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## 2012 Division of Medicinal Chemistry Award Address: Trekking the Cannabinoid Road: A Personal Perspective

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### Abstract

My involvement with the field of cannabinoids spans close to three decades, and covers a major part of my scientific career. It also reflects the robust progress in this initially largely unexplored area of biology. During this period of time, I have witnessed the growth of modern cannabinoid biology, starting from the discovery of its two receptors and followed by the characterization of its endogenous ligands and the identification of the enzyme systems involved in their biosynthesis and biotransformation. I was fortunate enough to start at the beginning of this new era and participate in a number of the new discoveries. It has been a very exciting journey. By covering some key aspects of my work during this period of “modern cannabinoid research,” this perspective, in part historical, intends to give an account of how the field grew, the key discoveries, and the most promising directions for the future.

### History

On October 30-31, 1986, the National Institute on Drug Abuse organized a “Technical Review” event in Building 1 of the National Institutes of Health (NIH) campus in Bethesda, MD, whose purpose was to bring together scientists involved in the field of cannabinoids to discuss the most current research efforts. I had just received my first cannabinoid grant and was invited by its organizer, Dr. Rao Rapaka, who had recently joined the extramural program of the National Institute on Drug Abuse, to help put together such an event. It was generally felt that scientific activity in this important aspect of drug abuse was at a low point and such an event may help energize the field.

By all accounts, this effort by the National Institute on Drug Abuse (NIDA) was very successful in achieving its goals. Publication of the conference proceedings<sup>2, 17</sup> was followed by a number of key discoveries, including the identification, cloning, characterization and imaging of the CB1 receptor; the discovery of the key endocannabinoids, anandamide and 2-arachidonylglycerol, and the design and synthesis of novel ligands that enabled the elucidation of the cannabinoid biochemical system and

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established its major role in mammalian physiology. It is now universally recognized that cannabinoids are a very active research area. Also, because of its pleiotropic nature and its drug-friendly targets, the endocannabinoid system has excellent prospects in serving as a basis for drug discovery.

The three decades preceding this conference had witnessed a great deal of exciting work aimed at accessing the therapeutic properties of cannabis and its ingredients and developing novel therapeutic medications. This involved the development of new chemistries for the synthesis of terpenoid analogs with structural similarities to cannabis' endogenous constituents. The effort was led by Alexander Todd from University of Manchester<sup>35</sup> and Roger Adams from the Noyes Chemical Laboratory at the University of Illinois, Urbana-Champaign,<sup>37</sup> and produced new molecules with pronounced physiological effects when tested in different animal species. A major boost to the field was the isolation of the key bioactive constituent of cannabis and its subsequent synthesis by Raphael Mechoulam in Israel.<sup>38, 39</sup> This gummy non-crystallizable compound, which was identified as a tricyclic terpene encompassing a middle pyran ring, a phenolic hydroxyl, and a linear 3-pentyl side chain attached to the aromatic ring, was named (-)-<sup>1</sup>-tetrahydrocannabinol and later renamed (-)-<sup>9</sup>-tetrahydrocannabinol (<sup>9</sup>-THC). Its structure served as a prototype for additional synthetic efforts by a number of academic laboratories, including the Mechoulam, as well as the Razdan and Pars laboratories in Cambridge, MA.<sup>40</sup>

The above efforts were paralleled by significant programs within the pharmaceutical industry to develop cannabinoid-based medications principally as non-opioid effective analgesic agents. Notably, Lilly's efforts had led to the synthesis and development of the drug Nabilone,<sup>41</sup> which has been used by patients receiving cancer chemotherapy. Also, companies such as Abbott and Arthur D. Little Inc. were developing nitrogen containing analogs (eg. Nabitan) that were deemed to have more drug-like properties.<sup>40</sup> One of the major programs was undertaken at Pfizer in Groton, Connecticut. The effort for the discovery of cannabinoid analgesics was led by two talented medicinal chemists, Larry Melvin and Ross Johnson, whose work led to their first clinical candidate, Levonantradol,<sup>42</sup> a compound that was less lipophilic than the key phytocannabinoid <sup>9</sup>-THC, and also was 10- to 100-fold more potent in analgesia tests. In their systematic SAR, they had developed a series of analogs lacking the middle ring of the tricyclic terpenoid structure which they named non-classical cannabinoids,<sup>17</sup> the most prominent of which was CP-55940,<sup>17</sup> a 3 $\beta$ -hydroxycyclohexane phenol in which the 3-pentyl chain of <sup>9</sup>-THC was substituted with a 1',1'-dimethylheptyl chain.

Melvin and Johnson were among the participants of this historic 1986 event, where they described the detailed SAR obtained from testing the non-classical cannabinoids for their analgesic effects.<sup>17</sup> Their results underscored the remarkable correlation between analgesic potency with their respective absolute and relative stereochemistries,<sup>17</sup> as well as subtle structural modifications. The results argued for the existence of a specific site of interaction through which the new cannabinoids were producing their effects. Coincidentally, an interesting presentation during the meeting was from a young investigator, Allyn Howlett,<sup>17</sup> who had been testing some of the phytocannabinoids for their effects on modulating the levels of cAMP. She showed that the most biologically-active of these compounds,

including  $^9$ -THC, dose-dependently reduced the levels of cAMP, suggesting an inhibitory effect on the enzyme adenylyl cyclase. During that meeting, the Pfizer scientists offered to develop a tritiated analog from their non-classical cannabinoid series, which they provided to Howlett's laboratory. She and her graduate student William Devane tested a variety of synthetic cannabinoid analogs, in a heterologous displacement assay, using the non-classical Pfizer analog CP55940 as the radioligand, and obtained high quality sigmoid curves that accurately reflected the *in vivo* potencies of the compounds.<sup>43</sup>

These successful results were immediately followed by the autoradiographic imaging of the putative receptor in rat brain by Miles Herkenham at the NIH, using the same radioligand,<sup>28</sup> and its cloning by Lisa Matsuda in Tom Bonner's laboratory,<sup>44</sup> which was next to that of Herkenham's, thus allowing for a great deal of communication between the two laboratories.

My own involvement with cannabinoid chemistry and biochemistry had started in the early eighties, when the prevalent theory for cannabinoid activity was one invoking drug-induced specific membrane perturbations that modulated the properties of functional proteins within the cell membrane. Based on this approach, I was awarded a NIDA grant, which involved detailed structural and conformational studies aimed at developing correlations between ligand structure and function with their effects on model and cellular membrane systems. A turning point in my approach came when, in 1985, I heard a presentation by a Pfizer scientist describing their very extensive medicinal chemistry with their new cannabinoid-like ligands and the striking differences in the pharmacological properties based on subtle structural modifications. The results strongly argued for the presence of a yet to be identified functional protein, in all likelihood a G-protein coupled receptor.

With accumulating evidence for the existence of one or more cannabinoid receptors, I started a research program whose goals were to design and develop new biochemical and pharmacological tools that would allow us to characterize these new GPCRs and also decipher the circuitries of what was anticipated to be an intriguing and extensive biochemical system. An early effort in my laboratory to identify this putative receptor was through the design and synthesis of novel photoaffinity and electrophilic probes. This initial work by my student Avgui Charalambous led to a family of photoactivatable  $^8$ -tetrahydrocannabinol analogs designed to covalently attach to the receptor either through a carbene or an aliphatic nitrene mechanism.<sup>1</sup> One of these, 5'-azido-  $^8$ -THC<sup>1</sup>, was subsequently radioiodinated and served as the first covalent probe for the CB1 receptor.<sup>2</sup> This work was being carried out in collaboration with my colleague Sumner Burnstein, contemporaneously with the characterization of the CB1 receptor in the Howlett and Bonner laboratories. When rat brain membranes were allowed to photoreact with our  $^{125}$ I-labeled azido probe, the gel, when developed, showed two bands reflecting the presence of the corresponding proteins. This radiolabeling was prevented when the preparation was pretreated with  $^9$ -THC. The experiment provided the first chemical evidence for the presence of a ligand-cannabinoid receptor complex. Other information obtained from this early simple labeling experiment was the identification of a second, fainter band of lower molecular weight which allowed us to speculate on the presence of another cannabinoid receptor. This first photoaffinity labeling opened the door for future experiments in my

laboratory to use covalent chemistry to characterize the structural, biochemical and biophysical properties of the cannabinoid receptors.

The story of the cannabinoid biochemical system was further expanded by the identification of the second cannabinoid receptor, designated as CB2, and initially described as peripheral because of its presumed absence in mammalian brain.<sup>45</sup> More recently evidence was obtained for its CNS presence, albeit in low concentrations.<sup>46, 47</sup> We also know now that CB2 levels can be very significantly enhanced in non-homeostatic conditions such as inflammation, neurodegeneration and cancer.

## The AM Compounds

Through the use of target-based design and focused medicinal chemistry, over the past three decades, we have developed a wide variety of structurally-distinct analogs, the most interesting of which have found applications in pharmacology, biochemistry, and biophysics, as well as serving as early leads or advanced preclinical candidates for drug development. Under the AM acronym, many of these compounds are universally used by research laboratories. As pharmacological probes, AM compounds have played a substantial role in elucidating the physiological role of the endocannabinoid system. In this medicinal chemistry effort, we were assisted by an excellent collaboration with Marcus Tius at the University of Hawaii. Initial *in vivo* testing of key AM compounds were provided by a number of very productive collaborations with the laboratories of Toby Järbe (Northeastern University), John Salamone (University of Connecticut), Philip Malan (University of Arizona), Frank Porreca (University of Arizona), Andrea Hohmann (University of Indiana), Linda Parker (University of Guelph), and Keith Sharkey (University of Calgary).

## Endocannabinoids

What are the endogenous molecules that engage and activate the two receptors? The first endocannabinoid anandamide (AEA) was isolated from pig brain in Mechoulam's laboratory.<sup>48</sup> This was followed by the characterization of 2-arachidonylglycerol (2AG) as the second endogenous cannabinoid.<sup>49, 50</sup> We now know that the endocannabinoid system is modulated by a large family of structurally-related lipid mediators belonging to either the amide or ester families, all of which are involved in the activation of the cannabinoid receptors. Additionally, there is evidence that these same lipid molecules may also modulate other systems such as the vanilloid receptor 1 (VR1) and other transient receptor potential vanilloid (TRPV) channels, as well as the largely unexplored GPR55, GPR35, GPR18 and GPR119 receptors. This property of pleiotropic action by lipid modulators is an important feature of the cannabinoid system, and distinguishes it from other non-lipid neurotransmitters. Arguably, the levels of individual modulators, as an aggregate within a human or animal biological sample, may serve as a unique descriptor of the organism's physiological state. This realization prompted us to develop an accurate LC/MS/MS-focused lipidomic assay for the measurement of the larger family of these endocannabinoid lipids, which we named the "endocannabinoid metabolome."<sup>51-54</sup> This assay expands the family of endocannabinoid lipid modulators beyond its two major players AEA and 2AG, and affords us a more accurate description of the endocannabinoid system by providing quantitative

information on its individual components. Such measurements can find utility in identifying endocannabinoid fluctuations that are drug-related or the result of cannabinoid-associated pathologies, such as depression and schizophrenia, where they could serve as important biomarkers.

As mentioned above, the endocannabinoid family comprises a variety of ethanolamides and glycerol long chain fatty acid esters. However, among those, AEA,, also known as anandamide, and 2AG are the most potent and best studied. AEA and 2AG are substrates for the enzymes fatty acid amide hydrolase (FAAH) and monoacylglycerol lipase (MGL), respectively, as well as other hydrolytic enzymes, and consequently have relatively short *in vivo* half lives, a property that limits their usefulness as probes for the *in vivo* pharmacological characterization of the endocannabinoid system. An early goal in my cannabinoid journey was to develop more stable analogs, encompassing the key structural features of the endogenous ligands. Design of the metabolically-stable anandamides was based on introducing substituents in the vicinity of the sessile amide bond to impede access of the ligand to the enzyme's catalytic site. Alternatively, AEA can be modified in a manner which maintains all features required for the recognition of CB1 and CB2 receptors while impeding its fit within the enzyme's catalytic domain. These approaches led to the design of ligands with significantly enhanced stability toward the key deactivating enzyme FAAH. The first of these, AM356, an anandamide analog substituted at the headgroup with a chiral methyl group, and also known as *R*-methanandamide, was a successful probe with significantly higher metabolic stability and substantially improved CB1 potency.<sup>55-57</sup> AM356 has gained popularity among pharmacologists and is universally used as a surrogate for the endogenous AEA. A second generation of such probes was inspired by our early observation that the inverse amides or retroamide analogs of anandamide were significantly more resistant to enzymatic hydrolysis.<sup>57</sup> Experimentation around that concept resulted in the development of AM1346,<sup>58, 59</sup> a very potent and metabolically-stable anandamide, which we named “mahanandamide” or superanandamide, a designation contributed by Dr. Rapaka. Ongoing efforts in our laboratory toward the development of hydrolytically-stable 2AG have led to the dimethylated headgroup analog AM10336 (K. Vadivel and A. Makriyannis, unpublished).

## The Role of Cell Membrane in Cannabinoid Activity

Prior to the discovery of the cannabinoid receptors, the dominant postulate was that these compounds act by perturbing the cell membrane, thus, indirectly affecting the function of key membrane proteins. This concept coincided with my first entry into the cannabinoid field, during which time we adapted or developed a variety of biophysical methods to study the effects of cannabinoids on model and natural membranes. The methods included solution<sup>60, 61</sup> and deuterium solid state NMR,<sup>29, 62</sup> as well as X-ray<sup>31, 62, 63</sup> and neutron small angle diffraction.<sup>64, 65</sup> These approaches, which were initially used to study the molecular mechanism of cannabinoid action, were subsequently directed to a more general program aimed at understanding the interaction of lipophilic and amphipathic ligands, including cannabinoids and endocannabinoids with cell membranes.

