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Chemical Tools for Studying Lipid-Binding Class A G Protein–Coupled Receptors

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Abstract—Cannabinoid, free fatty acid, lysophosphatidic acid, sphingosine 1-phosphate, prostanoid, leukotriene, bile acid, and platelet-activating factor receptor families are class A G protein-coupled receptors with endogenous lipid ligands. Pharmacological tools are crucial for studying these receptors and addressing the many unanswered questions surrounding expression of these receptors in normal and diseased tissues. An inherent challenge for developing tools for these lipid

receptors is balancing the often lipophilic requirements of the receptor-binding pharmacophore with favorable physicochemical properties to optimize highly specific binding. In this study, we review the radioligands, fluorescent ligands, covalent ligands, and antibodies that have been used to study these lipid-binding receptors. For each tool type, the characteristics and design rationale along with in vitro and in vivo applications are detailed.

I. Introduction

Cannabinoid (CB), free fatty acid (FFA), lysophosphatidic acid (LPA), sphingosine 1-phosphate (S1P), prostanoid (DP, EP, FP, IP, TP), leukotriene [leukotriene B(4) (LTB4), cysteinyl leukotriene (CysLT), oxoeicosanoid (OXE), formyl peptide receptor (FPR)2], bile acid receptor (GPBA receptor), and platelet-activating factor (PAF) receptors are class A G protein-coupled receptors (GPCRs) that are activated by lipid-derived endogenous ligands. GPCRs are the largest family of transmembrane-signaling proteins in the human genome and convey signals from a wide range of stimuli, including neurotransmitters, peptide hormones, and autocrine factors (Katritch et al., 2012), thus offering huge potential for disease therapy (Jacobson, 2015). Chemical tools are invaluable for interrogating GPCR structure and physiologic role, which facilitates efficacious drug development. GPCRs can mediate different signaling pathways and are dynamic, flexible, and sensitive to many proximal stimuli (Kobilka and Deupi, 2007); therefore, studies using live cells in native environments often reveal more relevant information. However, scientists face challenges with GPCR research, including very low receptor expression levels in native endogenous cells and tissues (Fredriksson and Schiöth, 2005). In addition, GPCRs are difficult to crystallize due to conformational flexibility, a fluid native membrane environment, and lack of stability in detergents (Salom et al., 2013). In spite of this, the number of reported GPCR crystal structures has exploded in recent years, opening up opportunities for rational structure-based design of drugs and tools (Cooke et al., 2015; Ghosh et al., 2015). Although X-ray crystallography offers invaluable insights into

GPCR structure, the static nature somewhat limits the use in unraveling dynamic receptor processes. The chemical tools for CB, FFA, LPA, S1P, prostanoid, leukotriene, GPBA, and PAF receptors discussed in this review can help elucidate receptor physiologic role and dynamic signaling events in live cells and in native environments, thus complementing available structural information.

A. Cannabinoid Receptor

Two CB receptor subtypes (CB₁ and CB₂), endocannabinoid (ECB) endogenous ligands, and the corresponding regulatory enzymes are part of the ECB system (Katona and Freund, 2012). CB₁ receptor (the then only identified CB receptor) was characterized in 1988 through investigation of binding of the known CB receptor radioligand [³H]CP-55,940 (Fig. 1; Table 1) in rat brain membranes (Devane et al., 1988), and it was cloned in 1990 (Matsuda et al., 1990). CB₁ receptor is abundant in the brain, particularly on central and peripheral neurons in the presynapse (Kano et al., 2009), postsynaptic cells, and astrocytes (Castillo et al., 2012), and is found in low levels in peripheral organs (Engeli et al., 2005). CB₁ receptor can activate multiple, diverse signaling pathways; for example, it can couple with G_i, G_s, and/or G_q proteins (Bosier et al., 2010) and modulate calcium and potassium channels. CB₂ receptor was cloned from spleen in 1993 (Munro et al., 1993) and is expressed predominantly in immune cells, lymphoid tissues, such as the spleen and tonsils (Galiege et al., 1995), and at low levels in central nervous system (CNS) tissues such as microglial cells (Savonenko et al., 2015). Two crystal structures of the human CB₁ receptor have recently been published, one complexed with a modified rimonabant-based

ABBREVIATIONS: 2-AG, 2-arachidonylglycerol; 2-AGE, 2-arachidonyl glyceryl ether; ALX, lipoxin A4; AEA, anandamide; BBB, blood brain barrier; BRET, bioluminescence resonance energy transfer; CB, cannabinoid; CNS, central nervous system; CysLT, cysteinyl leukotriene; DBT, delayed brain tumor; ECB, endocannabinoid; FAAH, fatty acid amide hydrolase; FFA, free fatty acid; FPR, formyl peptide receptor; GPBA receptor, bile acid receptor; GPCR, G protein-coupled receptor; gp, guinea pig; h, human; HEK, human embryonic kidney; IHC, immunohistochemistry; LPA, lysophosphatidic acid; LTB4, leukotriene B(4); mAb, monoclonal antibody; MS, multiple sclerosis; NBD, nitrobenzoxadiazole; NIR, near-infrared; OXE, oxoeicosanoid; pAb, polyclonal antibody; PAF, platelet-activating factor; PET, positron emission tomography; PG, prostaglandin; P-gp, P-glycoprotein; r, rat; S1P, sphingosine 1-phosphate; SAR, structure-activity relationship; SPECT, single-photon emission computed tomography; t_{1/2}, half-life; THC, Δ⁹-tetrahydrocannabinol; TMH, transmembrane helix.

antagonist (Hua et al., 2016) and the other with the inverse agonist taranabant (Shao et al., 2016).

CB receptors are implicated in an array of physiologic processes and disorders, not limited to the following: pain, inflammation, anxiety, obesity, anorexia, drug addiction, cardiovascular disease, stroke, epilepsy, Alzheimer's disease, Huntington's disease, motor neuron disease, multiple sclerosis (MS), Parkinson's disease, and cancer (reviewed in Pacher and Kunos, 2013). Selective targeting of CB₂ receptor holds promise for treating chronic pain perhaps without (or with reduced) the CNS-mediated psychoactive side effects often associated with CB₁ receptor activation (Dhopeswarkar and Mackie, 2014). This, along with promise in other therapeutic areas, is therefore driving

development of chemical tools to better understand how CB receptor expression and function can vary between cell types and healthy and diseased tissue.

1. Ligand Classes. The ECBs anandamide (AEA) (Devane et al., 1992) and 2-arachidonylglycerol (2-AG) (Mechoulam et al., 1995; Sugiura et al., 1995) are endogenous CB receptor ligands. Cannabis has been known for its medicinal properties for thousands of years, and in 1964 the major psychoactive component Δ^9 -tetrahydrocannabinol (THC), a partial CB receptor agonist, was characterized (Gaoni and Mechoulam, 1964). Many synthetic high-affinity selective agonists, antagonists, and some allosteric ligands for CB receptors have been developed and have been reviewed

Fig. 1. CB, FFA1, S1P, prostanoid, leukotriene, and PAF receptor radioligand tools for in vitro studies. Note: structures are not shown for radioligands with unknown radioisotope position.

TABLE 1
CB, FFA, S1P, leukotriene, prostanoid, and PAF receptor radioligands for in vitro experiments

Tool	Data	Function	Commercially Available	Reference
CB₁ Receptor–Selective Ligands				
[³ H]SR141716A	rCB ₁ $K_d = 0.61 \pm 0.06$ nM hCB ₁ $K_d = 1.05 \pm 0.19$ nM	Inverse agonist	Yes	Rinaldi-Carmona et al., 1996 Niehaus et al., 2007
[³ H]BMS725519	hCB ₁ $K_d = 3.95$ nM	Antagonist		Yang et al., 2011
[³ H]OMAR	Not reported	Inverse agonist	Yes	Volk et al., 2014
[³ H]MePPEP	hCB ₁ $K_i = 0.163 \pm 0.021$ nM	Inverse agonist	Yes	Suter et al., 2010
CB₂ Receptor–Selective Ligands				
[³⁵ S]SCH225336	hCB ₂ $K_d = 0.065$ nM	Inverse agonist	No	Gonsiorek et al., 2006
CB Receptor–Nonselective Ligands				
[³ H]CP-55,940	rCB ₁ $K_d = 0.133$ nM hCB ₁ $K_d = 0.54 \pm 0.07$ nM hCB ₂ $K_i = 2.55 \pm 0.19$ nM	Agonist	Yes	Devane et al., 1988 Bouaboula et al., 1995 Felder et al., 1995
[³ H]WIN55212 (<i>R</i> and <i>S</i> enantiomeric mixture)	rCB ₁ $K_d = 15.0 \pm 4.12$ nM hCB ₁ $K_d = 11.9 \pm 1.9$ nM hCB ₂ $K_d = 2.1 \pm 0.2$ nM	Agonist	Yes	Jansen et al., 1992 Song and Bonner, 1996 Slipetz et al., 1995
[³ H]HU243	rCB ₁ $K_d = 0.045$ nM hCB ₂ $K_d = 0.061 \pm 0.05$ nM	Agonist	Yes	Devane et al., 1992 Bayewitch et al., 1995
[³ H]BAY38-7271	hCB ₁ $K_d = 0.87 \pm 0.13$ nM hCB ₂ $K_d = 4.24 \pm 0.18$ nM	Agonist	No	Mauler et al., 2003 Mauler et al., 2003
FFA1 Receptor Ligands				
[³ H]AMG837	hFFA1 $K_d = 3.6$ nM	Partial agonist	Yes	Lin et al., 2012
[³ H]AM1638	hFFA1 $K_d = 13$ nM	Agonist	Yes	Lin et al., 2012
[³ H]TAK-875	Binding ratio ^a $\approx$ sevenfold more	Agonist	No	Bertrand et al., 2016a
FFA2 Receptor Ligands				
[³ H]GLPG0974	hFFA2 $K_d = 7.5 \pm 0.4$ nM	Antagonist	Yes	Sergeev et al., 2016
S1P Receptor Ligands				
[¹⁴ C]BAF312	hS1P ₁ $K_i = 1.5 \pm 1.3$ nM ^b hS1P ₃ $K_i > 10,000$ nM hS1P ₄ $K_i = 550 \pm 39$ nM ^b hS1P ₅ $K_i = 1.4 \pm 1.3$ nM ^b	Agonist Agonist Agonist Agonist	No	Briard et al., 2015
[¹⁴ C]18	hS1P ₁ $K_i = 3 \pm 1.3$ nM ^b hS1P ₃ $K_i = 9900 \pm 100$ nM ^b hS1P ₄ $K_i = 200 \pm 100$ nM ^b hS1P ₅ $K_i = 2.3 \pm 1.1$ nM ^b	Agonist Agonist Agonist Agonist	No	Briard et al., 2015
DP₁ Receptor–Selective Ligands				
[³ H]BWA868C	hDP ₁ $K_d = 1.45 \pm 0.01$ nM	Antagonist	Yes	Sharif et al., 2000
DP₂ Receptor–Selective Ligands				
[³ H]TRQ11238	hDP ₂ $K_d = 1.0 \pm 0.1$ nM	Antagonist	No	Ulven et al., 2007
[³ H ₃]QAW039	hDP ₂ $K_d = 1.14 \pm 0.44$ nM	Antagonist	No	Luu et al., 2015; Sykes et al., 2016
[³ H]OC-459	hDP ₂ $K_d = 2.44 \pm 0.58$ nM	Antagonist	No	Sykes et al., 2016
FP Receptor–Selective Ligands				
[³ H]9 β -(+)-fluprostenol	hFP $K_d = 33.8 \pm 2.9$ nM	Agonist	No	Sharif et al., 1999
IP Receptor Ligands				
[³ H]iloprost	hIP $K_d = 3.3$ nM hEP ₁ $K_i = 11 \pm 1$ nM hEP ₃ $K_i = 56 \pm 6$ nM hEP ₄ $K_i = 284 \pm 9$ nM	Agonist Partial agonist Partial agonist Partial agonist	Yes	Katsuyama et al., 1994 Abramovitz et al., 2000; Whittle et al., 2012 Abramovitz et al., 2000; Whittle et al., 2012 Abramovitz et al., 2000; Whittle et al., 2012
TP Receptor Ligands				
[³ H]SQ-29548	hTP $K_d = 4.5$ nM	Antagonist	Yes	Hedberg et al., 1988
[³ H]U46619	hTP $K_d = 108 \pm 56$ nM hFP $K_i = 241 \pm 8$ nM	Agonist	No	Kattelman et al., 1986 Abramovitz et al., 2000
[¹²⁵ I]BOP	hTP $K_d = 2.2 \pm 0.3$ nM mFP $K_i = 100$ nM mEP ₂ $K_i = 220$ nM mEP ₃ $K_i = 100$ nM	Agonist	Yes	Morinelli et al., 1989 Kiriyaama et al., 1997 Kiriyaama et al., 1997 Kiriyaama et al., 1997
[¹²⁵ I]ISAP	hIP $K_d = 0.468 \pm 0.049$ nM	Antagonist	Yes	Naka et al., 1992

(continued)

TABLE 1—Continued

Tool	Data	Function	Commercially Available	Reference
[³ H]GR32191	hIP $K_d = 2.8 \pm 0.03$ nM	Antagonist	No	Armstrong et al., 1993
[³ H]S-145	hTP $K_d = 0.75 \pm 0.25$ nM	Antagonist	No	Hanasaki et al., 1989
[³ H]ONO3708	hTP $K_d = 3.7 \pm 1.4$ nM	Antagonist	No	Hanasaki et al., 1989
[¹²⁵ I]I-S-145-OH	hTP $K_d = 5.72$ nM	Not determined	No	Ushikubi et al., 1989
[¹²⁵ I]PTA-OH	hTP $K_d = 22.1 \pm 2.3$ nM	Antagonist	No	Narumiya et al., 1986
[³ H]BAYU3405	hTP $K_d = 6 \pm 2.5$ nM	Antagonist	No	Theis et al., 1992
	hDP ₂ $K_d = 7.2 \pm 2.5$ nM			Sugimoto et al., 2005
LTB ₄ Receptor Ligands				
[³ H]CGS23131	hLTB ₄ $K_d = 13 \pm 1$ nM	Antagonist	No	Jackson et al., 1992;
CysLT ₁ Receptor Ligands				
[³ H]ICI198615	gpCysLT ₁ $K_d = 0.08 \pm 0.04$ nM	Antagonist	No	O'Sullivan and Mong, 1989; Alexander et al., 2013
PAF Receptor Ligands				
[³ H]dihydrokadsurenone	rbtPAF $K_d = 16.81 \pm 0.57$ nM	Antagonist	No	Hwang et al., 1986
[³ H]52770RP	hPAF $K_d = 4.2 \pm 0.3$ nM	Antagonist	No	Marquis et al., 1988
[³ H]WEB2086/[³ H]apafant	hPAF $K_d = 6.1$ nM	Antagonist	Yes	Ukena et al., 1988
[³ H]L-659989	rbtPAF $K_d = 1.60 \pm 0.20$ nM	Antagonist	No	Hwang et al., 1989
[³ H]SR27417	gpPAF $K_d = 0.094 \pm 0.007$ nM	Antagonist	No	Herbert, 1992

^aRatio of binding of [³H]TAK875 to GPR40-HEK (HEK 293 cells expressing FFA1 receptor) relative to binding of [³H]TAK875 to wild-type HEK 293, which do not natively express FFA1 receptor.

^bAffinity data for cold analogs (corresponding [¹²C] derivative).

elsewhere (Pertwee, 2000; Pertwee et al., 2010; Vemuri and Makriyannis, 2015). It must be noted that, although these ligands themselves can be considered chemical tools that can be used to study CB receptor, they have not been included in this review. However, some of these ligands are the basis of chemical tools, such as radioligands and fluorescent and covalent ligands, which are reviewed in this work.

There are a handful of approved drugs; for example, the CB receptor agonists dronabinol (THC) and nabilone (THC analog) are prescribed for nausea and vomiting in cancer chemotherapy, and Sativex (THC and cannabidiol) for neuropathic pain and spasticity in MS (Pertwee, 2012). In 2006, the CB₁ receptor inverse agonist rimonabant was licensed in the European Union and several South American countries (but never in the United States) to treat obesity but was withdrawn in 2009 due to side effects such as depression and suicidal ideation.

B. Free Fatty Acid Receptor

There are four characterized FFA receptor subtypes. Each receptor can transduce signals via coupling to various G proteins and through G protein-independent signaling, such as via β -arrestin pathways (reviewed in Milligan et al., 2017). FFA1 receptor, known at the time as GPR40, was identified in 2003 (Briscoe et al., 2003; Itoh et al., 2003; Kotarsky et al., 2003) and is expressed in high levels in pancreatic β and α cells, monocytes, and various enteroendocrine and nervous system cells (reviewed in Mancini and Poitout, 2013). FFA1 receptor expression and its role in the brain is an area of ongoing interest (Khan and He, 2017). FFA2 and FFA3 receptors were identified in 2003 as the then GPR43 and

GPR41, respectively (Brown et al., 2003; Le Poul et al., 2003), and are expressed in gut, immune cells, adipose tissue, and the peripheral nervous system. FFA4 receptor, which shares the least sequence homology compared with FFA1–3 receptors, was reported in 2005 (Hirasawa et al., 2005) as the then named GPR120 and has been found in many tissues such as intestinal, gastric, adipose, and liver. The crystal structure of FFA1 receptor bound to the allosteric agonist ligand TAK-875 (Fig. 1) has been reported (Srivastava et al., 2014). The relatively recent deorphanization of FFA receptors means their role in disease and therapeutic intervention has not yet been fully realized. Recognition of dietary FFAs and how these enable cross-talk between energy metabolism (Hara et al., 2014) and the immune system has implications for many conditions such as type II diabetes and obesity (reviewed in Milligan et al., 2014; Alvarez-Curto and Milligan, 2016).

