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Effects of atropine on megakaryocytic differentiated K562 leukemia cells

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ABSTRACT

Objective: Non-neuronal cholinergic system signaling pathways play a significant role in various malignancies, including leukemia, lung, colon, brain, and breast cancer. This study aims to investigate the effect of atropine on megakaryocytic differentiated cells, as well as to identify the apoptosis mechanisms. We also studied the effect of cholinergic drugs on muscarinic receptors and caspase gene expressions, cell proliferation and caspase activities in differentiated cells.

Materials and Methods: K562 cells were induced into megakaryocytic differentiation using phorbol 12-myristate 13-acetate (PMA). The effects of agonists/antagonists on differentiated K562 cells were examined using cell viability and 5-Bromo-2-deoxy-uridine (BrdU) assays. Caspase activities were detected by the caspase assay kit. Protein expression levels were detected by western blotting.

Results: Atropine reversed the effects of carbachol (CCh) on increased megakaryocytic differentiated leukemia cell survival and proliferation. The protein expression of M_1 *and* M_4 muscarinic receptors was upregulated by CCh, an effect that was reversed by atropine. CCh alone did not significantly change levels of M_2, M_3, *and* M_5 muscarinic receptor proteins in megakaryocytic differentiated K562 leukemia cells.

Conclusion: M_2, M_3 muscarinic receptors and caspase 9 may have important functions in preventing the progression of leukemia and may also make important contributions to targeted therapies in leukemia.

Keywords: Atropine, Leukemia, Megakaryocytic differentiation, Muscarinic receptors, Caspases

1. INTRODUCTION

Acetylcholine (ACh) plays a role in cell proliferation, differentiation and apoptosis-related signaling pathways in different cell types [1-3]. It was previously thought that ACh was only synthesized and secreted by neurons. However, recent studies have revealed that many other cell types, including non-neuronal and tumor cells, can also produce ACh. In previous studies, various components of the cholinergic system were detected in megakaryocytes and platelets [1-4]. ACh has effects on both nicotinic ACh receptor (nAChR) and muscarinic ACh receptors (mAChR) [3]. ACh has a paracrine or autocrine role in many different cell types. M_1-M_5 mAChRs are found at high levels in the central nervous and peripheral parasympathetic nervous systems [3]. Activation of mAChRs can result in increased or decreased activity of signaling pathways involved in tumor progression and metastasis, providing a broad perspective on the contribution of these pathways to different carcinogenesis and the development of new antitumor targets [3]. There is some research evidence that mAChR agonists/antagonists inhibit or induce, cell proliferation and also change apoptosis in different types of cells [5]. Previously, different researchers have reported that ACh causes changes in muscarinic M_2 and M_3 mAChR mRNA levels in response to cholinergic agonist treatment in cerebellar granule cells, Chinese hamster ovary cells, lymphocytes and immune cells, and human neuroblastoma cell line SH-SY5Y [1, 6-8]. mAChRs also participate in tumorigenesis such as colon, breast, prostate, ovary and liver cancers [9]. ACh released from T lymphocytes has been shown to interact with the M_3 mAChR. It triggers nuclear signaling pathways and regulates the expression of gene in T and B lymphocytes [5].

Muscarinic receptor antagonists have inhibitory effects in many types of cancer, including the breast, melanoma, lung, stomach, colon, pancreas, ovarian, prostate and brain cancer [10]. Non-neuronal ACh regulates some cell functions such as an increase in cell number, differentiation, cell death, migration, angiogenesis, immune functions, secretion, cytoskeletal organization, and ciliary activity [11, 12]. Carbachol (CCh) is synthetic cholinergic agonist that has similar roles of ACh on both muscarinic and nicotinic receptors. Previously, we

