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Therapeutic modulation of cannabinoid lipid signaling: metabolic profiling of a novel antinociceptive cannabinoid-2 receptor agonist

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Abstract

Aims—AM-1241, a novel, racemic cannabinoid-2 receptor (CB2) ligand, is the primary experimental agonist used to characterize the role of CB2-mediated lipid signaling in health and disease, including substance abuse disorders. In vivo pharmacological effects have been used as indirect proxies for AM-1241 biotransformation processes that could modulate activity. We report the initial pre-clinical characterization of AM-1241 biotransformation and in vivo distribution.

Main methods—AM-1241 metabolism was characterized in a variety of predictive in vitro systems (Caco-2 cells, mouse, rat and human microsomes) and in the mouse in vivo. Liquid chromatography and mass spectrometry techniques were used to quantify AM-1241 tissue distribution and metabolic conversion.

Key findings—AM-1241 bound extensively to plasma protein/albumin. A pharmacological AM-1241 dose (25 mg/kg, i.v.) was administered to mice for direct determination of its plasma half-life (37 min), following which AM-1241 was quantified in brain, spleen, liver, and kidney. After p.o. administration, AM-1241 was detected in plasma, spleen, and kidney; its oral bioavailability was ~21%. From Caco-2 permeability studies and microsomal-based hepatic clearance estimates, in vivo AM-1241 absorption was moderate. Hepatic microsomal metabolism of AM-1241 in vitro generated hydroxylation and demethylation metabolites. Species-dependent differences were discovered in AM-1241's predicted hepatic clearance. Our data demonstrate that AM-1241 has the following characteristics: a) short plasma half-life; b) limited oral bioavailability; c) extensive plasma/albumin binding; d) metabolic substrate for hepatic hydroxylation and demethylation; e) moderate hepatic clearance.

Significance—These results should help inform the design, optimization, and pre-clinical profiling of CB2 ligands as pharmacological tools and medicines.

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Conflict of Interest Statement

The authors declare that there are no conflicts of interest.

Keywords

biotransformation; cannabinoid; half-life; liver; microsomes; metabolism; mouse; seven-transmembrane receptor

Introduction

The endocannabinoid system is a ubiquitous cellular communication network including a family of endogenous signaling lipids (endocannabinoids) that function as agonists by engaging and activating at least one of two principal cannabinoid G protein-coupled receptors (GPCRs), designated CB1 and CB2 (De Petrocellis and Di Marzo 2009; Di Marzo and Petrosino 2007; Mackie 2008). Both CB1 and CB2 are expressed at key sites involved in pain perception and transduction (Mackie 2008; Vemuri et al. 2008). Small-molecule CB1 and non-selective CB1/CB2 agonists exert therapeutic analgesic effects in pre-clinical models of acute inflammatory, neuropathic, neoplastic, and chemotherapy-related pain (Cheng and Hitchcock 2007; Nackley et al. 2004; Pertwee 2009; Vemuri et al. 2008), which supports the conclusion that pharmacotherapeutic potentiation of cannabinergic signaling is a viable approach for pain management, a major medical need that remains unfulfilled due to the suboptimal efficacy and/or severe adverse effects associated with existing pain medicines (Stone and Molliver 2009). Unwanted psychoactivity and abuse liability mediated by central CB1 activation have hindered the clinical application of synthetic CB1-selective or non-selective CB1/CB2 agonists for pain relief (Cheng and Hitchcock 2007; Rahn and Hohmann 2009; Rahn et al. 2007). In contrast, the mainly peripheral localization of CB2 greatly reduces the risk of centrally-mediated side effects from selective CB2 agonists and offers the potential to separate the analgesic effects of cannabinergic agents from psychobehavioral liability (Anand et al. 2009; Malan et al. 2002; Whiteside et al. 2007).

The aminoalkylindole AM-1241, [(R,S)-(2-iodo-5-nitrophenyl)-[1-((1-methyl-piperidin-2-yl)methyl-1H-indol-3-yl)-methanone], (Fig.1), is a racemic CB2 ligand with low nanomolar affinity and high (up to 340-fold) selectivity for CB2 vs. CB1 (Beltramo 2009; Bingham et al. 2007; Mancini et al. 2009; Nackley et al. 2003; Quartilho et al. 2003; Yao et al. 2006). In cell systems over-expressing recombinant CB2, variable, species-dependent pharmacological functions have been attributed to AM-1241, leading some to characterize this agent as a “protean agonist” capable of changing its apparent mode of action (Beltramo 2009; Bingham et al. 2007; Mancini et al. 2009; Yao et al. 2006), whereas AM-1241 acts pharmacologically in native biological systems as a CB2 agonist (Beltramo 2009; Gutierrez et al. 2007; Hohmann et al. 2004; Ibrahim et al. 2005; Nackley et al. 2003; Nackley et al. 2004; Quartilho et al. 2003; Rahn et al. 2008). Although (R)-AM-1241 displays ~40-fold greater CB2 binding affinity than does (S)-AM-1241, the vast majority of investigations with AM-1241 in animal models have utilized the racemic compound (Beltramo 2009; Bingham et al. 2007; Rahn et al. 2008).

