

Adenosine Induces Autophagy in Cholangiocarcinoma Cells

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Abstract. *Background/Aim:* Cholangiocarcinoma is a lethal disease with increasing incidence worldwide. New therapeutic compounds are urgently needed for this disease. Here, the inhibitory effect of adenosine on cholangiocarcinoma cells was studied. *Materials and Methods:* Western blot analysis was used to study autophagy and flow cytometry to analyze cell death and the cell cycle. *Results:* Cholangiocarcinoma and immortalized cholangiocytes responded to adenosine differently, and adenosine inhibited cholangiocarcinoma cell growth by inducing autophagy. Adenosine failed to activate adenyl cyclase in cholangiocarcinoma cell lines, but activated this enzyme in immortalized cholangiocytes. Adenosine treatment activated AMPK and led to phosphorylation of its downstream proteins including ULK and Raptor. In addition, autophagy induced by adenosine appeared to be a survival mechanism. The combination of adenosine with autophagy inhibitors greatly increased cell death, as compared to the use of either agent alone. Interestingly, immortalized cholangiocytes were more resistant to adenosine. *Conclusion:* Adenosine may have potential for application in cholangiocarcinoma treatment.

Cholangiocarcinoma (CCA) is a lethal disease originating from epithelial cells of the bile ducts. Although the incidence of CCA is much lower in Western countries than in Southeast Asian countries, the incidence of this disease is increasing worldwide (1, 2). Risk factors for CCA include exposure to chemicals both in industrial and agricultural environments (3-11), infection by HBV virus (12-14), a combination of smoking and alcohol consumption (7) and liver fluke infection (15, 16). CCA is often resistant to current chemotherapeutic drugs (17, 18) and new strategies are being examined in the search for new CCA treatment options.

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Key Words: Cholangiocarcinoma, adenosine, autophagy, AMPK.

Adenosine is a compound that has been recently reported to inhibit cell proliferation and invasion of prostate cancer cell lines (19), and CCA cell lines (20). Extracellular adenosine can bind to adenosine receptors on the cell surface triggering an increase in cAMP and the consequent activation of the cAMP-dependent Protein Kinase A (PKA). However, adenosine can also be transported into the cell via transporter proteins resulting in the production of AMP and the activation of AMPK. In hepatoblastoma, adenosine treatment is known to induce autophagy (21), a process in which regions of the cytoplasm, protein aggregates, and mitochondria, are encapsulated in vesicles that fuse with lysosomes, enabling cell survival. However, autophagy can also be important in cell death depending on cell type and cell context. The role of adenosine in autophagy is not well understood. Recently, adenosine was reported to induce autophagy and apoptosis in human hepatoblastoma HepG2 cells at high doses in the millimolar range. The role of adenosine-induced autophagy in HepG2 cells was reported to be a survival mechanism (21). Activation of AMPK can increase autophagy through inactivation of the mTOR complex-1 (mTORC1) and by phosphorylation of the autophagy inducing kinase ULK1. The molecular mechanism that links adenosine treatment to the inhibition of CCA cell proliferation and invasion remains elusive, but a receptor-independent mechanism has been suggested based on the absence of receptor expression in CCA cells (20).

Herein, adenosine-induced autophagy in CCA cell lines that involved activation of AMPK signaling pathways was demonstrated.

Materials and Methods

Cell culture. The intrahepatic cholangiocarcinoma cell line, HuCCA-1, was established by Prof. Stitaya Sirisinha at Chulabhorn Research Institute (22) and was also purchased from Japanese Collection of Research Bioresources (JCRB) Cell Bank (Osaka, Japan). HuCCA-1 was derived from a male Thai patient whose serum was positive for the *O. viverrini* antigen (22). The RMCCA-1 cell line, established from a peripheral cholangiocarcinoma specimen surgically obtained from a male Thai patient, was kindly

provided by Prof. Rutaiwan Tohtong at Mahidol University (23). An immortalized cholangiocyte cell line, MMNK-1, was purchased from JCRB Cell Bank. All cell lines were maintained in Dulbecco's modified Eagle's medium (DMEM) (SH30243.02, Hyclone, Pittsburgh, PA, USA) supplemented with 10% fetal bovine serum (FBS) (10270-106, Gibco, Grand Island, NY, USA) and 1% MEM non-essential amino acids (11140-050, Gibco). All media were supplemented with 1% penicillin/streptomycin (15140-122, Gibco). All cell lines were maintained at 37°C with 5% CO₂.

Cell starvation. Starvation was induced by replacing culture media with phosphate buffer saline (PBS), followed by incubation for 4 h at 37°C with 5% CO₂.

MTT assay. Cells were plated in tissue culture treated 96-well plates. After overnight adhesion to the plate, the treatment group was incubated with the indicated concentrations of adenosine (A4036 Sigma-Aldrich, St. Louis, MO, USA), On day 2, MTT reagent (M6494, ThermoFisher Scientific, Hampton, NH, USA) was added to a final concentration of 0.5 mg/ml, followed by incubation for 2.5 h at 37°C. A half volume of stop solution [10% Sodium dodecyl sulfate (SDS) in 50% dimethylformamide in dH₂O] was added and mixed thoroughly before reading the absorbance at 560 nm on a plate reader.

Cyclic AMP assay. Cells were seeded in tissue culture treated 96-well plates and allowed to attach overnight. Adenosine was added at a final concentration at 500 µM. Cyclic AMP was detected using Cyclic AMP Competitive ELISA Kit (ab133051, Abcam, Cambridge, MA, USA) according to the manufacturer's protocol. Cells were treated with adenosine for 0, 15, 30, 45, and 60 min before the assay.

