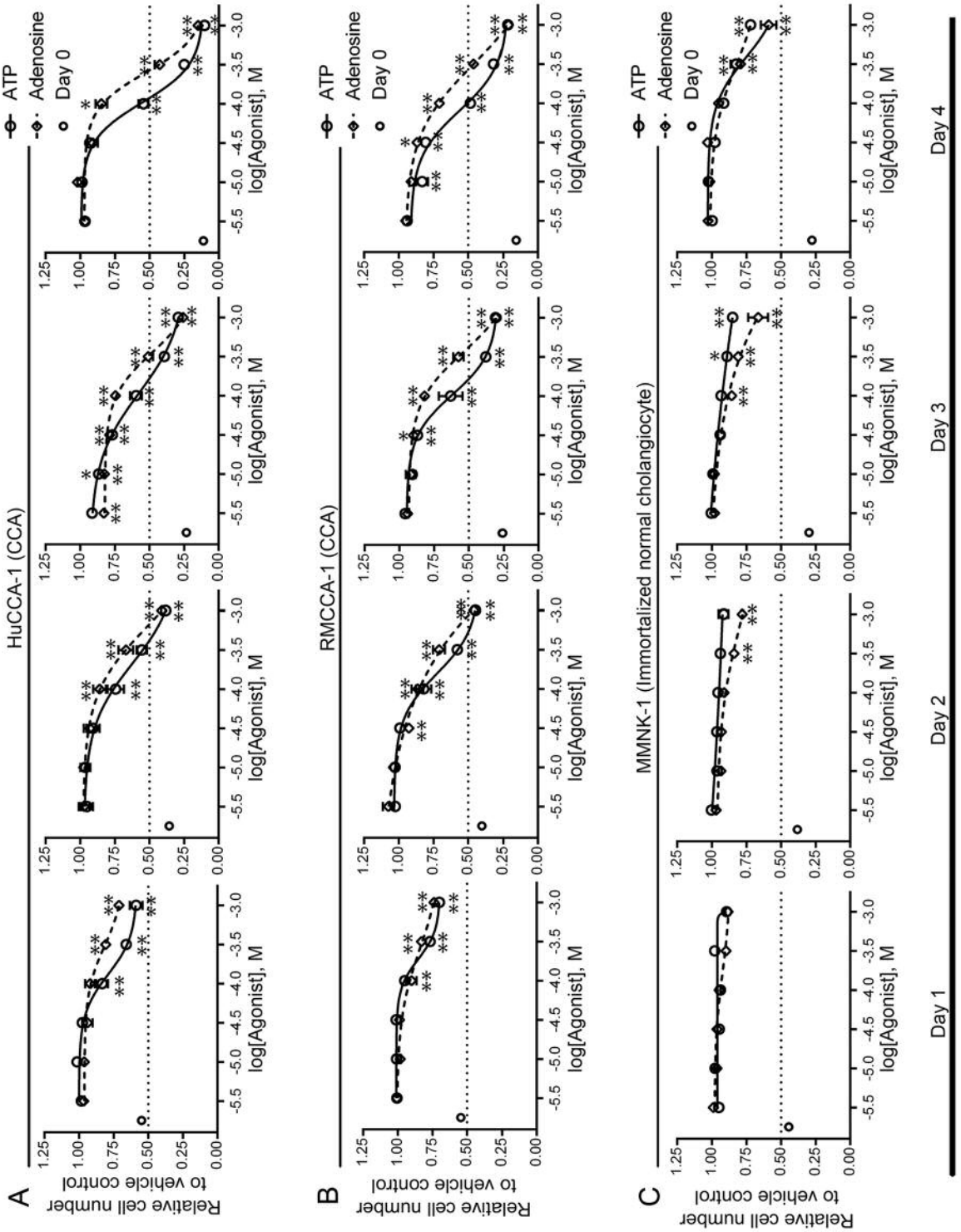

Figure 1. *ATP and adenosine inhibited cell proliferation of CCA and immortalized cholangiocytes. The concentration was in the range of log[agonist]=–5.5 M (3.16 μM) and log[agonist]=–3.0 M (1000 μM) in a half log increment. Cell proliferation inhibition was demonstrated in (A) HuCCA-1, (B) RMCCA-1, and (C) MMNK-1. Day0 represent the relative cell number at the initiation of the experiment as compared to the control after 4 days of treatment. All data were presented as mean±SEM from 3 replicates. Graphs were plotted by using Prism Graphpad 6 with log(inhibitor) vs. response –Variable slope (four parameters) function. Experiments were performed in triplicates. *p<0.05, **p<0.01.*

electrophoresed in 1.5% agarose gel at 100 V for 45 min at room temperature. 100bp DNA ladder (Bio-Helix, Beverly, MA, USA) was used to compare product size. Positive controls were performed by using HuCCA-1 genomic DNA.