The primacy of AM-1241 as a research tool for interrogating CB2 pharmacology in health and disease shows no sign of abating (Beltramo 2009; Gutierrez et al. 2007). Across a broad range of rodent models, AM-1241 dose-dependently alleviates neuropathic pain and the allodynia/hyperalgesia induced by thermal, mechanical (e.g., nerve injury), or chemical (e.g., capsaicin, carageenan, chemotherapeutic drugs) provocation without apparent adverse side effects (Anand et al. 2009; Beltramo 2009; Bingham et al. 2007; Gutierrez et al. 2007; Rahn et al. 2010; Rahn et al. 2008; Whiteside et al. 2007). Therapeutic effects of AM-1241 have also been reported in murine models of inflammatory bowel disease (Storr et al. 2009), bone cancer (Lozano-Ondoua et al. 2010), and amyotrophic lateral sclerosis (Kim et al. 2006; Shoemaker et al. 2007). Additionally, AM-1241 has proven itself as a key research

tool for defining the importance of CB1 vs. CB2 in psychobehavioral disorders, including substance abuse/drug addiction (Adamczyk et al. 2012; Gamaledin et al. 2012a; Gamaledin et al. 2012b).

Since AM-1241 is well established as the primary CB2 agonist, its thorough preclinical characterization would help optimize the pharmacology of this chemical class, assist in AM-1241 dose titration and interpreting dose-related activity across the diverse systems in which it continues to be tested, and inform the design of future CB2 agonists as potential medicines. These characteristics of AM-1241 have yet to be described, possibly due to the routine administration of AM-1241 in acute single-dose studies that evaluate only pathological and disease-related endpoints (Beltramo 2009). The apparent involvement of as yet undefined peripheral mechanisms in AM-1241's analgesic action (Ibrahim et al. 2003; Rahn et al. 2010) further invites evaluation of AM-1241 biodistribution and biotransformation. This report constitutes the initial characterization of AM-1241 in vivo oral bioavailability, biodistribution and metabolism, and presents complementary in vitro studies of AM-1241 plasma stability, plasma-protein binding, predictive human oral and microsomal metabolism. A single-dose protocol was used to maintain congruency with the wealth of behavioral studies that have employed a single, acute AM-1241 administration. Although many of these studies used intraperitoneal (i.p.) or subcutaneous (s.c.) injection, our study focuses on the more clinically tractable intravenous and oral routes. The aggregate data offer the first insights into important pharmacological characteristics of AM-1241, the canonical CB2 agonist.

Materials and Methods

Materials

Unless otherwise indicated, standard buffers, and reagents were purchased at the highest available grade from commercial sources. HPLC-grade acetonitrile, methanol, and chloroform were purchased from Fisher Scientific (Pittsburg, PA, USA). High-purity gases and liquid nitrogen were purchased from Med Tech Gases (Medford, MA, USA). AM-1241 was synthesized at the Center for Drug Discovery (Fig. 1) (Rahn et al. 2010). Free base was converted by titration with hydrochloric acid to a water-soluble hydrochloride salt (AM-1241*HCl), which was obtained as an amorphous lyophilized solid.

Animals and dosing protocol for biodistribution analysis

Male CD1 mice initially weighing 16–18 g (Charles River Laboratories, Willmington, MA, USA) were acclimated to vivarium conditions for one week prior to experimentation; water and food were provided ad libitum. On the experimental day, the mice (25–30 g body weight) were administered AM-1241*HCl (25 mg/kg in a dosing volume of 0.1 mL water) either i.v. (bolus dose injection into the lateral tail vein) or p.o. (via feeding needle). These studies were conducted in accordance with the Guide for the Care and Use of Laboratory Animals.

Collection and storage of biological samples

Biological samples were taken at various time points: 5 minutes to 2 hours after i.v. administration or 15 minutes to 2 hours after p.o. administration. Blood was collected through retro-orbital bleed (Hale et al. 2002), while necropsy and tissue harvest were performed after cervical dislocation according to protocols approved by the institutional Animal Care and Use Committee in accordance with public health and safety policies. All samples were frozen and stored at –20°C until analysis.

Sample preparation for HPLC

Plasma proteins were precipitated by diluting thawed plasma 1:5 (v/v) with acetonitrile followed by centrifugation at 20,000 g for 5 minutes; the resulting supernatant was evaporated to dryness under nitrogen. All tissues were homogenized in TME buffer (25 mM Tris base, 5 mM MgCl₂, 1 mM EDTA, pH 7.4) using a motorized Duall-type pestle homogenizer. Brain and spleen homogenates were deproteinized with acetonitrile as described for plasma. Kidney, liver and intestine homogenates were extracted using a modified Folch method (Cluny et al. 2010; Folch et al. 1957). All samples were reconstituted in a solution of 20 mM KH₂PO₄ (pH 3.0)/acetonitrile (1:1, v/v) prior to HPLC analysis.

