Supporting information

Role of the NMDA Receptor in the Antitumor Activity of Chiral 1,4-Dioxane Ligands in MCF-7 and SKBR3 Breast Cancer Cells

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Chemistry

General

Melting points were taken in glass capillary tubes on a Büchi SMP-20 apparatus and are uncorrected. IR and NMR spectra were recorded on PerkinElmer 297 and Varian Mercury AS400 instruments, respectively. Chemical shifts are reported in parts per million (ppm) relative to tetramethylsilane (TMS), and spin multiplicities are given as s (singlet), d (doublet), dd (double doublet), t (triplet), or m (multiplet). IR spectral data (not shown because of the lack of unusual features) were obtained for all compounds reported and are consistent with the assigned structures. Mass spectra were obtained using a Hewlett Packard 1100 MSD instrument utilizing electron-spray ionization (ESI). The microanalyses were recorded on FLASH 2000 instrument (ThermoFisher

Scientific). The elemental composition of the compounds agreed to within ±0.4% of the calculated value. Optical activity was measured at 20 °C with a Perkin-Elmer 241 polarimeter. When the elemental analysis was not included, crude compounds were used in the next step without further purification. All reactions were monitored by thin-layer chromatography (TLC) using silica gel plates (60 F254; Merck), visualizing with ultraviolet light. Chromatographic separations were performed on silica gel columns (Kieselgel 40, 0.040e0.063 mm, Merck) by flash chromatography. The term "dried" refers to the use of anhydrous sodium sulfate. Compounds were named following IUPAC rules as applied by ChemBioDraw Ultra (version 11.0) software for systematically naming organic chemicals.

Synthesis of the enantiomers (*R*)-(+)-1 and (*S*)-(-)-1

*(R)-(+)-(6,6-Diphenyl-1,4-dioxan-2-yl)methanamine [(R)-(+)-**1**].*

NaN_3 (0.13 g, 2.0 mmol) was added to a solution of (*S*)-(+)-**4**[23] (0.40 g, 0.94 mmol) in DMF (5 mL). The mixture was stirred at 100 °C for 3 h and then poured into H_2O and extracted with Et_2O. The organic layer was washed with brine, dried over Na_2SO_4 and concentrated in vacuo to give (*R*)-(+)-**5** (0.25 g, 95% yield), which was employed in the next step without further purification.

A solution of the azide (*R*)-(+)-**5** (0.25 g, 0.89 mmol) in Et_2O (10 mL) was added dropwise, at 0 °C, to a suspension of $LiAlH_4$ (0.36 g, 9.49 mmol) in Et_2O (10 mL). The mixture was stirred at room temperature for 4 h. The reaction was quenched with saturated Na_2SO_4 solution and filtered. After evaporation of the filtrate, the residue was purified by column chromatography eluting with $CHCl_3$/MeOH (95:5) to obtain (*R*)-(+)-**1** as a colorless oil (0.73 g, 78% overall yield): $[\alpha]_{20}^{D} = +48.0$ ($CHCl_3$). The ^1H NMR spectrum was identical to that of (±)-**1**.[24] The free base was transformed into the oxalate salt that was crystallized from EtOH: mp 211−212 °C, ESI/MS: m/z 270.1 ($M + H^+$), 296.2 ($M + Na^+$). Anal. Calcd for $C_{17}H_{19}NO_2 \cdot H_2C_2O_4$: C 63.50, H 5.89, N 3.90. Found: C 63.42, H 5.97, N 3.98.

*(S)-(-)-(6,6-Diphenyl-1,4-dioxan-2-yl)methanamine [(S)-(-)-**1**]*

This compound was prepared starting from (*R*)-(-)-**4**[23] following the procedure described for (*R*)-(+)-**1**. An oil was obtained: 79% overall yield; $[\alpha]_{20}^{D}$ = - 48.4 ($CHCl_3$). The ^1H NMR spectrum was identical to that of (*R*)-(+)-**1**. The free base was transformed into the oxalate salt that was crystallized from EtOH: mp 211−212 °C, ESI/MS: m/z 270.1 ($M + H^+$), 296.2 ($M + Na^+$). Anal. Calcd for $C_{17}H_{19}NO_2 \cdot H_2C_2O_4$: C 63.50, H 5.89, N 3.90. Found: C 63.36, H 5.79, N 4.01.

