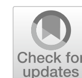
Naunyn-Schmiedeberg's Archives of Pharmacology
https://doi.org/10.1007/s00210-023-02418-4

RESEARCH



Potential antiproliferative and apoptotic effects of pilocarpine combined with TNF alpha in chronic myeloid leukemia cells

Zehra Kanlı[1] · Hülya Cabadak[2] · Banu Aydın[2]

Received: 14 October 2022 / Accepted: 2 February 2023
© The Author(s), under exclusive licence to Springer-Verlag GmbH Germany, part of Springer Nature 2023

Abstract

Pilocarpine is a selective M_1/M_3 agonist of muscarinic acetylcholine receptor subtypes. Muscarinic acetylcholine receptors are G protein-coupled receptors. These receptors are different drug targets. The aim of the present work was to investigate the effect of pilocarpine on the expression of M_3 muscarinic acetylcholine receptor, the AChE activity, IL-8 release response, and proliferation in K562 cells, via muscarinic receptor activation. Human chronic myeloid leukemic cell cultures were incubated with drugs. Proliferation assays were performed by BrdU assay. Expression of M_3 muscarinic acetylcholine receptor and apoptosis proteins such as bcl, bax, cyt C, and caspases was assessed with the semiquantitative Western blotting method. Pilocarpine inhibits chronic myeloid cell proliferation and M_3 muscarinic acetylcholine receptor protein expression. Pilocarpine increases caspase-8 and -9 expression levels, upregulating the proapoptotic protein Bax and downregulating the expression levels of the antiapoptotic protein Bcl-2. The apoptotic activity of pilocarpine is associated with an increase in AChE activity. M_3 muscarinic acetylcholine receptors can activate multiple signal transduction systems and mediate inhibitory effects on chronic myeloid K562 cell proliferation depending on the presence of 1% FBS conditions. This apoptotic effect of pilocarpine may be due to the concentration of pilocarpine and the increase in AChE level. Our results suggest that inhibition of cell proliferation by inducing apoptosis of pilocarpine in K562 cells may be one of the targets. M_3 selective agonist may have therapeutic potential in chronic myeloid leukemia.

Keywords Cholinergic receptors · M_3 muscarinic receptor agonist · TNFα · Leukemia cells

Zehra Kanlı and Hülya Cabadak contributed equally to this work

Highlights

- Pilocarpine increased acetylcholinesterase activity levels and apoptosis.
- TNFα increased muscarinic acetylcholine receptor M_3 expression in chronic myeloid leukemic cells.

✉ Hülya Cabadak
hcabadak@gmail.com

Zehra Kanlı
zehraknli@gmail.com

Banu Aydın
banuaydin@gmail.com

[1] Institute of Health Sciences, Marmara University, Basibuyuk-Maltepe, Istanbul 34854, Turkey

[2] Marmara University, School of Medicine, Department of Biophysics, Basic Medical Sciences Building, Maltepe, Istanbul 34854, Turkey

Abbreviations

AChR	Acetylcholine receptors
mAChRs	Muscarinic receptors
G proteins	Guanine nucleotide-binding proteins
cAMP	Cyclic adenosine monophosphate
EGFR	Epidermal growth factor receptor
AML	Acute myeloid leukemia
CML	Chronic myeloid leukemia
Bax	Bcl-2-associated X-protein
α7 nAChRs	α7 Nicotinic ACh receptors
CCh	Carbachol
ATR	Atropine
Cyt C	Cytochrome C
ACh	Acetylcholine
BrdU	5-Bromo-2′-deoxyuridine
ALL	Acute lymphocytic leukemia
CLL	Chronic lymphocytic leukemia
MLA	Methyllycaconitine

Published online: 13 February 2023

Introduction

Human chronic myeloid leukemia (CML) is a very slow-growing leukemia but can be transformed into a fast-growing acute leukemia. Therefore, CML should be treated, and this transformation should be prevented. Some chemotherapy drugs are used in chronic myeloid leukemia but do not completely treat. Leukemia is a group of heterogeneous neoplastic malignancies. Leukemia is a process that develops in the bone marrow and affects normal hematopoiesis. There are four main types of leukemia. They are classified according to their cellular origin, myeloid or lymphoid origin, and the course of the disease. Leukemia is classified as acute myeloid leukemia (AML), acute lymphocytic leukemia (ALL), chronic myeloid leukemia (CML), and chronic lymphocytic leukemia (CLL) Jin et al. 2016). In 1999, Wessler et al. defined the non-neuronal cholinergic system in non-neuronal cells. It was defined that a system is formed by expressed acetylcholine, choline acetyltransferase, acetylcholinesterase, and cholinergic acetylcholine receptors (1999). Cholinergic receptors are divided into two groups, muscarinic and nicotinic. Muscarinic acetylcholine receptors (mAChRs) are seven-transmembrane domains. These are the single subunit 50–70 kDa glycoproteins. They interact with G proteins (Bonner 1989; Bonner et al. 1987; Felder 1995). In terminology, muscarinic acetylcholine receptors are named M_1–M_5R (Caulfield & Birdsall 1998; Eglen 2012; Wess 2003). Activation of muscarinic M_1, M_3, and M_5 receptors causes phosphoinositide (PI) hydrolysis and an increase in cellular calcium concentration, while activation of M_2 and M_4 receptors causes inhibition of adenylyl cyclase activity and decreased cellular cyclic adenosine monophosphate (cAMP) levels (Carruthers et al. 2015; Clader & Wang 2005). These receptors mediate many essential physiological functions which are as follows: heart rate and strength, relaxation of blood vessels, constriction of airways in the lungs, increased secretion, mobility of various organs in the gastrointestinal tract, contraction of smooth muscles, and secretion of lacrimal glands in the organism (Carruthers et al. 2015; Clader & Wang 2005; Wess 2003). Pilocarpine is a cholinergic agonist that specifically binds to muscarinic receptors (M_1 and M_3) and causes an increase in free Ca^{2+} in the cytosol (Pronin et al. 2017). The slowly hydrolyzed muscarinic agonist pilocarpine has no nicotinic effect. Different researchers have reported that pilocarpine possesses anti-apoptotic activities (Reina et al. 2010). Activation of M_3 muscarinic acetylcholine receptors in various cancer cells stimulates complex signaling pathways that stimulate gene transcription leading to cell proliferation via epidermal growth factor receptor (EGFR)-dependent and independent signaling pathways. EGFR is a member of the tyrosine kinase receptor family. Epidermal growth factor receptors (EGFR) are glycoproteins (Finot et al. 2012; Huang et al. 2014; Natarajan et al. 2007; Sibilia et al. 2007). Activation of EGFR leads to protein tyrosine phosphorylation and the formation of intracellular phosphotyrosine-dependent protein complexes (K. Cheng et al. 2008; K. Cheng et al. 2003; Von Rosenvinge & Raufman 2011). The EGFR pathway is important for solid tumors such as colon cancer cell proliferation (Felton et al. 2018). The epidermal growth factor can stimulate cell proliferation, survival, and differentiation (Finot et al. 2012; Huang et al. 2014; Zhang et al. 2012). EGF and EGFR are overexpressed in many cancers such as breast, lung, and liver cancer (Breindel et al. 2013; Finot et al. 2012; Huang et al. 2014; Masuda et al. 2012). EGF stimulation causes cell proliferation, cell division, wound healing, cancer formation, and tumor development by biochemical changes (Citri & Yarden 2006; Finot et al. 2012; Huang et al. 2014).

Different studies have shown that acetylcholine production and expression of mAChRs in non-neuronal cells regulate important cell functions such as cell proliferation, migration, and differentiation (Albuquerque et al. 2009; K. Cheng et al. 2008; Karlin 2002; Paleari et al. 2008; Wessler et al. 1999; Yu et al. 2017). Moreover, these receptors have been detected in different tumor cells derived from the lung, colon, and brain, and their activation plays a role in cancer progression (Song et al. 2007; Ukegawa et al. 2003; Zuchner et al. 2006). Receptors such as M_3 mAChR have been shown to play an important role in the proliferation, differentiation, and apoptosis of leukemic cells (Jin et al. 2016). Apoptosis destroys damaged cells, preserving the integrity of normal tissues and organs in response to DNA damage, cellular stress, or oncogene expression, thereby blocking cell proliferation and tumor growth (Croce 2008; Shirjang et al. 2019; Slee et al. 1999; Tabas & Ron 2011; Verbrugge et al. 2010). In intrinsic (or mitochondrial) apoptotic pathway, DNA damage is triggered by a variety of intracellular stimuli, including cytotoxic drug therapy, growth factor deprivation, and/or oxidative stress. Several Bcl-2 family members, including Bax (Bcl-2-associated X-protein), Bak, Bcl-2, Bcl-xL, Mcl-1, Bid, and Bim, cytochrome c (Cyt C) release by regulating mitochondrial membrane permeability. Bcl-2 (anti-apoptotic) suppresses cell death, and Bax (pro-apoptotic) induces apoptosis. Pro-apoptotic factors such as Cyt C leak from mitochondria through these pores and play an important role in inducing apoptosis (Celepli 2020). Cytochrome C is a multifunctional enzyme located on the inner surface of mitochondria and is an important mediator and biomarker in the mitochondrial apoptotic pathway (Renz et al. 2001). The human chronic myeloid leukemia cell line (K562) was developed by Lozzio et al. in 1975 in pleural fluid in the blastic crisis phase of a chronic myeloid leukemia patient (Lozzio & Lozzio 1975). K562 cell line represents the early differentiation phase of the granulocyte line. Our previous studies have shown that M_2, M_3, and M_4 muscarinic acetylcholine receptors are functionally expressed in K562 cells, which are human chronic myeloid leukemia cells (Aydin et al. 2013; Cabadak et al. 2011; Cabadak et al. 2009). We also demonstrated that muscarinic acetylcholine receptors are functional in these cells by detecting changes

