Characterization of Esmethadone and Other NMDAR Channel Blockers on Human Heterodimeric N-methyl-D-aspartate Receptors

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INTRODUCTION

- Dysregulated Ca^{2+} currents via N-methyl-D-aspartate receptors (NMDARs) have been implicated in a multiplicity of diseases and disorders[1].
- Uncompetitive NMDAR channel blockers such as ketamine, memantine, and dextromethorphan are emerging as a new class of clinically tolerated drugs with demonstrated efficacy for the treatment of major depressive disorder (MDD), Alzheimer's disease, and pseudobulbar affect, respectively.
- Among different NMDAR channel blockers, unique molecule-specific interactions at NMDARs are likely to determine differential clinical effects[2].
- REL-1017 (esmethadone), a novel NMDAR uncompetitive channel blocker, is currently in Phase 3 clinical trials for MDD [6,7].

OBJECTIVE

- To characterize the NMDAR interactions of REL-1017 relative to other NMDAR channel blockers in clinical use.

METHODS

- CHO cells stably expressing recombinant diheteromeric human NMDARs, co-expressing hGluN1 with hGluN2A, hGluN2B, hGluN2C, or hGluN2D subunits were used in this study.
- Protein accession numbers were NP_015566, NP_000824, NP_000825, NP_000826, NP_000827 for GluN1, GluN2A, GluN2B, GluN2C, GluN2D, respectively.
- Fluorometric imaging plate reader (FLIPR, Molecular Devices) cell-based assays were performed in 384 well plate format, using Fluo-4 fluorescent indicator of intracellular free calcium ion concentration.
- Whole cell manual patch clamp electrophysiology was also used for REL-1017 IC_{50} determination, as well as for onset, offset, and trapped block parameters determination [3].

DISCLOSURES

This research was sponsored by Relmada Therapeutics, Inc. Drs. Folli, Stahl, Pappagallo, Inturrisi, Pani, and Manfredi are paid consultants of Relmada Therapeutics. Dr. Traversa is a current employee of Relmada Therapeutics. Drs. Bettini, De Martin, Mattarei, Carignani, Sgrignani, Locatelli, Cavalli, and Bifari are employed or have received fees from companies or Universities that have received payments or grants from Relmada. Drs. Inturrisi and Manfredi are inventors on esmethadone patents and other patents and patent applications.

RESULTS

Figure 1. REL-1017 CRC in FLIPR in presence of 10 mM L-glutamate and in absence of magnesium

REL-1017 CRCs were performed to calculate IC_{50} values in FLIPR calcium assay, relative to four different heterodimeric human NMDA receptors: hGluN1/hGluN2A, hGluN1/hGluN2B, hGluN1/hGluN2C, hGluN1/hGluN2D. Esmethadone IC_{50} is the esmethadone concentration able to induce a 50% reduction of the effect elicited by a selected agonist in a selected assay. We used 10 mM L-glutamate and 10 mM glycine as co-agonists, in absence of magnesium and we measured intracellular calcium levels with FLIPR. Calculated IC_{50} values are reported in Table 1.

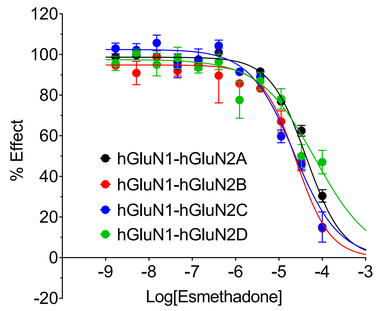

Figure 2. Esmethadone CRC in manual patch clamp in presence of 1 mM L-glutamate and 1 mM magnesium

Esmethadone was more potent in blocking NMDAR containing hGluN2D subunit (green dots and trace) in the below described manual patch clamp assay conditions. The graph represents % current recorded in the presence of 1, 3, 10, 30, or 100 µM esmethadone and normalized with respect to control, and relative fittings in four different NMDAR cell lines. Recordings were obtained at -60 mV, in presence of 1 mM L glutamate, 10 mM glycine, and 1 mM $MgCl_2$ at the end of a 120 s incubation period with L-glutamate and esmethadone. Data are mean ± SEM. IC_{50} and Hill slope values of every fitting are reported in Table 1.