Because of their lipophilic properties, cannabinergic compounds have very limited aqueous solubility, raising the question of how they can access their sites of action within the respective membrane-bound proteins? Our studies provided evidence that cannabinoids and amphipathic ligands in general, assume a preferred orientation within the membrane bilayer.<sup>9, 33, 66, 67</sup> In early works, we introduced the concept that amphipathic cannabinoids favorably partition into the membrane and, through fast lateral diffusion, interact with the receptor.<sup>9, 66, 67</sup> We postulated that the ligand aligns itself within the bilayer, in a favorable orientation and conformation which optimizes its ability for a productive interaction with the respective functional protein.<sup>9, 67</sup> To provide experimental evidence for our postulate, we used high-resolution NMR to study the conformational properties of select cannabinergic ligands.<sup>60, 61, 68</sup> We also developed <sup>2</sup>H-solid state NMR,<sup>33, 69, 70</sup> small angle x-ray<sup>31, 62, 71, 72</sup> and neutron diffraction approaches<sup>65</sup> described in a series of publications that allowed us to identify the manner by which the ligand aligns itself within the membrane.<sup>63, 72-75</sup> Our studies demonstrated that because of their amphipathic properties, cannabinergic ligands align themselves in an orientation in which the polar components of the molecule interact with the respective membrane headgroups. Such an orientation enhances their ability to engage in fast lateral diffusion within the bilayer and allows for a productive collision at the receptor site.<sup>9, 33</sup> We, thus, demonstrated that <sup>9</sup>-THC aligns itself in an “awkward” orientation, in which its phenolic hydroxyl interacts with the polar headgroup within the bilayer, while its hydrophobic tricyclic component orients with its access orthogonal to those of phospholipid chains.<sup>29, 31, 72</sup> Cannabinoids with two or more hydroxyl groups such as the Pfizer analog CP55940 align in a manner in which all three OHs interact with the polar membrane components.<sup>9</sup> We also showed that this “amphipathic reciprocal alignment” of amphipathic ligands within a membrane bilayer has general applicability with endogenous and exogenous lipid-friendly ligands such as steroids, including neurosteroids<sup>63, 74, 76</sup> and estrogens. Conversely, hydrophobic ligands with no polar groups capable of hydrogen bonding (e.g. OH, NH<sub>2</sub>), such as O-methyl- <sup>9</sup>-THC, align with their long axis parallel to the membrane bilayer chains.<sup>71, 72, 74</sup> The endocannabinoid anandamide aligns with its extended arachidonyl chain parallel with the membrane chains and its polar ethanolamide group interacting with the phospholipid headgroups.<sup>33</sup>

In the above series of biophysical experiments, we provided substantial experimental evidence supporting our postulate that hydrophobic and amphipathic ligands access their sites of action by fast lateral diffusion after preferentially partitioning in the cellular membrane bilayer.<sup>9, 33</sup> This “through membrane” access of ligands to their respective GPCR binding domains is now generally well-accepted within the GPCR scientific community.

### **Endocannabinoid Transport Across the Cell Membrane**

Endocannabinoids have been shown to access their intracellular targets through a yet to be fully identified transport mechanism.<sup>10, 77</sup> The search for an endocannabinoid transport mechanism was spearheaded by the discovery of AM404,<sup>10</sup> and subsequently AM1172,<sup>19, 77</sup> both lipophilic ligands that were shown to inhibit the transport of endocannabinoids across the cell membrane. This area of research, which we pursued through a close collaboration with Daniele Piomelli, provided excellent insights on the physiological effects resulting from modulating the intracellular levels of endocannabinoids and the potentially very

promising opportunities for utilizing this approach for therapeutic gain.<sup>71-74, 78</sup> To date, the endocannabinoid transport mechanism has remained quite elusive, with different postulates being proposed.<sup>79</sup> Notwithstanding this uncertainty, the value of developing transport inhibitors as pharmacological probes, and potentially as useful medications, remains high.

### Cannabinoid Diffusion across the Cell Membrane

Xenobiotic cannabinoids have been shown to diffuse passively across the cell membrane. Using well-targeted biophysical experiments with representative ligands and a number of different membrane preparations, we were able to obtain evidence on how cannabinergic compounds engage in this diffusion process and, thus, provide a basis for understanding the structural features within a ligand required for brain penetration and oral bioavailability. The above series of experiments involved studies on the conformation, location, and orientation of a carefully-selected group of cannabinergic ligands in model and cell membranes. We showed that hydrophobic molecules with no hydrogen bearing heteroatoms, such as O-methyl-THC, initially partition within the outer membrane leaflet and reside below the polar surface. When this partitioning becomes thermodynamically unstable, a fraction of the drug partitions in the middle of the bilayer, while remaining in equilibrium with the ligand population near the polar headgroup. In an interesting study using solid state <sup>2</sup>H NMR, we demonstrated the presence of these two drug populations within the membrane and, thus, provided a basis for a mechanism by which hydrophobic compounds cross the cell membrane. Conversely, amphipathic compounds with hydrogen-bearing heteroatoms (e.g. <sup>8</sup>-THC), first partition in the outer membrane leaflet from where they interact through H-bonding with polar membrane components and subsequently engage in a flip-flop action that allows them to translocate to the inner membrane leaflet where they can access the cytosolic cellular sites. Our work also demonstrated that this flip-flop mechanism, involving amphipathic ligands, is substantially faster than the diffusion of hydrophobic molecules.<sup>23</sup>

### Cannabinergic Carrier Proteins

To address the question of how cannabinoids and endocannabinoids can cross the “aqueous” intercellular space between two adjacent cells (e.g. neural synapses), we invoked the action of specialized proteins such as albumin. In a series of biophysical experiments, we provided experimental evidence of how ligands can be “picked up” and carried by albumin from a cell membrane surface and subsequently deposited on the adjacent cell surface. In an experiment, initially carried out using <sup>2</sup>H-NMR,<sup>27</sup> and later continued using <sup>19</sup>F-NMR, we were able to identify the binding domains of our ligand on albumin.<sup>21, 80, 81</sup> More recently, my laboratory has been involved in studying the role of another class of carrier proteins, known as fatty acid binding proteins (FABPs). These intracellular proteins are believed to have a role in transporting endocannabinoid ligands from the inner membrane leaflet to other intracellular functional proteins including FAAH, the endocannabinoid deactivating enzyme. We recently cloned one of these proteins and used <sup>15</sup>N-labelling to obtain high resolution NMR spectra, with which we can study in significant detail the interactions of this protein with endocannabinoids and xenocannabinoids.<sup>4</sup>

## CB2 Selective Ligands

The initial concept that cannabinoid-induced analgesia was an exclusively CB1 associated response was challenged in early work published by Porreca and Makriyannis.<sup>82</sup> Subsequently, the analgesic effects of CB2 agonists were established with the synthesis of CB2 selective ligands including HU308 from the Mechoulam laboratory<sup>83</sup> and our own more potent and *in vivo* more efficacious AM1241.<sup>84-86</sup> In a series of detailed publications involving collaborative work with Phil Malan at the University of Arizona and Andrea Hohmann at the University of Indiana, we documented the role of CB2 activation in peripheral and chronic analgesia, as well as inflammation.<sup>87-94</sup> More recently, in collaboration with Andrea Hohmann, using three different CB2 selective chemotypes, including AM1241, AM1710, and AM1714, we demonstrated that CB2 activation effectively relieves chemotherapy-induced neuropathy.<sup>95-98</sup> We also showed that the protective and therapeutic effects of these compounds were superior to those of gabapentin (Neurontin), the most commonly used medication for the different neuropathies. Our early success demonstrating the analgesic and anti-inflammatory effects obtained through activation of the CB2 receptor motivated a number of pharmaceutical companies to establish active research programs for the development of therapeutically-useful CB2 agonists. Unfortunately, the limited data obtained from testing CB2-selective agonists in humans did not fully validate the animal-based results. It can be argued that the limited clinical trials in which CB2 agonists were tested for their analgesic properties involved suboptimal therapeutic indications. I propose that more successful clinical results may be expected by choosing better targeted therapies.

Moreover, the development of novel CB2-selective agonists within academic and industrial laboratories opened the door for understanding the role of CB2 activation as a potential target for other therapeutic indications, such as bone disorders.<sup>99</sup> It has also become clear that, contrary to earlier findings, CB2 is expressed in brain.<sup>46</sup> Although brain expression under homeostatic conditions is limited, it becomes much more pronounced in pathological conditions, such as inflammation, neurodegeneration and cancer, where the CB2 is highly overexpressed both in brain and the periphery, suggesting that CB2 is a suitable target for such conditions as amyotrophic lateral sclerosis<sup>100</sup> and colitis.<sup>101</sup>

Very recent evidence has identified a role for CB2 activation in addiction,<sup>102</sup> which, arguably, can be considered a drug-induced inflammatory syndrome, and offers opportunities for additional therapeutic intervention.

## Ligand Assisted Protein Structure

Following their discovery, the structure and function of GPCRs has been a major goal of biologists and pharmacologists. Although these proteins were cloned and expressed over three decades ago, their structural and functional characterization remained elusive and relied mostly on indirect biophysical approaches. Based on crystal structure determination, first of bacterial rhodopsin, and later of rhodopsin,<sup>103</sup> computational methods were developed to obtain homology models of individual GPCRs, and their respective ligand receptor complexes based on the rhodopsin structure. Only recently were the crystal

structures of pharmacologically-interesting GPCRs achieved. First among these was the beta adrenergic receptor, an important accomplishment through collaborative efforts between the Brian Kobilka and Ray Stevens laboratories, which required significant protein engineering of the GPCR protein.<sup>104</sup> In parallel, Chris Tate and his collaborators at MRC, UK,<sup>105</sup> used extensive receptor mutations to obtain crystallizable GPCR samples.

In my laboratory over the past ten years, we have been developing a novel approach aimed at obtaining experimental information on the binding motifs and functional characteristics of cannabinergic ligands with their respective receptors. This approach, which we named “Ligand Assisted Protein Structure” (LAPS), relies on the availability of covalent CB1 and CB2 probes. To identify the binding motif of individual ligands within CB1 and CB2, we use a dual methodology which includes the development of targeted CB1 and CB2 mutants and a focused proteomic characterization of the complex using LC/MS/MS.

### CB1/CB2 Targeted Mutants

Our initial targets for CB1 and CB2 labeling were the cysteine residues within the seven transmembrane helical receptor domain, and developed comprehensive libraries of single or multiple cysteine mutants for each receptor. In early work, we showed that the isothiocyanate group was the optimal electrophile that reacted exclusively with cysteine residues near or at the ligand's binding domain under the experimental conditions we used.<sup>34, 106-108</sup> In parallel, for a photoaffinity-based approach, we chose aliphatic azide groups as suitable moieties capable of covalently labeling the receptor upon irradiation.<sup>6, 109</sup> By placing the reactive groups in different positions within a ligand, we were able to identify the respective reactive amino acid residues involved in its binding. Such experimentally-derived information was subsequently used to model the ligand-receptor complex. Conversely, our data were used to refine further existing CB1 and CB2 computational models.

Our first success story was with AM841, a classical cannabinoid analog carrying an isothiocyanate group at the end carbon of its dimethylheptyl chain.<sup>34</sup> By sequentially mutating the individual cysteines within the seven transmembrane helical domain of the CB1 receptor to other isosteric residues that were unreactive with the NCS group, for example serine or alanine, we demonstrated that the reactive cysteine was the one within the conserved CWXP motif of helix 6.<sup>5</sup> AM841 was also shown to attach to the same cysteine within the Hx6 of CB2, although through an overall different binding motif.<sup>20</sup> For both receptors, the functional potencies of this ligand exceeded by 20-50 fold that of its non-covalent counterparts, an observation we recently confirmed in *in vivo* tests. For this reason, AM841 was designated as a “megagonist.”<sup>20</sup>

The above approach, targeting individual cysteines within the intramembrane sites in CB1 and CB2, was used to characterize the binding motifs of cannabinergic ligands from a variety of distinct chemotype classes, and supplemented with ligands carrying other reactive electrophiles, such as the carbamate and nitrate groups.<sup>5, 20, 36</sup>

## CB1/CB2 Proteomic Characterization

To complement our targeted mutant approach, we use LC/MS/MS methodology to identify more directly the site(s) of covalent ligand attachment within the GPCR structure. Such approaches have been used very effectively in my laboratory to characterize the structures and functions of different cannabinergic enzymes, including rat and human FAAH and MGL,<sup>110, 111</sup> and more recently human N-acylethanolamine-hydrolyzing acid amidase (NAAA).<sup>30, 112</sup> However, its application to GPCRs proved to be more laborious and experimentally demanding.

Our proteomic studies with CB1 and CB2 required developing efficient methods for receptor expression, purification under non-denaturing conditions, and further proteomic analysis of the targeted proteins. For these studies, we expressed the receptors in baculovirus and developed methods for obtaining purified samples of functionally-competent receptors capable of selectively and effectively interacting with our covalent ligands. The preparations were subsequently subjected to selective enzymatic digestion and the resulting peptides analyzed using LC/MS/MS methods.<sup>113-116</sup> We have been successful in perfecting this approach, and have used it to characterize the sites of attachment for a number of electrophilic ligands.<sup>16</sup>

Currently, we are expanding the LAPS approach to include a wider variety of covalent probes aimed at multiple amino acid residues within the orthosteric or allosteric sites of functional proteins. We are also exploring the use of newly designed homo- and heterobifunctional probes. These interesting covalent ligands target two points of attachment within the GPCR by incorporating two reactive groups that are either identical (eg. *bis*NCS; homobifunctional) or different ones (eg. NCS/N<sub>3</sub>; heterobifunctional) within the same molecule and are intended to identify more accurately the ligands' binding motifs.

## CB1/CB2 Ligand Motifs and Functional Selectivity

To date, the LAPS approach has been extended to a number of ligand chemotypes,<sup>36</sup> and has targeted both CB1 and CB2. Our early data provide robust evidence that the CB1 and CB2 covalent ligands we have tested clearly have distinctive receptor bidding motifs, as well as differentiated signaling profiles. It is tempting to propose that the CB1 and CB2 functional selectivity observed with our covalent ligands can be correlated with the different motifs of the ligand-receptor complexes. According to this postulate, individual classes of ligands induce distinct, active receptor conformations that are represented by different signaling profiles. This opens the door for developing functionally selective CB1 and CB2 compounds by using the individual ligand-receptor complexes as pharmacophoric templates for our design. Such an effort could lead to novel medications possessing either reduced undesirable side effects or more selective pharmacological profiles.

