Introduction

It is well established that acetylcholine (ACh) acts as a neurotransmitter in the central and peripheral nervous systems and acts via activation of the nicotinic (nAChR) and muscarinic (mAChR) receptors. The mAChRs consist of five genetically distinct subtypes: M1, M2, M3, M4, and M5. Among these mAChRs, M1, M3, and M5 are coupled with the Gq type of G proteins that activate phospholipase C to commence the phosphatidylinositol triphosphate-signaling cascade. M2 and M4 mAChRs, on the other hand, are coupled with the Gi type of G proteins that inhibit adenylate cyclase activity (1).

It has recently been found that ACh is present in a variety of non-neuronal cells, such as keratinocytes (2), surface epithelium of bronchi, small and large intestine, gall bladder, vagina, skin, and pulmonary pleura (3). The presence of ACh in non-neuronal cells suggests that ACh acts not only as a neurotransmitter but also has other functions, such as activation of the immune system (4) and induction of cell proliferation and differentiation (5).

ACh is also found in cancer cells such as small cell lung carcinoma (SCLC) (6), non-small cell lung carcinoma (7), and colon cancer (8). In cell lines of these particular cancers, expression of mAChRs, nAChRs, choline acetyltransferase (ChAT) that synthesizes ACh, and acetylcholine esterase (AChE) that degrades ACh to

Full Paper

Selective M3 Muscarinic Receptor Antagonist Inhibits Small-Cell Lung Carcinoma Growth in a Mouse Orthotopic Xenograft Model

Nozomi Ami1,#1,* Kazumi Koga1,#2 Hiroshi Fushiki1,#3 Yoko Ueno1,#3 Yoshio Ogino1,#4 and Hisashi Ohta1,#5

1Department of Pharmacology, Tsukuba Research Institute, Banyu Pharmaceutical Co., Ltd., 3 Ohkubo, Tsukuba-shi, Ibaraki 300-2611, Japan

Received December 1, 2010; Accepted March 10, 2011

Abstract. In small cell lung carcinoma (SCLC), acetylcholine (ACh) is synthesized and secreted, and it acts as an autocrine growth factor through activation of its receptors, muscarinic receptor (mAChR) and nicotinic receptor (nAChR). Alteration of tumor growth by blockade of M3 mAChR in a human SCLC cell line, NCI-H82, was investigated in the present study. We used a highly selective M3 muscarinic antagonist, N-2-[3-[3R]-1-(cyclohexylmethyl)-3-piperidinyl]methylamino-2-oxoethyl)-3,3,3-triphenyl-propioamide (J-115311). Our results show that J-115311 inhibited the increased intracellular calcium elicited by carbachol, a muscarinic agonist, in SCLC cells. J-115311 also inhibited SCLC cell growth in vitro. In a mouse orthotopic xenograft model, J-115311 dose-dependently reduced tumor growth when NCI-H82 cells were inoculated into the upper left lobe of the lung. These findings indicate that blockade of M3 mAChR can suppress tumor growth in SCLC, suggesting the potential therapeutic utility of M3 muscarinic antagonists as anti-cancer agents.