HPLC conditions

Chromatographic separation for AM-1241 was achieved on an Alliance HPLC equipped with a dual wavelength ultraviolet detector (Waters Corporation, Milford, MA, USA) and outfitted with a Supelco C₁₈ Discovery column (250 × 4.6 mm) and guard column (Sigma-Aldrich, St. Louis, MO, USA). The mobile phase consisted of acetonitrile (solvent A), 20 mM KH₂PO₄ (pH 3.0):acetonitrile (60:40 v/v; solvent B), and water (solvent C). The injection volume was 20 µL; detection wavelengths were 204 and 220 nm. AM-1241 was well resolved from endogenous tissue constituents with a retention time of 14.5 minutes. External standards in acetonitrile were used to quantify AM-1241 in biological samples with linear concentration-peak area relationships ($r = 0.996$) within the 5–50 µM concentration range (acetonitrile or mouse plasma). The calculated lower limit of detection was 3 µM; the calculated limit of quantitation was 11 µM (Cunico et al. 1998). Plasma/tissue AM-1241 concentrations are averages from replicate biological samples taken at each sampling time and non-compartmental analysis was conducted using WinNonlin software (Pharsight Corp; Mountain View, CA).

Stability studies

To determine AM-1241 plasma stability, solutions of AM-1241 free base in acetonitrile and mouse plasma (20 µM final concentrations) were incubated at 37°C. Samples were taken hourly from each mixture and processed for HPLC quantification (above). Solutions of AM-1241*HCl in distilled water at 20°C (room temperature) and 4°C were used to determine AM-1241 thermal stability. The most concentrated aqueous solution (1.0 mg/mL) was prepared by dissolving AM-1241*HCl solid in distilled water and adjusting the solution pH to >5 with diluted HCl. The resulting AM-1241*HCl stock was serially diluted with water to obtain the lower concentrations (0.3, 0.03, 0.003 mg/mL) studied. Samples were taken from the thermal stability incubations at days 0, 3, 7, and 14 for HPLC quantification (above).

Caco-2 permeability assay

A cultured Caco-2 cell-monolayer system was used to estimate AM-1241 absorption across the gastrointestinal tract according to previously published methods (Woolf 1999). After incubation for 2 hours at 37°C in a shaking water bath, samples were taken from both the apical and basolateral sides, after which monolayer integrity was verified as <3% Lucifer yellow transfer to the basolateral side (Kasuga et al. 1998). Samples were subjected to solid phase extraction cartridges containing 50 mg C₁₈ adsorbent (Alltech) and prewashed with methanol. After sample loading, each cartridge was washed with water, and the bound AM-1241 was eluted with 5 mL methanol. Methanol eluants were dried under nitrogen and reconstituted in 20 mM KH₂PO₄ (pH 3):acetonitrile (1:1,v/v) for HPLC analysis. Apparent permeability was calculated as previously described (Yamashita et al. 2000).

Plasma binding

Binding of AM-1241 to plasma proteins was quantified by incubating various concentrations of AM-1241*HCl (0.2 to 50 μ M) in undiluted mouse plasma, aqueous human serum albumin (0.4 or 1.0%, w/v) or water at 37°C. Blank samples of each protein-containing matrix were also incubated and studied to ensure integrity of the analysis. After 30 minutes, samples were introduced into Amicon Centrifree filter assemblies (30,000 MW cutoff; Millipore Corporation, Billerica, MA, USA), which were then centrifuged at 1,500 g, 30 minutes and 4°C. The ultrafiltrate was recovered and diluted with 2 volumes of methanol prior to LC-MS/MS analysis. For this purpose, we used an Agilent 1100 series HPLC (Agilent Technologies, Wilmington, DE, USA) as the front end for a Thermo Finnigan Quantum Ultra triple quadrupole mass spectrometer (Thermo Electron, San Jose, CA, USA). Samples (10 μ L) were injected onto a Gemini C₁₈ column (2 $\times$ 50 mm, 5 μ ; Phenomenex, Torrance, CA, USA). The mobile phase consisted of 0.1% formic acid (v/v) in water (solvent A) and methanol (solvent B) with a flow rate of 0.3 mL/min. AM-1241 eluted at 6.27 minutes and was ionized in positive atmospheric pressure chemical ionization (APCI+) with detection in single reaction monitoring (SRM) mode for the transition mass-to-charge ratio (m/z) 504 to 98.

