Cannabinoid Receptor Antagonist-Induced Striated Muscle Toxicity and Ethylmalonic-Adipic Aciduria in Beagle Dogs


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Ibipinabant (IBI), a potent cannabinoid-1 receptor (CB1R) antagonist, previously in development for the treatment of obesity, causes skeletal and cardiac myopathy in beagle dogs. This toxicity was characterized by increases in muscle-derived enzyme activity in serum and microscopic striated muscle degeneration and accumulation of lipid droplets in myofibers. Additional changes in serum chemistry included decreases in glucose and increases in non-esterified fatty acids and cholesterol, and metabolic acidosis, consistent with disturbances in lipid and carbohydrate metabolism. No evidence of ketonuria or ketonemia was observed. We propose that IBI-induced toxic myopathy in beagle dogs is consistent with an inhibition of the mitochondrial flavin-containing enzymes including dimethyl glycine, sarcosine, isovaleryl-CoA, 2-hydroxyglutarate, and multiple acyl-CoA (short, medium, long, and very long chain) dehydrogenases. All of these enzymes converge at the level of electron transfer flavoprotein (ETF) and ETF oxidoreductase. Urinary ethylmalonate was shown to be a biomarker of IBI-induced striated muscle toxicity in dogs and could provide the ability to monitor potential IBI-induced toxic myopathy in humans. We propose that IBI-induced toxic myopathy in beagle dogs is not caused by direct antagonism of CB1R and could represent a model of ethylmalonic-adipic aciduria in humans.

Key Words: metabonomics; biomarkers; muscle toxicity; pharmacological; cannabinoid receptor; electron transfer flavoprotein.

Obesity is a human health problem of epidemic proportions and has been demonstrated to predispose individuals to type 2 diabetes, coronary artery disease, hypertension, and osteoarthritis (Flegal et al., 1998; National Task Force on the Prevention and Treatment of Obesity, 2000). Endocannabinoids appear to have a pivotal role in the control of energy homeostasis (Di Marzo et al., 2004), as demonstrated by genetically engineered cannabinoid-1 receptor (CB1R) deficient mice, which are hypophagic, lean, and resistant to diet-induced obesity (Ravinet Trillou et al., 2004). Therefore, the CB1R has been identified as a potential pharmaceutical target to treat obesity and in support of this target, CB1R antagonists have been shown to elicit an anorexigenic effect in animals (Ravinet Trillou et al., 2003). As part of its non-clinical development, the toxicity of CB1R specific antagonist ibipinabant (IBI) was evaluated in beagle dogs. As expected, at pharmacologic doses, IBI caused appetite suppression accompanied by weight loss or failure to gain weight in young growing dogs. Unexpectedly, daily oral doses of IBI caused striated muscle fiber degeneration accompanied by increases in serum alanine aminotransferase (ALT) and aspartate aminotransferase (AST) activities.

In an effort to provide a better understanding of the biochemical basis for IBI-induced myopathy in beagle dogs, a series of in vivo and in vitro investigative studies were conducted. These studies included evaluation of CB1R expression and radioligand binding in dog muscle and brain, further characterization of the lesions using light and electron microscopy, clinical pathology, and urine metabonomic evaluations of IBI-treated dogs. These studies revealed lipid accumulation in striated muscle accompanied by disturbances in lipid and carbohydrate metabolism, consistent with the human inherited condition, ethylmalonic-adipic aciduria. We concluded that IBI-induced myopathy in beagle dogs has features comparable to ethylmalonic-adipic aciduria and was not directly related to pharmacologic antagonism of CB1R.
Tissue CB1R Expression Profiling

mRNA—qPCR. Total RNA was extracted from snap-frozen cerebellum, left ventricular free wall, diaphragm, and semitendinosus samples collected from five IBI-naïve adult male beagles (Marshall Farms, North Rose, NY) with TRIzol Reagent (Invitrogen, Carlsbad, CA) following the manufacturer’s recommendations until the phase separation step. The RNA was then further purified and eluted with RNasey spin columns (Qiagen, Valencia, CA) according to the manufacturer’s recommendations. The RNA concentration in each sample was calculated by measuring the absorbance at 260 nm (A260). RNA purity was assessed by calculating the A260/A280 ratio, with a ratio between 1.9 and 2.1 indicative of high-purity samples. RNA integrity for each sample was assessed by comparing the 18S and 28S ribosomal RNA peaks using an Agilent 2100 Bioanalyzer. RNA was then reverse transcribed to generate cDNA with the TaqMan High Capacity cDNA Reverse Transcription kit and random primers (Applied Biosystems, Foster City, CA). Each reaction contained 40 ng/µl RNA. Negative controls containing no reverse transcriptase enzyme were generated for each sample to monitor for genomic DNA amplification. The cDNA and “No RT” negative controls were used as templates for qPCR. Standard curves for the target and endogenous control genes were generated with serial dilutions of canine cerebellum template cDNA (0.0015–100 ng per 25 µl reaction). Reactions were carried out in 96-well plates on an Applied Biosystems 7900 HT Sequence Detection System with SDS 2.1 software. Gene-specific pre-designed TaqMan probe and primer set for eukaryotic 18S rRNA was purchased from Applied Biosystems. A TaqMan probe and primer set for dog ctna1 was designed using Applied Biosystems’ Assays-By-Design service using the sequence information from the predicted canine CB1R sequence (accession XM_539034). The TaqMan assays used are as follows: 18S rRNA: cat no. Hs99999901_s1 (proprietary; sequences not available) CB1R (dog): forward primer: CAGGCCCCCTGCTTTAATAAGAG; reverse primer: GTGCCACATCGGCAATAAGC; TaqMan probe: TIGGGCGCTGGTGACAAT. The PCR thermocycling parameters used were 50°C for 2 min (1 cycle), 95°C for 10 min (1 cycle), and 95°C for 15 s + 60°C for 1 min (40 cycles). Data collected by SDS 2.1 were exported into Microsoft Excel and analyzed using the relative quantification standard curve method (Livak, 1997). Gene expression (mRNA) levels in each tissue were reported as Log 10 RQ (relative quantity) relative to mRNA levels in white adipose after normalization to 18S rRNA.

