EVALUATION OF THE *IN VIVO* AND *EX VIVO* BINDING OF NOVEL CB1 CANNABINOID RECEPTOR RADIOTRACERS

ASHLEY MILLER[A], JOHN GATLEY, PH.D.[B], AND ANDREW GIFFORD, PH.D.[B]

ABSTRACT

The primary active ingredient of marijuana, Δ9-tetrahydrocannabinol, exerts its psychoactive effects by binding to cannabinoid CB1 receptors. These receptors are found throughout the brain with high concentrations in the hippocampus and cerebellum. The current study was conducted to evaluate the binding of a newly developed putative cannabinoid antagonist, AM630, and a classical cannabinoid Δ8-tetrahydrocannabinol as potential PET and/or SPECT imaging agents for brain CB1 receptors. For both of these ligands *in vivo* and *ex vivo* studies in mice were conducted. AM630 showed good overall brain uptake (as measure by %IA/g) and a moderately rapid clearance from the brain with a half-clearance time of approximately 30 minutes. However, AM630 did not show selective binding to CB1 cannabinoid receptors. *Ex vivo* autoradiography supported the lack of selective binding seen in the *in vivo* study. Similar to AM630, Δ8-tetrahydrocanibol also failed to show selective binding to CB1 receptor rich brain areas. The Δ8-tetrahydrocanibol showed moderate overall brain uptake and relatively slow brain clearance as compared to AM630.

Further studies were done with AM2233, a cannabinoid ligand with a similar structure as AM630. These studies were done to develop an *ex vivo* binding assay to quantify the displacement of [131I]AM2233 binding by other ligands in Swiss-Webster and CB1 receptor knockout mice. By developing this assay we hoped to determine the identity of an unknown binding site for AM2233 present in the hippocampus of CB1 knockout mice. Using an approach based on incubation of brain slices prepared from mice given intravenous [131I]AM2233 in either the presence or absence of AM2233 (unlabelled) it was possible to demonstrate a significant AM2233-displacable binding in the Swiss-Webster mice. Future studies will determine if this assay is appropriate for identifying the unknown binding site for AM2233 in the CB1 knockout mice.

INTRODUCTION

The hemp plant, *Canabis sativa*, has been used for medicinal and recreational purposes for many centuries. Its popularity is derived primarily from its ability to alter mood and behavior. Known also as marijuana, the extracts of the hemp plant have a wide variety of therapeutic effects including the ability to act as an antiemetic, anti-inflammatory, antiglaucoma, analgesic, and appetite-enhancing agent (Felder, 1998). These physiological effects are mediated by the active compound in marijuana, Δ9-tetrahydrocannabinol (Δ9-THC), for which specific receptors were identified in the brain (Devane, 1988). High levels of these receptors were reported in substantia nigra, globus pallidus, hippocampus, and cerebellum. Other regions, including the brain stem and the thalamus, contain low or negligible concentrations of this receptor. These concentrations have been proven through *in vitro* autoradiographic studies with radiolabeled high-affinity ligands (Herkenham, 1990). Based on the areas of high concentration of CB1 receptors in the brain and the well-known behavioral effects of cannabinoid agonists (Mechoulam, 1986), it is likely that this receptor regulates short-term memory, coordination of movement and emotions.

The relatively high densities of the CB1 receptors in the brain have turned interests towards developing radiotracers capable of imaging CB1 receptors *in vivo* using PET or SPECT imaging. The imaging of these receptors *in vivo* would be of potential value in addressing several research questions. Such questions include determining the degree of occupancy of cannabinoid receptors necessary to produce therapeutic actions of cannabinoids, determining if new therapeutic agents posses significant binding to cannabinoid receptors *in vivo*, determining if cannabinoid receptors are up or down regulated as a result of chronic drug use or psychiatric conditions, and monitoring the loss of neuronal cell types possessing cannabinoid receptors.