1. Ligand Classes. As the name suggests, FFAs are ligands for FFA receptors. Medium- and long-chain FFAs have higher potency at FFA1 and FFA4 receptors, whereas shorter-chain FFAs have higher potency at FFA2 and FFA3 receptors (Brown et al., 2003; Stoddart et al., 2008; Christiansen et al., 2015; Milligan et al., 2017). FFAs can be released in the body as the result of triglyceride breakdown and bacterial-mediated carbohydrate breakdown, or consumed in the diet. Despite some structure–activity relationship (SAR) trends, many FFAs bind to more than one FFA receptor subtype, thus making characterizing and understanding FFA receptors challenging (Hudson et al., 2011). A greater number of synthetic ligands for FFA1 receptor has been reported compared with other receptor subtypes, and, of those, there are many more agonists than antagonists (reviewed

in Milligan et al., 2015, 2017; Tikhonova and Poerio, 2015). This mirrors research efforts that suggest agonists of FFA1 receptor may be of benefit therapeutically. TAK-875 (or fasiglifam) (Fig. 1), a FFA1 receptor agonist, underwent Phase III clinical trials and showed improved glycaemic control in type II diabetic patients, but was not progressed further due to safety concerns (Kaku et al., 2015).

C. Lysophospholipid Receptors

LPA and S1P receptors were first classified as endothelial differentiation gene receptors and later divided into two classes based on endogenous ligand preference (Chun et al., 2002). There are five characterized S1P receptors (S1P₁–S1P₅) and six characterized LPA receptors (LPA₁–LPA₆). Originally named endothelial differentiation gene 1, S1P₁ receptor was first characterized in 1990 (Hla and Maciag, 1990). S1P₁–S1P₃ receptors are ubiquitously expressed (Blaho and Hla, 2014), whereas S1P₄ and S1P₅ receptors are found in distinct cell types such as lymphoid, hematopoietic oligodendrocyte lineage cells, and in lung tissue (Gräler et al., 1998; Terai et al., 2003). As is the case for LPA receptors, different S1P receptor subtypes all couple to different combinations of G proteins (as reviewed in Kihara et al., 2015), and presumably also use yet to be fully characterized G protein-independent signaling mechanisms. The first of the LPA receptors was identified in 1996 (Hecht et al., 1996), and the expression and signaling have been recently reviewed (Contos et al., 2000; Choi et al., 2010; Fukushima et al., 2015; Stoddard and Chun, 2015). In brief, LPA₁ and LPA₃–LPA₆ receptors are widely expressed, whereas LPA₂ receptor expression is comparatively more limited, with higher levels found in testis and leukocytes. The crystal structure of S1P₁ receptor (Hanson et al., 2012) and, more recently, LPA₁ receptor (Chrencik et al., 2015) has been reported, both using a high-affinity antagonist.

S1P₁ receptor signaling regulates human B cell tissue distribution, thereby opening avenues to treat MS, leukemia, and certain immunodeficiencies (Sic et al., 2014; Farez and Correale, 2016). S1P or LPA receptor modulation (reviewed in Mutoh et al., 2012) may be of therapeutic benefit for many areas, not limited to the following: treatment of cancer and autoimmune disorders such as systemic sclerosis and arthritis (Stoddard and Chun, 2015); fibrotic disease of the kidney, liver, lung, and skin (Kihara et al., 2015); infectious disease (Arish et al., 2016); ischemic vascular disease (Abdel-Latif et al., 2015); and neurotrauma and Alzheimer's disease (Choi and Chun, 2013). A greater understanding of receptor expression and dynamic signaling is required to unlock this potential—for which development of new chemical tools is key.

1. Ligand Classes. S1P (reviewed in Ghasemi et al., 2016) and LPA (reviewed in Stoddard and Chun, 2015;

Yung et al., 2015) are the endogenous lysophospholipids for S1P and LPA receptors, respectively. In brief, both S1P and LPA mediate an enormous array of physiologic process, and, like the ECBs, production of these lipids is tightly regulated in a complex network involving many enzymatic transformations (Tang et al., 2015). Development of synthetic S1P receptor ligands has been most intensive for S1P₁ receptor; however, selective agonists and some antagonists of other S1P receptors have been reported and reviewed elsewhere (Blaho and Hla, 2014; Delgado and Martínez-Cartro, 2016). Synthetic ligands for S1P₁ receptor notably include the nonselective agonist prodrug FTY720 (fingolimod), which was the first orally available treatment of relapsing MS (Chun and Hartung, 2010). Predominantly, antagonists have been reported as synthetic ligands for LPA₁ and LPA₃ receptors; however, many gaps exist in terms of lack of selective agonists and antagonists for the various LPA receptor subtypes (Llona-Minguez et al., 2015). Currently, there are no marketed drugs targeting LPA receptors; however, the LPA₁ receptor antagonist BMS-986020 is currently in Phase II clinical trials to treat idiopathic pulmonary fibrosis.

D. Prostanoid Receptor

There are currently five recognized prostanoid receptor subtypes, DP, EP, FP, IP, and TP receptor, which have been categorized based on the potency of the principal endogenous ligands prostaglandin (PG) or thromboxane, and then further divided into receptor subtypes (DP₁–DP₂, EP₁–EP₄ receptor). First to be cloned was TP receptor (Hirata et al., 1991) and, most recently, DP₂ receptor (Nagata et al., 1999), which, despite sharing PGD₂ as an endogenous ligand, possesses little sequence homology to DP₁ receptor and has very different functions (Hirai et al., 2001). Prostanoid receptor expression, distribution, and diverse G protein-signaling pathways are reviewed by Woodward et al. (2011). Briefly, DP₁ receptor is expressed in the small intestine and platelets, and at particularly high levels in the retina, whereas DP₂ receptor expression is widespread across the digestion system, heart, spinal cord, and blood. EP₁ receptor distribution in humans is limited when compared with murine species. In contrast, EP₂, EP₃, EP₄, and FP receptors have widespread distribution in humans. TP receptor is expressed in platelets, the cardiovascular system, and smooth muscle. To date, crystal structures have not been reported for prostanoid receptors.

EP receptors have been implicated in tumorigenesis, and, as such, the diverse signaling pathways of EP receptor subtypes are being explored as cancer drug targets (O'Callaghan and Houston, 2015). EP₂ receptor has also been investigated as a target for treating inflammation in CNS disease, stroke, and ocular disease (Ganesh, 2014), whereas EP₄ receptor has been shown to have roles in immune modulation,

inflammation, and tissue development (Konya et al., 2013). DP₂ receptor is a promising drug target for the treatment of asthma and allergic rhinitis (Santini et al., 2016; Santus and Radovanovic, 2016).

1. Ligand Classes. The endogenous prostaglandins, PGD₂, PGE₂, pGF₂α, PGI₂, and thromboxane A₂/precursor PGH₂, are involved in a variety of functions, including smooth muscle contraction and relaxation and platelet aggregation. These endogenous ligands are promptly metabolized and therefore act as paracrine or autocrine factors (Smith et al., 2011; Korbecki et al., 2014). There are selective, synthetic agonists, and antagonists for all nine prostanoid receptors (reviewed in (Flesch et al., 2013; Lamers et al., 2013; Santini et al., 2016; Santus and Radovanovic, 2016; Marković et al., 2017) and approved TP receptor antagonists on the market, including seratrodist for the treatment of asthma (Dogne et al., 2002) and ridogrel for the prevention and treatment of thromboembolism (Vandewerf et al., 1994). Terutroban was investigated as an antiplatelet agent, but the trial was suspended as it was found to be no better than aspirin (Bousser et al., 2011). EP receptor-targeted drugs have a variety of uses, such as misoprostol and sulprostone for ulcer treatment, medical abortion and labor induction (Van Mensel et al., 2009), and iloprost (also targets IP receptor) for treatment of hypertension (Olschewski et al., 2002). EP receptors are being explored as an alternative target for development of anti-inflammatory therapies because specific cyclo-oxygenase-2 (which is involved in the same pathway) inhibitors have been shown to exhibit serious side effects such as myocardial infarction and gastric ulceration (Ganesh, 2014; O'Callaghan and Houston, 2015).

E. Leukotriene Receptor

The leukotriene family of receptors is classified according to its specificity for the endogenous leukotrienes and consists of two LTB₄, two CysLT, one OXE, and one FPR2/lipoxin A₄ (ALX) receptor, with LTB₄₁ receptor the first to be cloned (Yokomizo et al., 1997). Both subtypes of LTB₄ receptor couple to G_i and G_q, stimulate phospholipase C, and inhibit adenylate cyclase, whereas CysLT₁₋₂ receptors are G_q-coupled and OXE and FPR2/ALX receptor are G_i-coupled (Bäck et al., 2011; Powell and Rokach, 2013). All leukotriene receptors are expressed in leukocytes (as reviewed by Bäck et al., 2011), with LTB₄₁ receptor also expressed in smooth muscle and umbilical cord endothelial cells and LTB₄₂ receptor expressed in spleen, liver, and ovary. CysLT₁ receptor is expressed in lung, spleen, and smooth muscle, and CysLT₂ receptor in adrenal gland, heart, spleen, brain, and spinal cord. OXE receptor is expressed in the digestive system, heart, and lungs, whereas FPR2/ALX receptor is expressed in intestinal epithelial cells, lung, kidney, spleen, and placenta (Bäck et al., 2014). A leukotriene receptor crystal structure is

yet to be reported. The leukotriene receptors have a major role in inflammation and have been implicated in CNS disorders (Ghosh et al., 2016), cancer (Burke et al., 2016), allergy (Liu and Yokomizo, 2015), and asthma (Singh et al., 2013b).

1. Ligand Classes. Leukotrienes are the endogenous ligands of these receptors and are primarily inflammatory mediators. Leukotriene levels are tightly regulated by enzymes, including lipoxygenase that converts arachidonic acid to leukotrienes (Murphy and Gijon, 2007). There are a number of selective synthetic agonists and antagonists for the leukotriene receptors, including anti-inflammatory agonists for FRP2/ALX receptor (Corminboeuf and Leroy, 2015; Stalder et al., 2017) and CysLT₁ receptor antagonists for modulation of immune cell function (Theron et al., 2014). There are three CysLT₁ receptor antagonists currently on the market for treatment of asthma: Pranlukast (Keam et al., 2003), Zafirlukast (Kelloway, 1997), and Montelukast (Paggiaro and Bacci, 2011).

F. Bile Acid Receptor

GPBA receptor, often called TGR5 receptor, shows the closest sequence homology (28%) to S1P₁ receptor and signals via endogenous bile acids (Maruyama et al., 2002). This receptor is coupled to G_s, controlling adenylate cyclase stimulation and protein kinase A activation (Coppole and Li, 2016). GPBA is widely expressed in gall bladder, intestine, liver, and enteric neurons (Duboc et al., 2014). GPBA receptor mediates the action of bile acids on glucose homeostasis and inflammation, and is therefore an interesting target for inflammatory and metabolic disorders such as diabetes (Duboc et al., 2014; Lieu et al., 2014). There is no crystal structure available for GPBA receptor.

1. Ligand Classes. The endogenous ligands of GPBA receptors are the bile acids (for example, chenodeoxycholic acid, cholic acid, deoxycholic acid, and lithocholic acid), which principally enable digestion of fats in the intestine, but also mediate insulin secretion, energy expenditure, and immune cell function through GPBA receptor signaling (Coppole and Li, 2016). There have been a number of selective synthetic agonists developed for GPBA receptor (reviewed in Xu, 2016); however, no drugs have yet come to market that specifically target this receptor.

G. Platelet-Activating Factor Receptor

The PAF receptor was first cloned in 1991 (Honda et al., 1991). Activation by the endogenous agonist PAF signals via a complex network of G protein-dependent (Honda et al., 1994) and -independent (Lukashova et al., 2001; Chen et al., 2002) transduction pathways. PAF receptor is widely expressed in immune cells such as neutrophils, macrophages, and B cells, and also in other cell types such as keratinocytes, and, as the origin of the name suggests, platelets (Chao and

Olson, 1993; <http://www.guidetopharmacology.org/GRAC/FamilyIntroductionForward?familyId=55>). Several studies have shown the existence of intracellular PAF receptors, with differences in receptor cellular localization most likely resulting in distinct functions (Bhosle et al., 2016), thus adding an extra layer of complexity and potential for drugs targeting a specific receptor population. Historically, most therapeutic intervention via PAF receptor antagonism has been directed at treating conditions associated with inflammation and immunity, for example, asthma (Kasperska-Zajac et al., 2008), allergic conditions (Paľgan and Bartuzi, 2015), and autoimmune diseases (Edwards and Constantinescu, 2009). Due to the extensive pathophysiological role of this receptor, there has been recent interest in targeting PAF receptor for other indications, for example, in reducing human immunodeficiency virus–related comorbidities (Kelesidis et al., 2015) and in the treatment of dengue infections (Souza et al., 2009). A crystal structure of PAF receptor has not yet been reported.

1. Ligand Classes. The endogenous ligand is the proinflammatory phospholipid PAF, which in the 1960s was identified via its role in platelet aggregation and histamine release from activated platelets (Barbaro and Zvaifler, 1966). As is the case for other endogenous lipid-signaling molecules, the production and metabolism of PAF are highly regulated and complex (as reviewed in Prescott et al., 2000); for example, therapeutic intervention using PAF acetylhydrolase inhibitor darapladib failed to meet Phase III endpoints (Marathe et al., 2014). Clinical applications of PAF receptor modulation have driven the development of many selective antagonists (reviewed in Koltai and Braquet, 1994 and Feuerstein et al., 1997), and natural products have been reported as PAF receptor antagonists (reviewed in Singh et al., 2013a), of note ginkgolide B (also known as BN 52021). Rupatadine is a dual histamine-1 and PAF receptor antagonist clinically used to treat allergic rhinitis and urticarial (Gonzalez-Nunez et al., 2016), whereas several other PAF receptor antagonists are still in clinical trials (e.g., modipafant, which was discontinued for asthma indication but is now under investigation for dengue fever) or have failed in clinical trials/been discontinued (e.g., apafant, lexipafant, foropafant).

H. Orphan G Protein–Coupled Receptors

Future classification of orphan GPCRs into existing endogenous lipid-binding GPCR families or as new lipid-binding GPCR families is possible. GPR55 is found in CNS tissue, vasculature, and osteoclasts, and is thought to play a role in nociception, cancer, and metabolism (Henstridge, 2012). The endogenous ligand for GPR55 is reported as lysophosphatidylinositol, but sensitivity toward endogenous CBs and some synthetic CB receptor ligands has also been shown (reviewed in

Henstridge et al., 2016), whereas some chemical tools developed for CB receptors turned out to have affinity for GPR55 (*Fluorescent Tools for In Vitro Experiments*). Endogenous lipid-like ligands have been shown to bind to other class A orphans such as GPR18, GPR84, GPR119, GPR132, and GPR174 in more than one publication (<http://www.guidetopharmacology.org/GRAC/GPCRListForward?class=A>).

I. Rational Design of Tools and Considerations for Use

A well-characterized ligand with high affinity and selectivity for the target receptor is often selected as the starting point for designing a radioligand, fluorescent, or covalent ligand. GPCRs can exist in multiple conformations (e.g., R and R*), and each conformation may have a different affinity for ligands, such as antagonists, agonists, or allosteric modulators; therefore, end biologic use should be considered early in the design process. Often tool design is aided by structural information, either crystallographic or homology receptor models, and/or knowledge from biologic techniques such as site-directed mutagenesis. For receptors with endogenous or synthetic lipophilic ligands, it is important to balance the inherent requirement for some lipophilicity of the orthosteric pharmacophore to retain receptor affinity and/or efficacy but without undesirable properties such as high levels of nonspecific membrane binding. Promiscuous binding to other GPCRs and cellular proteins also contributes to undesirable nonspecific binding and should be considered in the design process. The receptor selectivity of a chemical tool should be considered in a context-specific manner to enable meaningful use, as selectivity measured in different cell lines overexpressing a given receptor subtype in vitro may not necessarily translate into in vivo selectivity if relative receptor subtype expression is vastly different due to tissue- or disease-induced differences.

Ligand entry into lipid-activated CB (Makriyannis and Rapaka, 1990; Tian et al., 2005; Hurst et al., 2010) and S1P (Hurst et al., 2013) receptors is proposed to be via a lipid portal rather than direct extracellular entry as for other class A GPCRs. This has implications for access of chemical tools such as radioligands, covalent, and fluorescent ligands into the receptor binding site. The generation of antibodies against an antigen is of course a very different process to that of designing chemical tools and requires stable purified protein, often in a certain functional conformation, which can be quite challenging for flexible membrane-embedded GPCRs. Depending on availability and suitability, in vitro GPCR research can use cells or tissue from many different species; for example, discussed in this manuscript are human (h), mouse (m), rat (r), bovine (b), guinea pig (gp), and rabbit (rbt) cell lines/tissues. Small variations in sequence can lead to significant differences in potency of a ligand for a receptor (Hudson et al., 2013); therefore, when analyzing

tools, it is extremely important to consider the receptor species.

II. Radioligands

A. Characteristics and Design Rationale

Radioligands contain a radioisotope such as ^3H , ^{123}I , ^{125}I , ^{35}S , ^{11}C , and ^{18}F , which undergoes nuclear decay to emit β particles, positrons, or γ radiation that can be detected and quantified. Sensitivity and shelf life are determined by specific activity and half-life ($t_{1/2}$) of the radioisotope; for example, ^{125}I has a specific activity of 2200 Ci/mmol and a $t_{1/2}$ of 60 days compared with ^3H with a specific activity of 120 Ci/mmol and a 12-year $t_{1/2}$. It should be noted that these specific activities are representative and will vary across batches. More commonly, ^3H radioligands have been used to study CB, FA, LPA, S1P, prostanoid, leukotriene, GPBA, and PAF receptors, due to commercial availability and the ^3H – ^1H interchange leading to little chemical change, thus preserving biologic activity and better relative safety over other radioisotopes. Radioisotopes are chemically incorporated, for example, through reaction of a phenolic ligand with [^{11}C]methyl iodide or catalytic reduction of an alkene with tritium gas.