Keywords: selective M3 muscarinic antagonist, M3 muscarinic receptor, small cell lung carcinoma, orthotopic xenograft model, non-neuronal cholinergic system
choline and acetic acid (1, 9, 10) indicate that ACh is locally synthesized and metabolized. Although various subtypes of the mAChRs are expressed in some of these tumor cells, M3 mAChR appears to be mainly involved in tumor progression (11). For example, muscarinic activation induces cell proliferation, and blocking of M3 mAChR inhibits cell proliferation, in vitro and in vivo (7, 12, 13). Knockdown of M2 mAChR using small interfering RNAs (siRNAs) also results in blocked ACh-induced increase in [Ca2+]i, whereas knockdown of M1 and M3 mAChRs appears to have no effect on calcium response (12). Furthermore, the inhibitory potency of mAChR subtype-selective antagonists and M3 mAChR siRNAs on ACh-increased intracellular calcium and phosphorylation of mitogen-activated protein kinase (MAPK) and Akt has also been demonstrated (12). Furthermore, both tumor number and volume of colon cancer induced by azoxymethane were reduced in M3 mAChR–deficient tumor cells, M3 mAChR appears to be mainly involved (11). For example, muscarinic activity induces cell proliferation, and blocking of M3 mAChR inhibits cell proliferation, in vitro and in vivo (7, 12, 13). Knockdown of M2 mAChR using small interfering RNAs (siRNAs) also results in blocked ACh-induced increase in [Ca2+]i, whereas knockdown of M1 and M3 mAChRs appears to have no effect on calcium response (12). Furthermore, the inhibitory potency of mAChR subtype-selective antagonists and M3 mAChR siRNAs on ACh-increased intracellular calcium and phosphorylation of mitogen-activated protein kinase (MAPK) and Akt has also been demonstrated (12). Furthermore, both tumor number and volume of colon cancer induced by azoxymethane were reduced in M3 mAChR–deficient mice compared with wild-type mice (14).

A muscarinic antagonist has been reported to have anti-tumor activity, inhibiting SCLC growth in an NCI-H82 xenograft model. In this model, darifenacin, which is clinically used to treat urinary incontinence, inhibited tumor growth at doses of 1 – 3 mg/kg per day after 5 weeks of administration (12). Darifenacin demonstrated potent antagonistic activity against M3 mAChR, though its selectivity against other mAChRs was poor (15). The anti-tumor efficacy of darifenacin might be mediated by blockade of M3 mAChR, although the involvement of other subtypes of mAChRs cannot be excluded.

Recently, a potent selective M3 muscarinic antagonist, J-115311 (N-(2-[3-[(3R)-1-(cyclohexylmethyl)-3-piperidinyl]methylamino)-3-oxopropyl]amino-2-oxoethyl)-3,3,3-triphenyl-propioamide), was discovered (15). The binding affinity of J-115311 for human M3 mAChR is 0.31 nM with high selectivity over other mAChRs (ratio of binding affinities: M1/M3, 380; M2/M3, 98; M4/M3, 45; M5/M3, 120). Selective blockade of M3 mAChR by J-115311 is also demonstrated in vivo (M1/M3, >200; M2/M3, 45; M5/M3, 120) (15).

In order to further understand the role of M3 mAChR in cancer cells and to explore the possible therapeutic utility of M3 muscarinic antagonists as antitumor agents, we evaluated the effect of a selective M3 muscarinic antagonist, J-115311, in a mouse orthotopic xenograft model of SCLC.

Materials and Methods

Drugs

J-115311 was synthesized at the Tsukuba Research Institute of Banyu Pharmaceutical Co., Ltd. (15). Darifenacin hydrobromide was purchased from Waterstone Technology, LLC. (Carmel, IN, USA).

Cell culture

The human SCLC cell line NCI-H82 (American Type Cell Collection (ATCC), Manassas, VA, USA) was cultured in RPMI 1640 (ATCC) supplemented with 10% fetal bovine serum (Invitrogen, Carlsbad, CA, USA) and 100 units/ml penicillin and 100 μg/ml streptomycin (Invitrogen). The cells were incubated at 37°C with 5% CO2 in a humidified atmosphere.

Calcium fluorometry

Changes in [Ca2+]i were measured in NCI-H82 cells using FLIPR TETRA (Molecular Devices, Sunnyvale, CA, USA). NCI-H82 cells were seeded in 96-well plates coated with poly-d-lysine (Becton, Dickinson and Company, Franklin Lakes, NJ, USA) at a density of 5 × 10^4 cells/well and cultured overnight. The cells were incubated with 2 μM Fluo-4 (Invitrogen) in assay buffer (Hank’s Balanced Salt Solution, 10 mM Hepes, pH 7.4) containing 1% fetal bovine serum and 2.5 mM probenecid (Sigma-Aldrich, St. Louis, MO, USA) for 1 h. After the incubation, the cells were washed four times with the assay buffer to remove excess Fluo-4.