Statistical analysis. Data were graphed as mean±SEM by using Prism Graphpad software. Statistical analysis was performed using ANOVA with Dunnett's test by using JMP software. All experiments were performed in triplicates.

Results

ATP and adenosine inhibited CCA cell proliferation. ATP and adenosine inhibited CCA cell proliferation in a dose- and time-dependent manner. Time dependent response of ATP could be seen in all dosages for HuCCA1 and RMCCA-1. One hundred micromolar ATP (log[ATP]=–4.0) was able to inhibit HuCCA-1 approximately 20% at 1 day after treatment. The inhibitory effect increased to 25%, 35%, and 50% at days 2, 3, and 4 respectively (Figure 1A). A similar trend was observed in RMCCA-1 with asmaller degree of inhibition (Figure 1B). However, IC_{50} of ATP on HuCCA-1 and RMCCA-1 at 4 days was equal at 120 μM. On the other hand, adenosine showed less cell proliferation inhibition on HuCCA-1 (Figure 1A) and RMCCA-1 (Figure 1B), compared to ATP. Adenosine had IC_{50} at 260 μM on both HuCCA-1 and RMCCA-1. Interestingly, ATP had no inhibitory effect on MMNK-1 cells until 3 days after treatment (Figure 1C) and reached only 25% inhibition after 4 days with the highest dose tested. Similarly, adenosine was unable to inhibit MMNK-1 cell proliferation below 50% (Figure 1C). Therefore, the IC_{50} of both ATP and adenosine for MMNK-1 was not obtained in this study.

ATP and adenosine did not synergize on CCA. A combination of ATP and adenosine at low doses (10 μM and lower) did not show any synergistic inhibitory effect on RMCCA-1 and HuCCA-1 cells (Figure 2A and 2B), while it stimulated RMCCA-1 cell proliferation (Figure 2B). On the other hand, their combination showed a synergistic inhibitory effect on MMNK-1 (Figure 2C). The combination inhibited MMNK-1 cell proliferation to lower than 50% at 1000 μM while either ATP or adenosine alone had no effect.

ATP and adenosine inhibited CCA cell motility. ATP and adenosine inhibited both cell migration on cell culture disc and cell invasion through Matrigel. ATP at IC_{50} (120 μM), obtained from Figure 1, slowed-down HuCCA-1, RMCCA-1, and MMNK-1 cell migration to 86%, 83%, and 82% respectively as compared to the vehicle control group at 18 h after treatment (Figure 3A). A stronger inhibitory effect was observed in adenosine treatment at the IC_{50} concetration (260 μM). Cell migration of HuCCA-1, RMCCA-1, and MMNK-1 at 18 h of adenosine treatment was inhibited to 82%, 64%, and 75% respectively compared to the vehicle control group (Figure 3A). Furthermore, ATP inhibited HuCCA-1 and RMCCA-1 cell invasion through Matrigel to approximately 66% compared to the vehicle control group. ATP had a greater inhibitory effect on MMNK-1 cell invasion (Figure 3B). Notably, adenosine had a greater effect on cell invasion as compared to ATP in all 3 cell lines. HuCCA-1 cell invasion was decreased to approximately 11% in HuCCA-1, 26% in RMCCA-1, and 16% in MMNK-1 cells (Figure 3B).