Metabolism of AM-1241 and LC-MS/MS metabolite analysis

For in vitro hepatic metabolism studies, AM-1241*HCl (100 μ M, final concentration) was incubated at 37°C for 30 minutes with rat or human liver microsomes (1 mg/mL protein; Celsis In Vitro Technologies, Chicago, IL, USA) in 50 mM potassium phosphate buffer with or without (control) 1 mM NADPH. Analytes were extracted from the reaction mixtures with chloroform:acetone (1:1, v/v) and centrifugation at 15,000g for 10 minutes at 4°C. The organic layer was evaporated to dryness under nitrogen, reconstituted in methanol and analyzed with the LC-MS/MS system described above outfitted with a Zorbax Stablebond C-8 column (2.1 $\times$ 50 mm; Agilent Technologies, Santa Clara, CA, USA). Data dependent scans allowed detection of AM-1241 and putative metabolites, the later represented by NADPH-dependent peaks.

Intrinsic hepatic clearance (Cl_H)

The in vitro metabolic-stability approach of Obach and Mohutsky was used with modification for predicting intrinsic hepatic clearance (Cl_H) (Mohutsky et al. 2006; Obach 1999). Microsomal half-life was determined in triplicate incubations containing 1 μ M AM-1241*HCl and 0.5 mg/mL mouse, rat or human microsomal protein in 50 mM potassium phosphate, pH 7.4. After a 3 minute pre-incubation at 37 °C, either buffer (control) or NADPH (1 mM final concentration) was added to initiate the reaction. At various time points, aliquots were diluted with equal volumes of methanol containing internal standard (chemically similar CB2 agonist), centrifuged at 13,000 g for 5 min at 4 °C and analyzed by LC-MS/MS (see above). Intrinsic clearance (Cl_{int}), in vitro T_{1/2} and predicted hepatic clearance (Cl_H) using a “well-stirred” venous-equilibrium model of hepatic elimination (Mohutsky et al. 2006; Obach 1999) were calculated using the following values for gLiver/kg: 25.7 (human), 40 (rat), 87.5 (mouse) (Davies and Morris 1993).

Results

Validation of AM-1241 HPLC quantification in biological samples

We first established conditions that allowed reliable AM-1241 recovery and quantification from plasma and various biological tissues that exhibit significant, therapeutically-relevant CB2 expression (Beltramo 2009; Cheng and Hitchcock 2007; Mackie 2008; Pertwee 2009; Rahn and Hohmann 2009; Whiteside et al. 2007). As determined from appropriately spiked

samples in concentrations ranging from 5–100 μM , we achieved excellent AM-1241 recovery from all biological matrices examined: plasma, brain and spleen >90%, and from kidney, liver and intestine >70%. Representative chromatograms of AM-1241 alone, a blank mouse plasma extract, and a mouse plasma extract from an animal administered AM-1241 (Fig. 2), illustrate that the HPLC conditions we developed resolved the parent compound from all other ultraviolet-absorbing plasma constituents without interfering peaks. Comparable HPLC resolution of AM-1241 was achieved from the constituents of each tissue studied (data not shown).

AM-1241 solution and plasma stability

AM-1241 was stable in acetonitrile for at least 24 hours and in mouse plasma for at least 6 hours, as demonstrated by the recovery of >95% AM-1241 after incubation at 37°C. Aqueous AM-1241 samples at concentrations of 0.003, 0.03, 0.3, and 1 mg/mL were stable at room temperature and 4°C for up to 14 days.

Estimation of in vivo AM-1241 absorption

Caco-2 cell permeability studies gave an average P_{app} value of $(6.2 \pm 0.01) \times 10^{-6} \text{ cm/s}$ (mean $\pm$ standard deviation) from two individual determinations, each performed in quadruplicate.

AM-1241 plasma-protein binding

Plasma proteins bound AM-1241 extensively. After a 30 minute incubation of AM-1241 (up to 50 μM final concentration) in mouse plasma at 37°C, virtually all the compound (99% in triplicate determinations) was bound to plasma protein. When AM-1241 (50 μM final concentration) was incubated in 0.4% or 1.0% (w/v) aqueous human serum albumin, the most prevalent protein in plasma, 56% and 85% of the compound bound to albumin, respectively, making albumin a prime candidate for much of the AM-1241 protein binding in whole plasma.

Responses of mice to AM-1241 administration

Behaviorally, mice remained alert and apparently unaffected following p.o. administration of AM-1241*HCl, except for a transient gaping response in two of the twenty-nine animals observed. Following i.v. administration of 25 mg/kg AM-1241*HCl, mice were initially somewhat hypoactive, but behaved normally by 90 minutes post-dose. Mice receiving vehicle alone showed neither behavioral response. No gross organ or tissue abnormalities were evident from end-point necropsy after any AM-1241 treatment (data not shown).