Immunohistochemistry. Cerebellum from positive-control CB1R wild-type (WT, +/-) mice and negative-control CB1R knockout (KO, −/−) mice and cerebellum, heart, and skeletal muscle (diaphragm and semitendinosus) from normal beagle dogs (3) were snap-frozen, embedded in Tissue-Tek OCT compound (Sakura, Tarrytown, NY), and allowed to air-dry, and stored below −70°C until use. Immunoperoxidase staining was conducted utilizing an avidin-biotin complex procedure. Endogenous peroxidase activity was quenched by incubating cryosections in a solution containing sodium azide (1mM), glucose (10mM), and glucose oxidase (1 U/ml) for 60 min at 35°C, and non-specific binding of reagents was blocked by sequential incubation of avidin and biotin solutions (15 min each) followed by a protein solution (20% normal goat serum and 3% casein) for 60 min. Cryosections were cut at approximately 5µm, allowed to air-dry, and stored below −70°C until staining. Slides were fixed in cold 2% paraformaldehyde for 3 min just prior to staining. Immunoperoxidase staining was conducted utilizing an avidin-biotin complex procedure. Endogenous peroxidase activity was quenched by incubating cryosections in a solution containing sodium azide (1mM), glucose (10mM), and glucose oxidase (1 U/ml) for 60 min at 35°C, and non-specific binding of reagents was blocked by sequential incubation of avidin and biotin solutions (15 min each) followed by a protein solution (20% normal goat serum and 3% casein) for 60 min. Cryosections were then incubated 24°C with 1 µg/ml of rabbit polyclonal anti-CB1R antibody (cat no. ab23703, Abcam Inc., Cambridge, MA). Negative control slides were produced by substituting the primary antibody with concentration-matched non-immune rabbit polyclonal serum. All slides were incubated with biotinylated goat anti-rabbit IgG secondary antibody (1:500, Jackson ImmunoResearch Laboratories, West Grove, PA) for 30 min, followed by incubation with the “ABC” Elite reagent (Vector Laboratories, Burlington, CA) for an additional 30 min. Next, 3,3′-diaminobenzidine (DAB) was applied for 15 min as substrate for the peroxidase reaction. Slides were counterstained with hematoxylin and coverslipped for light microscopic evaluation. Localization of CB1R staining was tabulated using non-statistical methods. Staining intensity was graded semiquantitatively as negative, equivocal, weak positive, moderate positive, or strong positive.

Western blotting. Dog cerebellum, left ventricle free wall, interventricular septum, diaphragm, semitendinosus, and liver tissues were collected and snap frozen in a container of 2-methylbutane floating on liquid nitrogen. Corresponding rat tissues were collected, placed into tubes, and snap frozen directly in liquid nitrogen. All tissues were held on dry ice before storage at −70°C until use. Protein was prepared from these tissues as well as from WT and CB1R-null mouse brain tissues using T-PER (Pierce, Rockford, IL) with Halt protease inhibitor cocktail with EDTA (Pierce). Mouse brain tissue collection and genotyping data were recorded in PRI notebook pages N63645:081,086. Protein lysates were stored at ≤−70°C. The total protein concentration of each sample was evaluated using the BCA protein assay kit (Pierce).

Twenty-five micrograms of reduced protein from each sample were electrophoresed on 4–12% Bis-Tris polyacrylamide gels (Invitrogen) with NuPAGE MOPS SDS running buffer (Invitrogen). Ten micrograms of BenchMark Pre-Stained Standard (Invitrogen) were included on each gel, and this marker was subsequently calibrated with MagicMark Standard (Invitrogen). The samples were electrophoresed for approximately 1–2 h at 120 mA. Following electrophoresis, the proteins were transferred to nitrocellulose membranes (Invitrogen) for approximately 1–2 h at 160 mA. To verify uniformity and completeness of the transfer, each gel was stained with Simply Blue SafeStain (Invitrogen), and each membrane was stained with Ponceau S staining solution (Sigma-Aldrich).

After destaining, the membranes were incubated for 1 h at room temperature in SuperBlock BLOTTO (Pierce) with 0.05% Tween 20 to block non-specific antibody binding. To detect CB1R protein, the blot was incubated overnight at 4°C with a rabbit polyclonal antibody to CB1R (Abcam, Cambridge, MA; cat no. ab23703) shown to specifically react with rat CB1R and predicted to react with dog CB1R (due to 100% identity with immunogen) diluted 1:500 in blocking buffer. Following incubation with primary antibody, membranes were washed in Tris-buffered saline and Tween 20 (TBST) and then incubated for 1 h at room temperature in goat anti-rabbit IgG (H+L)-horseradish peroxidase secondary antibody (Pierce cat no. 31460) diluted to 1:20,000 in blocking buffer. Membranes were then washed with TBST at room temperature and incubated for 5 min at room temperature in SuperSignal West Pico solution (Pierce) followed by exposure to Hyperfilm ECL (Amersham).

