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Cytotoxicity of methoctramine and methoctramine-related polyamines

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ABSTRACT

Methoctramine and its analogues are polymethylene tetramines that selectively bind to a variety of receptor sites. Although these compounds are widely used as pharmacological tools for receptor characterization, the toxicological properties of these polyamine-based structures are largely unknown. We have evaluated the cytotoxic effects of methoctramine and related symmetrical analogues differing in polymethylene chain length between the inner nitrogens against a panel of cell lines. Methoctramine caused cell death only at high micromolar concentrations, whereas its pharmacological action is exerted at nanomolar level. Increasing the spacing between the inner nitrogen atoms resulted in a significative increase in cytotoxicity. In particular, an elevated cytotoxicity is associated to a methylene chain length of 12 units dividing the inner amine functions (compound **5**). H9c2 cardiomyoblasts were the most sensitive cells, followed by SH-SY5Y neuroblastoma, whereas HL60 leukaemia cells were much more resistant. Methoctramine and related compounds down-regulated ornithine decarboxylase, the first enzyme of polyamine biosynthesis even at non-toxic concentration. Further, methoctramine and compound **5** caused a limited up-regulation of spermine/spermidine N-acetyltransferase, suggesting that interference in polyamine metabolism is not a primary mechanism of toxicity. Methoctramine and its analogues bound to DNA with a higher affinity than spermine, but the correlation with their toxic effect was poor. The highly toxic compound **5** killed the cells in the absence of caspase activation and caused an increase in p53 expression and ERK1/2 phosphorylation. Compound **5** was directly oxidized by cell homogenates producing hydrogen peroxide and its toxic effect was partially subdued by the inhibition of its uptake, by the NMDA ligand MK-801, and by the antioxidant N-acetylcysteine, suggesting that compound **5** can act at different cellular levels and lead to oxidative stress.

1. Introduction

The natural polyamines putrescine, spermidine, and spermine are ubiquitous, cationic molecules that play essential roles in many biological processes, including cell growth and proliferation [1]. It is well known that polyamines interact with a variety of cellular macromolecules, such as nucleic acids and membrane phospholipids mainly through the formation of ionic bonds, since at physiological pH, protonation of amino groups is nearly complete [2]. Furthermore, the binding of polyamines to a variety of receptors, ion channels and other defined recognition sites, has constantly been reported [3].

The concept that polyamines may represent a universal template in the receptor recognition process is embodied in the design of ligands of different biological targets. In fact, it was demonstrated that the insertion of different pharmacophores onto the polymethylene backbone as well as an appropriate distance separating the amine functions, can tune both affinity and selectivity for any given receptor [4,5]. The application of this approach led to the discovery of methoctramine [6], the most extensively studied polyamine as selective muscarinic M_2 receptor antagonist. Methoctramine has been thoroughly used as a tool in muscarinic receptor characterization and classification, due to its high affinity for muscarinic M_2 receptors, low affinity for muscarinic M_3 receptors, and intermediate affinity for muscarinic M_1 receptors [7]. Furthermore, appropriate structural modifications performed on the structure of methoctramine led to the creation of new polyamines endowed with high affinity and selectivity for muscarinic receptor subtypes,

Abbreviations: DAPI, 4′,6-diamidino-2-phenylindole; DENSPM, N^1,N^{11}-diethylnorspermine; ERK, extracellular regulated kinase; NAC, N-acetylcysteine; NMDA, N-methyl-D-aspartate; ODC, ornithine decarboxylase; SSAT, spermidine/spermine N^1-acetyltransferase.

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G proteins [8], muscle-type nicotinic receptors [9] and vanilloid receptors [10].

Many synthetic compounds with a polyamine backbone have a strong toxic effect. In fact, since polyamines are strictly related to cell proliferation, several cytotoxic polyamine analogues have been synthesized with the aim to generate anticancer drugs [1,11]. However, although methoctramine and its analogues are widely used as pharmacological tools for receptor characterization [6], the general toxicological properties of these polyamine-based structures are largely unknown. The lack in information regarding these compounds has led us to study methoctramine and related tetramines to define the impact of these substances on cell survival.

2. Materials and methods

2.1. Materials

Methoctramine and derivatives were synthesized and characterized following our methods previously reported [8,9,12,13]. N^1,N^{11}-Diethylnorspermine (DENSPM) was provided by Tocris (Bristol, UK); monoclonal antibodies against ERK 1/2 and phospho-ERK 1/2 were from Cell Signaling Technology, Danvers, MA; anti-p53 or anti-β-actin was purchased from Santa Cruz Biotechnology, Santa Cruz, CA; polyclonal anti-SSAT was from Abcam, Cambridge, MA. All other chemicals were from Sigma–Aldrich.