Cell imaging. Live cells were imaged with an inverted phase contrast microscope (Nikon eclipse TS2, Tokyo, Japan) under 20X objective lens.

Cell cycle analysis. Cells were plated in tissue culture treated 96-well plates. After overnight adhesion to the plate, cells were treated with adenosine at a final concentration of 500 µM for 48 h. Cell pellet was collected and resuspended in ice-cold 0.003% (0.03 mg/ml) deoxyribonuclease I (Sigma Aldrich, DN25) in PBS and incubated on ice for 5 min. After 3 washes, cell pellets were incubated in ice-cold PBS:70% ethanol in a ratio 1:9. The cell suspension was kept in -20°C for 18 h. Before analysis, cell pellet was resuspended in 1 ml of propidium iodine DNA staining solution (200 µg/ml DNase-free RNase A (12091-021, Life Technologies) and 20 µg/ml propidium iodine (P1304MP, Life Technologies) for 1 h. DNA content was assessed using the BD FACSCanto™ Flow Cytometer machine and data were analyzed with ModFit LT software version 3.0.

Cell death analysis. Cells were seeded in tissue cultured treated 6-well plates. Autophagy inhibitors, 2.5 mM 3-methyladenine (ab120841, Abcam), 30 µM hydroxychloroquine (ab120827, Abcam), and 100 nM bafilomycin A1 (ab120497, Abcam) were added 1 h prior to adenosine. Adenosine was added to a final concentration of 500 µM and incubated for 48 h. To examine apoptosis, cells were stained with Muse™ annexin V & dead cell kit (MCH100105, MilliporeSigma, Burlington, MA, USA) according to the manufacturer's protocol. Samples were read by using Muse™ cell analyzer (0500-3115, MilliporeSigma) to determine the percentage of apoptotic and dead cells. Data were analyzed by using Muse™ software version 1.4. To examine cell death,

calcein-acetoxymethyl (C3100MP, Fisher Scientific) and propidium iodide (P1304MP, Fisher Scientific), at the final concentrations of 0.125 µM and 1.5 µM, respectively, were added 30 min before imaging. Images were obtained using a Nikon Eclipse T2S phase contrast inverted fluorescent microscope. Live cells were stained with calcein and showed a green fluorescent signal, while dead cells were stained with propidium iodine and showed a red fluorescent signal. Green and red fluorescence pixels were counted. Percentage of dead cells was calculated as the percentage of dead to the total number of cells.

Western blot analysis. A total of 20 µg protein was separated by electrophoresis and transferred to a nitrocellulose membrane (GE, Boston, MA, USA) at 23°C. Membranes were blocked in blocking buffer [4% bovine serum albumin (BSA) w/v in Tris-buffered saline, 0.1% Tween 20 (TBST)], and then incubated with primary antibodies overnight at 4°C on a rocking shaker. All antibodies were diluted in blocking buffer. Primary antibodies were 1:1,500 anti-LC3B antibody (3868, Cell Signaling Technology, Danvers, MA, USA), 1:1,500 anti-AMPK T172 antibody (2535, Cell Signaling Technology), 1:1,500 anti-AMPK antibody (5832 Cell Signaling Technology), 1:1,500 anti-pRaptor S792 antibody (2083 Cell Signaling Technology), 1:1,500 anti-Raptor antibody (2280 Cell Signaling Technology), 1:1,500 anti-pULK1 antibody (5869 Cell Signaling Technology), 1:1,500 anti-ULK antibody (8054, Cell Signaling Technology), 1:1,500 anti-phosphor AMPK substrates antibody (5759, Cell Signaling Technology), 1:5,000 anti-GAPDH antibody (8884, Cell Signaling Technology) and 1:5,000 anti-β-actin antibody (A2066, Sigma Aldrich, St. Louis, MO, USA). Then, membranes were washed with TBST followed by incubation for 75 min on a rocking shaker at 23°C with 1:5,000 Anti-rabbit IgG, HRP-linked antibody (7074, Cell Signaling Technology). Signal was visualized by autoradiography using enhanced chemiluminescence (ECL) and a horseradish peroxidase (HRP) chemiluminescent substrate, followed by exposure to Hyperfilm (28906838, GE Healthcare, Boston, MA, USA). Band intensity was analyzed using ImageJ software.

Lactate dehydrogenase (LDH) releasing assay. Cells were plated in 96-well plates with DMEM media and allowed to adhere overnight. Cells were treated with either adenosine alone (final concentration 500 µM) or with autophagy inhibitors, 2.5 mM 3-methyladenine (ab120841, Abcam), 30 µM hydroxychloroquine (ab120827, Abcam), and 100 nM bafilomycin A1 (ab120497, Abcam). Released LDH level was assessed using Cytotoxicity Detection KitPLUS LDH (04744934001, Roche, Singapore). After 48 h, lysis buffer was added to the lysis control group. Released LDH level was assessed according to the manufacturer's protocol.

Statistical analysis. Data were calculated as mean±standard deviation (SD). Statistical analyses were performed using ANOVA with Dunnett's test by using JMP software version 16. All experiments were performed in triplicate.