To verify uniformity in protein amount loaded between samples, each blot was stripped and probed for a loading control protein, β-actin. The membranes were incubated in Restore Western Blot Stripping Buffer (Pierce) for 20 min at room temperature to strip them of antibodies. To determine if all secondary antibody was removed, membranes were incubated in SuperSignal West Pico solution and exposed to film for approximately 2 min. The membranes were then incubated in secondary antibody for 1 h at room temperature, washed, incubated in SuperSignal West Pico solution and exposed to film to verify that all primary antibody was removed. When stripping was complete, membranes were blocked again and probed following the procedure above with a rabbit polyclonal β-actin primary antibody (Abcam; cat no. ab8227) diluted to 1:1000 in blocking buffer.

Radioligand binding. Tissue membrane protein was dispensed into 96-well assay plates in binding buffer containing 25mM hydroxethyl piperazineethanesulfonic acid pH 7.4, 150mM NaCl, 1mM ethylenediaminetetraacetic acid, 2mM MgCl2, and 0.25% bovine serum albumin. One microliter of compound in dimethyl sulfoxide (DMSO) or DMSO alone was added to the wells. The binding reaction was started by the administration of radioligands [3H]-CP55,940 (160 Ci/mmol, NET1051, NEN), [3H]-SR141716A (38 Ci/mmol, TRK1028, Amersham), [3H]-nitrindipine (84 Ci/mmol, NET741, NEN), or [3H]-scopolamine (82 Ci/mmol, NET636, NEN). The total volume of the reaction was 75 µl, and the incubation was carried out at room temperature for 3h. The unbound tracer ligands were removed by rapid filtration through a presoaked (0.3% polyethyleneimine) GF/B plate with a Packard Cell Harvester. The filters were washed 8 times with 250 µl phosphate-buffered saline (pH 7.4)
Define nature and time-course of muscle lesion development. In the 3-month investigative study (Study 1 [300 mg/kg/day dose group only]), a 2-month recovery arm was included to assess the recovery period. Serum samples were collected from fasted dogs, once prior to the first dose and prior to evaluation by high-resolution liquid chromatography-mass spectrometry (LC/MS)-based background subtraction (Zhang et al., 2010). In Study 3, plasma samples were collected from fasted dogs, once prior to the first dose and prior to a daily dose on days 3, 7, 10, and 14 and weekly thereafter. Plasma was prepared and stored at −20°C until samples were shipped on dry ice to RTI International for evaluation of total and free carnitine and acylcarnitines using a validated LC/MS/MS method. Internal standard working solutions were prepared for carnitines in double distilled water and methanol (25 mM HCl, 0.75 M MeOH solvent) and for carnitine in water. Samples were evaluated using a validated LC/MS/MS system consisting of an Agilent liquid chromatograph coupled with an Applied Biosystems’ triple quadrupole mass spectrometer. Instrument control, data collection, and data processing were handled by the Analyst (version 1.3.2) software package from Applied Biosystems. The calibration curve was obtained by performing linear regression analysis on a plot of analyte concentration versus analyte/internal standard peak area ratio. The mobile phase on the high-performance liquid chromatograph (HPLC) consisted of 80:20 (v/v) water:methanol.

Urinary organic acids and metabonomics. In Study 3, two procedures were employed in urinary metabonomics analyses.

Urinary organic acids and acylglycines were analyzed as their trimethylsilyl derivatives using gas chromatography-mass spectrometry (GC-MS) essentially as described in the recent guidelines for organic acid analysis as a confirmatory test for the diagnosis of inherited organic acidurias in the newborn period (Bennett, 2006). Essentially, an internal standard of undecanedioic acid was

<table>
<thead>
<tr>
<th>Study no.</th>
<th>Primary study objectives</th>
<th>Oral dosages (mg/kg/day)</th>
<th>No. of dogs per dose group</th>
<th>Duration of dosing (weeks)</th>
<th>Serum or plasma</th>
<th>Urine</th>
<th>Sudan black histochemistry</th>
<th>Electron microscopy</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Define nature and time-course of muscle lesion development</td>
<td>0, 300</td>
<td>18</td>
<td>13</td>
<td>—</td>
<td>—</td>
<td>✓</td>
<td>✓</td>
</tr>
<tr>
<td>2</td>
<td>Identify biomarkers of muscle lesion development</td>
<td>0, 300</td>
<td>12</td>
<td>6</td>
<td>✓</td>
<td>—</td>
<td>✓</td>
<td>—</td>
</tr>
<tr>
<td>3</td>
<td>Define dose-response relationship and correlation of biomarkers to muscle lesion development</td>
<td>0, 1, 10, 300</td>
<td>8</td>
<td>13</td>
<td>✓, ✓</td>
<td>✓</td>
<td>✓</td>
<td>—</td>
</tr>
</tbody>
</table>

*Dogs were euthanized after 2, 6, 9, or 13 weeks of treatment and 4 or 8 weeks after cessation of treatment.

*Analyses included NEFA, HBA, carnitines and plasma acylcarnitines (C12, C14, and C16), lactate, and pyruvate.

*Analyses were performed using a TopCount scintillation counter (Packard Instrument) after the addition of a scintillation reagent, MicroScint20.