For GPCRs with lipid-like endogenous ligands, it is often challenging to design suitable radioligands that possess sufficient polarity to negate high levels of nonspecific membrane binding. For example, most CB receptor radioligands for in vivo imaging are lipophilic compounds with $\log D_{7.4}$ in the range of 3.3–6.0. High-affinity radioligands with subnanomolar K_d values are preferred, as they can be used effectively at lower concentrations (Bigott-Hennkens et al., 2008). As with many other chemical tools, the researcher can select a tool that is most advantageous to his or her study—this could be an agonist, antagonist, or inverse agonist. Currently, there are reported radiolabeled allosteric tools for studying FFA1 receptor but not for other lipid-binding GPCRs discussed in this work; future development, for example, of a radiolabeled ORG27569 (Fig. 6), a CB receptor allosteric ligand, would prove very interesting.

B. Applications of Radioligands

Radioligands have been used extensively to study GPCR pharmacology, for example, distribution, signaling, oligomerisation, allosteric modulation, and in binding assays for screening novel ligands. Radioligands have been used in saturation, competition, or kinetic binding assays to determine various ligand–receptor parameters such as K_d , K_i , and B_{max} . There are also in vivo applications such as positron emission tomography (PET) and single-photon emission computed tomography (SPECT). High-affinity and excellent radioligand receptor subtype specificity are especially important with low levels of receptor expression and to avoid

toxicity and radiation hazards. Limitations inherent to radioligands include legal and safety requirements surrounding handling, disposal, synthesis, and/or storage, and the need for specialized equipment and facilities to address this, along with natural radioactive decay that limits shelf life.

1. *Radioligands for In Vitro Experiments.* Rather than an exhaustive commentary of all radioligands and uses, this review describes the commonly used in vitro radioligands based on synthetic scaffolds (Fig. 1; Table 1) and describes selected studies that showcase the utility of these radioligands. It should be noted that many radiolabeled lipids are commercially available and have been used to study receptor and endogenous ligand trafficking, for example [^{33}P]S1P (Jo et al., 2012), [^3H]LPA (Thomson et al., 1994), [^3H]AEA (Hillard et al., 1997), [^3H]PGE₂ (Nemoto et al., 1997), [^3H]PGD₂ (Matsuo and Cynader, 1993), [^3H]LTB₄ (Toda, 1999), [^3H]5-oxo-ETE (O’Flaherty et al., 1998), and [^3H]PAF (Nakamura et al., 1991). Although these labeled endogenous ligands can be useful tools for interrogating receptor pharmacology (Hecht et al., 1996; Parrill et al., 2000; Van Brocklyn et al., 2000), they often suffer from rapid enzymatic degradation even in in vitro experiments. In this study, we review only radiolabeled synthetic ligands.

In 1964, THC was identified as a CB₁ receptor ligand, and soon after it was ^{14}C -labeled to provide the first CB receptor radioligand [^{14}C]THC (Miras, 1965). Since then a number of THC-based CB receptor radioligands have been developed (Aguirell et al., 1969; Pitt et al., 1980; Nye et al., 1985); however, nowadays these are not commonly used due to high nonspecific membrane binding.

Autoradiographic studies of receptor distribution can be carried out in vitro; for example, an early report using the CB receptor radioligand [^3H]CP-55,940 (Fig. 1; Table 1) found the highest CB₁ receptor density in substantia nigra, basal ganglia, globus pallidus, cerebellum, and hippocampus regions that are known to control cognitive and motor functions (Herkenham et al., 1990; Glass et al., 1997). An interesting recent example of a competition-binding assay using [^3H]CP-55,940 is the evaluation of approximately 50 compounds from the herbal mixture known as “Spice,” previously shown or assumed to be a CB₁ agonist (Hess et al., 2016). Many new structural analogs of cannabinoids have emerged in recent years designed to circumvent legally defined chemical classes, with little accompanying pharmacological evaluation. Hess et al. (2016) carried out radioligand-binding assays using [^3H]CP-55,940, which revealed most compounds had low to subnanomolar affinity for CB₁ and CB₂ receptors, and further functional assays showed these to be agonists.

CB receptor affinity determined by a radioligand competition-binding assay is often the first parameter by which ligands are ranked despite the sometimes little structural similarity between radioligand and test

ligand. A recent report has highlighted some important things to note when using a universal radioligand for investigating binding of ligands to CB₂ receptor (Smoum et al., 2015). Smoum et al. (2015) reported that HU-433, the enantiomer of the well-studied HU-308, had 25-fold reduced binding affinity to CB₂ receptor (as measured using a competition assay with [³H]CP-55,940) but had much higher potency in osteoblast proliferation and anti-inflammatory experiments compared with HU-308. It was proposed that HU-433 and HU-308 may bind to CB₂ receptor in different orientations, thus turning on different downstream signaling pathways and competing with [³H]CP-55,940 non-equally. This study serves as a reminder that measured compound affinity for a receptor is only ever in relation to the reference, and sometimes the choice of reference or radioligand tool can influence not just the raw number but also the rank order of ligands.

Radioligands are crucial tools for investigating the existence of multiple GPCR states. For example, it was estimated that CB receptors are present in a 70% inactive:30% active conformation in rat cerebellar membrane using the agonist [³H]CP-55,940 that bound only to active rat (r) CB₁ receptor and the inverse agonist [³H]SR141716A (Fig. 1; Table 1) that bound to rCB₁ receptors in both active and inactive states (Kearn et al., 1999). Radioligands are also important tools for identifying key receptor amino acids, for example, direct comparison of radioligand binding to wild-type versus mutant receptor. In one study, modified human (h) CB₁ receptors were constructed with residues D2.63 and K373 mutated to Ala or the reciprocal Asp/Lys mutation to investigate the importance of an ionic interaction (Marcu et al., 2013). [The residue numbering system used is based on the Ballesteros–Weinstein numbering scheme, whereby the single-letter code of the amino acid is followed by the transmembrane helix (TMH) number, and then the amino acid's position relative to the TMH's most conserved residue (assigned as 50)]. The K_d , B_{max} , and K_i of [³H]SR141716A were measured for wild-type receptor and for each receptor mutant, and, interestingly, no significant difference in binding was observed. However, a GTP γ S-binding assay indicated signaling was impaired in each Ala receptor mutant, but not in the reciprocal Asp/Lys mutant, with the authors suggesting the ionic interaction between D2.63 and K373 influences the EC-3 loop and is important for CB receptor signaling.

The physiologic relevance of GPCR oligomerization is not yet fully understood, and radioligands have been used as tools to shed light on this; for example, a selective adenosine_{2A} receptor agonist CGS21680 decreased the B_{max} of the CB₁ receptor radioligand [³H]SR141716A in synaptosomal membrane of rat brain from 3.23 ± 0.17 pmol.mg⁻¹ to 2.68 ± 0.18 pmol.mg⁻¹ (Ferreira et al., 2015). This was attributed to inhibition of CB₁ receptor signaling by adenosine_{2A} receptor, with

the authors suggesting that dual targeting of these receptors may prove therapeutically beneficial. Despite radioligands having utility for investigating GPCR oligomerization, fluorescent tools are often superior for this line of investigation, as bioluminescence resonance energy transfer (BRET) and fluorescence resonance energy transfer experiments can provide precise spatial information (*Applications of Small-Molecule Fluorescent Tools*). Allosteric binding sites of CB, FFA, prostanoid, and S1P receptors have been interrogated using radioligand tools. In one example, a CB₁ receptor radioligand-binding assay carried out in the presence of compounds such as ORG27569 (Fig. 6) increased the binding affinity of the agonist [³H]CP-55,940, whereas a decrease in the binding affinity for the inverse agonist [³H]SR141716A was observed (Price et al., 2005).

Distinct FFA1 receptor binding sites have been identified using the partial allosteric agonist [³H]AMG837 (position of the ³H radioisotope was not reported) and the full allosteric agonist [³H]AM1638 (Table 1) in experiments with and without endogenous FFA docosahexaenoic acid and other unlabeled FFA1 receptor agonists (Lin et al., 2012). Three allosterically linked binding sites were proposed, with the authors noting these ligand–receptor interactions were far more complex than previously recognized. These findings are important in the context of the role of FFA1 receptor in glucose-stimulated insulin secretion and may provide a novel multiligand treatment therapy for type 2 diabetes, in which a reduced dose of drug can be used due to ligand cooperativity. Another research group has reported the synthesis and use of [³H]TAK-875 (Fig. 1; Table 1) as a FFA1 receptor radioligand agonist (Bertrand et al., 2016a), based on the TAK-875 drug that failed Phase II clinical trials. After demonstrating that [³H]TAK-875 bound to GPR40–human embryonic kidney (HEK) cells in higher levels than in wild-type HEK cells, the research team developed a [¹⁸F]TAK-875 radioligand (*Radioligands for In Vivo Experiments*).

The first reported radioligand-binding assay for FFA2 receptor used [³H]GLPG0974 (Table 1; position of the ³H radioisotope not reported) as the competing ligand (Sergeev et al., 2016). This radiolabeled antagonist was characterized using saturation equilibrium-binding assays across several different FFA2 receptor mutants and then analyzed in kinetic binding assays, in particular to determine on and off rates of unlabeled agonists and antagonists. Data from these experiments led to the suggestion that an agonist requires interaction with both arginine residues (Arg-180^{5,39} and Arg-255^{7,35}), whereas, for an antagonist, interaction with only one of these two arginines is required. This provided valuable information to aid rational structure-based design of novel tools and drugs for this therapeutically relevant receptor. Radioligands based on synthetic, selective scaffolds for FFA3 and FFA4 receptors have not yet been reported; however, researchers

have used labeled fatty acids such as [^{33}P]S1P to probe the binding site(s) of unlabeled synthetic agonists (Jo et al., 2012).

To eventually develop iodinated derivatives for in vivo imaging of S1P receptor (*Radioligands for in vivo experiments*), Briard et al. (2015) made two radioligands—[^{14}C]BAF312 and a cold iodinated derivative of this [^{14}C]18 (Fig. 1; Table 1) (Briard et al., 2015). These two ligands were used in autoradiography studies to compare biodistribution of the MS drug BAF312 (Fig. 1) to the iodinated derivative [^{127}I]MS565 with the goal of making [^{123}I]MS565. Whole-body autoradiography studies in rats revealed similar biodistribution profiles of [^{14}C]BAF312 and [^{14}C]18.

Radioligands based on synthetic LPA receptor ligands are not yet reported despite the availability of several high-affinity synthetic ligands for these receptors (Ohta et al., 2003; Qian et al., 2012). It is expected that development of radioligands based on synthetic ligands for studying LPA receptors will see rapid growth. This development will be fueled by the need to prepare novel ligands targeting these receptors for various disease conditions.

Selective synthetic radioligands are available for prostanoid family DP₁, DP₂, FP, and TP receptors. The selective DP₁ receptor antagonist [^3H]BWA868C (Table 1) has been used for autoradiographic studies in human eye sections (Sharif et al., 2000). This study revealed high levels of DP₁ receptor expression in ciliary epithelium/processes, ciliary muscles, the iris, and the retinal choroid, which was similar to that observed previously using the endogenous ligand [^3H]PGD₂ (Matsuo and Cynader, 1993). Selective radiolabeled DP₂ receptor antagonists have been reported: [^3H]TRQ11238 (Ulven et al., 2007), [^3H]QAW039 (Luu et al., 2015), and [^3H]OC-459 (Sykes et al., 2016) (Fig. 1; Table 1). Consideration of ligand–receptor kinetics is crucial in a drug discovery program, and often drug dissociation time from the receptor is clinically relevant. Sykes et al. (2016) therefore used [^3H]QAW039 and [^3H]OC-459 (tritiated versions of the Phase III asthma treatments fevipiprant and timapiprant, respectively) to directly measure drug kinetics and function in parallel to unravel the drug mechanism of action, concluding that QAW039 should competitively inhibit disease-relevant DP₂ receptor-mediated responses in human cells (Sykes et al., 2016).

Sharif et al. (1999) used the selective FP receptor agonist [^3H]9 β -(+)-fluprostenol (Fig. 1; Table 1), a carboxylic acid derivative of the glaucoma drug travoprost, to study prostanoid receptor distribution in the human eye (Sharif et al., 1999), which revealed FP receptor distribution consistent with that obtained using tritium-labeled endogenous ligand [^3H]PGF_{2 α} (Matsuo and Cynader, 1992; Davis and Sharif, 1999).

[^3H]Iloprost (Fig. 1; Table 1), the tritiated analog of the marketed agonist drug iloprost, has been used to

investigate IP receptor expression and function, although it also possesses modest potency for EP₃ and EP₁ receptors (Abramovitz et al., 2000). Decreased saturation binding of [^3H]iloprost in platelets of humans with type 2 diabetes compared with healthy humans has been demonstrated (Knebel et al., 2015). This finding along with other functional measurements led the authors to suggest that this lower IP receptor expression in platelets of patients with type 2 diabetes might lead to increased platelet aggregation, which may be the reason for the increased risk of thrombosis observed in such patients.

Among prostanoid receptors, the greatest number of radioligands has been developed to study TP receptor (Fig. 1; Table 1), probably due to its importance in thrombosis and related cardiovascular disorders. Selective antagonist [^3H]SQ-29548 (Hanasaki et al., 1988; Hedberg et al., 1988) (Fig. 1; Table 1) is frequently used in competition-binding assays. For example, in one study, the antibody C-EL2Ab, which binds to the C-terminal of second extracellular loop of TP receptor, competitively inhibited the binding of [^3H]SQ-29548 to TP receptor and also inhibited TP receptor–mediated platelet aggregation, which showed the importance of this portion of the receptor structure in platelet activation and the potential of C-EL2Ab as an alternative antiplatelet agent (Murad et al., 2012).

Compared with the prostanoid receptor family, there are far fewer synthetic scaffold-based radioligands reported for the leukotriene receptor family, and therefore radiolabeled endogenous ligands are frequently used. There is one report of the synthesis and use of the antagonist [^3H]CGS23131 (Fig. 1; Table 1) to characterize the single LTB₄ receptor subtype recognized at that time on human polymorphonuclear neutrophils (Jackson et al., 1992) and several early reports on the use of [^3H]ICI198615 (Fig. 1; Table 1) (Aharony et al., 1988; O'Sullivan and Mong, 1989) to study the single recognized CysLT receptor. Pranlukast, a selective antagonist for CysLT₁ receptor that is used clinically to treat asthma, has been tritium labeled and used for autoradiographic studies in human nasal inferior turbinates (Shirasaki et al., 2006). These studies revealed high distribution of CysLT₁ receptor in vascular endothelium and the interstitial cells; however, the K_d of [^3H]pranlukast was not reported.

There are many examples of synthetic high-affinity antagonists for PAF receptor, several of which have been radiolabeled and used as tools, for example [^3H]dihydrokadsurenone (Hwang et al., 1986), [^3H]52770-RP (Robaut et al., 1987; Marquis et al., 1988), [^3H]WEB2086 (also known as [^3H]apafant) (Ukena et al., 1988), [^3H]L-659989 (Hwang et al., 1989) (Fig. 1; Table 1), and [^3H]SR27417 (Herbert, 1992) (Table 1). Determination of the degree of competition that a synthetic radioligand has with the endogenous receptor agonist is important; for example, it was shown that [^3H]WEB2086, but not

[³H]52770-RP, interacted with the same binding site as [³H]PAF in human platelets (Ukena et al., 1988). Now commercially available, [³H]WEB2086 has been used several times as a tool to study PAF receptor; for example, radioligand-binding assays showed that human B lymphoid cell line LA350 expressed high levels of PAF receptor (Zhuang et al., 2000). To date, synthetic radiolabeled ligands for GPBA receptor have not been reported; however, it is likely this will be an area of future interest as drugs targeting this receptor (Xu, 2016) are developed.

2. Radioligands for In Vivo Experiments. In vivo radioligand experiments require consideration of blood brain barrier (BBB) permeability (if required) and ligand metabolism to radiometabolites that can complicate analysis. As discussed in *Introduction*, selectivity of a chemical tool in vivo requires careful consideration of receptor subtype selectivity (which is often first measured in vitro), versus relative in vivo expression levels of the subtypes/other receptors. Much effort has been directed toward the development of in vivo radioligands to study CB₁ receptor in brain to investigate pain and addiction pathways. Despite PET radioisotopes having a shorter $t_{1/2}$ (¹¹C $t_{1/2}$ = 20 minutes, ¹⁸F $t_{1/2}$ = 110 minutes), Miller et al. (2008) compared with those used in SPECT (¹²³I terminal $t_{1/2}$ = 13 hours; γ ray = 159 keV) (Kung et al., 2003). PET has been used extensively to study CB receptors due to higher resolution and sensitivity. A major challenge is that the short radioligand $t_{1/2}$ requires on demand synthesis with fast purification. PET and SPECT imaging time, which is also related to radioligand $t_{1/2}$, is usually insufficient to allow radioligand–receptor binding to reach equilibrium; therefore, appropriate kinetic models should be used to correct this. In vivo imaging of the ECB system using PET (Horti et al., 2014), brain imaging of CB receptors using PET and SPECT (Casteels et al., 2013), and synthesis of such radioligands (Ahamed et al., 2013) have recently been extensively reviewed. Of note and described in these reviews are radioligands [¹¹C]OMAR, [¹¹C]MePPEP, [¹⁸F]FMPEP-*d*2, and [¹⁸F]MK-9470, which have been used in preclinical imaging of CB receptors in humans. Since the publication of these reviews, additional novel radioligands have been reported and are discussed in this work along with more recent applications of commonly used in vivo radioligands.

[¹¹C]OMAR (Table 2) is a structural analog of SR141716A (Fig. 1), in which a methyl and chlorine of SR141716A have been replaced with cyano and ¹¹C-labeled methoxy to increase polarity (Fan et al., 2006). [¹¹C]OMAR has been used in human PET studies to characterize hCB₁ receptor expression in cannabis dependence (D'Souza et al., 2016), post-traumatic stress disorder (Neumeister et al., 2013), and threat perception in trauma (Pietrzak et al., 2014). [¹⁸F]MK-9470 (Table 2) is a high-affinity inverse agonist for CB₁ receptor (Burns et al., 2007), which to date is the most

widely used radioligand for in vivo CB₁ receptor studies. Since the area was last reviewed (Horti et al., 2014), [¹⁸F]MK-9470 has been used in PET experiments to analyze hCB₁ receptor expression in schizophrenia (Ceccarini et al., 2013), prostate carcinoma (Emonds et al., 2013), Alzheimer's disease (Ahmad et al., 2014), alcohol dependence (Ceccarini et al., 2014), cannabis dependence (Ceccarini et al., 2015), functional dyspepsia (Ly et al., 2015), food intake disorders (Ceccarini et al., 2016b), and Huntington's disease (Ceccarini et al., 2016a).