In vitro biological assays

Binding studies

Membrane Preparation and Cell Culture

The pig brains for the performance of the binding assay to the PCP-binding site of the NMDA receptor were a kind donation of the local slaughterhouse (Coesfeld, Germany). Homogenizers: Elvehjem Potter (B. Braun Biotech International, Melsungen, Germany) and Soniprep 150, MSE, London, UK). Centrifuges: Cooling centrifuge model Rotina 35R (Hettich, Tuttlingen, Germany) and High-speed cooling centrifuge model Sorvall RC-5C plus (Thermo Fisher Scientific, Langenselbold, Germany). Multiplates: standard 96-well multiplates (Diagonal, Muenster, Germany). Shaker: self-made device with adjustable temperature and tumbling speed (scientific workshop of the institute). Harvester: MicroBeta FilterMate-96 Harvester. Filter: Printed Filtermat Typ A and B. Scintillator: Meltilex (Typ A or B) solid state scintillator. Scintillation analyzer: MicroBeta Trilux (all Perkin Elmer LAS, Rodgau-Jügesheim, Germany).

Preparation of Membrane Homogenates from Pig Brain Cortex

Fresh pig brain cortex was homogenized with the potter (500-800 rpm, 10 up-and-down strokes) in 6 volumes of cold 0.32 M sucrose. The suspension was centrifuged at 1,200 x g for 10 min at 4 °C. The supernatant was separated and centrifuged at 31,000 x g for 20 min at 4 °C. The pellet was

resuspended in 5-6 volumes of TRIS/EDTA buffer (5 mM/1 mM, pH 7.5) and centrifuged again at 31,000 x g for 20 min at 4 C. The final pellet was resuspended in 5-6 volumes of buffer and frozen (−80 °C) in 1.5 mL portions containing about 0.8 mg protein/mL.

Protein Determination

The protein concentration was determined by the method of Bradford[32] modified by Stoscheck.[33] The Bradford solution was prepared by dissolving 5 mg of Coomassie Brilliant Blue G 250 in 2.5 mL of EtOH (95%, v/v). 10 mL deionized H_2O and 5 mL phosphoric acid (85%, m/v) were added to this solution, the mixture was stirred and filled to a total volume of 50.0 mL with deionized water. The calibration was carried out using bovine serum albumin as a standard in 9 concentrations (0.1, 0.2, 0.4, 0.6, 0.8, 1.0, 1.5, 2.0 and 4.0 mg/mL). In a 96-well standard multiplate, 10 μL of the calibration solution or 10 μL of the membrane receptor preparation were mixed with 190 μL of the Bradford solution. After 5 min, the UV absorption of the protein-dye complex at λ = 595 nm was measured with a plate reader (Tecan Genios, Tecan, Crailsheim, Germany).

Performance of the Binding Assays

The test compound solutions were prepared by dissolving approximately 10 μmol (usually 2-4 mg) of test compound in dimethyl sulfoxide (DMSO) so that a 10 mM stock solution was obtained. To obtain the required test solutions for the assay, the DMSO stock solution was diluted with the respective assay buffer. The filtermats were presoaked in 0.5% aqueous polyethylenimine solution for 2 h at room temperature before use. All binding experiments were carried out in duplicates in the 96-well multiplates. The concentrations given are the final concentration in the assay. Generally, the assays were performed by addition of 50 μL of the respective assay buffer, 50 μL test compound solution in various concentrations (10^{-5}, 10^{-6}, 10^{-7}, 10^{-8}, 10^{-9} and 10^{-10} mol/L), 50 μL of corresponding radioligand solution and 50 μL of the respective receptor preparation into each well of the multiplate (total volume 200 μL). The receptor preparation was always added last. During the incubation, the multiplates were shaken at a speed of 500-600 rpm at the specified temperature. The assays were terminated after 120 min by rapid filtration using the harvester. During the filtration,

