Tissue Distribution and Characterization of Drug-Related Material in Rats and Dogs after Repeated Oral Administration of Casopitant

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ABSTRACT:

Casopitant [1-piperidinecarboxamide,4-(4-acetyl-1-piperazinyl)-N-((1R)-1-(3,5-bis(trifluoromethyl)phenyl)-ethy])-2-(4-fluoro-2-methylphenyl)-N-methyl-[2(R,4S)] has been shown to be a potent and selective antagonist of the human neurokinin 1 receptor, the primary receptor for substance P. During long-term toxicity studies conducted in rat and dog, evidence of cardiomyopathy and increased cardiac weight were observed. The distribution and metabolism of casopitant were studied in both species evaluating the accumulation of drug-related material (DRM) after repeat dosing and its potential relationship with pathological findings observed in myocardium. After repeat oral administration of [14C]casopitant to rats (20 days) and dogs (14 days), DRM was quantifiable in all of the tissues examined with lung and liver containing the highest level of radioactivity. The concentration of radioactivity was significantly higher in tissues than in plasma, declining slowly and still quantifiable after a recovery period of 20 days. The principal circulating components identified in both species were casopitant, M12 (oxidized deacetylated), M13 (hydroxylated piperazine), and M31 and M134 (two N-dealkylated piperezines). In tissues, a similar metabolic pattern was observed, in which casopitant, M31, M134, M76 (N-deacetylated), and M200 (N-deacetylated N,N-deethylated) were the major components quantified. After a 26-week repeat dose study in dog, casopitant and M13 were the major circulating components, whereas in myocardium, M200 and M134 were the major ones and their levels increased over time, reaching considerable concentrations (millimolar magnitude). After a washout period, all circulating derivatives decreased to undetectable levels, whereas M200 was still the major component in myocardium. Overall DRM in plasma did not correlate with the respective concentrations in tissues.

Introduction

Neurokinin subtype-1 (NK-1) receptors are widely distributed in the peripheral and central nervous systems, including areas thought to be involved in the regulation of affective behavior and neurochemical response to stress (Leslie, 1985; Kramer et al., 1998; Holmes et al., 2003). NK-1 receptors are also found in non-neural tissues such as endothelial and inflammatory cells as well as gastrointestinal, respiratory, and genitourinary tissues. Blocking of NK-1 neurotransmitter receptors has been demonstrated to be effective for the treatment of major depressive disorder and one or more anxiety disorders (Kramer et al., 2004; Furmark et al., 2005) and to prevent chemotherapy-induced and postoperative nausea and vomiting (Warr et al., 2005; Quartara et al., 2009).

Casopitant [1-piperidinecarboxamide,4-(4-acetyl-1-piperazinyl)-N-((1R)-1-(3,5-bis(trifluoromethyl)phenyl)-ethy])-2-(4-fluoro-2-methylphenyl)-N-methyl-[2(R,4S); GW679769] is a piperidine derivative that has been shown to be a potent and selective antagonist of the human NK-1 receptor, the primary receptor of substance P, both in vitro and in vivo with good brain penetration properties (Minthorn et al., 2008). On the basis of this mode of action, it has been evaluated for the prevention of chemotherapy-induced and postoperative nausea and vomiting (Herrstedt et al., 2009; Khojasteh et al., 2009). In addition, casopitant has been investigated in a number of chronic dosing indications in which the NK-1 receptor is believed to play a role, such as anxiety, depression, insomnia, and overactive bladder.

The nonclinical safety package of casopitant included appropriate investigations for both acute and chronic indications. During long-term repeat dose toxicity studies in rats (6 months) and dogs (9 months), evidence of cardiomyopathy (namely myocardial necrosis, degeneration, and inflammation) and increased heart weight, with no significant sex differences, were recorded; these had never been detected in the previous shorter toxicity studies. These changes were accompanied by increases in plasma levels of cardiac troponin I and creatine kinase MB-mass isoenzyme concentrations, biomarkers for cardiac damage. In addition, transmission electron microscopy showed ultrastructural changes in the hearts of both preclinical species, considered suggestive of phospholipid accumulation. Based on the authors’ experience of NK-1 receptor antagonists (unpublished data) and on published preclinical data of this class of drugs (http://www.accessdata.fda.gov/drugsatfda_docs/NDA/2008/022023s000TOC.cfm), it is necessary to conduct investigations for the characterization of drug-related material (DRM) in both species.

The nonclinical safety package of casopitant included appropriate investigations for both acute and chronic indications. During long-term repeat dose toxicity studies in rats (6 months) and dogs (9 months), evidence of cardiomyopathy (namely myocardial necrosis, degeneration, and inflammation) and increased heart weight, with no significant sex differences, were recorded; these had never been detected in the previous shorter toxicity studies. These changes were accompanied by increases in plasma levels of cardiac troponin I and creatine kinase MB-mass isoenzyme concentrations, biomarkers for cardiac damage. In addition, transmission electron microscopy showed ultrastructural changes in the hearts of both preclinical species, considered suggestive of phospholipid accumulation. Based on the authors’ experience of NK-1 receptor antagonists (unpublished data) and on published preclinical data of this class of drugs (http://www.accessdata.fda.gov/drugsatfda_docs/NDA/2008/022023s000TOC.cfm), it is necessary to conduct investigations for the characterization of drug-related material (DRM) in both species.

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ABBREVIATIONS: NK-1, neurokinin-1; GW679769, 1-piperidinecarboxamide,4-(4-acetyl-1-piperazinyl)-N-((1R)-1-(3,5-bis(trifluoromethyl)phenyl)-ethy])-2-(4-fluoro-2-methylphenyl)-N-methyl-[2(R,4S)]; DRM, drug-related material; HPLC, high-performance liquid chromatography; MS/MS, tandem mass spectroscopy; QC, quality control; AUC, area under the plasma concentration-time curve; PL, phospholipidosis; CAD, cationic amphiphilic drug.