The CB₂ receptor in vivo radioligand toolbox is underdeveloped compared with that of CB₁ receptor, perhaps due to the timeline of receptor subtype characterization and the intense interest in CNS-expressed CB₁ receptor. The 2-oxoquinoline-based [¹¹C]NE40 (Table 2) (Evens et al., 2009) was the first radioligand used for in vivo PET imaging of hCB₂ receptor (Ahmad et al., 2013) and will be an important tool for studying CB₂ receptor in pathologic conditions. Ahmad et al. (2013) showed rapid brain uptake and washout of [¹¹C]NE40, along with major uptake in lymphoid tissue in agreement with known expression of hCB₂ receptor.

Hortala et al. (2014) developed a triazine-based radioligand [¹⁸F]d₂-3 (Fig. 2; Table 2), which was used for PET imaging of CB₂ receptor in rhesus monkey and baboon models of neuroinflammation (Hortala et al., 2014). [¹⁸F]5 (Fig. 2; Table 2) is another triazine-based radioligand, which was developed to study in vivo distribution of a series of triazine-based CB₂ receptor agonists (Yrjölä et al., 2015). Other radioligands with an oxoquinoline scaffold have been reported and used for imaging CB₂ receptor in rats and mice, for example, [¹¹C]KD2 (Mu et al., 2013), [¹¹C]RSR-056 (Slavik et al., 2015a), [¹¹C]RS-016 (Slavik et al., 2015b), [¹⁸F]RS-126 (Slavik et al., 2016), and [¹¹C]KP23 (Mu et al., 2014) (Fig. 2; Table 2). These oxoquinoline-based radioligands all exhibited similar patterns of biodistribution with high specific binding to spleen (concluded from excess cold ligand–blocking experiments) and low brain uptake, along with high nonspecific binding to liver and small intestine. It is unclear whether low brain uptake is solely a reflection of proportionally lower CB₂ receptor expression in brain compared with spleen or also a function of poor BBB passage. In the case of [¹¹C]RSR-056 and [¹¹C]RS-016, an increase in brain radioligand uptake in a neuroinflammatory mice model was observed as compared with healthy mice. However, it remains ambiguous whether this increase is a result of the increased expression of CB₂ receptor or a result of increased brain permeability due to inflammation-induced disruption of the BBB.

Both *cis* and *trans* isomers of the naphthyridin-based radioligand [¹⁸F]CB91 were evaluated (Fig. 2; Table 2) with the *cis/trans* mixture then used for imaging CB₂ receptor in mice and showed distribution in spleen and gut, consistent with known CB₂ receptor distribution,

but also in kidneys, pancreas, and brown adipose tissue. High liver accumulation of radioligand indicated rapid clearance by the hepatobiliary route (Saccomanni et al., 2015). [^{11}C]AZD1940 (Fig. 2; Table 2) is a radiolabeled analog of a peripherally restricted CB receptor agonist AZD1940, which was a drug investigated for treatment of neuropathic pain. [^{11}C]AZD1940 was used for PET studies in monkeys to study the distribution of AZD1940 and revealed low CNS exposure; however, further experiments are required to determine the

contribution of unbound versus CB receptor-bound ligand in brain given the nanomolar CB₁ receptor affinity of [^{11}C]AZD1940 (Schou et al., 2013). Synthesis of PET radioligands based on aminoalkyl indole (Gao et al., 2014a) and benzenesulfonamide (Gao et al., 2014b) scaffolds has been reported; however, biologic data for these ligands are not yet reported. Most reported CB₂ receptor radioligands exhibit high lipophilicity and metabolic susceptibility or lack CB₂ receptor specificity, and much work still needs to be

TABLE 2

Commonly used and new CB receptor radioligands for in vivo use reported since (Casteels et al., 2013; Horti et al., 2014); FFA, S1P, prostanoid, leukotriene, and PAF receptor radioligands for in vivo use

Tool	Data for Corresponding Cold Ligand	Function	Reference
CB₁ Receptor–Selective Ligands			
[^{11}C]OMAR	hCB ₁ $K_i = 11 \pm 7$ nM	Inverse agonist	Fan et al., 2006
[^{18}F]MK-9470	hCB ₁ $\text{IC}_{50} = 0.7$ nM hCB ₂ $\text{IC}_{50} = 44$ nM	Inverse agonist	Burns et al., 2007
CB₂ Receptor–Selective Ligands			
[^{11}C]NE40	hCB ₁ $K_i > 1000$ nM hCB ₂ $K_i = 9.6$ nM	Inverse agonist	Evens et al., 2009
[^{18}F]-d ₂ -3	hCB ₁ $\text{IC}_{50} = 180$ nM hCB ₂ $\text{IC}_{50} = 1.4$ nM	Agonist	Hortala et al., 2014
[^{18}F]5	hCB ₂ $\text{EC}_{50} = 18.0\text{--}62.0$ nM	Agonist	Yrjölä et al., 2015
[^{11}C]KD2	hCB ₁ $K_i > 10,000$ nM hCB ₂ $K_i = 1.7 \pm 2.0$ nM	Not determined	Mu et al., 2013
[^{11}C]KP23	hCB ₁ $K_i > 1000$ nM hCB ₂ $K_i = 6.8 \pm 5.8$ nM	Not determined	Mu et al., 2014
[^{11}C]RSR-056	hCB ₂ $K_i = 2.5$ nM	Not determined	Slavik et al., 2015a
[^{11}C]RS-016	hCB ₁ $K_i > 10,000$ nM hCB ₂ $K_i = 0.7 \pm 0.6$ nM	Not determined	Slavik et al., 2015b
[^{18}F]RS-126	hCB ₁ $K_i > 10,000$ nM hCB ₂ $K_i = 1.2 \pm 0.8$ nM	Not determined	Slavik et al., 2016
[^{18}F]CB91 (<i>trans</i> isomer)	hCB ₁ $K_i = 300$ nM hCB ₂ $K_i = 9.0$ nM	Not determined	Manera et al., 2009
[^{18}F]CB91 (<i>cis</i> isomer)	hCB ₁ $K_i = 200$ nM hCB ₂ $K_i = 0.7$ nM	Not determined	Manera et al., 2009
Nonselective CB Receptor Ligands			
[^{11}C]AZD1940	hCB ₁ $K_i = 11.75$ nM hCB ₂ $K_i = 0.87$ nM	Agonist	Schou et al., 2013
FFA Receptor Ligands			
[^{18}F]TAK875	hFFA1 2.4-fold more potent than TAK875	Agonist	Bertrand et al., 2016a
S1P Receptor Rigands			
[^{18}F]17	hS1P ₁ Activity ^a = 95	Agonist	Shaikh et al., 2015
[^{11}C]TZ3321	hS1P ₁ $\text{IC}_{50} = 2.13 \pm 1.63$ nM hS1P ₂ $\text{IC}_{50} > 1000$ nM hS1P ₃ $\text{IC}_{50} > 1000$ nM	Not determined	Jin et al., 2017
[^{18}F]28c	hS1P ₁ $\text{IC}_{50} = 2.63 \pm 0.27$ nM hS1P ₂ $\text{IC}_{50} > 1000$ nM hS1P ₃ $\text{IC}_{50} > 1000$ nM	Not determined	Rosenberg et al., 2016
[^{11}C]5a	hS1P ₁ $\text{IC}_{50} > 1000$ nM hS1P ₂ $\text{IC}_{50} = 9.52 \pm 0.70$ nM hS1P ₃ $\text{IC}_{50} > 1000$ nM	Not determined	Yue et al., 2015
DP₁ Receptor Ligands			
1-[^{14}C]	hDP ₁ $K_i = 1.5 \pm 0.57$ nM hTP $K_i = 0.84 \pm 0.19$ nM hEP ₂ $K_i = 410 \pm 320$ nM hEP ₃ $K_i = 470 \pm 46$ nM	Antagonist	Sturino et al., 2007
2-[^{14}C]	hIP $K_i = 400 \pm 100$ nM hDP ₁ $K_i = 1.1 \pm 0.22$ nM hTP $K_i = 14 \pm 2.7$ nM hEP ₂ $K_i = 310 \pm 82$ nM	Antagonist	Sturino et al., 2007
LTB₄ Receptor Ligands			
SG380	LTB ₄ $\text{IC}_{50} = 18$ nM	Antagonist	Harris, 2008
PAF Receptor Ligands			
[^{14}C]WEB2086	hPAF $K_i = 30 \pm 2.1$ nM	Antagonist	Parent et al., 1996
[^{14}C]E6123	hPAF $K_i = 2.7$ nM	Antagonist	Tsunoda et al., 1990; Sakuma et al., 1991

^aPercentage of activity is percentage reduction in peripheral blood CD4⁺ cells mediated by S1P₁ receptor in mice treated with ligand as compared with control (untreated) mice.

done to enable intricate in vivo imaging of CB₂ receptor in humans.

As already discussed, there are very few reported FFA receptor radioligands. In the same body of work as [³H]TAK-875 (*Radioligands for In Vitro Experiments*), Bertrand et al. (2016a) also reported the synthesis of [¹⁸F]TAK-875 (Fig. 3; Table 2). The cold analog [¹⁹F]TAK-875 was also synthesized and showed similar FFA1 receptor agonist activity to TAK-875 (Fig. 1), and in vitro and in vivo studies are underway.

There are a greater number of in vivo radioligands for S1P compared with FFA and LPA receptors, a trend perhaps reflective of the number of marketed and under development drugs for S1P receptor. The synthesis and evaluation of FTY720 analogs with a cold ¹²⁷I appended to varying positions as a lead-in to making [¹²³I] or [¹²⁴I] radioligands for SPECT or PET S1P receptor imaging have been reported (Briard et al., 2011). The 2-iodo derivative, BZM055, was identified as a lead candidate for radiolabeling, and the authors comment that imaging studies are ongoing. Briard et al. (2015) also took a similar approach and synthesized an iodinated analog of the S1P receptor ligand BAF312 (Fig. 1). Distribution of cold analog [¹²⁷I]MS565 was analyzed using the equivalent [¹⁴C]18 tool (*Radioligands for In Vitro Experiments*) and is suitable for development of hot [¹²³I]MS565 as a SPECT tool (Briard et al., 2015).

Cold fluorinated derivatives of the S1P₁ receptor antagonist W146 have been synthesized, with the most promising in vitro derivative radiolabeled to [¹⁸F]24 (Fig. 3; binding affinity not reported) (Prasad et al., 2014). Although stable in serum, in vivo PET imaging of S1P₁ receptor in mice using [¹⁸F]24 showed accumulation of radioactivity in bone, most likely due to metabolic defluorination, thus limiting its use. The same laboratory subsequently reported [¹⁸F]17, a derivative of FTY720, for imaging S1P receptors (Fig. 3; Table 2) (Shaikh et al., 2015). [¹⁸F]17 induced peripheral blood lymphopenia (a measure of S1P₁ receptor downstream regulation) at a comparable level to FTY720 and did not undergo defluorination during mice PET studies; however, rapid clearance of [¹⁸F]17 was observed. Thus, further optimization of these PET radioligands is required.

[¹¹C]TZ3321 (Fig. 3; Table 2), based on a selective S1P₁ receptor oxadiazole scaffold, has been reported and used in MicroPET imaging studies in a femoral artery wire-injury mouse model (Jin et al., 2017). These studies revealed high expression of S1P₁ receptor, in line with the proposal that higher levels of this receptor are observed in vascular smooth muscle cells following intimal lesions usually caused by in-stent restenosis. The same research group then reported synthesis of the PET radioligand [¹⁸F]28c (Fig. 3; Table 2), also based on oxadiazole scaffold (Rosenberg et al., 2016). S1P₁ receptor expression is thought to increase in inflammation, and in vivo PET imaging indeed showed increased

Fig. 2. CB receptor radioligand tools for in vivo studies reported since (Casteels et al., 2013; Horti et al., 2014). Note: structures are not shown for radioligands with unknown radioisotope position.

binding of [¹⁸F]28c in the liver of lipopolysaccharide-treated mice compared with control mice. This radioligand could be an important tool for monitoring S1P₁ receptor expression as a measure of inflammation.

Radioligand tools have been developed for imaging S1P₂ receptor, for example, [¹¹C]5a (Fig. 3; Table 2) (Yue et al., 2015), which is a derivative of the S1P₂ receptor antagonist JTE-013. PET studies in mice showed high uptake of [¹¹C]5a in S1P₂ receptor-rich regions such as heart, lung, kidney, and liver. Low brain levels were observed, with the authors suggesting that this could be due to radioligand efflux by P-glycoprotein (P-gp), as an increase in brain penetrability was observed on treatment with P-gp inhibitor cyclosporine A. Further improvements in brain uptake of this radioligand are required before utility in PET imaging of MS and neuroinflammation conditions is achieved. S1P₃ receptor radioligands have also been developed for in vivo imaging. [¹⁸F]1, a mixture of two compounds (Fig. 3), was synthesized based on a known [¹⁹F]-containing indole-based ligand for S1P₃ receptor (Rokka et al., 2013); however, to date pharmacological data for this radioligand have not been reported.

Four [¹⁴C]-labeled DP receptor antagonists, including 1-[¹⁴C] and 2-[¹⁴C] (Fig. 3; Table 2) (Berthelette and Wang, 2007), along with three [³H]-labeled analogs (Scheiget et al., 2004), have been synthesized and used

to study the extent of covalent protein labeling in vitro (Sturino et al., 2007). These [^{14}C]-labeled DP receptor antagonists may prove useful tools for in vivo DP receptor studies in the future, although DP receptor selectivity in particular over TP receptor will require further optimization. [^{14}C]-Fluprostenol has been used to analyze the disposition and metabolism of an intramuscular injection of fluprostenol in horses (Chapman et al., 1980), although DP receptors were not directly investigated.

An optimized [$^{99\text{m}}\text{Tc}$]RP517-containing formulation has been developed as a [$^{99\text{m}}\text{Tc}$]-labeled analog of the hydrazinonicotinamide-conjugated LTB $_4$ receptor antagonist SG380 (Table 1; unchelated ligand) for imaging infection and inflammation (Liu et al., 2002). A study by Riou et al. (2002) examined [$^{99\text{m}}\text{Tc}$]RP517 uptake in an ischemia-reperfusion-induced myocardial inflammation model, and found that postreperfusion [$^{99\text{m}}\text{Tc}$]RP517 uptake correlated with myeloperoxidase (a specific neutrophil enzyme) levels. Ex vivo imaging of heart slices postreperfusion also showed [$^{99\text{m}}\text{Tc}$]RP517 localized within the area of inflammation. To investigate whether these results might be LTB $_4$ receptor specific, Riou et al. (2002) also performed in vitro studies using [F]-RP517, an undisclosed CY3 fluorescently tagged analog of RP517, which showed a 44% displacement with LTB $_4$ and complete displacement with nonfluorescent RP517 on isolated neutrophils. [$^{99\text{m}}\text{Tc}$]RP517 has also been used as a tool to study *Escherichia coli* infection in rabbits; however, although accumulation of [$^{99\text{m}}\text{Tc}$]RP517 occurred in the abscess, there was also significant accumulation in the intestines, leading the authors to propose development of a more hydrophilic tool (Brouwers et al., 2000). This research group then reported ^{111}In labeling of the more hydrophilic LTB $_4$ receptor antagonist DPC11870-11 (Van Eerd et al., 2003). [^{111}In]-DPC11870-11 showed specific receptor interactions, localized to infection foci rapidly after injection and showed minimal accumulation in the intestines.

[^{14}C]WEB2086 (also called [^{14}C]apafant) (Table 2) was first reported in a patent in 1989 (Birke and Stiasni, 1989) and has since been used to show apafant is a substrate for P-gp but has not been used to directly interrogate PAF receptor (Leusch et al., 2002; Fuchs et al., 2014). Again, in a pharmacokinetic rather than direct PAF receptor study, [^{14}C]E6123 (Table 2) has been reported and used to probe metabolic enzymes of PAF antagonist E6123 (Kusano et al., 1993).

PET radioligands are valuable tools for studying the pharmacokinetics of investigational drugs and in vivo imaging of GPCRs. In particular, many in vivo radioligands have been reported for CB receptor, and it is anticipated in the near future more radioligands for the other class A lipid-binding GPCRs will be developed, thus facilitating an understanding of the role these receptors have in disease conditions.

III. Small-Molecule Fluorescent Tools

A. Characteristics and Design Rationale

Small-molecule-based fluorescent tools have been used to study molecular targets, such as receptors, enzymes, proteins, small molecules, and ion channels, and are increasingly being used to study GPCRs (Vernall et al., 2014; Stoddart et al., 2015). Other fluorescent-based techniques, such as yellow fluorescent protein and green fluorescent protein genetic tags, antibodies, nanoparticles, and tagged proteins (Cottet et al., 2013; Ciruela et al., 2014; Ma et al., 2014; Sridharan et al., 2014; Stoddart et al., 2016), are also used but are outside the scope of this review.

