September 2014

AM-2232

Binding and Functional Activity at Cannabinoid CB1 Receptors

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DEA-VA Interagency Agreement Title: "In Vitro Receptor and Transporter Assays for Abuse Liability Testing for the DEA by the VA"

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Binding Assay Results for AM-2232

Receptor [^{3}H]Ligand	IC_{50} (nM)	K_i (nM) ± SEM	Hill Slope ± SEM
[^{3}H]CP-55,940 HEK-CB1 cell membranes	119 ± 32	63 ± 16	-1.29 ± 0.03

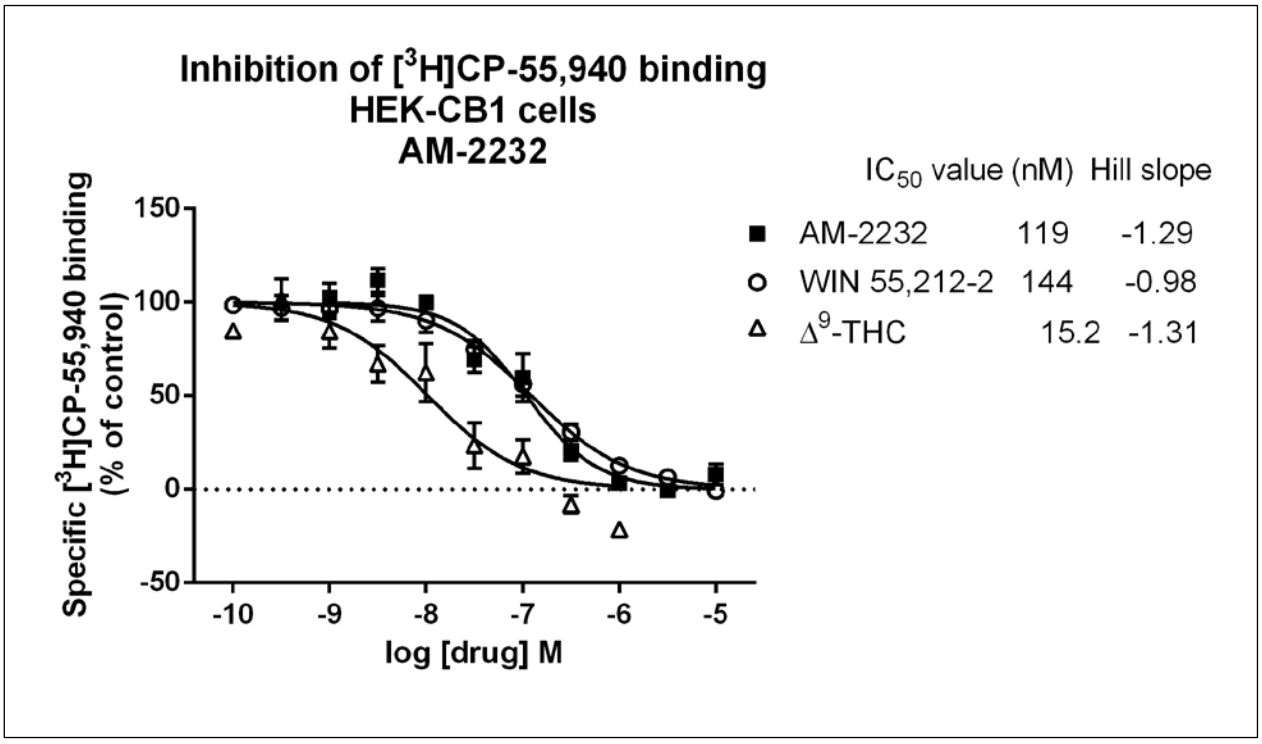
CB1 cannabinoid receptor binding and functional results

		Standard	Standard
HEK-CB1	**AM-2232**	**WIN 55,212-2**	**Δ^9-THC**
[^{3}H]CP-55,940 Binding Ki (nM)	63 ± 16	80.1 ± 6.3	8.3 ± 1.6
Hill coefficient	-1.29 ± 0.03	-0.98 ± 0.06	-1.31 ± 0.07
Number of experiments	3		
Adenylate cyclase	**AM-2232**	**WIN 55,212-2**	**Δ^9-THC**
Forskolin-stimulated cAMP			
Agonist inhibition EC_{50} (nM)	7.9 ± 1.0	32 ± 12	36 ± 16
Maximal effect (% WIN max)	80 ± 14%	95.4 ± 4.6%	74 ± 13%
Number of experiments	3		