## CB1 Receptor Antagonists

The development of rimonabant (SR141716A, Acomplia), a selective CB1 antagonist, by Sanofi as a potential antiobesity medication was initially greeted enthusiastically by cannabinoid researchers. When tested in humans, the drug was shown to reduce body weight and to ameliorate dyslipidemias, diabetes and metabolic syndrome. However, this

enthusiasm was dampened by later observations of undesirable side effects, which included nausea, anxiety, depression, and in some cases, suicidal tendencies, which led to its withdrawal from the market in October 2008.<sup>117, 118</sup>

In my laboratory, we have been developing CB1 receptor antagonists since the late nineties,<sup>119-123</sup> among which, AM251 and AM281 received special attention as useful pharmacological probes with inverse agonist pharmacological profiles.<sup>119, 120, 122</sup> AM281 was later used to demonstrate the first *in vivo* imaging of the receptor in non-human primates using SPECT technology.<sup>119-121</sup> In tandem, and anticipating potential CNS side effects resulting from CB1 antagonism, we also made serious efforts to develop novel compounds with no or reduced side effect profiles. We followed two distinct approaches. The first was to develop effective CB1 antagonists with no inverse agonist or weak inverse agonist properties. This approach led to a family of compounds with *in vitro* neutral antagonist profiles, i.e. no or minimal effect on cAMP levels. The best known of these, AM4113 and AM6527,<sup>124-135</sup> when tested in rodents, reproduced all of the therapeutic effects associated with rimonabant and other inverse CB1 antagonists being developed by the pharmaceutical industry, including reduction in food consumption, weight loss, and ability to antagonize the effects of stimulant and nicotine addiction. When tested in animals, these compounds did not exhibit any of the undesirable side effects of inverse agonists in a number of animal models. The testing for side effect profiles included nausea, using emesis experiments in ferrets<sup>127</sup> and rats,<sup>126, 128</sup> gastrointestinal motility in mice, as well as anxiety,<sup>129, 130</sup> depression and anhedonia<sup>132</sup> in rats. The above results are very encouraging, but require further validation to determine how the data can be translated into improved and novel CB1 antagonists with favorable therapeutic index profiles.

Our second approach for obtaining CB1 antagonists with reduced undesirable side effect profiles was the development of peripherally-active compounds. This effort was reinforced by existing clinical data from the rimonabant trials, suggesting that some of the drug's key therapeutic effects were peripherally mediated through a mechanism involving modulation in lipid metabolism and energy balance. The most prominent result of our effort was AM6545, a peripherally-acting neutral antagonist. When tested in rodents, this compound was found to produce weight loss, as well as improved lipid profile and insulin sensitivity. Currently, AM6545 is in advanced preclinical testing and being considered for development for the treatment of non-alcoholic fatty liver disease (NAFLD), as well as liver fibrosis.<sup>136-139</sup> Current SAR work on the 2-pyrrolidinone scaffold has led to AM10009 or molecule with distinct chiral features (J. Garcia et al, unpublished).

## Cannabinoid Agonists with Controlled Deactivation

A program we recently initiated based on the “soft drug” approach aims at developing safer and more effective cannabinergic compounds with controllable deactivation, improved druggability and an overall safer pharmacological profile.

To this end, we incorporate a metabolically vulnerable ester group within the structure of a successful cannabinergic ligand.<sup>140, 141</sup> Hydrolysis at the esteratic site leads to products devoid of pharmacological activity and low or no toxicity. In our controlled deactivation/

detoxification design, the compound's systemic half-life is determined by two factors. The first is the extent to which the ligand is sequestered within the body before it is released for systemic circulation (depot effect). This process is dependent on the compound's physicochemical properties and can be modulated by adjusting log P and PSA. The second parameter is the rate of enzymatic hydrolysis by blood esterases. This can be calibrated by incorporating suitable stereochemical features in the vicinity of the hydrolyzable group (enzymatic effect). In recent publications involving analogs with an ester group at the side chain or as a lactone structure within the cannabinoid C-ring of the cannabinoid structure, we demonstrated our ability to modulate the rates of hydrolysis, as well as the drug's depot effects. Key compounds synthesized in our laboratory show great promise as pharmacological probes and potential drug leads. Currently, we are pursuing this approach for the design of peripherally-acting analgesic agents, as well as potential medications in THC substitution therapy.

## Endocannabinoid Deactivating Enzymes

### Fatty Acid Amide Hydrolase

The first enzyme to be identified was a specific amidase involved in the hydrolysis of anandamide and other long-chain fatty acid amides and ethanolamides. The role of this intriguing enzyme was first identified by Dale Deutsch, with whom we collaborated and developed the first inhibitors for this enzyme, the most prominent of which was the sulfonyl fluoride AM374, a relatively simple compound with surprisingly high selectivity for FAAH and capable of crossing the BBB.<sup>142, 143</sup> To enhance our ability to test FAAH inhibitors, we developed two assays.<sup>144, 145</sup> We also were first to purify this enzyme from rat brain membranes using an affinity column designed by my student Andreas Goutopoulos.<sup>146</sup> The enzyme which we had initially named anandamide amidase was later given the name fatty acid amide hydrolase (FAAH).

Our efforts to characterize this important enzyme were subsequently superseded by the work of Ben Cravatt, who cloned and purified it, and subsequently, through a collaboration with Ray Stevens, produced informative crystal structures of the inhibitor enzyme complex.<sup>147</sup> The development of transgenic mice in which the enzyme was deleted was a big step in understanding the enzyme's pharmacology. This very productive effort by the Scripps investigators opened a new chapter in cannabinoid research.

A major effort by the academic and industrial research communities led to a plethora of novel FAAH inhibitors, which allowed for the exploration of the therapeutic value of indirect activation of the cannabinoid receptors. A prominent early inhibitor, URB597 by Daniele Piomelli,<sup>148</sup> was used to demonstrate the pharmacological effects associated with FAAH inhibition and enhancement of endocannabinoid tone and the potential for using such agents in depression. Our own AM3506 and AM5206 were shown to have potent neuroprotective properties using a model involving glutamatergic excitotoxicity in collaboration with my colleague Ben Bahr.<sup>12, 18, 149-152</sup> Subsequently, a productive program by the Pfizer group led to a high quality series of FAAH inhibitors encompassing electrophilic ureas, of which PF-04457845 was the first to advance to clinical trials.<sup>153</sup> Unfortunately, when this compound was tested in humans for osteoarthritis, it failed to show

substantial efficacy. Notwithstanding the unsatisfactory clinical results, I am convinced that there is a promising future for the development of FAAH inhibitor medications by targeting more relevant therapeutic indications.

This key endocannabinoid presynaptically-localized enzyme is an esterase involved in the hydrolytic deactivation of the endocannabinoid ester 2AG, which also is its optimal endogenous substrate. Chemical inactivation of this enzyme leads to an increase in 2AG levels with the concomitant enhancement of cannabinoid activity. The human enzyme was cloned by a number of laboratories and expressed in *E. coli*. Additionally, a number of crystal structures have been produced for the enzyme-ligand complexes.<sup>154, 155</sup> Our involvement with MGL stemmed from an interest in obtaining detailed information on its structure and function beyond what was available in the literature, and in using it to design and synthesize novel inhibitors covering a broad spectrum of potency, reversibility, and selectivity profiles. Additionally, we were interested in exploring MGL species differences with the intent of developing novel successful inhibitors that were equally potent in human and rodent enzymes to ensure that if successful, these compounds could be advanced beyond the preclinical stage.

The work involved cloning and expression of the enzyme in *E. coli* and purification using affinity chromatography.<sup>26, 110, 111, 156</sup> We subsequently used MGL as a lead project to develop biophysical methods which we plan to apply to other endocannabinoid targets. Structural experiments were based principally on the combined use of mass spectrometry and high resolution NMR,<sup>14, 156</sup> accompanied by computer modeling. For example, we identified, at a distance of 22 Å from the reactive serine, a tryptophan residue, which, when substituted with other residues, rendered the enzyme practically inactive. The NMR experiments provided detailed information on the H-bonding network required to enhance the nucleophilic properties of the catalytic serine,<sup>14</sup> while maintaining the active enzyme conformation. The work opened the door for designing allosteric enzyme inhibitors by targeting key enzyme residues away from the catalytic site, but distantly involved in the catalytic process. The work also identified the two conformations (active and inactive) observed with other esterases, where the enzyme lid is respectively in the open or closed conformations. Thus, we were able to develop specific conditions under which each of the two enzyme conformational states could be isolated and structurally characterized in solution.<sup>156</sup>

Although a cytosolic enzyme, MGL's activity is enhanced by its association with a membrane environment. To study these MGL membrane interactions, we used nanodiscs in combination with Hydrogen/Deuterium Exchange Mass Spectrometry (HXMS) technology.<sup>26</sup> In parallel, we have produced triply-labeled (<sup>2</sup>H, <sup>13</sup>C, <sup>15</sup>N) enzyme preparations for the purpose of identifying residue resonances through a 3D-analysis. This work, which will serve as a basis for using SAR by NMR methods to study inhibitor-enzyme interactions and design optimized inhibitors, is currently underway.

Early evidence from the Cravatt laboratory and ours has suggested a level of synergy between the two key endocannabinoid deactivating enzymes FAAH and MGL.<sup>15, 157, 158</sup> To explore these findings, we designed and synthesized a family of dual MGL/FAAH

inhibitors. To date, our program on novel FAAH, MGL and MGL/FAAH inhibitors (eg. AM6701) has identified promising leads, which are being pursued as potential medications for neurodegenerative diseases and addiction.

## Paracannabinoid Targets

Because of the pleiotropic nature of its endogenous ligands, cannabinoid biology is connected with other biochemical systems involving lipid modulators, such as the VR1 ion channel, a number of recently deorphanized GPCRs, including GPR55, as well as the arachidonic acid metabolizing enzyme cyclooxygenase-2 and the more recently identified NAAA. Because of their functional proximity with the endocannabinoid system, some of these targets are being explored in my laboratory. Recently, we have cloned, expressed, and purified GPR55, and are prepared to subject it to our LAPS approach in order to gain more detailed structural and functional information.

Also, we have cloned, expressed, and purified hNAAA, a lysosomal amidase which has shown promise as a therapeutic target for inflammation. Our mammalian expression system has allowed us to produce pure enzyme in milligram quantities.<sup>112</sup> We used proteomic approaches to gain information on the catalytic mechanism of this unique cysteine amidase, where the apoenzyme is activated through an intramolecular scission, leading to two chain components, which form the active enzyme heterodimer.<sup>30, 112</sup> Although NAAA hydrolyzes anandamide, its optimal native substrate is palmitylethanolamide, a lipid modulator generally associated with the nuclear receptor PPAR-alpha.

Recently, we have obtained good quality <sup>1</sup>H NMR spectra of the purified enzyme, which allows us to follow the enzymatic catalysis in real NMR time, and to design novel inhibitors. The most successful of these, AM9053, is a chemically- and metabolically-stable compound with substantial *in vitro* potency ( $K_i = 30$  nM). Very recently, its *in vivo* efficacy was demonstrated in a colon inflammation model.

## Now and the future

Almost three decades after its discovery, the endocannabinoid system offers rich opportunities for the chemist. It has been identified as a major biochemical system, playing major roles in brain function, autonomic physiology, the immune system, and as a key player in maintaining homeostasis. Much of this biology needs to be further elaborated and new, related pathways remain to be discovered. The medicinal chemist should have a major role in developing or participating in the development of molecular tools in this exciting endeavor. These include selective pharmacological probes, *in vivo* imaging agents for PET and SPECT, as well as *in vitro* fluorescent and radiolabelling reagents. There continues to be a need for new ligands for structural biology work involving NMR, x-ray, and mass spectrometry that will assist in understanding the function of some key endocannabinoid proteins, including the two GPCRs and the enzymes and proteins involved in endocannabinoid biosynthesis, metabolism, and transport. The pleiotropic nature of the endocannabinoids encourages the exploration of noncannabinoid functional proteins associated with the endocannabinoid system, and identified in this perspective as paracannabinoid targets, as potential therapeutic targets. These may include other GPCRs

and channels that are modulated by lipid ligands, as well as enzymes involved in their biotransformations.

As a therapeutic target, the endocannabinoid system has found to date only modest success, and the failure of Sanofi's CB1 antagonist Acomplia was a major setback in this endeavor. However, a number of new findings, coupled with current efforts for cannabinoid-based drug development, point to a more successful future. CB1 antagonist approaches, including the peripherally-acting compounds, show promise as medications for metabolic disorders, liver damage and fat metabolism. In this regard, early candidates, such as AM6545, with combined neutral antagonist/peripheral profiles, may reduce the peripheral side effect profile in this class of compounds. As for the brain penetrant neutral CB1 antagonists, these may find usefulness in addiction disorders, including those from nicotine, opioids, and alcohol.

Notwithstanding the initial unsatisfactory results from the limited *in vivo* trials, CB2 agonists continue to show promise as potential medications for inflammatory and neuropathic pain, as well as in neurodegenerative conditions, including ALS and multiple sclerosis. Arguably, the development of CB1 agonists with reduced side effects, either through peripheralization or the controlled deactivation approaches I have outlined earlier, still holds great promise as analgesic medications for pain management, lacking some of the side effects associated with opioid analgesia.

Therapeutic approaches involving indirectly-acting cannabinoid agonists, such as FAAH inhibition, are attractive because of their more favorable side effect profiles, notably the absence of THC-like effects in the CNS. However, initial *in vivo* trials with a FAAH inhibitor for osteoarthritis have not proven successful. Notwithstanding this failure, such compounds still offer promise as anti-inflammatory and neuroprotective medications, if properly targeted. MGL inhibitors, acting, at least in part, through a non-cannabinoid mechanism, are being developed as potential anticancer medications. Also, the observed synergy accompanying the simultaneous inhibition of both deactivating enzymes FAAH and MGL invites the development of optimized dual inhibitors. Indirectly-acting cannabinoid agonists also hold promise in drug abuse as potential substitution therapies. Early results identify NAAA inhibitors as potential anti-inflammatory agents in conditions such as irritable bowel syndrome. Recent data with our selective NAAA inhibitor AM9053 are very exciting. Such compounds may also prove useful as peripherally-acting analgesics.

Finally, CB1 positive or negative allosteric modulators offer interesting prospects for developing medications lacking CB1-related undesirable side effects. Although substantial research has identified several scaffolds that exhibit *in vitro* allosteric profiles, *in vivo* experiments have shown only modest efficacy results. This is an area which would benefit from novel or improved scaffolds.

The ubiquitous presence of the endocannabinoid system presents difficulties in targeting it for therapeutic gain. For this reason, it is important that indications for cannabinergic drug development be explored very thoughtfully. In this regard, a better understanding of cannabinoid receptor-related functional selectivity should assist in the development of drugs with safer pharmacological profiles, and also identify additional therapeutic opportunities.

Thus, compounds belonging to the same pharmacological class could be optimized for different indications, i.e. developing drugs from the same family with many flavors to address specific needs. Notwithstanding some of the earlier failures in developing cannabinoid-related medications, there is ample accumulated knowledge to predict that the endocannabinoid system offers great promise as a potential source of future therapies.