Results

Cholangiocarcinoma cells and immortalized cholangiocytes respond differently to extracellular adenosine. It has been shown that adenosine inhibits cholangiocarcinoma cell growth (20), but the details of this inhibition have not been elucidated. Hence, the mechanism of this inhibition was

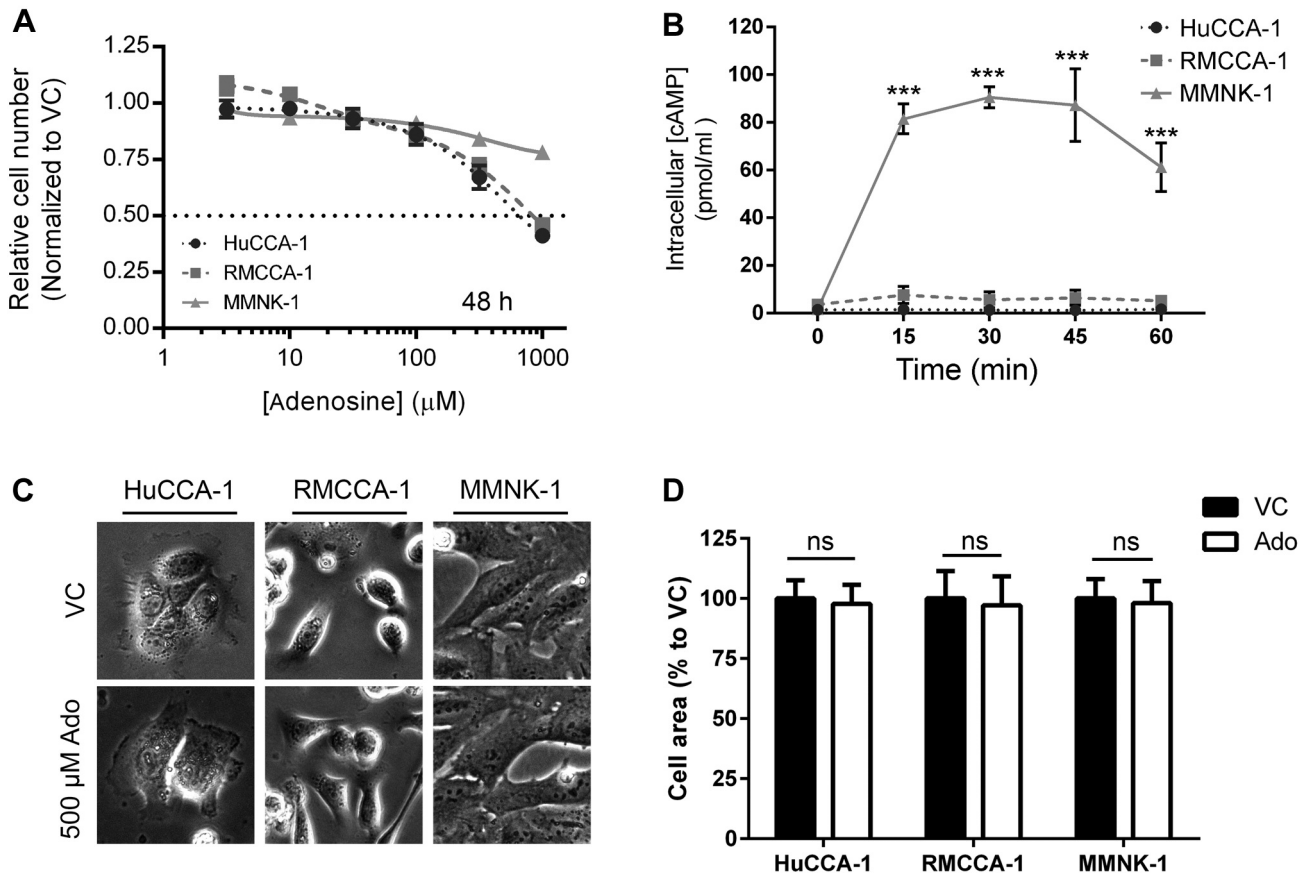


Figure 1. Cholangiocarcinoma (CCA) cells and immortalized cholangiocytes (imCho) responded to adenosine differently. (A) Adenosine inhibited cell growth in CCA cell lines and immortalized cholangiocyte cell line, as measured by the MTT assay after 2 days treatment. (B) Adenosine up-regulates cAMP production in imCho but not in CCA cells. (C) Adenosine treatment did not alter cell morphology after 2 days of treatment. (D) Cell area analysis from images of (C). Ado: Adenosine; VC: vehicle control; ns: not significant. $*p < 0.05$, $**p < 0.01$, $***p < 0.001$. All experiments were performed at least in triplicate and with at least three biological replicates. Graphs were plotted as mean \pm SD. Ado was used at the concentration of 500 μM unless otherwise stated.

examined by using 2 cholangiocarcinoma (CCA) cell lines and 1 immortalized cholangiocytes (imCho) cell line. The results demonstrated that these two types of cells responded differently to adenosine, with the imCho cells being more resistant to adenosine than CCA cells (Figure 1A). One millimolar adenosine decreased CCA cell growth to 41% and 45% in HuCCA-1 and RMCCA-1, respectively (Figure 1A), while the same concentration of adenosine only decreased MMNK-1 cell growth to 78% (Figure 1A). In addition, adenosine treatment did not significantly alter cAMP levels in CCA cell lines, but caused significant increase in cAMP levels in MMNK-1 cells, reaching a plateau at 15-45 min, before declining at 60 min (Figure 1B).

Adenosine did not damage cells, induce apoptotic cell death or arrest of the cell cycle. Since fewer cells and lower MTT signals were observed in the adenosine-treated group of CCA

cells (Figure 1A), the mechanism of the inhibition in CCA cells was studied by examining cell morphology. The results showed that adenosine did not visually alter cell morphology of either CCA or imCho cell lines (Figure 1C), or alter the cell area of either CCA or imCho cells (Figure 1D). Further study of apoptotic cell death showed that 500 μM adenosine, a concentration inhibiting growth of CCA cells to 75% (Figure 1A), did not induce apoptosis in either HuCCA-1 cells (Figure 2A) or RMCCA-1 cells (Figure 2B). In addition, 500 μM adenosine did not cause cell cycle arrest in CCA cells (Figure 2C). Further investigation on whether adenosine damaged cells and disrupted cell membrane integrity by measuring the level of released lactate dehydrogenase (LDH) was performed. The results showed that the level of released LDH in the adenosine-treated group did not differ from vehicle control treated group, while the level in the lysis control groups was significantly higher (Figure 2D).