2.2. Cell culture and treatment

HL60 (human promyelocytic leukemic cells) were maintained in exponential growth in RPMI 1640 medium supplemented with 10% fetal bovine serum, 5% glutamine and 5% antibiotics. The cells were diluted to a density of 5×10^5 ml^{-1} in fresh medium immediately before the treatments. SH-SY5Y neuroblastoma cells were cultured in HAM'S F-12/MEM containing 10% fetal bovine serum, 5% glutamine, 1% non-essential amino acids and antibiotic solution. These cells were seeded in 10 cm diameter Petri plates (105×10^3 cells in 7 ml). The experiments were performed after cells were grown for 2 days, when their density was about 4×10^5 ml^{-1}. H9c2 cardiomyoblasts (embryonic rat-heart derived cells) were cultured in Dulbecco's modified Eagle's medium (DMEM, Celbio) supplemented with 10% heat inactivated fetal calf serum, 5% glutamine and antibiotics. Subcultured cells (70×10^3 cells in 7 ml in 10 cm diameter Petri plates) were grown for 48 h, reaching a density of about 4×10^5 ml^{-1} before treatments. Primary cardiomyocytes were obtained from newborn Wistar rats as described [14] and 1×10^6 cells in a 3.5 cm plates were used for the experiment. All cells types were routinely maintained at 37 °C in a humidified atmosphere containing 5% CO_2.

All tested compounds were dissolved in phosphate-buffered saline (PBS), diluted to a 100× solution in PBS and added to cell cultures (1% with respect to the total volume) in order to obtain the required concentration in the medium. Control cells received the corresponding volume of PBS.

2.3. Cell death and cell survival

Cell viability was determined by trypan blue exclusion by counting living cells and stained dead cells with a Burker hemocytometer. Samples were done in triplicate, and at least 10 fields were counted for each sample. At the end of the incubation, once the number of living cells in samples was measured, cell survival was calculated as the percentage of living viable cells in treated samples in respect to the number of viable cells in control samples. The IC_{50} value is the concentration of toxic compound required to reduce cell survival to 50%. To determine this value, the cells were incubated in the presence of the following micromolar concentration of the tested compounds: 0, 1, 5, 10, 20, 50, 100. After 24 h, viable cells were counted. A dose–response curve was plotted and concentrations that yielded 50% survival were calculated using the GraphPad Prism3 software.

The assays used to detect apoptosis were: caspase activation, DNA fragmentation, and nuclear morphology.

The activity of Caspase protease enzymes was measured by the cleavage of the fluorogenic peptide substrate AcDEVD-AMC that represents a substrate for caspase 3 and other members of the caspase family. At the indicated time points, cells were washed in phosphate-buffered saline, harvested in 0.4 ml of lysis buffer, and subjected to two cycles of freeze–thawing. The lysates were centrifuged for 10 min at 28,000 × g at 4 °C and the supernatant was then used to assay in duplicate enzyme activity. Extract (10 μl) was incubated for 15 min at 37 °C in a final volume of 30 μl to determine caspase activity as described [15].

DNA fragmentation was detected by agarose gel electrophoresis as previously detailed [15].

The effect of treatments on the number of apoptotic nuclei was determined by the visualization and counting of 4′,6-diamidino-2-phenylindole (DAPI)-stained nuclei. At the indicated time point, cells grown in triplicate on glass coverslips were washed twice with cold PBS, fixed in ice-cold methanol/acetic acid (1:1, v/v) for 15 min, rinsed with PBS, and then stained with 0.1 mg/ml of DAPI. After staining, cells were mounted on standard glass slides and observed with a IX50 Olympus inverted microscope (Olympus, Tokyo, Japan) [16]. Apoptotic cells were identified by nuclear features characteristic of apoptosis (nuclear shrinkage and/or fragmentation, chromatin condensation). For each data point, done in triplicate, 20 fields were examined. The percentage of fragmented nuclei was determined and the results presented as the mean ± S.E.M. for the three replicates of any point.

2.4. Polyamine metabolism

To measure the activity of ornithine decarboxylase (ODC), the cells were washed with PBS and scraped in a buffer consisting of 0.1 mM EDTA, 0.02 mM pyridoxal phosphate, 2.5 mM dithiothreitol in 10 mM sodium phosphate buffer, pH 7.2. The cells were disrupted by freeze–thawing three times and then centrifuged at 11,000 rpm for 15 min. ODC activity in the supernatant was measured in duplicate by estimating the release of $^{14}CO_2$ from [^{14}C-Carboxyl]-ornithine during a 60 min incubation [14,15]. Specific ODC activity is expressed as units/mg protein, where 1 unit corresponds to 1 nmol of CO_2/h of incubation.

The activity of spermidine/spermine N^1-acetyltransferase (SSAT) in cytosolic extracts was determined measuring in duplicate the incorporation into spermidine of radioactive acetyl groups deriving from [Acetyl-1-^{14}C] Acetyl Coenzyme A during a 10 min incubation period as previously detailed [15].

Enzymatic oxidation of spermine and compound **5** was assayed in whole cell homogenates as described by Wang et al. [17]. In this assay, homogenates obtained by freeze–thawing of H9c2 cells were incubated in triplicate with the polyamine (0.25 mM) and the H_2O_2 formed due to oxidation of spermine or compound **5** was detected by converting homovanillic acid into a highly fluorescent compound in the presence of horseradish peroxidase. Oxidative activity is expressed as the increase in fluorescence emission at 426 nm, with excitation at 323 nm (arbitrary units) during 1 h incubation.

Polyamines and methoctramine derivatives were determined in acidic cellular extracts by reversed phase HPLC after derivatization with dansyl chloride [18]. To elute the strongly apolar methoctramine analogues, the column (25 cm × 4.6 mm Gemini 5μ from Phenomenex) was eluted with a 12 min linear gradient from water/methanol/acetonitrile (30:35:35) at flow 1.0 ml/min to