Changes in fluorescence were measured before and after the application of 300 μM carbachol (Sigma-Aldrich). The M3 muscarinic antagonists were added to the cells 5 min prior to the application of carbachol.

Data analyses were performed using Prism (version 4.03; GraphPad Software, La Jolla, CA, USA). Data were plotted as the fluorescence changes between the relative fluorescence unit (RFU) at the maximum responses after the application of carbachol with or without several concentrations of the M3 muscarinic antagonists. Curve fitting was achieved using nonlinear regression.

Cell proliferation and viability

Cell proliferation and viability was measured using the WST-8 assay. NCI-H82 cells were seeded at 5 × 10^3 cells/well in a 96-well plate and cultured overnight. M3 muscarinic antagonists dissolved in dimethylsulfoxide (DMSO; Nacalai Tesque, Kyoto), at a final DMSO concentration of 0.5%, were added to the cells. The final medium volume in each well was 200 μl. At 72 h after drug application, 20 μl of WST-8 reagent (Kishida Chemical, Osaka) was added to each well. The cells were incubated for an additional 3 h and then the plates were read at an optical density of 450 nm on a microplate reader (SpectraMax® 190, Molecular Devices).

Cell cycle analysis

The effect of J-115311 on cell cycle distribution was
analyzed using BD Cycletest™ Plus DNA Reagent Kit (Becton, Dickinson and Company). NCI-H82 cells were seeded at 6 × 10^4 cells/well onto 6-well plates and allowed to adhere for 24 h. Cells were incubated with J-115311 for 48 h and were harvested by trypsinization. Cells were centrifuged for 3 min at 1200 rpm and resuspended in 1 ml of buffer solution (dimethylsulfoxide in sucrose-sodium citrate) and stored at −80°C until analysis. Cells were thawed at room temperature and centrifuged for 3 min at 5000 rpm. Supernatant was removed and 125 μl of Solution A (trypsin in spermine tetrahydrochloride detergent buffer) was added. One hundred microliters of Solution B (RNase A and trypsin inhibitor in spermine buffer) was added after 10-min incubation at room temperature. Ten minutes later, 100 μl of cold Solution C (propidium iodide in spermine buffer) was added and the cells were incubated for 15 min at 4°C. Subsequently, cell cycle distribution was analyzed by flow cytometry (BD FACSCalibur™, Becton, Dickinson and Company).

**Animal experiments**

Female mice (C.B-17/Icr-scid/scidJcl; CLEA Japan, Tokyo) were housed in microisolators under controlled temperature and humidity (23 ± 2°C, 55 ± 15%), and on a 12-h light/dark cycle. Mice had access to tap water and food ad libitum. Food was prepared as a paste by mixing the powder form of CE-2 (CLEA Japan) with 1.5 times the volume of water depending on the weight of the powder (e.g., 100 g/150 ml) because suppression of food intake can be caused by a mechanism-based dry mouth induced by M3 mAChR antagonism (16, 17, 18). All experimental procedures were approved by the Banyu Institutional Animal Care and Use Committee and were undertaken in an AAALAC-approved animal facility.

NCI-H82 cells were cultured as described above and resuspended in fresh PBS buffer at a concentration of 1 × 10^6 cells/ml. A 20-μl aliquot of this cell suspension was injected into the upper lobe of the left lung orthotopically (19). Tumors were allowed to grow for 1 week. MicroCT images were obtained using an eXplore Locus microCT scanner (GE Healthcare, Chalfont St. Giles, UK) under the conditions of 93-μm isotropic resolution, 80 kV, and 450 μA under isoflurane anesthesia (1% – 2%) in order to confirm the growth of the tumors in the lung (20). Mice were divided into three groups (n = 7 – 8) to obtain the same mean tumor volume based on the CT images. J-115311 was dissolved in 50% DMSO and 50% distilled water and administered subcutaneously at doses of 25 and 50 mg/kg for 10 days. At the end of the study, the lungs and tumors were harvested and weighed. Tumor diameters were measured with a digital caliper, and tumor volume was calculated using the following formula:

\[
\text{Tumor volume (mm}^3) = \text{length (mm)} \times \text{width (mm)}^2 \times 0.5
\]

**Statistical analysis**

Results are expressed as the mean ± S.E.M. Data analysis was performed using SAS software (SAS Institute, Cary, NC, USA). One-way analysis of variance followed by William’s test was used. Values of P < 0.05 were considered statistically significant. Values for IC₅₀ in calcium fluorometry and cell proliferation assays were calculated by using Prism (version 4.03, GraphPad Software).