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unlikely that all of the above findings could be explained by NK-1 receptor antagonist mechanism of action.

As a consequence of this cardiac finding, a battery of supplementary preclinical studies were conducted to determine the time to onset of the cardiovascular effect, to assess the progression of lesions, to investigate the reversibility of these effects, and to understand whether accumulation of or a high concentration of DRM in myocardium could be correlated with the observed toxicological findings. After a single oral administration in rats and dogs, casopitant has been shown to be extensively metabolized, widely distributed with quite a long retention time of DRM in tissues and slow rate of elimination mainly in dog (Miraglia et al., 2010).

This article provides an overview of a series of disposition experimental studies conducted to investigate 1) the distribution and accumulation of DRM in tissues and plasma after repeat oral administration of casopitant in rats and dogs, 2) the residual concentration of DRM in tissues after different washing periods, and 3) the nature of metabolites detected and the quantification of the most relevant ones in plasma and selected tissues, myocardium in particular. Casopitant was administered as its mesylate salt (using either unlabeled or \(^{14}\text{C}\)-labeled salt), and the doses selected were within the range of cardiotoxic doses.

Materials and Methods

Chemicals. Casopitant mesylate (coded as GW679769B), standards of metabolites M12 (coded as GSK631832), M13 (coded as GSK525060), M31 (coded as GSK517142), M15 (coded as GSK1497960), M76 (coded as GW679979), M134 (coded as GSK2110277), and M200 (coded as GSK2174599) were all supplied by Chemical Development, GlaxoSmithKline. Metabolite structures are reported in Table 4.

Radiolabeled \(^{14}\text{C}\)casopitant mesylate (Fig. 1), \(^{2}\text{H}_3\text{H}_3\text{C}\)casopitant, \(^{13}\text{C}\)M13, \(^{1}\text{H}_3\text{C}\)M12, and \(^{2}\text{H}_3\text{C}\)M31 were synthesized by GlaxoSmithKline Isotope Chemistry (Stevenage, UK). The specific activities of \(^{14}\text{C}\)casopitant mesylate were 111 kBq/mg (radiochemical purity of 99%) for the rat study and 11.1 kBq/mg (radiochemical purity of 99.7%) for the dog study.

Liquid scintillation cocktails were obtained from PerkinElmer Life and Analytical Sciences (Waltham, MA). All other chemicals used were reagent grade or higher and were obtained from approved commercial suppliers.

Animals. Female Sprague-Dawley rats (189–231 g) were obtained from Charles River Ltd. (Margate, Kent, UK). Male beagle dogs (10–16 kg) were obtained from Harlan Ltd. (Blackthorn, UK).

The animals were kept under standard environmental conditions using routine methods of animal husbandry. Water from the domestic supply and a standard diet were provided to animals. Water was available ad libitum throughout the study period.

Rats were not fasted before drug administration, whereas dogs were. For dogs, food was withheld overnight during collection of samples for clinical pathology and before scheduled necropsy.

Rat and dog studies with \(^{14}\text{C}\)casopitant were conducted at Huntingdon Life Sciences Ltd. (Huntingdon, Cambridgeshire, UK), whereas the dog study with the unlabeled version was conducted at GlaxoSmithKline (Verona, Italy). All in-life experiments described in this article complied with national legislation and with the company policy on the care and use of laboratory animals and with related codes of practice.

Dose Preparation. For distribution and metabolism studies, oral doses were prepared daily, shortly before administration, dissolving an appropriate amount of \(^{14}\text{C}\)casopitant mesylate in the appropriate volume of water for injections (with sonication as necessary). The formulation obtained was continually mixed using a magnetic stirrer until completion of dosing. Aliquots of the formulation were analyzed by radio-HPLC to determine the radiochemical purity and confirm the stability of \(^{14}\text{C}\)casopitant in the dose formulation during both preparation and over the dosing period. The mean radiochemical purity of \(^{14}\text{C}\)casopitant was always >99%.

For the 26-week oral investigational cardiotoxicity study in dog, an aliquot of unlabeled casopitant mesylate was dissolved in sterile water for injections and stored at room temperature protected from light. The solution obtained was stable for 7 days.

Repeat Dose Studies with \(^{14}\text{C}\)Casopitant in Rats and Dogs. Rat. Twelve female rats received a single oral administration of \(^{14}\text{C}\)casopitant at a target dose level of 60 mg of free base/kg. Blood samples were collected from each rat at 2 and 24 h (terminal sample) postdose. Another 24 female rats received the same single daily dose for 20 consecutive days. Twelve of the rats were sacrificed at 24 h after administration on day 20, after collection from each rat of blood samples at 2 h (nonterminal sample obtained via a tail vein) and 24 h (terminal sample obtained via cardiac puncture under isoflurane anesthesia) postdose. Six rats were sacrificed after a recovery period of 7 days following cessation of dosing, and the remaining six rats were sacrificed after a recovery period of 20 days. On both occasions, a single terminal blood sample was obtained from each rat.

All blood samples were collected into K\(_2\)EDTA tubes as anticoagulant. After removal of aliquots for radioanalysis, the remaining blood samples were centrifuged at 2000g for 10 min at 4°C to separate plasma samples. The myocardium, liver, skeletal muscle (quadriiceps), lungs, and kidneys were removed from each of the rats after terminal sampling at 24 h postdose, washed in ice-cold saline, blotted dry, weighed, and frozen.

Dog. Four male dogs received repeat oral administrations of \(^{14}\text{C}\)casopitant as single daily doses on 14 consecutive days at a target dose level of 40 mg of free base/kg/day. Both dogs were sacrificed after collection of blood samples at 2 and 24 h after the final occasion of dosing (day 14), and the other two were sacrificed after collection of blood samples after a recovery period of 20 days following cessation of dosing.