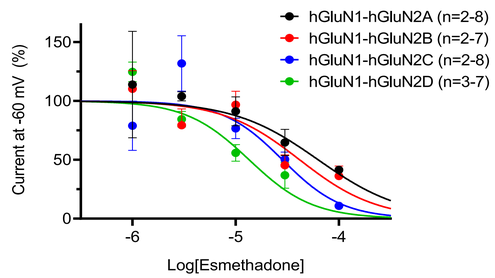

Table 1 - NMDAR channel blockers IC_{50} values by FLIPR or manual patch clamp

IC_{50} values of five selected NMDAR channel blockers were obtained in FLIPR assay, are shown in the lower portion of Figure 1. We also show (in green) IC_{50} values of esmethadone obtained in manual patch clamp (manual), as described in Figure 2. A low IC_{50} value indicates a high potency for a test item, since it means that lower concentrations of the test item are required to elicit a 50% inhibition of agonist response.

Test item	Assay	IC_{50} (µM) by NMDAR type			
		hGluN1-hGluN2A	hGluN1-hGluN2B	hGluN1-hGluN2C	hGluN1-hGluN2D
Esmethadone	Manual/ 1 mM MgCl2	63	42	28	14
Esmethadone	FLIPR	43	25	23	68
Memantine	FLIPR	34	10	3.6	7.3
(±)-Ketamine	FLIPR	30	6.3	3.4	11
(+)-MK 801	FLIPR	0.29	0.07	0.58	0.76
Dextromethorphan	FLIPR	51	15	5.2	28

Figure 3. Esmethadone onset and offset kinetic in manual patch clamp

CHO cell line stably expressing hGluN1/hGluN2C was used for this study. Recordings occurred at -70 mV fixed voltage equal to holding potential in absence of extracellular magnesium. A protocol was set up to evaluate how fast esmethadone blockade of NMDAR mediated currents can be established (onset kinetic), and how fast this blockade can be removed (offset kinetic), by perfusion with a buffer containing agonist L-glutamate, but devoid of esmethadone. Test item application protocol diagram (top) and sample traces (bottom) of test item onset and offset kinetic experiments with 10 µM esmethadone treated cell (left), or 1 µM (±)-ketamine treated cell (right). I_0 , I_1 and I_2 were the currents measured at the end of the first 5s 10 µM/10 µM L-glutamate/glycine application, the 30 s co-application with test item, the final 50 s co-agonists application, respectively. Current values obtained from different experiments during the onset and offset phases were normalized and averaged, to derive tau-on and tau-off parameters reported in Table 2.