AM-1241 biodistribution and clearance profiles

As calculated directly from the plasma area-under-the-curve (AUC) ratio between p.o. and i.v. administration, the AM-1241 oral bioavailability was ~21% (Table 1). The half-life of AM-1241 after p.o. administration was over 2-fold longer than after i.v. administration, and both the volume of distribution and rate of clearance were markedly greater after p.o. than after i.v. dosing. AM-1241 was present in plasma, brain, spleen, kidney, liver and intestine following i.v. administration; maximal concentrations within these compartments were observed within 5–15 minutes (Fig. 3). Thereafter, AM-1241 tissue concentrations decreased to become undetectable by 240 minutes in plasma, intestine, spleen and kidney; by 120 minutes in brain; and by 90 minutes in liver. Following p.o. administration, AM-1241 was detected at lower concentrations relative to plasma in spleen, kidney and intestine, with no obvious kinetic pattern, but not in brain or liver (Fig. 3). The fraction of the total AM-1241 dose in each analyzed tissue at the time of maximum observed concentration is given in Table 2. Among the organs examined, brain and kidney evidenced

the greatest acute exposure to AM-1241 after i.v. administration, whereas kidney contained the greatest fraction of the total AM-1241 p.o. dose.

An in vitro metabolic-stability approach with mouse, rat and human liver microsomes was utilized to estimate the in vitro Cl_{int} of AM-1241 (Mohutsky et al. 2006; Obach 1999). Table 3 summarizes the rate constant of substrate loss (k_{loss}), the in vitro $T_{1/2}$, the scaled Cl_{int} and the resulting predicted total hepatic clearance (Cl_H) from this analysis.

AM-1241 biotransformation

As quantified by analytical HPLC, the acute decrease in the liver and kidney contents of AM-1241 after i.v. administration (Fig. 3) was accompanied, within 15 minutes post-dosing, by the appearance of one (in kidney) or more (in liver) discrete, well-defined chromatographic peak(s) (Fig. 4, a–d). For kidney, a prominent new peak was observed at the retention time of ~13.0 minutes, whereas liver evidenced multiple new peaks in the 8–13 minute region, among which the peak at 12.4 minutes dominated. Since these peaks were not endogenous to either tissue itself, they were considered indicative of in vivo AM-1241 metabolites. The predominant AM-1241 metabolite in kidney reached highest levels at 15 and 45 minutes post-dose and was not detectable at 120 minutes, whereas the predominant liver metabolite reached a maximum at 10 minutes post-dose and was not detectable at 90 minutes post-dose (Fig. 4e).

In order to gain some insight into the origin and identities of the putative in vivo AM-1241 metabolites, AM-1241 was reacted with rat and human liver microsomes, and the reaction mixtures were analyzed by LC-MS/MS. New molecular species appeared in the LC-MS/MS total-ion chromatogram that originated from the NADPH-dependent transformation of AM-1241. The product spectrum from the rat microsomal reaction mixtures evidenced a species with the m/z value indicative of the AM-1241 substrate itself (Fig. 5a) and two other species consistent with the formation of specific AM-1241-derived demethylation (Fig. 5b) and hydroxylation (Fig. 5c) products (Table 4). Thus, AM-1241 phase I microsomal metabolism occurred primarily on the piperidine moiety. Incubation of AM-1241 with human liver microsomes resulted in the appearance of the same metabolites, but the demethylated metabolite was even more prevalent than it was from the reaction of AM-1241 with rat liver microsomes (data not shown). Formation of AM-1241-derived hydroxylation and demethylation metabolites was independent of the sex of the rats from which microsomes were obtained (data not shown).

Discussion

Thorough preclinical characterization of GPCR orthosteric ligands displaying salutary pharmacological actions in vivo has helped establish GPCRs as the single most successful class of drug targets and GPCR ligands as important medicines (Congreve and Marshall 2010). A paramount example of a GPCR ligand with well-established preclinical therapeutic efficacy is the CB2 agonist AM-1241, which continues to be the most widely-used research tool for studying CB2 (patho)physiology, particularly the role of specific endocannabinoid-system receptors in pain transmission, antinociception and substance abuse/drug addiction (Adamczyk et al. 2012; Anand et al. 2009; Beltramo 2009; Gamaledin et al. 2012a; Gamaledin et al. 2012b; Gutierrez et al. 2007; Rahn et al. 2010; Rahn et al. 2008; Whiteside et al. 2007). Optimal use of AM-1241 as a pharmacological agent and template for CB2-agonist drug design necessitates appreciation of its in vivo distribution and metabolism. These properties of AM-1241 are of particular interest in light of the predominantly peripheral disposition of CB2 in vivo, and the contributions of both central and peripheral mechanisms to CB2-mediated pain relief (Cheng and Hitchcock 2007; De

Petrocellis and Di Marzo 2009; Mackie 2008; Pertwee 2009; Rahn et al. 2010; Vemuri et al. 2008).