Plasma samples were prepared as described above, and selected tissues (myocardium, liver, skeletal muscle, single lung, and kidney) were removed from the carcasses. All samples were treated as described previously.

Repeat Dose Study with Unlabeled Casopitant in Dog. In a 26-week investigative cardiotoxicity study with interim kills, male dogs were administered casopitant once daily at the dose of 40 mg of free base/kg/day. Designated dogs received a daily oral gavage dose of casopitant (three dogs for each occasions) or vehicle (two dogs for each occasions) for 6, 13, 20, or 26 weeks. In addition, an equal number of animals were similarly treated for 13 weeks followed by a 22-week recovery period and then were sacrificed. Plasma samples obtained from blood collected into K\(_2\)EDTA tubes at nominal times of predose, 0.5, 1, 2, 4, 6, 8, 10, and 24 h after dosing on the day before each necropsy phase (weeks 6, 13, 20, and 26) were stored at approximately –20°C until analyzed. In addition, samples of 3 to 5 g (each) of myocardium from all animals were collected during each occasion of necropsy, weighed, briefly washed in ice-cold saline, and frozen on solid carbon dioxide.

Assay of Total Radioactivity. Aliquots of plasma samples were mixed with Ultima Gold scintillation cocktail. Tissues samples were weighed, an appropriate amount of water was added, and the total weight was recorded before homogenization on ice. Aliquots of each homogenate and blood samples were
Metabolite Profiling in Plasma and Tissue Homogenate by Radio-HPLC-MS/MS. Samples were analyzed by using a Packard TriCarb 2100 TR liquid scintillation counter (PerkinElmer Life and Analytical Sciences) with automatic quench correction by an external standard method (Botta et al., 1985).

HPLC method 1 (used for analysis of rat and dog plasma and tissue homogenate samples). The chromatographic instrument consisted of an Agilent 1100 binary pump, autosampler, and column oven (45°C) (Agilent Technologies) with a Synergi Hydro RP column (250 × 4.6 mm, 4 μm; Phenomenex, Torrance, CA). The mobile phase consisted of 5 mM ammonium formate aqueous (pH 5 adjusted with formic acid) (solvent A) and 50 mM ammonium formate aqueous (pH 5) in acetonitrile (10:90 buffer-acetonitrile) (solvent B) at a flow rate of 1 ml/min. A gradient was used, starting at 37% B with a linear change to 57% B over 60 min, followed by further two linear increases to 80% B at 62 min and to 100% B at 80 min. These conditions were maintained for a further 5 min.

HPLC method 2 (used for estimation of relative abundance of metabolites M31, M134, and M169 in dog plasma and tissues homogenate samples). The chromatographic instrument used consisted of an Agilent 1100 binary pump, autosampler, and column oven (35°C) (Agilent Technologies) with a Synergi Polar RP column (250 × 4.6 mm, 4 μm; Phenomenex). The mobile phase consisted of 2.5 mM ammonium acetate aqueous (native pH) (solvent A) and 2.5 mM ammonium acetate aqueous (native pH) in acetonitrile (5:95 water-acetonitrile) (solvent B) at a flow rate of 1 ml/min. A gradient was used, starting at 10% B with a linear change to 80% B over 82 min, followed by a linear increase to 95% at 82.1 min, with these conditions being maintained for a further 5 min.

HPLC method 3 (used for estimation of relative abundance of metabolites M12, M31, M69, M76, M134, M169, and casopitant in rat plasma and tissue homogenate samples). The chromatographic instrument used consisted of an Agilent 1100 binary pump, autosampler, and column oven (45°C) (Agilent Technologies) with a Gemini C18 RP column (150 × 4.6 mm, 3 μm). The mobile phase consisted of 10 mM ammonium carbonate aqueous (pH 10) (solvent A) and 100 mM ammonium formate aqueous (pH 10) in acetonitrile-methanol (10:63:27 buffer-acetonitrile-methanol) (solvent B) at a flow rate of 1 ml/min. A gradient was used, starting at 10% B with a linear change to 80% B over 55 min, followed by a further linear increase to 95% at 82.1 min, with these conditions being maintained for a further 5 min.

For all methods, HPLC column recoveries were determined on selected samples by collecting the total HPLC column eluate for the appropriate run and assaying the radioactivity to assess recovery of injected radioactivity. Recoveries (≥85%) of radioactivity were obtained from the HPLC eluate collected.

### TABLE 1
Concentration of radioactivity in female rat plasma and tissues after single (day 1) and repeat (day 20) oral administration of [14C]casopitant and at 7 (day 27) and 20 (day 40) days after completion of repeat dosing at 60 mg/kg/day