each well was washed five times with 300 μL of water. Subsequently, the filtermats were dried at 95 °C. The solid scintillator was melted on the dried filtermats at a temperature of 95 °C for 5 min. After solidifying of the scintillator at room temperature, the trapped radioactivity in the filtermats was measured with the scintillation analyzer. Each position on the filtermat corresponding to one well of the multiplate was measured for 5 min with the [^{3}H]-counting protocol. The overall counting efficiency was 20%. The IC_{50}-values were calculated with the program GraphPad Prism® 3.0 (GraphPad Software, San Diego, CA, USA) by nonlinear regression analysis. Subsequently, the IC_{50} values were transformed into K_i-values using the equation of Cheng and Prusoff.[34] The K_i-values are given as mean value ± SE from three independent experiments.

The assay was performed with the radioligand [^{3}H]-(+)-MK-801 (22.0 Ci/mmol; Perkin Elmer). The thawed membrane preparation of pig brain cortex (about 100 µg of the protein) was incubated with various concentrations of test compounds, 2 nM [^{3}H]-(+)-MK-801, and TRIS/EDTA buffer (5 mM/1 mM, pH 7.5) at room temperature. The nonspecific binding was determined with 10 µM unlabeled (+)-MK-801. The K_d-value of (+)-MK-801 is 2.26 nM.

Biological studies on MCF-7 and SKBR3 cell lines

Cell lines

The MCF-7 e SKBR3 breast cancer cell lines used are from Type Culture Collection, ATCC, (Rockville, MD, USA). MCF-7 cells were cultured in RPMI 1640 supplemented with 10% FBS, 2 mM L- Glutamine, 100 IU/ mL penicillin, 100 µg streptomycin. SKBR3 cells were cultured in Dulbecco's modified Eagle's Medium (DMEM) supplemented with 10% FBS, 2 mM L- Glutamine, 100 IU/ mL penicillin, 100 µg streptomycin. Both cells lines were grown in incubator at 37°C, 5% CO_2 and 95% of humidity.

Reagents

DCFDA (10 µg/mL), DMSO (used as vehicle), NAC (10 mM), PI, SRB, trichloroacetic acid (TCA), acetic acid were from Sigma Aldrich (Sant Louis, MO, USA). JC-1 (10 µg/mL) was from

Invitrogen (Carlsband, CA, USA). Annexin V-FITC (5 µL/mL) was purchased from Enzo Life Sciences (Farmingdale, NY, USA).

Cell growth inhibition

To determine the effects of compounds **1-3**, (*S*)-(+)-ketamine, MK-801, enantiomer (*S*)-**1** and enantiomer (*R*)-**1** on MCF-7 and SKBR3 breast cancer cell lines, an SRB assay was performed. Cells were seeded at $5x10^3$ cells/well in a 96-well microplate. 24 h later, cultures were treated with increasing concentrations of compounds for 48 h at 37 °C in a 5% CO_2 atmosphere and 95% relative humidity. At the initiation of each experiment (t=0), and after drug treatments, 100 µL of 10% (w/v) TCA were added to each well, incubated for 1 h at 4°C, washed with deionized water, and dried at room temperature. One hundred microliters of SRB solution were added to each well, incubated for 10 min at room temperature, rinsed four times with 1% (v/v) acetic acid, and allowed to dry at room temperature. Finally, 100 µL of 10 mM Tris base solution (pH 10.5) was added to each well, and the absorbance was measured at 515 nm in a microplate reader (BioTek Instruments, Winooski, VT, USA). The absorbance at t=0 was compared with the absorbance at the end of the experiment to determine cell growth in treated cells compared with control cells. The antitumor activity was estimated by measurements of three parameters: Growth Inhibition 50 (GI_{50}), the drug concentration (μM) required to inhibit 50% net of cell growth; Total Growth Inhibition (TGI), the drug concentration (μM) required to inhibit 100% of cell growth; Lethal Concentration 50 (LC_{50}), the drug concentration (μM) required to kill 50% of the initial cell number.