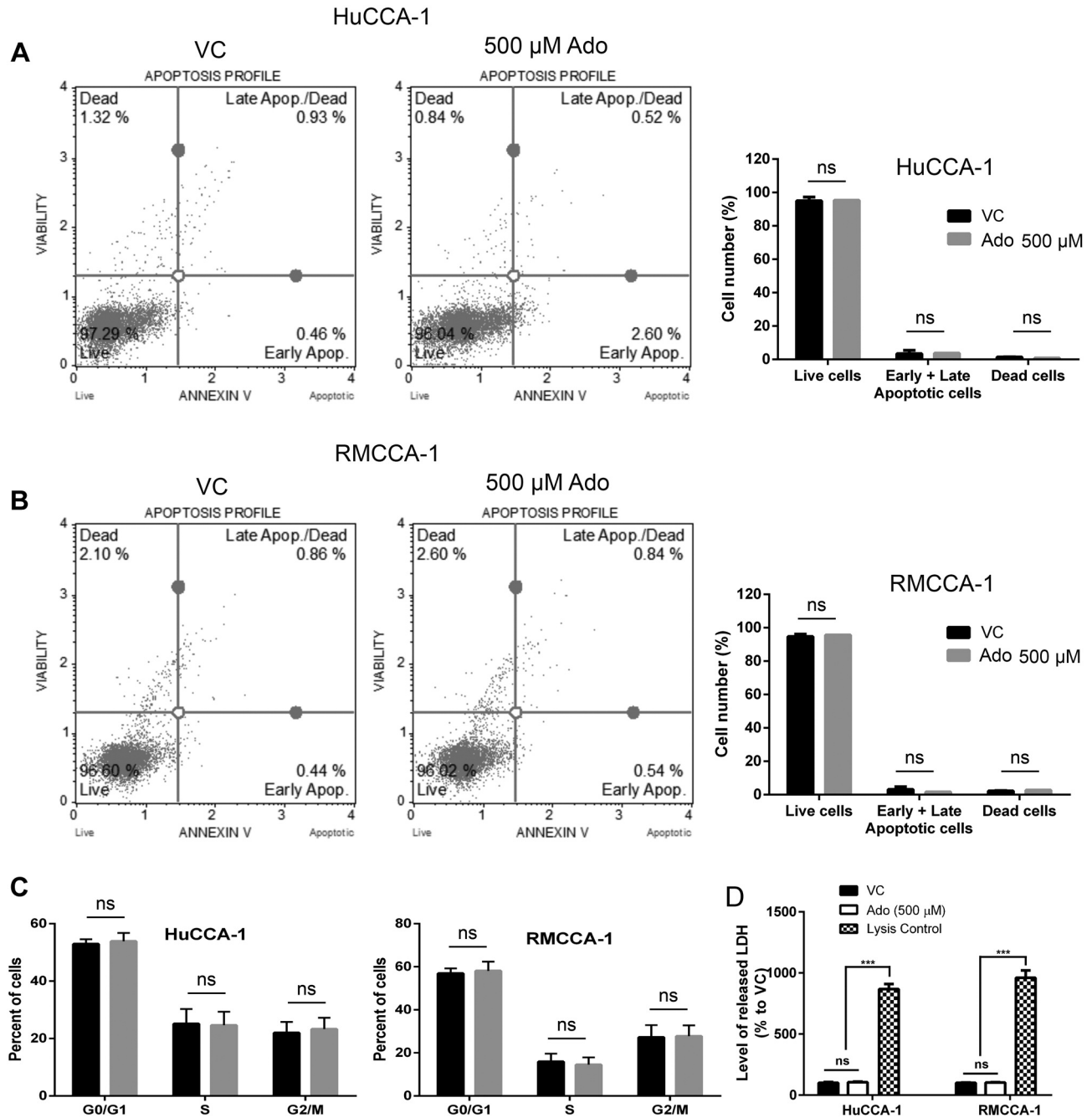


Figure 2. Adenosine did not induce apoptosis, cell damage and cell cycle arrest in cholangiocarcinoma cells. Adenosine at 500 μ M did not induce apoptosis in (A) HuCCA-1 and (B) RMCCA-1. (C) No cell cycle arrest was observed in CCA cell lines treated with 500 μ M adenosine. (D) Level of released lactate dehydrogenase in 500 μ M adenosine-treated group was not different from that of vehicle control-treated group. Ado: Adenosine; VC: vehicle control; LDH: lactate dehydrogenase; ns: not significant. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$. All experiments were performed at least in triplicate and with at least three biological replicates. Graphs were plotted as mean \pm SD. Ado was used at the concentration of 500 μ M unless otherwise stated.

Adenosine induced autophagy in cholangiocarcinoma cells. Since the lower cell numbers after adenosine treatment did not appear to be due to apoptotic cell death or cell damage, autophagy was studied after adenosine treatment.