The primary classes of chemical compounds that have been found to be active at CB1 cannabinoid receptors are the classical and non-classical cannabinoids, anandamides, aminoalkylindoles, and pyrazoles (Gifford, manuscript in preparation). To date, pyrazoles have been mostly targeted as lead compounds for the development of CB1 cannabinoid PET and SPECT radiotracers. These compounds are antagonists and/or inverse agonists at the CB1 receptor and are typified by SR141516A (Gifford, manuscript in preparation). Pyrazole derivatives developed so far for *in vivo*

A: University of Connecticut, Storrs, CT; B: Brookhaven National Laboratory, Upton, NY

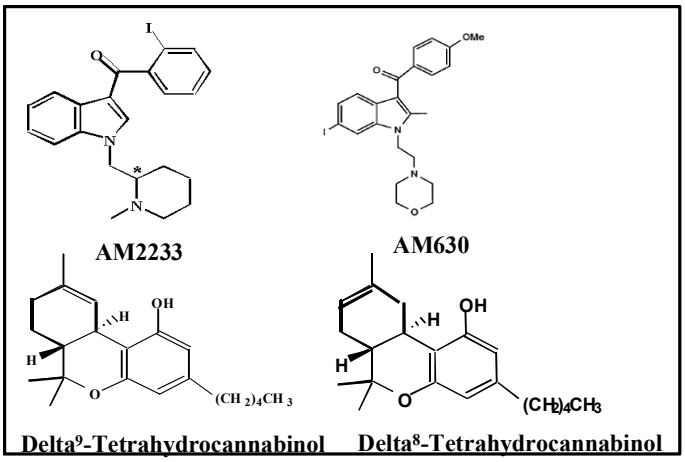

Figure 1: Structures of cannabinoid ligands evaluated in this study.

imaging have included [123I]AM281 for SPECT (Gatley, 1998) and [18F]SR144385 and [18F]SR147963 for PET (Mathews, 2000). These radiotracers have shown reasonable specificity in binding to CB1 receptors *in vivo*, but they have mediocre brain uptake which suggests they have limited potential as SPECT or PET radiotracers.

In addition to the pyrazole derivatives, the aminoalkylindoles have also shown promise for developing *in vivo* imaging agents for CB1 receptors. Similar to the pyrazole derivatives, the aminoalkylindoles have a relatively low lipophilicity compared to that of the classical and non-classical cannabinoids. However, where the pyrazole derivatives are antagonists/inverse agonists, the aminoalkylindoles are generally agonists (Compton, 1992). Because of this, the information obtained from these radiotracers will be different since agonist radiotracers will mostly bind to receptors in the high agonist affinity state where as the antagonists will bind to both high and low agonist affinity states. Inverse agonists bind preferentially to the low agonist affinity states.

One of the most potent aminoalkylindoles, developed to date, at the CB1 receptor is WIN 5512-2 (D'Ambra, 1992). This compound is useful in pharmacological and behavioral studies, but it lacks an iodine or fluorine group to make it useful for *in vivo* imaging of the CB1 receptor. The present study was conducted to evaluate a newly developed putative cannabinoid antagonist, AM630, based on an aminoalkylindole structure with an iodine group, and a classical cannabinoid, Δ8-THC, as imaging agents. Also, further study was done on AM2233, an aminoalkylindole with a higher affinity than WIN 5512-2, and an iodine group, making it potentially useful as an *in vivo* radiotracer.

MATERIALS AND METHODS

Male mice (Swiss-Webster strain) were purchased from Taconic Farms. CB1 receptor knockout mice were bred at Brookhaven National Laboratory. Federal guidelines for the care and use of animals were strictly followed. Studies were approved by the institutional review committee.

Δ8-THC was obtained from NIDA. AM630 and AM2233 were generously provided by Alexandros Makriyannis of the University of Connecticut.

Synthetic and Labeling Chemistry

[131I]AM630 and [131I]AM2233 were prepared by radioiododestannylation of their respective tributyltin precursors and purified by HPLC. AM2233 and its precursors were prepared as described by Deng (manuscript in preparation).

AM630 and Δ8-THC *In Vitro* Brain Uptake

Mice were injected via a tail vein with [131I]AM630 and [3H]Δ8-THC dissolved in .2 mL 40% 2-hydroxypropyl-β-cyclodextrin and 3-5% bovine serum albumin. Animals were killed by decapitation at 5, 15, 30 minutes, 1 and 2 hours, and the cerebellum, brain stem, and hippocampi were dissected out. These were weighed, solubulizer was added, after dissolving, scintillate was added and levels of [131I] were determined with a γ-counter. After the appropriate number of half-lives, the vials were again counted via liquid scintillation counting for [3H] levels.