NMDA receptor gene silencing

DSiRNATriFECTa kit hs.RI.GRIN1.13 was purchased from TemaRicerca (Castenaso, Italy), siCONTROL non-targeting siRNA (siGLO) used as negative control was purchased from Thermo Scientic-Dharmacon (Lafayette, CO, USA). For gene silencing experiments, MCF-7 and SKBR3 cells were plated at the density of 1.25 x 10^5 cells/mL and GRIN1 (or siNMDA R) or siGLO was added to the wells, following the TransiT-X2 Dynamic Delivery System transfection protocol (TemaRicerca).

RNA extraction and reverse transcription

RNA extraction was performed by the Single Shot Cell Lysis Kit (Milan, BioRad). Total RNA samples were eluted in the appropriate buffer. cDNA was synthesized using the High-Capacity cDNA Archive Kit (Applied Biosystems, Foster City, PA, USA) according to the manufacturer's instructions.

Quantitative real time polymerase chain reaction (qRT-PCR)

Quantitative RT-PCR was performed by using a IQ5 Multicolor real-time PCR detection system (BioRad, Hercules, CA, USA). The reaction mixture contained the RT2 SYBR R Green qPCRMastermix (Qiagen, Milan, Italy). Human NMDAR primers sequence: forward 5'-CCAGTCAAGAAGGTGATCTGCAC-3'; reverse 5'- TTCATGGTCCGTGCCAGCTTGA-3'. GAPDH primers sequence: forward 5'- AGAAAAACCTGCCAAATATGATGAC-3'; reverse 5'-TGGGTGTCGCTGTTGAAAGTC-3'. Primers were purchased from OriGene Technologies (Rockville, MD, USA). The PCR parameters used were 10 min at 95 °C followed by 40 cycles of 95 °C for 15 seconds and 60 °C for 40 seconds. All samples were assayed in triplicate in the same plate. The relative amount of target mRNA was calculated using GAPDH as a housekeeping gene.

Western blot assay

Cells were lysed in a lysis buffer (1 M Tris pH 7.4, 1 M NaCl, 10 mM EGTA, 100 mM NaF, 100 mM Na_3V0_4, 100 mM fluoridefenilmetansulfonile, 2% deoxycholate, 100 mM EDTA, 10% triton X-100, 10% glycerol, 10% SDS, 0.1 M $Na_4P_2O_7$) supplemented with a protease inhibitor cocktail (Sigma Aldrich). Lysates were separated on 6.5/12% SDS polyacrylamide gel and transferred onto Hybond-C extra membranes (GE Healthcare). Non-specific binding sites were blocked with 5% low-fat dry milk, 1% bovine serum albumin (BSA) in PBS with 0.1% tween 20 for 1 h at room temperature. Then membranes were incubated overnight at 4 °C in primary antibodies (Abs): anti-human caspase 3 (1:1000, Cell Signaling, Pero, Italy), anti-human NMDA R (1:1000, TemaRicerca) and anti-human GAPDH (1:5000, Sigma Aldrich), followed by the incubation (room temperature, 1 h) with HRP-conjugated anti-rabbit or anti-mouse secondary Abs. Peroxidase

activity was visualized with the LiteAblot ®PLUS and LiteAblot® TURBO (EuroClone, Milan, Italy) kits and densitometric analysis was carried out by a Chemidoc using the Quantity One software (BioRad).

Immunofluorescence and FACS analysis

Cells were fixed with 4% paraformaldehyde and then stained with anti-NMDA receptor Ab (1:100) in permeabilization buffer (PBS, 1% FBS, 0.1% NaN_3 and 1% saponin). After an incubation of 1 h at 4 °C, cells were then incubated with FITC-conjugated anti-rabbit Ab and analyzed using a FACScan cytofluorimeter with CellQuest software.