<table>
<thead>
<tr>
<th>Sample</th>
<th>Time Point</th>
<th>Day 1 (n = 12)</th>
<th>Day 20 (n = 12)</th>
<th>Day 27 (Recovery Phase) (n = 6)</th>
<th>Day 40 (Recovery Phase) (n = 6)</th>
<th>µg Eq casopitant free base/g</th>
</tr>
</thead>
<tbody>
<tr>
<td>Plasma</td>
<td>2</td>
<td>6.06 ± 1.26</td>
<td>18.5 ± 2.6</td>
<td>1.92 ± 0.42</td>
<td>0.246 ± 0.018</td>
<td></td>
</tr>
<tr>
<td>Blood</td>
<td>24</td>
<td>5.60 ± 0.97</td>
<td>14.5 ± 4.7</td>
<td>7.67 ± 1.78</td>
<td>3.63 ± 0.14</td>
<td></td>
</tr>
<tr>
<td>Myocardium</td>
<td>24</td>
<td>34.6 ± 6.5</td>
<td>79.6 ± 16.2</td>
<td>32.9 ± 6.3</td>
<td>26.7 ± 6.0</td>
<td></td>
</tr>
<tr>
<td>Lungs</td>
<td>24</td>
<td>135 ± 55</td>
<td>405 ± 109</td>
<td>136 ± 6.0</td>
<td>96.8 ± 14.2</td>
<td></td>
</tr>
<tr>
<td>Kidneys</td>
<td>24</td>
<td>59.1 ± 6.8</td>
<td>256 ± 91.6</td>
<td>64.6 ± 15.7</td>
<td>39.2 ± 8.4</td>
<td></td>
</tr>
<tr>
<td>Liver</td>
<td>24</td>
<td>121 ± 14.5</td>
<td>545 ± 92.8</td>
<td>240 ± 52.2</td>
<td>90.4 ± 31.7</td>
<td></td>
</tr>
<tr>
<td>Skeletal muscle</td>
<td>24</td>
<td>18.3 ± 3.1</td>
<td>28.1 ± 5.4</td>
<td>10.7 ± 1.5</td>
<td>7.74 ± 0.76</td>
<td></td>
</tr>
</tbody>
</table>

### TABLE 2
Concentration of radioactivity in male dog plasma and tissues after single (day 1) and repeat (day 14) oral administration of [14C]casopitant and at 20 (day 34) days after completion of repeat dosing at 40 mg/kg/day

<table>
<thead>
<tr>
<th>Sample</th>
<th>Time Point</th>
<th>Day 1</th>
<th>Day 14</th>
<th>Day 34 (Recovery Phase)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Plasma</td>
<td>2</td>
<td>20.8</td>
<td>69.5 ± 4.1</td>
<td></td>
</tr>
<tr>
<td>Blood</td>
<td>24</td>
<td>9.68</td>
<td>49.2 ± 5.0</td>
<td>11.6</td>
</tr>
<tr>
<td>Myocardium</td>
<td>24</td>
<td>11.3</td>
<td>47.4 ± 2.5</td>
<td>9.95</td>
</tr>
<tr>
<td>Lungs</td>
<td>24</td>
<td>9.40</td>
<td>35.3 ± 3.4</td>
<td>96.2</td>
</tr>
<tr>
<td>Kidneys</td>
<td>24</td>
<td>N.D.</td>
<td>243</td>
<td>345</td>
</tr>
<tr>
<td>Liver</td>
<td>24</td>
<td>N.D.</td>
<td>905</td>
<td>102</td>
</tr>
<tr>
<td>Skeletal muscle</td>
<td>24</td>
<td>N.D.</td>
<td>1050</td>
<td>612</td>
</tr>
</tbody>
</table>

N.D., not determined. **n = 4.**
Radio-HPLC data were captured off-line (Bruin et al., 2006): chromatographic fractions (150 μl each) were collected using a fraction collector model FC LH200 (Intek Services, Surrey, UK) onto 96-deep well LumaPlates (microwell plates) containing yttrium silicate solid scintillant (PerkinElmer Life and Analytical Sciences). Radioactivity was determined by scintillation counting (TopCount NXT counter; PerkinElmer Life and Analytical Sciences).

**Structural Identification of Metabolites.** Structural characterization was performed on selected samples by radio-HPLC-mass spectrometry using hybrid a quadrupole/time-of-flight Q-TOF Ultima (Waters MS Technologies, Manchester, UK) tandem mass spectrometer (Morris et al., 1996). Electrospray ionization, in positive and/or negative mode, was used. The HPLC flow was split (1:5) between the mass spectrometer and a fraction collector (model FC 204; Gilson Inc., Middleton, WI), which was used for off-line radiodetection.

Metabolites were identified on the basis of charged molecular ions, mass accuracy, and their collision-induced dissociation fragmentation (Oliveira and Watson, 2000). Authentic standards, when available, were used to compare chromatographic retention times and fragmentation patterns. For some metabolites definitive structures had been confirmed by 1H NMR (Plumb et al., 1999) in clinical study previously (Pellegatti et al., 2009). Fully characterized metabolites were designated by the letter M followed by a number; when a synthetic standard was available a GSK code number was assigned.

**Casopitant, M12, M13, M31, M76, M134, and M200 Quantification.**

Quantification of casopitant and selected metabolites in dog plasma and myocardium homogenate was performed by using two analytical methods (A and B) based on protein precipitation with acetonitrile, followed by HPLC-MS/MS analysis. The HPLC system used consisted of an Agilent 1100 binary pump (Agilent Technologies, Waldbronn, Germany) and an HTS PAL (CTC Analytics AG, Zwingen, Switzerland) autosampler.

HPLC-MS/MS validated method A (used for analysis of casopitant, M12, M13, and M31 in dog plasma samples). Casopitant, M12, M13, and M31 were extracted from 50 μl of dog plasma by protein precipitation using acetonitrile (150 μl) containing [1H,13C]casopitant, [1H,13C]M12, [1H,13C]M13, and [1H,13C]M31 as internal standards, at a concentration of 200 ng/ml each. After vortex mixing, 100 μl of water was added, and the deproteinized samples were centrifuged for 10 min at approximately 3000 g. The supernatant was separated using a Hypersil Gold column (50 x 3.0 mm, 5.0 μm) and eluted at a flow rate of 0.7 ml/min. The isotropic mobile phase consisted of 35% 5 mM ammonium acetate (native pH) and 65% acetonitrile. The column eluate was introduced into the TurboIonSpray source of an API-4000 triple quadrupole mass spectrometer (Applied Biosystems, Streetsville, ON, Canada) operated in positive ion mode. The temperature of the source heater was maintained at 600°C with a curtain gas setting of 25 and collision gas setting of 5. Casopitant, M13, M12, and M31 were monitored by multiple reaction monitoring of m/z 617 to 167, 633 to 479, 589 to 453, and 591 to 184, respectively. [1H,13C]casopitant, [1H,13C]M13, [1H,13C]M12, and [1H,13C]M31 were monitored by multiple reaction monitoring of m/z 621 to 171, 637 to 483, 593 to 493, and 595 to 184, respectively.