Hydroxychloroquine was used to disrupt autophagosome degradation, so that the combination of adenosine and hydroxychloroquine would lead to an accumulation of the autophagosome, if adenosine induces autophagy. The results

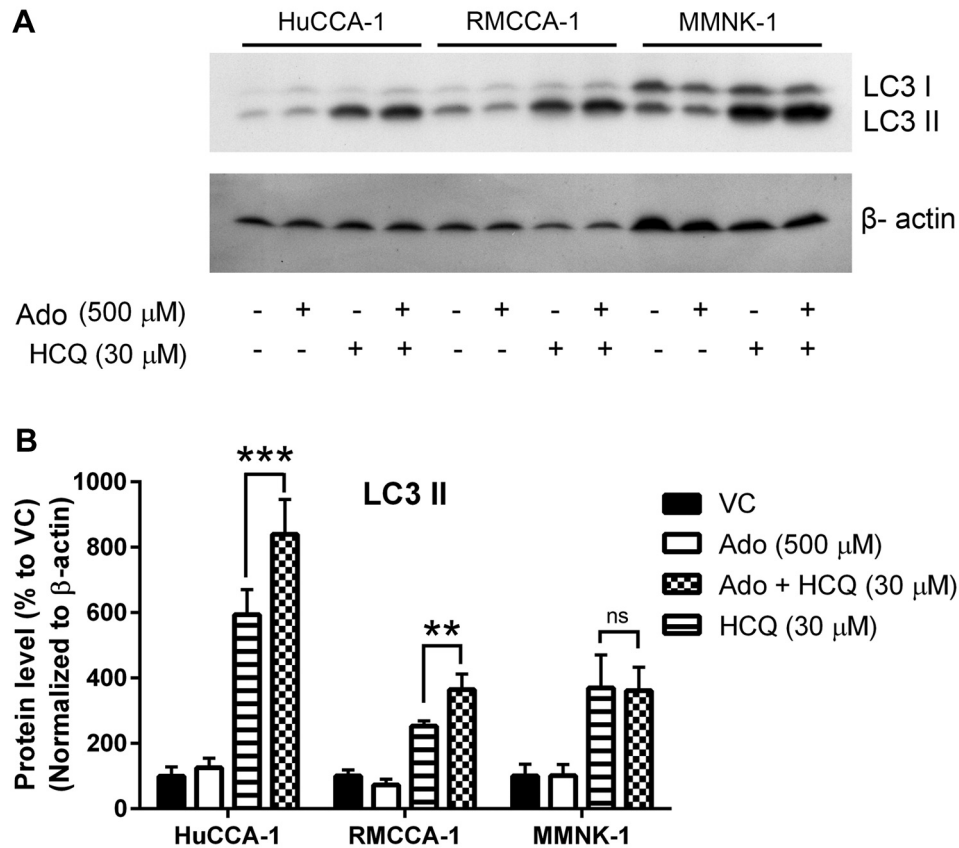


Figure 3. Adenosine induced autophagy in cholangiocarcinoma (CCA) cells. (A) Adenosine up-regulated LC3-II level in CCA cell lines but not in immortalized cholangiocytes (*imCho*). (B) Quantitative analysis of protein levels in (A). Ado: Adenosine; VC: vehicle control; HCQ: hydroxychloroquine; ns: not significant. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$. All experiments were performed at least in triplicate and with at least three biological replicates. Graphs were plotted as mean \pm SD. Ado was used at the concentration of 500 μ M and HCQ at 30 μ M unless otherwise stated.

showed that levels of the autophagosome marker, LC3-II, were increased significantly in HuCCA-1 and RMCCA-1 cells, but not in MMNK-1 cells, after treatment with 500 μ M adenosine and 30 μ M HCQ as compared to the HCQ treated group (Figure 3A and B). Treatment of HuCCA-1 and RMCCA-1 cells with both 500 μ M adenosine and 30 μ M HCQ increased LC3-II marker levels to 840% and 365% of the vehicle control group, respectively. In contrast, HCQ alone increased LC3-II marker levels to only 590% and 250% in HuCCA-1 and RMCCA-1 cells, respectively (Figure 3B). However, the combination of adenosine and HCQ did not increase LC3-II marker levels in MMNK-1 cells, as compared to cells treated with HCQ alone (Figure 3B).

Furthermore, the molecular pathway by which adenosine induces autophagy in CCA cells was studied by probing for phosphorylated AMPK α -substrates. The results showed that the levels of the phosphorylated forms of several AMPK substrates were increased after 500 μ M adenosine treatment (Figure 4A). Further study of the downstream pathway of AMPK, including

Raptor and ULK, showed that adenosine up-regulated the phosphorylated AMPK α T172 levels in both HuCCA-1 and RMCCA-1 cells to 195% and 125%, respectively (Figure 4B and C). Furthermore, phosphorylated Raptor S792 was up-regulated to 122% and 196% in HuCCA-1 and RMCCA-1 cells, respectively, while phosphorylated ULK-1 S555 was increased to 130% and 134%, respectively (Figure 4B and C).

Adenosine-induced autophagy in CCA cell lines is a survival mechanism. Autophagy may either be a survival or a death mechanism depending on many factors such as treatment type, duration, and cellular context. Further study of the role of autophagy in adenosine treatment was performed by using autophagy inhibitors. The results showed no difference in the percentage of dead cells with adenosine treatment alone in all CCA cell lines tested (Figures 2, 5A and B). However, when autophagy inhibitors were added 1 h prior to adenosine treatment, the percentage of dead cells was greatly increased (Figure 5A and B), while the number of live cells was also