In Vivo Studies in Dogs

An overview of the experimental designs for the three in vivo investigative studies (Studies 1–3) performed in female beagle dogs is presented in Table 1.

Animal source and husbandry. Female beagle dogs, 6–9 months old, were obtained from Marshall Farms, North Rose, NY, housed in masonry runs, and fed Harlan Diet no. 2025C: certified 25% protein dog diet. In these studies, dogs were provided an enriched environment (food enrichment, chew toys, and social interaction). The studies were 3 months (Studies 1 and 3) and 6 weeks (Study 2) in duration. In the 3-month investigative study (Study 1 [300 mg/kg/day dose group only]), a 2-month recovery arm was included to assess the reversibility of IBI-related effects. IBI was dosed once daily by oral gavage as a suspension in 1% methylcellulose/0.5% poloxamer 188. The studies were conducted in accordance with the National Institute of Health (NIH) Guide for the Care and Use of Laboratory Animals (NIH Publication No. 85-23, revised 1996) and approved by the Bristol-Myers Squibb Institutional Animal Care and Use Committee at New Brunswick, NJ (BMS-ACUC).

Clinical pathology. Blood samples for clinical pathology (Studies 1–3) were obtained from the external jugular vein from fasted dogs, once or twice prior to the first dose, twice weekly prior to a daily dose in the first 2–3 weeks of dosing, then weekly thereafter in the dosing period and monthly (Study 1) during the recovery period. Serum parameters determined on the Hitachi 917, Roche Diagnostic Systems, Inc. included AST, ALT, gamma glutamyltransferase, alkaline phosphatase, and creatine kinase (CK) activities, total bilirubin, total protein, albumin, globulin, total cholesterol, triglycerides, glucose, urea nitrogen, creatinine, calcium, phosphorus, sodium, potassium, chloride, and bicarbonate. Serum samples for the evaluation of β-hydroxybutyric acid (HBA) and non-esterified fatty acids (NEFA) in Studies 2 and 3 (pretreat and weekly evaluation in Study 2) were stored at −70°C before subsequent analysis with the Hitachi 912, Wako Chemicals USA, Inc. at Bristol-Myers Squibb Co., Department of Clinical Pathology, Princeton, NJ. Analysis of serum samples for lactate (Hitachi 917, Roche Diagnostic Systems, Inc.) and pyruvate (LabCorp, Preclinical Department, 1904 Alexander Drive, Research Triangle Park, NC) was performed twice pretreat and prior to a daily dose on days 24 and 31 in Study 2. Blood samples were also collected in EDTA tubes, centrifuged to obtain plasma (0.5 ml aliquots), stored at −20°C, and subsequently analyzed for creatinine and acylcarnitine levels (Studies 2 and 3, refer to Plasma Carnitine and Acylcarnitines section). Urine samples were collected in EDTA tubes, centrifuged to obtain plasma (0.5 ml aliquots), stored at −70°C, and subsequently evaluated for osmolality, total carnitine, free carnitine, organic acids, and/or acylglycines.

Urinary organic acids and metabonomics. In Study 3, two procedures were employed in urinary metabonomics analyses.

Urine organic acids and acylglycines were analyzed as their trimethylsilyl derivatives using gas chromatography-mass spectrometry (GC-MS) essentially as described in the recent guidelines for organic acid analysis as a confirmatory test for the diagnosis of inherited organic acidurias in the newborn period (Bennett, 2006). Essentially, an internal standard of undecanedioic acid was...
added to a volume of urine equivalent to 0.2 mg of creatinine and then sub-
jected to acidification to pH 2 and extracted 3 times with 2 volumes of ethyl acetate. The organic phase was pooled and dried down under a stream of nitro-
gen at room temperature. The dried material was derivatized by the addition of 100 µl of bis(trimethylsilyl) trifluoroacetamide:trimethylchlorosilane (99:1) (Pierce Chemical Co., Rockford, IL), capped, and heated for 30 min at 70°C in a heating bath. After cooling to room temperature, the product was injected into a GC-MS system (Agilent) programmed to run from 70°C to 250°C at a rate of 5°C/min and a bake out time of 10 min at 300°C. Data acquisition was in the full scan mode collecting all data in the range of m/z 50–550. Peak identification was by library search and comparison to authentic standards.

Quantification was against the internal standard following preparation of stand-
ard curves. Because there is a lack of normal data for these metabolites in dogs, each study group acted as internal controls. The control group was assumed to be the closest equivalent to normal animals.

Urine samples for nuclear magnetic resonance (NMR) were prepared by add-
ing 0.5 parts of a 0.2M, pH 7.4, sodium phosphate buffer in H₂O:D₂O 80:20, containing 0.5mM sodium 3-trimethylsilyl-(2,2,3,3-²H₃)-1-propionate (TSP). The NMR spectra were measured on a Bruker 600 MHz spectrometer equipped with a TCI z-gradient 5 mm cryoprobe at 300 K using the gradient Noesyp1d pre-
saturation pulse scheme for water suppression with 256 scans per spectrum and a 2-s relaxation delay. All NMR spectra were manually phased, baseline corrected, and referenced to the internal standard TSP by a single operator. A superposition of all spectra was used to manually define integration regions for the data. The spectra were initially scaled to a constant spectral integral to compensate for vari-
ation in urine volumes and NMR amplification. Scaling was refined by scaling to a constant integral of the least variable regions. Prior to PCA analysis, values of all variables were mean centered and variance stabilization was accomplished by dividing the mean-centered values by the SD (UV scaling) unless noted oth-
ewise. There was no indication of NMR signals from the drug or its metabolites.