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have shown that M_2, M_3 and M_4 mAChRs were determined in chronic myeloid leukemic K562 cells. Additionally, it was determined that M_3 mediates nitric oxide (NO) signaling in these cells. CCh treatment in undifferentiated K562 cells altered muscarinic M_2, M_3, and M_4 receptor gene expression as well as M_2 and M_3 protein expression and also increased cyclic adenosine monophosphate (cAMP) levels [13]. K562 cells also express alpha 7 nicotinic acetylcholine receptor (*α7-nAChR*). It has been stated that ACh causes an increase in Ca^{2+} level via *α7-nAChR* in K562 chronic myelogenous leukemic cells [14]. Schedel et al., showed that functional *α7-nAChR*s were expressed in platelets [15]. Apoptosis is the mechanism that ensures the formation of tissue shapes during development and tissue homeostasis by destroying harmful or dysfunctional cells. Different researchers have demonstrated the important role of the caspase family in mediating apoptosis signaling pathways and the occurrence of apoptosis [15, 16]. The mechanism of apoptosis is classically induced via the intrinsic and the extrinsic pathways [15]. Each pathway is associated with unique stimuli and independent signal transduction steps. Different caspases are participated in the apoptosis. Caspase family members are required for the continuation of cellular functions such as apoptotic cell death and remodeling. For example, *caspase 8* is activated in the extrinsic apoptotic pathway, and *caspase 9* is activated in the intrinsic apoptotic pathway and these two pathways activate *caspase 3* [15, 16]. Cancer cells can regulate apoptotic pathways by different mechanisms [15]. In cancer, impairment of apoptotic signaling, especially the activation of anti-apoptotic pathways, prevents cancer cells from undergoing apoptosis and contribute to the proliferation of tumor cells by causing uncontrolled proliferation to go out of control. Different investigators have shown that muscarinic receptor-mediated activation can increase or decrease cell growth and apoptosis in conjunction with cell metabolic activity levels [17-19]. Chronic myeloid leukemia (CML) is one of the types of myeloproliferative neoplasms. These cells are characterized by the development of the Breakpoint cluster region-Abelson murine leukemia virus *(Bcr)-(Abl)* fusion oncogene. *(Bcr)-(Abl)* fusion oncogene translates into an active tyrosine kinase. This kinase causes too many immature myeloid cells to enter the circulation [20, 21]. Therapy strategies through induction of differentiation are reported to be interesting in the treatment of leukemia and various cancers in recent studies. Multipotent K562 cells can differentiate into erythroid or megakaryocytic cells when exposed to different agents. Differentiation of K562 cells with phorbol 12-myristate13-acetate (PMA) alters structural and functional properties of the cell [22]. In this study, the effects of cholinergic drugs on mAChR protein expression, proliferation and apoptosis in megakaryocytic differentiated K562 leukemia cells were investigated.

2. MATERIALS and METHODS

Materials

Atropine, CCh and Roswell Park Memorial Institute 1640 (RPMI-1640) medium were purchased from Sigma Chemical Co, St. Louis, MO, USA. 5-Bromo-2-deoxy-uridine (BrdU) Labeling and Detection Kits were supplied from Roche Company (Mannheim, Germany). Caspase assay kits were purchased from Invitrogen (Waltham, Massachusetts, USA). Fetal bovine serum (FBS) was obtained from Gibco (Grand Island, NY, USA). Nitro Blue Tetrazolium/5-Bromo-4-Chloro-3-indolyl – phosphate (NBT/BCIP) was purchased from Promega (Madison, WI, USA). All other chemicals were obtained from Sigma, unless stated otherwise.

Cell culture

K562 cells (a frozen cell line CCL-243) were taken from the American Type Culture Collection ATCC, (Manassas, VA, USA). Cells were grown in RPMI-1640 containing 10% fetal bovine serum (FBS), 100 U/ml penicillin and 100 µg/ml streptomycin in a humidified atmosphere with 5% CO_2 at 37 °C.

Antibodies

M_1-M_3, M_5 mAChRs, *caspase 3, caspase 8, caspase 9* and *Beta-actin* (β-actin), specific antibodies were supplied from Santa Cruz Biotechnology, (Santa Cruz Biotechnology, Dallas, Texas, CA, USA). M_4 muscarinic receptor antibody was supplied from Life Span, St Louis, MO, USA. The following secondary antibody and dilutions were used alkaline phosphatase-conjugated goat anti rabbit 1:10,000 (Sigma, St Louis, MO, USA).