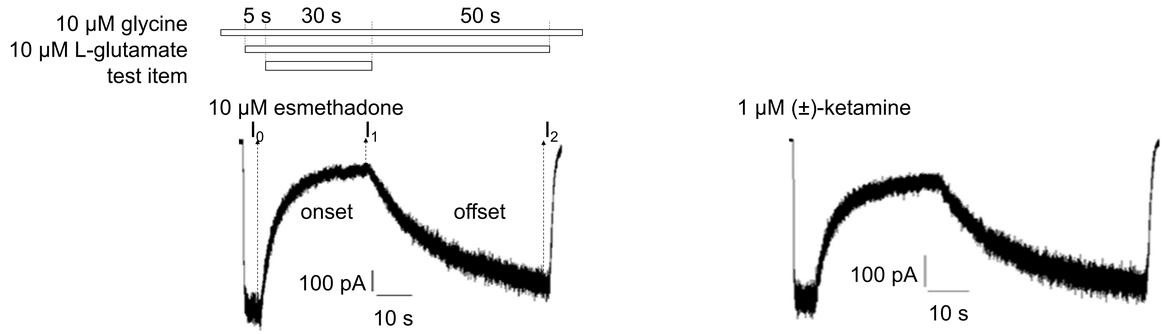

Figure 4. Esmethadone trapped block in manual patch clamp

Trapping experiments were designed to measure trapped block (B_T) of 10 µM esmethadone or 1 µM (±) -ketamine, that is % ratio between residual block (B_R) after extensive (85 s) cell wash with glycine alone and initial test item block (B), as previously described [1]. Test item application protocol diagram (top) and sample trace (bottom) of a trapping experiments with 10 µM esmethadone treated cell is shown.

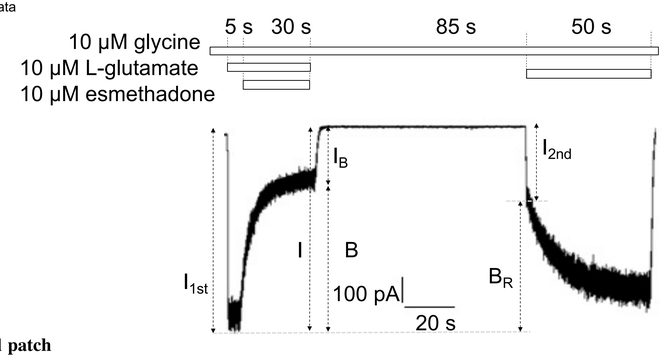

Table 2 - Onset, offset, and trapping parameters [2]

Onset kinetic constant (tau-on) of a NMDA receptor antagonist represents the time required for the test item to reach approximately 63.2% of its current blocking effect. Tau-on (s) is reported as mean ± SEM. **** is P<0.0001 from Tukey's test after one-way ANOVA. Offset kinetic constant (tau-off) of an NMDA receptor antagonist represents the time required for the removal of approximately 63.2% of test item current blocking effect, in continuous perfusion with a buffer containing the agonist L-glutamate, but not the test item. Tau-off (s) is reported as mean ± SEM. Tau-off values were not significantly different, by Tukey's test after one-way ANOVA. Trapped block (B_T) resulted similar comparing 10 µM esmethadone and 1 µM (±)-ketamine. Experiments were carried out as exemplified in Figures 3 and 4.

Test item	Tau-on (s)	Tau-off (s)	B_T (%)
10 µM esmethadone	4.7 ± 0.21	17.7 ± 1.0	85.9 ± 1.9
1 µM (±)-Ketamine	4.7 ± 0.14.	15.2 ± 0.6	86.7 ± 1.8
10 µM (±)-Ketamine	0.99 ± 0.05****	17.2 ± 3.0	Not determined

CONCLUSIONS

The presented in vitro studies of REL-1017 showed:

- GluN1-GluN2D subtype preference, in the presence of physiological concentration of Mg^{2+}, and of 1 µM L-glutamate
- Ketamine-like trapping
- Lower potency compared to other uncompetitive NMDAR channel blockers (5-10-fold lesser potency compared to ketamine)

The slow esmethadone onset kinetic and its preference for GluN1-GluN2D subtypes suggests that esmethadone and other uncompetitive channel blockers may modulate tonic ambient L-glutamate effects [2,4,5] with relative sparing of phasic NMDAR activity.

Esmethadone trapped parameters, similar to ketamine, might be functional to blocking the effects excessive ambient glutamate, which in turn might be relevant for its therapeutic potential in MDD.

The 5-10-fold lower potency of esmethadone compared to ketamine (FLIPR and manual patch results) might explain its lack of dissociative effects in healthy subjects[6] and in patients MDD[7].

Hyperactive GluN1-GluN2D NMDAR subtypes may be central to the pathophysiology of MDD and may be the target of NMDAR channel blockers with therapeutic efficacy as rapid acting antidepressants.

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