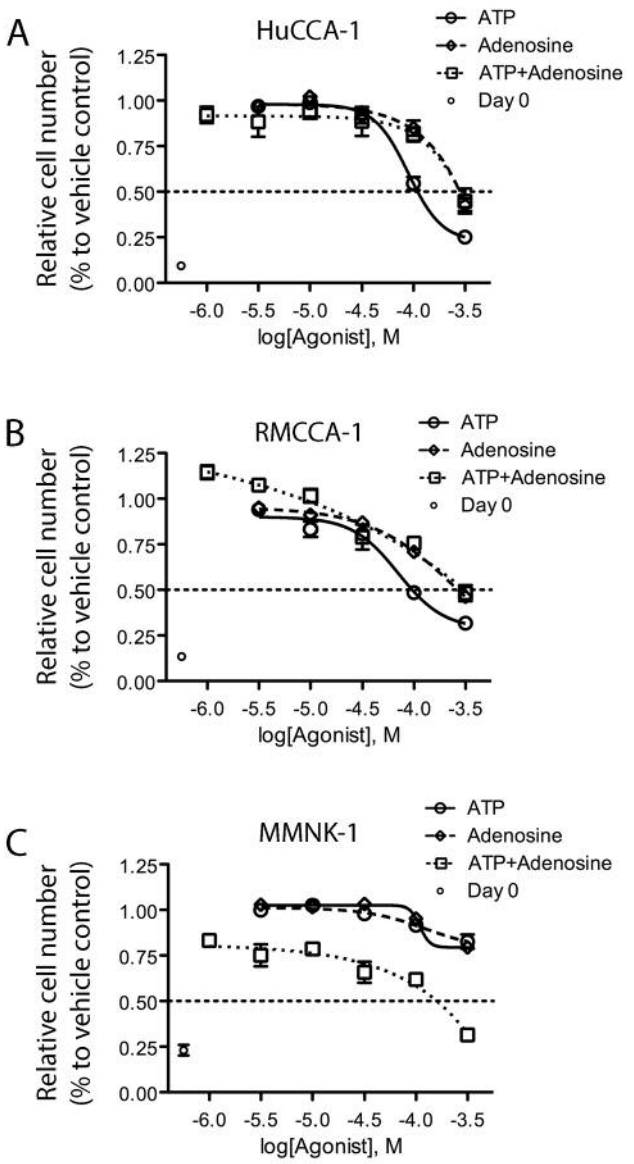

Figure 2. *ATP and adenosine inhibitory effect did not synergize. The inhibitory effect on cell proliferation did not synergize on CCA cells, (A) HuCCA-1 and (B) RMCCA-1, but (C) the synergistic effect was observed on immortalized cholangiocytes (MMNK-1). Graphs were plotted by using Prism Graphpad 6 with one-phase decay function. Experiments were performed in triplicates.*