Megakaryocytic differentiation of K562 cells with PMA

For megakaryocytic differentiation, K562 cells (1×10^5 cells/ml) were treated with 10 nM PMA (Calbiochem, Darmstadt, Germany) for 48 h. Megakaryocytic differentiation with PMA changed K562 leukemia cell morphology. PMA induced the differentiation of K562 cells into megakaryocytes. Changes in the morphology of undifferentiated K562 cells and megakaryocyte differentiated K562 cells were observed in 2D cell culture conditions. Images were captured from undifferentiated K562 cells and megakaryocyte differentiated K562 cells using an Phase-contrast light microscope (100 X), the scale bar is 20µM (Olympus, Tokyo, Japan) [23].

Megakaryocyte differentiation marker is integrin β3. We previously showed that PMA treatment in K562 cells leads to the induction of *β3-integrin* protein expression in the megakaryocytic differentiation process. Cells were homogenized, protein levels were detected and western blotting was performed as described previously [24]. The differentiation of K562 cells into megakaryocytes was demonstrated by western blotting analysis of *β3-integrin* protein levels [23].

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Cell viability and proliferation

Cell viability was determined by 0.4% trypan blue dye exclusion test using the cell counter (TC-20 BioRad, Hercules, CA, USA). In our previous study, the megakaryocyte differentiation protocol and drug concentrations were described in detail. [22]. K562 cells were incubated in RPMI 1640 culture medium containing FBS with 1% penicillin/streptomycin at 37°C with 5% CO_2. K562 cells (1×10^5 cells/ml) were treated with 10 nM PMA (Calbiochem, Darmstadt, Germany) for 48 h. Megakaryocytic differentiation led to a change in the morphology of K562 cells. $1x10^4$ cells/100µL megakaryocytic differentiated K562 leukemia cells were plated in 96-well plates containing fetal bovine serum-free RPMI-1640 medium. After 24 hour, these cells were placed in 1% FBS serum containing medium. Cholinergic drugs were then placed to the cell culture medium: Atropine (10 µM) was added and after 30 minutes, CCh (100 µM) was placed and incubated for 24 hours and 48 hours. Cell numbers were determined with the BrdU commercial kit (Roche Company, Mannheim, Germany). The optical density (OD) was determined using a Microplate Reader (a Thermo Multiskan Ascent, California, USA) set to 405-490 nm.

Western blot analysis

Protein level detection in whole cell lysates [23] and western blot analyses were explained in our previous studies with minor modification [13]. Equal amounts of protein extracts (100 µg/line) were separated by 10% SDS-PAGE gel and transferred to nitrocellulose membranes. Then the membranes were blocked in 1% bovine serum albumin for 1hour. The M_1, M_2, M_3, M_5 mAChRs, *caspase 3, caspase 8, caspase 9* and *β-actin* antibodies (Santa Cruz Biotechnology Inc., Dallas, Texas, USA) and M_4 mAChR (Life Span, St Louis, MO, USA) were diluted 1:100 and incubated overnight. Membranes were incubated with alkaline phosphatase-conjugated secondary antibody (Sigma, St Louis, MO, USA) for 1 hour at room temperature. This was followed by enhancement with NBT/ BCIP (Invitrogen, Waltham, USA). The M_1, M_2, M_3, M_4, M_5 mAChRs, *caspase 3, caspase 8, caspase 9* and *β-actin* molecular weights are 52 kDa, 75 kDa, 75 kDa, 74 kDa 60 kDa, 32 kDa, 55 kDa, 46 kDa and 47 kDa respectively. Total amount of protein in each lane was normalized to the internal control *β-actin*. Blots were imaged and band intensities were quantified using Bio-Rad Molecular Analyst software (Free Edition, www.totallab.com).