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## Biography

Alexandros Makriyannis is the George Behrakis Trustee Chair in Pharmaceutical Biotechnology at Northeastern University, Professor of Pharmaceutical Sciences, Chemistry, and Chemical Biology, and Director of the Center for Drug Discovery. He received his Ph.D. in medicinal chemistry at the University of Kansas and postdoctoral training in synthetic organic chemistry at the University of California, Berkeley. At the University of Connecticut, he rose to the rank of Distinguished Professor, before joining Northeastern University and establishing its Center for Drug Discovery. His work is characterized by being at the interface of chemistry and biology, encompassing over 450 publications, 40 patents, and numerous awards, most recently the Northeastern Award for Excellence in Research and Creativity (2012) and the ACS Medicinal Chemistry Hall of Fame (2013).

## Nonstandard Abbreviations Used

<b>2AG</b>	2-arachidonoylglycerol
<b>AEA</b>	endocannabinoid anandamide
<b>DMPC</b>	dimyristoylphosphatidyl choline
<b>DPPC</b>	dipalmitoylphosphatidyl choline
<b>FABP</b>	fatty acid binding protein
<b>MGL</b>	monoacylglycerol lipase
<b>NAAA</b>	N-acylethanolamine-hydrolyzing acid amidase
<b>TRVP</b>	transient receptor potential vanilloid
<b>VR1</b>	vanilloid receptor 1

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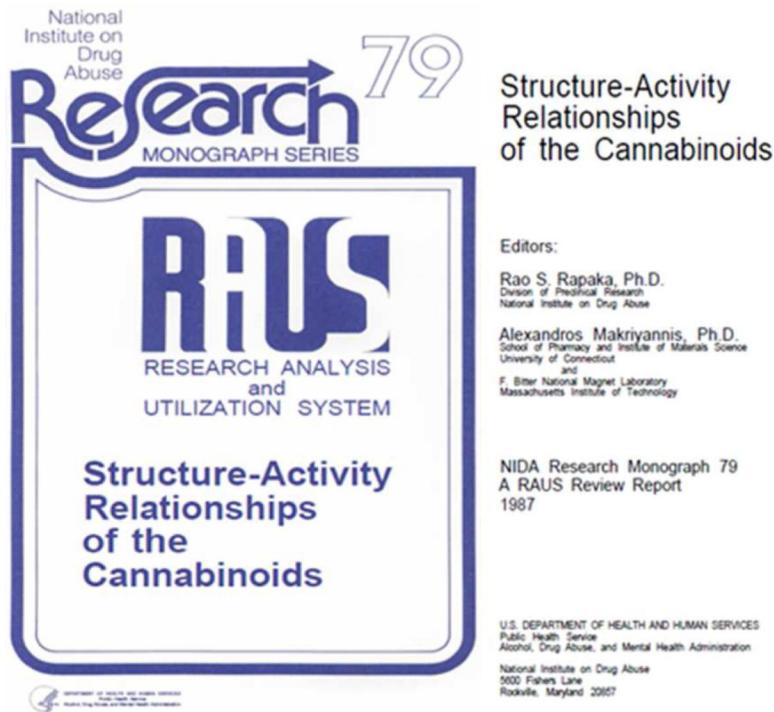
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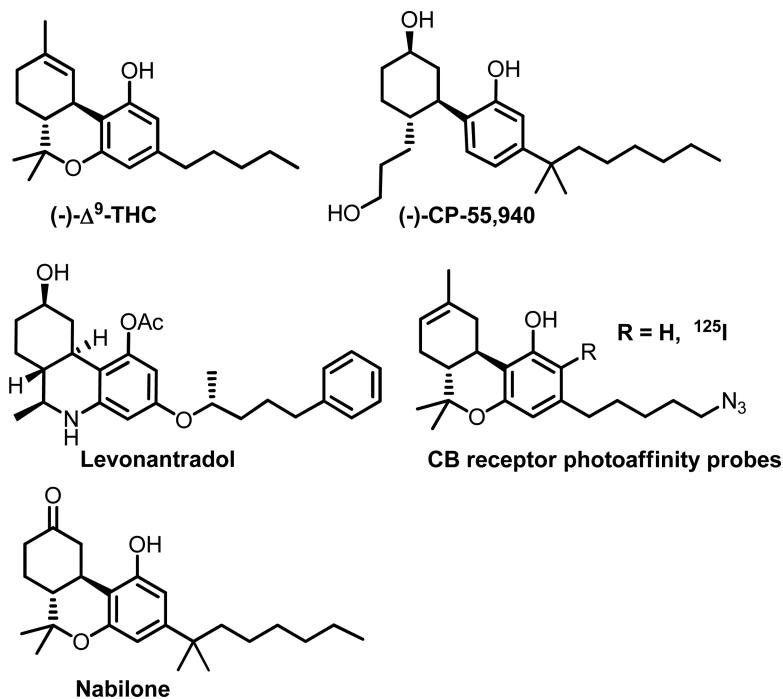
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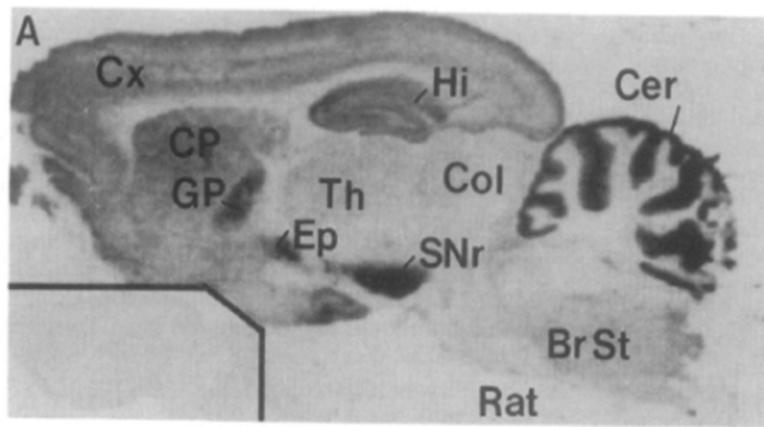
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**Figure 1.**  
Technical Review Proceedings Cover Page<sup>17</sup>

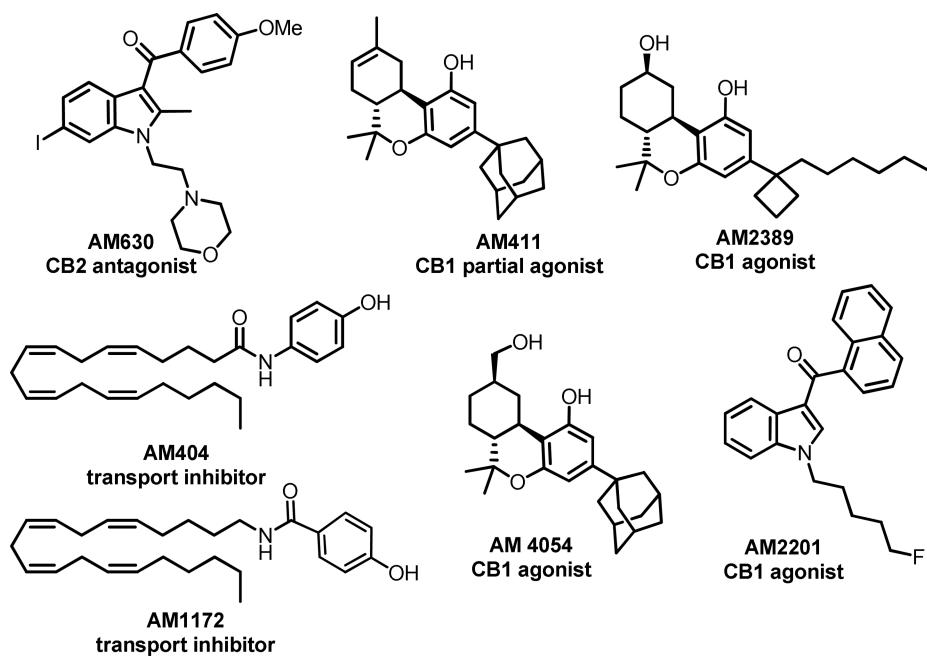


**Figure 2.**  
Phyto- and Xeno-Cannabinoids<sup>1,2</sup>

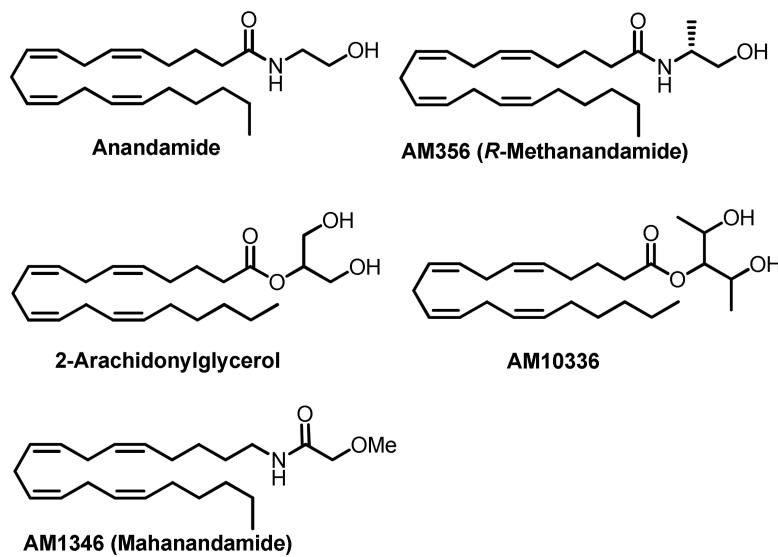


**Figure 3.**

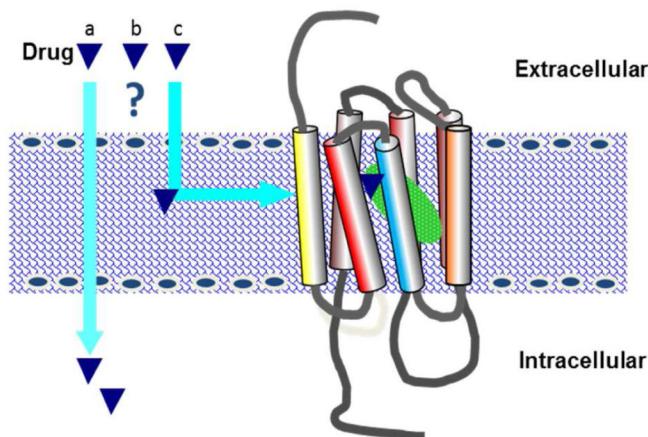
Autoradiography of 10 nM [ $^3\text{H}$ ]CP55,940 binding in a sagittal section of rat brain. Br St, brainstem; Cer, cerebellum; Col, colliculi; CP, caudate-putamen; Cx, cerebral cortex; Ep, entopeduncular nucleus (homolog of GPi); GP, globus pallidus (e, external; I, internal); Hi, hippocampus; Th, thalamus.<sup>28</sup>

**Figure 4.**

Select AM Compounds. AM630 is the first CB2 antagonist.<sup>3,4</sup> AM411 is the first adamantyl cannabinoid,<sup>8</sup> while AM4054 is a later generation analog.<sup>11</sup> Both are early CB1 receptor agonists. AM2389 is a very potent long-acting CB1 agonist;<sup>13</sup> AM404 is the first early transporter inhibitor,<sup>10</sup> while AM1172 is a second generation transport inhibitor.<sup>19</sup> AM2201 is a very potent aminoalkylindole CB1 agonist.<sup>22</sup>



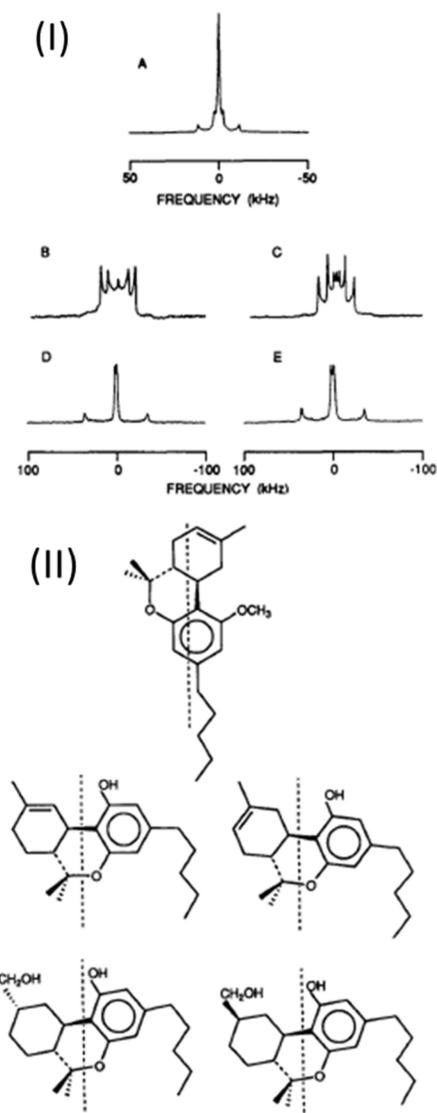
**Figure 5.**  
Endocannabinoid Analogs



### The Role of Cell Membrane in Cannabinoid Activity

**Figure 6.**

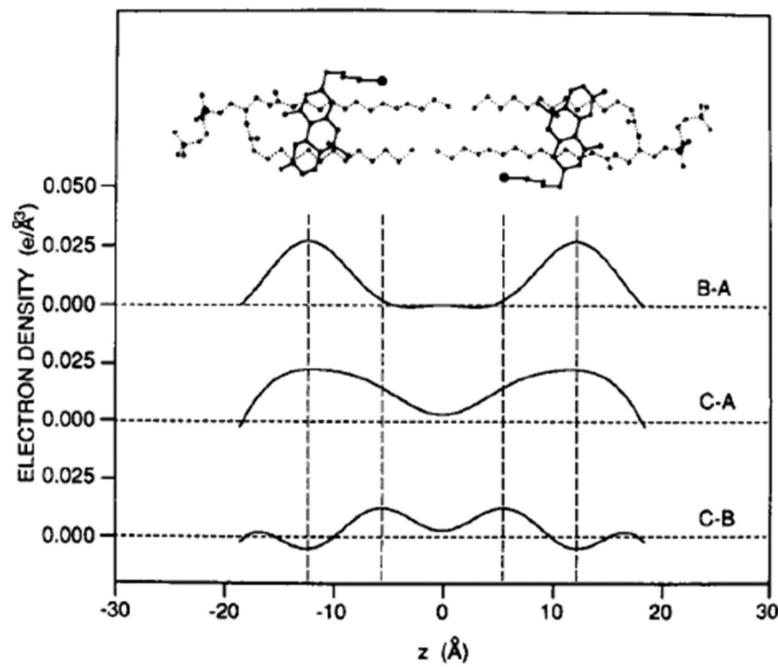
Drug and endogenous ligands partition within the cellular membrane where they can: (a) diffuse passively across the bilayer to enter the intracellular space; (b) are translocated intracellularly through a yet to be fully identified transport mechanism; (c) interact with the receptor active site through fast lateral diffusion.<sup>27</sup>



