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Validation of Sigma I Receptor Occupancy with Antipsychotic Ligands: A Molecular Perspective

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ABSTRACT

Sigma-1 receptors are unique and distinct class of receptors widely expressed in the central nervous system with involvement in regulation of various neutransmitters and are often over expressed in tumor cell lines of various tissues, such as melanoma, breast cancer, small lung carcinoma and prostate cancer. Accordingly, sigma ligands display anticancer activity in- vivo and in-vitro. In the present study, an attempt has been made to validate sigma-1receptor and study its antipsychotic ligand interactions in a molecular perspective. C6 cells (rat glioma) grown in monolayers were exposed to haloperidol (sigma-1 antagonist) and other anti-psychotic ligands and their relative cytotoxicity was determined by MTT assay to be 42.79 %, 18.96 %, 24.95 and 22.72% for haloperidol, ligand 1, ligand 2 and ligand 3 respectively. Occupancy of sigma receptors with ligands through DNA laddering studies reveals the antipsychotic ligands modulate psychosis via sigma-1receptor.

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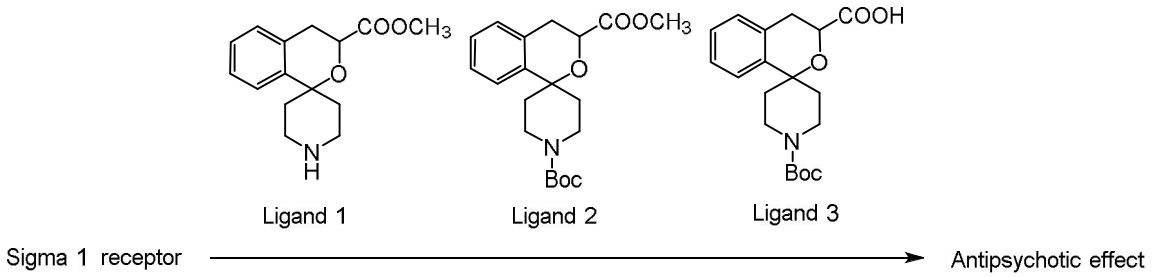
GRAPHICAL ABSTRACT



1. Introduction

Sigma receptors are distinct and unique non-opiod trans-membrane receptors widely expressed in central nervous system and have been implicated to modulate various neurotransmitters.[1-2] In addition, they are found in endocrine, immune, and reproductive tissues, and they are also found in high abundance in heart, spleen, liver and kidney.[3] Also, mitochondria is enriched with sigma receptors. In mitochondria, they initiate TCA cycle through complex formation with IP3 and increase calcium levels which inturn induces cell hyper-metabolisation resulting in neuroprotection and neurite outgrowth.[4-8] Furthermore, the sigma-1receptor has been reported to play roles in the regulation of gastro-intestinal effects, modulation of opioid analgesia, atten-uation of cocaine-induced loco-motor activity and toxicity and so on.[5] Heterogeneous nature of several sigma ligands suggested the presence of various subtypes in which sigma-1receptor is a trans-membrane receptor with three hydro-phobic domains in the central, N and C termini of the protein. Recent study shows that sigma-1 and 2 receptors are over expressed in tumor cell lines of various tissues such as melanoma, breast cancer, small lung carcinoma and prostate cancer and are suggested to play an important role in cell proliferation and adhesion of breast cancer cells.[6] Hence sigma receptors would be suitable targets for developing tumor selective agents in cancer treatment.

As sigma receptors are reported to modulate several physio-logical and pathophysiological processes, their ligands can be used to influence these processes and treat depression, schizophrenia, amnesia and mental improvement, cocaine abuse as well as other psychiatric disorders.[7] The endogenous ligands for sigma receptors include progesterone, dimethyl-tryptamine (DMT) and sphingosine etc.[8] A wide variety of psychotropic drugs with diverse chemical structures have been reported to exhibit high affinity for σ-1 receptors[9] and this could be attributed to the lipophilic sterol-binding domain on the protein which is capable of accommodating a large array of lipophilic ligands.

Among them the antipsychotic compound haloperidol (Haldol) has the highest affinity for the σ-1 receptor.[10] Haldol

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(Haloperidol), the first generation antipsychotic is a tranquiliser and a neuroleptic drug belonging to butyrophenone neuroleptics. The sigma ligands (haloperidol and rimcazole) were also shown to inhibit growth of both cultured cancer cells under in-vitro and in-vivo and they do not interfere in the proliferation of non-cancer tissues.[11-13] But, the side effects of haloperidol in terms of mortality in elderly patients, dementia related psychosis and others require development of alternate safe efficient anti-psychotic agents for therapeutic use. Spiro cyclic piperidine derivatives (1-4) exhibit high affinity for sigma receptors (Fig. 1). Our earlier bioinformatics study has predicted favorable interaction between sigma-1 receptor protein and three antipsychotic ligands synthesized in our laboratory (data to be published). The present paper describes validation of sigma-1receptor occupancy with the possible antipsychotic ligands synthesized in our laboratory and its interactions in a molecular perspective.