Effect of CCh/Atropine on caspase activity in megakaryocytic differentiated K562 leukemia cells

Megakaryocytic differentiated K562 leukemia cells (1×10^6 cells/ml) were incubated for 24 hours in cell 96 well plates, atropine (10 µM) and /or CCh (100 µM), added to culture medium respectively. The cell lysate was prepared and caspase activities were determined with ELISA kit (*caspase 3,8, and 9* assay kit) (Invitrogen, Waltham, MA). OD was determined at 405 nm using a Thermo Multiskan Ascent Microplate Reader (Thermo Fisher Scientific, Waltham, MA, USA). Caspase assay kits were used according to the manufacturer's instructions. Each measurement was made at least in six independent experiments.

Statistical Analysis

The data are expressed as the Standard Error of the Mean (SEM) of at least three independent experiments. All statistical analyses were performed using two-way analysis of variance (Two-way ANOVA) followed by Tukey's multiple comparison test, using GraphPad Software Inc.'s Prism version. A significance level of $p<0.05$ was considered statistically significant.

3. RESULTS

Effect of atropine on megakaryocyte differentiated K562 leukemia cell proliferation

Inducing K562 cells to differentiate into megakaryocytes with PMA changed cell morphology**.** Images were captured from undifferentiated K562 and megakaryocyte differentiated K562 leukemia cells using a Phase-contrast light microscobe (Olympus, Tokyo, Japan) (Fig.1A).

100 µM CCh caused an increase in the proliferation of megakaryocytic differentiated K562 leukemia cells at 24 and 48 hours. CCh, which activates cholinergic receptors, increased BrdU incorporation to 139% and 96% of control in 24 and 48 hours, respectively. Atropine partly reversed CCh (100 µM)-induced increase of BrdU incorporation (Fig. 1B). Blocking mAChRs with atropine reversed the effects of CCh on increased megakaryocytic differentiated leukemia cell survival and cell numbers in 24 and 48 hours.

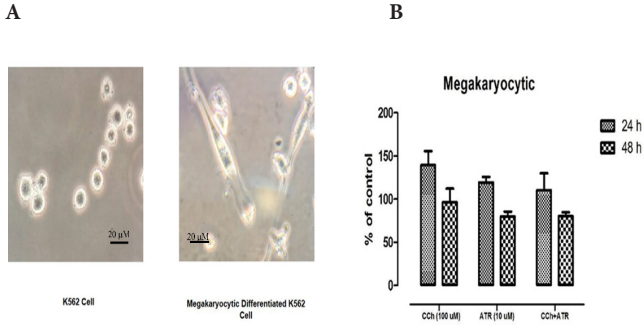


Figure 1. *Effect of atropine and CCh on proliferation, A) Representative images of undifferentiated K562 cells, megakaryocyte differentiated K562 cells were seen using Phase-contrast light microscobe (100 X), the scale bar is 20µM B) Effects of CCh 100 µM with or without atropine at 24 hours or 48 hours on megakaryocytic differentiated K562 cell proliferation. Proliferation detected with BrdU assay (absorbance at 405 nm) The difference between the 24 and 48 hours was found to be statistically significant (*p<0.0106). Data represent the mean +/– SEM of 3 experiments. ATR:Atropine, CCh: Carbachol.*

Effects of *cholinergic agonists/antagonists on muscarinic receptor expression in megakaryocyte differentiated K562 leukemia cells*

Megakaryocyte differentiated K562 leukemia cells were incubated with CCh/Atropine for 24 hours. M_1-M_5 muscarinic receptors were identified by western blot analysis. CCh (100 µM) treatment significantly increased M_1 and M_4 mAChRs expression (*p<0.05) (Fig 2A,3B).