The concentrations of casopitant and metabolites present in plasma samples were determined from calibration curves constructed from analysis of samples spiked with known concentrations of casopitant, M13, M12, and M31 with weighted 1/x2 linear regression applied in each case over the range of 15 to 15,000 ng/ml.

HPLC-MS/MS method B (used for analysis of M76, M134, and M200 in dog plasma and for all analytes in myocardium homogenate samples). M76, M134, and M200 were extracted from 2 μl of dog plasma by protein precipitation as described for method A. The supernatant was analyzed using the same HPLC-MS/MS conditions described previously.

Individual dog myocardium samples were weighed after thawing, an appropriate amount of water was added [tissue-water, 1:3, w/w], and total weight was recorded before homogenization on ice by Ultra-Turrax (IKA-Werke GmbH & Co. KG, Staufen, Germany). Then homogenate samples were analyzed for casopitant, M13, M31, M76, M134, and M200 by using analytical method B with the same extraction procedures and HPLC conditions used for plasma. Casopitant, M13, M12, M31, M76, M134, and M200 were monitored by multiple reactions monitoring of m/z 617 to 167, 633 to 479, 589 to 453, 591 to 184, 575 to 125, 506 to 370, and 549 to 141, respectively.

For both analytical methods detailed quality control (QC) samples, prepared in plasma and myocardium homogenate at three different analyte concentra-

![Graph](image)
either single or repeated oral administration of $^{14}$C]casopitant mesylate,
it was observed (Fig. 4).

<table>
<thead>
<tr>
<th>Peak Identification</th>
<th>Rat Plasma</th>
<th>Dog</th>
<th>% of sample radioactivity (μg equivalents of $^{14}$C]GW8776996)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Casopitant (P)</td>
<td>1.5 (+ M76 + M111) (0.176)</td>
<td>1.9 (0.960)</td>
<td>3.7 (+ M200) (9.05)</td>
</tr>
<tr>
<td>M12</td>
<td>1.2 (0.135)</td>
<td>4.1 (+ M69) (2.03)</td>
<td>2.4 (+ M111 + M200) (2.33)</td>
</tr>
<tr>
<td>M13</td>
<td>0.3 (0.037)</td>
<td>4.8 (2.41)</td>
<td>2.1 (5.12)</td>
</tr>
<tr>
<td>M15</td>
<td>0.2 (0.021)</td>
<td>0.9 (0.424)</td>
<td>0.5 (1.19)</td>
</tr>
<tr>
<td>M31</td>
<td>4.1 (0.469)</td>
<td>4.8 (2.41)</td>
<td>16.3 (39.7)</td>
</tr>
<tr>
<td>M134 + M169</td>
<td>N.D.</td>
<td>N.D.</td>
<td>N.D.</td>
</tr>
<tr>
<td>M57 + M155</td>
<td>N.D.</td>
<td>N.D.</td>
<td>N.D.</td>
</tr>
<tr>
<td>M76</td>
<td>1.5 (+ M111 + P) (0.176)</td>
<td>0.8 (0.380)</td>
<td>3.4 (8.22)</td>
</tr>
<tr>
<td>M200</td>
<td>N.D.</td>
<td>BQL</td>
<td>3.7 (+ P) (0.05)</td>
</tr>
<tr>
<td>M201</td>
<td>N.D.</td>
<td>∞</td>
<td>0.7 (1.79)</td>
</tr>
<tr>
<td>M202</td>
<td>N.D.</td>
<td>∞</td>
<td>0.7 (1.79)</td>
</tr>
<tr>
<td>M203</td>
<td>N.D.</td>
<td>N.D.</td>
<td>2.0 (4.76)</td>
</tr>
<tr>
<td>M206</td>
<td>4.6 (0.527)</td>
<td>0.7 (0.345)</td>
<td>0.4 (0.877)</td>
</tr>
</tbody>
</table>

N.D., not detected; BQL, below quantification limit, set to 25 cpm as peak area; ∞, observed by HPLC-MS/MS only.

The quantification of M31, M134, and M169 in all matrices was grouped together because of coeluting chromatography with HPLC method 1. Further analyses performed with HPLC method 2 and/or 3 revealed that the majority of this radio peak corresponded to M134 for all dog matrices and to M31 for rats.

Toxicokinetic Analysis. Analysis was performed by noncompartmental pharmacokinetic analysis using WinNonlin (version 4.1; Pharsight Corporation, Mountain View, CA). All computations used the nominal plasma collection interval, except for the systemic exposure to casopitant in all matrices was grouped together because of coeluting chromatography with HPLC method 1. Further analyses performed with HPLC method 2 and/or 3 revealed that the majority of this radio peak corresponded to M134 for all dog matrices and to M31 for rats.

Results

Plasma and Tissue Distribution of Radioactivity. Rat. After either single or repeated oral administration of $^{14}$C]casopitant mesylate to female Sprague-Dawley rats for 20 days at a target dose level of 60 mg of free base/kg/day and after recovery periods of 7 (day 27) and 20 (day 40) days following cessation of the repeated administration, radioactivity was quantifiable in all of the tissues examined with the highest levels detected in lung and liver as shown in Table 1 and Fig. 2.

DRM accumulated in plasma, blood, and in the tissues after repeat oral administration of casopitant. On day 20 at 24 h postdose, the mean concentrations of radioactivity were 2- to 3-fold higher for plasma, blood, myocardium, lungs, and skeletal muscle and approximately 4- to 5-fold higher for kidneys and liver than those measured after a single dose (Table 1).