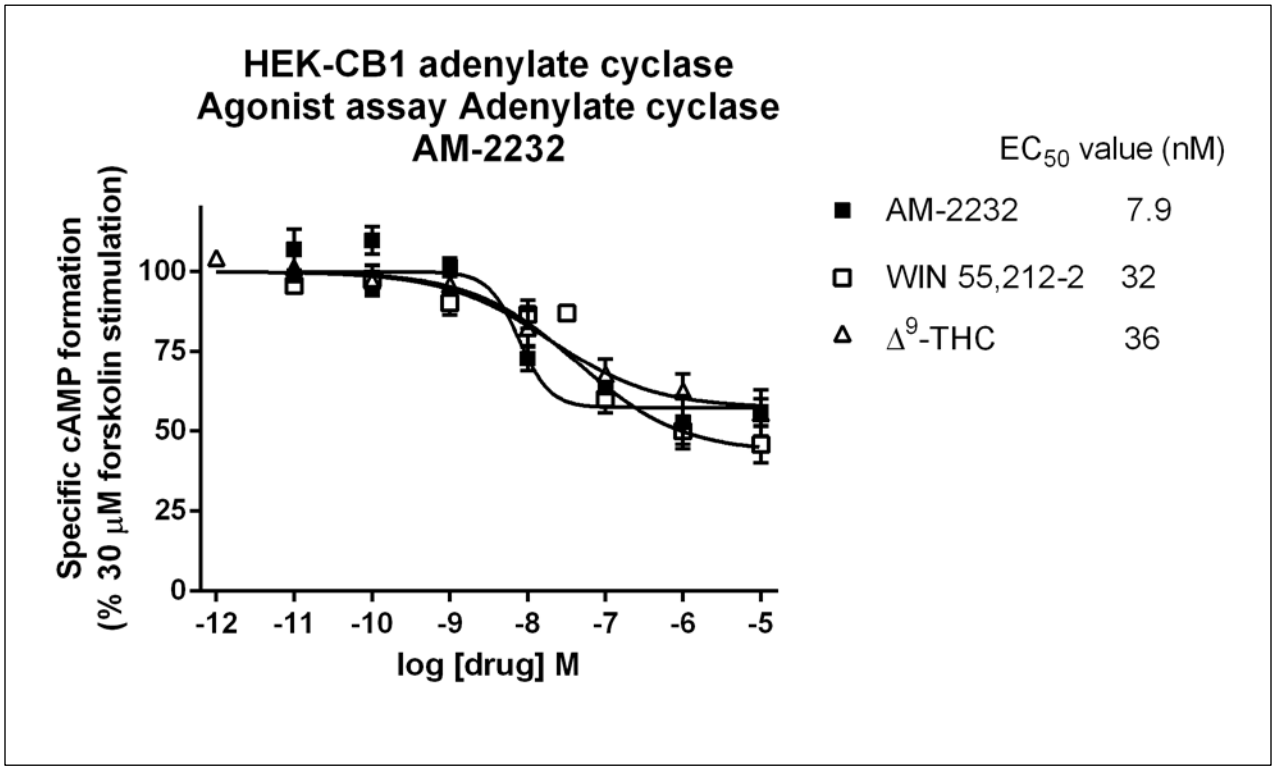
Numbers represent the means ± SEM from at least three independent experiments, each conducted with duplicate determinations. When the K_i or the IC_{50} for the AM-2232 is greater than 10 µM, only two experiments are conducted and no standard error or hill slope is reported. A Hill coefficient other than one suggests complex interactions with binding sites. The standards are (R)-(+)-WIN 55,212-2 and Δ^9-tetrahydrocannabinol (Δ^9-THC).

For the cAMP functional assays, adenylate cyclase activity is stimulated with 30 µM forskolin. CB1 agonists inhibit the forskolin-stimulated production of cAMP. Data for agonists presented in the table are normalized to the maximal inhibition of forskolin-stimulated cAMP formation by (R)-(+)-WIN 55,212-2, a full agonist.

CB1 receptor binding results



CB1 receptor functional results



Data are shown as percent of 30 μM forskolin-stimulated cAMP formation.

METHODS

Synthetic cannabinoids are weighed and dissolved in DMSO to make a 10 mM stock solution. An initial dilution to 25 μM in assay buffer or water for binding is made. Subsequent dilutions are made with assay buffer supplemented with DMSO, maintaining a final concentration of 0.1% DMSO in all wells. Pipetting is conducted using a Biomek 2000 robotic workstation.

