Esmethadone (REL-1017) Compares With NMDA Receptor Antagonists in FLIPR-Calcium Assay

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INTRODUCTION

- N-methyl-D-aspartate receptor (NMDAR) channel blockers such as ketamine and esketamine are emerging as a new drug class with potentially rapid and effective antidepressant activity.
- However, the adoption of intravenous ketamine and intranasal esketamine has been limited by dissociative psychotomimetic effects requiring clinical patient supervision during and post-administration.
- Esmethadone (REL-1017; dextromethadone; DXT) is a low affinity, low potency NMDAR channel blocker. It binds to the MK-801 site of the NMDAR with low micromolar IC_{50} value.[1] Esmethadone has 20 fold lower affinity for mu opioid receptors (MORs) compared to levomethadone[2] and does not appear to contribute in a clinically meaningful way to the opioid effects of racemic methadone.[3,4] According to a recent DEA statement on racemic methadone, esmethadone "lacks significant respiratory depressant action and abuse liability".[5]
- In a recent phase 2 MDD trial, [6] esmethadone showed robust, rapid and sustained antidepressant effects and very favourable safety, tolerability and pharmacokinetic (PK) profiles in patients with inadequate responses to standard antidepressant treatments.

OBJECTIVES

- To characterize esmethadone *in vitro* functional effect on heterodimeric NMDA receptors, by calculating esmethadone IC_{50} values in presence of 10 μM L-glutamate, as well as by estimating K_B, the equilibrium dissociation constant, in FLIPR calcium assay.

METHODS

- Fluorometric imaging plate reader (FLIPR, Molecular Devices) cell-based assays was performed in 384 well plate format, using Fluo-4 fluorescent indicator of intracellular free calcium ion concentration.
- Assay buffer composition included 145 mM NaCl, 5 mM KCl, 2 mM $CaCl_2$, 1 g/liter D-(+)-glucose, 20 mM HEPES (pH adjusted to 7.3 with NaOH).
- Test items were added, without pre-incubation, together with indicated concentration of L-glutamate and 10 μM glycine, but in absence of magnesium.
- Area under the curve (AUC) values of fluorescence readings were measured for 5 minutes after L-glutamate addition, and normalized to readings obtained in presence of 10 μM L-glutamate plus 10 μM glycine (100%) and buffer (0%).
- In FLIPR concentration response curve (CRC) experiments, every test item was assessed at 11 final concentrations: 100-33-11-3.7-1.2 μM, then 412-137-46-15-5.1-1.7 nM. L-glutamate and glycine were both used at 10 μM final concentration in CRC experiments.
- Aptuit CHO cell lines, expressing human heterodimeric NMDA receptors, were used: hGluN1/hGluN2A, hGluN1/hGluN2B, hGluN1/hGluN2C and hGluN1/hGluN2D.
- Protein accession number of NMDAR subunits are:
  - hGluN1 NP_015566
  - hGluN2A NP_000824
  - hGluN2B NP_000825
  - hGluN2C NP_000826
  - hGluN2D NP_000827
- Four parameter logistic equation were used to calculate L-glutamate pEC_{50} or test item pIC_{50}.
- Operational equation for allosteric modulators [7,8] was created in Prism 8 (GraphPad) software to estimate K_B and α parameters for every test item:

$$Y = E_{MAX}\frac{\frac{\tau[A]}{EC_{50}(\tau+1)}}{\left(\left(\frac{[A]}{EC_{50}(\tau+1)}\right)+\left(\frac{\tau[A]}{EC_{50}(\tau+1)}\right)\right)*\left(1+\frac{\alpha[B]}{K_B}\right)+\frac{[B]}{K_B}+1}$$

Y is % effect of L-glutamate in presence of test item. [A], E_{MAX}, EC_{50} and τ (efficacy value) are L-glutamate parameters, while [B], K_B and α are test item parameters, corresponding to test item molar concentration, estimated test item equilibrium dissociation constant, and α or cooperativity term, respectively.

RESULTS

Figure 1 - L-glutamate CRC

L- glutamate concentration response curve (CRC) was obtained to characterize the four NMDAR cell lines by calculating L-glutamate EC_{50} in the different cell lines. L- glutamate CRC was performed in FLIPR assay in presence of 10 μM glycine, but in absence of magnesium, using CHO cell lines expressing different heterodimeric human NMDA receptors. L-glutamate CRC included following 10 final concentrations: 1 mM, 100 μM, 10 μM, 3.3 μM, 1.1 μM, 370 nM, 123 nM, 41 nM, 13.7 nM. 4.6 nM. L-glutamate EC_{50} resulted 0.25, 0.13, 0.087 and 0.034 μM on hGluN1/hGluN2A, hGluN1/hGluN2B, hGluN1/hGluN2C, hGluN1/hGluN2D receptors, respectively.