Mitochondrial transmembrane potential (ΔΨm)

Mitochondrial transmembrane potential (*ΔΨm)* was evaluated by JC-1 staining. Briefly, MCF-7 and SKBR3 cells were plated at the density of 1.25 x 10^5 cells/mL and treated for different time with (*S*)-**1**. At the end of the treatment, cells were incubated for 10 min at room temperature with 10 μg/mL of JC-1. JC-1 was excited by an argon laser (488 nm), and green (530 nrn)/red (>570 nrn) emission fluorescence was collected simultaneously. Carbonyl cyanide chlorophenylhydrazone protonophore, a mitochondrial uncoupler, was used as a positive control (data not shown). Samples were analyzed by a FACScan cytofluorimeter using the CellQuest software.

ROS production

The fluorescent probe DCFDA was used to assess oxidative stress levels. Briefly, MCF-7 and SKBR3 cells were plated at the density of 1.25 x 10^5 cells/mL and treated for different time with (*S*)-**1**. In some experiments, cells were pre-treated for 1 h with 10 mM NAC. At the end of treatments, cells were incubated with DCFDA (Life Technologies Italia) for 20 min prior to the harvest time point at 37 °C, 5% CO_2, and analyzed by FACScan cytofluorimeter using the Cell Quest software.

Cell death analysis

Cell death was evaluated using FITC-conjugated Annexin V and PI staining followed biparametric FACS analysis. Briefly, untransfected, siNMDA R and siGLO MCF-7 and SKBR3 cells were

plated at the density of 1.25 x 10^5 cells/mL. At the end of the treatments, cells were incubated with 5 µL Annexin V-FITC and 10 µL PI. The cells were than analyzed by flow cytometry using CellQuest software.

Statistical analysis

The statistical significance was determined by Student's t-test. The statistical analysis of GI_{50}, TGI, LC_{50} levels was performed using GraphPad Prism® 3.0. No statistically significant difference was found between untransfected and siGLO transfected MCF-7 and SKBR3 cells (data not shown).

In vitro ADME studies

The enantiomers (*R*)-**1** and (*S*)-**1** were evaluated for their *in vitro* ADME profile. For both compounds, hepatic *in vitro* intrinsic clearance, determined in rat and human liver microsomes, and permeability studies in Caco-2 cell line were assessed according to previously reported procedures.[30-32] The results, reported in Table S1, showed that in rat liver microsomes both compounds were highly cleared through cytochrome P450, although at higher extent for enantiomer (*S*)-**1** (145.4 mL/min/kg), than enantiomer (*R*)-**1** (77.7 mL/min/kg). Nevertheless, when evaluated in human liver microsomes, they both showed to be slowly metabolically cleared, with *in vitro* intrinsic clearance determined at 13.4 mL/min/kg and half-life greater than 139.8 min, therefore suggesting a potential for low *in vivo* hepatic clearance in humans.

When tested in Caco-2 cell line, both enantiomers showed good apparent permeability without relevant impact of P-gp efflux transporter, with efflux ratios measured at 1.07 and 0.73 for (*R*)-**1** and (*S*)-**1**, respectively.

Overall, apparent permeability from Caco2 cell lines and *in vitro* intrinsic clearance from liver microsomes suggest a potential for good oral bioavailability in human.

Table S1. ADME parameters of enantiomers (*R*)-**1** and (*S*)-**1**

		(*R*)-**1**	(*S*)-**1**
In vitro Hepatic Intrinsic Clearance (CLi) in Rat Liver Microsomes	CLi (μL/min/mg protein)	33.7	63.0
	CLi (mL/min/g Liver)	1.8	3.3
	CLi (mL/min/ kg)	77.7	145.4
	Half-life (min)	41.2	22.0
In vitro Hepatic Intrinsic Clearance (CLi) in Human Liver Microsomes	CLi (μL/min/mg protein)	9.90	9.90
	CLi (mL/min/g Liver)	0.500	0.500
	CLi (mL/min/ kg)	13.4	13.4
	Half-life (min)	>139.8	>139.8
Permeability[a]	Papp A-B (nm/sec)	156	195
	Papp B-A (nm/sec)	170	107
	Papp A-B inhibitor (nm/sec)	147	138
	Papp B-A inhibitor (nm/sec)	152	122
	Efflux Ratio (B-A/A-B)	1.07	0.73