Concentrations of **AM-2232** tested

Assay	Concentration Range
Binding:	
CB1	1 nM-10μM
Function:	
CB1	0.01 nM-10μM

1. CB1 receptor: [^{3}H]CP55940 binding assay:

Cell preparation: The methods were adapted from previously published methods [Farrens et al., 2003, J Peptide Res 60: 336-347]. HEK cells expressing the CB1 receptor served as the tissue source. The cells were grown until confluent on 15 cm plates. Media was removed, cells were washed with phosphate-buffered saline (PBS), scraped into 7 ml PBS and centrifuged at 10,000 rpm for 10 min. The pellet was resuspended in 5 ml hypotonic buffer (5 mM Tris, 2 mM EDTA, pH 7.4 at 4^{o}C with protease inhibitor III (Calbiochem)) and homogenized with a Polytron for 10 sec. The homogenate was centrifuged at 16,500 rpm for 20 min. The pellet was washed with 5 ml of assay buffer (20 mM Tris, 5 mM MgC_2, 2 mM EDTA, pH 7.4 with protease inhibitors), centrifuged at 16,500 rpm for 20 min, and covered with 2 ml of assay buffer and stored at -80^{o}C until used. Final resuspension volume was 3 ml of assay buffer per original cell culture plate.

The binding assay included drug or buffer, membrane preparation, [^{3}H]CP-55,940 (~1.3 nM) and assay buffer supplemented with 5 mg/ml bovine serum albumin (BSA) in a final volume of 500 µl. Specific binding was defined as the difference between total binding and binding in the presence of 10 µM WIN 55,212-2. The reaction was incubated for 1 hr at 30^{o}C, and terminated by filtration with ice cold assay buffer supplemented with 1 mg/ml BSA through Perkin Elmer A filtermats presoaked in 0.2% polyethylenimine using a Tomtec 96-well harvester. Radioactivity remaining on filters was counted in a Perkin Elmer microbetaplate reader. IC_{50} values were calculated using GraphPad Prism.

2. CB1 cannabinoid receptor: adenylate cyclase assay

The method was adapted from previously published methods for inhibition of adenylate cyclase by dopamine D4.4 receptor agonists [Janowsky et al., 2014, Psychopharmacology 231:2771-2783]. HEK-CB1 cells were plated at a density of 200,000 cells per well in 48 well plates in DMEM supplemented with 10% FetalClone and pen-strep. After ~36 hours, the medium was changed to DMEM supplemented with 10% charcoal-stripped FetalClone and pen-strep. The medium was removed ~18 hr later. For agonist assays, 0.8 ml EBSS (116 mM NaCl, 22 mM glucose, 15 mM HEPES, 8.7 mM NaH_2PO_4, 5.4 mM KCl, 1.3 mM $CaCl_2$, 1.2 mM $MgSO_4$, 1 mM ascorbic acid, 0.5 mM IBMX [3-isobutyl-1-methyl-xanthine] and 2% BCS, pH 7.4 at 37^{o}C) was added, cells were incubated 20 min, agonists were added, and, after 20 min incubation, 30 µM forskolin was added in a final volume of 1 ml. For antagonists, 0.7 ml EBSS was added, cells were incubated for 10 min, antagonists were added, cells were incubated 10 min, 2 nM WIN-55,212-2 (final) was added, and after 20 min incubation, 30 µM forskolin was added in a final volume of 1 ml. For all conditions, after 20 min incubation with forskolin, the reaction was terminated by aspiration of the buffer, and 0.1 ml 3% trichloroacetic acid was added. Plates were incubated for 2 hr on a rotator. Adenylate

cyclase activity was measured using a cyclic AMP EIA kit (Cayman). Aliquots (20-30 µl) of each well were diluted to 200 µl with EIA buffer from the kit, and 50 µl of the dilution was added to the EIA plate. After addition of tracer and antiserum, the EIA plates were incubated for 18 hr at 4^{o}C. The reaction was aspirated, plates were washed 5x300 µl with wash buffer, and Ellman's reagent was added. After two hour incubation in the dark on a rotator, the plates were read at 410 nm. Basal cAMP was subtracted from all values. CB1 receptor agonists inhibit forskolin-stimulated cAMP formation, maximal inhibition is defined with 10 µM WIN55212-2.

3. Data analysis: For binding, data were normalized to the binding in the absence of drug. Three or more independent competition experiments were conducted with duplicate determinations. GraphPAD Prism was used to analyze the ensuing data, with IC_{50} values converted to K_i values using the Cheng-Prusoff equation ($K_i=IC_{50}/(1+([drug^*]/K_d\ drug^*))$), where drug* is the labeled ligand used in the binding assays The K_d value used in the equation was 1.543 nM for [^{3}H]CP55,940 at CB1 receptors.

For functional assays, GraphPAD Prism was used to calculate either EC_{50} (agonists) or IC_{50} (antagonists) values, with data initially expressed as percent of forskolin-stimulated cAMP formation, as shown in the graph "CB1 receptor functional results." Maximal agonist effects were then normalized to the maximal inhibition by WIN-55,212-2 as reported in the tables.