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Antiproliferative effect induced by novel imidazoline S43126 in PC12 cells is mediated by ROS, stress activated MAPKs and caspases

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ABSTRACT

Background: Some imidazoline compounds have pleiotropic effects including cell death *in vitro*. We examined the antiproliferative action of a novel imidazoline compound S43126, and the role of the I_1-imidazoline receptor, ROS, MAPKs and caspases in S43126-induced cell death.
Methods: PC 12 cells were treated with various concentrations of S43126 in the presence or absence of several ligands, and the effects on cell proliferation, ROS levels, and apoptosis were evaluated using Trypan Blue, Alamar Blue, Western blot and microscopy.
Results: We showed that S43126 reduced PC12 cell proliferation by greater than 50%, increased cell death by greater than 40% and increased apoptotic body formation. These effects were reversed by I_1R-antagonist, efaroxan. S43126 also increased intracellular ROS levels by greater than 2.5-fold relative to vehicle-treated control. These effects were significantly inhibited by N-acetyl-cysteine. In addition, pharmacologic inhibitors of ERK, JNK and p38 MAPK, significantly reduced S43126-induced antiproliferative activity. Caspases 3, 8 and 9 were all activated in a time-dependent manner by S43126. Pan caspase inhibitor z-VAD-fmk, ameliorated the effects of S43126 on cell death and cell proliferation.
Conclusion: Our data showed that the effects of S43126 on PC12 cell death were partly mediated by ROS production, MAPK and caspase activation. These results further indicate an emerging role for I_1R in apoptotic processes.

Introduction

Imidazoline compounds have been shown to affect proliferation of various cell types [1–4]. These compounds bind to imidazoline receptors of which there are at least three subtypes [5–7]. I_1-receptors are present in brain, liver, kidney, adipose tissue and pancreas, and are labeled by [^{3}H] clonidine [8,9]. The I_2-receptors are labeled by [^{3}H] idazoxan and are located in the mitochondria as a novel regulatory site on monoamine oxidase [10]. The third type of imidazoline receptor, I_3 is closely related to the I_1-receptor and is found in the pancreas, where it is involved in insulin regulation and secretion [11].

We have shown that S43126 (2-[4′-methoxyphenyl]-4, 5-dihydro-1H-imidazole), a novel, I_1-imidazoline receptor agonist, does not cause contraction of tail artery, suggesting that it is not active at alpha (α)-adrenergic receptors (α-AR) [12]. This fact is supported by radioligand binding studies [13], where S43126 has a $pK_i < 5$ at α_1 or α_2 – AR, and $pKi = 7$ at I_1-receptors. This compound is functionally active at I_1-imidazoline receptor since it lowers blood pressure when injected into the rostral ventrolateral medulla (RVLM) of spontaneously hypertensive rats (SHR) [14], and activates the I_1-signaling pathway. S43126 is selective for I_1-imidazoline receptors compared to alpha (α)-adrenergic receptors, and has the potential to advance the field of imidazoline research. Therefore, S43126 may be used as an imidazoline agonist without concern for the confounding influence of adrenergic actions that is seen with many other imidazoline compounds.

Previous studies have shown that some imidazoline compounds such as idazoxan, moxonidine, agmatine, efaroxan, benazoline and

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RX871024 can induce cell death *in vitro* at micro and millimolar concentrations [1–4]. Idazoxan was shown to be cytotoxic to Lewis lung carcinoma (3LL) and prostate cancer cells (DU145 cells) [4]. Moxonidine, idazoxan, agmatine and efaroxan were all shown to be cytotoxic to PC12 cells at micromolar to millimolar concentrations [2]. By using benazoline, it was demonstrated that the I_1-receptor might be involved in some apoptotic processes [1] and RX87104 induced death of pancreatic MIN6 cells by activation of JNK [15].

In this paper we investigated the antiproliferative effect of S43126 on PC12 cells. PC12 cells are a clonal pheochromocytoma cell line derived from a tumor of rat adrenal medullary chromaffin cells. These cells express I_1-imidazoline receptor but lack α_2-AR, as shown by radioligand binding as well as molecular approaches [15,16]. Since many imidazoline compounds also bind to α_2-AR, it is advantageous to use S43126 in our studies, since this ligand has greater selectivity for I_1-receptor compared to α_2-adrenergic receptor, and allows us to have cleaner studies, decrease reliance on inhibitors to separate imidazoline effects from adrenergic effects, and potentially a better side effect profile if developed for clinical use. There are no studies to date which have demonstrated the ability of S43126 to induce cell death *in vitro*. Therefore we examined the effect of S43126 on PC12 cell proliferation and the role of the I_1-imidazoline receptor, reactive oxygen species (ROS), mitogen activated protein kinases (MAPK) and caspases in S43126-induced cell death. The results from our study will serve to illuminate the anti-proliferative mechanism of action of S43126 in PC12 cells.