### *How Cannabinoids Access their Receptors*

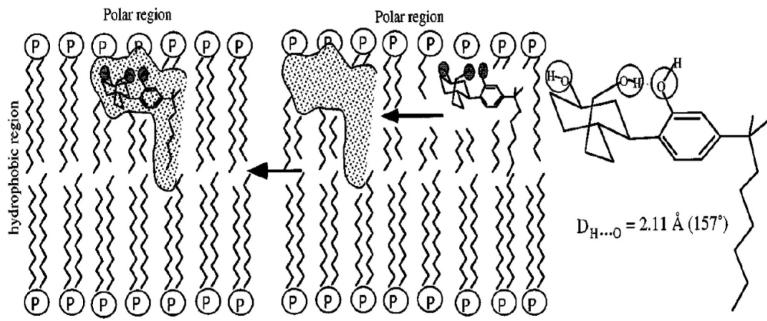
**Figure 7.**

(I) Representative solid state <sup>2</sup>H-NMR spectra from dipalmitoylphosphatidyl choline (DPPC) model membranes (42°C) containing five cannabinoids each having two deuterium labels at 2 and 4-positions: 2,4-d<sub>2</sub>-Me-<sup>8</sup>-THC(A), 2,4-d<sub>2</sub>-<sup>8</sup>-THC(B), 2,4,-d<sub>2</sub>-<sup>9</sup>-THC(C), 2,4-d<sub>2</sub>-11-OH-9α-HHC(D) and 2,4-d<sub>2</sub>-11-OH-9β-HHC(E). (II) Orientations of Me-<sup>8</sup>-THC (top), <sup>8</sup>-THC and <sup>9</sup>-THC (middle), 11-OH-9α-HHC and 11-OH-9β-HHC (bottom) in hydrated DPPC bilayers. The dashed lines represent the direction of the lipid acyl chains.<sup>29</sup>



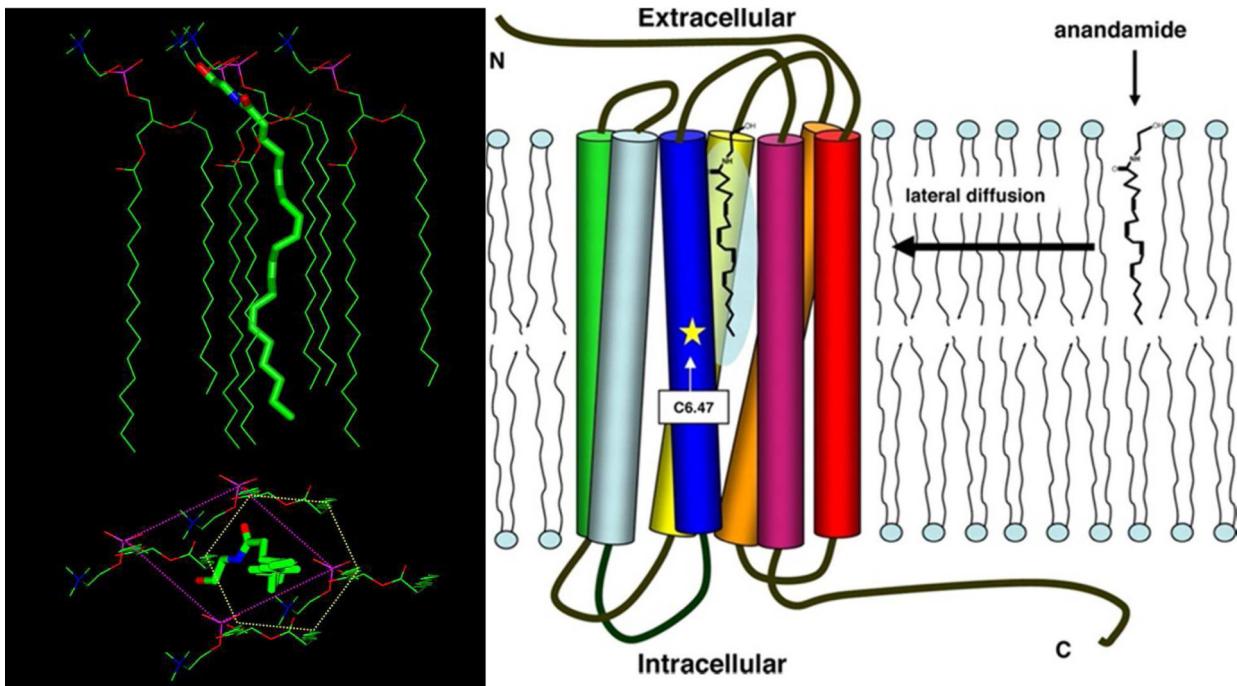
**Figure 8.**

Electron density profile differences inside the bilayer: Curve B-A is the difference between profiles of dimyristoylphosphatidyl choline (DMPC) +  $^{8}\text{-THC}$  and DMPC, curve C-A is the difference between those of DMPC + 5'-I-  $^{8}\text{-THC}$  and DMPC, and curve C-B is the difference between those of DMPC + 5'-I-  $^{8}\text{-THC}$  and DMPC +  $^{8}\text{-THC}$ . The outer pair of the vertical dashed lines indicates the peaks in curves B-A and the inner pair indicates the peaks in C-B. They represent the positions of the center of  $^{8}\text{-THC}$  and the iodine atom of 5'-I-  $^{8}\text{-THC}$  in the bilayer, respectively.<sup>31</sup>



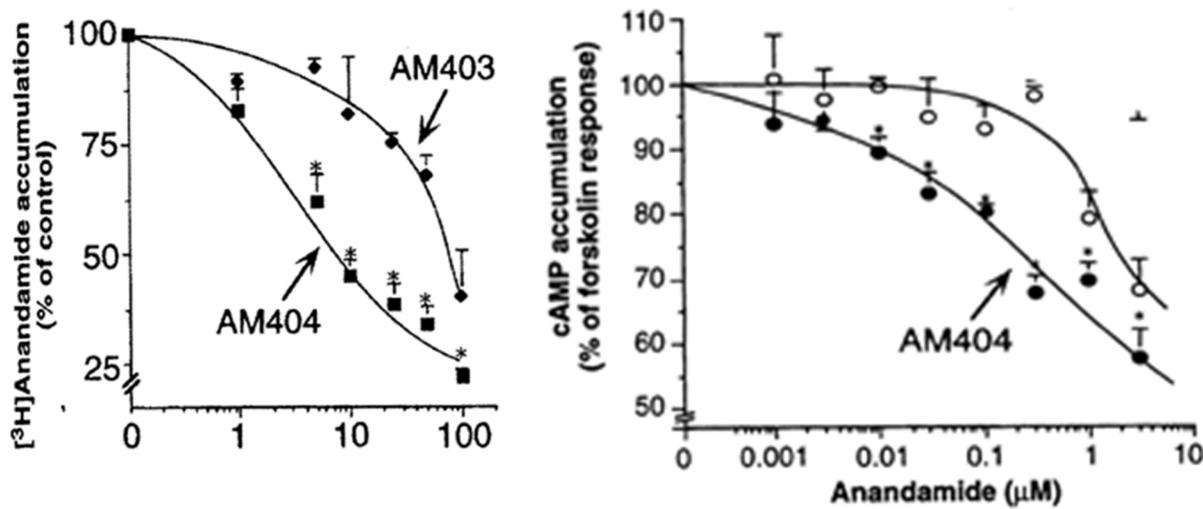
**Figure 9.**

A ligand-membrane-receptor model representing the trans-membrane diffusion of CP55940 en route to interacting with the cannabinoid receptor. According to our hypothesis, the ligand preferentially partitions in the membrane bilayer where it assumes a proper orientation and location allowing for a productive collision with the active site.<sup>4, 9</sup>



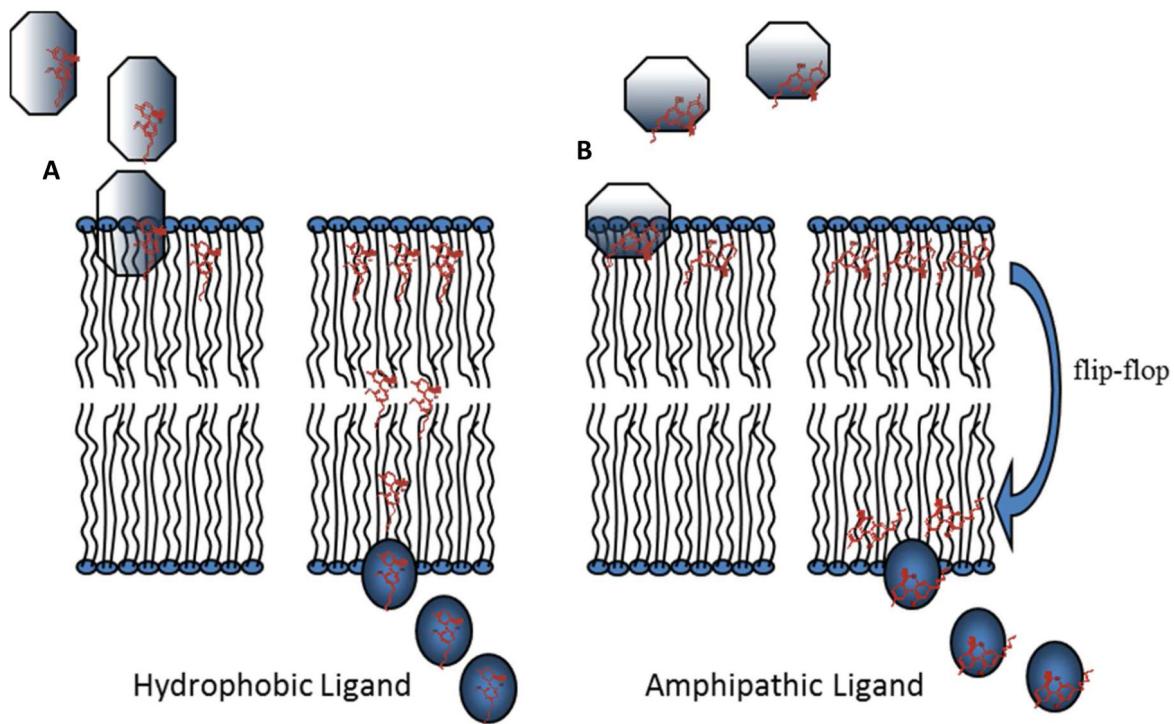
**Figure 10.**

<sup>2</sup>H-solid state NMR experiments identifying the endocannabinoid anandamide assuming an extended conformation in the bilayer with its polar group at the same level as the polar phospholipid head groups. It diffuses laterally to the binding site of the CB1 receptor and interacts with a hydrophobic groove formed by helices 3, 5, and 6. The data were used to model the location of anandamide within the bilayer and its approach to the cannabinoid receptor site through fast lateral diffusion. The C-20 anandamide segment was shown to interact with C6.47 of CB1 Hx6,<sup>33</sup> using covalent ligand labeling experiments<sup>34</sup> and is designated by a yellow star.

**Figure 11.**

On the left, inhibition of  $[^3\text{H}]$ anandamide accumulation by astrocytes by AM404. On the right, effects of AM404 on anandamide-induced inhibition of adenylyl cyclase activity in cortical neurons. AM403 is a control inactive ligand. In all experiments, cells were incubated with the inhibitors for 10 min before the addition of  $[^3\text{H}]$ anandamide for an additional 4 min.<sup>10</sup>

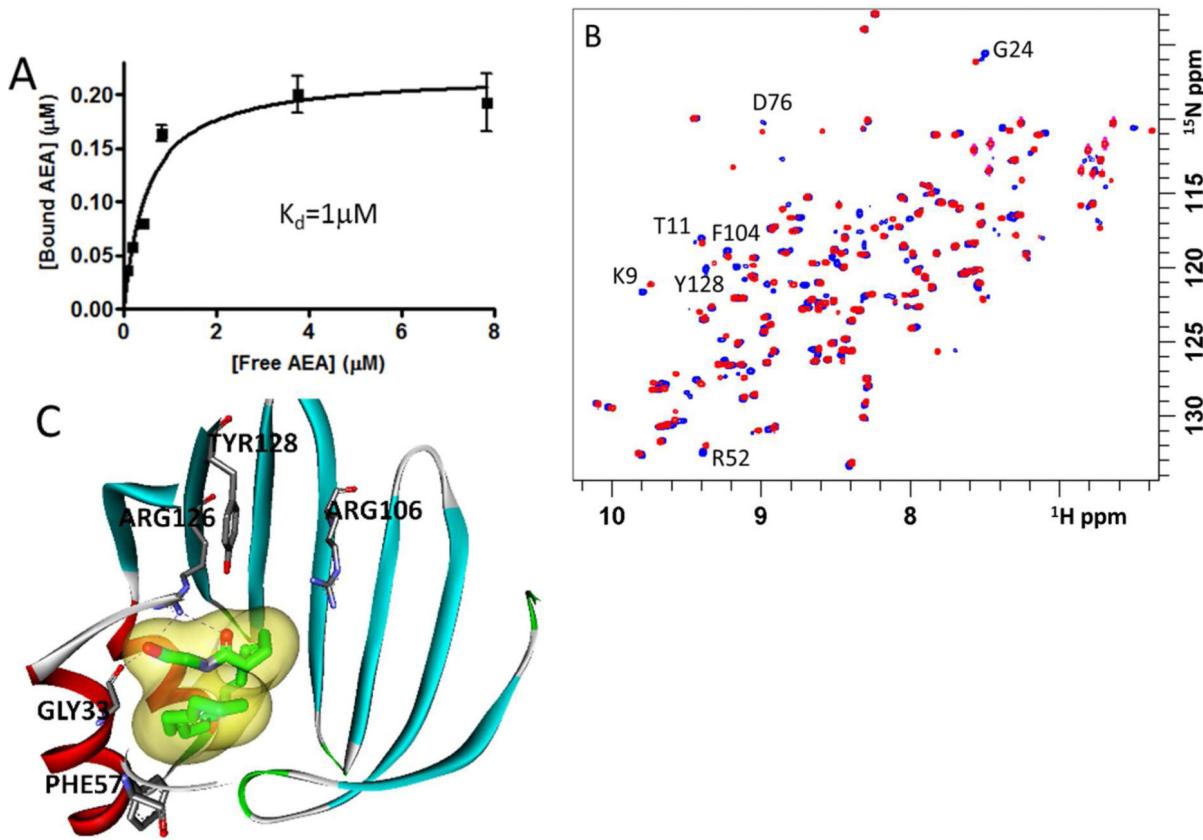
### Cannabinoid Diffusion across the Cell Membrane