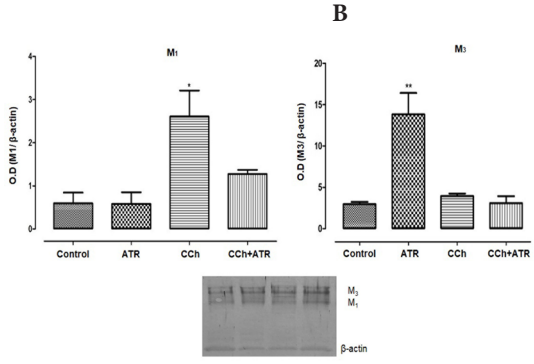


Figure 2. *Cholinergic agents modify expression of the M_1, M_3 mAChR proteins in megakaryocytic differentiated K562 leukemia cells. Megakaryocytic differentiated K562 leukemia cells expressing muscarinic receptors were treated with CCh and/or atropine (10 µM) for 0–24 hours. M_1 and M_3 mAChR proteins were detected by western blotting using specific antibodies in the same membranes. The β-actin confirmed equal amount of loading. The data are expressed as the means ± SEM of three independent experiments. The estimated molecular weights were 52 kDa (M_1) 75 kDa (M_3) and b actin 47kD. (*p<0.05, ** p<0.05). ATR:Atropine, CCh: Carbachol.*

To investigate the effect of atropine on protein expression of muscarinic receptors, megakaryocytic differentiated K562 cells were pretreated with 10 µM atropine for 30 minutes and then added to CCh. Western blot data showed that atropine increased significantly M_2 muscarinic receptor protein expressions relative to the untreated group (Fig 3A). Atropine also caused increase in the expression of M_3 muscarinic receptor protein compared to the untreated group (Figure 2B).

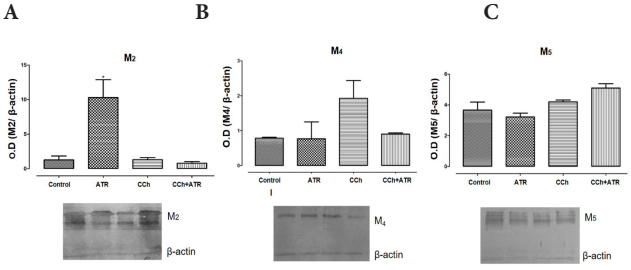


Figure 3. *Cholinergic agonists and antagonists alter M_2, M_4, and M_5 mAChR protein expressions in megakaryocytic-differentiated K562 leukemia cells. M_2, M_4 and M_5 mAChR proteins were detected by western blotting using specific antibodies. The β-actin confirmed equal amount of loading. The data are expressed as the means ± SEM of three independent experiments (* p<0.05). A. Molecular weights were 75 kDa (M_2), B. 74 kDa (M_4), C. 60 kDa (M_5) and b-actin 47kD. ATR:Atropine, CCh: Carbachol.*

As shown above, in the megakaryocytic differentiated K562 leukemia cells, M_1 and M_4 mAChR protein expressions were significantly increased in CCh compared to untreated controls (Fig. 2; 3B). Atropine reversed the effect of CCh on upregulation of M_1 and M_4 mAChRs protein expression (Fig. 2; 3B). The expression of M_5 mAChRs was not significantly changed by CCh versus untreated controls (Fig.3C).

Roles of cch and/or atropine in apoptosis signaling

We investigated the expression of proteins associated with the apoptosis signaling pathway. Expression of *caspase 3, caspase 8* and *caspase 9* was assayed by western blotting in megakaryocytic differentiated K562 leukemia cells. A significant difference in *caspase 3* and *caspase 8* expression was observed in CCh (100 µM) group cells compared to untreated control cells. *Caspase 3* expression decreased by 25% with atropine, 67% with CCh, and 52% with CCh+ATR compared to untreated control cells. (*P<0.05). (Fig. 4 A, B).