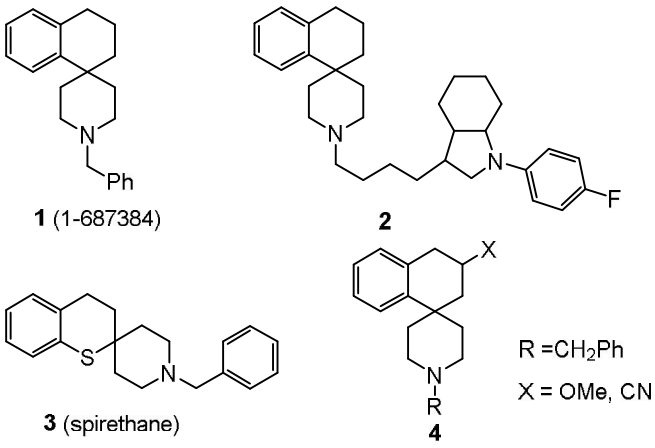


Fig 1. Spiro cyclic piperidine derivatives.

2. Results and Discussion

As Rat glioma cell line contained high densities of sigma 1 and 2 receptors, it was used for various binding studies, ligand uptake studies and cytotoxicity analysis.[12] C6 cells are used as an *in vitro* model for the validation of sigma-1receptor for antipsychotic ligand interactions measured through the occupancy of sigma-1receptor and the cytotoxic effects of antipsychotic ligands. Previous studies indicated that antipsychotic drugs bring about cell death of most cancer cell lines including C6 rat glioma cell lines. We have used an *In-silico* approach to evaluate the binding ability of sigma receptor protein and spiropiperidine derivatives. The three dimensional structure of sigma-1 receptor protein was predicted using I-Tasser, a structure prediction server and its interaction with ligands **1**, **2** and **3** were analyzed using docking softwares in our earlier study. All the three ligands showed favorable interactions within the predicted active site of sigma-1 protein (data to be published). Having established the favorable binding affinity for sigma-1 receptor, cytotoxicity of the antipsychotic ligands was also performed using MTT assay compared with the standard antipsychotic (haloperidol) drug and the results are tabulated in Table 1. Cytotoxicity results revealed that among the three ligands studied, ligand **2** exhibited the high toxicity of 24.95% as compared to 22.72% and 18.96% exhibited by ligand **3** and **1** respectively.

Table 1. Cytotoxicity of antipsychotic ligands

Concentration /Sample No.	1	2	3	Haloperidol
25µg	7.839388	1.147228	14.88209	11.72721
50µg	10.99426	7.648184	15.67878	29.79605
75µg	14.37221	19.91714	16.69853	33.04653
100µg	18.96112	24.9522	22.72148	42.79796

As expected the toxicity of antipsychotic ligands increased with increase in concentration, which is depicted in Fig 2. C6 Rat glioma cell lines were treated with haloperidol and antipsychotic ligands. The treated cell lines and untreated control cell line samples were subjected to caspases and DNA fragmentation assay. DNA fragments were observed in the cells treated with haloperidol and ligand **3**. DNA laddering indicates the interaction of sigma-1 receptor and antipsychotic ligands (Fig 4). Increased level of caspase **3** expression in the treated glioma cells confirms the interaction of antipsychotic ligands to sigma-1 receptor as well as cytotoxicity of the ligands. Caspase levels were relatively high in cells treated with haloperidol and ligand 3 when compared to ligand **1** and **2** (Fig. 3). These chosen ligands act as antagonist for the activation of sigma-1 receptor involved in various psychotic diseases. The above mentioned results also suggest that the

antipsychotic ligands mediate cell death in cancer cells via sigma-1 receptors.

3. Conclusion

In conclusion, the present study establishes the interaction of sigma-1receptor with synthesized antipsychotic spiropiperidine derivatives through caspase assay and DNA fragmentation studies. Spiropiperidine ligand **2** displayed high toxicity of 24.95% as compared to 22.72% and 18.96% exhibited by the ligands 3 and 1, respectively. As expected, the cytotoxicity in C6 cell lines was observed in dose dependent manner for haloperidol and the antipsychotic ligands. These ligands act as antagonist for the sigma-1 receptor that is proposed to be involved in various psychotic diseases. Further the present study also suggests that the antipsychotic ligands mediate cell death in cancer cells via sigma-1 receptors. The obtained results clearly indicate that spiropiperidine ligand **3** would be a potential candidate for anticancer activity studies.