**Figure 12.**

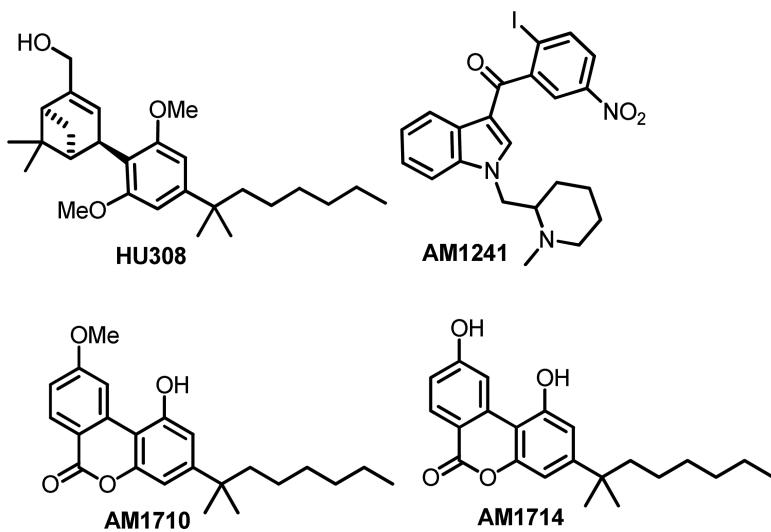
Lipophilic ligands are transported intracellularly by means of extracellular carrier proteins (e.g. albumin) and deposited initially at the outer membrane leaflet. Hydrophobic ligands (A, e.g. Me-<sup>9</sup>-THC) initially occupy a location in the outer leaflet of the membrane below the phospholipid headgroups. Subsequently, a second population of the ligand is found at the center of the bilayer where the two ligand populations are in equilibrium with each other. This suggests a mechanism for the hydrophobic ligands to reach the inner membrane leaflet where they can be taken up by specialized carrier proteins. Amphipathic ligands (B, e.g. <sup>9</sup>-THC) initially partition at the outer membrane leaflet with their polar groups interacting with the phospholipid headgroups. Through a flip-flop mechanism, the ligands are translocated to the corresponding site at the inner membrane leaflet.<sup>23</sup>

### Cannabinergic Carrier Proteins

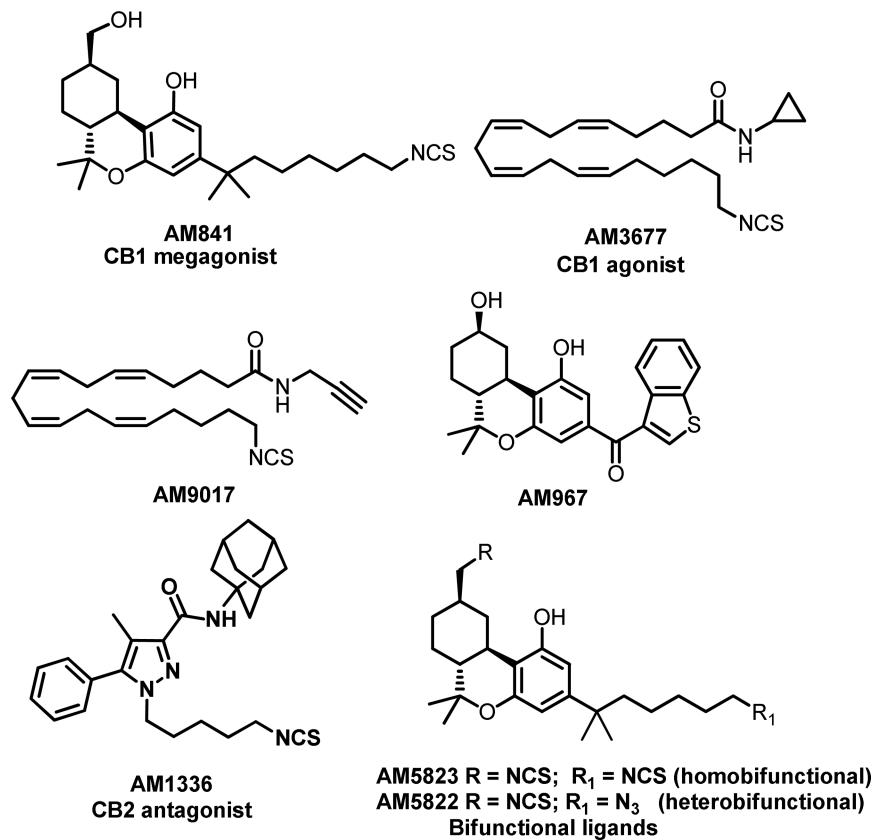


**Figure 13. Human Fatty Acid Binding Protein (FABP7)**

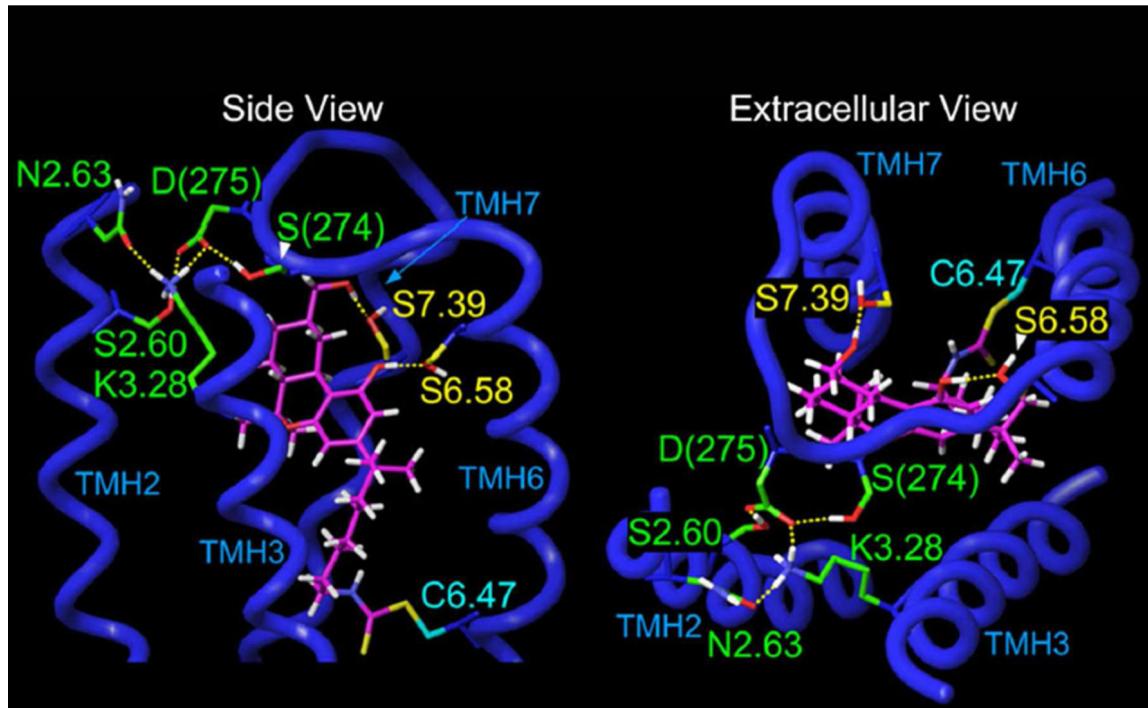
**A.** The binding of  $[^3\text{H}]\text{-anandamide (AEA)}$  to human brain fatty acid binding protein (FABP7). **B.** Overlay of 2D  $^1\text{H}$ - $^{15}\text{N}$  Heteronuclear Single Quantum Coherence (HSQC) NMR spectra of free  $^{15}\text{N}$ -labeled human FABP7 (blue) and a sample with the addition of anandamide (1:5) (red). **C.** Docking of AEA into the FABP7 crystal structure reveals three potential H-bond interactions: one between the AEA carbonyl group and the guanidinium group of Arg126, and the other two between the AEA hydroxyl group with the guanidinium group of Arg126.<sup>21</sup>



**Figure 14.**  
CB2 Agonists

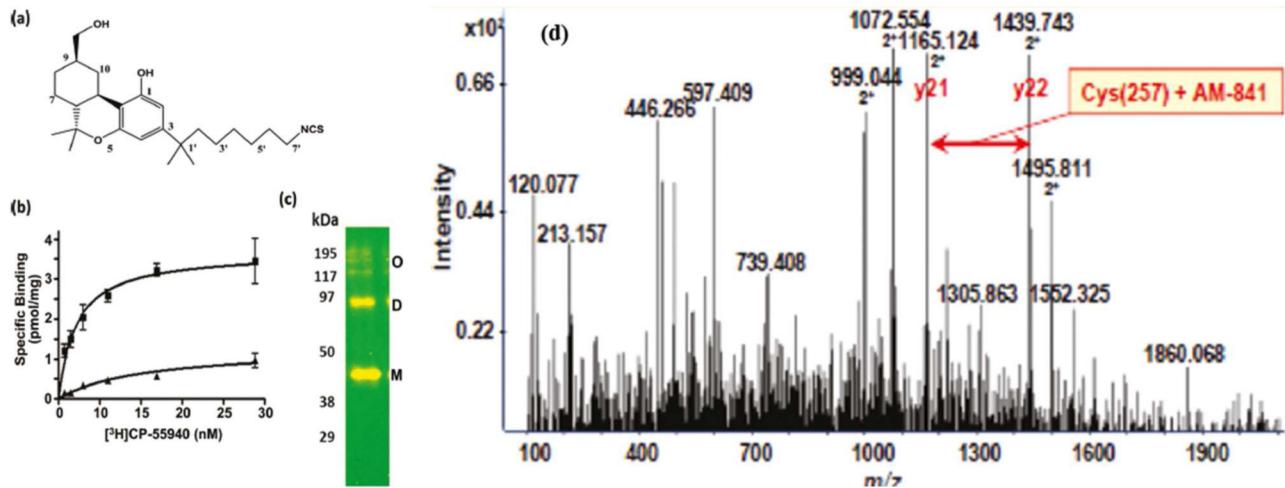
**Figure 15.**

Covalent Ligands. AM841 is a CB1/CB2 covalent megagonist.<sup>5</sup> AM3677 is an anandamide covalent CB1 receptor agonist;<sup>6</sup> AM9017 is an anandamide covalent CB2 agonist (K. Vadivel and A. Makriyannis, unpublished); AM967 is a photoactivatable CB2 covalent probe;<sup>32</sup> AM1336 is a covalent CB2 receptor antagonist.<sup>36</sup> AM5822 and AM5823 are covalent homo- and heterobifunctional CB1 ligands (Y. C. Leung and A. Makriyannis, unpublished).

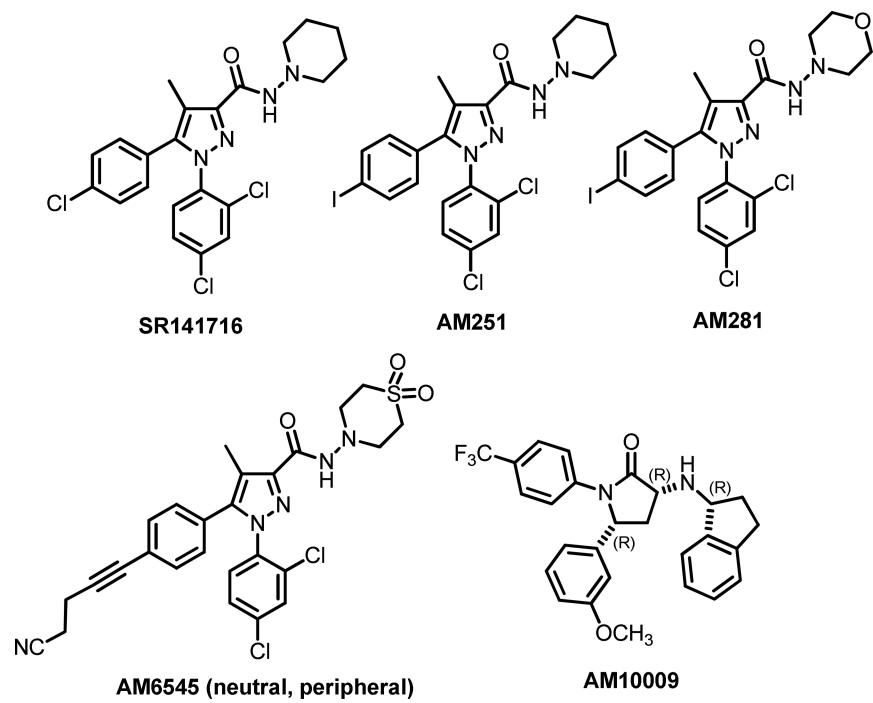


**Figure 16. Illustration of the CB2 R\*/AM841 Binding Site from Modeling Studies**

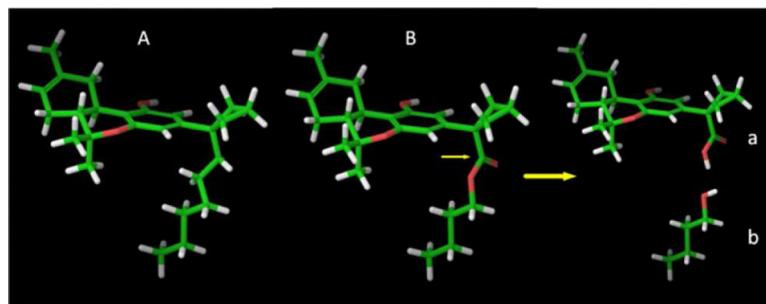
TMHs 1, 4, and 5 have been omitted from this view for simplicity. In the energy-minimized CB2 R\*/AM841 complex in which AM841 is covalently attached to C6.47(257), the carbocyclic ring CH<sup>2</sup>OH of AM841 hydrogen-bonds with S7.39(285) ( $d = 2.62 \text{ \AA}$ ; O-H-O angle = 175°), while the phenolic hydroxyl of AM841 hydrogen-bonds with S6.58(268) ( $d = 2.61 \text{ \AA}$ ; O-H-O angle = 176°). Also illustrated here is the formation of a salt bridge between D275 in EC3 and K3.28(109), a residue that is crucial for classical CB binding in the CB1 receptor, but not CB2. In the final, energy-minimized complex illustrated here, K3.28(109) is involved in a salt bridge with D275 of the EC3 loop ( $d = 2.55 \text{ \AA}$ ; N-H-O angle = 171°) and in a hydrogen bond with N2.63(93) ( $d = 2.66 \text{ \AA}$ ; N-H-N angle = 168°). D275 also forms a hydrogen bond with S274 in EC3 ( $d = 2.67 \text{ \AA}$ ; O-H-O angle = 170°) and with S2.60(90) ( $d = 2.63 \text{ \AA}$ ; O-H-O angle = 160°), a residue that is accessible from within the binding pocket in the CB2 receptor due to the helix distortion produced by S2.54(84).<sup>20</sup>