**Results**

**Functional analysis of M₃ mAChR in NCI-H82 cells**

To confirm the expression of functional M₃ mAChR in NCI-H82 cells, carbachol-stimulated \([Ca^{2+}]\) influx was measured. The muscarinic receptor agonist carbachol caused concentration-dependent increases of \([Ca^{2+}]\), in NCI-H82 cells, with an EC₅₀ value of 11 μM (Fig. 1A). As shown in Fig. 1B, both J-115311 and darifenacin were able to completely inhibit 300 μM carbachol–induced \([Ca^{2+}]\), influx in a dose-dependent manner. IC₅₀ values for J-115311 and darifenacin were 31 and 24 nM, respectively.

**Effects of M₃ muscarinic antagonists on cell proliferation and viability**

The proliferation of NCI-H82 cells was suppressed in a concentration-dependent manner by the addition of J-115311. An IC₅₀ value of 6.2 μM was obtained, and at over 17 μM, all cells were dead. Darifenacin also suppressed cell proliferation in a concentration-dependent manner with an IC₅₀ value of 22 μM, but did not cause total cell death (Fig. 2).

**Effect of the M₃ muscarinic antagonist on cell cycle**

The effect of J-115311 on the cell cycle was analyzed in NCI-H82 cells. As shown in Table 1, treatment with 10 μM of J-115311 induced cell death. The percentage of cells in G1, S, and G2/M phases were decreased due to increased cell death.

**Effects of M₃ muscarinic antagonists on tumor growth in vivo**

Treatment of mice with J-115311 did not affect their body weight compared with vehicle-treated mice. Body weights at the end of the study were 20.6 ± 0.4, 20.4 ± 0.3, 20.2 ± 0.3 g for the vehicle and 25 and 50 mg/kg for J-115311.

NCI-H82 cells inoculated into the upper lobe of the left lung orthotopically were detectable with a microCT...
scan 1 week after injection (Fig. 3, upper panel). A total of \(2 \times 10^6\) NCI-H82 cells transplanted orthotopically reached a size of \(88.2 \pm 23.0\) mm\(^3\) approximately 3 weeks after inoculation (Fig. 3, lower panel).

As shown in Fig. 4, J-115311 dose-dependently suppressed both the tumor weight (71.6 ± 13.5, 57.5 ± 17.0, and 45.2 ± 6.8 mg for the vehicle and 25 and 50 mg/kg for J-115311, respectively) and tumor volume (88.2 ± 23.0, 76.0 ± 24.0, and 50.9 ± 7.9 mm\(^3\) for the vehicle and 25 and 50 mg/kg for J-115311, respectively). The tumor volumes in the mice treated with 50 mg/kg of J-115311 were significantly reduced when compared with the vehicle-treated group \((P < 0.05)\), yet reduction in tumor weights in mice treated with 50 mg/kg of J-115311 did not reach statistical significance \((P < 0.09, \text{compared with the vehicle-treated group})\).