in different signaling pathways such as PKC, NO, cAMP, and Ca^{+2} via muscarinic receptors (Aydin et al. 2013; Cabadak et al. 2011, 2009; Kanli et al. 2019). Onder et al. showed that K562 cells express the alpha-7 nicotinic receptor. They also showed the effects of cholinergic agonist ACh and/or atropine (ATR), nicotinic antagonist, MLA on cell proliferation, and intracellular Ca^{2+} level (Onder Narin et al. 2021). Recent studies indicate that AChE is also a promising tumor suppressor (Xi et al. 2015). Our previous studies showed that the M_3 mAChR receptor was actively expressed, and AChE activity increased in response to ACh in K562 cells; as a result, K562 cells may be affected by pilocarpine that is M_3 agonist. Tumor necrosis factor alpha (TNFα) is a multifunctional and multicellular cytokine. It plays an important role in apoptosis and cell survival, as well as inflammation and immunity (Goukassian et al. 2007; Limb et al. 1996; Maddahi et al. 2011). TNFα has been shown to have antitumor properties as well as a broad spectrum in different diseases (van Horssen et al. 2006). TNFα is produced by a variety of cells, including macrophages, lymphoid cells, mast cells, endothelial cells, fibroblasts, and neuronal tissue (Bremer 2013; Maddahi et al. 2011; Micheau & Tschopp 2003). Acetylcholine also mediates the release of interleukin-8 (CXCL8). It is synthesized by peripheral blood mononuclear cells, fibroblasts, epithelial cells, endothelial cells, tumor cells, and keratinocytes. The CXCL8/IL-8 chemokine family and its receptors are involved in inflammatory diseases (Russo et al. 2014). Chemokines such as epidermal growth factor (EGF), IL-8, IL-12, IL-6, and IL-1 can stimulate tumor cells to produce a variety of inflammatory factors, chemoattractors such as monocytes, neutrophils, or lymphocytes tumor cells, and leukocytes in the circulation of tumor tissues in the inflammatory environment can contribute to its regulation (Huang et al. 2014; Liu et al. 2008; Wong et al. 2009). On the other hand, activated cancer cells secrete inflammatory mediators secondarily in the regulation of tumor progression (Huang et al. 2014; Shirabe et al. 2012). The IL-8 protein secreted by tumor cells promotes tumor migration, invasion, and angiogenesis (Huang et al. 2014).

We aimed to demonstrate the roles of pilocarpine in K562 cell proliferation and apoptosis. We also compared the effects of pilocarpine, 4DAMP, and TNFα, on IL-8 release (CXCL8), and AChE activity in K562 cells. Molecular mechanisms and targeted cellular signaling pathways involved in cell proliferation and apoptosis were investigated at protein levels.

Material and methods

Cell lines, cell culture, and reagents

Human erythroleukemia K562 cell lines were purchased from the American Type Culture Collection (ATCC), Manassas, VA, USA, obtained in the form of a frozen cell line CCL-243. Cells were cultivated in an incubator under standard conditions (37 °C, 5% CO_2) in RPMI-1640 medium (Life Technologies, Darmstadt, Germany), containing 10% fetal bovine serum and 100 U/ml penicillin and 100 µg/ml streptomycin (all Biochrom, Berlin, Germany). The antibodies were purchased from Santa Cruz Biotechnology Inc. (Santa Cruz, CA, USA). Pilocarpine, TNFα, and 4-diphenylacetoxy-N-methyl-piperidine (4-DAMP) were purchased from Sigma-Aldrich (Schnelldorf, Germany).

Cell proliferation assay

K562 cells (1×10^6) were seeded into 96-well plates containing RPMI-1640 medium to serum-free conditions. After 24 h, these "starved cells" were placed into a medium containing 1% FBS. The cells were grown in RPMI 1640 medium supplemented with 2 mM L-glutamine, 1% heat-inactivated FBS, 100 U/mL penicillin, and 100 mg/mL streptomycin, at 37 °C under a humidified condition of 5% CO_2. K562 cells were exposed to pilocarpine in the presence or absence of the M_3 muscarinic selective antagonists 4DAMP. 4DAMP were treated 30 min before pilocarpine, TNFα, and EGF were added to the culture medium. Cells were collected, and cell proliferation was assessed by BrdU (5-bromo-2′-deoxyuridine; Roche, Mannheim, Germany) assays, respectively, according to the manufacturer's protocols. Samples at a wavelength of 370 nm were read on a BioTek Synergy H1 unit (Synergy H1, BioTek, Winooski, VT, USA). Cell viability and proliferation were also evaluated by the trypan blue exclusion test and cell counter (TC-20 BioRad, Hercules, CA, USA).

Measurement of CXCL8 release

CXCL8 release into the supernatants was measured using a Millipore Human IL-8 ELISA kit in accordance with the manufacturer's instructions.

Measurement of AChE activity

AChE activity of human erythroleukemia cells was determined by the Choline/Acetylcholine Assay kit (DACE-100) after the cells were incubated with TNFα, agonists, and antagonists for 24 h.

Western blotting

Caspase 3, 8, and 9, bcl, bax, cyt C, and M_3 muscarinic receptor proteins were evaluated by semiquantitative Western blotting. K562 cells were treated with drugs for 24 h in a medium with 1% serum. Cells were harvested and washed in PBS for 15 min at 400 g, and the pellet was frozen at −80 °C. Protein level detection

in whole lysates and Western blot analyses described our previous studies with minör modification (21). Band intensities were quantified by optical density using the free edition of ImageJ software. β-actin was used as reference proteins (loading control) in each blot. The apparent molecular weights of M_3 mAChR, Caspase 3, 8, and 9, bcl, bax, cyt C, and β-actin are 75 kDa, 20 kDa, 55 kDa, 46 kDa, 26 kDa, 23 kDa, 15 kDa, and 47 kDa, respectively.

Statistical analysis

All figures show the standard error of the mean (±SEM) of at least four/six times independent experiments. Statistically significant differences were determined by using the one-way analysis of variance followed by Dunnett's posttests. All statistical tests were performed with the Prism program (Graphpad Prism 6), and ($P<0.05$) was considered significant.

Results

The change in the proliferation of human erythroleukemia cell proliferation treated with mitogens

Mitogenic roles of FBS, TNFα, EGF, and pilocarpine were detected in K562 cells. K562 cells were treated for 24, 48, and 72 h to determine their mitogenic effects in the presence of 0%, 1%, and 10% FBS, EGF, and TNFα. Cell proliferation curves were shown in Fig. 1.

Serum-free conditions (%0 FBS) proliferation of K562 was increased time-dependent manner being significant at 24, 48, and 72 h. Mitogens increased K562 proliferation at 24, 48, and 72 h. The mitogenic effectiveness at 48 h was the following: 10% FBS > 5% FBS > EGF (10 ng/mL) > TNFα (1 ng/ ml). When the 0% FBS vs other groups were compared, the increase in the number of cells was found significant (****$p<0.0001$). In the cells treated with epidermal growth factor for 24 h, the number of cells increased significantly compared to cells without FBS. But K562 cells treated with epidermal growth factor for 72 h decreased compared to cells without FBS. A significant difference was found between both the EGF and TNFα groups compared to 10% FBS (****$p<0.0001$). FBS is the best mitogen for K562 cells. The pro-inflammatory cytokine, TNFα, significantly decreased compared to cells with 10% FBS at 48 h.

The effect of FBS, EGF, TNFα, agonist, and antagonists in human erythroleukemia cell proliferation

The effect of FBS, EGF, TNFα, and agonists in human chronic myeloid leukemia cell proliferation is shown in Fig. 2.