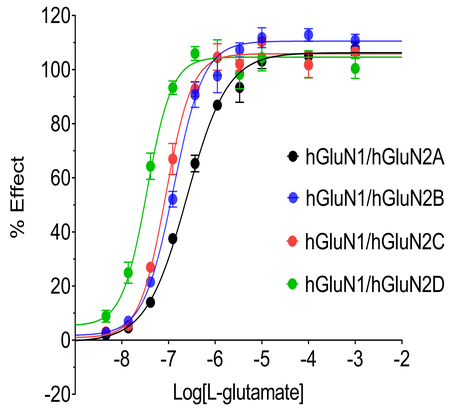

Figure 2 - Esmethadone CRC

Esmethadone CRCs were performed to calculate IC_{50} values in FLIPR assay, relative to four different heterodimeric human NMDA receptors: hGluN1/hGluN2A, hGluN1/hGluN2B, hGluN1/hGluN2C, hGluN1/hGluN2D. Esmethadone IC_{50} is esmethadone concentration able to induce a 50% reduction of the effect elicited by a selected agonist in a selected assay. We used 10 μM L-glutamate and 10 μM glycine as co-agonists, and we measured intracellular calcium levels by FLIPR calcium assay. Esmethadone CRC traces in FLIPR calcium assay are shown here below, while calculated IC_{50} values are reported in Table 1.

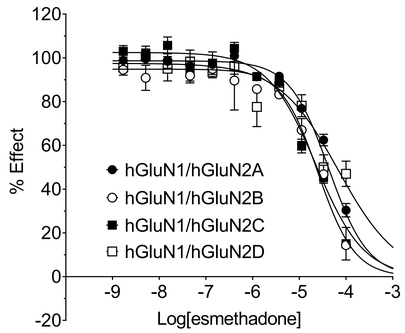

Figure 3 - Esmethadone effect on L-glutamate CRC

Esmethadone effect on L-glutamate CRC was obtained in FLIPR calcium assay, to estimate esmethadone K_B. Esmathadone K_B is the apparent equilibrium dissociation constant of the esmethadone-receptor complex, that is esmethadone concentration required to occupy 50% of the total NMDA receptor population. Below graphs are showing L- glutamate CRCs, alone (◆) or in presence of 6 different concentrations of esmethadone, (● 50 μM, ○ 12.5 μM, ■ 3.1 μM, □ 781 nM, ▲ 195 nM, △ 49 nM) using four different CHO cell lines expressing heterodimeric human NMDA receptor: hGluN1/hGluN2A, hGluN1/hGluN2B, hGluN1/hGluN2C, hGluN1/hGluN2D. Operational equation for allosteric modulators was used to estimate esmethadone K_B, and is reported in Table 2. Usourmontable profile of esmethadone is also apparent from below graphs, since inhibition induced by high concentration of esmethadone (e.g. ● 50 μM) cannot be surmounted even by agonist concentration as high as 1 mM L-glutamate.