Ex Vivo Autoradiography

Mice were injected via a tail vein with either [131I]AM630 or [131I]AM2233 dissolved in .2 mL 40% 2-hydroxypropyl-β-cyclodextrin. Animals were killed by decapitation after 40 minutes. The hippocampal region of the brain was dissected out, immersed in ice-cold saline, glued to a plastic block, and slices were cut 300 μm thick using a vibratome from the fresh brain tissue. After the sections were cut, they were placed on glass coverslips and allowed to air-dry on a slide warmer. Following drying, the sections were exposed to a phosphoimager plate (Molecular Dynamics) and the plates scanned after an exposure time of 12 –20 hours.

AM223 *Ex Vivo* Assay

Mice were injected via a tail vein with [131I]AM2233 dissolved in .2 mL 40% 2-hydroxypropyl-β-cyclodextrin. Animals were killed by decapitation after 30 minutes. The hippocampus was dissected out and was sliced in 300 μm slices using a tissue chopper. Then one hippocampi was add to a vial containing cold AM2233 and 5 mL of 50 mM Tris-HCL (pH 7.4), from now on referred to as buffer, and the other to a vial containing only 5 mL of the buffer. The vials were then counted in a γ-counter. After incubation in a 30°C water bath for 30 minutes, the vials were removed and either underwent filtration or centrifugation.

Filtration

After being removed from the water bath, each vial was added to a larger vial containing 10 mL of the ice-cold buffer. The smaller vial was rinsed with approximately 1 mL of ice-cold buffer, then the solution was homogenized using a tissue tearer for 20 seconds. The contents of the vial were then sucked up into a 20 cc syringe and filtered through a glass fiber filter. The filter was then washed with 20 mL of ice-cold buffer, removed and placed in another vial. Scintillate was then added to the vial and after sitting for four hours, the vials were counted in a liquid scintillation γ-counter.

Centrifugation

After being removed from the water bath, each vial was added to a large centrifugation tube containing 10 mL of ice-cold buffer.

The smaller vials were rinsed with approximately 1 mL of ice-cold buffer, and then the solution was homogenized using a tissue tearer for 20 seconds. The tubes were then placed in the centrifuge and spun at 8000 rpms for 3 minutes. After they had been spun the supernatant was poured off and the pellet resuspended in 1 mL of ddH_2O. Then the solution was pipetted into a large vial, scintillate was added, and the vials were counted in a liquid scintillation counter.

RESULTS

Time-Course of Brain Uptake

Mice were given [131I]AM630 intravenously and the time course of brain uptake of the radiotracer followed Figure 2a. Maximal brain uptake of [131I]AM630 was already reached by the first sacrifice time point at 1 minute post-injection. Thereafter, radioactivity declined moderately rapidly, reaching about half of its peak value after 30 minutes. Uptake was not significantly higher in the hippocampus or cerebellum, areas with high densities of cannabinoid receptors, relative to the brain stem, an area with a low density of receptors.

Mice were given [3H]Δ8-THC intravenously and the time course of brain uptake of the radiotracer was followed in Figure 2b. Maximal brain uptake of [3H]Δ8-THC was reached by the first sacrifice time point 5-minute post-injection in the hippocampus and the brain stem, however the cerebellum did not reach maximal brain uptake until 30-minute post-injection. Uptake of [3H]Δ8-THC was moderate compared to [131I]AM630, and its clearance from the brain was relatively slow compared to [131I]AM630. Again the hippocampal and cerebellum values were similar to the values for the brain stem.

Ex Vivo Autoradiography

Mice were injected intravenously with [131I]AM630 and sacrificed at 30-minute time points after radiotracer administration (Figure 3a). *Ex vivo* autoradiography showed generally a uniform brain distribution, with only a very weak indication of localization to CB1 receptor brain areas, as suggested by *in vitro* binding assays.

Mice were injected intravenously with [131I]AM2233 and sacrificed at 20- and 40-minute time points after radiotracer administration (Figure 3b). *Ex vivo* autoradiography indicated a strong regional localization in the brain radioactivity that closely paralleled that of the brain CB1 receptor, as shown in Gifford (manuscript in preparation).