TNFα and pilocarpine caused inhibition compared to 0% FBS **$p<0.005$ and **$p<0.05$. K562 cell proliferation was significantly inhibited by pilocarpine and TNF alpha in FBS-free conditions. Pilocarpine caused decreased cell proliferation compared to the FBS-free

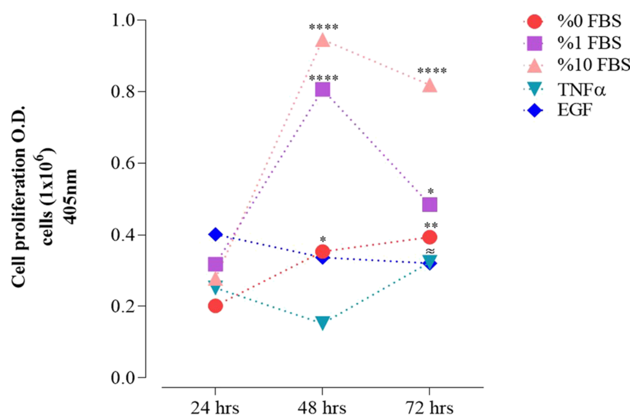


Fig. 1 Effects of FBS, EGF, and TNFα on K562 cell proliferation time course. K562 were cultured for 24, 48, and 72 h in a medium without FBS (%0 FBS; ●), in the presence of 1% FBS (■), 10% FBS (▲), EGF 10 ng/mL (◆), TNFα 1 ng/mL (▼). Data is the mean ± SEM of $n=6$ experiments for triplicate. Proliferative responses of each mitogen were significant compared to 0% FBS (basal) ****$p<0.0001$,**$p<0.04$, *$p<0.05$, ≈$p<0.019$

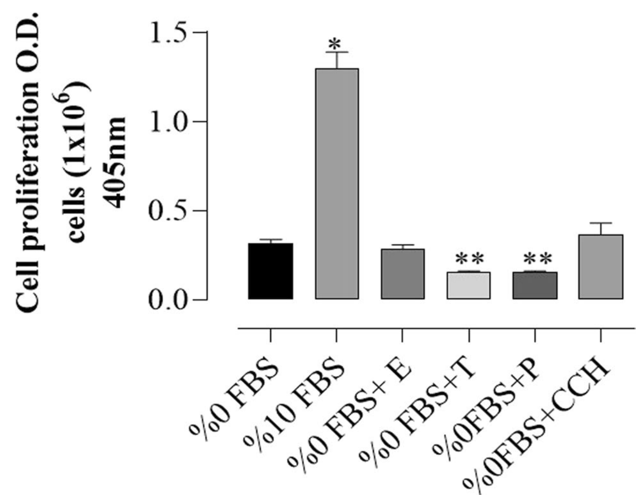


Fig. 2 Effects of mitogens and cholinergic agonists on K562 cell proliferation. K562 were cultured in the presence of RPMI-1640 medium (%0 FBS, 10% FBS, EGF (10 ng/mL), TNFα (1 ng /mL, pilocarpine 100 μM, and CCh 100 μM). Data is the mean ± SEM of $n=6$ experiments for triplicate. The proliferation responses of each mitogen concentration compared to %0 FBS were significant **$p<0.005$; *$p<0.05$

group (%0 FBS), ***$p < 0.0004$, and also, similar effects were seen on the (%1 FBS) plus pilocarpine group compared to the (%1 FBS) group (****$p < 0.0001$) (Fig. 3a). Cell proliferation increased by 1% FBS compared to the %0 FBS group (**$p < 0.001$). A total of 1% serum and pilocarpine group and 1% serum + 4DAMP + pilocarpine group caused inhibition compared to the 1% serum group (*$p < 0.001$) (Fig. 3a).

The effect of TNFα, M_3 muscarinic receptor agonists, and M_3 muscarinic receptor antagonists on acetylcholine esterase (AChE) activities in human chronic myeloid leukemia cells

The AChE activity levels have been found to be increased in response to pilocarpine treatment in human chronic myeloid cells (Fig. 3b).

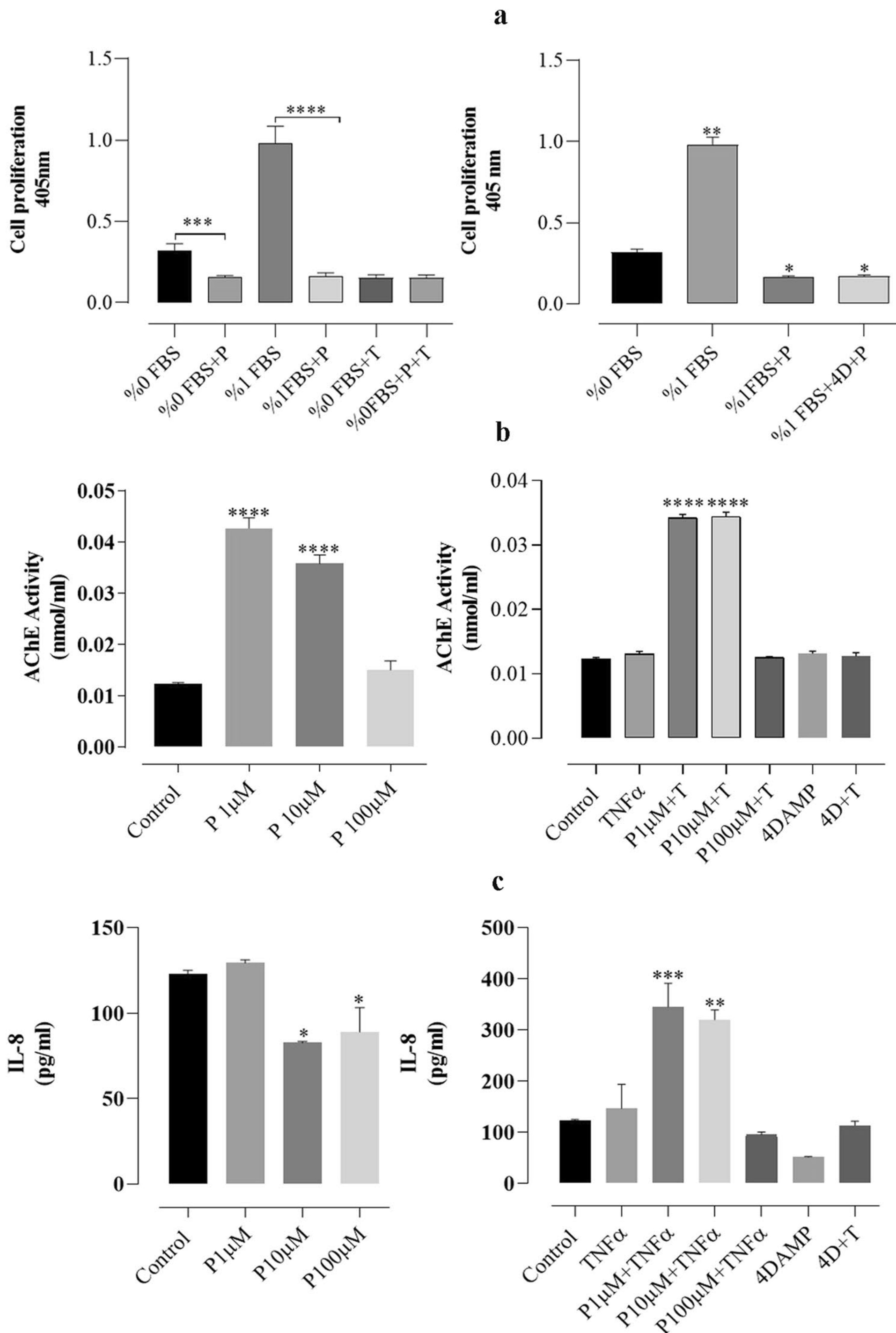


Fig. 3 The effects of pilocarpine and mitogens (FBS, TNFα) on K562 cell proliferation. **a** K562 cells were cultured in RPMI-1640 medium %0 FBS, pilocarpine 100 µM, 1% FBS, serum-free TNFα 1 ng/mL, serum-free pilocarpine + TNFα during 48 h. Each mitogen responses compared to %0 FBS were found significant ***$p < 0.0004$ and ****$p < 0.0001$. **b** The effects of pilocarpine in K562 cell proliferation. K562 cells were cultured in RPMI-1640%0 FBS, %1 FBS, pilocarpine 100 µM, and 4DAMP 10 µM during 48 h. %1 FBS were significant compared to %0 FBS group **$p < 0.001$. Pilocarpine 100 µM, pilocarpine 100 µM, and 4DAMP compared to %1 FBS were found significant *$p < 0.05$. Data is the mean ± SEM of $n = 6$ experiments for triplicate. **b** The effect of pilocarpine and other mitogens on acetylcholine esterase (AChE) activity in human erythroleukemia cells. The bars represent the mean of 4 experiments (****$p < 0.0001$) different from control groups. Pilocarpine 1 µM, 10 µM, and 100 µM. TNFα 1 ng/mL, 4DAMP 10 µM. **c** The effect of pilocarpine, TNFα 1 ng/mL, and/or 4DAMP 10 µM combinations treatment on the on IL-8 level. The bars represent the mean of 6 experiments. *$p < 0.006$; **$p < 0.0001$, ***$p < 0.0001$ significantly different from control. Pilocarpine 1 µM, 10 µM, and 100 µM

It is a new finding that pilocarpine exhibits antiproliferative effects in the presence of 1% and 0% FBS. Antiproliferative effects of pilocarpine were not reversed by 4DAMP (M_3 AChR antagonist).

The AChE activity levels have been found to be increased in response to pilocarpine treatment in human chronic myeloid cells (Fig. 3b).

The AChE levels increased compared to the control (****$p < 0.0001$), but only the pilocarpine 100 μM group was found to be not significantly different from that of the controls. A significant increase in the AChE levels was found in the 1 μM and 10 μM concentrations of pilocarpine and the presence of TNFα compared to the control groups (****$p < 0.0001$).

Effect of TNFα, agonists, and antagonists on interleukin-8 (IL-8) activity in human erythroleukemia cells

Incubation of K562 cells with two pilocarpine concentrations caused inhibition of IL-8 release. The mean increases in IL-8 release caused by a combination of pilocarpine and TNF were about three fold higher than the control (Fig. 3c).