**Figure 17.**

(a) Chemical structure of AM841. (b) Saturation-binding assay of [<sup>3</sup>H] CP-55940 radioligand of FLAG-hCB2R-6His in membranes from *Sf*-21 cells overexpressing this receptor. Membrane incubation with AM841 prior to [<sup>3</sup>H] CP-55940 binding (▲) reduced the receptor  $B_{max}$  by ~75% relative to the  $B_{max}$  in membranes not exposed to AM841 (■). Data are means  $\pm$  SEM of at least two independent experiments performed in duplicate. (c) Western blot analysis of the purified FLAG-hCB2R-6His preparations with anti-FLAG antibody detection. M, monomer; D, dimer; O, oligomers, (d) MS/MS analysis of Hx6 peptide carrying AM841.<sup>16</sup>

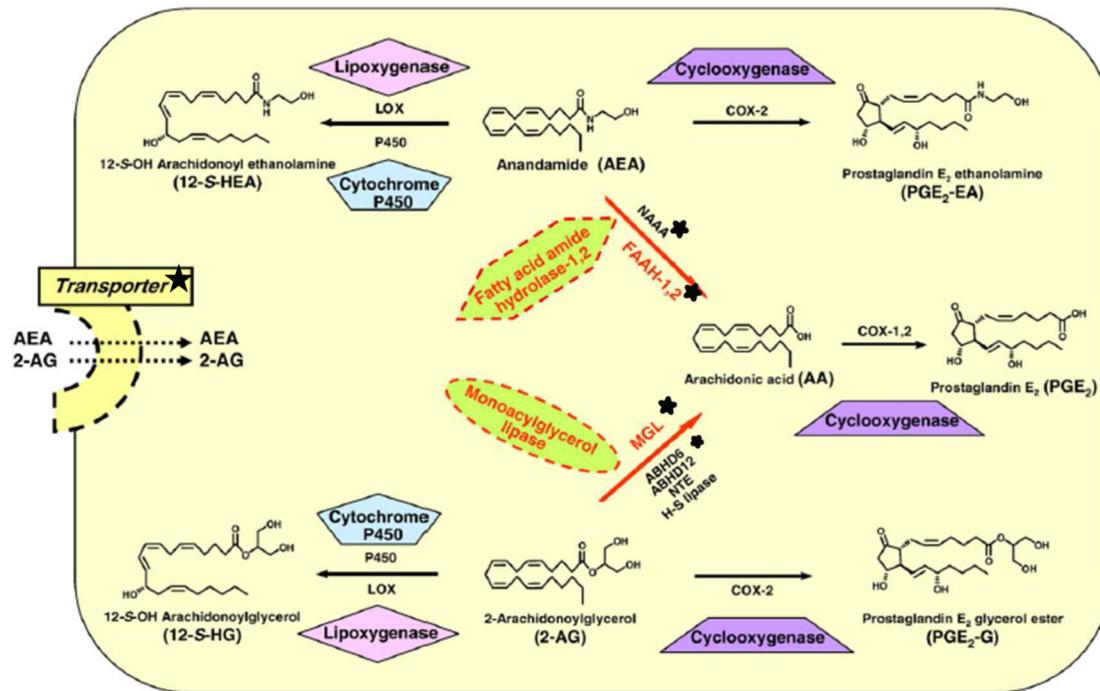


**Figure 18.**  
CB1 Antagonists

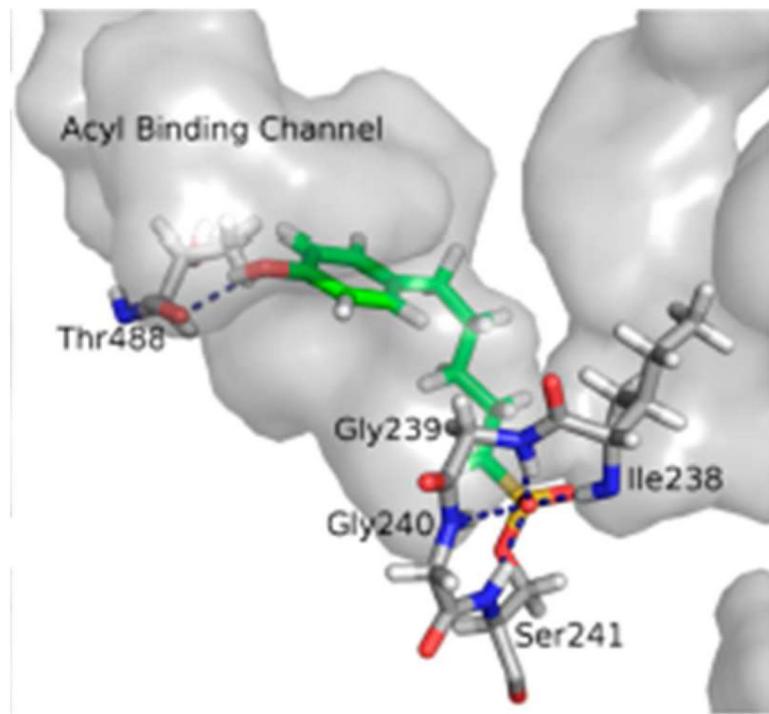


**Figure 19. Example of controlled-deactivation cannabinergic ligand**

Compound **A** [AMG38, (*6aR-trans*)-3-(1-hexylcyclobutyl)-*6a,7,10,10a*-tetrahydro-*6,6,9*-trimethyl-*6H*-dibenzo[*b,d*]pyran-1-ol] is a potent CB1 agonist ( $K_i = 1.5 \text{ nM}$ )<sup>7</sup> while compound **B** is its corresponding analog with similar pharmacophoric groups which also encompasses a key ester group in its side chain that is available for enzymatic cleavage. Through the action of esterases, **B** yields two fragments (**a** and **b**) that are shown to have negligible cannabinergic activity.

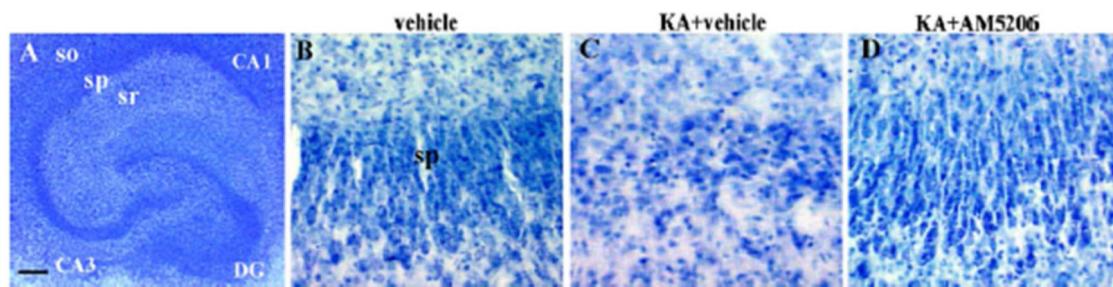
**Figure 20.**

Diagrammatic representation of the major hydrolytic inactivation and oxidative biotransformation pathways implicated in anandamide and 2-arachidonoylglycerol catabolism. (•) Endocannabinoid proteins being studied by AM.



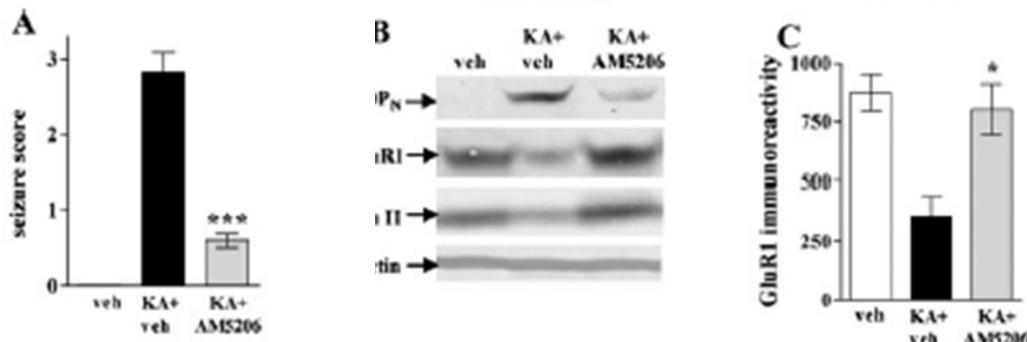
**Figure 21.**

AM3506 was covalently docked to the catalytic Ser241 of rFAAH in the acyl chain binding channel. There is significant hydrogen bonding with the oxyanion hole (formed by the backbone of Ile238, Gly239, Gly240, and Ser241), and also with the backbone carbonyl of Thr488. Hydrogen bonds are denoted by a blue dashed line, and the surface of the binding channels is shown in gray.<sup>12</sup>

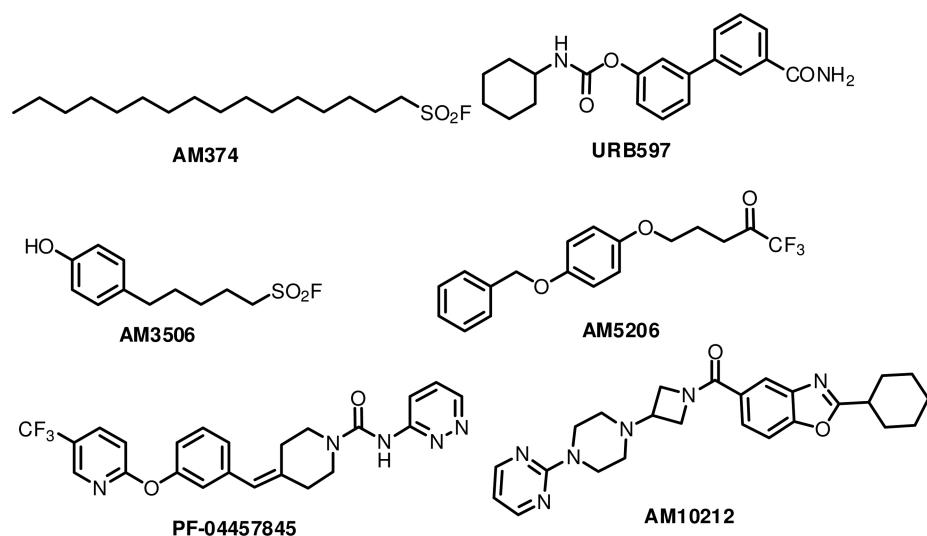


**Figure 22.**

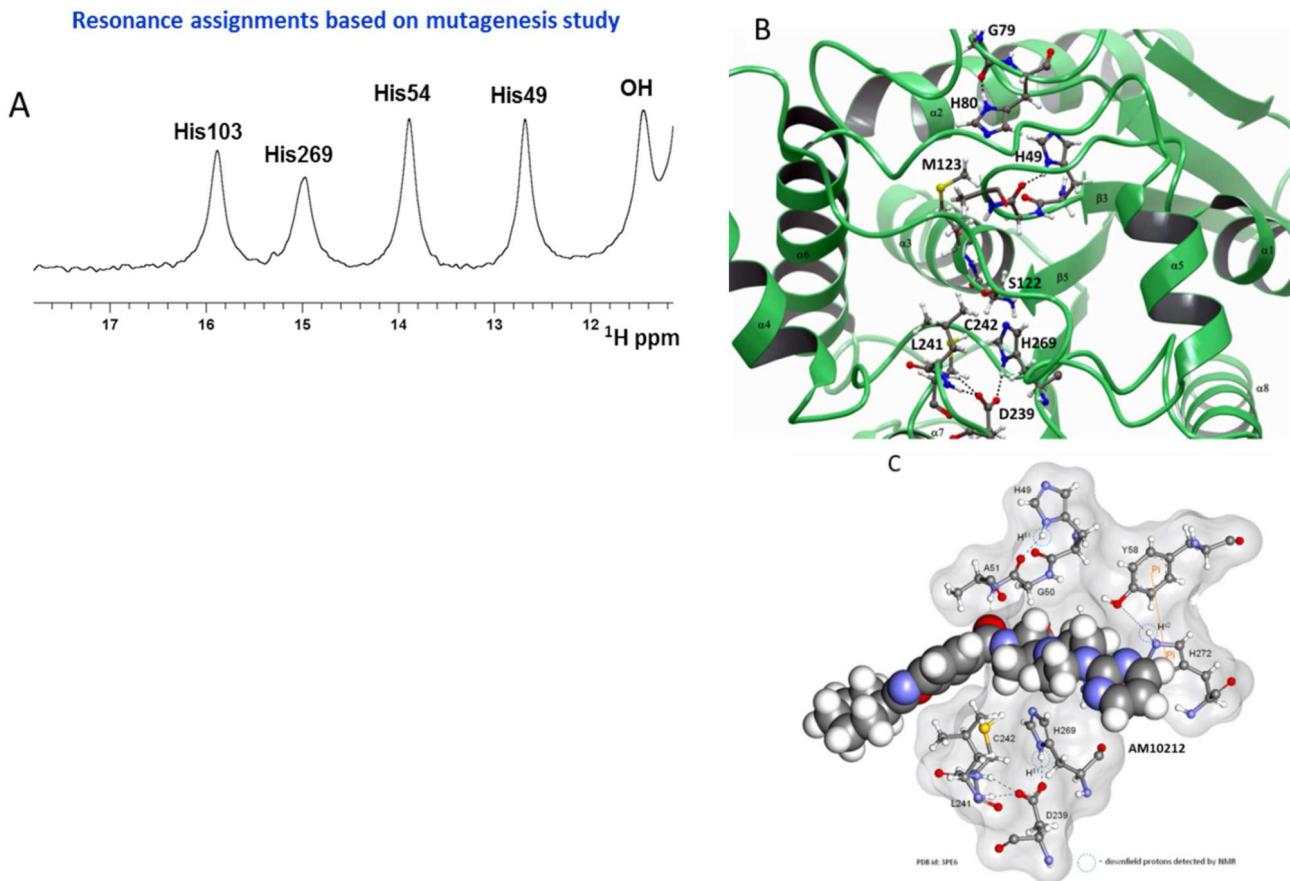
AM5206 affords neuronal protection in the hippocampus after KA-induced excitotoxicity *in vitro*. Organotypic hippocampal slice cultures were used, and a low-power photomicrograph shows their characteristic maintenance of native cellular organization. (A) Nissle staining was also used to assess cellular integrity across treatment groups. CA3 pyramidal zones are shown from slices treated consistently with vehicle (B), those pretreated with vehicle before a 2-h exposure to 60  $\mu$ M KA (C), and slices pretreated with 10  $\mu$ M AM5206 before the KA insult (D). After a washout step, the cultured slices were then incubated with vehicle or AM5206 for 24 h before the tissue was fixed, sectioned, and stained. The KA insult resulted in neuronal loss and obvious pyknotic changes that were reduced by the FAAH inhibitor. DG, dentate gyrus; so, stratum oriens; sp, stratum pyramidale; sr, stratum radiatum. Scale bar: a, 400  $\mu$ M, b-d, 45  $\mu$ M.<sup>18</sup>