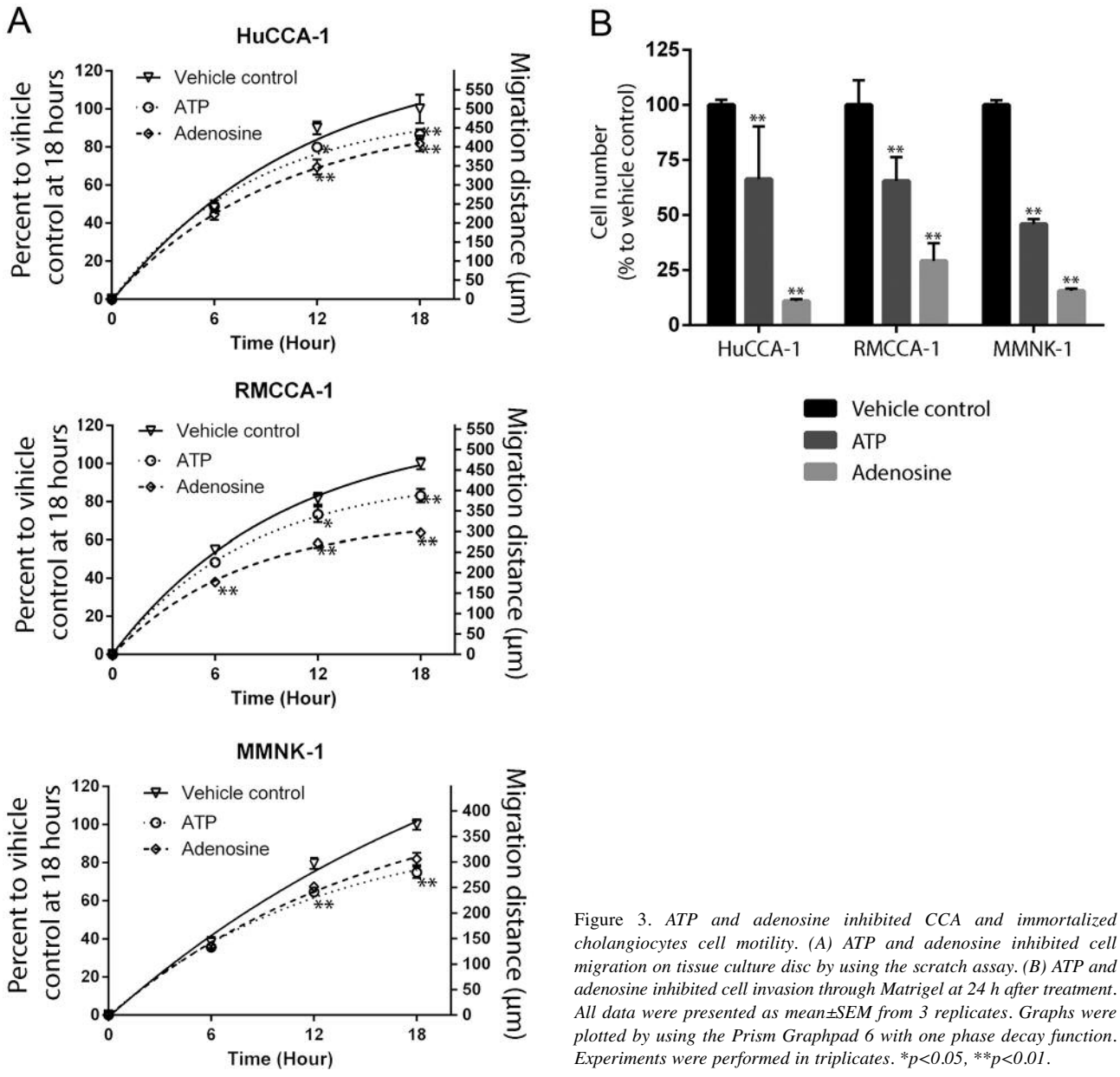

Figure 3. *ATP and adenosine inhibited CCA and immortalized cholangiocytes cell motility. (A) ATP and adenosine inhibited cell migration on tissue culture disc by using the scratch assay. (B) ATP and adenosine inhibited cell invasion through Matrigel at 24 h after treatment. All data were presented as mean±SEM from 3 replicates. Graphs were plotted by using the Prism Graphpad 6 with one phase decay function. Experiments were performed in triplicates. *p<0.05, **p<0.01.*

Non-hydrolysable form of ATP had a greater effect than ATP. ATPγS, a non-hydrolysable ATP analog, was used to verify that ATP, not its degraded byproducts ADP or AMP, inhibited CCA cell proliferation. ATPγS showed greater inhibition on cell proliferation at both 10 μM and 100 μM compared to ATP on HuCCA-1 and RMCCA-1 cells. MMNK-1 cells did not respond to 10 μM of both ATP and ATPγS as expected. One hundred micromolar of ATPγS, however, showed a significantly greater inhibition as compared to ATP on MMNK-1 (Figure 4A). Furthermore, apyrase was used to degrade ATP to ADP and AMP in order to examine the inhibitory effects of these degraded byproducts. Apyrase reduced the inhibitory effect of ATP (120 μM) significantly in all cell lines tested (Figure 4B).

Transcriptional profile of purinergic receptors on CCA cell lines and MMNK-1. Two CCA cell lines (HuCCA-1 and RMCCA1) and one immortalized cell line (MMNK-1) expressed several purinergic receptors (Figure 5). P2Y2 and P2X3 receptors were expressed in all cell lines while P2Y6, P2Y13, and P2X7 receptors were expressed only in CCA cell lines but not in MMNK-1 cells. P2X4 was expressed on