When 1 ng/mL TNFα was added to the 1 μM and 10 μM pilocarpine groups, an increase in IL-8 levels was determined compared to the control groups (***$p < 0.0001$; **$p < 0{,}0004$).

M_3 muscarinic receptor expression in human chronic myeloid leukemia cells

The effect of TNFα, pilocarpine, and/or 4DAMP on M_3 mAChR expression in K562 cells is shown in Fig. 4.

Cells were treated with pilocarpine, TNFα, and/or 4DAMP. M_3 mAChR expression increased in cells co-stimulated with 4DAMP and pilocarpine compared to the control. The increase in M_3 mAChR expression with TNFα and pilocarpine was statistically significant (*$p < 0.01$) compared to pilocarpine. A significant increase in M_3 mAChR expression was observed when 4DAMP + pilocarpine was compared to the control (****$p < 0.0001$). A significant increase in M_3 mAChR expression was found when 4DAMP + pilocarpine was compared to 4DAMP (***$p < 0.0001$).

Effects of muscarinic M_3R agonist, antagonist, and TNFα on Bax/Bcl-2, cytocrome C, and Caspase 3, 8, and 9 expression in human chronic myeloid leukemia cells

A total of 1 μM and 10 μM pilocarpine increased Bax/Bcl-2 ratio (Fig. 5a). Increase in Bax/Bcl-2 ratio (more than twofold) was revealed after 24-h incubation. PD153035 is an EGFR inhibitor that prevents epidermal growth factor receptor activation. When compared to control levels, PD153035 caused a significantly increase in Bax/Bcl-2 ratio (**$p < 0.001$). Our

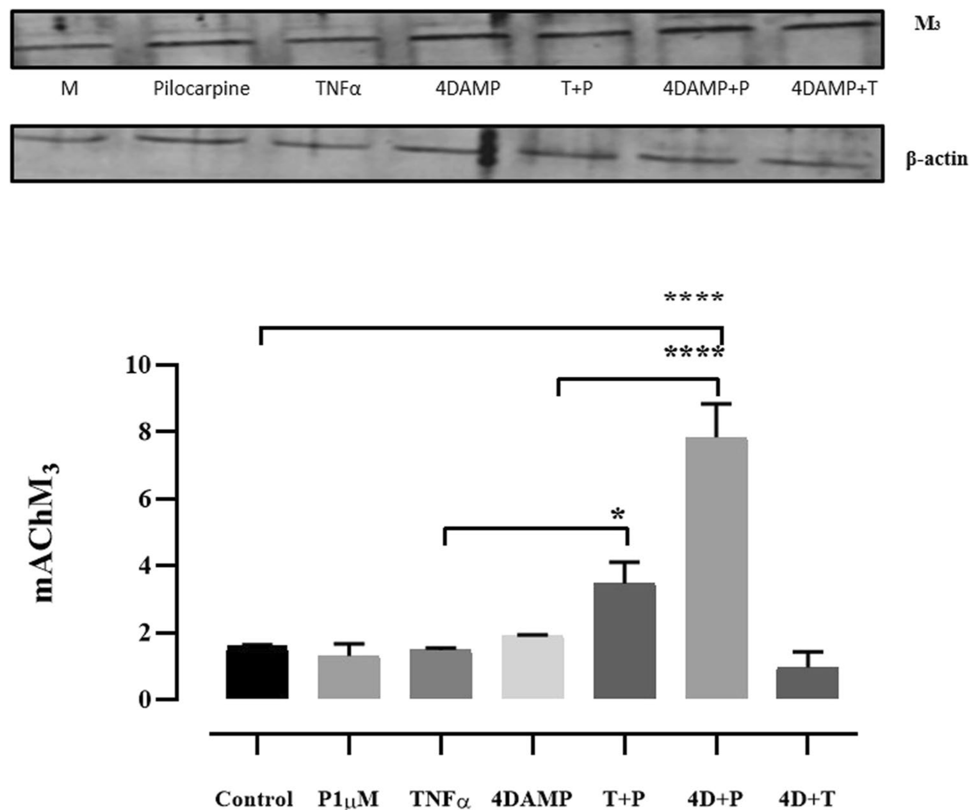


Fig. 4 Effects of pilocarpine on muscarinic M_3 receptor expression in K562 cells. **a** Western blotting. The representative images of nitrocellulose membranes were obtained from Western blotting analyses showing protein expressions of M_3 expression in the K562 cells. The estimated molecular weights were M_3 75 kDa and beta-actin 47 kDa. **b** Densitometric analysis of results is averaged from 4 experiments and shown as (± SEM) (****$p < 0.0001$, (*$p < 0.01$)

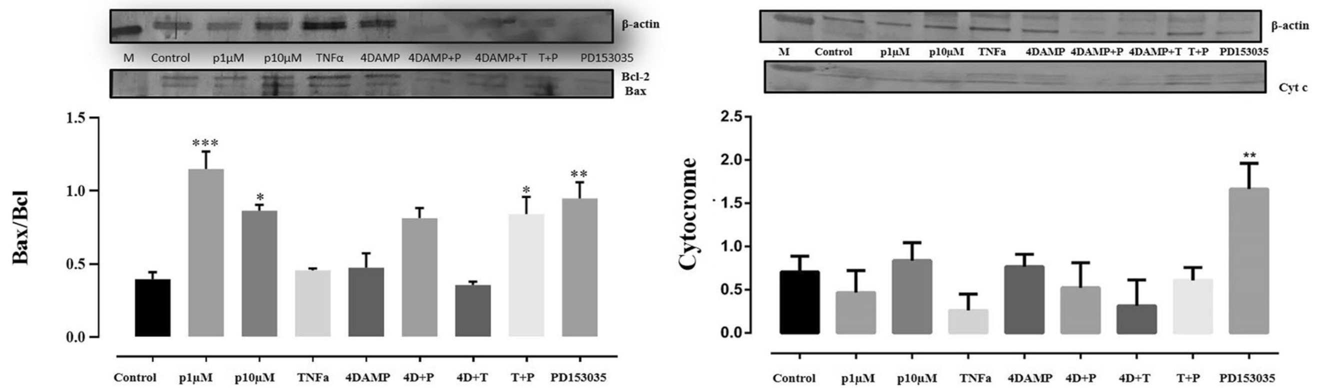


Fig. 5 **A** Effects of muscarinic M_3R agonist, antagonist, and TNFα on Bax/Bcl-2 expression in K562 cells. K562 cells were pre-treated with 4DAMP for 30 min before the addition of pilocarpine, TNFα, and PD153035 as indicated. Levels of Bax and Bcl-2 were determined by Western blot analysis as described in "Material and methods." The estimated molecular weights were Bax 23 kDa, Bcl-2 26 kDa, and beta-actin 47 kDa. The upper panel shows a the representative images of blot. Blots were analyzed by densitometry, and the values are expressed as the means ± SEM of three independent experiments. Pilocarpine 1 μM; 10 μM $***p < 0.0001$;$*p < 0.01$, significantly different from the control; TNFα + P vs control $*p < 0.01$; PD153035 vs control group $**p < 0.001$). **B** Effects of muscarinic M_3R agonist, antagonist on, and 1 ng/mL TNFα on cytocrome c expression. The estimated molecular weights were Cyt C 15 kDa and beta-actin 47 kDa. PD153035 $**p < 0.0001$ vs control group

results showed that PD153035 increased Bax/Bcl-2 ratio, and 4DAMP reversed pilocarpine effects on Bax/Bcl-2 ratio in K562 cells. A high Bax/Bcl-2 ratio may be characteristic of apoptotic cells. Cytocrome C expression increased with PD53035 compared to control ($**p < 0.0001$) (Fig. 5b).

Imbalances in the ratios of proapoptotic (Bax) and antiapoptotic (Bcl 2) Bcl-2 family members may predispose to cell death with pilocarpine.

Caspase 3, 8, and 9 were detected by Western blotting in K562 cells. The cells were treated with pilocarpine, 4DAMP, TNFα, and PD153035 for 24 h. Caspase 3, 8, and 9 expressions were demonstrated after drug treatment compared to the untreated control cells (Fig. 6).

Depending on the cell type and conditions, caspase-8 can potentiate or suppress tumor malignancy. Researchers have developed some drugs that can increase Caspase-8 expression. EGF (10 ng/mL) has mitogenic effects on K562 cells.

In this study, an increase in Bax/Bcl-2 ratio and caspase-8 and -9 expressions was significantly increased with 1 μM pilocarpine compared to the control ($p < 0.001$). A total of 1 ng/mL TNFα has been shown to cause increased caspase-3 expression levels but decreased caspase-9 expression levels compared to the control.