Concentrations of radioactivity in all the tissues analyzed were higher than concentrations in plasma throughout (Table 1). In particular, on day 20, corresponding tissue/plasma concentration ratios were approximately 6, 33, 21, 44, and 2 for myocardium, lung, kidney, liver, and skeletal muscle, respectively.

After cessation of repeat dosing, levels of radioactivity in the tissues declined slowly. On day 27, mean concentrations of radioac-

![Fig. 4](https://example.com/fig4.png)

**Fig. 4.** A representative reconstructed radiochromatogram of pooled dog myocardium, after a repeat oral administration of $^{14}$C]casopitant at a target dose of 40 mg of free base/kg for 14 days.

![Fig. 5](https://example.com/fig5.png)

**Fig. 5.** A representative reconstructed radiochromatogram of pooled rat myocardium, after a repeat oral administration of $^{14}$C]casopitant at a target dose of 60 mg of free base/kg for 20 days.
TABLE 4  
Relevant metabolites of [14C]casopitant  
Structures of the underlined metabolites in the table have been confirmed with synthetic standards.

<table>
<thead>
<tr>
<th>Proposed Structure</th>
<th>Positive [M + H]^+ or Negative [M + H]^+ Ion and Major Fragment Ions (m/z)</th>
</tr>
</thead>
</table>
| ![Casopitant](#) | 617 [M + H]^+  
489, 481, 346, 327, 298, 277, 241, 210, 167 |
| ![GSK631832](#) (M12) | 589 [M + H]^+  
489, 453, 249, 241, 182, 139 |
| ![GW525060](#) (M13) | 633 [M + H]^+  
615, 497, 489, 479, 461, 275, 183, 165 |
| ![GSK1497960](#) (M15) | 631 [M + H]^+  
495, 489, 353, 327, 241, 184, 175, 141 |
| ![M57](#) | 607 [M + H]^+  
548, 489, 412, 327, 298, 241, 141 |
| ![GW679979](#) (M76) | 575 [M + H]^+  
489, 439, 241 168, 125 |
| ![GSK2110277](#) (M134) | 506 [M + H]^+  
370, 315, 298, 241, 175 |
| ![M155](#) | 607 [M + H]^+  
489, 241, 200, 157 |
| ![M169](#) | 550 [M + H]^+  
414, 327, 298, 279, 272, 241, 210, 184, 175, 143, 100 |
| ![GSK174599](#) (M200) | 549 [M + H]^+  
489, 413, 315, 241, 175, 142 |
tivity in the myocardium, lungs, kidneys, liver, and skeletal muscle (quadriceps) had only declined to values between 25 and 44% of those at 24 h after the final dose (day 20) and on day 40, further declines were between 38 and 81% of the corresponding values on day 27. On day 40, tissue concentrations of radioactivity were between 38- and 400-fold higher than in plasma (Table 1).

Dog. In male beagle dogs, after repeat oral administration of [14C]casopitant for 14 days at a target dose level of 40 mg of free base/kg/day, the concentrations of radioactivity in plasma were higher on day 14 than at the corresponding times on day 1, with concentrations at 2 h postdose being greater than those at 24 h postdose on both occasions (Table 2).

After a recovery period following cessation of dosing of 20 days (day 34), radioactivity in plasma was still quantifiable but had declined to approximately 24% of the corresponding values at 24 h postdose (day 14) (Table 2). On day 14, radioactivity was quantifiable in all the tissues examined with the rank order liver > postdose (day 14) (Table 2). On day 14, radioactivity was quantifiable but had de-

Concentrations of radioactivity in tissues were higher than those in plasma at both sampling times (day 14 and day 34) as shown in Fig. 3. On day 34, tissue levels had declined relatively slowly to 21 and 58% of the corresponding values on day 14 (Table 2).

Metabolic Profile After Repeat Oral Administration of [14C]Casopitant. The major metabolites of casopitant were identified and quantified in rat plasma and myocardium samples and dog plasma and myocardium, skeletal muscle, lung, and kidney samples, obtained from the distribution studies described previously.

Rat. After a single oral administration of [14C]casopitant, the metabolic pattern observed in female rat plasma was equivalent to that fully detailed and reported recently (Miraglia et al., 2010), in which casopitant, the hydroxylated piperazine derivative M13 and the N-deethylated piperazine M31 were the major circulating components. On day 20, at 2 h postdose, casopitant was still the principal component in plasma, accounting for approximately 17% of plasma radio-

N.A., toxicokinetic parameters not available because plasma levels were below the limit of quantification (50 ng/ml) at all time-points considered.

*Mean was not calculated where n <3; therefore, individual toxicokinetic parameters are reported.
at 2 and at 24 h postdose, respectively. Other notable circulating metabolites detected at both time points were M13 (5–7% of plasma radioactivity), the deacetylated oxidized derivative M12 (4% of plasma radioactivity), and M134 (3–5% of plasma radioactivity). All relevant circulating metabolites have already been identified as major components in dog plasma after a single oral administration (Miraglia et al., 2010).

In myocardium, skeletal muscle, kidney, and lung, the metabolic pattern was similar. The major metabolite was M134, which represented 16, 27, 20, and 50% of myocardium, skeletal muscle, kidney, and lung radioactivity, respectively (Table 3). M76 accounted for approximately 3 and 8% of myocardium and skeletal muscle radioactivity, respectively; in coelution with casopitant it represented 7 and 12% of kidney and lung radioactivity, respectively. After 20 days of recovery from the last dosing (day 34), a number of radiometabolites were still detectable in myocardium and skeletal muscle extracts, among which the piperazine-modified derivatives M31, M134, M200, and M203 were the most relevant ones (data not shown). As reported in Table 3, all relevant metabolites detected in tissues, with the exception of M203, were also found circulating but at much higher concentrations than in plasma. Proposed metabolite structures and supporting spectral data for all matrices are shown in Table 4.