Small-molecule-based fluorescent tools are commonly synthesized by tethering a known pharmacophore/ligand to a fluorophore via a covalent linker. Pharmacophore selection is guided by target receptor affinity, selectivity, and the desired utility of the fluorescent ligand (antagonist, agonist, etc.). The linker is positioned in a benign area of the pharmacophore and is of an appropriate length such that the binding of the pharmacophore to the receptor is not perturbed. However, this is often based on experimental trial and error rather than on predictive comparable functionality to the parent pharmacophore. Fluorophore selection is dependent on choice of wavelength and equipment filter

Fig. 3. FFA, S1P, and prostanoïd receptor radioligand tools for in vivo studies. Note: structures are not shown for radioligands with unknown radioisotope position. The structures of [$^{99\text{m}}\text{Tc}$]RP517 and [^{111}In]-DPC11870-11 are not shown because of size.

path length, quantum yield, resistance to photo bleaching, and appropriate physicochemical properties. There are examples of a fluorophore designed as part of a pharmacophore (*Fluorescent Tools for In Vitro Experiments*) and a fluorophore acting as a biologically active ligand itself (May et al., 2010; Dale et al., 2012). Another design approach is to join a fluorophore to a suitably tagged ligand in situ during the biologic experiment (*Fluorescent Tools for In Vitro Experiments*). Much like radioligands, the physicochemical characteristics of a fluorescent tool must allow for specific receptor binding and minimal nonspecific interactions such as with the membrane, to obtain a high signal to noise ratio. This is especially challenging for receptors with endogenous lipid ligands and lipophilic synthetic ligands. To this end, the linker and fluorophore could be used to tune the overall physicochemical properties of fluorescent tools, although the proposed entry via a lipid portal into some of these receptors (Rational Design of Tools and Consideration for Use) must not be forgotten.

B. Applications of Small-Molecule Fluorescent Tools

The increasing availability of small-molecule fluorescent tools, both commercially and via published synthesis, is progressing the use of techniques such as fluorescence confocal microscopy, scanning confocal microscopy, flow cytometry, high-throughput screening, fluorescence correlation spectroscopy, fluorescence resonance energy transfer and BRET assays, and diagnostic imaging to study receptor pharmacology. Once a pharmacophore-linker-fluorophore ligand is successfully developed, it is a lead point for attachment of another entity in place of the fluorophore, for example, another pharmacophore for a bivalent ligand, a magnetic resonance-based ligand, or theranostic agent. Many of the reported fluorescent tools have not been fully pharmacologically characterized for functional activity, that is, agonist, antagonist, or inverse agonist, which can be an important consideration as the functionality and application of a fluorescent tool are often different from the parent pharmacophore.

1. *Fluorescent Tools for In Vitro Experiments.* Early reports of fluorescent small molecules for lipid-binding GPCRs were of labeled endogenous receptor agonists, for example, a benzoxadiazole-based fluorophore linked to anandamide (Koga et al., 1995) or to S1P (Hakogi et al., 2003; Yamamoto et al., 2008). Bile acid derivatives have been linked to nitrobenzoxadiazole (NBD) fluorophores and used to study cellular uptake (Májér et al., 2012), and fluorescent LTB₄ linked to two AlexaFluor fluorophores has been used specifically to study LTB₄ receptor (Sabirsh et al., 2005). An early report of a flow cytometry assay for FFA1 receptor used the commercially available fluorescent ligand C1-BODIPY-C12 (Hara et al., 2009). Many endogenous ligands for CB, FFA, and S1P receptor are

now commercially available attached to a range of fluorophores.

In 2008, a biotin-AEA tool was developed to study the biodistribution of AEA, which was visualized in a two-step process using an anti-biotin monoclonal antibody (mAb), followed by a green fluorescent anti-mouse secondary antibody (Fezza et al., 2008). Martin-Couce et al. (2011) described modification of the ECBs AEA, 2-AG, and 2-arachidonyl glyceryl ether (2-AGE) to contain a biotin or alkyne tag for subsequent direct, selective in situ fluorescent labeling with a streptavidin-fluorophore or a click reaction, respectively. The 2-AGE ECB tagged with an alkyne was the most potent tool, but it lacked receptor subtype selectivity ($K_i = 84.7 \pm 0.8$ nM at hCB₁; 84.9 ± 0.6 nM at hCB₂), whereas 2-AGE-biotin-3b (Fig. 4; Table 3) showed some selectivity ($K_i = 221 \pm 8$ nM at hCB₁, 450 ± 11 nM at hCB₂). Imaging of hCB₁ receptor in mouse hippocampal cell lines (HT-22) transfected with CB₁ receptor has been carried out by reaction of 2-AGE-biotin-3b with streptavidin-Alexa488 fluorophore in situ. Some background fluorescence was observed in control experiments using either nontransfected cells or with a high concentration of the CB₁ and CB₂ receptor agonist HU210, indicating some nonspecific binding. The ligand 2-AGE and other ECBs are lipids, so using these as a basis for chemical tools means large amounts of nonspecific membrane binding are probable along with modest, if any, CB receptor subtype selectivity, driving the need for fluorescent, synthetic ligands with improved selectivity and physicochemical properties.

The first reported fluorescent tools based on a non-endogenous CB pharmacophore were dansyl derivatives of THC (Forrest et al., 1971), and these were used for analytical tracing of cannabinoid ligands (Just et al., 1972). However, as this was prior to CB receptor characterization, these fluorescent compounds were not evaluated explicitly as CB receptor ligands. About 30 years later, a fluorescent naphthoyl indole CB₂ receptor agonist designed around JWH-015 was developed using a CB₂ receptor homology model and ligand docking that indicated a naphthoyl-linker position may be tolerated and the NBD fluorophore could act as a second pharmacophore with the receptor (Yates et al., 2005). Unfortunately, this fluorescent compound had significant loss of affinity (10 μ M gave 25% displacement of [³H]CP-55940 from CB₂ receptor) compared with parent indole agonist JWH-015, cytosolic accumulation, and nonspecific binding. In another report, replacement of part of an orthosteric pharmacophore with a fluorescent moiety has been reported to generate a fluorescent CB₂ receptor tool (Petrov et al., 2011). The morpholine of isatin acylhydrazones, believed to interact with the lipophilic cavity of CB₂ receptor, was substituted with NBD to provide the CB₂ receptor-selective NMP6 (Fig. 4; Table 3). Confocal microscopy demonstrated specific binding of NMP6 to CB₂ receptor on CD4⁺ T cells, as binding was inhibited by

Fig. 4. CB receptor fluorescent tools.

preincubation with the selective CB₂ receptor agonist GW842166X. Further utility of NMP6 was demonstrated using flow cytometry to study CB₂ receptor expression on mouse lung mononuclear B cells.

Use of commercially available T1117 (tocrifluor 1117) (Fig. 4; Table 3) was first reported in 2008 (Daly et al., 2008). T1117 was developed by linking AM251 [a CB₁ receptor-selective inverse agonist, IC₅₀ = 4 ± 1 nM (Gatley et al., 1997)] to a tetramethylrhodamine fluorophore. There are conflicting reports regarding pharmacological characterization—early studies reported T1117 to bind to GPR55 (increased Ca²⁺ response in HEK293 cells expressing GPR55) with only weak affinity at CB₁ receptor (Daly et al., 2010). However, in a different study, T1117 exhibited moderate binding at CB₁ receptor (K_d = 460 ± 80 nM at rCB1) and was used in a CB₁ receptor competition-binding assay (Bruno et al., 2014). The

fluorescence of T1117 was quenched upon CB₁ receptor binding and restored upon displacement by unlabeled test compounds. T1117 was then used as the competitive tracer in a binding assay, providing IC₅₀ values for anandamide and AM251 in agreement with literature values. Bruno et al. (2014) further demonstrated utility of T1117 as a drug discovery tool by the appropriate identification of the CB₁ receptor allosteric ligand ORG27569 (Fig. 6). However, use of T1117 to study CB₁ receptor in native cell environments is limited, as it exhibits nonspecific binding to membrane most likely due to high lipophilicity (Bruno et al., 2014). This limitation is probe specific and not a general feature of fluorescent ligands, as demonstrated by fluorescent ligands for FFA1 receptor (discussed in this work) and for other class A GPCRs (reviewed in Vernall et al., 2014) that can be used to study GPCRs in native cell environments.

TABLE 3
CB, FFA, and prostanoid receptor fluorescent ligands

Tool	Data	Reference
CB₁ Receptor and GPR55 Receptor Ligands		
T1117	hGPR55-mediated Ca ²⁺ response in HEK293 cells ^a mCB ₁ 10% displacement of [³ H]CP-55,940 with 1 μM T1117 hCB ₁ K _d = 460 ± 80 nM	Daly et al., 2010 Bruno et al., 2014
CB Receptor Ligands		
2-AGE-biotin-3b ^b	hCB ₁ K _i = 221 ± 8 nM hCB ₂ K _i = 450 ± 11 nM	Martin-Couce et al., 2011
NMP6	hCB ₁ < 40% at 10 μM hCB ₂ K _i = 387 nM	Petrov et al., 2011
Biotin-HU210-1 ^a	hCB ₁ K _i = 2.4 ± 0.4 nM hCB ₂ K _i = 1.6 ± 0.4 nM	Martín-Couce et al., 2011
Biotin-HU308-3 ^a	hCB ₁ K _i > 5000 nM hCB ₂ K _i = 44 ± 4 nM	Martín-Couce et al., 2011
NIR-mbc94	mCB ₂ K _i = 260 nM	Sexton et al., 2011
NIR760-mbc94	mCB ₂ K _d = 26.9 ± 3.7 nM	Zhang et al., 2013
NIR760-Q	hCB ₂ K _d = 75.51 ± 27.97 nM	Wu et al., 2014b
ZW760-mbc94	mCB ₂ K _d = 53.9 ± 13.0 nM	Wu et al., 2014a
NIR760-XLP6	mCB ₁ K _d > 10,000 nM	Ling et al., 2015
IR700DX-mbc94	mCB ₂ K _d = 169.1 ± 66.09 nM mCB ₂ K _d = 42.0 ± 19.6 nM	Zhang et al., 2014
FFA Receptor Ligands		
TUG-905-NBD-4	hFFA1 K _d = 7.4 ± 1.3 nM	Christiansen et al., 2016
F-TAK-875A	hFFA1 K _d = 15.1 ± 3.2 nM	Ren et al., 2016
TAK-875-Alexa488-16	hFFA1-mediated Ca ²⁺ response equipotent to TAK-875	Bertrand et al., 2016b
EP₃ Receptor Ligands		
Pyryllium-labeled cinnamon acid derivative 8	hEP ₁ K _i = 68,625 ± 12,735 nM hEP ₂ K _i = 2943 ± 279 nM hEP ₃ K _i = 135 ± 23 nM hEP ₄ K _i = 902 ± 226 nM	Tomasch et al., 2012

^aHEK293 cells stably expressing GPR55.

^bBiotin-tagged ligand (precursor for fluorescent tool) that conjugates with streptavidin-fluorophore in situ during the biologic experiment to provide corresponding fluorescent tool.

CB receptor agonists HU210 and HU308 were derivatized with a biotin tag at the ethoxy position, suitable for subsequent in situ conjugation to a fluorophore (Martín-Couce et al., 2012). The biotin-derivatized HU210-1 exhibited high affinity but little subtype selectivity for CB receptors, whereas biotin-derivatized HU308-3 exhibited selectivity for CB₂ receptor (Fig. 4; Table 3). Endogenous CB₁ and CB₂ receptor expression was studied in neurons and microglia using biotin-HU210-1 and biotin-HU308-3 by addition of streptavidin-Alexa488 fluorophore, and receptor-specific binding was then confirmed by using unlabeled HU210. Martín-Couce et al. (2012) also used biotin-HU210-1 in flow cytometry to study CB receptor expression in the monocytic cell line THP-1 at the single-cell level. The same research group has used biotin-HU210-1 in conjugation with streptavidin-Alexa488 to show there is high CB₁ receptor expression in B, T, plasmacytoid dendritic, and myeloid dendritic cells from donors with allergic rhinitis, atopic dermatitis, or food allergies (Martin-Fontecha et al., 2014).

Recently, within the space of a few months, there were three independent reports of fluorescent ligands for FFA1 receptor. In one of these reports, fluorescent ligands based on the TUG-770 or TUG-905 pharmacophore linked to a NBD fluorophore were constructed (Christiansen et al., 2016). Of these, TUG-905-NBD-4 (Fig. 5; Table 3) showed only a small reduction in potency compared with TUG-905

and retained FFA1 receptor agonism. A BRET assay paired with NanoLuciferase-tagged FFA1 receptor was established using TUG-905-NBD-4, with low levels of nonspecific fluorescence allowing for measurement of ligand kinetic parameters, and also a robust competition-binding assay was established. In a different study, a fluorescein-conjugated TAK-875 (Fig. 1) ligand (F-TAK-875A) (Fig. 5; Table 3) was synthesized as a racemic mixture and used in flow cytometry competition assays, which showed that FFA carbon-chain length was correlated with binding potency to FFA1 receptor (Ren et al., 2016). In the third report, Bertrand et al. (2016b) synthesized and evaluated a series of TAK-875-linker-fluorophore conjugates containing different types of linkers and fluorophores. In live cells overexpressing hFFA1 receptor, the lead probe TAK-875-Alexa488-16 (Fig. 5; Table 3) showed specific labeling of FFA1 receptor. This fluorescent agonist may prove especially valuable for studying dynamic receptor processes as 90-minute post-incubation fluorescent internalization was observed. Endogenously expressed FFA1 receptor in pancreatic β cells was then visualized using a combination of TAK-875-Alexa488-16 and an Alexa488 antibody to amplify the signal. These reports collectively highlight the importance of both linker position and physicochemical properties for a successful fluorescent imaging tool. All three of these reports were based on a FFA1 receptor-selective parent

pharmacophore (TAK-875 or TUG-905 core); however, the affinity and/or function of the fluorescent tool at the other FFA receptors were not provided, and it can be predicted, but not assumed, that the fluorescent tool and parent pharmacophore have a comparable receptor subtype selectivity profile.

To our knowledge, the only report of high-affinity synthetic ligand fluorescent conjugates for prostanoid, leukotriene, PAF, or GBPA receptors is by Tomasch et al. (2012), who synthesized a series of cinnamic acid antagonists containing various fluorophores. The most promising fluorescent compound when accounting for both EP₃ receptor affinity and a desirable emission wavelength above that of tissue autofluorescence was pyrylium-labeled cinnamon acid derivative 8 (Fig. 5; Table 3). Although with approximately threefold reduced affinity for EP₃ receptor compared with the parent, nonfluorescent pharmacophore, in different cell lines with respective recombinant EP₁₋₄ receptor subtypes, eight showed selectivity for EP₃ receptor over EP₁ (550-fold), EP₂ (24-fold), and EP₄ receptor (seven-fold). Labeling of EP₃ receptors in HT-29 cells using eight was demonstrated, with binding determined as specific by displacement of eight with excess nonfluorescent selective EP₃ receptor ligand. Further utility of eight was demonstrated in murine kidney, human brain tissue, and human platelets, with the authors commenting that

future studies will aim to improve EP₃ over EP₄ receptor selectivity.

In the same body of work reporting photoactivatable ginkgolide derivatives as PAF receptor tools (*Photo-activatable Covalent Tools*), two ginkgolide derivatives tagged with a dansyl fluorophore at different positions were reported (Strømgaard et al., 2002). The most promising of these fluorescent conjugates had moderate PAF receptor affinity [$K_i = 0.96 \mu\text{M}$, guinea pig (gp) PAF]; however, it was not evaluated further for PAF receptor fluorescent labeling or photoactivated covalent binding. Nevertheless, future development of fluorescently tagged ginkgolide derivatives as fluorescent tools may prove useful for PAF receptor interrogation.

2. Near-Infrared Wavelength Fluorescent Tools for In Vivo Imaging. Both the optical properties of tissues, including endogenous chemical components present, and choice of fluorophore can influence the depth that a suitable signal-to-noise fluorescent signal can be detected in vivo (reviewed in Pansare et al., 2012; Hong et al., 2017). Longer wavelength fluorophores such as those in the near-infrared (NIR) region (700–900 nm) and the now termed NIR-II region (1000–1700 nm) are well suited to in vivo imaging and typically can be detected at a tissue depth of up to 5–7 mm with good signal-to-noise resolution. As is the case with in vivo radioligands, the absorption, distribution, metabolism, and excretion of the tool in relation to the intended use need to be considered, along with careful characterization of in vivo versus in vitro selectivity.

There has been particular interest surrounding in vitro use/characterization and also in vivo use of NIR fluorescent tools to study CB₂ receptor expression in malignant tumors. Synthesis of selective CB₂ receptor SR144528 derivatives with linkers introduced to the pyrazole or 4-chloro phenyl position abolished CB₂ receptor-binding affinity; however, incorporation of a linker at the benzylic position was more successful, resulting in the SR144528 linker derivative mbc94 ($K_i = 15 \text{ nM}$ at mCB₂) (Fig. 4) (Bai et al., 2008; Sexton et al., 2011). Attachment of the fluorescent IRDye 800CW to mbc94 to give NIR-mbc94 (Fig. 4; Table 3) led to some loss in affinity ($K_i = 260 \text{ nM}$ at mCB₂). This fluorescent tool was successfully used in a multiwell high throughput screening assay using intact CB₂-mid delayed brain tumor (DBT) cells (mouse DBT cells transfected with CB₂ receptor); however, imaging experiments using primary microglia cells revealed a high level of NIR-mbc94-nonspecific binding (Sexton et al., 2011).

The same group of researchers developed NIR760-mbc94 (Fig. 4; Table 3) using the same CB₂ receptor pharmacophore-linker mbc94 but a different fluorophore (NIR760) (Zhang et al., 2013). When CB₂-mid DBT cells were incubated with NIR760-mbc94 in the presence and absence of the control nonfluorescent pharmacophore SR145528, the fluorescent intensity was reduced by only 40% compared with the control,

Fig. 5. FFA and prostanoid receptor fluorescent tools.

indicating some nonspecific binding of the fluorescent tool, which the authors suggested was due to nonspecific protein binding because NIR760-mbc94 has a net negative charge. In a cancer model, CB₂-mid DBT cells were injected subcutaneously into the right flank of healthy mice, and tumors were allowed to develop for 10 days. Zhang et al. (2015) then injected mice with NIR760-mbc94 and observed fluorescence on the whole body, followed by tumor-specific localization 48–72 hours postinjection. Injection with SR144528 1 hour prior to NIR760-mbc94 was carried out to determine specific CB₂ receptor binding of NIR760-mbc94, and after 72 hours a 31% reduction in fluorescence of the tumor area/normal area ratio was observed. As CB₂ receptor plays an important role in inflammation, this fluorescent tool has also been used for in vivo imaging of mCB₂ receptor expression in a complete Freund's adjuvant-induced inflammation mouse model (Zhang et al., 2015). Low specific binding of NIR760-mbc94 was observed with inflammation-specific fluorescence visible only 36 hours postinjection.