Discussion

The canonical downstream signaling M_1, M_3, and M_5 muscarinic acetylcholine receptors stimulate phospholipase A2, phospholipase D, and tyrosine kinase, as well as a calcium channel; M_2 and M_4 acetylcholine receptors are coupled to G proteins of Gi/o class. Other signaling pathways activation cause M_2 and M_4 receptors also can activate phospholipase A2 (Caulfield & Birdsall 1998; Felder 1995; Hulme et al. 1990). Muscarinic acetylcholine receptors interact with multiple effectors in signal transduction pathways (Felder 1995). An increase in rat ASMC (atypical smooth muscle cell) cell proliferation at 24, 48, and 72 h at 5–10% 10 ng/mL TNFα concentrations was determined over time. At 72 h, mitogenic activity was found 10% FBS > 5% FBS = EGF 10 ng/mL = TNFα 1 ng/mL. TNFα in rat ASMC cells was found to increase cell proliferation and also trigger apoptosis. They confirmed that FBS exhibited the best mitogenic effect in these cells. Accordingly, they suggested that TNFα have a weak mitogenic effect. EGF has been reported to stimulate the growth and proliferation of ASMC cells as a mitogen (Placeres-Uray et al. 2013). In our present study, 1% and 10% FBS has a mitogenic effect in K562 cells at 24, 48, and 72 h. At 72 h, mitogenic activity was found high level 10% FBS > 1% FBS, but EGF 10 ng/mL = TNFα 1 ng/mL low-level increased cell proliferation as well as weak mitogenic effects. EGF has the best mitogenic effect at 24 h in K562 cells. TNFα caused the lowest effect on cell proliferation at 48th than other agents. Pilocarpine has a role in K562 cell proliferation: (1) inhibitory effect by itself and (2) synergistic inhibitory effect at a concentration of mitogens TNFα 1 ng/mL in the presence and absence of 1% FBS. In previous studies in K562 cell proliferation, the inhibitory effect of carbachol at 24 h was shown to be reversed with atropine and 4DAMP (Cabadak et al. 2011). For the first time in this study, pilocarpine was shown to cause inhibition of cell proliferation in the presence and absence of 1% FBS. This effect could not be reversed with 4DAMP. Pilocarpine

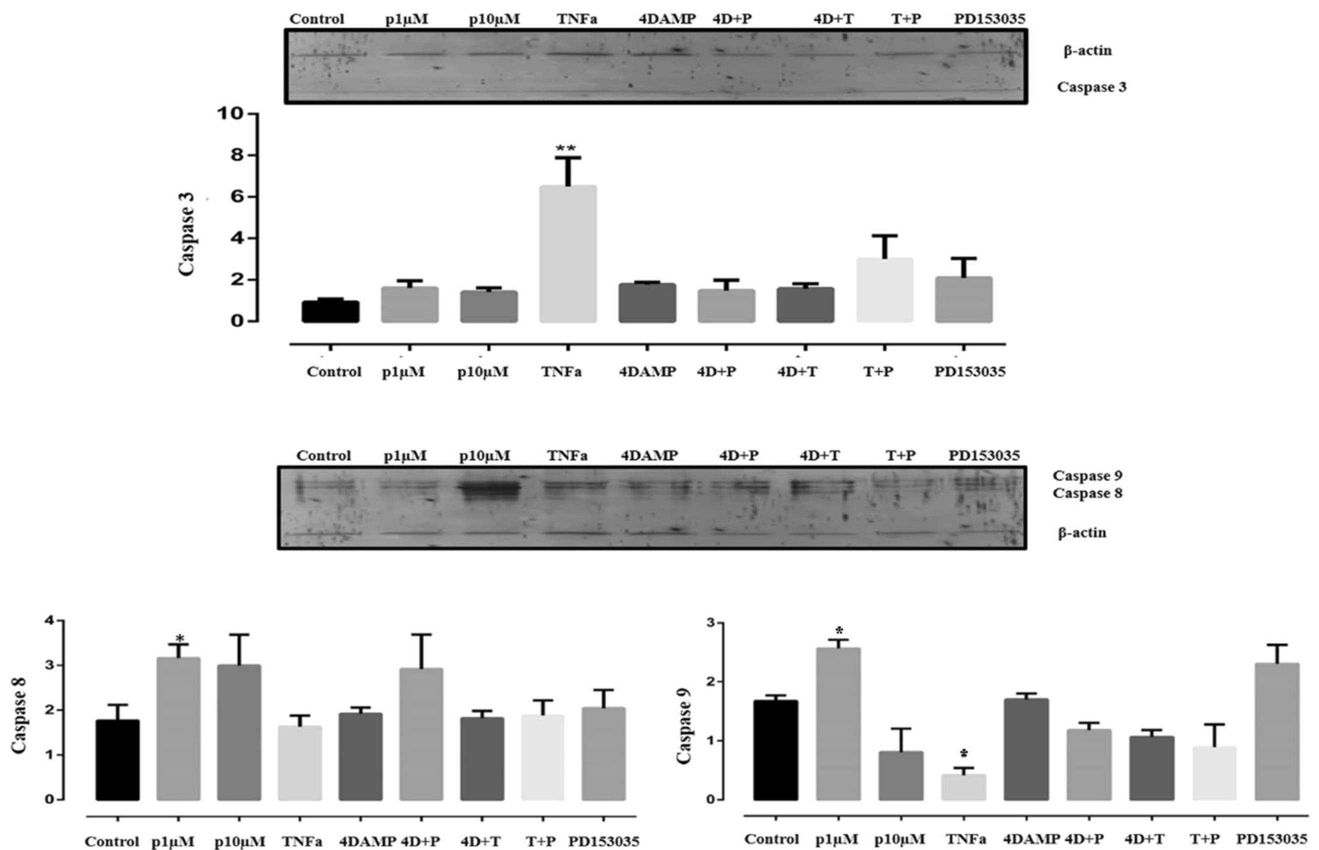


Fig. 6 Effects of muscarinic M_3R agonist, antagonist on, and TNFα on caspase 3, 8, and 9 expression in K562 cells. K562 cells were pre-treated with 4DAMP for 30 min before the addition of pilocarpine, TNFα, PD153035 as indicated. Levels of caspase 3, 8, and 9 were determined by Western blot analysis as described in "Material and methods." The estimated molecular weights were Caspase 3: 32 kDa, Caspase 8: 55 kDa, Caspase 9: 46 kDa, and beta-actin: 47 kDa. The upper panel shows the representative images of blot. Blots were analyzed by densitometry, and the values are expressed as the means ± SEM of three independent experiments. **A** $**p < 0.003$, significantly different from the control. **B** $*p < 0.001$ significantly different from the control. **C** $*\ p < 0.001$ significantly different from the control

inhibited cell proliferation at 48 h in K562 cells. TNFα and pilocarpine had a similar effect on cell proliferation. It was determined that the rat ASMC cell proliferation had the same mitogenic effect as carbachol 5% FBS in 24 h, and 5 ng EGF had a similar effect. It has been shown that in the presence of 10% FBS in these cells, carbachol causes dose-dependent inhibition. Placeres-Uray et al. determined that this reduction was due to cell death. AF-DX-116 (M_2 antagonist) and 4DAMP were found to be more effective in reversing the inhibitory effect of carbachol in the presence of 10% FBS in rat airway smooth muscle cells. Their data showed that M_2 receptors are more effective (Placeres-Uray et al. 2013). Xu et al. showed that pilocarpine has been found to have synergistic effects in TNFα and CXCL8 (Xu et al. 2013). In the 16-HBE cells, the cigarette extract not only improved the increase in the expression of M_2 and M_3 stimulated by TNFα but also had an effect at a hundred times lower concentration of acetylcholine. Studies have shown that M_3 mAChR is widely expressed in gastrointestinal tumors and may play an important role in cancer proliferation, differentiation, and progression (Frucht et al. 1999; Y. S. Park & Cho 2008; Raufman et al. 2008; Ukegawa et al. 2003; Wegener et al. 2004). Feng et al. found that pilocarpine changes the proliferation and migrations of cholangiocarcinoma cells, increasing the expression of M_3-mAChR in cholangiocarcinoma (Y. Feng et al. 2018; Y. J. Feng et al. 2012). According to our study pilocarpine, TNFα, and 4DAMP showed any change in M_3 protein expression level; while pilocarpine and 4DAMP were administered together, a significant increase in M_3 mAChR protein expression was determined compared to the control. In different cancer cells, pilocarpine has been found to inhibit cell proliferation (Xu et al. 2013). In many studies, acetylcholine has been shown to be expressed in various tumors, including lung cancer, and common malignant tumors in various organs (Y. J. Feng et al. 2012; Song et al. 2003; Trombino et al. 2004). Reina et al. reported that pilocarpine triggered apoptosis in human skin fibroblast cells.

Pilocarpine accelerated apoptosis by increasing the IP_3 level by activating M_1 and M_3 receptors in these cells (Reina et al. 2010). Studies in human alveolar epithelial cell line A549 cells have shown that M_3 receptor stimulation leads to ACh release and thus to an increase in TNFα-mediated CXCL8 secretion. TNFα caused an increase in the expression of M_2 and M_3 receptors and a decrease in the M_1 receptor in A549 cells (Y. J. Feng et al. 2012). It was determined that TNFα increased cell proliferation and also triggered apoptosis in rat ASMC cells. They confirmed that FBS showed the best mitogenic effect in these cells. Accordingly, they suggested that TNFα has a weak mitogenic effect and may regulate cell proliferation (Keslacy et al. 2007; Placeres-Uray et al. 2013; Stewart et al. 1995). Although the expression of M_2 and M_3 receptors increased in human alveolar epithelial cell line A549 cells, only 4DAMP caused a decrease in CXCL8 secretion with TNFα stimulation. In contrast, the selective antagonists for M_1 and M_2 receptors had no significant effects when concentrations of 0.1 µM methoctramine and 1 µM pirenzepine were applied (Gosens et al. 2009; Preiksaitis et al. 2000; Watson et al. 1999). In addition, pilocarpine-induced CXCL8 release associated with M_3 receptors was blocked by 4 DAMPs. In Bühling et al. in A549 cells, no significant response was observed in response to acetylcholine in CXCL8 release (Buhling et al. 2007). It was determined that pilocarpine-induced CXCL8 release was weak but reproducible and varied depending on the concentration in these cells. Pilocarpine was found to have a synergistic effect on TNFα and CXCL8 (Xu et al. 2013). Muscarinic stimulation with methacholine and synergistic effects of TNFα on CXCL8 release has been reported in human bronchial smooth muscle cells (Oenema et al. 2010). In 16-HBE cells, however, cigarette extract not only promoted the increase in TNFα-induced M_2 and M_3 expression but also showed an effect at a 100-fold lower concentration of acetylcholine. They show that blocking M_3 receptors with 4DAMP alone or with nonspecific muscarinic blockade reduced TNFα-stimulated IL-8 release (Profita et al. 2011).