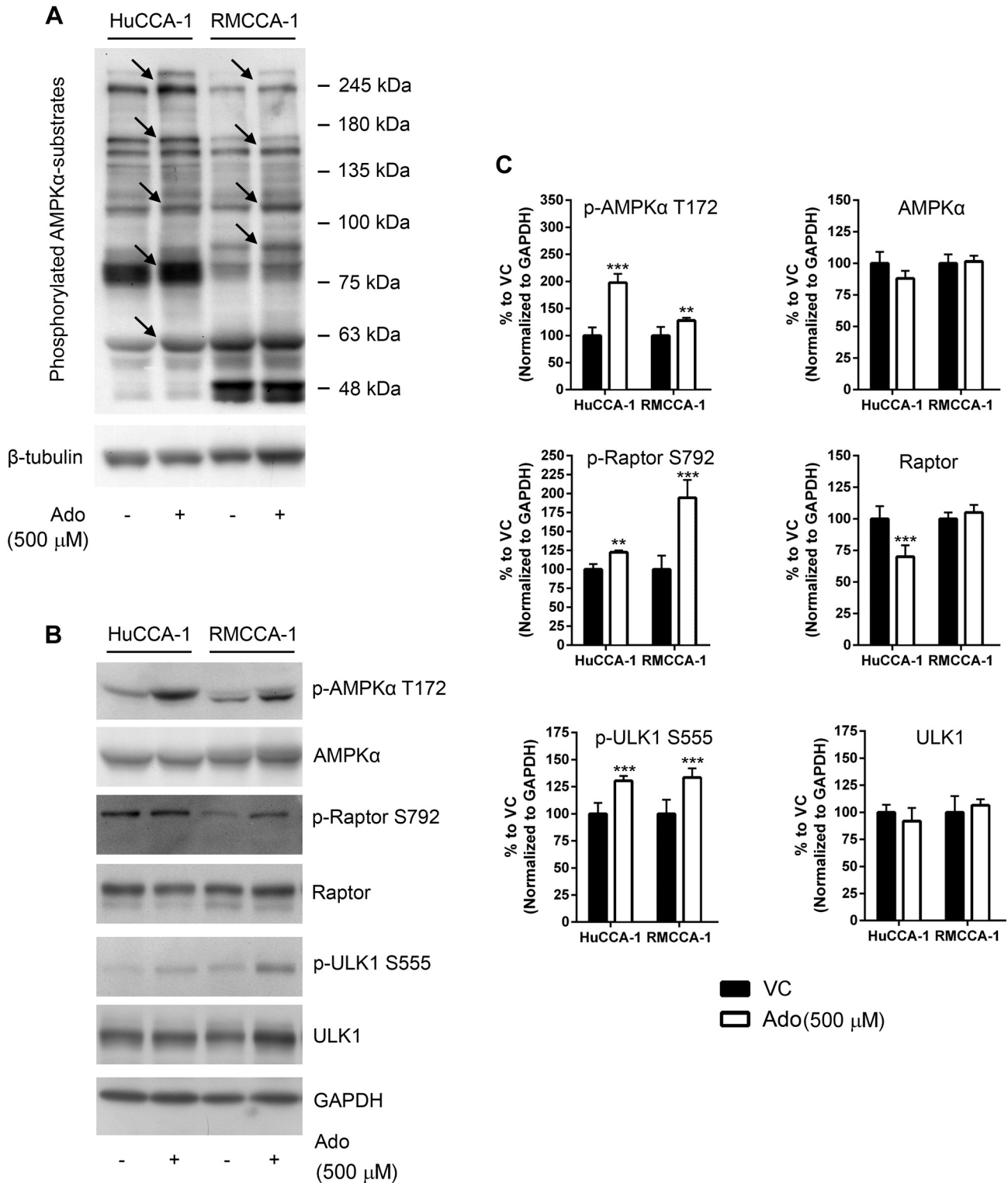


Figure 4. Adenosine activated AMPK in cholangiocarcinoma cells. (A) Treatment with 500 μ M adenosine for one day up-regulated AMPK kinase activity and increased phosphorylated AMPK substrates. (B) Treatment with 500 μ M adenosine for one day up-regulated phosphorylated AMPK, phosphorylated Raptor, and phosphorylated ULK1. (C) Quantitative analysis of protein levels in (B). Ado: Adenosine; VC: vehicle control. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$. All experiments were performed at least in triplicate and with at least three biological replicates. Graphs were plotted as mean \pm SD. Ado was used at the concentration of 500 μ M unless otherwise stated.