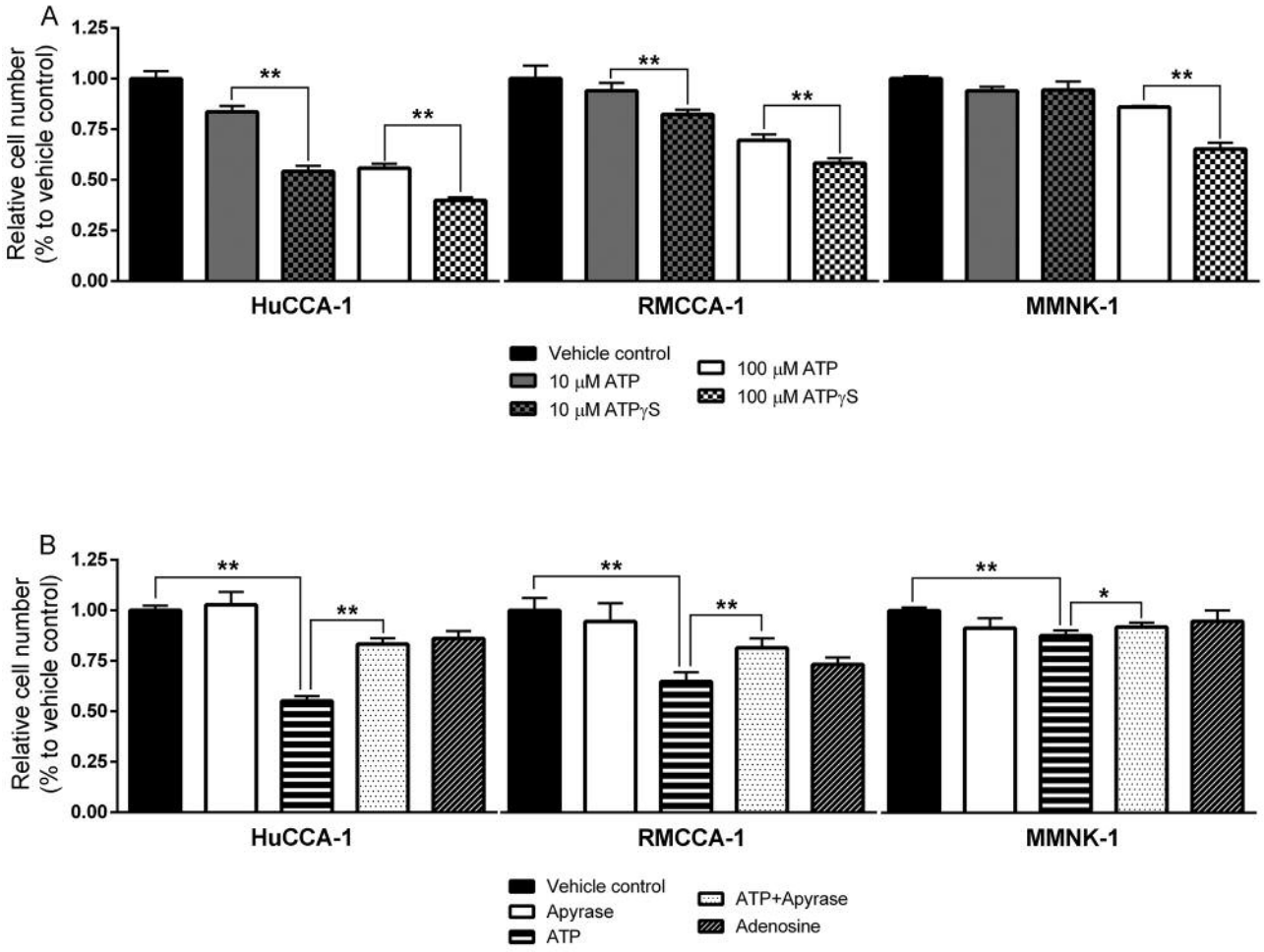

Figure 4. *ATP, not its degraded by products, inhibited CCA. MTT assay was performed after 4 days of treatment. (A) A non-hydrolysable ATP analog, ATPγS, showed greater inhibitory effect than ATP. (B) Apyrase, which hydrolyzes ATP ADP and AMP finally, significantly reduced inhibitory effects of ATP on CCA cell proliferation. Experiments were performed in triplicates. *p<0.05, **p<0.01.*

RMCCA-1 and MMNK-1, and P2X6 was expressed only on RMCCA-1 cells. No adenosine receptor was expressed in these cell lines (Table II). Primer sequences are listed in Table I.