The same group of researchers then conjugated a quinolone-based ligand with the NIR760 fluorophore to give NIR760-Q (Fig. 4; Table 3) (Wu et al., 2014b). Nonspecific binding was observed in experiments with Jurkat cells in the presence of the unlabeled control ligand 4Q3C, which the authors suggest may be due to the total negative charge of NIR760. The same research group subsequently developed the zwitterionic NIR-fluorophore-containing ZW760-mbc94 (Fig. 4; Table 3) (Wu et al., 2014a). Specificity of ZW760-mbc94 for CB₂ receptor was determined in vitro by incubating CB₂-mid DBT cells in the absence or presence of the unlabeled control ligand 4Q3C, which afforded a 50% reduction in fluorescence intensity. This is a moderate improvement compared with negatively charged NIR760-mbc94, and development of fluorescent tools with improved in vivo selective binding is an ongoing challenge. The fluorescent tool ZW760-mbc94 was further evaluated by injection into mice 10 days after CB₂-mid DBT cell inoculation in the right flank, and images showed high fluorescence in the liver as well as fluorescence throughout the whole body that persisted 72 hours after ZW760-mbc94 injection. Ex vivo analysis revealed mice treated with the control blocking ligand 4Q3C showed a 47% reduced tumor:normal tissue fluorescence ratio as compared to ZW760-mbc94-treated mice without blocking ligand, indicating some observed fluorescence was CB₂ receptor-dependent.

Another fluorescent tool developed by the same research group is NIR760-XLP6 (Fig. 4; Table 3), which consists of a pyrazolopyrimidine pharmacophore conjugated via a linker to the NIR760 fluorophore (Ling et al., 2015). Experiments were carried out to determine in vivo receptor selectivity, which showed higher fluorescence (40% higher) in mice with CB₂-mid DBT tumors as compared with CB₁-mid DBT (DBT cells

transfected with CB₁ receptor) tumors. This research group has also developed a CB₂ receptor-targeted photosensitizer IR700DX-mbc94 (Fig. 4; Table 3) by attaching a NIR fluorophore, IR700DX, to mbc94 (Zhang et al., 2014). In vivo experiments in mice revealed that IR700DX-mbc94 inhibited growth of CB₂ receptor-positive tumors following light (wavelength 670–710 nm) irradiation, but not that of the CB₂ receptor-negative tumors (Jia et al., 2014). This is an interesting novel approach to treating tumors overexpressing CB₂ receptor. A patent application (Bornhop et al., 2013) has been published by the same group of researchers that describes the use of SR144528 derivatives tethered to an IRDye800CW fluorophore, a topoisomerase inhibitor (such as etoposide), and a gadolinium chelate. The potential applications of these SR144528 derivatives claimed by the patent include use as a molecular imaging tool and as a targeted drug delivery system.

The development and use of fluorescent tools for GPCRs are a rapidly developing field. There are several reports of selective CB₂ receptor fluorescent tools; however, there is a lack of selective CB₁ receptor fluorescent tools, and no doubt this is an area of intensive research. Selective fluorescent tools for the other FFA, S1P, LPA, prostanoid, leukotriene, PAF, and GPBA receptors based on synthetic pharmacophores will most likely be developed in the future, which will facilitate some exciting biologic experiments.

IV. Covalent Tools

A. Characteristics and Design Rationale

A covalent ligand (or affinity label) is a tool that, once in place at the target receptor, is able to form a covalent bond with one or more amino acid side chains located at or near the ligand binding site (Weichert and Gmeiner, 2015). Covalent tools should ideally be able to first interact with the receptor noncovalently with high affinity and then engage in a controllable and/or specific reaction(s). High affinity will increase the concentration of covalent ligand at the binding site and therefore increase covalent labeling.

Covalent tools can be classified as photoactivatable and/or electrophilic, each of which can be irreversible or reversible. Photoactivatable ligands (or photoaffinity labels) possess a chemically inert moiety such as an azide or benzophenone that can be irradiated to a highly reactive nitrene or biradical, which can form a covalent bond with a nearby amino acid. Electrophilic ligands contain a reactive electrophilic functional group that can react with a nucleophilic amino acid side chain to form a covalent bond. Investigating affinity and specificity for the target receptor, as well as stability of the covalent ligand, is important for the validation of a covalent probe. Covalent binding is usually quantified using a radioligand-binding assay by calculating the decrease in radioligand $B_{\max}$ after incubation of receptors

with the covalent ligand and washing to remove unreacted ligand (Picone et al., 2002). There has been reinvigorated interest in covalent drugs (Singh et al., 2011; Nussinov and Tsai, 2015) and studying the kinetics of irreversible and reversible covalent ligand binding. A number of studies have investigated potency and modulation of drug–protein residence times by adjusting the covalent reactivity of drugs such as kinase inhibitors (Flanagan et al., 2014; Krishnan et al., 2014; Bradshaw et al., 2015), a concept that is also applicable to covalent tools for GPCRs.

B. Applications of Covalent Tools

Covalently linked tools have been used to investigate key amino acid residues involved in ligand binding and receptor activation, as well as in receptor-signaling studies and in vivo physiologic research. When combined with in silico modeling and site-directed mutagenesis, covalent tools can enable characterization of the spatial orientation and topography of receptor ligand binding site(s). Indeed, covalent tools have been employed in the mapping of ligand binding sites of dopamine transporters (Vaughan et al., 2005), GPCRs such as α_2 -adrenoceptor (Matsui et al., 1989) and adenosine receptor (Kennedy et al., 1996), and enzymes such as the 2-AG–deactivating monoacylglycerol lipase (Zvonok et al., 2008). A covalent ligand–receptor interaction has aided in the isolation and purification of other class A GPCR subtypes (Dohlman et al., 1988) and improved receptor stability for GPCR X-ray crystal structure determination (Palczewski et al., 2000; Choe et al., 2011). Covalent ligands have also been useful in establishing the receptor targets of biologically active compounds (Sumranjit and Chung, 2013).

Antibodies, peptide sequencing, or mass spectrometry is often used to analyze a covalent ligand–GPCR complex. By comparison with fluorescent ligands and radioligands, covalent tools discussed in this work do not possess the functionality for direct use in imaging studies and for the most part have been used in cell-based studies. When designing an in vivo covalent ligand, it is important to consider the safety profile of irreversible binding, as off-target interactions could result in, for example, potentially immunogenic extracellular protein–ligand adducts (Johnson et al., 2010).

There are numerous reports of the utility of covalent tools for CB receptors and to a lesser extent leukotriene, prostanoid, and PAF receptors; however, to date there have been no reports of covalent ligands for FFA, S1P, or LPA receptors. A large number of covalent tools based on classic, nonclassic, and ECB CB receptor scaffolds have been developed by the Makriyannis group (ligands named with the prefix “AM”). Their approach, termed ligand-assisted protein structure, is to develop a range of ligands with various reactive moieties at different ligand positions to elucidate the role of different receptor amino acid side chains, thus

characterizing the ligand binding site of CB receptor (Picone et al., 2002).

1. Photoactivatable Covalent Tools. The most commonly used inert groups capable of photoactivation are azides, benzophenones, and diazirines. An advantage of photoactivated ligands is that the highly reactive species generated in situ is able to form a covalent bond with any amino acid nearby, compared with electrophilic covalent ligands that are limited to reaction with only one or two nucleophilic amino acid side chains (Sumranjit and Chung, 2013). The highly reactive species generated can covalently bind to the receptors through insertion into bonds (e.g., C-H) in the peptide backbone and amino acid side chains (Cavalla and Neff, 1985). Desirable properties of a photoactivated ligand include high receptor affinity, activatable at a wavelength that does not cause damage to the target receptor or biosystem, the generation of a reactive group with a short lifetime, indiscriminate or finely-tuned formation of a covalent bond with any nearby amino acid, and formation of a stable irreversible adduct (Vodovozova, 2007).

Initial efforts to develop covalent tools for CB receptors focused on analogs of Δ^8 -THC, which has a similar affinity for CB₁ receptor, but is more stable than the naturally more prevalent Δ^9 -THC isomer. A photoactivatable azido group was positioned at the THC aliphatic chain terminus, as SAR showed this position to be tolerant of bulky groups (Charalambous et al., 1992). This ligand, 5'-azido- Δ^8 -THC AM91 (Fig. 6; Table 4), exhibited approximately twofold increased affinity to that of Δ^8 -THC ($K_i = 35 \pm 11$ nM at rCB₁). AM91 was developed shortly after cloning of CB₁ receptor with the intention of aiding receptor isolation and characterization and was a prototype for the next generation of photoactivatable ligands. Increasing interest in CB receptors and the need for tools to identify receptor subtypes and enable receptor isolation, purification, and characterization drove the development of subsequent CB receptor covalent tools.

The same research group radioiodinated AM91 by introducing [¹²⁵I] *ortho* to the phenolic group to give 2-iodo-5'-azido- Δ^8 -THC (2-[¹²⁵I]-AM91) (Fig. 6; Table 4) (Burstein et al., 1991). The 2-[¹²⁵I]-AM91, equilibrated with tissue and exposed to UV light to induce azide to nitrene conversion, could detect CB receptor expression in mouse cerebral cortex ($K_d = 5.60$ pM) and mouse lymphoma cells ($K_d = 9.38$ pM). Samples of mouse brain analyzed by SDS-PAGE and autoradiography showed formation of a covalent bond between ligand and CB receptor. This experiment provided the earliest evidence of a covalent ligand–CB receptor entity and provided a clue to the existence of receptor subtypes as a fainter, lower molecular weight band on the gel was also observed. This other band was later shown to be CB₂ receptor (Makriyannis, 2014).

Distinct chemical classes of photoaffinity labels can provide insight into the ligand-binding mode and the

topography of the CB receptor ligand binding site(s). A series of photoactivatable CB receptor ligands featuring a heteroaroyl group at C3 in place of an alkyl chain were synthesized, including a CB₂ receptor-selective 3-benzothiophenyl-derivative AM967 ($K_i = 34.2$ nM at mCB₂, 124.8 nM at hCB₂, 1254 nM at rCB₁) (Fig. 6; Table 4) (Dixon et al., 2012). Photolysis of aryl phenones with UV light at approximately 350 nm generates a highly reactive triplet-state ketone intermediate, which can form covalent bonds through insertion in C-H bonds (Vodovozova, 2007). Receptor photolabeling of CB₂ receptor using AM967 was measured at 67% (reduction in specific binding of [³H]CP-55,940) in HEK293 membrane preparations expressing mCB₂ receptor (Dixon et al., 2012). This covalent series provided interesting SAR data on heteroaroyl-containing CB₂ receptor ligands. Future experiments with AM967 could elucidate key amino acids specifically involved in the binding of arylphenone analogs, expanding the structural understanding of CB₂ receptor and further enabling structure-based design of subtype-selective drugs.

The classic CB-based tool AM993, which has a C3 adamantyl group, has been synthesized to inform receptor molecular recognition of this fixed conformation adamantyl moiety (Ogawa et al., 2015). AM993 (Fig. 6; Table 4) behaved as an agonist at rCB₁ receptor with an $EC_{50} = 2.4$ nM and $E_{max} = 45\%$ and as a neutral antagonist at hCB₂ receptor. Successful covalent labeling of CB receptors following pretreatment with 10-fold above the K_i of AM993 was demonstrated with a 67% reduction in specific binding of [³H]CP-55,940 at rCB₁ and a 60% reduction at hCB₂ receptor. A series of electrophilic and photoactivatable aryl pyrazole compounds based on the high-affinity CB₁ receptor

antagonist rimonabant have been developed (Howlett et al., 2000). However, most of these compounds did not maintain the affinity of the parent compound, and the best in this series irreversibly labeled rCB₁ receptor at $IC_{50} = 28$ nM. Modified ECBs have also been developed as CB receptor covalent ligands. AM3661 is based on the structure of AEA and incorporates a cyclopropylamide moiety at the head group for improved CB₁ affinity and a photoactivatable azide at the terminal alkyl chain. AM3661 (Fig. 6; Table 4) was shown to irreversibly label 68% of rCB₁ receptor when used at 18 nM (Li et al., 2005). Binding assays were carried out in the presence of a serine protease inhibitor to avoid AM3661 hydrolysis by fatty acid amide hydrolase (FAAH), a key enzyme in ECB metabolism. ECB-based covalent tools can be used to reveal key information regarding the binding and signaling of AEA and 2-AG as well as ECB-like ligands.

There are also a number of CB receptor photoactivatable tools reported in review literature but without published primary experimental data, which are summarized below. The tool 7'-N₃-1',1'-dimethylheptyl- Δ^8 -THC, the 1'-geminal dimethyl and seven carbon aliphatic chain analog of AM91, was reported to give improved affinity ($K_i = 0.4$ nM at CB₁) compared with AM91 (Picone et al., 2002). This improved affinity would be advantageous when working in native cell lines with low CB receptor expression. The (-)-11-hydroxy-7'-azido- Δ^8 -THC (AM836) was ascribed an $IC_{50} = 0.16$ nM at CB₁ receptor (Palmer et al., 2002; Thakur et al., 2005). Two tools containing an azido instead of the 10-methyl of 1',1'-dimethylheptylhexahydrocannabinol have been reported. AM869 contains an iodide at the terminus of the seven-carbon chain ($K_i = 0.67$ nM at CB₁, 0.72 nM at

Fig. 6. CB receptor photoactivatable, electrophilic, and bifunctional covalent tools.

TABLE 4
CB, leukotriene, prostanoid, and PAF receptor covalent ligands

Tool	Data	Function	Covalent Binding ^a	Reference
CB Receptor Photoactivatable Ligands				
AM91 2-[¹²⁵ I]-AM91	rCB ₁ K _i = 19 ± 6 nM mCB ₁ K _d = 5.6 pM, 9.38 pM		85% at 1 μM	Charalambous et al., 1992 Burstein et al., 1991
AM967	rCB ₁ K _i = 1254 nM hCB ₂ K _i = 124.8 nM mCB ₂ K _i = 34.3 nM		67% at 342 nM	Dixon et al., 2012
AM993	rCB ₁ K _i = 4.4 nM hCB ₂ K _i = 9.6 nM mCB ₂ K _i = 26.4 nM	Agonist Antagonist	67% at 44 nM 60% at 96 nM	Ogawa et al., 2015
AM3661	rCB ₁ K _i = 0.9 ± 0.2 nM mCB ₂ K _i = 57.6 ± 19 nM		68% at 18 nM	Li et al., 2005
CB Receptor Electrophilic Ligands				
AM708 7'-NCS-DMH-THC AM841	rCB ₁ IC ₅₀ = 1.6 ± 0.3 nM rCB ₁ IC ₅₀ = 0.66 nM hCB ₁ K _i = 9.05 ± 2.06 nM	Agonist Agonist	80% at 10 nM ~100% at 100 nM 83% at 3.3 nM	Guo et al., 1994 Morse et al., 1995 Picone et al., 2005
AM994	hCB ₂ K _i = 1.51 nM rCB ₁ K _i = 3.0 nM, EC ₅₀ = 0.8 nM and E _{max} = 94%	Agonist	63% at 30 nM	Pei et al., 2008 Ogawa et al., 2015
	mCB ₂ K _i = 34.6 nM hCB ₂ K _i = 10.3 nM, EC ₅₀ = >400 nM and E _{max} = -91%	Inverse agonist	74% at 103 nM	
AM3677	rCB ₁ K _i = 1.3 ± 0.2 nM mCB ₂ K _i = 48.5 ± 13 nM	Agonist	58% at 26 nM	Li et al., 2005
AM1336 Isothiocyanate 12 GAT100	hCB ₂ K _i = 0.54 nM rCB ₁ IC ₅₀ = 160 nM hCB ₁ EC ₅₀ = 174 nM (cAMP assay) hCB ₁ EC ₅₀ = 2.09 nM (β-arrestin assay)	Inverse agonist Agonist Negative allosteric modulator	60% at 5.4 nM 70% at 1 μM	Mercier et al., 2010 Yamada et al., 1996 Kulkarni et al., 2016
Methyl arachidonyl fluorophosphonate	rCB ₁ IC ₅₀ = 20 nM	Antagonist		Fernando and Pertwee, 1997
CysLT Receptor Photoactivatable Ligands				
[¹²⁵ I]-azido-LTD ₄	gpCysLT ₁ K _i = 1.7 nM ^b , K _d = 0.3 nM			Metters and Zamboni, 1993
[¹²⁵ I]- L-745310	gpCysLT ₁ IC ₅₀ = 27 nM ^b , 53 nM ^a	Antagonist		Gallant et al., 1998
7Z,9E LTD ₄ Aryldiazonium derivative	gpCysLT ₂ K _i = 80 ± 9 nM gpCysLT ₁ K _i = 8 ± 0.9 μM			Klotz et al., 1993
7E,9E LTD ₄ Aryldiazonium derivative	gpCysLT ₂ K _i = 110 ± 14 nM gpCysLT ₁ K _i = 40 ± 12 μM			
LTB₄ Receptor Photoactivatable Ligands				
Aryl azide LTB ₄ derivative 4bα	hLTB ₄ IC ₅₀ = 0.7 μM	Antagonist	40% at 1:1 molar ratio to h-LTB ₄	Durand et al., 2000
PAF Photoactivatable Ligands				
[¹²⁵ I]AAGP	rbtPAF EC ₅₀ = 3.2 ± 1.9 nM ^a , K _d = 2.4 ± 0.7 nM	Agonist		Chau et al., 1989
Tetrafluorophenylazide ginkgolide B derivative	gpPAF K _i = 90 nM	Antagonist	Not demonstrated	Strømgaard et al., 2002
TP Receptor Photoactivatable Ligands				
I-APA-PhN ₃	hTP K _i = 290 nM		58% at 20 μM (reduction in specific binding of U46619)	Arora et al., 1987; Kattelman et al., 1987
[¹²⁵ I]PTA-azido I-PTA-PON ₃	hTP K _d = 11 nM hTP K _d = 9.5 nM	Antagonist	52% at 163 nM, 77% at 326 nM (reduction in I-PTA-OH B _{max})	Mais et al., 1989 Mais et al., 1990
[¹²⁵ I]SAP-N ₃	hTP K _d = 382 ± 41 pM			Mais et al., 1991
IP Receptor Photoactivatable Ligands				
[³ H]APNIC	mIP K _d = 4.7 nM		80% of specific binding of [³ H] APNIC at 13 nM	Ito et al., 1992
EP Receptor Photoactivatable Ligands				
[³ H]azido-PGE ₂	bEP IC ₅₀ = 400 nM ^a			Michalak et al., 1990

^aCovalent binding for all CB photoactivatable and electrophilic ligands was measured as a percentage reduction in [³H]CP-55,940-specific binding.