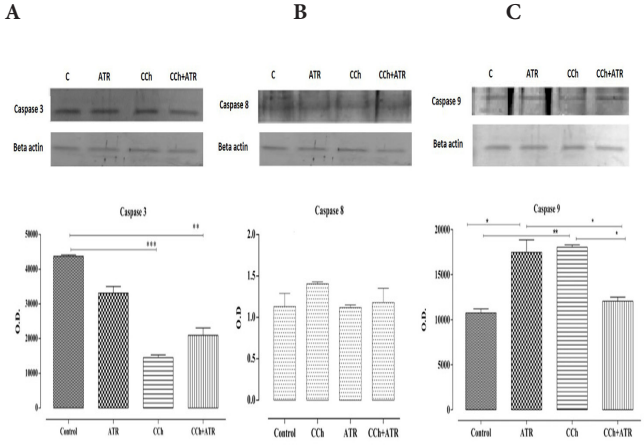


Figure 4. *Changes of caspase 3, caspase 8 and caspase 9 protein expression in megakaryocytic differentiated K562 cells exposed to cholinergic agonist /antagonist. Caspase 3, caspase 8 and caspase 9 proteins were detected by western blotting using specific antibodies. The β-actin confirmed equal amount of loading. A representative image from experiment (of 3) is shown. The data are expressed as the means ± SEM of three independent experiments when compared to untreated group. A. 32kD (caspase 3), B.55 kDa (caspase 8), C. 46kD (caspase 9). 47kD (β-actin) (*P<0.05, (**P<0.005), (***P<0.004). ATR:Atropine, CCh: Carbachol.*

Caspase 8 expression level was reduced by 6% with atropine, 75% with CCh, and 56% with CCh+Atropine compared to untreated group (*P<0.05). *Caspase 9* expression was upregulated with CCh respect to untreated group (*P<0.05) (Fig. 4 C). *Caspase 9* expression levels relative to the untreated control cells; atropine caused an increase of 62%, CCh 67%, and CCh+Atropine 12% (*P<0.05). The stimulatory effect of CCh was reversed when CCh and atropine were given together. (*P<0.05; **P<0.05). These data demonstrated that atropine could also increase and induce *caspase 9*, the apoptosis-associated protein in these differentiated leukemia cells.

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Effects of CCh and/or Atropine on caspase 3, 8 and 9 activity

The effect of cholinergic agents on caspase activity was evaluated in megakaryocytic differentiated K562 leukemia cells. The apoptotic effect of CCh (100 µM) at 24 hours triggered an increase in *caspase 3* and *caspase 8* activity, but it was not significant (p>0.05) (Fig.5 A, B). *Caspase 9* activity was significantly increased in response to atropine in megakaryocytic differentiated K562 leukemia cells (****p<0.05) (Fig.5 C).

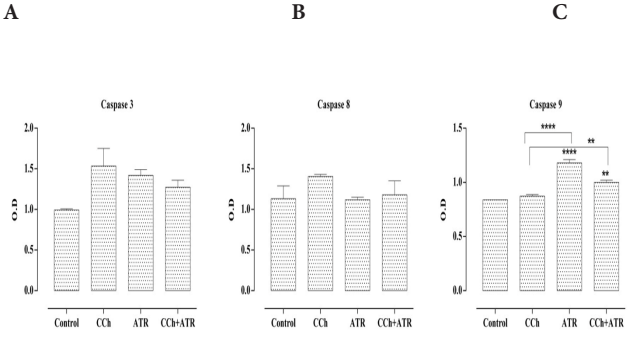


Figure 5. *Effect of CCh alone or in the presence of atropine on caspase 3, caspase 8 and caspase 9 activity in megakaryocytic differentiated K562 leukemia cells. The cells were incubated with CCh (100 µM); Atropine (10 µM) and/ or combination for 24 hours. Caspase 3,8 and 9 activities were detected using commercial ELISA kits. The data are expressed as the means ± SEM of three independent experiments (****p<0.05), (**p<0.05). ATR:Atropine, CCh: Carbachol.*

Our result showed that atropine alone significantly caused an increase in *caspase 9* activity level versus untreated control group in megakaryocytic differentiated K562 leukemia cells (****p<0.05). There is a significant difference in *caspase 9* activity levels between CCh and CCh+Atropine (**p<0.05) and also a significant difference was found between CCh and atropine (****p<0.05) (Fig. 5 C). Figure 6 shows the flow chart of the study design.