Discussion

Herein, ATP and adenosine were reported for the first time to inhibit CCA cell proliferation and motility at doses of 100 μM or higher. Other studies have shown that ATP and adenosine could either inhibite or stimulate cancer cell proliferation and motility. The key factor might be the dose of ATP and adenosine to which cells were exposed. Many studies have shown that a concentration of ATP of 100 μM or higher inhibited cancer cell proliferation; while a concentration of ATP lower than 10 μM may stimulate cancer cell proliferation. Our group has recently reported the growth inhibition induced byATP at 32 μM (log[ATP]=–4.5) and higher doses on prostate cancer cell lines (29). In addition, ATP at a very high dose in the range of milli molar was reported to induce cytotoxicity and apoptosis in many cancer types such as hepatoma, adenocarcinoma, and cervical cancer cells (24, 30-33). On the other hand, some studies have showed that 10 μM ATP and lower doses stimulated growth and motility of ovarian cancer (34), hepatoma (19), breast cancer (23), lung cancer (25), and prostate cancer cells (20, 35). To utilize ATP and/or adenosine *in vivo*, one must consider the final dose of the compounds at the tumor sites. In this study, CCA and immortalized cholangiocyte cell proliferation was not stimulated by either ATP or adenosine alone at low doses (3.16 μM and 10 μM) but RMCCA-1 was stimulated by a combination of ATP and adenosine at 10 μM and lower