^bAffinity data for cold analogs.

CB₂), whereas AM1708 has both an alkene and ¹²⁵I at the terminus of the alkyl chain ($K_i = 0.8$ nM at CB₁, 0.85 nM at CB₂) (Khanolkar et al., 2000).

Initial characterization of CysLT₁ receptor was aided by a radioiodinated photoactivatable azido analog of LTD₄. [¹²⁵I]-azido-LTD₄ [$K_i = 1.7$ nM (nonradioactive analog)] (Fig. 7; Table 4) was able to selectively covalently label a 45-kDa protein in guinea pig lung membranes (Metters and Zamboni, 1993). LTD₄, LTE₄, LTC₄, and MK-0571 (a CysLT₁ antagonist) were able to inhibit the photolabeling of the 45-kDa protein by [¹²⁵I]-azido-LTD₄ with similar potencies to their IC₅₀ values at CysLT₁ receptor, providing evidence that this protein is CysLT₁ receptor. However, [¹²⁵I]-azido-LTD₄ was also found to nonselectively label guinea pig serum albumin. Subsequently, the more synthetically accessible photoaffinity probe [¹²⁵I]-L-745310, an analog of CysLT₁ receptor antagonist montelukast, containing a trifluoromethyl diazirine as the photoactivatable group, was developed (Fig. 7; Table 4) (Gallant et al., 1998). The reactive carbene generated upon protolysis (350 nm) of the diazirine can insert into C-H bonds. [¹²⁵I]-L-745310 was able to label the same 45-kDa protein in guinea pig lung preparations, acting as an antagonist (IC₅₀ = 27 nM, 53 nM at gpCysLT₁). High levels of nonspecific labeling were reduced with addition of a detergent and optimization of irradiation time and temperature.

The endogenous leukotrienes possess some innate photoactivatable labeling properties due to the presence of conjugated double bonds, and this has been exploited (Falk et al., 1989; Müller et al., 1991; Slipetz et al., 1993; Nicosia et al., 1995). However, this process requires cryofixation of the receptor–ligand complex to reduce significant nonspecific labeling.

Photoactivatable peptidoleukotriene analogs for CysLT₂ receptor have been developed, containing either 7Z,9E or 7E,9E dienic moieties in an effort to improve stability of the endogenous leukotrienes (Klotz et al., 1993). The aryldiazonium-substituted dienic LTD₄ derivatives (Fig. 7; Table 4) showed greater selectivity for CysLT₂ receptor than the less potent arylazido derivatives. In addition, irradiation of the azido analogs at 245 nm overlaps with the absorption of the dienic moiety (235 nm), resulting in simultaneous decomposition of both azide and diene, whereas only a single reactive species is produced when the diazonium analogs are irradiated at 360 nm.

Characterization of PAF receptor was aided by a similar approach using [¹²⁵I]AAGP, a photoactivatable, radiolabeled derivative of PAF (Fig. 7; Table 4) (Chau et al., 1989). [¹²⁵I]AAGP showed saturable, high-affinity binding in rabbit platelet membranes ($K_d = 2.4 \pm 0.7$ nM, $B_{\max} = 1.1 \pm 0.2$ pmol/mg; EC₅₀ of nonradioactive analog = 3.2 ± 1.9 nM), and photolabeling identified a protein of 52 kDa as the PAF receptor. In another study, a series of ginkgolide B and ginkgolide C derivatives

were synthesized containing varying photoactivatable groups (Strømgaard et al., 2002). These were all found to act as antagonists at PAF receptor with the highest affinity compound a ginkgolide B derivative containing a tetrafluorophenylazide ($K_i = 90$ nM at gpPAF) (Fig. 7; Table 4) and being more potent than ginkgolide B ($K_i = 0.56$ μM at gpPAF receptor). This ligand could potentially be used to investigate ginkgolide interactions with PAF receptor; however, as yet covalent binding has not been demonstrated.

The first prostanoid receptor covalent tool developed was a diazonium salt of 9,11-dimethylmethano-11,12-methano-16-(4-aminophenoxy)13,14-dihydro-13-aza-15 alpha beta-omega-tetranor TXA₂, PTA-POA, which was shown to irreversibly bind to hTP receptor, but affinity was unable to be determined (Mais et al., 1986). An iodinated azide derivative of 13-azaprostanoic acid, I-APA-PhN₃ (Fig. 7; Table 4), was able to irreversibly bind TP receptor with moderate affinity ($K_i = 290$ nM at hTP) and irreversibly inhibit human platelet aggregation (Arora et al., 1987; Kattelman et al., 1987). The TP receptor photoaffinity probe, [¹²⁵I]PTA-azido (Fig. 7; Table 4), had good affinity ($K_d = 11$ nM at hTP) and was able to label three protein bands of 43, 39, and 27 kDa, but it was not clear which of these was TP receptor (Mais et al., 1989). [¹²⁵I]PTA-azido was used to determine the isoelectric point of TP receptor (Mais and Halushka, 1989).

Another TP receptor photoaffinity probe, I-PTA-PON₃ (Fig. 7; Table 4), was used to inhibit human platelet aggregation and irreversibly label hTP receptor (Mais et al., 1990). A radioiodinated photoaffinity probe [¹²⁵I]SAP-N₃ (Fig. 7; Table 4) irreversibly labeled human platelet TP receptor with high affinity (Mais et al., 1991). [¹²⁵I]SAP-N₃ was used with SDS-PAGE autoradiography to identify TP receptor with a molecular weight of 50–51 kDa, and subsequent digestion of this photoaffinity-labeled receptor identified two N-linked glycosylation sites (Mais et al., 1992). Proteolytic cleavage studies of purified, [¹²⁵I]SAP-N₃-labeled TP receptor were carried out to localize the ligand binding domain to amino acids 99–192 (True and Mais, 1994).

A PGI₂ analog, [³H]APNIC (Fig. 7; Table 4), was used to characterize IP receptor. [³H]APNIC had good affinity ($K_d = 4.7$ nM, $B_{\max} = 0.58$ pmol/mg protein, mouse mastocytoma P-815 cells) and photolabeled IP receptor with good efficiency (80% of specific binding of [³H]APNIC at 13 nM), and was able to identify a 43-kDa protein in mouse mastocytoma and a 45-kDa protein in porcine platelets as IP receptor (Ito et al., 1992; Suzuki et al., 1992).

A moderate affinity azidophenacyl ester of PGE₂ (azido-PGE₂) (Fig. 7; Table 4) was synthesized for use as a tool to isolate and identify EP receptor (Michalak et al., 1990). The radiolabeled analog, [³H]azido-PGE₂, was able to covalently label a protein of 100 kDa in isolated bovine cardiac sarcolemmal vesicles, and this photolabeling could be inhibited with excess unlabeled

Fig. 7. Leukotriene, PAF, and prostanoid receptor photoactivatable covalent tools.

PGE₂ and azido-PGE₂. Another EP receptor photoaffinity probe has been reported, (15*S*)-17-(4-azidophenyl)-18,19,20-trinorprostaglandin E₂, which had moderate receptor affinity (IC₅₀ = 300 nM chicken spinal cord EP receptor), but covalent binding was not demonstrated (Kawada et al., 1991). A series of photoactivatable PGF_{2α} derivatives were synthesized with the purpose of developing an FP receptor probe. The highest affinity derivative in the series had a *K_i* of 49 nM (ovine luteal cells); however, covalent binding wasn't demonstrated (Golinski et al., 1992).

A number of other photoactivatable ligands have been developed for lipid-binding receptors that, due to low-moderate affinity, will have limited utility as covalent tools, but may still have some application. For instance, labeling with moderate affinity aryl azide LTB₄ derivative photoaffinity probe 4bα (IC₅₀ = 0.7 μM at hLTB₄) (Fig. 7; Table 4) was carried out in conjunction with amino acid sequencing and mass spectrometry to determine the binding residues as Cys 97, Ser 100, Met 101, and Ser 104 of TM-III and Trp 234 and Tyr 237 in TM-VI, providing initial characterization of the LTB₄ receptor ligand binding site (Durand et al., 2000).

2. Electrophilic Covalent Ligands. A variety of reactive moieties has been used in covalent labels for class

A GPCRs, including isothiocyanates, halomethylketones, reactive thiols, Michael acceptors, and nitrogen mustards (Weichert and Gmeiner, 2015). The isothiocyanate is a popular choice for electrophilic tools as it is readily prepared from primary amines (Wong and Dolman, 2007), is stable in water, but is reactive with amino acids containing a thiol, imidazole, or amine under physiologic conditions (Guo et al., 1994).

AM708 and the closely related analog 7'-NCS-DMH-THC were the first two high-affinity electrophilic covalent ligands reported for CB₁ receptor (Fig. 6; Table 4). Affinity of AM708 (IC₅₀ = 1.6 ± 0.3 nM at rCB₁) was measured with a [³H]CP-55,940 displacement assay (Guo et al., 1994). Treatment with 10 nM AM708 led to 80% reduction in the available rCB₁ receptor binding sites, whereas 100 nM treatment practically depleted all the rCB₁ receptor binding sites. Time- and AM708 concentration-dependent irreversible ligand-CB receptor binding was demonstrated for the first time. Guo et al. (1994) postulated that there must be an amine, thiol, or imidazole amino acid side chain present in the CB receptor binding site to which the covalent ligand bound. The more hydrophobic 7'-NCS-DMH-THC (IC₅₀ = 0.66 nM at rCB₁) demonstrated irreversible binding with incubation at five times the

apparent IC_{50} leading to an 83% reduction in specific binding of [3H]CP-55,940 at rCB_1 receptor (Morse et al., 1995). Taking all evidence into account, it was postulated that covalent binding of AM708 and 7'-NCS-DMH-THC indicates the likelihood of a lysine or cysteine residue in proximity to the alkyl side chain terminus in the CB_1 receptor-binding pocket (Picone et al., 2002). The CB receptor ligand AM960 (Fig. 6) [$IC_{50} = 25$ nM at rCB_1 (preliminary biologic experiment, no further full experiment reported)] was designed in an effort to introduce an iodo group while maintaining the covalent binding ability of AM708 (Chu et al., 2003). Incorporation of a C6 3-iodopropyne group was informed by previous SAR showing that an iodopropyl group at this position conferred high affinity and the iodo group offers potential for [^{125}I] radiolabeling.

Concurrent use of covalent ligands and site-directed mutagenesis is a powerful way to study ligand-binding orientation and determine which amino acids are involved in binding of a specific ligand. AM841 (Fig. 6; Table 4) was the first CB_1 receptor covalent tool to be used in conjunction with site-directed mutagenesis and provided important information about ligand activation sites (Picone et al., 2005). It was proposed using CB_1 receptor homology modeling and ligand docking that a cysteine residue present on helix 6 C6.47 (residue 355), part of the highly conserved CWXP-binding motif in class A GPCRs, was the most likely site of covalent attachment of AM841. Mutation of Cys C6.47 to Ser, Ala, or Leu to reduce or eliminate the nucleophilicity of residue 355 was carried out to test this hypothesis. The affinity of AM841 for hCB_1 C6.47 Ser ($K_i = 10.46 \pm 0.88$ nM) and Ala ($K_i = 11.32 \pm 0.29$ nM) mutants was similar but was reduced for the C6.47 Leu mutant ($K_i = 58.09 \pm 11.69$ nM). In spite of retaining some receptor affinity, AM841 did not irreversibly bind to any of the three mutants. It is therefore worth considering the contribution of measured binding affinity versus covalent bond formation—high ligand affinity is not dependent on covalent bond formation, although measurement can be biased depending on the experiment used to measure K_i . Also of consideration is the effect site-directed mutagenesis has on overall receptor shape/misfolding. In this study, the CB_1 receptor global conformation of the mutants is likely to be maintained as it is in the wild-type receptor because AM841 displayed comparable affinity.

There have been multiple studies utilizing AM841 to probe CB receptor structure and function. Pei et al. (2008) used AM841 to demonstrate distinctions in ligand-binding motifs between hCB_1 and hCB_2 receptor subtypes via complementary site-directed mutagenesis and ligand-docking studies. Mutagenesis of hCB_2 receptor indicated that C6.47 is the site of covalent attachment of AM841 and ruled out two cysteines on helix seven, C7.38 and C7.42. Activation of hCB_2 receptor by AM841 (leading to inhibition of forskolin-

stimulated cAMP production) was achieved with much higher potency compared with the noncovalent analog of AM841 (wherein the NCS group is replaced by an H atom) or at hCB_1 receptor. The highly conserved CB_1 receptor Lys K3.28, which has been shown to be important in recognition of multiple ligands by hCB_1 receptor (Song and Bonner, 1996), was demonstrated as having little effect on hCB_2 receptor-AM841 binding. Pei et al. (2008) also postulated that AM841 accesses the CB_2 receptor-binding pocket through the lipid bilayer (discussed in *Leukotriene Receptor*). Despite a potential lipid bilayer entry, covalent ligands such as AM841 are thought unlikely to bind to random nucleophilic amino acids as a high-affinity binding interaction needs to occur before significant covalent labeling can occur. This specific covalent reactivity can be shown using mass spectrometry, as demonstrated by Szymanski et al. (2011), who further characterized the covalent interaction of AM841 with hCB_2 receptor residue C6.47. Multiple reaction mass spectrometry monitoring showed that covalent modification of hCB_2 receptor by AM841 was exclusive to TMH6, and high-resolution mass spectrometry of the TMH6 tryptic peptide confirmed this covalent labeling to be selective for C6.47 (Szymanski et al., 2011). This report shows the power of combining mass spectrometry-based proteomics and site-directed covalent labeling in the elucidation of GPCR ligand binding sites. Taken together, this information provides valuable insight into CB receptor ligand-binding pocket(s) and requirements for high-affinity, selective ligand design.

Highly specific and potent covalent ligands have also been used to investigate the physiologic and pathophysiological roles of GPCRs. AM841 has been used in an in vivo study of inflammatory bowel disease to show the involvement of both central and peripheral mCB_1 and mCB_2 receptors in the anti-inflammatory action of cannabinoids (Fichna et al., 2014). The utility of AM841 in research and therapeutics has been further demonstrated by examining gastrointestinal motility in healthy and stressed mice (Keenan et al., 2015). In this study, AM841 was found to act as a peripherally restricted ligand, normalizing accelerated gastrointestinal motility through action on CB_1 receptor in the small and large intestine. These studies demonstrate the therapeutic potential of covalent ligands such as AM841. It would be interesting to see what the equivalent noncovalent ligand would do in the same experiments to dissect the importance of the covalent attachment in the observed therapeutic effects.

An electrophilic adamantyl-substituted covalent tool (AM994) (Fig. 6; Table 4) has also been developed with good affinity for both CB_1 and CB_2 receptors, which behaved as an agonist at rCB_1 receptor and an inverse agonist at hCB_2 receptor (Ogawa et al., 2015). Successful labeling of CB receptor following pretreatment with 10-fold the K_i of AM994 was demonstrated with a 63%

reduction in specific binding of [3 H]CP-55,940 at rCB₁ receptor and a 74% reduction at hCB₂ receptor.

The same research group that developed photoactivatable AM3661 has also synthesized an isothiocyanate-functionalized AEA tool AM3677 (Fig. 6; Table 4), which irreversibly labels 58% of rCB₁ receptor when used at 26 nM (binding assays carried out in presence of serine protease inhibitor as with AM3661) (Li et al., 2005). Further structural and functional profiling was carried out by Janero et al. (2015), which showed that AM3677 forms a covalent bond to hCB₁ C6.47. AM3677 was found to function as an agonist, inhibiting cellular cAMP formation and stimulating irreversible internalization of rCB₁ receptor. The authors propose AM3677 could be used as a tool in the study of ECB-induced CB₁ receptor activation and associated signaling.

Functionalization of a known biarylpyrazole (AM6731) with an isothiocyanate group generated AM1336 (Fig. 6; Table 4), a covalent hCB₂ receptor inverse agonist (Mercier et al., 2010). AM1336 irreversibly bound 60% of available hCB₂ receptor when administered at 5.4 nM. Mutation of Cys to Ala or Ser at single and multiple points in hCB₂ receptor and analysis using tool AM1336 revealed two residues in TMH7 (C7.38 residue 284 and C7.42 residue 288) as key for inverse agonist binding. C1.39 (residue 40) of TMH1 was also found to modulate hCB₂ receptor ligand affinity, and residue C137 from intercellular loop 2 was shown to affect the maximum efficacy of AM1336.

Exploration of the aminoalkylindole CB chemical scaffold as an electrophilic ligand has been carried out with synthesis of analogs containing an isothiocyanate on the indole ring or at various C3 naphthyl positions (Yamada et al., 1996). The most potent derivative (isothiocyanate 12) (Fig. 6; Table 4), containing indole 6-isothiocyanate substitution, demonstrated irreversible binding at sixfold the IC₅₀ with 70% reduction in specific binding of [3 H]CP-55,940 at rCB₁ receptor. This depletion of [3 H]CP-55,940 binding indicates that the aminoalkylindole class and nonclassic CBs (e.g., CP-55,940) may have overlapping binding sites.