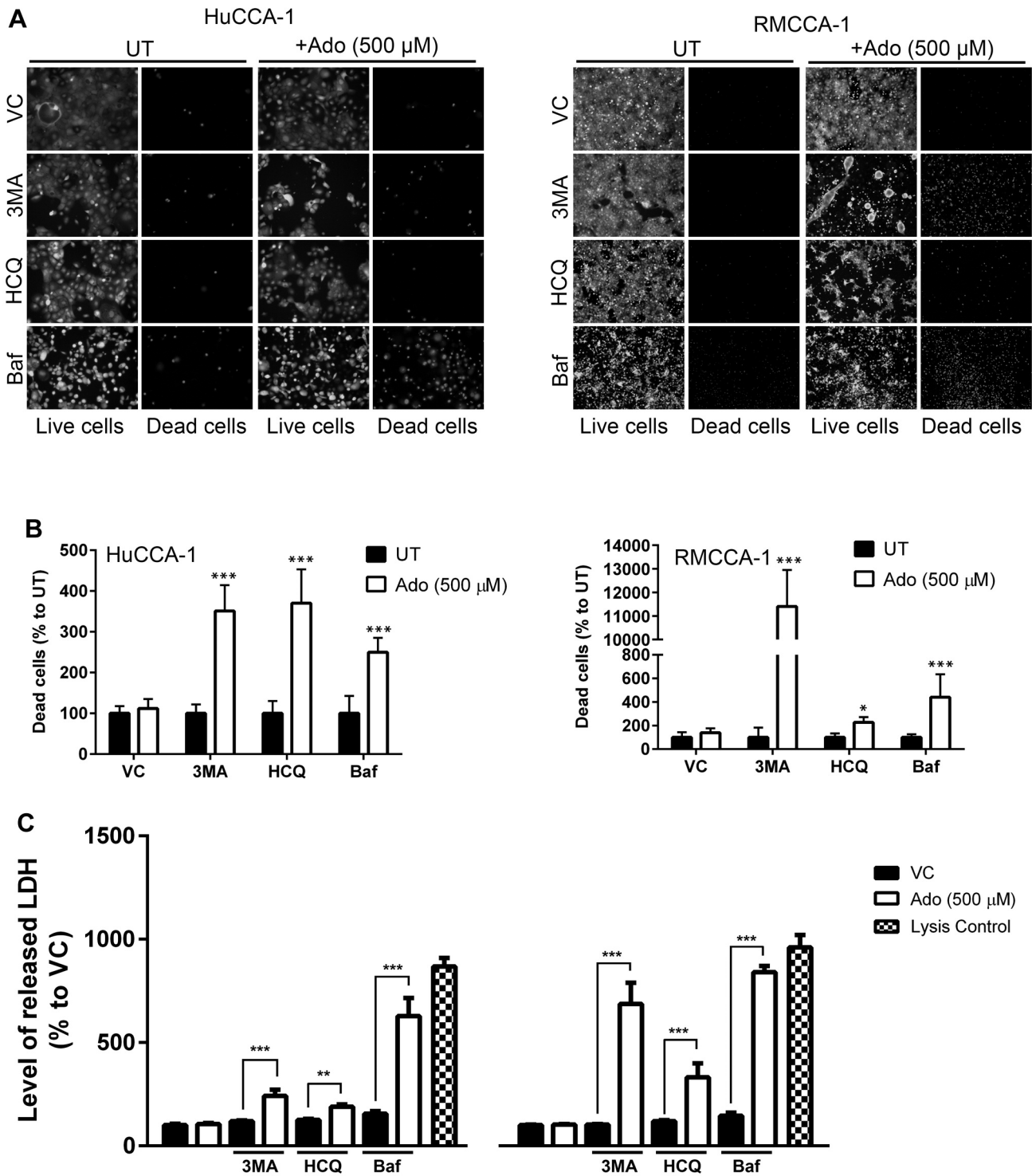


Figure 5. Adenosine-induced autophagy is a survival mechanism of CCA cells. (A) A combination of autophagy inhibitors with adenosine induced cell death, as shown after propidium iodine staining, in all CCA cell lines tested. (B) Percent of dead cells significantly increased when both adenosine and autophagy inhibitors were added to CCA cells. (C) Combination of 500 μ M adenosine with autophagy inhibitors increased the level of released lactate dehydrogenase, while adenosine or autophagy inhibitor alone did not. VC: Vehicle control, 3MA; 3-Methyladenine, HCQ; hydroxychloroquine, Baf; bafilomycin A1, $*p<0.05$, $**p<0.01$, $***p<0.001$. All experiments were performed in at least triplicate and with at least three biological replicates. Graphs were plotted as mean \pm SD. Ado was used at the concentration of 500 μ M, HCQ at 30 μ M, 3MA at 2.5 mM, and Baf at 100 nM unless otherwise stated.