Data preparation, visualization, and analysis were performed using

XwinNMR, ACD 1HNMR, the R statistics package, Partek Pro 6.1, JMP
(SAS Institute), and Spotfire (Spotfire Inc.) and Simca-P11+ (Umetrics Inc.). Identification of the metabolites included matching the 1D spectra with refer-
ce compounds in a proprietary spectral database, 2D COSY and 2D HMBC, and ultimately by the addition of known standards to the sample. Full details are of all methods are presented elsewhere (Ott and Aranibar, 2006).

**Anatomic pathology.** At designated time points during each study (Studies 1–3), dogs were fasted overnight and euthanized according to a pro-
tocol approved by the BMS-ACUC. Gross necropsy was performed, and re-
presentative samples of skeletal muscle (diaphragm, semitendinosus, sartorius/ quadriceps, triceps brachii, masseter, and/or latissimus dorsi) and heart were collected, fixed in 10% neutral buffered formalin, processed, and sectioned. All sections were stained with hematoxylin and eosin (H&E) and examined by light microscopy for any IBI-related or spontaneous lesions. Additional stain-
ing of frozen skeletal muscle (diaphragm, semitendinosus, and/or sartorius/ quadriceps) and heart were performed using Sudan black histochemistry.

In Study 1, transmission electron microscopy (TEM) was performed on re-
presentative samples of the semitendinosus muscle and left ventricular myocar-
dium from dogs (n = 2 or 3 per group) treated with IBI at 0 (control) or 300 mg/ kg/day for up to 13 weeks followed by up to 8 weeks of postdose recovery. Fresh samples were immersed in McDowell-Trap fixative and then postfixed in 1% osmium tetroxide, dehydrated, and embedded in PolyBed 812. Blocks were semi-thin sectioned at approximately 1 µm, stained with toluidine blue, and examined by light microscopy. A subset was selected for ultra-thin sectioning, stained with uranyl acetate and lead citrate, and examined using a FEI Tecnai G Spirit BioTWIN transmission electron microscope. Representative digital images were captured by an Advanced Microscopy Techniques (AMT) Corp. camera system and saved electronically in a tagged image file (tif) format.

**RESULTS**

**Tissue CB1R Expression Profiling**

By QPCR, immunohistochemistry (IHC), Western blotting, and radioligand binding evaluations, no evidence of CB1R expression was detected in striated muscle samples of beagle dogs (Table 2, Figs. 1 and 2). The validity of these assays was confirmed by demonstrating strong CB1R expression in the cerebellum of beagle dogs and the brain of WT (CB1R +/+ ) mice, and lack of CB1R expression in the cerebellum of CB1R KO mice (Fig. 2). Although low levels of CB1R mRNA were detected in the left ventricular myocardium, the only evidence of CB1R protein expression in this tissue was by IHC which identified focal CB1R immunoreactivity associated with neural and/or vascular cells but not in cardiac myocytes. Of note, CB1R protein expression was not identified by IHC in neural or vascular cells of skeletal muscle.

**In Vivo Studies in Dogs**

**Clinical Pathology.** In Study 3, IBI-induced striated muscle toxicity correlated with increases in serum AST, ALT, and/or CK activities at 10 and 300 mg/kg/day (Fig. 3). In this study, substantial alterations in the metabolic profile also occurred in these IBI-treated dogs and included changes in serum glucose, cholesterol, lactate, NEFA, HBA, and bicarbonate (Table 3). This profile was consistent with that seen in Studies 1 and 2, which established the time course of these enzymatic and metabolic changes in dogs given IBI at 300 mg/kg/day. There were transient minor IBI-related increases in serum glucose at ≥ 10 mg/kg/day (up to 1.4× pretest) preceding decreases in serum glucose (down to 62% pretest) at 300 mg/kg/day (Study 1 changes demonstrated in Fig. 4). In Study 3, there were mild increases in serum cholesterol (1.3–1.4× control, individually up to 2.0× pretest) at 300 mg/kg/day, sporadic increases in NEFA at 10 mg/kg/day (up to 3.8× pretest) and at 300 mg/kg/
day (up to 3.6× pretest), and transient increases in serum HBA at 10 mg/kg/day (up to 8.8× pretest) and at 300 mg/kg/day (up to 24.5× pretest). There was a sporadic IBI-related decrease in serum bicarbonate in all dogs at 300 mg/kg/day (down to 44% of pretest), consistent with metabolic acidosis. In the 6-week study (Study 2), there was also increased serum lactate at 300 mg/kg/day on days 24 and/or 31, consistent with a titration acidosis (Studies 1–3, Supplementary pathology tables and appendices).

**Plasma Carnitine and Acylcarnitines.** In Study 3, total and free carnitine levels were increased in the plasma of all dogs treated with IBI at ≥ 10 mg/kg/day in a dose- and time-dependent manner (Fig. 5). The increases in total carnitine were predominantly due to a proportionately greater increase in acylcarnitines compared with carnitines (Studies 2 and 3, Supplementary pathology tables and appendices).

