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Functional assays to define agonists and antagonists of the sigma-2 receptor

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ABSTRACT

The sigma-2 receptor has been identified as a biomarker in proliferating tumors. To date there is no well-established functional assay for defining sigma-2 agonists and antagonists. Many sigma-2 ligands with diverse structures have been shown to induce cell death in a variety of cancer cells by triggering caspase-dependent and independent apoptosis. Therefore, in the current study, we used the cell viability assay and the caspase-3 activity assay to determine sigma-2 agonists and antagonists. Three classes of sigma-2 ligands developed in our laboratory were evaluated for their potency to induce cell death in two tumor cell lines, mouse breast cancer cell line EMT-6 and human melanoma cell line MDA-MB-435. The data showed that the EC_{50} values of the sigma-2 ligands using the cell viability assay ranged from 11.4 μM to >200 μM, which were comparable with the EC_{50} values obtained using the caspase-3 assay. Based on the cytotoxicity of a sigma-2 ligand relative to that of siramesine, a commonly accepted sigma-2 agonist, we have categorized our sigma-2 ligands into agonists, partial agonists, and antagonists. The establishment of functional assays for defining sigma-2 agonists and antagonists will facilitate functional characterization of sigma-2 receptor ligands and sigma-2 receptors.

The sigma receptor was originally defined pharmacologically long before its molecular identity was known [1]. It is the specific binding site for a group of compounds, which were later named as sigma ligands. The sigma receptor was once thought to be a subset of the opioid receptor [2], but was subsequently revealed to be a distinct class of receptor system [1]. Radioligand binding studies and biochemical analyses have shown that there are at least two types of sigma receptors, sigma-1 and sigma-2. The sigma-1 receptor gene has been cloned [3–5] from guinea pig, human and rodent origins. The most prominent action of sigma-1 receptors in biological systems is the regulation and modulation of voltage-regulated and ligand-gated ion channels, including Ca^{2+}, K^+, Na^+, Cl^-, and SK channels, and NMDA and IP3 receptors [6]. The sigma-2 receptor has been identified as a biomarker in proliferating tumors [7,8]. It regulates cell growth and is an emerging target for cancer diagnosis and therapeutics [9]. Recently, the progesterone receptor membrane component 1 (PGRMC1)[2] protein complex has been identified as the putative sigma-2 receptor binding site [10].

To date numerous sigma-2 selective ligands have been developed [9,11–19]. These ligands were generally characterized by receptor binding assays and determination of agonist/antagonist has been awaiting proper functional assays. Some sigma receptor ligands are referred in the literature as agonists/antagonists based on behavioral studies. For example, haloperidol and pentazocine were called sigma agonists because they have antipsychotic activity and analgesic activity, respectively, in clinical use [20]. BD1047 and BD1063 were called sigma receptor antagonists because they had no effects on their own but attenuated the dystonia produced by DTG and haloperidol in rats [21]. Other sigma-2 ligands were defined as agonists/antagonists using cell-based assays. For example, CB-64D was called a sigma-2 receptor agonist because it elicited calcium release in human neuroblastoma cells [22] and induced cell death in the breast tumor cell line [20]. However, there is no well-established functional assay for defining agonists/antagonists for sigma-2 receptors. This is mainly because the molecular identity of the sigma-2 receptor was unknown until recently, and the mechanism of ligand-receptor interaction is largely unclear.

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[2] *Abbreviations used:* ABN, azabicylononane; EGFR, epidermal growth factor; PGRMC1, progesterone receptor membrane component.

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Sigma-2 receptor-mediated cell death is one of the most active areas in sigma-2 receptor research. Many sigma-2 ligands with diverse structures kill a variety of cancer cells at concentrations in the micromolar range [17,20,23–25]. It is suggested that sigma-2 ligands bind to sigma-2 receptors and trigger caspase-independent and caspase-dependent apoptosis. Therefore, in the current study, we propose to use cell viability and caspase-3 activity, a hallmark of apoptosis, as functional assays to define the agonist/antagonist properties of sigma-2 receptor ligands. Three classes of sigma-2 ligands developed in our laboratory were evaluated in these two assays in two tumor cell lines: mouse breast cancer cell line EMT-6 and human melanoma cell line MDA-MB-435. Based on the potency of these ligands to induce cell death in cancer cells relative to the well-accepted sigma-2 agonist siramesine, we were able to categorize the sigma-2 ligands into the traditional terms used to describe intrinsic activity at a receptor: agonists, partial agonists, and antagonists. The establishment of functional assays for defining sigma-2 agonists/antagonists will facilitate the functional characterization of sigma-2 receptor ligands and sigma-2 receptors.