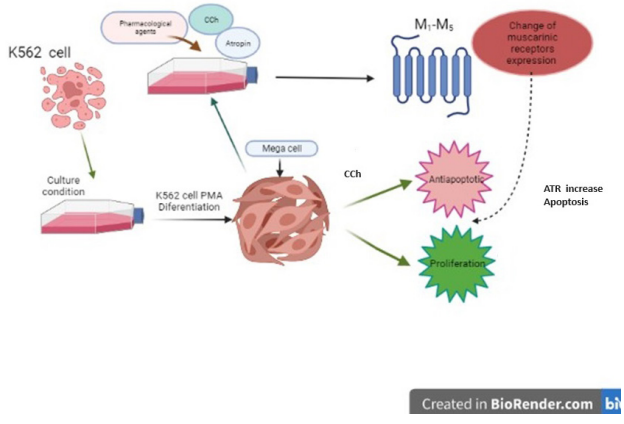


Figure 6. *Flow chart of the study design*

4. DISCUSSION

K562 cells are a widely used cell model for hematopoietic cell growth and differentiation [25]. G-protein-coupled receptors (GPCRs) are an important drug target. mAChRs are involved in the GPCR family. GPCRs can induce stimulatory or inhibitory effect on apoptosis through different types of receptors in different cells. Therefore, clarifying the role of cholinergic agents and potential therapeutic targets has been the focus of different cancers. Different research groups have reported that mAChRs mediate different cellular signalling pathways in hematopoietic cells [5, 13]. In this study, we investigated the effect of atropine on proliferation and apoptosis in megakaryocytic differentiated K562 leukemia cells. The main new findings of this research are 1) CCh stimulates cell proliferation in megakaryocytic differentiated K562 cells and 2) CCh alters the expression of M_1-M_5 muscarinic receptors and *caspase 3, caspase 8, and caspase 9* in these cells. M_3 and M_5 receptor protein expression was found to be approximately threefold higher than other mAChRs in megakaryocytic differentiated K562 cells. 3) Atropine stimulates M_2, M_3 and *caspase 9* expression in megakaryocytic differentiated K562 cells. Cabadak et al., demonstrated the presence of M_2-M_4 receptor expression in undifferentiated K562 cells by using western blot and RT-PCR techniques [26]. We showed that undifferentiated K562 cell proliferation largely depends on the amount of FBS. CCh in serum-free conditions caused a significant increase in DNA synthesis [13, 26]. DNA synthesis was inhibited when undifferentiated K562 cells were exposed to CCh in the presence of 1% or 10% serum [13]. CML is diagnosed more frequently in older people. The prevalence of CML is approximately 15% of all leukemia cases [27]. Cholinergic receptors and their functions are important in neuronal and non-neural cells and tissues. Cholinergic agents modulate the acetylcholine-related mechanism. Cholinergic mechanisms are also important in different types of cancer cells. For example, stimulation of muscarinic M_3 receptors in lung cancer cells increases their proliferation [28]. Different muscarinic M_3 receptor antagonists such as 4-DAMP (4-diphenyl-acetoxy-N-methyl-piperidine) and p-F-HHSiD (para-Fluorohexahydrosila-difenidol), inhibited the growth of small cell lung cancer (SCLC) cells [28, 29]. Rosemond and colleagues found that M_3 mAChRs were expressed in H508 human colon cancer cells [9]. CCh increased cell growth and cell cycle progression in these cells. Atropine completely reversed the effects of CCh [30]. Our previous research also showed that curare induced an increase in cell proliferation of undifferentiated and megakaryocyte-differentiated K562 cells [24]. Aydin et al., reported that eserine with or without atropine affected megakaryocyte differentiated K562 cell proliferation [24]. mAChR subtypes are found in different types of cancer, including colon, bladder, cervix, pancreas, and lung cancer [9, 10, 28, 31, 32]. Preclinical studies have suggested that cholinergic drugs may be useful in the treatment of cancer, particularly bladder and prostate cancer [9]. Gutkind et al., suggested that M_1, M_3 and M_5 mAChRs may serve as conditional oncogenes when NIH 3T3 cells are exposed to CCh [33]. Baumgold et al., reported that CCh inhibited DNA synthesis in M_1-transfected