**Discussion**

Reports show that ChAT and M\(_3\) mAChR are expressed in NCI-H82 cells \((9, 12)\). In the present study, we observed carbachol-induced \([Ca^{2+}]\) influx in NCI-H82 cells, indicating the presence of mAChRs, because carbachol is known to selectively activate mAChRs despite its lack of selectivity among the mAChRs. We demonstrated that both J-115311 and darifenacin were able to inhibit the increased \([Ca^{2+}]\) influx caused by carbachol in NCI-H82 cells. J-115311 and darifenacin have similar binding affinities for human M\(_3\) mAChR, and their \(K_i\) values are 0.31 and 0.84 nM, respectively \((15)\). Since the
selectivity of darifenacin to M₁ mAChR over M₂ and M₅ mAChRs is poor (M₁/M₃, 6.5; M₅/M₃, 2.7) (15), inhibition by darifenacin might also be mediated by M₁ mAChR or by M₅ mAChR or both. On the other hand, J-115311 has high selectivity to M₃ mAChR over M₁ and M₅ mAChRs (M₁/M₃, 380; M₅/M₃, 120) (15). In the present study, the IC₅₀ values of J-115311 and darifenacin to inhibit [Ca²⁺]ᵢ influx were similar, 31 and 24 nM in NCI-H82 cells, respectively, thus indicating that [Ca²⁺]ᵢ influx in NCI-H82 cells might be mediated solely by M₃ mAChR. This selective contribution of M₃ mAChR in NCI-H82 cells is supported by the finding that knockdown of M₃ mAChR RNA alone blocks acetylcholine-induced [Ca²⁺]ᵢ increase (12).

Both J-115311 and darifenacin suppressed cell proliferation in a dose-dependent manner. It is reported that endogenous ACh can be detected in the medium of cultured NCI-H82 cells incubated in the presence of neostigmine, an AChE inhibitor (6, 9). Thus, both J-115311 and darifenacin could exert their inhibitory effects on cell proliferation stimulated by endogenous ACh. J-115311 was about three times more potent than darifenacin (IC₅₀ values: 6.2 and 22 µM for J-115311 and darifenacin, respectively) at inhibiting cell proliferation. However, J-115311 and darifenacin do have a similar potency for inhibiting carbachol-induced [Ca²⁺]ᵢ influx in the same cell line. It is known that the release of ACh from the parasympathetic nerves is controlled by M₂ autoreceptors located on the nerves (21, 22) and that inhibition of these autoreceptors leads to the release of ACh. It is also reported that the M₂ mAChR antagonist significantly increases proliferation of H1694 cells, an SCLC cell line, which suggests that the presence of the M₂ autoreceptor regulates cell proliferation in lung cancer cells (23). Furthermore, it is reported that forskolin, which induces the elevation of cAMP level by activating adenylate cyclase, induces lung cancer cell proliferation (24, 25). M₂ mAChR is coupled with the Gi type of G protein that inhibits adenylate cyclase activity, which leads to a decrease of cAMP levels. Thus, antagonism of M₂ mAChR leads to cell proliferation. Expression of M₂ mAChR mRNA in NCI-H82 cells by reverse transcription-PCR has been reported under normal conditions (26). Darifenacin exerts relatively strong antagonism on M₂ mAChR compared with that of J-11531 (M₂/M₃ = 56 and M₂/M₃ = 98 for darifenacin and J-115311, respectively) (15). The difference in IC₅₀ values of J-115311 and darifenacin to inhibit cell proliferation might be due to the difference in selectivity to M₁ mAChR–regulating cell proliferation.

In the present study, discrepancy of IC₅₀ values between the [Ca²⁺]ᵢ influx assay and the cell proliferation assay was observed. Song et al. showed that darifenacin totally inhibited ACh-induced [Ca²⁺]ᵢ influx in NCI-H82 cells at the concentration of 300 nM (26). In their study, the IC₅₀ value for the cell proliferation assay should be
Darifenacin totally inhibited carbachol-induced \([\text{Ca}^{2+}]_i\) influx at around 100 nM. The IC50 value for cell proliferation was 22 \(\mu\)M after 3 days of treatment with darifenacin. The IC50 value for the cell proliferation assay was equal between the two studies.