Increases occurred in short (C2–C6), medium (C8–C10), and long or very long (C12–C22) chain acylcarnitines, including
butyrylcarnitine or isobutyrylcarnitine (C4), isovaleryl or 2-methylbutyrolycarnitine (C5), hexanoylcarnitine (C6), and/or octanoylcarnitine (C8) in all dogs ≥ 10 mg/kg/day. No changes were found at the lowest dose evaluated (1 mg/kg/day), but detectable amounts (when none was evident pretest) to substantial increases in plasma acylcarnitines (C2–C22) were present in 26 of 39 parameters at 10 mg/kg/day and 30 of 39 parameters at 300 mg/kg/day at one or more time points. The increases began later at 10 mg/kg/day than 300 mg/kg/day and generally persisted for the remainder of the study.

**Urinary Organic Acids and Metabonomics.** In Study 3, dose-dependent increases in urinary total and free carnitine and acylcarnitines were evident in two of eight dogs at 10 mg/kg/day and all dogs at 300 mg/kg/day by week 5. Additional dose-dependent IBI-related changes included detectable levels to substantial increases in adipate, suberate, sebacic acid, ethylmalonate, methylsuccinate, 2-hydroxyglutarate, hexanoylglycine, isovalerylglycine, and/or lactate in four of seven dogs at 10 mg/kg/day and up to all eight dogs at 300 mg/kg/day at all time points evaluated (weeks 1, 5, 9, and 13) during this 3-month study except for week 1 (at 300 mg/kg/day, there were detectable levels of ethylmalonate in a few dogs during week 1 but they were below the limits of quantitation or < 2 mg/g creatinine, Table 4). Also at 300 mg/kg/day, there were increases in unsaturated sebacic acid, unsaturated suberate, and butyrylglycine in one to five of eight dogs (Study 3, Supplementary pathology tables and appendices).

In addition, a pairwise comparison of the NMR metabonomic analysis on weeks 5 and 9, but not week 13, indicated increased levels of acetylcholine, acetyl carnitine, pyruvate, N,N-dimethylglycine, and sarcosine. The most consistent and abundant altered (i.e., increased) parameter in both the 10 and 300 mg/kg/day dose groups was ethylmalonate.

The findings were similar whether the analyses were performed by NMR standardized to background levels of common urinary metabolites or by MS standardized to creatinine. Urinary metabolite composition in vehicle control and at 1 mg/kg/day remained stable over the course of the study with no significant outliers.

**Anatomic Pathology**

In Studies 1–3, the incidence and severity of skeletal muscle degeneration at 10 and/or 300 mg/kg/day IBI correlated well with increased serum muscle enzyme activity noted in these groups as assessed in Studies 1–3 (refer to Clinical Pathology section). Muscle groups were similarly affected with myofiber

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**TABLE 3**

Interpretation of Results from In Vivo Study No. 3 (Beagle Dogs Treated with IBI for 13 Weeks)*

<table>
<thead>
<tr>
<th>Interpretation</th>
<th>Serum</th>
<th>Urine</th>
<th>Histopathology and/or ultrastructural pathology</th>
</tr>
</thead>
<tbody>
<tr>
<td>Disturbances in glucose utilization associated with striated muscle degeneration</td>
<td>Increases in AST, ALT, and CK activities (resolution generally associated with decreases in glucose) Increases in glucose (days 2–8) Decreases in glucose (variable during days 22–92)</td>
<td>n/a</td>
<td>Degeneration of skeletal and cardiac muscles (H&amp;E)</td>
</tr>
<tr>
<td>Disturbances in lipid metabolism associated with lipid accumulation in striated muscle</td>
<td>Increases in cholesterol, acylcarnitines (C2–C22), and free carnitine</td>
<td>Increases in carnitines (total, free, and acylated) Altered organic acid and acylglycine profile Increases in specific organic acids</td>
<td>Lipid accumulation in skeletal and cardiac muscles (H&amp;E, Sudan black, TEM) n/a</td>
</tr>
<tr>
<td>Metabolic acidosis</td>
<td>Decreases in bicarbonate, increases in lactate, and sporadic increases in NEFA and HBA</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

**Note.** n/a, not applicable.

*BBI-related effects were observed at dosages of 10 and 300 mg/kg/day, but not at 1 mg/kg/day.

**FIG. 4.** Time-dependent changes in serum glucose levels were observed in dogs treated with IBI (Study 1). Increases occurred during the first week followed by decreases in weeks 3–12. *p < 0.05 and **p < 0.01 indicate significantly different from time-matched controls based on the Dunnett’s multiple comparison t-test procedure.
degeneration and regeneration despite fiber composition (diaphragm and semitendinosus) except for masseter muscle, which was affected to a lesser degree. In the left ventricular myocardium (Studies 2 and 3), slight myofiber degeneration accompanied by small numbers of inflammatory cells was also observed at ≥10 mg/kg/day.

In all studies, gross changes in skeletal muscle in affected IBI-treated dogs included slight-to-moderate mottled tan to diffuse pale discoloration, ranging from individual muscle groups to generalized muscle pallor. This pallor correlated with widespread lipid vacuolation of myofibers characterized as widely dispersed clear round spaces (Fig. 6). These vacuoles stained blue-black with Sudan black histochemistry. By TEM, lipid droplets in skeletal and cardiac myofibers were located within the sarcoplasm, interspersed among mitochondria, both beneath the sarcolemma and between myofibrils, and generally ranged from 0.15 to 1.50 µm in diameter. Larger lipid droplets (near the upper end of the size range) tended to abut and distort adjacent mitochondria.