4. Materials and Methods

4.1. Antipsychotic ligands

Ligand 1: 3-Methylcarboxylate-(3,4- dihydrospiro [2- benzopyran])-1, 4' – piperidine (Scheme 1).

Ligand 2: 1'-Tertiarycarboxy carbonyl (3,4- dihydrospiro [(2) benzopyran-1,4'–piperidine]-3-methylcarboxylate (Scheme 1).

Ligand 3: 1'-Tertiary- Butoloxycarbonyl [(2) benzopyran-1, 4' – piperidine] -3- methylcarboxylic acid (Scheme 1).

The spiro piperidine derivatives (**Ligand 1, 2, 3**) synthesized in our laboratory were chosen for the validation of sigma-1receptor's interaction with ligands and compared with the standard sigma-1 antagonist (Haloperidol).

4.2. MTT Assay

Cytotoxicity analysis of the spiro piperidine derivatives were performed on C6 Rat glioma cells using MTT assay and compared with haloperidol as standard [12]. Approximately 5x10³cells/well (cell line) were seeded into 96 well plate, 100µl of DMEM medium was added and incubated at 37ºC. After 24h the medium was discarded and fresh medium containing 25, 50, 75 and 100µg of spiro piperidine derivatives (1, 2, 3) were added. The plates were incubated for 48h at 37ºC in a CO_2 incubator. Once again the medium was discarded after incubation period and fresh 100µl medium containing 10µl of 3-(4, 5-Dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide (MTT 5mg/ml) was added. After incubation at 37ºC in a CO_2 incubator for 4h, the medium was discarded and 200µl of DMSO was added to dissolve the formazon crystals. Then the absorbance was read in a microplate reader at 570nm and cell survival and cell toxicity were calculated by the following formulae.

- Viability % = (Test OD/Control OD) X 100
- Cytotoxicity % = 100 – viability%

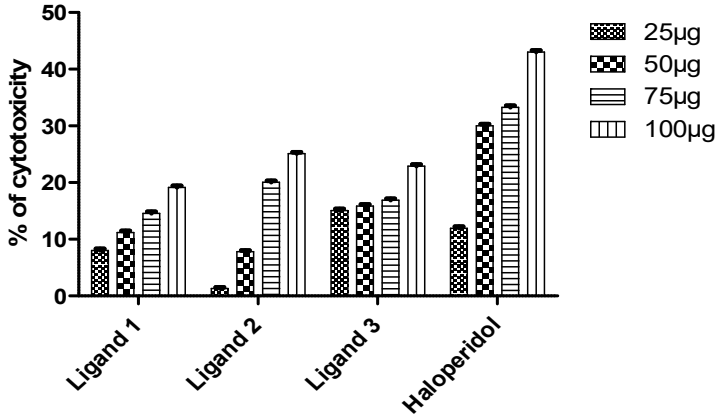


Fig 2. Cytotoxicity of 3 antipsychotic ligands in comparison to haloperidol at various concentrations. Y axis indicates the % of cytotoxicity of antipsychotic ligands and haloperidol. The graph represents dose dependent cytotoxicity effect. The results represent the mean ± SD (bars) values of independent experiments (n = 3).

4.3. CASPASE quantification assay

CASPASE activities by chromogenic assays were determined as per standard protocol using CASPASE-3 activation kits (Calbiochem, Merck). The cells were treated with various concentrations of spiro-derivatives (1, 2 3) and haloperidol and subjected to lysis using lysis buffer (50mM HEPES, 100mM NaCl, 0.1% CHAPS, 1mM DTT, 100mM EDTA). The resulting lysates were centrifuged at 10,000×g for 1 min and the supernatant was used to estimate protein concentration by Lowry's method using BSA as a standard. 100µg protein (cellular extracts) was diluted in 50 µl cell lysis buffer for each assay. Next, in 96-well microtiter plates the cellular extracts were incubated with 5 µl of the 4mM p-nitroanilide (pNA) substrates, DEVD-pNA (caspase-3 activity) for 2h at 37°C. CASPASE activity is the measure-ment of

free pNA formed by cleavage of the substrates at 405nm in a microtiter plate reader. Relative CASPASE-3 activity was calculated using the following formula were v- volume of sample in ml, d- dilution factor and t- reaction time in minutes

$$\text{Activity, µmol pNA/min/ml} = \frac{\text{ODxd}}{\varepsilon\text{mMxtxv}}$$

4.4. DNA Fragmentation assay

The occupancy of sigma-1 receptor with antipsychotic spiro-derivatives was characterized biochemically by the activation of a nuclear endonuclease which results in cleavage of DNA into multimers having approximately 180-200 base pairs that can be visualized as an 'oligosomal ladder' by standard agarose gel electrophoresis. Cells seeded in 24 well plate and kept in CO_2 incubator were treated with 100µg of antipsychotic ligands for 48h. At the end of incubation period, the cells were centrifuged for 1000rpm for 3mins at 14°C. The pellet was resuspended in a lysis buffer (10 mMTris-HCI, pH 8.0, 10 mMNaCl, I0 mM EDTA, 20mg/ml Proteinase K, 10% SDS), and incubated at 37°C.