[a]The P-gp inhibitor used in permeability studies is Elacridar 10 μM, Papp = passive membrane permeability, A-B = apical to basolateral, B-A = basolateral to apical

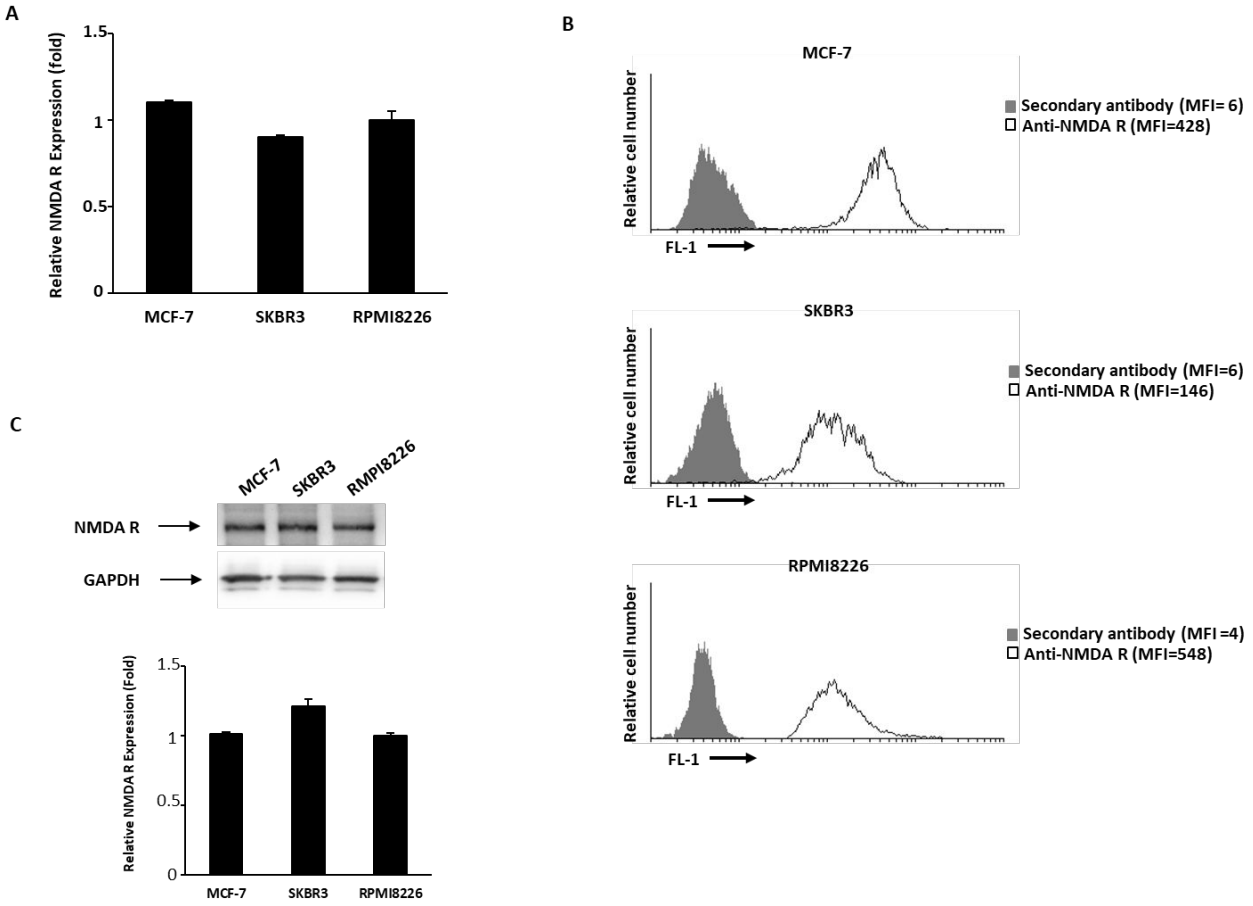

Figure S1. NMDA receptor expression in human MCF-7 and SKBR3 breast cancer cell lines. (A) Analysis of NMDA receptor gene expression was performed by quantitative RT–PCR. RPMI8226 was used as positive control. Relative NMDA receptor expression was normalized to GAPDH levels and calculated using $2^{-\Delta\Delta Ct}$ method. All the data shown are the mean ± SD of at least three separate experiments. (B) NMDA receptor expression was analyzed by flow cytometry on MCF-7, SKBR3 and RPMI8226 cell lines. Light lines represent NMDA receptor expression, gray histogram secondary