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A9L cells [33]. Renz et al., demonstrated that downregulation of the MAPK and PI3K/AKT signaling pathway in cancer cells through the M_1 mAChR could directly inhibit pancreatic tumor development [34]. Bennett et al., suggested that ACh inhibited platelet activation and NO played a role in platelet inhibition [35]. Our study also focused on whether CCh or atropine alters the expression of mAChRs. Our study showed for the first time that CCh/Atropine causes changes in the expression of mAChR subunits in megakaryocytic differentiated K562 cells. CCh increased M_1 and M_4 mAChRs expression compared to untreated control megakaryocytic differentiated K562 cells. However, CCh alone did not significantly change M_2, M_3, and M_5 mAChRs protein levels in megakaryocytic differentiated K562 leukemia cells. Atropine increased M_2 and M_3 mAChRs protein levels and also increased *caspase 9* protein levels in differentiated K562 leukemia cells. These results indicated that atropine inhibited cell proliferation and induced apoptosis through muscarinic M_2 and M_3 receptors. Reina et al., showed that M_1 and M_3 mAChRs in skin fibroblast cells could cause stimulation apoptotic cell death and proliferation [36]. Pilocarpine, M_3 mAChR agonist, consistently induced apoptotic pathways in human neonatal skin fibroblasts [36]. Recent studies have indicated that atropine is involved in in epithelial-mesenchymal transition regulation and can reduce colony formation of metastatic breast cancer cells and subsequently alleviate stemness [37]. Interestingly in our results, atropine increased *caspase 9* expression in megakaryocytic differentiated K562 cells. The atropine treated group for *caspase 9* activity resulted in an increase compared to basal activity in control megakaryocytic differentiated K562 cells. Blocking the function of mAChRs by atropine not only turns off the intracellular signaling of CCh that stimulates cell growth but may also potentiate the increase in apoptosis.

Our data are the first to establish that CCh increases the proliferation of megakaryocytic differentiated K562 cells by upregulating M_1 and M_4 mAChRs. These results in megakaryocytic differentiated K562 cells are found to be different from undifferentiated K562 cells. M_1 and M_4 mAChRs may contribute to growth-stimulating mechanisms in megakaryocytic differentiated K562 leukemia cells. Atropine caused an increase in the expression of M_2, M_3 mAChRs , *caspase 9* as well as *caspase 9* activity in megakaryocyte differentiated K562 leukemia cells. Atropine reduced megakaryocytic differentiated K562 leukemia cell proliferation. Atropine may be also an additive effect on chemotherapy drugs. To clarify the detailed mechanism of the proliferation of megakaryocytic differentiated K562 leukemia cells induced by cholinergic agonists/antagonists, we need to further investigate the relationship between mAChRs and apoptosis cell signaling pathway mechanisms under cholinergic stimulation.

Acute megakaryoblastic leukemia (AMKL), a subtype of acute myeloid leukemia (AML), affecting megakaryocytes, is a rare malignancy affecting platelet-producing megakaryocytes found in the bone marrow in adults. In the future, different signaling pathways should be investigated to determine the contribution of the non-neuronal cholinergic system to the treatment of AMKL in the megakaryocytic differentiated K562 leukemia cell model.

In conclusion, the results of these studies showed that atropine reversed the effects of CCh on the survival and proliferation of megakaryocytic differentiated leukemia cells. M_2, M_3 muscarinic receptors and *caspase 9* may have important functions in preventing the progression of leukemia and may make important contributions to targeted therapies in leukemia.

Compliance with Ethical Standards

Ethical approval: Ethical approval was not sought for the present study because this article does not contain any studies with human or animal subjects.

Conflict of interest: The authors declare that there are no conflicts of interest.

Financial support: This study received no specific grant from any funding agency in the public, commercial or not-for-profit sector.

Author contributions: HC, ZK and BA: Conceived and planned the experiments. HC, ZK and BA: Carried out the experiments, ZK and BA: Contributed to sample preparation, HC and BA: Contributed to the interpretation of the results, ZK and BA: Took the lead in writing the manuscript, HC: Provided critical feedback and helped shape the research, analysis and manuscript. All authors discussed the results and commented on the manusctipt and approved the final version.

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