AM2233 *Ex Vivo* Assay

Mice were injected intravenously with [131I]AM2233 and sacrificed at 30 minutes after radiotracer administration. The hippocampi were then incubated with and without cold AM2233. The tissue was then either centrifuged or filtered to maintain the receptors. Centrifugation did not give a large signal to noise ratio (Figure 4a), as seen by the close values of total and nonspecific binding. Filtration, however, gave a two-fold difference between total and non-specific binding in Swiss-Webster mice.

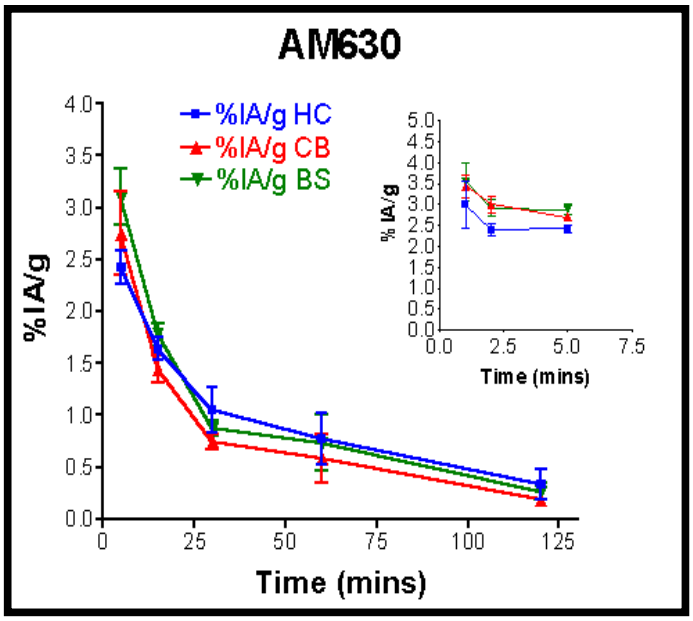

Figure 2a. Time activity curves.

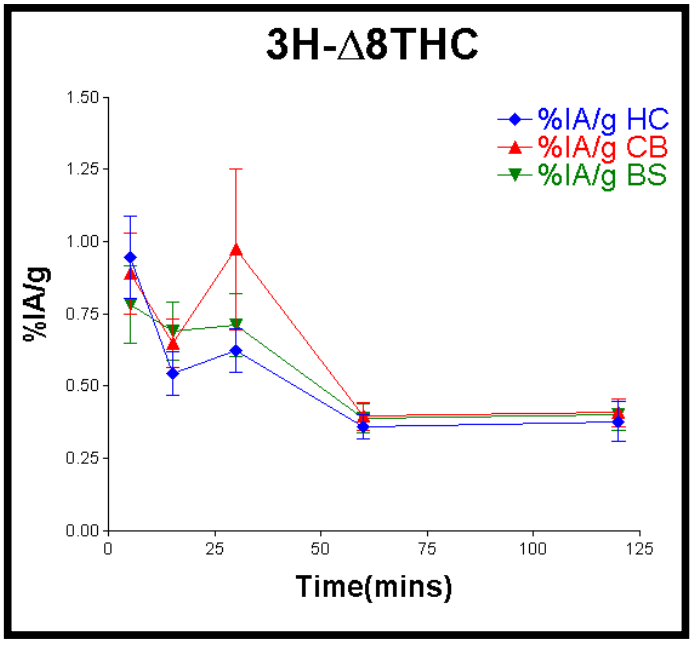

Figure 2b. Time activity curves.

DISCUSSION AND CONCLUSION

The present study was an attempt to show labeling of CB1 receptors using an aminoalkylindole cannabinoid antagonist, and a classical cannabinoid agonist. Earlier attempts by our group at labeling brain CB1 receptor with a cannabinoid agonist employed fluorine-18 labeled Δ8-THC. In both mice and baboon experiments, this radiotracer did not show selective localization in CB1 receptor-rich brain regions, presumably because of too low an affinity combined with a high non-specific binding. This proved to be true with tritium labeled Δ8-THC, as well.

The aminoalkylindoles have more suitability for labeling CB1 cannabinoid receptors *in vivo* than classical cannabinoids and

other classes of cannabinoid agonists because of their good receptor affinity combined with a significant lower lipophilicity. In earlier studies, AM2233 proved to be a good candidate for *in vivo* imaging because of its high CB1 receptor affinity combined with the fact that it possesses a SPECT-labelable iodine group (Gifford, manuscript in preparation). AM630 is an aminoalkylindole like AM2233 and also possesses a SPECT-labelable iodine group, however it did not show CB1 receptor affinity *in vivo* or in *ex vivo* autoradiography.