These considerations prompted us to conduct this initial characterization of AM-1241 biodistribution and biotransformation. Because rodents are routinely utilized in pre-clinical compound profiling, including study of AM-1241 pharmacological activity (Beltramo 2009; Gutierrez et al. 2007; Lozano-Ondoua et al. 2010; Rahn and Hohmann 2009; Rahn et al. 2007; Rahn et al. 2010; Rahn et al. 2008; Shoemaker et al. 2007; Storr et al. 2009), we selected the mouse as our *in vivo* system. We also employed a single-dose *in vivo* protocol, since the vast majority of AM-1241 studies in disease models have used an acute, single-dose paradigm (Beltramo 2009; Gutierrez et al. 2007; Ibrahim et al. 2003; Kim et al. 2006; Lozano-Ondoua et al. 2010; Nackley et al. 2004; Rahn et al. 2010; Shoemaker et al. 2007). Furthermore, a single-dose experimental paradigm constitutes a solid basis for designing future studies involving variations in administration protocol. We use a standardized dose of 25 mg/kg for both *i.v.* and *p.o.* AM-1241 administration. As summarized (Beltramo 2009), most studies on AM-1241 (and other CB2 agonists) in rodent pain models have administered AM-1241 *i.p.* as a single *i.p.* dose up to 50 mg/kg. Maximal AM-1241 analgesic efficacy in a standard hind paw model of thermal sensitivity was reached after local (intraplantar) administration of 3.3 mg/kg (Malan et al. 2001), although lower intraplantar and *i.p.* AM-1241 doses can elicit analgesia in models of carageenan- and capsaicin-induced pain (Beltramo 2009; Gutierrez et al. 2007; Hohmann et al. 2004; Nackley et al. 2003). Nonetheless, *i.p.* or *s.c.* analgesic doses of AM-1241 in the 10–30 mg/kg are commonly utilized in such models and in an incision model of post-operative pain (Bingham et al. 2007; LaBuda et al. 2005; Yao et al. 2008) and AM-1241 treatment at a daily dose of 20–40 mg/kg, *i.p.* was required to reduce experimental colitis in a murine model (Storr et al. 2009). Doses of AM-1241 up to 6 mg/kg *i.v.* and of other CB2 agonists up to 30 mg/kg *p.o.* have been reported efficacious in various preclinical pain models (Beltramo 2009; Beltramo et al. 2006; Ohta et al. 2008). These data support the conclusion that the 25 mg/kg AM-1241 dose we used is a pharmacological one. Indeed, the tissue distribution of GW405833, a CB2-selective aminoalkylindole agonist that exhibits maximal effective antihyperalgesic and analgesic activities at 1–3 mg/kg, *i.p.* (Beltramo 2009), has been characterized at 100 mg/kg, *i.p.* (Valenzano et al. 2005).

Whether administered orally or systemically (*i.v.*) at 25 mg/kg, AM-1241 was not toxic, for post-experiment necropsies were unremarkable. Following AM-1241 *i.v.*, but not *p.o.*, administration, transient hypomotility was noted. Although hypomotility is a behavioral response associated with cannabinoid agonists, their motor effects in rodents are variable and may exhibit a triphasic, dose-dependent pattern: lower doses induce transient hypoactivity similar to what we observed, whereas higher doses stimulate movement until the hallmark signs of muscular rigidity and fixed posture of catalepsy emerge (McLaughlin et al. 2005; Valenzano et al. 2005). Cannabinoid agonist-induced motor effects have been mainly attributed to central CB1 stimulation (McLaughlin et al. 2005; Monory et al. 2007). An acute “spillover” effect of AM-1241 on CB1 in the CNS might thus account for the transient hypomotility we observe after *i.v.* dosing, a conjecture congruent with our detection of AM-1241 in brain after *i.v.* (but not *p.o.*) AM-1241 administration. However, the appreciable (up to 340-fold) selectivity of AM-1241 for mouse CB2 vs. CB1 (Beltramo 2009; Hohmann et al. 2004; Nackley et al. 2003; Shoemaker et al. 2007) and the ability of two non-selective cannabinoid-receptor agonists, the aminoalkylindoles AM-2233 and WIN 55,212-2, to induce profound catalepsy at only 1.0 mg/kg *i.v.* (Dhawan et al. 2006; Petit et al. 1999) suggest that factors other than an off-target AM-1241 response are involved. Since CB1 stimulation by the endocannabinoid 2-arachidonoylglycerol (2-AG) is primarily responsible for hypomotility in mice, and 2-AG is a full agonist at both CB1 and CB2 (Long et al. 2009; McLaughlin et al. 2005; Monory et al. 2007; Morgan et al. 2009), AM-1241

associated hypomotility could reflect a shift in the intrinsic poise of 2-AG signaling at CNS synapses containing CB1 and CB2 consequent to AM-1241 CB2 engagement that favors CB1 stimulation by 2-AG. Two out of 29 mice receiving 25 mg/kg AM-1241 p.o. exhibited gaping, a surrogate response for nausea/emesis in rodents incapable of vomiting (Parker and Limebeer 2006). Since rodent gaping is commonly observed after ingestion of an aversive chemical (e.g., lithium) and can be induced by CB1 antagonists/inverse agonists, but not by CB1 neutral antagonists or CB2 agonists (Parker and Limebeer 2006; Salamone et al. 2007), the gaping we observed in only a few mice after AM-1241 administration p.o. likely reflects a negative taste reaction to the AM-1241 dosing solution.