antibody controls. MFI, mean fluorescence intensity. (C) A representative western blot analysis of NMDA receptor protein levels in MCF-7 and SKBR3 breast cancer cell lines. Densitometry values were normalized to GAPDH used as loading control.

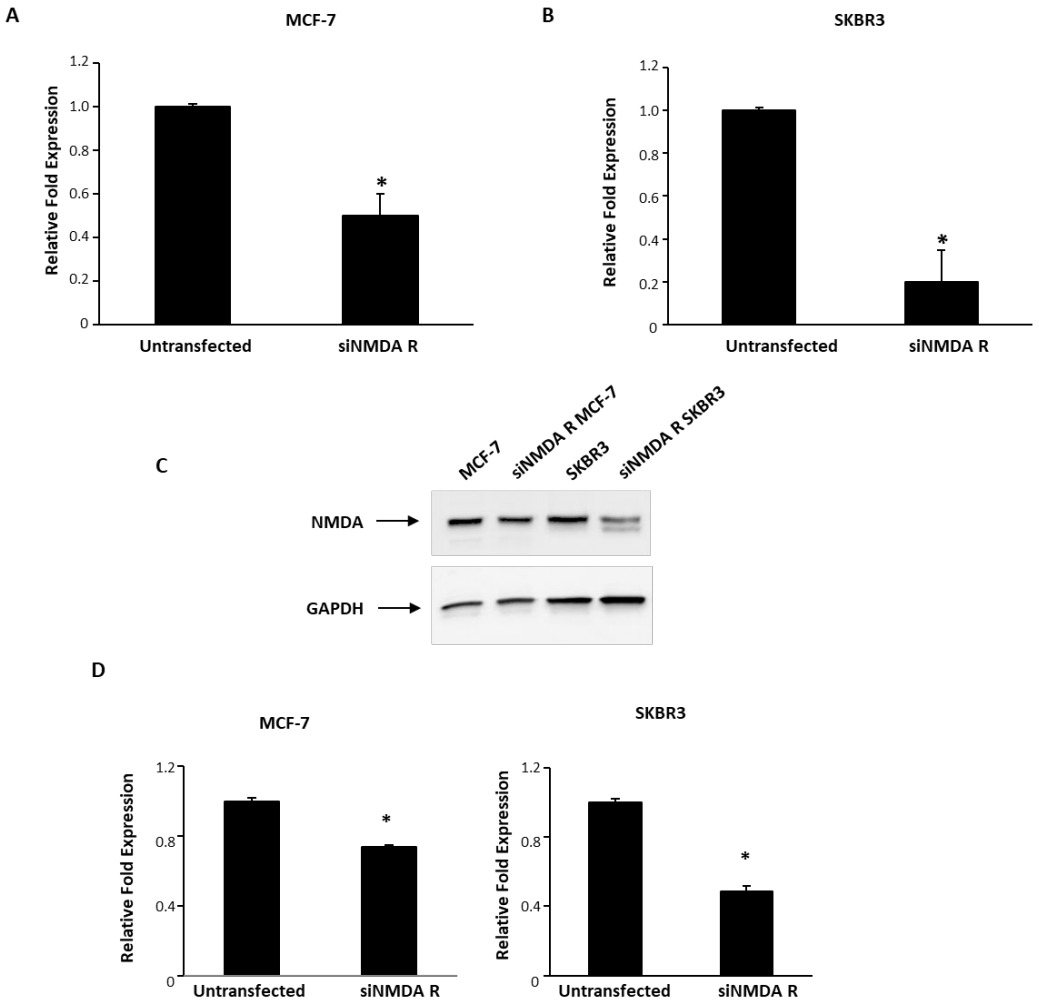

Figure S2. Silencing of NMDA GluN1 subunit in MCF-7 and SKBR3 cell lines. (A,B) NMDA receptor mRNA levels, evaluated by qRT-PCR, were normalized to GAPDH levels and calculated using $2^{-\Delta\Delta Ct}$ method. NMDA receptor protein levels were evaluated using western blot analysis (C) followed by densitometric analysis (D). GAPDH protein level was used as loading control. Data shown are the mean ± SD of at least three separate experiments. *$p<0.01$ siNMDA R transfected vs untransfected cells.