Quantification of Casopitant and Selected Metabolites in Dog Plasma and Myocardium during an Investigative Toxicity Study.

In male beagle dogs, after a repeat oral dose of 40 mg/kg/day casopitant for 26 weeks, plasma systemic exposure (in terms of AUC0 and Cmax) to casopitant and its circulating metabolites M13, M12, M31, and M134 were generally similar between weeks 6 and 26; therefore, only toxicokinetic parameters measured at week 26 are reported and discussed (Table 5). Metabolites M76 and M200 were detected but only were occasionally quantified because their plasma levels (M200 in particular) were very close to the quantification limit (50 ng/ml).

As reported previously, the major circulating metabolite was M13, giving exposure similar to that of the parent compound. Equivalent exposure (but lower than that of the parent) was observed for M12 and M134 and for M31 and M76 (when quantified). As shown in Fig. 6, plasma levels of M12, M134, M31, and M76 remained essentially constant throughout the 24-h dosing interval at week 26, suggesting an apparently long half-life.

Neither casopitant nor its metabolites was detected in the plasma samples taken during the recovery phase. Casopitant and the six selected metabolites were all detected and quantified in dog myocardium samples collected at all occasions of treatment evaluated as reported in Table 6. Among the drug-related components detected in the tissue, the most relevant was M200 (major one) and M134, with levels increasing over the time from 6 to 26 weeks of treatment as shown in Fig. 7. These two major metabolites were also the only components quantified after 22 weeks of dosing withdrawal after 13 weeks of treatment (Table 6). Overall, no correlation was observed between myocardium and plasma levels of casopitant and its relevant metabolites.

Discussion

During chronic safety assessment studies with casopitant in rat (6 months) and dog (9 months), cardiomyopathy and cardiovascular changes were the major findings observed in both genders at the same mean systemic exposure (≥50 μg · h/ml). The incidence of cardiac changes seemed to be dose- and time-related, and they were accompanied by an increase in plasma levels of biomarkers for cardiac damage such as cardiac troponin I and creatine kinase-MB mass. Moreover, transmission electron microscopy revealed evidence of ultrastructural changes in the hearts of treated animals, which were considered characteristic of phospholipidosis (PL) (Halliwell, 1997; Anderson and Borlák, 2006). In addition to myocardium, other tissues (i.e., skeletal muscle and lung) showed phospholipidotic features, which had also been detected in previous shorter toxicity studies.

PL was not an unexpected finding: casopitant and several notable metabolites are structurally consistent with cationic amphiphilic drugs (CADs), and such agents are known to induce phospholipidosis (Reasor et al., 2006). The phospholipidotic potential of casopitant and active metabolites M13 and M31 was also demonstrated in vitro, reflecting the in vivo observations (data not shown). The peculiarity was that myocardium is not a common PL target tissue; few drugs are known to cause cardiac phospholipidosis, which could be associated with the cardiotoxicity observed, although a mechanistic understanding of toxicity linked to PL was not elucidated (Shaikh and Downar, 1987; Roos et al., 2002; Vonderfecht et al., 2004; Rabkin, 2006).

Concomitant with cardiomyopathy, some hemodynamic effects that could be explained by a secondary pharmacological effect of casopitant or its metabolites on calcium physiology via inhibition of the L-type and T-type calcium channel on vascular smooth muscle were observed (Waitkus-Edwards et al., 2002; Scragg et al., 2004). This potential effect of DRM was supported by evidence of gingival changes seen in dogs during long-term study, a finding characteristic of some classes of calcium channel blockers in animals and humans (Heijl and Sundin, 1989; Ellis et al., 1999; Missouris et al., 2000). In vitro binding studies indicated a low calcium channel blockade by casopitant and M13, M12, and M31. Although activity was low, it could be meaningful if significant accumulation occurred in myocardium.

Based on the findings described and considering that they were manifest only after prolonged daily exposure, it was hypothesized that cardiac effects were probably related to physiochemical characteristics of casopitant or metabolite(s) and to accumulation of DRM with the potential to induce PL. In addition, some involvement of altered calcium physiology also seemed possible.

As a consequence of these observations the distribution, accumulation, and retention of DRM in selected tissues, after chronic dosing of [14C]casopitant, were investigated. The characterization and quantification of the most notable metabolites of casopitant in plasma, myocardium, kidney, lung, and skeletal muscle (the last three tissues for dog only) were also studied. Selected doses were within the range of cardiotoxic doses previously tested during long-term toxicity studies.

Overall, in both preclinical species, the concentration of radioactivity in tissues was much higher than that in plasma (Tables 1 and 2). After the recovery periods considered, radioactivity in plasma and tissues remained detectable and decreased very slowly with the rate of elimination from tissues generally slower than that from plasma in both rat and dog.

These results confirmed the long retention of DRM in tissues and the slow elimination previously reported after acute administration of casopitant (Miraglia et al., 2010). Myocardium was not the tissue with the highest concentration, accumulation, or retention of DRM (Figs. 2 and 3). Lung and liver contained the highest levels of radioactivity on all occasions (Tables 1 and 2). However, as reported (Monro, 1990), the presence and retention of a xenobiotic in tissue is not necessarily correlated to a toxicological effect.

In rat and dog, the main circulating components after repeat oral dosing were unchanged casopitant and metabolites M12, M13, M31, and M134. In addition, in rat only, M206 was notable after repeat oral administration and after the recovery periods.

In rat and dog myocardium, the metabolites identified involved multiple oxidations of the piperazine or piperidine rings, loss of the N-acetyl group, piperazine N-deethylation or loss of the piperazine ring, N-demethylation, and various combinations of these biotransformations. Furthermore, tissues showed a similar metabolite pattern, in
which M31, M76, M134, and M200 were the major metabolites, which was still observed after a 20-day recovery period in both species and still quantifiable in the rat (data not shown).