We developed and validated extraction and analytical HPLC methods for accurate, reliable AM-1241 quantification in murine plasma and in tissues where CB2 has been associated with important (patho)physiological roles (Beltramo 2009; Cheng and Hitchcock 2007; Mackie 2008; Pertwee 2009; Rahn and Hohmann 2009; Whiteside et al. 2007). This direct approach is in contrast to reliance upon time-course studies using in vivo pharmacological effects as proxies for tissue distribution/bioavailability (Sink et al. 2009). Quantification of AM-1241 in plasma after p.o. and i.v. administration allowed determination that its oral bioavailability is ~21%. This value is consistent with the AM-1241 P_{app} value of 6.2×10^{-6} cm/s we measured in a Caco-2 cell system, since P_{app} values in the range of $1-10 \times 10^{-6}$ cm/sec are indicative of moderate (i.e., 20–70%) in vivo oral bioavailability (Yee 1997). Among the very few CB2 agonists whose oral bioavailability has been determined experimentally in rodents, values of 0% for GW405833 (Valenzano et al. 2005), and 13–100% for a series of N-alkyldienearylcarboxamides (Ohta et al. 2007) have been reported at doses of 0.1–100 mg/kg.

The rapid AM-1241 hepatic clearance predicted from in vitro metabolic stability data with mouse, rat and human microsomes was substantiated by direct in vivo half-life profiling in the mouse. The half-life of AM-1241 after p.o. administration (82 minutes) is less than the 3–5 hour half-lives reported after p.o. administration of some N-arylamide-oxadiazole CB2 agonists (Cheng et al. 2008) and the CB2 agonist GW842166X (Giblin et al. 2007). AM-1241's moderate oral bioavailability and short in vivo half-life would favor its i.p. or i.v. administration or injection at an injury/inflammation site, as well as its use in acute nociceptive tests, congruent with many published applications (Beltramo 2009; Gutierrez et al. 2007; Ibrahim et al. 2005; Malan et al. 2002; Nackley et al. 2003; Quartilho et al. 2003; Rahn et al. 2010; Whiteside et al. 2007). The half-life of AM-1241 may also provide a rationale for employing multiple i.p. administrations over several days in order to attain therapeutic efficacy against more chronic injury states, such as experimental colitis (Storr et al. 2009), as well as the need for sustained AM-1241 i.p. administration to reduce spontaneous and evoked pain and bone loss or fracture in a murine bone cancer model (Lozano-Ondoua et al. 2010). Given its demonstrated tissue-protective and nociceptive effects (Beltramo 2009; Gutierrez et al. 2007; Lozano-Ondoua et al. 2010; Ohta et al. 2008; Rahn et al. 2008; Shoemaker et al. 2007; Yao et al. 2008), AM-1241 may serve as a valuable template for judicious structural modification aimed at enhancing its oral bioavailability and/or extending its half-life. This proposition is especially attractive in light of the excellent plasma exposure after p.o. and (especially) i.v. AM-1241 administration (as indexed by the AUC), which is comparable to that reported for some lead N-arylamide-oxadiazole and N-alkyldienearylcarboxamide CB2 agonists (Cheng et al. 2008; Ohta et al. 2007).

AM-1241 is susceptible to extensive phase I cytochrome P450 metabolism to generate demethylated and hydroxylated products. Based on the fragmentation patterns we observed by LC-MS/MS when AM-1241 is incubated with rat or human microsomal systems, AM-1241 was predominantly metabolized at its piperidine moiety, an observation useful in assessing potential chemical modifications that might alter AM-1241's metabolism.

Although the P450 family plays a critical role in xenobiotic detoxification in mice, rats, and humans, it remains to be established whether there are significant, species-dependent differences in AM-1241 microsomal metabolism (Martignoni et al. 2006). The CB2 antagonist/inverse agonist AM-630 and the CB1/CB2 agonist WIN-55212-2 are metabolized at their morpholino moiety, which corresponds to AM-1241's piperidine group (Zhang et al. 2002; Zhang et al. 2004), indicating that the P450 mediated biotransformation of all three aminoalkylindoles may be similar. The synthetic cannabinoid JWH-018 is likewise a substrate for phase I, P450 hydroxylation by human microsomes in vitro (Wintermeyer et al. 2010) and in vivo (Sobolevsky et al. 2010), as is the CB2 aminoalkylindole agonist JWH-015 in vitro (Zhang et al. 2006). These aggregate data suggest that P450 mediated hydroxylation may be inter-species metabolic route by which the lipophilicity of ligands that modify cannabinergic lipid signaling is reduced to facilitate compound elimination. The metabolites of AM-1241 may themselves prove to be very important pharmacologically: preliminary experiments indicate that the proposed demethylated metabolite of AM-1241 shares a similar selectivity for CB2 vs. CB1, but is somewhat less potent a CB2 agonist, as compared to the parent compound (data not shown).