**DISCUSSION**

Striated muscle toxicity is a relatively common problem in drug development and was viewed as a potential impediment to the development of IBI, particularly if direct CB1R antagonism was determined to be involved in the pathogenesis. In our studies, such on-target toxicity was shown to be unlikely because CB1R mRNA expression, protein, and CB1R ligand binding were lacking in canine striated muscles. In humans, which express CB1R in skeletal muscle, CB1R antagonism has been associated with evidence of increased oxidative pathways in muscle (Cavuoto et al., 2007), the opposite of that seen in IBI-treated dogs. Although possible indirect CB1R effects cannot be completely ruled out, our data indicate that IBI-induced muscle toxicity in dogs was likely an off-target toxicity, and we were able to provide further insight into potential mechanisms for this toxicity.

The myopathy evident in beagle dogs treated with IBI was characterized by lipid accumulation as well as muscle fiber degeneration. The myodegeneration was monitored by standard biomarkers of muscle injury (ALT, AST, and CK activities) which were increased following the administration of IBI to dogs. The lipid accumulation was associated with apparent dysregulation of fatty acid metabolism by striated muscle. The unique physiology of dogs (due to fiber-type composition of type 1, 2A, and 2X fibers) may make them particularly susceptible to this type of lesion. Dogs lack classical-type 2B (purely glycolytic) fibers (Snow et al., 1982) and thereby rely more on oxidative (fatty acid–based) metabolism for energy.
Furthermore, compared with other laboratory animal species, dog fast-twitch muscle fibers (types 2A and 2X) exhibit an unusually high mitochondrial content and oxidative capacity (Acevedo and Rivero, 2006), which likely explains the high level of endurance that dogs possess (Rivero et al., 1994). This high oxidative/low glycolytic capacity would be expected to increase the susceptibility of dog skeletal muscles to compounds which alter fatty acid β-oxidation. Thus, the skeletal myofiber degeneration observed in dogs given IBI is thought to have resulted from an acute energy deficit following diminished fatty acid β-oxidation coupled with an inadequate reserve of muscle with glycolytic capacity. The temporal correlation between muscle degeneration and subsequent decreases in serum glucose and increases in urinary and serum lactate are consistent with this hypothesis.

Lipid myopathy has been reported in animals with fatty acid oxidation defects (DiDonato and Taroni, 2004) and is similar to the lesions observed in dogs following administration of IBI. In reported cases, lipid accumulation results from the diminished capacity of muscle to effectively utilize fatty acids as a fuel source leading to the gradual accumulation of fatty acids in the form of triglycerides.

Changes in several blood and urine chemical parameters in dogs given IBI were also consistent with altered energy metabolism. These included decreased serum glucose, fluctuating increases in serum NEFA and ketones (HBA), increases in carnitine and acylcarnitines, plasma lactate, blood pyruvate, and lactate/pyruvate ratio, and urinary organic acids and in toto, was indicative of fatty acid oxidation perturbation.

**FIG. 6.** The CB1R antagonist IBI causes lipid storage myopathy in beagle dogs. Skeletal muscle from female dogs orally dosed with IBI at 300 mg/kg/day for 13 weeks (A) or 2 weeks (C), or with control vehicle (D–F). (A) Transverse histologic section shows numerous vacuolated, skeletal myofibers (brackets). H&E stain. (B) TEM of a skeletal myofiber shows numerous lipid droplets between myofibrils abutting and distorting adjacent mitochondria (arrow heads). Bar = 500nM. (C) Longitudinal histologic section shows necrotic myofiber with fragments of coagulated sarcoplasm infiltrated by macrophages (brackets). H&E stain. (D) Transverse histologic section shows normal skeletal myofibers. H&E stain. (E) TEM has normal skeletal myofiber with only one lipid droplet, which is noticeably smaller than those in Figure 2B. Bar = 500nM. (F) Longitudinal section shows normal skeletal myofibers. H&E stain.
During mitochondrial β-oxidation of fatty acids, acyl-CoA esters undergo repeated removal of two carbon units. The length of the fatty acid determines which acyl-CoA dehydrogenase (short, medium, and long or very long acyl-CoA dehydrogenase) deacylates the fatty acid. The altered plasma levels of acylcarnitines of all C chain lengths in dogs given IBI administration suggested that all of the major acyl-CoA dehydrogenases were affected, indicating a general inhibition of fatty acid oxidation. Metabonomic analysis of urine was conducted to further characterize the effects on lipid oxidation. Increases in urinary organic acid and acylglycine metabolites (ethylmalonic acid [EMA], methylsuccinic acid, adipate, suberate, sebacic acid, butyrylglycine, and hexanoylglycine) reflected renal excretion of plasma carnitine and acylcarnitines and were also consistent with inhibition of multiple acyl-CoA dehydrogenases.

In the presence of excess butyryl-CoA, propionyl-CoA carboxylase can convert butyryl-CoA to ethylmalonyl-CoA, resulting in the production and urinary excretion of the end-products methylsuccinic acid and free EMA rather than methylmalonic acid (Amendt et al., 1987; Corydon et al., 1996; Jethva and Ficicioglu, 2008; Jethva et al., 2008; Kalousek et al., 1980; Fig. 7), as seen with short-chain acyl-dehydrogenase (SCAD) deficiency (Fig. 7). Similarly, increases in sebacic acid, suberic acid, adipic acid, and hexanoylglycine have been associated with medium long chain acyl-dehydrogenase (MCAD) and very long chain acyl-dehydrogenase deficiencies in humans (Vianey-Liaud et al., 1987; Fig. 7). These metabolites are not generally found in substantial quantity in normal urine samples. However, when fatty acid oxidation of medium-to-very long chain fatty acids is impaired, alternate metabolism via microsomal ω oxidation occurs, resulting in formation of these organic acids and glycine conjugates.