In our study, 10 µM and 100 µM concentrations of pilocarpine caused a decrease in IL-8 levels. When 1 and 10 µM concentrations of pilocarpine were applied together with TNFα, it caused an increase in IL-8 release. Pilocarpine slightly inhibited IL-8 release, while 4DAMP fully inhibited it. Our results showed that blocking M_3 receptors with 4DAMP alone reduced IL-8 release, but TNFα partly reversed these effects in K562 cells. 4DAMP may have a role in regulating the release of factors such as IL-8, which has chemotactic potential. In K562 cells, pilocarpine may also reduce cell invasion by reducing IL-8 release. Our results also showed that the increase of AChE level by pilocarpine is mediated by the M_3 muscarinic receptor in K562 cells. Moreover, pilocarpine overstimulation of AChE in K562 cells can inhibit critical proliferation pathways. We know that AChE is also a promising tumor suppressor. Our results show that pilocarpine and TNF together contribute to the inhibition of K562 cell proliferation by increasing the AChE level. Different studies suggested that the elevation of AChE activity could be a promising anticancer therapeutic. We observed that the effect of pilocarpine on M_3 expression increased with the addition of TNFα. Our study is the first to demonstrate the effect of TNFα on M_3 muscarinic receptor expression and M_3 receptor-mediated inhibition of K562 cell proliferation. Furthermore is the fact that the addition of 4DAMP alters the effects of pilocarpine on M_3R expression in K562 cells. Bcl-2 prevents pore formation and cytochrome C release by binding to members such as pro-apoptotic Bax (E. H. Cheng et al. 2001), while the increase in Bax expression eliminates tumor cells and induces cell death (Wei et al. 2001). Our results show that pilocarpine increased Bax/Bcl-2 ratio but did not significantly change the Cyt C expression level in K562 cells. It was determined that the Bax/Bcl-2 ratio increased in K562 cells both in cells to which pilocarpine was added and in cells incubated with TNF alpha and pilocarpine. Induction of apoptosis by pilocarpine was mediated by the downregulation of Bcl-2 and the upregulation of Bax. Prokop et al. reported that decreased Bax/Bcl-2 ratio was associated with relapse in ALL patients ($n = 14$) (Prokop et al. 2000). PD tyrosine kinase inhibitor increased Bax/Bcl ratio but decreased Cyt C levels in K562 cells. Yuan et al. suggest that the pilocarpine-induced human corneal stroma (HCS) cell apoptosis might be triggered via both a death receptor-mediated pathway and a mitochondrion-dependent pathway (Yuan et al. 2016). Reina et al. showed that activation of M_1 and M_3 muscarinic receptors by pilocarpine resulted in apoptosis of human fibroblast cells (Reina et al. 2010). Inhibition of M_3 has a suppressive role in K562 cell proliferation by affecting the intrinsic pathways of apoptosis. Cholinergic agents targeting apoptosis have shown potential in antiproliferative effects in chronic myeloid leukemia cells. Pilocarpine may be effective in inducing apoptosis in chronic myeloid leukemia cells by increasing the level of AChE. AChE exerts its pivotal role in apoptosis by its participation in the formation of the apoptosome (S. E. Park et al. 2004). Pilocarpine may be a promising treatment strategy to improve the treatment of chronic myeloid leukemia. This work reveals the essential role of the M_3 receptor agonist, pilocarpine, in K562 cells and suggests it may be an additional target for anti-chronic myeloid leukemia therapy. Determining the importance of the non-neuronal cholinergic system in leukemia has revealed the need to investigate the interaction of different signaling pathways. According to the results of the study, the role of M_3 receptor-mediated signaling pathways in leukemia will be investigated in the future and may be important on leukemia treatment. The roles of muscarinic agonists in the antimitogenic signal transduction pathway may contribute to the development of new treatment combinations for chronic myeloid leukemia.

Acknowledgements This work has been supported by Marmara University Scientific Research Projects Coordination Unit.

Author contribution HC conceived and designed research. Material preparation, data collection, and analysis were performed by Zehra Kanlı and Banu Aydın. The first draft of the manuscript was written by Hulya Cabadak, and all authors commented on previous versions of the manuscript. All authors read and HC approved the final manuscript.

Funding This work was supported by Marmara University Scientific Research Projects Coordination Unit (Istanbul/Turkey) under grant number SAG-C-YLP-080415–0101, 8.04. to Hulya Cabadak.

Data availability The data that support the findings of this study are available from the corresponding author upon reasonable request.

Declarations

Competing interests The authors declare no competing interests.

Ethical approval No ethical approval is required.

Conflict of ınterest The authors declare no competing interests.

References

Albuquerque EX, Pereira EF, Alkondon M, Rogers SW (2009) Mammalian nicotinic acetylcholine receptors: from structure to function. Physiol Rev 89(1):73–120. https://doi.org/10.1152/physrev.00015.2008

Aydin B, Kan B, Cabadak H (2013) The role of intracellular pathways in the proliferation of human K562 cells mediated by muscarinic receptors. Leuk Res 37(9):1144–1149. https://doi.org/10.1016/j.leukres.2013.05.018

Bonner TI (1989) The molecular basis of muscarinic receptor diversity. Trends Neurosci 12(4):148–151. https://doi.org/10.1016/0166-2236(89)90054-4

Bonner TI, Buckley NJ, Young AC, Brann MR (1987) Identification of a family of muscarinic acetylcholine receptor genes. Science 237(4814):527–532. https://doi.org/10.1126/science.3037705

Breindel JL, Haskins JW, Cowell EP, Zhao M, Nguyen DX, Stern DF (2013) EGF receptor activates MET through MAPK to enhance non-small cell lung carcinoma invasion and brain metastasis. Cancer Res 73(16):5053–5065. https://doi.org/10.1158/0008-5472.CAN-12-3775

Bremer, E. (2013). Targeting of the tumor necrosis factor receptor superfamily for cancer immunotherapy.*ISRN Oncol,* 371854. https://doi.org/10.1155/2013/371854

Buhling F, Lieder N, Kuhlmann UC, Waldburg N, Welte T (2007) Tiotropium suppresses acetylcholine-induced release of chemotactic mediators in vitro. Respir Med 101(11):2386–2394. https://doi.org/10.1016/j.rmed.2007.06.009

Cabadak H, Aydin B, Kan B (2011) Regulation of M2, M3, and M4 muscarinic receptor expression in K562 chronic myelogenous leukemic cells by carbachol. J Recept Signal Transduct Res 31(1):26–32. https://doi.org/10.3109/10799893.2010.506484

Cabadak H, Cuadra AE, El-Fakahany EE, Kan B (2009) M2, M3, and M4 muscarinic receptors are expressed in the guinea pig gallbladder. J Recept Signal Transduct Res 29(1):63–66. https://doi.org/10.1080/10799890802687416

Carruthers SP, Gurvich CT, Rossell SL (2015) The muscarinic system, cognition and schizophrenia. Neurosci Biobehav Rev 55:393–402. https://doi.org/10.1016/j.neubiorev.2015.05.011

Caulfield, M. P., & Birdsall, N. J. (1998). International Union of Pharmacology. XVII. Classification of muscarinic acetylcholine receptors.*Pharmacol Rev, 50*(2), 279–290. Retrieved from https://www.ncbi.nlm.nih.gov/pubmed/9647869

Celepli, S., Bigat, İ., Celepli P., Karagin. P.H. (2020). '' Apoptoz ve Apoptotik Yolların Gözden Geçirilmesi''.*Güncel Gastroenteroloji Dergisi, Cilt 24 Sayı 3*(24/3–2020), Page; 103–111.