Materials and methods

Cell culture, reagents and drugs

PC12 cells were obtained from the American Type Culture Collection (ATCC). Cells were cultured in DMEM (Mediatech, Inc.; Manassas, VA) containing 10% FBS, 2 mM glutamine and 5% (10,000 units penicillin and 10 mg streptomycin/mL) antibiotic. Drug stock solutions were dissolved in 0.1% dimethyl sulfoxide (DMSO) [Sigma–Aldrich Corp., St. Louis, MO, USA] and stored protected from light at −20 °C until use. S43126 was a gift from Dr. Geneviève Baziard-Mouysset (Université Toulouse III, Faculté des Sciences Pharmaceutiques, Laboratoire de Chimie Pharmaceutique, Toulouse, France). Caspase substrates were purchased from EMB Chemicals, Inc. (San Diego, CA, USA). z-VAD-fmk, PD98059, and SP600125 were purchased from Enzo Life Sciences International, Inc. (Plymouth Meeting, PA, USA). Alamar Blue™ dye was obtained from AbD Serotec (Raleigh, NC, USA). Trypan Blue was purchased from Mediatech, Inc. (Manassas, VA, USA). Efaroxan, SB202190, 2′,7′-dichlorodihydrofluorescein diacetate, N-acetylcysteine and all other reagents were purchased from [Sigma–Aldrich Corp., St. Louis, MO, USA].

Alamar Blue™ assay

The cytotoxicity of S43126 in PC12 cells was determined using the Alamar Blue™ assay as previously described [17]. Briefly, cells were plated in 96-well plates at a density of 10,000 cells/well, in a total volume of 200 μL media. After a 24 h incubation, cells were treated with 0.1% DMSO (vehicle) or S43126 (10^{-8}–10^{-3} M) for 72 h before the addition of 20 μL of Alamar Blue™ dye. After 4 h of dye incubation, fluorescence was read using a FLx800 microplate fluorescence reader (excitation and emission = 530 and 590 nm respectively). In some experiments, cells were treated with either 0.1% DMSO, 1 mM S43126, 1 μM Efaroxan, 5 mM NAC, 1 μM PD98059, 1 μM SP600125, 1 μM SB202190 or 1 μM z-VAD-fmk for 72 h. Alternatively, cells were pretreated with either 1 μM Efaroxan, 5 mM NAC, 1 μM PD98059, 1 μM SP600125, 1 μM SB202190 or 1 μM z-VAD-fmk followed by treatment with 1 mM S43126 and subsequent evaluation of cytotoxicity.

Trypan Blue assay

Cytotoxicity was also determined by Trypan Blue exclusion assay. Briefly, cells plated in triplicate in 6 well plates were treated as described above. Dead cells have a distinctive blue color under the microscope.

Hoffman modulation phase contrast microscopy

Apoptosis was visualized using an Olympus IX70 inverted microscope equipped with Hoffman modulation phase contrast, and images were acquired using a SPOT digital camera system. Briefly, PC12 cells were plated overnight, and then treated as described above in 6 well plates in triplicates. Following treatment, cells were examined for apoptotic body formation.

Caspase activity assay

Apoptosis was also determined by caspase activation assays. PC12 cells were plated at a density of 2×10^4 cells/well, in 96-well Nunc black plates, and cultured overnight in the presence of 5% CO_2 at 37 °C. Cells were treated 24 h later with 1 mM S43126 for various times (24–72 h). Experiments were done in triplicate. At the end of each time point, a one-step cellular caspase activity assay was performed using the substrates (EMB Chemicals, Inc., San Diego, CA) as follows: DEVD-AMC for caspase 3, IETD-AMC for caspase 8 or LEHD-AMC for caspase 9 as described previously [18]. Briefly, following S43126 treatment, cells were incubated for 1 h at 37 °C with 50 μL/well of filtered 3X one-step caspase assay buffer (150 mM HEPES, pH 7.4, 450 mM NaCl, 150 mM KCl, 30 mM $MgCl_2$, 1.2 mM EGTA, 1.5% Nonidet P40, 0.3% CHAPS, 30% sucrose) supplemented with 150 μM of the appropriate caspase substrates, 30 mM DTT and 3 mM phenylmethanesulphonylfluoride. Caspase activity was measured (excitation at 360 nm and emission at 460 nm) using an FLx800 microplate fluorescence reader. The caspase activity for each time point was expressed as percent caspase activation relative to vehicle-treated control.

Measurement of intracellular reactive oxygen species level

Intracellular ROS level was measured using the fluorescent dye 2′,7′-dichlorodihydrofluorescein diacetate (H_2DCF-DA) and flow cytometry. H_2DCF-DA is a nonpolar, membrane permeable compound, which can be taken up into cells and then converted to a membrane impermeable, nonfluorescent polar compound, 2′,7′-dichlorodihydrofluorescein (H_2DCF). The conversion from H_2DCF-DA to H_2DCF is mediated by cellular esterases. Intracellular ROS oxidizes the non-fluorescent H_2DCF to the fluorescent 2′,7′-dichlorofluorescein DCF, which can be easily detected and measured by flow cytometry. PC12 cells were treated with 0.1% DMSO (negative control), or S43216. The DCF fluorescence intensity is proportional to the amount of ROS formed intracellular. As a positive control, 500 μM H_2O_2 for 30 min was used.

Measurement of intracellular glutathione levels

Cells were plated in 25 cm^2 flasks at 1×10^6 cells/flask, and cultured overnight in the presence of 5% CO_2 at 37 °C. Cells in triplicate were treated 24 h later with 1 mM S43126 for 72 h to induce apoptosis. At the end of the time point, intracellular GSH