Conclusion

The data presented constitute the initial characterization of the biodistribution and metabolism of AM-1241, the prototypic agonist used to gain insight into the (patho)physiological role of CB2-mediated signaling, as obtained from in vitro biochemical and cell-based systems and in vivo in mice. Our data show that AM-1241 has the following characteristics: a) short plasma half-life; b) limited oral bioavailability; c) extensive binding to plasma/albumin; d) subject to hepatic hydroxylation and demethylation; and e) moderate hepatic clearance. These results should aid in the design of detailed studies involving variations in AM-1241 dose and administration protocols, as well as the interpretation of already published efficacy data. The present work should also help inform the synthesis and optimization of future-generation CB2 agonists with therapeutic utility.

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Figure 2. Chromatograms illustrating the HPLC resolution of AM-1241 from endogenous constituents of mouse plasma: (a) AM-1241 in acetonitrile; (b) deproteinized blank mouse plasma; and (c) deproteinized mouse plasma sampled 10 minutes after i.v. administration of 25 mg/kg AM-1241*HCl.

a. Plasma; IV

e. Spleen; IV

i. Liver; IV

b. Plasma; oral

f. Spleen; oral

j. Liver; oral

Not detected

c. Brain; IV

g. Kidney; IV

k. Intestine; IV

d. Brain; oral

Not detected

h. Kidney; oral

l. Intestine; oral

Figure 3.

Distribution of AM-1241 among plasma and designated tissues after either i.v. or p.o. administration of 25 mg/kg AM-1241*HCl to mice. Values are the means $\pm$ standard deviation of three individual mice.

Figure 4.

HPLC chromatograms of tissue extracts suggestive of potential AM-1241 metabolites in kidney and liver 15 minutes after 25 mg/kg AM-1241*HCl i.v. administration to mice. Chromatograms of (a) kidney extract after AM-1241 administration, (b) kidney extract after vehicle administration, (c) liver extract after AM-1241 administration, (d) liver extract after vehicle administration. Note the appearance of discrete new peak(s) in the tissues from AM-1241-treated mice. Panel (e) is a graphical representation of the relative absorbance signal (as peak area) of the major potential AM-1241 metabolite in kidney (at ~13 minutes retention time) and liver (at ~12.4 minutes retention time).

Figure 5.

LC-MS spectra of: (a) AM-1241; (b) demethylated AM-1241 metabolite; (c) predominant hydroxylated AM-1241 metabolite. The spectra were obtained from LC-MS/MS analysis of organic extracts of AM-1241 incubated with rat liver microsomes for 30 minutes at 37°C. Based upon the observed m/z and those of potential AM-1241 metabolites (Table 4), the spectra were assigned to specific AM-1241 demethylated and hydroxylated metabolites.

Table 1
AM-1241 biodistribution parameters in mice

AM-1241*HCl (25 mg/kg) was administered i.v. or p.o. Data from three studies, each including 10 mice, were averaged prior to non-compartmental analysis using WinNonlin software.

PK parameter	25 mg/kg i.v.	25 mg/kg p.o.
C ₀ (mg/L)	6.0	NA
C _{max} (mg/L)	NA	0.80
AUC (min*mg/L)	350	74
Mean Residence Time (observed) (min)	54.7	113
Volume of distribution (observed) (mL)	110	950 (V _z /F)
Clearance (observed) (mL/min)	2.0	8.1 (Cl/F)

Table 2
Tissue distribution of AM-1241 in mice

Relative acute tissue exposure to AM-1241, indexed as percentage of total AM-1241 dosed (25 mg/kg) after administration by the route specified at the indicated time of maximal concentration.

Tissue (<i>route of administration</i>)	% Total dose/gram tissue at maximum concentration	Time of maximal concentration, post-dose (minutes)
Brain (i.v.)	1.27	15
Spleen (i.v.)	1.98	15
Spleen (p.o.)	0.12	60
Kidney (i.v.)	1.8	5
Kidney (p.o.)	0.18	15
Liver (i.v.)	0.05	10
Intestine (i.v.)	0.53	10
Intestine (p.o.)	0.44	15

Table 3**Prediction of AM-1241 clearance**

Data were obtained using the in vitro half-life approach from reactions of AM-1241 with human, rat or mouse liver microsomes.

	Human	Rat	Mouse
k_{loss} (per min)	0.118	0.051	0.138
$T_{1/2}$ (min)	5.9	13.7	10.6
Cl_{int} (ml/min/kg)	272	182	514
Cl_{H} (ml/min/kg) (Well Stirred)	2.39	1.77	9.66

Table 4
Rationalized fragments of AM-1241 used for metabolite identification

Based on these fragment assignments and their specified m/z 's, AM-1241 metabolism by hepatic microsomes occurs primarily on the piperidine ring.

Fragment
AM-1241 $R_1 = \text{CH}_3$ $R_2 = \text{H}$.	98	112	276		407
Demethylated AM-1241 $R_1 = \text{H}$ $R_2 = \text{H}$.	84	98	276	393	407
Hydroxylated AM-1241 $R_1 = \text{CH}_3$ $R_2 = \text{OH}$.	114	128	276	393	407