Cheng EH, Wei MC, Weiler S, Flavell RA, Mak TW, Lindsten T, Korsmeyer SJ (2001) BCL-2, BCL-X(L) sequester BH3 domain-only molecules preventing BAX- and BAK-mediated mitochondrial apoptosis. Mol Cell 8(3):705–711. https://doi.org/10.1016/s1097-2765(01)00320-3

Cheng K, Samimi R, Xie G, Shant J, Drachenberg C, Wade M, Raufman JP (2008) Acetylcholine release by human colon cancer cells mediates autocrine stimulation of cell proliferation. Am J Physiol Gastrointest Liver Physiol 295(3):G591-597. https://doi.org/10.1152/ajpgi.00055.2008

Cheng, K., Zimniak, P., & Raufman, J. P. (2003). Transactivation of the epidermal growth factor receptor mediates cholinergic agonist-induced proliferation of H508 human colon cancer cells. *Cancer Res, 63*(20), 6744–6750. Retrieved from https://www.ncbi.nlm.nih.gov/pubmed/14583469

Citri A, Yarden Y (2006) EGF-ERBB signalling: towards the systems level. Nat Rev Mol Cell Biol 7(7):505–516. https://doi.org/10.1038/nrm1962

Clader JW, Wang Y (2005) Muscarinic receptor agonists and antagonists in the treatment of Alzheimer's disease. Curr Pharm Des 11(26):3353–3361. https://doi.org/10.2174/138161205774370762

Croce CM (2008) Oncogenes and cancer. N Engl J Med 358(5):502–511. https://doi.org/10.1056/NEJMra072367

Eglen, R. M. (2012). Overview of muscarinic receptor subtypes. *Handb Exp Pharmacol*(208), 3–28. https://doi.org/10.1007/978-3-642-23274-9_1

Felder, C. C. (1995). Muscarinic acetylcholine receptors: signal transduction through multiple effectors. *FASEB J, 9*(8), 619–625. Retrieved from https://www.ncbi.nlm.nih.gov/pubmed/7768353

Felton J, Hu S, Raufman JP (2018) Targeting M3 muscarinic receptors for colon cancer therapy. Curr Mol Pharmacol 11(3):184–190. https://doi.org/10.2174/1874467211666180119115828

Feng Y, Hu X, Liu G, Lu L, Zhao W, Shen F, Zhang B (2018) M3 muscarinic acetylcholine receptors regulate epithelial-mesenchymal transition, perineural invasion, and migration/metastasis in cholangiocarcinoma through the AKT pathway. Cancer Cell Int 18:173. https://doi.org/10.1186/s12935-018-0667-z

Feng YJ, Zhang BY, Yao RY, Lu Y (2012) Muscarinic acetylcholine receptor M3 in proliferation and perineural invasion of cholangiocarcinoma cells. Hepatobiliary Pancreat Dis Int 11(4):418–423. https://doi.org/10.1016/s1499-3872(12)60201-x

Finot, F., Masson, R., Desmots, F., Ribault, C., Bichet, N., Vericat, J. A., Loyer, P. (2012). Combined stimulation with the tumor necrosis factor alpha and the epidermal growth factor promotes the proliferation of hepatocytes in rat liver cultured slices.*Int J Hepatol,* 785786. https://doi.org/10.1155/2012/785786

Frucht, H., Jensen, R. T., Dexter, D., Yang, W. L., & Xiao, Y. (1999). Human colon cancer cell proliferation mediated by the M3 muscarinic cholinergic receptor.*Clin Cancer Res, 5*(9), 2532–2539. Retrieved from https://www.ncbi.nlm.nih.gov/pubmed/10499630

Gosens R, Rieks D, Meurs H, Ninaber DK, Rabe KF, Nanninga J, Zuyderduyn S (2009) Muscarinic M3 receptor stimulation increases cigarette smoke-induced IL-8 secretion by human airway smooth muscle cells. Eur Respir J 34(6):1436–1443. https://doi.org/10.1183/09031936.00045209

Goukassian DA, Qin G, Dolan C, Murayama T, Silver M, Curry C, Losordo DW (2007) Tumor necrosis factor-alpha receptor p75 is required in ischemia-induced neovascularization. Circulation

115(6):752–762. https://doi.org/10.1161/CIRCULATIONAHA.106.647255

Huang P, Xu X, Wang L, Zhu B, Wang X, Xia J (2014) The role of EGF-EGFR signalling pathway in hepatocellular carcinoma inflammatory microenvironment. J Cell Mol Med 18(2):218–230. https://doi.org/10.1111/jcmm.12153

Hulme EC, Birdsall NJ, Buckley NJ (1990) Muscarinic receptor subtypes. Annu Rev Pharmacol Toxicol 30:633–673. https://doi.org/10.1146/annurev.pa.30.040190.003221

Jin, M. W., Xu, S. M., An, Q., & Wang, P. (2016). A review of risk factors for childhood leukemia.*Eur Rev Med Pharmacol Sci, 20*(18), 3760–3764. Retrieved from https://www.ncbi.nlm.nih.gov/pubmed/27735044

Kanli Z, Aydin B, Cabadak H (2019) Effects of cholinergic compounds and TNF-alpha on human erythroleukemia K562 cell proliferation and caspase expression. Marmara Medical Journal 32(1):20–26. https://doi.org/10.5472/marumj.518797

Karlin A (2002) Emerging structure of the nicotinic acetylcholine receptors. Nat Rev Neurosci 3(2):102–114. https://doi.org/10.1038/nrn731

Keslacy S, Tliba O, Baidouri H, Amrani Y (2007) Inhibition of tumor necrosis factor-alpha-inducible inflammatory genes by interferon-gamma is associated with altered nuclear factor-kappaB transactivation and enhanced histone deacetylase activity. Mol Pharmacol 71(2):609–618. https://doi.org/10.1124/mol.106.030171

Limb GA, Chignell AH, Green W, LeRoy F, Dumonde DC (1996) Distribution of TNF alpha and its reactive vascular adhesion molecules in fibrovascular membranes of proliferative diabetic retinopathy. Br J Ophthalmol 80(2):168–173. https://doi.org/10.1136/bjo.80.2.168

Liu H, Pan Z, Li A, Fu S, Lei Y, Sun H, Zhou W (2008) Roles of chemokine receptor 4 (CXCR4) and chemokine ligand 12 (CXCL12) in metastasis of hepatocellular carcinoma cells. Cell Mol Immunol 5(5):373–378. https://doi.org/10.1038/cmi.2008.46

Lozzio, C. B., & Lozzio, B. B. (1975). Human chronic myelogenous leukemia cell-line with positive Philadelphia chromosome.*Blood, 45*(3), 321–334. Retrieved from https://www.ncbi.nlm.nih.gov/pubmed/163658

Maddahi A, Kruse LS, Chen QW, Edvinsson L (2011) The role of tumor necrosis factor-alpha and TNF-alpha receptors in cerebral arteries following cerebral ischemia in rat. J Neuroinflammation 8:107. https://doi.org/10.1186/1742-2094-8-107

Masuda H, Zhang D, Bartholomeusz C, Doihara H, Hortobagyi GN, Ueno NT (2012) Role of epidermal growth factor receptor in breast cancer. Breast Cancer Res Treat 136(2):331–345. https://doi.org/10.1007/s10549-012-2289-9

Micheau O, Tschopp J (2003) Induction of TNF receptor I-mediated apoptosis via two sequential signaling complexes. Cell 114(2):181–190. https://doi.org/10.1016/s0092-8674(03)00521-x

Natarajan A, Wagner B, Sibilia M (2007) The EGF receptor is required for efficient liver regeneration. Proc Natl Acad Sci U S A 104(43):17081–17086. https://doi.org/10.1073/pnas.0704126104

Oenema TA, Kolahian S, Nanninga JE, Rieks D, Hiemstra PS, Zuyderduyn S, Gosens R (2010) Pro-inflammatory mechanisms of muscarinic receptor stimulation in airway smooth muscle. Respir Res 11:130. https://doi.org/10.1186/1465-9921-11-130

Onder Narin G, Aydin B, Cabadak H (2021) Studies on the role of alpha 7 nicotinic acetylcholine receptors in K562 cell proliferation and signaling. Mol Biol Rep 48(6):5045–5055. https://doi.org/10.1007/s11033-021-06498-4

Paleari L, Grozio A, Cesario A, Russo P (2008) The cholinergic system and cancer. Semin Cancer Biol 18(3):211–217. https://doi.org/10.1016/j.semcancer.2007.12.009

Park SE, Kim ND, Yoo YH (2004) Acetylcholinesterase plays a pivotal role in apoptosome formation. Cancer Res 64(8):2652–2655. https://doi.org/10.1158/0008-5472.can-04-0649

Park YS, Cho NJ (2008) Enhanced proliferation of SNU-407 human colon cancer cells by muscarinic acetylcholine receptors. BMB Rep 41(11):803–807. https://doi.org/10.5483/bmbrep.2008.41.11.803

Placeres-Uray FA, Febres-Aldana CA, Fernandez-Ruiz R, Gonzalez de Alfonzo R, Lippo de Becemberg IA, Alfonzo MJ (2013) M2 muscarinic acetylcholine receptor modulates rat airway smooth muscle cell proliferation. World Allergy Organ J 6(1):22. https://doi.org/10.1186/1939-4551-6-22

Preiksaitis, H. G., Krysiak, P. S., Chrones, T., Rajgopal, V., & Laurier, L. G. (2000). Pharmacological and molecular characterization of muscarinic receptor subtypes in human esophageal smooth muscle.*J Pharmacol Exp Ther, 295*(3), 879–888. Retrieved from https://www.ncbi.nlm.nih.gov/pubmed/11082420

Profita M, Bonanno A, Montalbano AM, Ferraro M, Siena L, Bruno A, Gjomarkaj M (2011) Cigarette smoke extract activates human bronchial epithelial cells affecting non-neuronal cholinergic system signalling in vitro. Life Sci 89(1–2):36–43. https://doi.org/10.1016/j.lfs.2011.04.025