In the *ex vivo* biodistribution studies, [131I]AM2233 binding showed a distribution typical of that for binding to brain CB1 receptors, suggesting that binding was mostly or wholly to this receptor. In CB1 knockout mice, specific binding was largely absent although some weak binding did appear to be present in the hippocampus. However, no regionally selective binding of AM2233 was observed in *in vitro* autoradiography on cryostat cut sections from CB1 knockout mice and thus the cause of the weak hippocampal binding in *ex vivo* studies in these mice is unclear.

The development of an *ex vivo* binding assay was performed to help quantify the displacement of [131I]AM2233 binding by other ligands in Swiss-Webster and CB1 receptor knockout mice. By quantifying this displacement we will be able to begin studies to determine the identity of the unknown binding site for [131I]AM2233 present in the hippocampus of CB1 knockout mice.

In conclusion, though AM630 possesses the structure to be a SPECT-labelable radioligand, *in vitro* binding studies and *ex vivo* autoradiography showed that it lacks the affinity for CB1 receptors necessary to make it an ideal SPECT radiotracer. Δ8-THC also lacked the affinity necessary, and showed low brain uptake consistent with lipophilic classical cannabinoid agonists, which prevents it from being an ideal SPECT radiotracer. The *ex vivo* binding assay developed to quantify the displacement of [131I]AM2233 by unlabelled AM2233, however, showed promise with a two-fold difference in total and non-specific binding utilizing filtration in Swiss-Webster mice. Further studies in CB1 receptor knockout mice will be necessary to identify the unknown binding site for [131I]AM2233 in the hippocampus of CB1 knockout mice.

ACKNOWLEDGMENTS

I thank the United States Department of Energy for the opportunity to participate in the ERULF program at Brookhaven National Laboratory in Upton, New York.

I would also like to thank my mentors Dr. Andrew Gifford and Dr. John Gatley along with the entire staff at the Gatley-Gifford Laboratory. I would like to give a special thank you to Alexis DiMaio and Jordan Plieskatt for all their help this summer.

The research described in this paper was performed at Brookhaven National Laboratory, a national scientific user facility

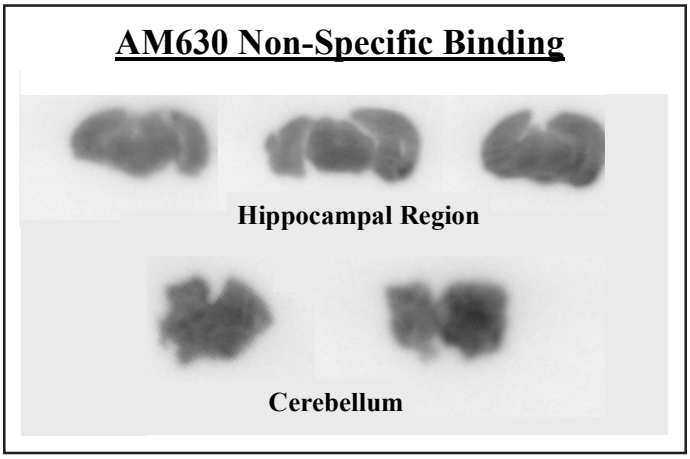

Figure 3a. Phosphoimager studies with AM630.

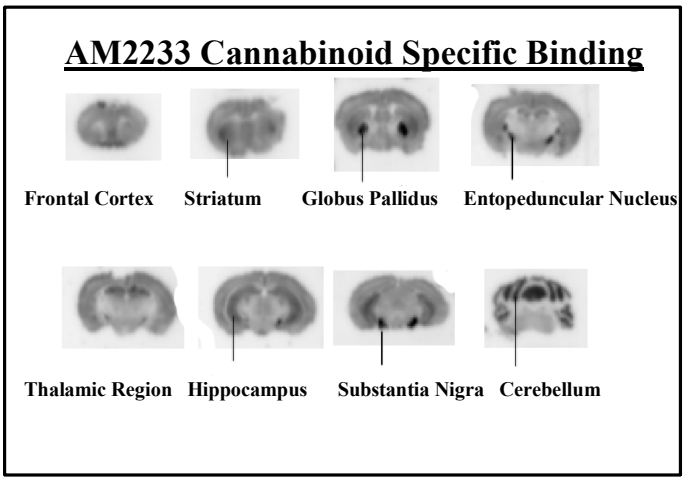

Figure 3b. Phosphoimager studies with AM2233.