**Figure 23.**

AM5206 affords seizure and neuronal protection after KA induced excitotoxicity *in vivo*. Seizures were induced by i.p. injection of 9.5 mg/kg KA (n=12 rats), and following the KA administration animals were immediately injected with either vehicle or 8 mg/kg AM5206. Vehicle-treated control rats (veh, n=11) did not receive KA or AM5206. Seizures were scored by blinded raters over a 4-h period (a) and mean scores  $\pm$  SEM are shown (ANOVA, P<0.0001). At 48 h post injection, hippocampal tissue was rapidly dissected, homogenized, and equal protein aliquots assessed by immunoblot for BDPN, GluR1, synapsin II (syn II), and actin (b). Mean integrated optical densities for GluR1 (c) are shown ( $\pm$ SEM; ANOVA, P<0.001). Post hoc tests compared to KA+ vehicle data, *single asterisk* P<0.05; *triple asterisk*, P<0.001.<sup>18</sup>

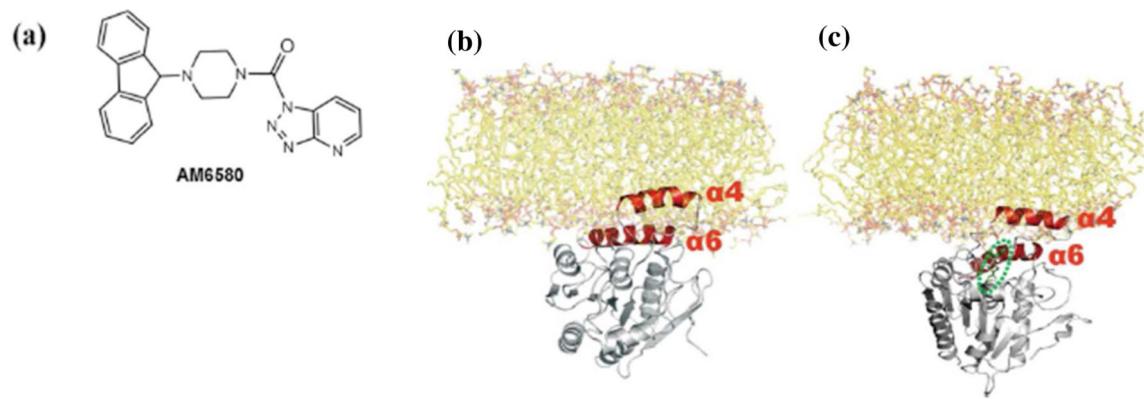


**Figure 24.**  
FAAH and MGL Inhibitors



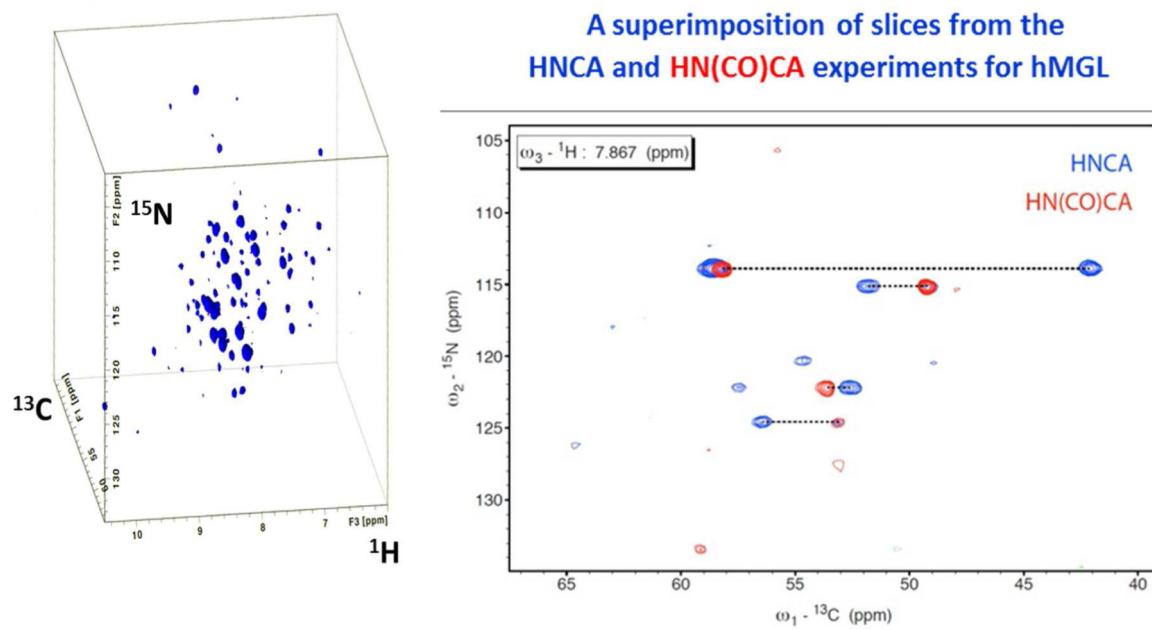
**Figure 25. Hydrogen bonding network in the active site of hMGL**

**A.** Downfield  $^1\text{H}$  NMR resonances corresponding to the H-bonds in the active site definitively assigned by comparing spectra of wt hMGL and different mutants. **B.** Active site hydrogen bonding network in hMGL identified using site-directed mutagenesis with total loss of activity observed with D239A, H269A and S122 mutants. The results shed light into the molecular details of hMGL catalysis. **C.** hMGL inhibitor AM10212 interaction at the active site. This compound was first synthesized at Johnson and Johnson.<sup>14</sup>



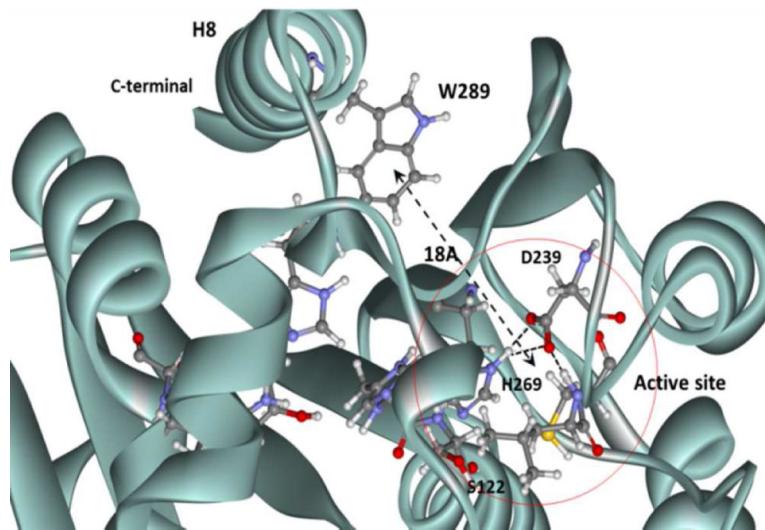
**Figure 26.**

MD simulation of hMGL interaction with membrane phospholipid bilayer. (a) Structure of AM6580. Snapshots of hMGL based on HXMS experimental derived data with a phospholipid bilayer membrane of the same composition as in experimental nanodiscs (POPC: POPG, 3:2 molar ratio) after 10 ns of MD simulation. The enzyme is depicted: (b) unliganded as *apo*-hMGL and (c) occupied with carbamylating inhibitor, AM6580, in the active site. Helix  $\alpha$ 4 in the lid domain and nearby helix alpha 6 are depicted in red, and AM6580 (c) is highlighted and depicted in green.<sup>26</sup>

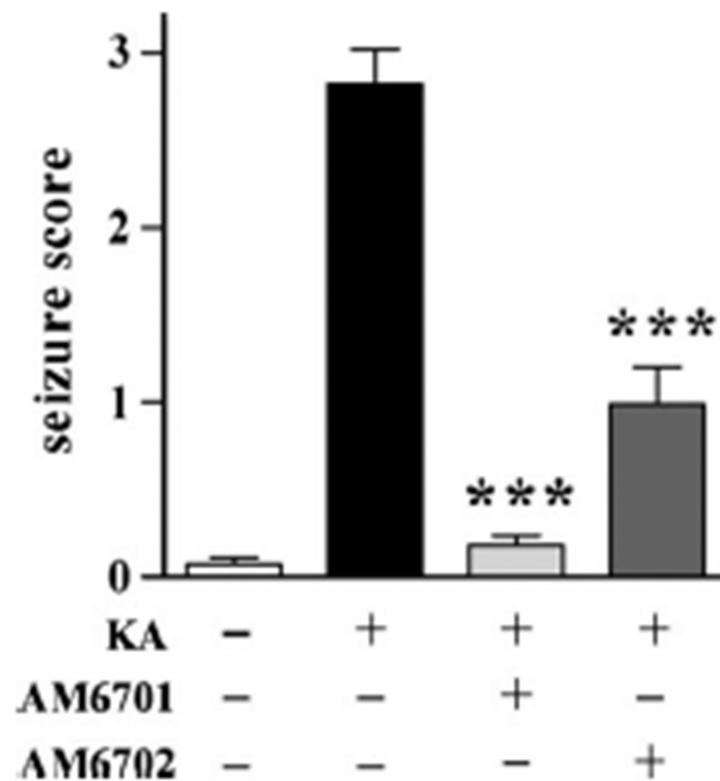


**Figure 27.**

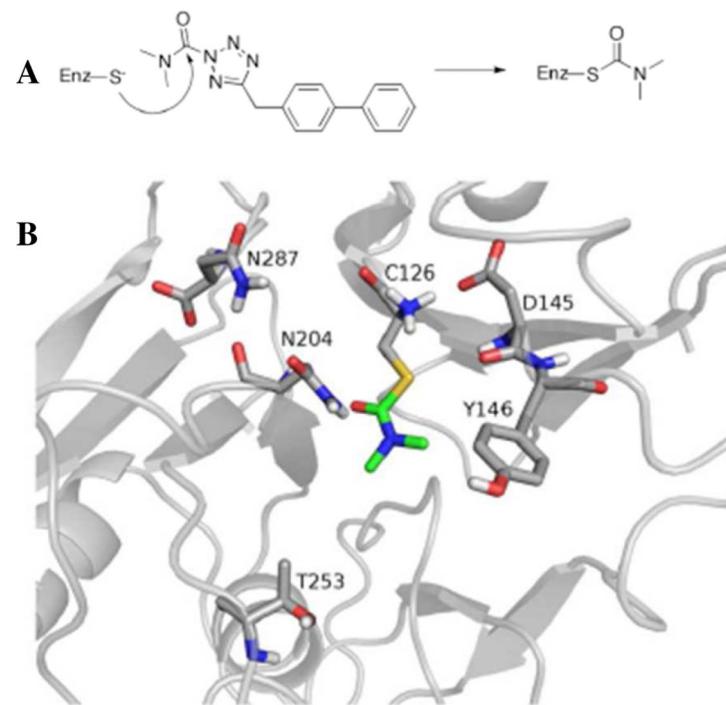
3D-cube from the HNCA spectrum of hMGL (900 MHz spectrometer with cryo-probe, 2 days experiment. Triple resonance HNCA and HN(CO)CA spectra were recorded at 310 K on a Bruker Avance 900 MHz NMR spectrometer for the assignment of backbone resonances. All spectra were processed using the Topspin software (Bruker Biospin). Together, these experiments reveal the CA chemical shift for each amino acid residue in hMGL, and provide information linking adjacent residues in the sequence.<sup>21</sup>



**Figure 28. Example of Long Range Interactions discovered in the hMGL enzyme**  
W289 is located in helix H8 at the C-terminal of hMGL separated by a distance of ~18Å between W289 and the binding site. Substitution of W289 with Leu leads to total loss of enzymatic activity. (PDB id: 3jw8)<sup>24, 25</sup>

**Figure 29.**

Reduction of KA-induced seizure severity by AM6701(FAAH, MGL inhibitor) and AM6702 (FAAH inhibitor). Seizures were initiated in rats with an intraperitoneal injection of 9.8 mg/kg KA. Immediately following the KA administration, animals were injected with vehicle (n=16) or 5 mg/kg AM6701 or AM6702 (n=6-8). No-insult control rats received 2 vehicle injections (n=13). Seizure scores were tabulated by blinding raters for a 4-h period following the injections, and the mean scores  $\pm$ SEM are shown. Analysis of variance  $p<0.0001$ ; post hoc test compared to KA only group: \*\*\* $p<0.0001$ .<sup>15</sup>



## Paracannabinoid Targets

**Figure 30.**

(A) Putative mechanism of irreversible inhibition of hNAAA by AM 6701 via thiocarbamylation of Cy126. (B) Representation of the active site of hNAAA after treatment of AM6701. Homology model illustrates thiocarbamylation of catalytic nucleophile Cys126 after treatment with AM6701.<sup>30</sup>