Overall after repeated administration of $[^{14}C]$casopitant, a large number of metabolites (approximately 36) were detected in rat and dog plasma and tissues. Radiolabeled components were present in the tissues at levels significantly higher than those in plasma; after a washout period following chronic dosing, piperazine-modified derivatives were still quantifiable in tissues but decreased to undetectable levels in plasma. A similar metabolic pattern, at least qualitatively, was observed in all tissues of both preclinical species that were examined, and no unique metabolite was detected in the myocardium.

Liquid chromatography-mass spectrometry assay development for the quantification of all metabolites detected in preclinical species was deemed technically not feasible. The agreed upon strategy was to focus on those derivatives that were notable or accumulating or showed long retention in both rat and dog tissues. The selected structures included those suggestive of calcium channel blockade or CAD-like. On the basis of these criteria, M13, M12, M31, M76, M134, and M200 were quantified together with casopitant in plasma and myocardium samples collected at different times during a 26-week oral investigative toxicity study in dogs dosed at 40 mg/kg/day with unlabeled casopitant. In all animals treated with casopitant, cardiac changes were initially noted over the first 6 weeks of treatment, progressing in severity with duration of dosing, but with adverse findings on light microscopy appearing only after 20 weeks of treatment. Although signs of reversibility were apparent, full recovery did not occur after the 22-week treatment-free period (Casartelli et al., 2010).

Systemic plasma exposure to casopitant and M12, M13, M31, and M134 was approximately equivalent in weeks 6 and 26, suggesting that a steady state was reached (Table 5). In contrast, M76 and M200 were only occasionally quantified (plasma levels were very close to the quantification limit) in few animals, mainly in week 26. Moreover, plasma levels of M12, M31, M134, and M76 remained constant throughout the 24-h dosing interval, suggesting an apparently long half-life. Twenty-two weeks after dosing cessation, no detectable levels of casopitant or any of the six metabolites were found, suggesting that their washout from plasma was largely complete.

In contrast to plasma in which M13 was the major metabolite (Table 5), in myocardium M200 was the major component, reaching considerable concentrations (up to 114,014 ng/g tissue corresponding to 0.2 mM) and showing a clear time-dependent increase from 6 to 26 weeks of treatment (Table 6). Other notable metabolites were M134 and M76, showing increased levels with time. After multiple dosing for 26 weeks, the accumulation of M200 and M134 was clearly evident in myocardium (Fig. 7), and in contrast to observations in plasma, the clearance of these metabolites was very slow with both still quantifiable after cessation of casopitant chronic administration. The progression of cardiac changes seen in life, and the continued presence of heart changes during and after the recovery period,

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**TABLE 6**

<table>
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<tr>
<th>Period</th>
<th>Animal No.</th>
<th>Casopitant</th>
<th>M12</th>
<th>M13</th>
<th>M31</th>
<th>M76</th>
<th>M134</th>
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N.Q., not quantifiable (below the limit of quantification of 50 ng/ml).
correlated with the cardiac concentrations of these metabolites (Casartelli et al., 2010). It is interesting to note that not only elimination from but also accumulation in myocardium was slow: in fact, after 14 days M200 was not the major metabolite (Table 3).

In conclusion, these investigative studies showed that plasma concentrations of DRM cannot be considered representative of tissue concentrations and could not be used to predict DRM tissue concentrations. Poor correlation between plasma and tissue concentrations of drug and metabolite(s) is not unusual because several factors influence tissue concentrations including the action of tissue transporters (Lin, 2006; The International Transporter Consortium, 2010). In contrast to plasma in which metabolite concentrations generally remained constant at different times, in myocardium major metabolites did not reach the steady state up to 26 weeks and showed a significant tendency to accumulate after chronic administration. This slow trend of tissue accumulation without attaining a steady state after chronic dosing despite stabilization of plasma metabolite levels is characteristic of many phospholipidosis-inducing CADs (Gum et al., 2001; Vonderfecht et al., 2004). After the washout period, all circulating components decreased to undetectable levels or were completely cleared, whereas in myocardium some metabolites were retained longer and were still detectable. Nevertheless, no unique metabolites were detected in myocardium: all relevant drug-related components were also present in other tissues. M200 was the major metabolite in dog myocardium with levels increasing over time (39,180–114,014 ng/g tissue corresponding to 0.07–0.2 mM) and remaining relevant (31,732 ng/g tissue corresponding to 0.06 mM) after 22 weeks of dosing withdrawal. Accumulation and slow elimination of ethylenediamine derivatives are not unusual (Breyer and Gaertner, 1974; Gaertner et al., 1975). However, the presence per se of a xenobiotic in a tissue is not necessarily deleterious (Monro, 1990; Lafuente-Lafuente et al., 2009). Potential M200 toxicity could be related to its chemical structure, being suggestive of a potential calcium antagonist (as proven for M31) or chelating activity (not investigated) (Qian and Guo, 2010). No further investigations were performed to confirm this hypothesis although M200 remains the most intriguing metabolite. Nevertheless, even if M200 were proven to be responsible for cardiotoxicity, it would not be easily monitored throughout plasma pharmacokinetics to provide a safety margin (Eichler and Muller, 1998).

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Authorship Contributions

Participated in research design: Pagliarusco, Martinucci, Miraglia, and Pellegratti.

Conducted experiments: Bordini, Miraglia, and Cufari.

Performed data analysis: Pagliarusco, Bordini, Miraglia, Cufari, and Ferrari.

Wrote or contributed to the writing of the manuscript: Pagliarusco, Martinucci, Bordini, Cufari, Ferrari, and Pellegratti.

References


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