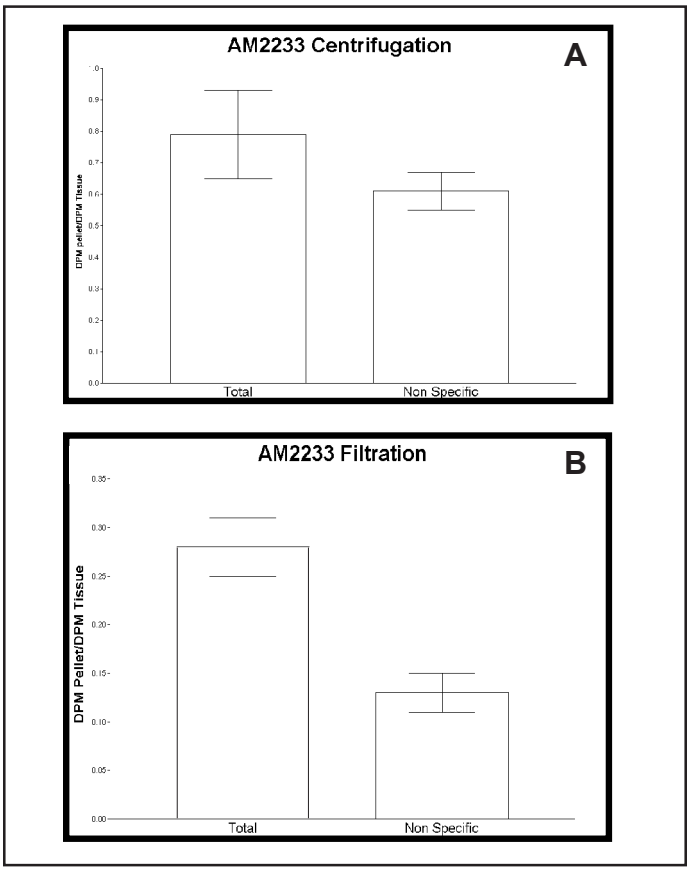

Figure 4. Development of an *ex vivo* binding assay for CB1 receptors.

sponsored by the United States Department of Energy's Office of Health and Environmental Research and located in Upton, New York.

REFERENCES

Compton Dr, Gold LH, Ward SJ, Balster Rl and Martin BR (1992) Aminoalkylindole analogs: Cannabimimetic activity of a class of compounds structurally distinct from delta 9-tetrahydroconnabinol. J.Pharmacol Exp Ther 263:1118-1126.

D'Ambra TE, Estep KG, Bell MR, Eissenstat MA, Josef KA, Ward SJ, Haycock DA, Baizman ER, Casiano FM, Beglin NC, et al. (1992) Conformationally restrained analogues of pravadoline: nanomolar potent, enantioselective, aminoalkylindole agonists of the cannabinoid receptor. J Med Chem 35(1):124-35.

Devane WA, Dysarz FA, Johnson MR, Melvin LS and Howlett AC (1988) Detmination and characterization of a cannabinoid receptor in the rat brain. Mol Pharmaco 34:605-613.
Felder CC, Glass M. (1998) Cannabinoid receptors and their endogenous agonists.Annu Rev Pharmacol Toxicol 38:179-200.

Herkenham M, Hynn AB, Little MD, Johnson MR, Melvin LS, DeCosta BR, Rice KC (1990) Cannabinoid receptor localization in brain. Proc Natl Acad Sci 87:1932-1936.

Mathews WB, Scheffel U, Fineley P, Ravert HT, Frank RA, Rinaldi-Caroma M, Barth F, Dannals RF (2000) Biodistribution of [18F] Sr 144385 and [18F] SR147963 selective radioligands for positron emission tomographic studies of brain cannabinoid receptors. Nuc Med and Bio 27:757-762.

Mechoulan R (1986) Cannabinoids as Therapeutic Agents. CRC Press, Boca Raton, Fl.