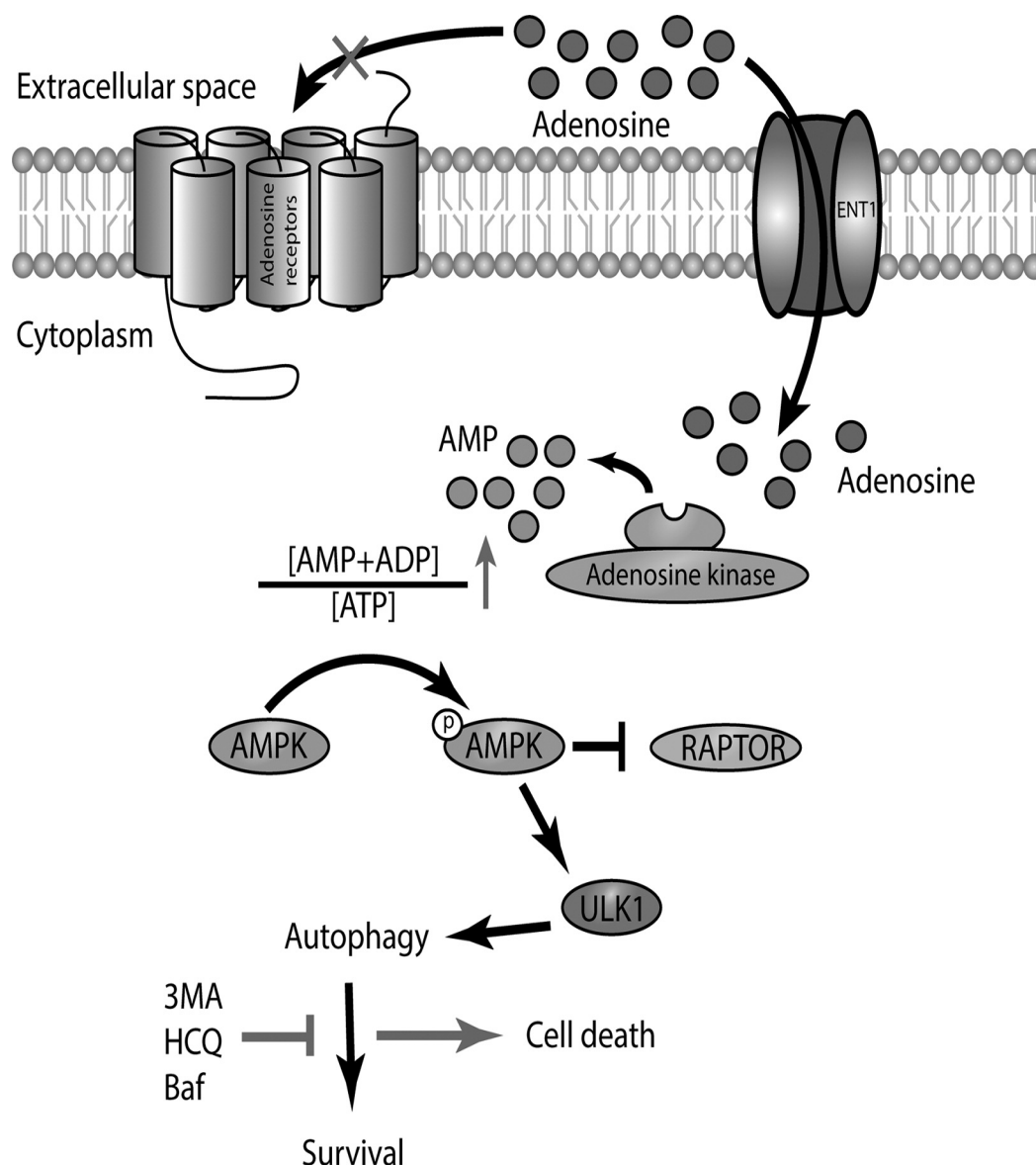


Figure 6. Proposed model of the molecular mechanism of the adenosine-induced growth inhibition and induction of autophagy in cholangiocarcinoma cells via the AMPK signaling pathways.

decreased significantly (Figure 5A). HuCCA-1 dead cells were increased to 348%, 368% and 255% when adenosine was added together with 2.5 mM 3-methyladenosine (3MA), 30 μ M hydroxychloroquine (HCQ) and 100 nM bafilomycin A1 (Baf), respectively, when compared to the group without adenosine treatment (Figure 5B). RMCCA-1 was more sensitive to 3MA, and the combination of 3MA and adenosine increased RMCCA-1 cell death to 11,450%, compared to the group not treated with adenosine. The combinations of HCQ or Baf with adenosine increased cell death to 232% and 444% in RMCCA-1, respectively (Figure 5B). In addition, cell damage was

studied with the LDH assay, where combinations of adenosine and autophagy inhibitors resulted in a significant increase in released LDH levels (Figure 5C). Therefore, it appeared that adenosine-induced autophagy is a survival mechanism that CCA cells exploit in order to survive the effect of adenosine.

Discussion

An inhibition of CCA cell proliferation and invasion by adenosine was recently reported (20, 24). This appears to involve a transporter-dependent mechanism, where

adenosine is transported into the cell and converted into AMP, as shown in the model proposed in Figure 6. In addition, adenosine has been reported to inhibit cell proliferation in many cancer types including hepatoma cells (21, 25), colon cancer (26), ovarian cancer (27), and prostate cancer (19). Another study also reported that adenosine at 4 mM induced cell cycle arrest and apoptosis in hepatoma cells (21). However, our results showed a very slight increase in the cell population in the G₀/G₁ phase after 2 days of 500 µM adenosine treatment in CCA cells (Figure 2C), with no apoptosis observed (Figure 2A and B), possibly due to differences in cell type and adenosine concentration. Although lower cell numbers and lower absorbance were found in the MTT assay, CCA cells treated with adenosine visually had similar cell morphology, as compared to the vehicle control treated cells (Figure 1C).

Since lower cell numbers were found in the adenosine treated group but no apoptosis and cell damage were observed, another cell death mechanism, autophagy, was studied. Recently, 2 mM adenosine was reported to induce autophagy in hepatoma cells (21). Here, our results show that adenosine, at doses as low as 500 µM, induced autophagy in CCA cell lines after 1-day treatment. Adenosine appeared to induce autophagy via AMPK activation.

The results demonstrate that adenosine-induced autophagy is a survival mechanism cancer cells exploit to avoid cell death. A combination of adenosine with autophagy inhibitor, particularly 3MA and bafilomycin a1, leads to CCA cell death. These combinations have potential for further study in animal experiments to determine the proper administration method and concentration. Adenosine has also been used in Phase III clinical trials for patients with coronary artery stenosis, using a standard intravenous infusion dose of adenosine at 140 mg/kg/min (ClinicalTrials.gov Identifier: NCT02350439). The trial reported that a higher infusion rate at 200 mg/kg/min showed more stable distal coronary pressure (Pd)/aortic pressure (Pa) ratio (28).

Therefore, adenosine has potential for the treatment of CCA, especially when combined with autophagy inhibitors such as 3MA or bafilomycin a1. However, further studies on these combinations in animal models are crucial for developing these compounds as adjuvant treatment for CCA patients.

Conflicts of Interest

The Authors declare no conflicts of interest in relation to this study.

Authors' Contributions

Conceptualization; J.L., Methodology; J.L., Validation; J.L., Formal Analysis; J.L., Investigation; J.L., Writing-Original Draft Preparation, J.L.; Writing-Review & Editing, J.L., J.S., and J.S., Supervision, J.S. and J.S.; Funding Acquisition, J.S. and J.S.

Acknowledgements

The Authors would like to thank Prof. Dr. Rutaiwan Tohtong for providing RMCCA-1 cell line.

Funding

This project was supported by funding from Chulabhorn Research Institute, Bangkok, Thailand.

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Received February 19, 2021

Revised July 1, 2021

Accepted July 2, 2021