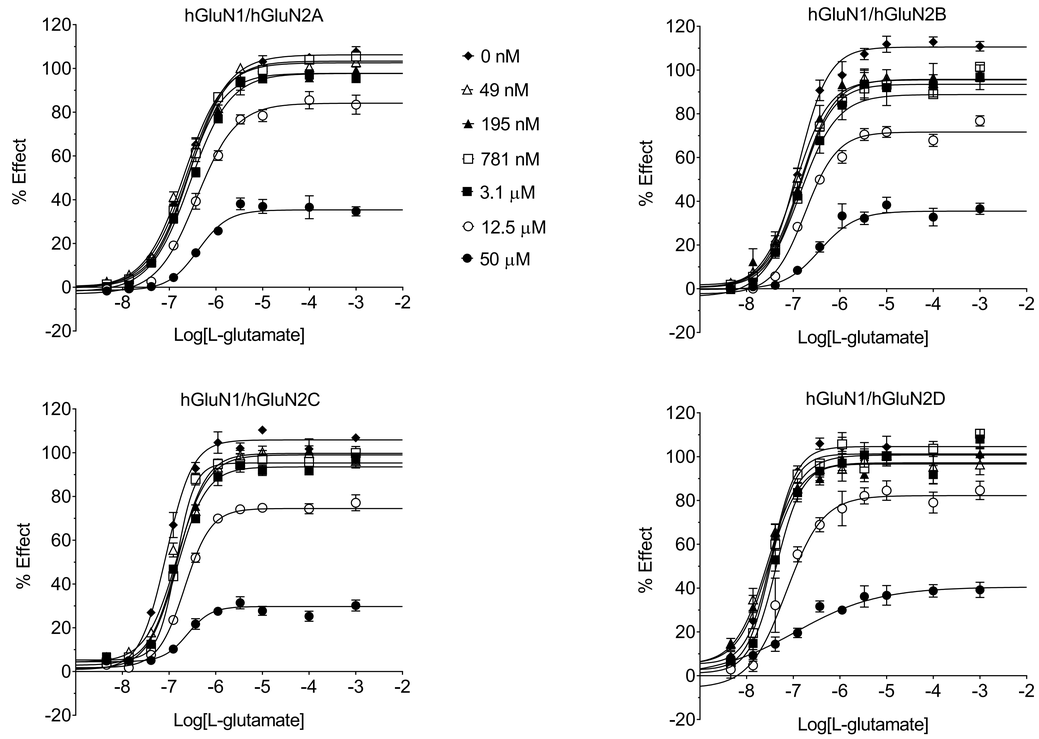

Table 1 - NMDAR channel blockers IC_{50} values

IC_{50} values of five selected NMDAR channel blockers obtained in FLIPR assay, as exemplified for esmethadone in Figure 2. An high IC_{50} value indicates a low potency for a test item, since it means that higher concentration of the test item are required to elicit a 50% inhibition of agonist response.

Test item IC_{50} (μM)	NMDAR type			
	hGluN1/ hGluN2A	hGluN1/ hGluN2B	hGluN1/ hGluN2C	hGluN1/ hGluN2D
Esmethadone IC_{50} (μM)	49	28	21	69
Memantine IC_{50} (μM)	35	8.8	3.3	6.9
(±)-Ketamine IC_{50} (μM)	27	6.3	3.4	11
(+)-MK 801 IC_{50} (μM)	0.28	0.061	0.57	0.78
Dextromethorphan IC_{50} (μM)	51	15	5.8	27

Table 2 - NMDAR channel blockers estimated K_B values

Estimated K_B values for five NMDAR pore blocker obtained in FLIPR by L- glutamate CRCs, alone or in presence of 6 different concentrations of test item. Experiments were conducted for the various test items as exemplified in Figure 3 for esmethadone. Operational equation for allosteric modulators was used to estimate esmethadone and other test items K_B, using the formula described in Methods section.

Test item K_B (μM)	NMDAR type			
	hGluN1/ hGluN2A	hGluN1/ hGluN2B	hGluN1/ hGluN2C	hGluN1/ hGluN2D
Esmethadone K_B (μM)	8.9	6.1	4.5	7.8
Memantine K_B (μM)	3.6	0.58	0.28	0.59
(±)-Ketamine K_B (μM)	4.3	1.1	0.46	1.4
(+)-MK 801 K_B (μM)	0.11	0.048	0.14	0.15
Dextromethorphan K_B (μM)	9.6	1.9	1.2	6.7

CONCLUSIONS

- Esmethadone inhibited NMDAR response to 10 μM L-glutamate in all four different tested receptor combinations, with similar potency (IC_{50} values). Esmethadone resulted with following IC_{50} rank order: hGluN2C≤hGluN2B≤hGluN2A≤hGluN2D (Figure 2 and Table 1).
- Remaining test items, i.e. (±)-ketamine, memantine, (+)-MK 801, (±)-ketamine, memantine and dextromethorphan, all showed IC_{50} values in line with their reported potencies, and with limited subunit preferences (Table 1).
- Esmethadone showed (Figure 3) an unsurmountable profile, when tested in presence of different L-glutamate concentrations, typical of NMDAR pore blockers.
- Esmethadone resulted (Table 2) with estimated K_B in the micromolar range with any of the studied NMDAR isoforms.
- Esmethadone potency range at different NMDARs, together with a favourable PK profile may be a key to its observed antidepressant effect, devoid of psychotomimetic side effects.

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DISCLOSURES

- This research was sponsored by Relmada Therapeutics, Inc. Drs. Inturrisi, Stahl, Pappagallo, and Manfredi are paid consultants for Relmada Therapeutics. Drs. Inturrisi and Manfredi are inventors on esmethadone patents and other patents and patent applications

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