Prokop A, Wieder T, Sturm I, Essmann F, Seeger K, Wuchter C, Daniel PT (2000) Relapse in childhood acute lymphoblastic leukemia is associated with a decrease of the Bax/Bcl-2 ratio and loss of spontaneous caspase-3 processing in vivo. Leukemia 14(9):1606–1613. https://doi.org/10.1038/sj.leu.2401866

Pronin AN, Wang Q, Slepak VZ (2017) Teaching an old drug new tricks: agonism, antagonism, and biased signaling of pilocarpine through M3 muscarinic acetylcholine receptor. Mol Pharmacol 92(5):601–612. https://doi.org/10.1124/mol.117.109678

Raufman JP, Samimi R, Shah N, Khurana S, Shant J, Drachenberg C, Cheng K (2008) Genetic ablation of M3 muscarinic receptors attenuates murine colon epithelial cell proliferation and neoplasia. Cancer Res 68(10):3573–3578. https://doi.org/10.1158/0008-5472.CAN-07-6810

Reina S, Sterin-Borda L, Passafaro D, Borda E (2010) Muscarinic cholinoceptor activation by pilocarpine triggers apoptosis in human skin fibroblast cells. J Cell Physiol 222(3):640–647. https://doi.org/10.1002/jcp.21981

Renz A, Berdel WE, Kreuter M, Belka C, Schulze-Osthoff K, Los M (2001) Rapid extracellular release of cytochrome c is specific for apoptosis and marks cell death in vivo. Blood 98(5):1542–1548. https://doi.org/10.1182/blood.v98.5.1542

Russo RC, Garcia CC, Teixeira MM, Amaral FA (2014) The CXCL8/IL-8 chemokine family and its receptors in inflammatory diseases. Expert Rev Clin Immunol 10(5):593–619. https://doi.org/10.1586/1744666X.2014.894886

Shirabe K, Mano Y, Muto J, Matono R, Motomura T, Toshima T, Maehara Y (2012) Role of tumor-associated macrophages in the progression of hepatocellular carcinoma. Surg Today 42(1):1–7. https://doi.org/10.1007/s00595-011-0058-8

Shirjang S, Mansoori B, Asghari S, Duijf PHG, Mohammadi A, Gjerstorff M, Baradaran B (2019) MicroRNAs in cancer cell death pathways: apoptosis and necroptosis. Free Radic Biol Med 139:1–15. https://doi.org/10.1016/j.freeradbiomed.2019.05.017

Sibilia M, Kroismayr R, Lichtenberger BM, Natarajan A, Hecking M, Holcmann M (2007) The epidermal growth factor receptor: from development to tumorigenesis. Differentiation 75(9):770–787. https://doi.org/10.1111/j.1432-0436.2007.00238.x

Slee EA, Harte MT, Kluck RM, Wolf BB, Casiano CA, Newmeyer DD, Martin SJ (1999) Ordering the cytochrome c-initiated caspase cascade: hierarchical activation of caspases-2, -3, -6, -7, -8, and -10 in a caspase-9-dependent manner. J Cell Biol 144(2):281–292. https://doi.org/10.1083/jcb.144.2.281

Song P, Sekhon HS, Lu A, Arredondo J, Sauer D, Gravett C, Spindel ER (2007) M3 muscarinic receptor antagonists inhibit small cell lung carcinoma growth and mitogen-activated protein kinase phosphorylation induced by acetylcholine secretion. Cancer Res 67(8):3936–3944. https://doi.org/10.1158/0008-5472.CAN-06-2484

Song P, Sekhon HS, Proskocil B, Blusztajn JK, Mark GP, Spindel ER (2003) Synthesis of acetylcholine by lung cancer. Life Sci 72(18–19):2159–2168. https://doi.org/10.1016/s0024-3205(03)00078-x

Stewart AG, Tomlinson PR, Fernandes DJ, Wilson JW, Harris T (1995) Tumor necrosis factor alpha modulates mitogenic responses of human cultured airway smooth muscle. Am J Respir Cell Mol Biol 12(1):110–119. https://doi.org/10.1165/ajrcmb.12.1.7529028

Tabas I, Ron D (2011) Integrating the mechanisms of apoptosis induced by endoplasmic reticulum stress. Nat Cell Biol 13(3):184–190. https://doi.org/10.1038/ncb0311-184

Trombino S, Cesario A, Margaritora S, Granone P, Motta G, Falugi C, Russo P (2004) Alpha7-nicotinic acetylcholine receptors affect growth regulation of human mesothelioma cells: role of mitogen-activated protein kinase pathway. Cancer Res 64(1):135–145. https://doi.org/10.1158/0008-5472.can-03-1672

Ukegawa JI, Takeuchi Y, Kusayanagi S, Mitamura K (2003) Growth-promoting effect of muscarinic acetylcholine receptors in colon cancer cells. J Cancer Res Clin Oncol 129(5):272–278. https://doi.org/10.1007/s00432-003-0433-y

van Horssen R, Ten Hagen TL, Eggermont AM (2006) TNF-alpha in cancer treatment: molecular insights, antitumor effects, and clinical utility. Oncologist 11(4):397–408. https://doi.org/10.1634/theoncologist.11-4-397

Verbrugge I, Johnstone RW, Smyth MJ (2010) SnapShot: extrinsic apoptosis pathways. Cell 143(7):1192–1192. https://doi.org/10.1016/j.cell.2010.12.004

Von Rosenvinge EC, Raufman JP (2011) Muscarinic receptor signaling in colon cancer. Cancers (basel) 3(1):971–981. https://doi.org/10.3390/cancers3010971

Watson N, Daniels DV, Ford AP, Eglen RM, Hegde SS (1999) Comparative pharmacology of recombinant human M3 and M5 muscarinic receptors expressed in CHO-K1 cells. Br J Pharmacol 127(2):590–596. https://doi.org/10.1038/sj.bjp.0702551

Wegener C, Hamasaka Y, Nassel DR (2004) Acetylcholine increases intracellular Ca2+ via nicotinic receptors in cultured PDF-containing clock neurons of Drosophila. J Neurophysiol 91(2):912–923. https://doi.org/10.1152/jn.00678.2003

Wei MC, Zong WX, Cheng EH, Lindsten T, Panoutsakopoulou V, Ross AJ, Korsmeyer SJ (2001) Proapoptotic BAX and BAK: a requisite gateway to mitochondrial dysfunction and death. Science 292(5517):727–730. https://doi.org/10.1126/science.1059108

Wess J (2003) Novel insights into muscarinic acetylcholine receptor function using gene targeting technology. Trends Pharmacol Sci 24(8):414–420. https://doi.org/10.1016/S0165-6147(03)00195-0

Wessler I, Kirkpatrick CJ, Racke K (1999) The cholinergic “pitfall”: acetylcholine, a universal cell molecule in biological systems, including humans. Clin Exp Pharmacol Physiol 26(3):198–205. https://doi.org/10.1046/j.1440-1681.1999.03016.x

Wong VW, Yu J, Cheng AS, Wong GL, Chan HY, Chu ES, Chan HL (2009) High serum interleukin-6 level predicts future hepatocellular carcinoma development in patients with chronic hepatitis B. Int J Cancer 124(12):2766–2770. https://doi.org/10.1002/ijc.24281

Xi HJ, Wu RP, Liu JJ, Zhang LJ, Li ZS (2015) Role of acetylcholinesterase in lung cancer. Thorac Cancer 6(4):390–398. https://doi.org/10.1111/1759-7714.12249

Xu ZP, Devillier P, Xu GN, Qi H, Zhu L, Zhou W, Cui YY (2013) TNF-alpha-induced CXCL8 production by A549 cells: involvement of the non-neuronal cholinergic system. Pharmacol Res 68(1):16–23. https://doi.org/10.1016/j.phrs.2012.10.016

Yu H, Xia H, Tang Q, Xu H, Wei G, Chen Y, Bi F (2017) Acetylcholine acts through M3 muscarinic receptor to activate the EGFR signaling and promotes gastric cancer cell proliferation. Sci Rep 7:40802. https://doi.org/10.1038/srep40802

Yuan XL, Wen Q, Zhang MY, Fan TJ (2016) Cytotoxicity of pilocarpine to human corneal stromal cells and its underlying cytotoxic mechanisms. Int J Ophthalmol 9(4):505–511. https://doi.org/10.18240/ijo.2016.04.05

Zhang Y, Wang L, Zhang M, Jin M, Bai C, Wang X (2012) Potential mechanism of interleukin-8 production from lung cancer cells: an involvement of EGF-EGFR-PI3K-Akt-Erk pathway. J Cell Physiol 227(1):35–43. https://doi.org/10.1002/jcp.22722

Zuchner T, Schliebe N, Schliebs R (2006) Zinc uptake is mediated by M1 muscarinic acetylcholine receptors in differentiated SK-SH-SY5Y cells. Int J Dev Neurosci 24(1):23–27. https://doi.org/10.1016/j.ijdevneu.2005.11.018

Publisher's note Springer Nature remains neutral with regard to jurisdictional claims in published maps and institutional affiliations.

Springer Nature or its licensor (e.g. a society or other partner) holds exclusive rights to this article under a publishing agreement with the author(s) or other rightsholder(s); author self-archiving of the accepted manuscript version of this article is solely governed by the terms of such publishing agreement and applicable law.