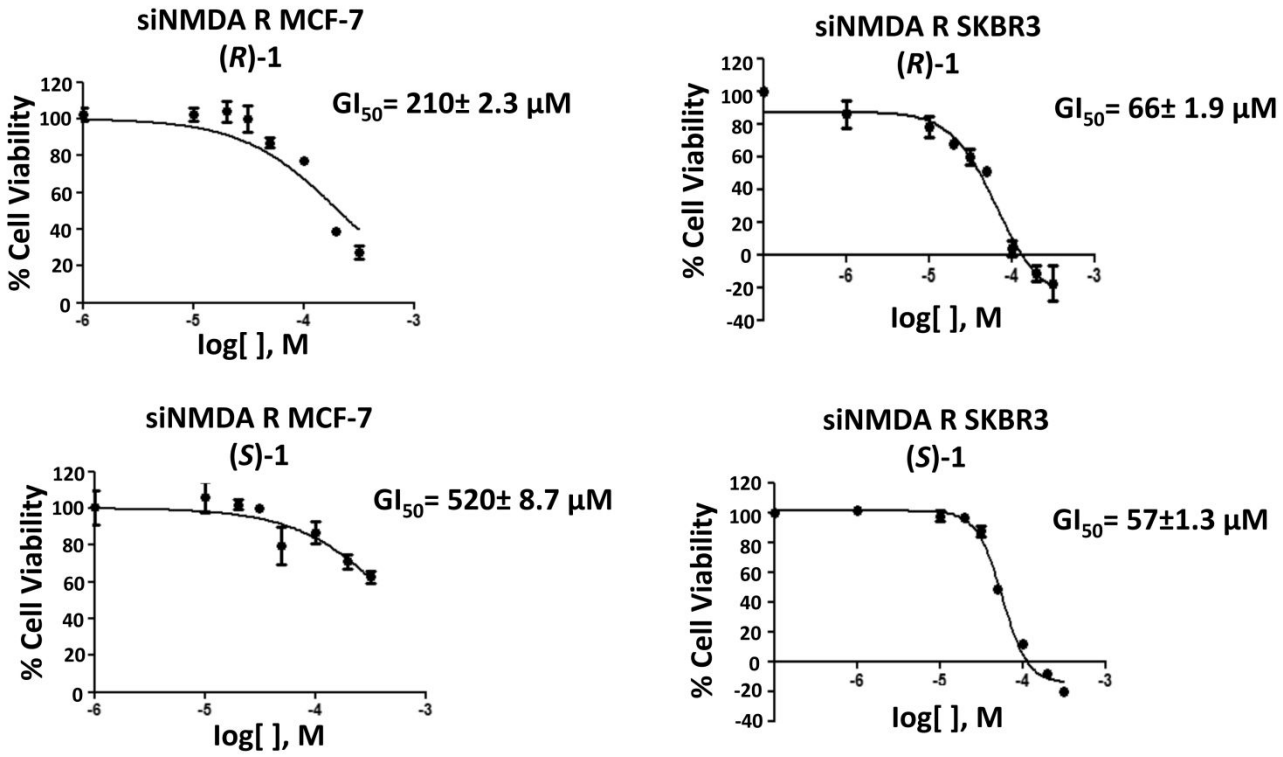

Figure S3. MCF-7 and SKBR3 siNMDA R transfected cells were treated for 48 h with different doses of (*R*)-**1** and (*S*)-**1**. Cell viability was determined by SRB assay. Data are expressed as mean ± SE of three separate experiments.

References

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