J-115311 caused cell death without causing cell cycle arrest. Therefore, cell death caused by inhibition of M3 mAChR appeared to be mediated by the inhibition of the intracellular signal transduction system independent of cell cycle regulation. It is reported that ACh induces phosphorylation of Akt in NCI-H82 cells and that the M3 mAChR antagonist 4-DAMP inhibits this phosphorylation (12). Activation of phosphatidylinositol 3-kinase (PI3K) converts phosphatidylinositol-bis-phosphate to phosphatidylinositol-tri-phosphate (PIP3). PIP3 leads to the recruitment of 3-phosphoinositide-dependent protein kinase-1 to the proximal side of the plasma membrane, which results in the activation of Akt (27). Involvement of the PI3K/Akt pathway in the regulation of lung cancer cell proliferation is reported (28, 29). Thus, the PI3K/Akt pathway might be involved also in the proliferation of NCI-H82 cells. It is also reported that carbachol activates MAPK phosphorylation in NCI-H82 cells, and the M3 mAChR antagonist inhibits this phosphorylation both in vitro and in vivo (12). The extracellular-signal-regulated kinase (ERK) pathway is reported to be one of the intracellular signaling pathways downstream of M3 mAChR in colon cancer cells, when epidermal growth factor receptor (EGFR) is co-expressed with M3 mAChR (30). In NCI-H82, it has been reported that expression of EGFR is below the detection level. In spite of low expression levels, the EGFR tyrosine kinase inhibitor gefitinib inhibited phosphorylation of ERK in NCI-H82 cells, suggesting the presence of a functional EGFR in NCI-H82 (31). Involvement of the ERK pathway is not excluded in the proliferation of NCI-H82 cells. In the present study, we did not measure the activity of these signaling mechanisms; further studies will be necessary to clarify the signaling mechanisms downstream of M3 mAChR blockade.

In general, growth rates of tumor cells transplanted subcutaneously are slow (32). It took 2 – 3 weeks for \(2 \times 10^7\) cells NCI-H82 cells inoculated into subcutaneous tissue to reach about 100 mm\(^3\) in size. However, fewer amounts of cells \((2 \times 10^6)\) transplanted orthotopically grew faster than those transplanted subcutaneously. This difference in growth speed of the cells between orthotopic and ectopic transplantation might be due to differences in the microenvironment of the tissues. It is well established that orthotopic xenograft mouse models of human tumors are capable of reproducing the histology and metastatic pattern of most human tumors (32 – 36). For example, the metastatic sites of human lung cancer are the bone, lung (contralateral), brain, and liver. In an orthotopic xenograft mouse model of lung cancer, the metastatic sites are the bone, lung (contralateral), lymph node, and pleura (34). In this study, the selective M3 muscarinic antagonist J-115311 inhibited tumor growth in an orthotopic xenograft model of SCLC, thus indicating that blockade of M3 mAChR was effective at suppressing tumor growth in a similar microenvironment to the primary target organ in humans.

Human breast tumor tissue obtained from cancer patients (stage I) was found to overexpress M3 mAChR,
which was not detectable in normal breast tissue (37). AChE activity was decreased in airway surface fluid obtained from squamous cell carcinoma patients (38). It is also reported that reducing AChE activity with AChE inhibitors led to tumor growth in a rat mammary tumor model (39). These results suggest that activation of local cholinergic tone positively regulates tumor growth, although it is not clear how/why the local cholinergic tone increases such growth. Activated cholinergic tone could be mediated by the mAChRs, especially M3 mAChR for tumor growth. Furthermore, involvement of M1 mAChR in SCLC cell adhesion and migration is also reported (40).

In summary, a selective muscarinic M3 antagonist, J-115311, reduced tumor growth not only in vitro but also in an orthotopic xenograft mouse model of SCLC. Therefore, muscarinic M3 antagonists could be potent therapeutic targets for cancer treatment in the future.

Acknowledgment

We would like to thank Satomi Yoshinaga for her technical assistance with the animal experiments.

References

12 Song P, Sekhon HS, Lu A, Arredondo J, Sauer D, Gravett C, et al. M3 muscarinic receptor antagonists inhibit small cell lung carcino-


Hoffman RM. Orthotopic is orthodox: why are orthotopic-transplant metastatic models different from all other models? J Cell Biochem. 1994;56:1–3.