Due to the psychotropic side effects associated with direct CB₁ receptor activation, alternative CB drug development strategies have been investigated. One area of promise is allosteric modulators, potentially allowing more efficacious control of downstream signaling effects. However, improved knowledge of any CB₁ receptor allosteric site(s) is critical to drug development. To this end, Kulkarni et al. (2016) have recently developed the covalent CB₁ receptor ligand GAT100 (Fig. 6; Table 4), by replacement of the chloro moiety of allosteric CB₁ receptor allosteric ligand ORG27569 (Fig. 6) with an isothiocyanate group. Further in-depth characterization showed GAT100 is a negative allosteric modulator of CP-55,940 (Fig. 1), AEA, and 2-AG across several signaling pathways and may interact with key residue C7.38(382) of CB₁ receptor (Laprairie

et al., 2016). It will be very interesting to see what structural knowledge of the CB₁ receptor allosteric site is gleaned by future use of this tool.

Methyl arachidonyl fluorophosphonate has been shown to act as an irreversible inhibitor of CB₁ receptor (IC₅₀ = 20 nM at rCB₁) (Fig. 6; Table 4), preventing subsequent binding of CP-55,940 and reducing the maximal responses of agonists WIN 55,212-2 and CP-55,940 (Deutsch et al., 1997; Fernando and Pertwee, 1997). Methyl arachidonyl fluorophosphonate, which is also a potent irreversible antagonist of FAAH (IC₅₀ = 2.5 nM at rFAAH), acts as a phosphorylation agent as the electrophilic fluorophosphonate group can covalently label nucleophilic residues such as serine.

There is a lack of electrophilic covalent probes for the other fatty acid-binding class A GPCRs when compared with CB receptors. An attempt at an electrophilic covalent ligand for the putative prostamide receptor(s) was made; however, biologic data have not yet been published (Shelnut et al., 2015).

3. Bifunctional Covalent Ligands. Bifunctional ligands with two reactive/photoactivatable functional groups are capable of forming two covalent bonds to one receptor. To this end, the homobifunctional ligand AM5823 with two isothiocyanate groups and the heterobifunctional ligand AM5822 with an isothiocyanate and an azide group have been developed (Fig. 7) (unpublished data) (Makriyannis, 2014). Another bifunctional tool, di-azido AM859, has been reported in a review (K_i = 1.6 nM at CB₁, 2.65 nM at CB₂) (unpublished primary experimental data) (Khanolkar et al., 2000). It is hoped that this approach will provide greater spatial accuracy in the classification of CB receptor ligand-binding orientation.

V. Antibodies

An antibody (or immunoglobulin) is a heterodimer glycoprotein consisting of two amino acid heavy chains and two light chains linked by disulfide bonds and hydrophobic interactions (Schroeder and Cavacini, 2010). These chains contain a constant sequence region at the C-terminal and a variable sequence region at the N-terminal, the latter of which selectively binds to a particular antigen. Significant obstacles to raising antibodies against membrane-embedded GPCRs include low receptor expression levels and the difficulty in isolating pure receptor in a stable and functionally relevant conformation (Hutchings et al., 2010). Methods are being developed to improve stability and overexpress GPCRs to aid in the production of functional antibodies (for example (Takeda et al., 2015)).

A. Characteristics of Antibodies

Antibodies from a monoclonal source (mAbs) are identical, as they are produced by a single clone of a B cell hybridoma and bind to a single epitope. Polyclonal

antibodies (pAbs) are produced by multiple B lymphocytes and thus are a mix of antibodies with varying affinity and specificity (Ritter, 2000). GPCRs exhibit a high degree of conformational flexibility (particularly in the extracellular loops), and this can impact antibody affinity if an epitope is more or less accessible in a particular conformer (Peeters et al., 2011). The affinity of a mAb toward a GPCR may also be sensitive to receptor conformation, polymorphism, and/or glycosylation state. The affinity of pAbs may be less affected by receptor conformation; however, there may be less specificity for GPCRs compared with mAbs (Lipman et al., 2005), and there can be batch to batch variability. Overall, the affinity, specificity, and reproducibility of mAbs over pAbs are hugely advantageous in the pursuit of reliable and consistent results (Singh et al., 2014).

B. Antibody Limitations

The major limitations with using antibodies as tools for GPCR localization studies are false positives and false negatives. False positives most often occur due to lack of antibody specificity, leading to the recognition of other proteins with similar or identical epitopes (Ivell et al., 2014). False negatives can likewise arise due to low antibody specificity for the target, but can also be caused by epitopes being hidden or in an altered conformation, or by unsuitable sample fixing procedures. In addition, use of antibodies can be especially challenging and unreliable in the study of GPCRs due to very low levels of receptor expression.

CB₁ receptor is historically regarded as the CNS CB receptor, whereas CB₂ receptor is the peripheral receptor. Reports of neuronal CB₂ receptor using antibody-based methods have challenged this notion (reviewed in Atwood and Mackie, 2010); however, there has long been debate in the CB receptor community surrounding the accuracy of reported receptor expression determined solely using antibodies. This fueled a spate of CB₂ receptor antibody validation studies (Baek et al., 2013; Cecyre et al., 2014; Marchalant et al., 2014), which showed that whereas many commercially available pAbs have sensitivity for CB₂ receptors, often specificity is lacking. Other validation studies have also raised doubts of the specificity of several CB₁ receptor pAbs (Grimsey et al., 2008; Morozov et al., 2013), as well as a S1P₁ receptor mAb and pAb (Talmont and Moulédous, 2014) and a FFA1 receptor mAb and two pAbs (Teutsch et al., 2014). Together, these validation studies illustrate the importance of complete characterization of tools for the individual applications intended. There are many positive and negative controls available for accurate confirmation of antibody specificity, and these are summarized well elsewhere (Rhodes and Trimmer, 2006; Moser et al., 2007; Bussolati and Leonardo, 2008; Lorincz and Nusser, 2008; Baek et al., 2013).

Antibodies can be purchased from a commercial source or made in-house; however, each option has

limitations. Specificity of commercial mAbs and pAbs has been shown to be highly variable (Berglund et al., 2008), so time-consuming, thorough validation of the antibody is necessary to ensure meaningful results. It has been estimated that poor characterization of antibodies costs biomedical research \$350 million each year in the United States (Bradbury and Pluckthun, 2015). Some academic groups produce their own antibodies to reliably secure sufficient reagent to complete their study, but this can be expensive and time consuming, with no guarantee of a functioning antibody (Rhodes and Trimmer, 2006). Reproducibility of antibody-based experiments is hampered by an estimated 44% of published articles (in all fields of research) failing to provide enough unique identifiers of the antibody used (i.e., a universal database identifier, or a vendor and catalog number, or protocols for reproduction) (Vasilevsky et al., 2013). There are numerous CB, FFA, S1P, LPA, prostanoïd, leukotriene, PAF, and GPBA receptor antibodies available for purchase and a number of comprehensive databases available that detail some of these antibodies (<http://www.antibodypedia.com/>, <http://antibodyregistry.org/> and <https://www.citeab.com/>). Only the specific details of the antibodies used in the examples cited have been given in this work.

C. Applications of Antibodies

Antibodies have been used to detect and quantify lipid-binding receptors using a variety of techniques such as Western blotting, immunohistochemistry (IHC), immunocytochemistry, flow cytometry, and immunofluorescence. Antibodies have been used to study localization and variation in receptor expression between different tissue types and disease states. Antibodies can be used in conjunction with radioligands to help identify structural motifs key to function, for example, C-EL2Ab and [³H] SQ-29548 described in *Applications of Radioligands* (Murad et al., 2012). Several recent examples are discussed in this review that demonstrate a range of techniques rather than an exhaustive summary, as the number of studies is extensive.

IHC is commonly used to study CB receptor expression across different cell and tissue types (Bouskila et al., 2013), between healthy and diseased tissue (Allen et al., 2009; Dowie et al., 2014), and in developing predictions of disease severity and outcome (Chung et al., 2009). IHC has also been used to localize GPBA receptor expression (Cipriani et al., 2013), to study CysLT₁ receptor expression in Hodgkin's lymphoma (Schain et al., 2008), and to localize EP₁, EP₂, EP₃, EP₄, and FP receptor expression in the human ureter (Oll et al., 2012).

Western blotting has been used to study variations in CB receptor expression (Alvaro-Bartolome and Garcia-Sevilla, 2013). IHC and Western blotting have been used to localize FFA1 and FFA4 receptor (Mizuta et al., 2015), FFA3 receptor (Nøhr et al., 2015), and S1P₁ receptor expression (Bradaric et al., 2011; Maeda et al.,

2014). Using antibodies, localization of elevated S1P₁ receptor helped demonstrate that the BBB-protective effects of the traditional antimalarial drug artesunate are mediated through S1P₁ receptor signaling (Zuo et al., 2017). In another study, Western blotting and immunocytochemistry were used to show that activation of S1P receptor induces cardiac hypertrophy (Robert et al., 2001). IHC and LPA receptor antibodies have been used as tools to study tissue expression of LPA receptors, for example, on cell membranes in muscle-invasive bladder cancer specimens (where greater LPA₁ receptor expression was observed), indicating this could be used as a diagnostic marker (Kataoka et al., 2015).

Machado et al. (2014) used immunofluorescence to monitor CB receptor activation in rat paw using fluorescently labeled CB₁ and CB₂ receptor antibodies (Proteimax Biotechnology, Cotia, São Paulo, Brazil) that are specific to the activated receptor conformation. This study monitored CB and opioid receptor interactions during treatment with the snake venom peptide crotalphine, concluding that the venom antinociceptive effects were dependent on activation of peripheral CB₂ receptor, causing release of dynorphin A and subsequent κ -opioid receptor activation. Immunofluorescence labeling has been used to investigate FFA1 receptor in the medulla oblongata in a study of the role that this receptor plays in pain control mechanisms (Nakamoto et al., 2015). Immunofluorescence has also been used to study EP₄ receptor expression in an investigation evaluating the potential of this receptor as a therapeutic target for leukemia and lymphoma (Gobec et al., 2014).

Flow cytometry has been used by Jean-Gilles et al. (2015) to quantify fluorescent antibody (PA1-745 and PA1-744; Affinity Bioreagents, Golden, CO) labeling of CB₁ and CB₂ receptors in the presence of proinflammatory cytokines in MS (Jean-Gilles et al., 2015). Agudelo et al. (2013) also used flow cytometry and primary polyclonal and secondary fluorescein-conjugated antibodies (sc-20754 and sc-25494, Santa Cruz Biotechnology, Santa Cruz, CA; 10224, Cayman Chemicals, Ann Arbor, MI, respectively) to quantify CB₁, CB₂ receptor, and GPR55 to study the effects of alcohol use on immune responses and dendritic cell modulation. This study concluded that alcohol upregulated CB₂ receptor and GPR55 in monocyte-derived dendritic cells. IHC and flow cytometry were used to study how variations in anatomic location of B cell non-Hodgkin's lymphoma can be related to the differential expression of S1P₁ and S1P₃ receptors (both which control lymphocyte exit from secondary lymphoid organs) and S1P₂ receptor (responsible for retention of lymphocytes within germinal centers) (Middle et al., 2015). Flow cytometry was used to investigate reduced LTB₄₁ receptor expression in chronic myeloid leukemia patients (Lucas et al., 2014) and increased LTB₄₂ receptor expression in a study on cisplatin-resistant ovarian cancer (Park et al., 2016).

Antibodies labeled with a heavy metal, most commonly gold, can be studied using electron microscopy. For example, the role of CB₁ receptor in epilepsy and neurodegenerative disease was examined by labeling first with rabbit polyclonal anti-CB₁ (ab23703; Abcam, Cambridge, UK) and then with goat anti-rabbit IgG conjugated to 1.4-nm gold grains (Nanoprobes, Stony Brook, NY) (Meng et al., 2014). Observations by electron microscopy demonstrated that CB₁ receptor expression was enhanced in the astrocytes of epileptic rats. Heterodimerization of GPCRs can have significant effects on receptor function, signaling and trafficking, and ligand pharmacology (Hiller et al., 2013). GPCR heterodimer-selective antibodies have been used to study the localization, prevalence, and function of GPCR heteromers (reviewed in Gomes et al., 2014). A mAb specific for δ opioid-CB₁ receptor heteromers was used by Bushlin et al. (2012) to detect increased δ opioid-CB₁ heteromer prevalence in the cortex upon induction of neuropathic pain. This study also found nonsignaling doses of CB₁ receptor agonists increased δ opioid receptor activity in neuropathic pain-afflicted animals *ex vivo*. The antibody was capable of blocking this heteromer-mediated activity, thus demonstrating the potential of heteromer-selective antibodies as therapeutic agents and δ opioid-CB₁ heteromer as a therapeutic target. In another study, an antibody selective for the CB₁-AT₁ receptor heteromer was used to investigate heteromer-mediated signaling (Rozenfeld et al., 2012). Production of fibrogenic proteins from activated hepatic stellate cells in alcohol-treated rat membranes was blocked by the heteromer-specific antibody, indicating the potential of CB₁-AT₁ heteromers as a therapeutic target for liver fibrosis therapies. However, it is important to note that the results from these studies were observed *in vitro*, and that there will be additional challenges in the therapeutic application of antibodies in the CNS, such as BBB penetrability. Strategies are in development for improving antibody brain penetration (Niewoehner et al., 2014) as well as the design of small-molecule bivalent ligands that target receptor heteromers.

D. Beyond Antibodies

Many GPCR antibody limitations such as selectivity, stability, solubility, and production are being addressed by the emergence of the next generation of tools. For example, bio-orthogonal coupling and release of either a drug or tracer to an antibody that targets tumor-associated glycoproteins have been demonstrated (Rossin et al., 2016). This approach has yet to be reported for antibodies targeting GPCRs, although no doubt will be reported in the future. Nanobodies are single-domain antibodies approximately 10% of the size of regular antibodies that have two heavy and two light chains. Nanobodies are increasingly used as diagnostics, targeting tools, and therapies (reviewed in De Meyer et al., 2014 and Chakravarty et al., 2014), and for GPCRs have

been useful for cocrystallization and X-ray structure determination, for example, stabilizing β_2 -adrenergic receptor in the active form (Ring et al., 2013). More recently, a negative allosteric nanobody for β_2 -adrenergic receptor has been reported and used to tease out the pharmacology of various ligands (Staus et al., 2016). Affibodies are smaller nonimmunoglobulin proteins that are increasingly used as both diagnostic tools and drugs (Feldwisch et al., 2012). Bio-orthogonal pretargeting followed by coupling of fluorophores and in vivo radioligands to an affibody that targets human epidermal growth factor receptor 2 has been reported (Altai et al., 2016). To date nanobodies or affibodies for CB, FFA, LPA, or S1P receptors have not been reported.

VI. Conclusions and Future Outlook

The discovery and characterization of CB, FFA, LPA, S1P, prostanoid, leukotriene, GPBA, and PAF receptors have been made possible by the use of tools such as covalent and fluorescent small-molecule ligands, radioligands, and antibodies. An ideal tool or technique should be able to provide information about the chemical and conformational structure of the receptor, changes occurring during receptor activation, and receptor-signaling cascades in the native cell environment. However, it is difficult to imagine a single tool or technique capable of providing all this information in a system as complex as GPCRs. Thus, our current understanding of receptor structure and function is based on information obtained using these tools in conjunction with other techniques such as molecular biology and receptor mutagenesis, X-ray crystallography, and homology modeling. Before the application of an individual tool to study a receptor, it is critical that the tool be subjected to rigorous pharmacological validation, as structural modifications can drastically alter the pharmacological characteristics in comparison with the untagged/unmodified ligand.

Radioligands, including those based on synthetic scaffolds reviewed in this work and also radiolabeled endogenous ligands, are the most developed tools for all the lipid-binding class A GPCRs. Preclinical in vivo imaging, in particular for CB₁ receptor, is increasingly used where signal depth/tissue penetration is a consideration. Radioligands have the advantage of sharing the highest structural similarity with the parent ligand as compared with other tools and cause minimum disturbance compared with the unlabeled ligand–receptor interaction. However, the drawbacks of radiation safety and shelf life fuel the continued development and interest in small-molecule fluorescent tools. Future development of synthetic ligands tethered to newly discovered NIR fluorophores will fuel the use of fluorescent probes for in vivo imaging as an alternative to radioligands, especially as longer wavelength NIR-II fluorophores allow for improved detection depth.

Although there can be issues with nonspecific background fluorescence and quenching by fluorescence chelators, the advantages of small-molecule fluorescent ligands (such as precise spatial information) usually far exceed these limitations. Nonspecific membrane interactions can be an issue for radioligands and fluorescent ligands, especially when the core pharmacophore/ligand is hydrophobic, as is often the case for lipid-binding GPCRs. A nonspecific membrane interaction is very much probe-specific, so researchers should select the best available tool for their experiment hypothesis, or alternatively there are huge scope and demand to design and synthesize novel probes with more favorable properties. Covalent ligands are powerful tools to probe the binding site of these lipid receptors especially when used in conjunction with mutation studies. Antibodies are very versatile and can be used in a number of different techniques and are theoretically very specific, yet the cost, pharmacokinetic properties (especially for in vivo study), and the questions surrounding specificity (in particular for CB₂ receptor antibodies) are limiting factors.

The toolbox available to researchers for some lipid-receptor subtypes is either empty or minimal. As drug discovery drives reports of new high-affinity selective small-molecule ligands, it is anticipated this in turn will facilitate development of new chemical tools with better selectivity and pharmacokinetic properties.

Continued advancement of knowledge regarding GPCR structure and ligand–receptor binding interactions, such as that provided by the recently solved CB₁ receptor crystal structures, will also fuel rational design of chemical tools. The use of biologics as selective tools is another expanding field, with researchers focusing on smaller ligands such as peptides, nanobodies, affibodies, and aptamers. Development of new tools will enable probing questions about CB, FFA, LPA, and S1P receptors to be addressed, in particular in complex processes such as receptor allosterism and oligomerization.

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