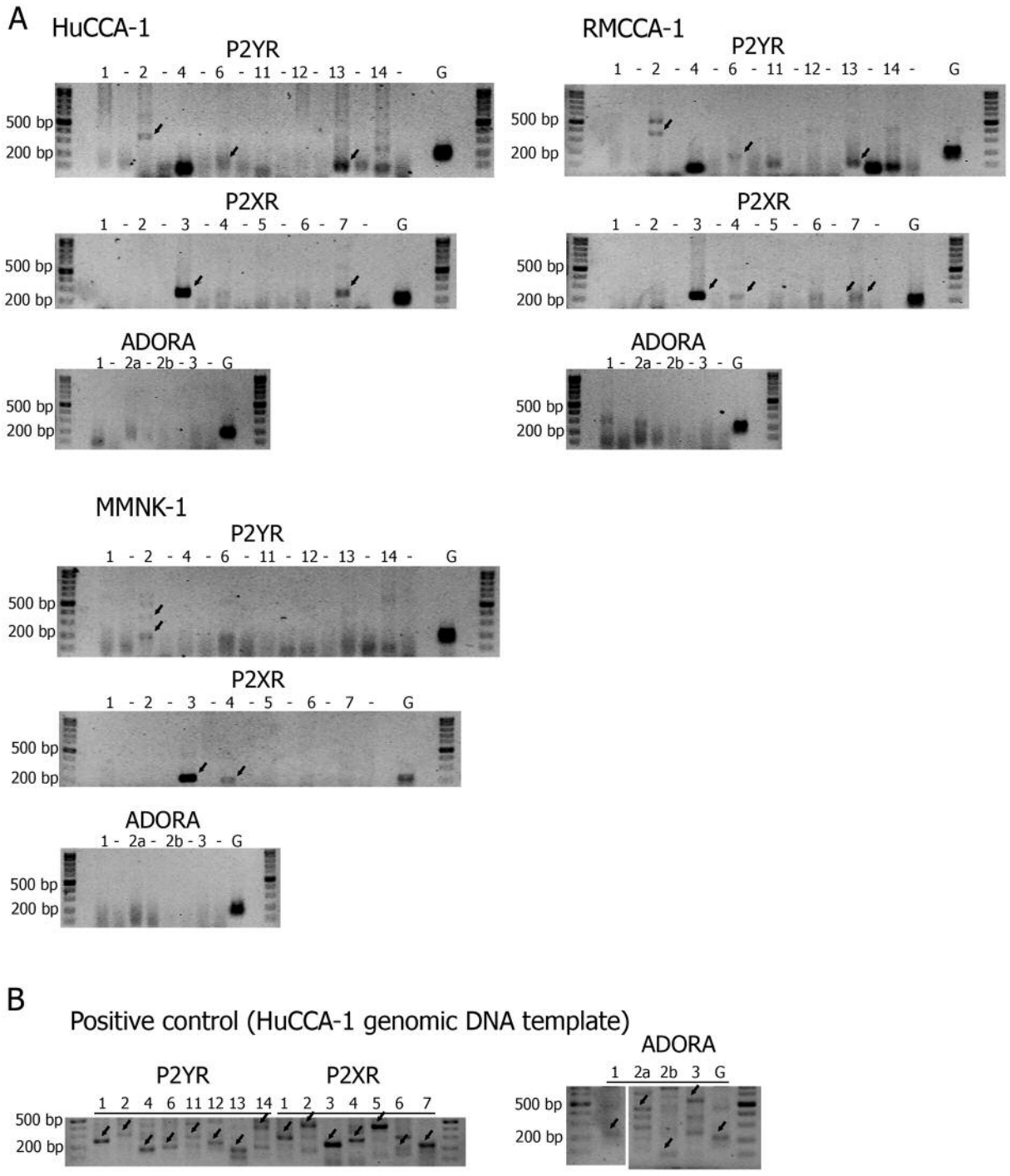

Figure 5. *Reverse transcriptase PCR showing transcriptional profile of P2Y receptors (P2YR), P2X receptors (P2XR), and adenosine receptors (ADORA) in CCA cell lines and immortalized cholangiocytes. Positive control used HuCCA-1 genomic DNA as a template. Arrows represent a positive expression of the specified gene. Experiments were performed in triplicates. Picture represents the three replicates.*

doses. Therefore, the effects of ATP and adenosine do not only depend on dose but also depend, at least in part, on cell types and the purinergic receptors expressed on the cells.

Furthermore, immortalized cholangiocytes showed resistance to ATP and adenosine treatments. These data can be used in order to find the proper conditions to apply

Table II. *Transcriptional profile of purinergic receptors on CCA cells. Transcriptional profile obtained from reverse transcription PCR. + indicates the presence of the specified gene expression.*

Cell line	P2RY Subtypes								P2RX Subtypes						
	1	2	4	6	11	12	13	14	1	2	3	4	5	6	7
HuCCA-1	–	+	–	+	–	–	+	–	–	–	+	–	–	–	+
RMCCA-1	–	+	–	+	–	–	+	–	–	–	+	+	–	+	+
MMNK-1	–	+	–	–	–	+	–	–	–	–	+	+	–	–	–

Cell line	ADORA Subtypes			
	A1	A2A	A2B	A3
HuCCA-1	–	–	–	–
RMCCA-1	–	–	–	–
MMNK-1	–	–	–	–

adenine compounds as a remedy for CCA. One factor that may be associated with this difference between CCA cells and immortalized cholangiocytes is the variation of purinergic receptor types expressed.

In this study, CCA cell lines and an immortalized cholangiocarcinoma cell line shared a few common P2Y receptors and P2X receptors, which were P2Y2 and P2X3. P2Y2 receptor was reported to either induce apoptosis (16) or stimulate cell proliferation and cell motility (19-21, 35). However, most studies demonstrated that P2Y2 stimulated cancer cell proliferation and motility rather than suppressed them or induced cell death. Therefore, it might not be the best candidate receptor for the inhibitory effects observed in this study. Although P2X3 receptor was also a common receptor among CCA cell lines and MMNK-1, it was recently reported to induce cell proliferation in hepatocellular carcinoma cell lines and to be associated with poor recurrence-free survival in patients (36). On the other hand, P2Y6, P2Y13, and P2X7 are the receptors that are expressed in CCA cells, but not in immortalized cholangiocarcinoma cells (MMNK-1). Herein, it was demonstrated that MMNK-1 cells showed some resistance to cell proliferation and cell motility inhibition by ATP and adenosine. Therefore, the receptors expressed in CCA cells but not in MMNK-1 cells might be responsible for the aforementioned inhibition. In addition, adenosine was shown to suppress cell proliferation and motility without its receptors expression in CCA cell lines used in this study. Therefore, receptor-independent inhibitory effects of adenosine on CCA cells were suggested. Some researches have demonstrated a receptor-independent inhibition of adenosine on other cancers. Breast and prostate cancer, for example, were shown to be inhibited by adenosine in a receptor-independent manner (37). Further studies are required in order to examine the mechanism of inhibition caused by adenosine on CCA cells.

Acknowledgements

The Authors would like to thank Assoc. Prof. Rutaiwan Tohtong, for her kindness in providing RMCCA-1 cell line. This study was supported by RD&E funding (SCH-NR2015-871) from National Science and Technology Development Agency, Thailand.

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Received May 11, 2017
Revised May 29, 2017
Accepted May 30, 2017