The DNA was extracted by phenol-chloroform method, precipitated overnight at -20°C in ethanol containing 0.3 M final concentration sodium acetate. Then the pellet was dissolved in TE buffer (0.1 M Tris-HCl, pH 8.0, 10 mM EDTA). DNA samples were electrophoretically separated on 2% agarose gel containing ethidium bromide (0.4µg/mL). The UV (302 nm) transilluminator was used for visualization of DNA (Emmy P. Rogakou*et al*, 2000; Gavriel*iet al*, 1992). Untreated cells were used as control while haloperidol treated cells were used as positive control.

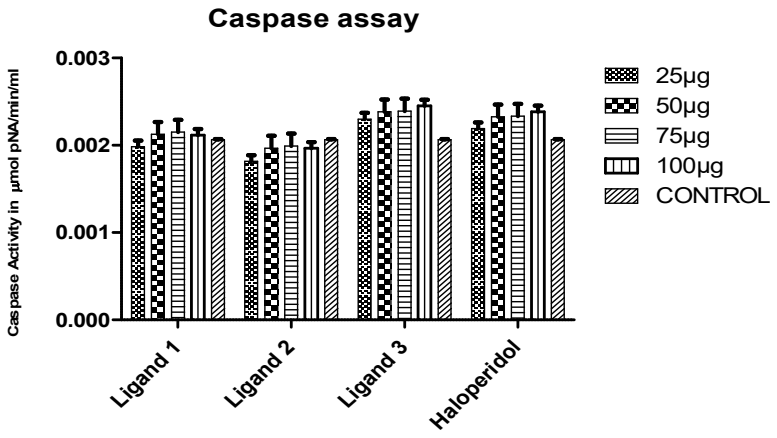
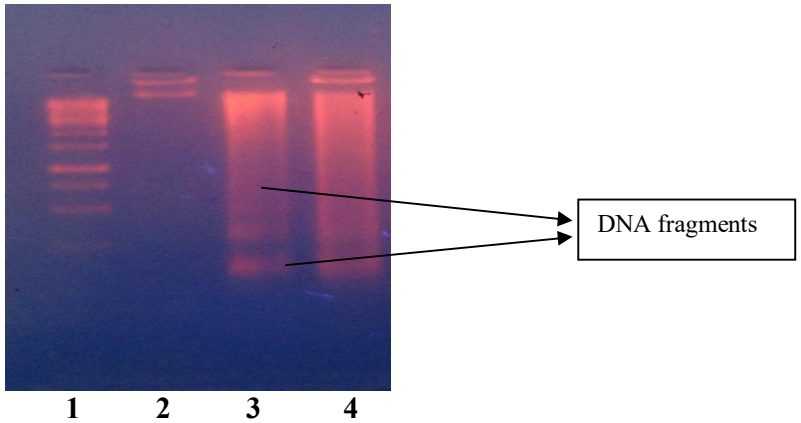
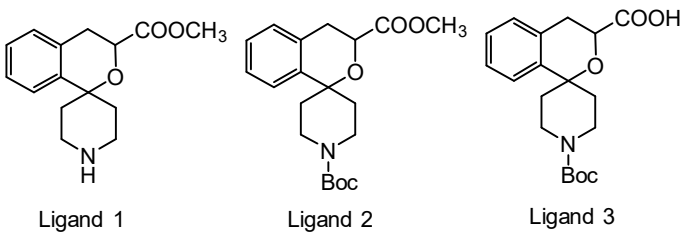


Fig 3. Level of Caspase expression on treated cells in comparison to haloperidol at various concentrations. Y axis indicates the caspase activity mediated via antipsychotic ligands and haloperidol. The graph represents cell death of cancer cells via antipsychotic ligands. The results represent the mean ± SD (bars) values of independent experiments (n = 3).



Lane 1: 1 Kb DNA ladder
Lane 2: DNA from control cells
Lane 3: DNA from cells treated with Haloperidol
Lane 4: DNA from cells treated with Antipsychotic ligand 3

Fig 4. DNA laddering pattern of Antipsychotic ligand (3) and haloperidol treated rat glioma cells in comparison to DNA of non-treated cells. Lower fragments in Lane 3 and 4 represents fragmentation of DNA via sigma receptors



Scheme 1. Antipsychotic ligands.

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