Materials and methods

Receptor binding assays

The sigma-1 and sigma-2 receptor binding affinities of sigma-2 ligands were determined as previously described [26]. Briefly, guinea pig brain (sigma-1 assay) or rat liver (sigma-2 assay) membrane homogenates (~300 μg protein) were diluted with 50 mM Tris–HCl, pH 8.0 and incubated with either ~5 nM [^{3}H](+)-pentazocine (34.9 Ci/mmol; sigma-1 assay) or 1 nM [^{3}H]RHM-1 (80 Ci/mmol; sigma-2 assay) in a total volume of 150 μl in 96 well plates at 25 °C. The concentrations of each compound ranged from 0.1 nM to 10 μM. After incubating for 60 min, the reactions were terminated by the addition of 150 μl of cold wash buffer (10 mM Tris–HCl, 150 mM NaCl, pH 7.4) using a 96 channel transfer pipette (Fisher Scientific, Pittsburgh, PA), and the samples harvested and filtered rapidly into a 96 well fiberglass filter plate (Millipore, Billerica, MA) that had been presoaked with 100 μl of 50 mM Tris–HCl, at pH 8.0 for 1 h. Each filter was washed three times with 200 μl of ice-cold wash buffer, and the bound radioactivity quantified using a Wallac 1450 MicroBeta liquid scintillation counter (Perkin Elmer, Boston, MA). Nonspecific binding was determined in the presence of 10 μM cold haloperidol.

Cell culture conditions

EMT-6 mouse breast cancer cells were grown in DMEM containing 10% fetal bovine serum, 100 units/ml penicillin and 100 μg/ml streptomycin. MDA-MB-435 human melanoma cells were grown in MEM containing 10% fetal bovine serum, 2 mM L-glutamine, 1 mM sodium pyruvate, 1% nonessential amino acids (Mediatech Inc, Manassas, VA), 2% MEM vitamins (Invitrogen, Carlsbad, CA), 100 units/ml penicillin, and 100 μg/ml streptomycin. Both cell lines were maintained at 37 °C in a humidified incubator with a 5% CO_2/95% air atmosphere.

MTS cell viability assay

The cytotoxicity of the compounds on EMT-6 and MDA-MB-435 cells was measured by using the CellTiter 96 Aqueous One Solution (Promega, Madison, WI), which contains a tetrazolium compound [3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2*H*-tetrazolium, inner salt; MTS], according to the manufacturer's protocol. Briefly, cells were plated 5×10^3 cells/well in 96-well plates 24 h prior to treatment with the compounds. Each compound was dissolved in DMSO and serially diluted in culture medium to acquire the desired concentrations. The final concentration of DMSO in the cell culture medium was no more than 1.0%. After a 24 or 48 h treatment with the various compounds, 20 μl of the CellTiter 96 AQueous One Solution Reagent was added to each well, and the plate was incubated for 1–2 h at 37 °C. The plate was then read at 490 nm in a Victor^3 plate reader (PerkinElmer Life and Analytical Sciences, Shelton, CT). Cell viability (%) and cytotoxicity (%) at each concentration of the compound were calculated by formulas (1) and (2), respectively:

$$\text{Cell viability}(\%) = 100\frac{\text{OD}_{490,\sigma 2}}{\text{OD}_{490,\text{control}}} \quad (1)$$

$$\text{Cytotoxicity}(\%) = 100 - \text{cell viability}(\%) \quad (2)$$

where $OD_{490,\ \sigma 2}$ is the absorbance at 490 nm for sigma-2 ligand-treated cells, and $OD_{490,\ \text{control}}$ is the absorbance at 490 nm for untreated cells. The EC_{50}, defined as the concentration of the sigma-2 ligand required to inhibit cell proliferation by 50% relative to untreated cells, was determined from the dose-response curves generated using GraFit software, version 5 (Erithacus Software Limited, UK). All compounds were assayed in triplicate, and the EC_{50} values presented as the mean ± SEM of three independent experiments.

Caspase-3 activation assay

The caspase-3 activity induced by the compounds in EMT-6 and MDA-MB-435 cells was measured using the Apo-ONE homogeneous caspase-3/7 Assay (Promega, Madison, WI). This assay utilizes a profluorescent substrate Z-DEVD-R110 specific for caspase-3/7 coupled with an optimized cell permeabilization buffer. Cleavage of the peptide sequence DEVD by active caspase-3/7 releases free Rhodamine 110 which when excited at 485 nm, becomes intensely fluorescent and can be detected at emission wavelength 535 nm. The amount of fluorescent product generated is directly proportional to the caspase-3/7 activity in each sample. The cells were plated 5×10^3 cells/well in 96-well black, clear-bottomed plates 24 h prior to treatment with the compounds. After a 24 h treatment with the various compounds, caspase-3 activity was assessed using the Apo-ONE homogeneous caspase-3/7 assay. Ten milliliters of buffer was pre-mixed with 100 μl of the caspase-3/7 substrate Z-DEVD-R110. One hundred microliters of the substrate-buffer mix was added to each well and the plate was placed on an orbital shaker for 5 min. The plate was then incubated at room temperature in the dark for up to 18 h. The plate was then read at excitation and emission wavelengths 485 and 535 nm, respectively, on a Victor^3 plate reader (PerkinElmer Life and Analytical Sciences, Shelton, CT). The EC_{50}, defined as the concentration of the sigma-2 ligand required to elicit the caspase-3 activity by 50% of the maximal caspase-3 activation values, was determined from the dose-response curves generated using GraFit software, version 5 (Erithacus Software Limited, UK). All the compounds were assayed in triplicate, and the EC_{50} values presented as the mean ± SEM of three independent experiments.

Results

Chemical structures and the sigma receptor binding affinities of the sigma-2 ligands

In the current study, 11 sigma-2 ligands developed in our laboratory including 6 azabicylononane (ABN) analogs, 4 benzamide analogs, and 1 tropane analog, were evaluated for the potency to