Additional increases in urinary metabolites excreted in IBI-treated dogs suggested that other mitochondrial enzymes were also inhibited by IBI. Urinary sarcosine and dimethylglycine (choline oxidation products) suggest an effect on the

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**FIG. 7.** Biochemical pathways for short- and long-chain fatty acyl-CoA metabolism. Arrows pointing to the left indicate normal metabolism, whereas arrows pointing to the right indicate alternative metabolism following inhibition of SCAD (A) and MCAD (B). The enzymes in the pathways are shown in italics, and metabolites that were observed following IBI administration to beagle dogs are shown in boxes.
enzymes sarcosine and dimethyl glycine dehydrogenases. Urinary isovalerylglycine (involved in branched chain [leucine] amino acid catabolism) is consistent with a deficiency in the enzyme isovaleryl-CoA dehydrogenase (Vockley and Ensenauer, 2006). Urinary 2-hydroxyglutaric acid indicates inhibition of 2-hydroxyglutarate dehydrogenase, a flavin adenine dinucleotide (FAD)-dependent mitochondrial enzyme (Rzem et al., 2004). All of these metabolites were detected following IBI administration.

The dimethyl glycine, sarcosine, isovaleryl-CoA, 2-hydroxyglutarate, and multiple acyl-CoA dehydrogenases are all mitochondrial enzymes that require the cofactor FAD and use electron transfer flavoprotein (ETF) as an electron acceptor. ETF and ETF oxidoreductase are involved in the sequential transfer of electrons from the cofactor FADH$_2$ to ubiquinone (CoQ10) of the electron transport chain (Fig. 8) for ATP production. Inhibition of ETF or ETF oxidoreductase would be expected to inhibit all mitochondrial flavin-containing dehydrogenases. In dogs with lipid storage myopathy, changes in urinary organic acids, plasma amino acids, and plasma, urine and muscle carnitine were deemed consistent with disorders of mitochondrial oxidative metabolism (Shelton et al., 1998). IBI-induced lipid myopathy is different from those described by Shelton because sebacic and adipic aciduria occurred in dogs treated with IBI but not in the other reported myopathies in dogs.

The metabonomic profile of IBI-treated dogs appears most consistent with EMA-adipic aciduria, a relatively mild form of the inherited multiple acyl-CoA dehydrogenation deficiency (MADD:M) disorders in humans (Corydon et al., 1996; Vianey-Liaud et al., 1987). A more severe form, glutaric aciduria II (MADD:S), is often associated with neonatal death, whereas EMA-adipic aciduria generally has a more variable clinical course with clinical manifestations occurring during periods of stress (Amendt and Rhead, 1986). Both forms of MADD are characterized by impaired mitochondrial ability to oxidize substrates such as [1-14C] octanoate and palmitoyl-CoA and decreased activities of mitochondrial flavin-containing dehydrogenase activity, but the impairment is more profound in MADD:S (Amendt and Rhead, 1986). Individuals with MADD have been identified with mutations in and/or deficient activity of ETF or ETF oxidoreductase. Individuals with MADD that respond to riboflavin supplementation may actually suffer from a disorder of mitochondrial flavin metabolism or transport.

Although recognized as an inherited genetic defect in humans, there is little published literature suggesting that xenobiotics can induce MADD. A condition similar to MADD has been reported in horses with rhabdomyolysis, increases in serum acyl carnitines, hyperglycemia, and urinary increases in lactic acid, EMA,
2-methylsuccinate, butyrylglycine, isovalerylglucose, and hexanoxylglycine (Westermann et al., 2008). This profile was similar to that observed in the urine in IBI-treated dogs. The muscles of affected horses had increased activities of short and medium acyl-CoA and isovaleryl-CoA dehydrogenases. Furthermore, rhabdomyolysis was accompanied by microvesicular lipodosis that predominately involved type 1 muscle fibers. The exact cause of these myopathies was not identified but a genetic basis was not established, and the underlying mechanism was postulated to be due to inhibition of ETF or ETF oxidoreductase.

A similar condition was observed in the urinary metabolic analysis of rats treated with the industrial solvent ethylene glycol monomethyl ether. The urinary profile was consistent with inhibition of choline oxidation, branched chain amino acid catabolism, and fatty acid beta-oxidation pathways (Takei et al., 2010). All of these pathways contain flavin-containing mitochondrial enzymes and use ETF as an electron acceptor. ETF oxidoreductase inhibition was speculated to be involved in ethylene glycol monomethyl ether toxicity, and it provides another example of a condition induced by a xenobiotic that is similar to MADD in humans.

This is the first evidence of MADD in dogs. In our study, EMA was identified as a significant biomarker of IBI-induced myopathy in dogs because it is not routinely found in urine. Increases in EMA preceded actual muscle damage and were considered more sensitive and specific than monitoring leakage enzymes such as CK and AST activities. In conclusion, IBI-induced myopathy in dogs was attributed to disrupted fatty acid metabolism, and despite the lack of data on enzyme activity or expression, the pattern of changes is very specific and most consistent with the inhibition of mitochondrial ETF-dependent flavin-containing dehydrogenases. Metabolomic technology was successfully used to investigate the pathogenesis of IBI-induced myopathy and identify specific biomarkers for monitoring this toxicity.

SUPPLEMENTARY DATA

Supplementary data are available online at http://toxsci.oxfordjournals.org/.

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