Fabp3 as a Biomarker of Skeletal Muscle Toxicity in the Rat: Comparison with Conventional Biomarkers

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Fatty acid binding protein 3 (Fabp3) has been used as a serological biomarker of cardiac injury, but its utility as a preclinical biomarker of injury to skeletal muscle is not well described. Fabp3 concentrations were determined for tissues from Sprague-Dawley rats and found to occur at highest concentrations in cardiac muscle and in skeletal muscles containing an abundance of type 1 fibers, such as the soleus muscle. Soleus is also a primary site of skeletal muscle (SKM) injury caused by lipid-lowering peroxisome proliferator–activated receptor alpha (PPAR-α) agonists. In rats administered repeat doses of a PPAR-α agonist, the kinetics and amplitude of plasma concentrations of Fabp3 were consistent with plasma compound concentrations and histopathology findings of swollen, hyalinized, and fragmented muscle fibers with macrophage infiltration. Immunohistochemical detection of Fabp3 revealed focal depletion of Fabp3 protein from injured SKM fibers which is consistent with increased serum Fabp3 concentrations in treated rats. We then assessed the predictivity of serological Fabp3 for SKM necrosis in short duration toxicology studies. Rats were treated with various doses of 27 different compounds, and the predictivity of serological biomarkers was assessed relative to histology in individual rats and in treatment groups. Under these study conditions, Fabp3 was the most useful individual biomarker based on concordance, sensitivity, positive and negative predictive values, and false negative rate. In addition, the combination of Fabp3 and aspartate aminotransferase (AST) had greater diagnostic value than the conventional combination of creatine kinase-MM isoenzyme (CK) and AST.

Key Words: biomarker; skeletal muscle; necrosis; predictivity; rat; tissue distribution.

However, these medications can also cause adverse side effects, including skeletal muscle (SKM) weakness and necrosis (Graham et al., 2004; Hodel, 2002; Rosenson, 2004). Extreme cases of SKM toxicity, called rhabdomyolysis, can be a serious adverse event; the severity and prevalence of statin-related SKM toxicity resulted in withdrawal of one statin from commercial use (Charatan, 2001). SKM necrosis is therefore an adverse event of particular interest during preclinical testing of new drug candidates for treatment of hyperlipidemia.

Serological biomarkers of SKM injury in people include the established markers creatine kinase-MM isoenzyme (CK) and aspartate aminotransferase (AST) (Panteghini et al., 2006). These biomarkers have been useful for identifying SKM toxicity, but may lack the sensitivity necessary to identify more subtle drug-related effects on SKM in patients. CK, AST, and to a lesser extent alanine aminotransferase (ALT) are used to detect and monitor striated muscle injury in animals (Bender, 2003; Hoffmann et al., 1999; Lumeij, 1997), but their diagnostic power in toxicology studies may be suboptimal when a sensitive biomarker of SKM injury is needed, especially when blood samples are collected outside the optimal window for individual biomarkers. Thus, more predictive and sensitive serological biomarkers that identify SKM injury early in its progression, for example, prior to the occurrence of histologic lesions, would be useful in preclinical safety testing.

A related objective for new SKM biomarkers is to identify the type of muscle fibers that are adversely affected by treatment with a compound that injures SKM. Slow-twitch (type 1) oxidative fibers are potentially sensitive to PPAR-α–mediated toxicity (DeSouza et al., 2006), whereas fast-twitch (type 2) glycolytic fibers are more sensitive to statin-induced toxicity (Smith et al., 1991; Westwood et al., 2005). Currently, the available biomarkers cannot diagnose in any species the identity of injured muscle groups and/or muscle fiber types based on serological biomarkers alone.

Less commonly used biomarkers of SKM injury that have the potential to improve our ability to identify SKM injury include myoglobin (Van Nieuwenhoven et al., 1995; Sayers and Clarkson, 2003), skeletal troponins (Sorichter et al., 1999),...
parvalbumin (Dare et al., 2002), and Fabp3 (Glatz et al., 2003, Pelsers et al., 2005). Serological fatty acid binding protein 3 (Fabp3) measurements in people have primarily involved patients suspected of having acute coronary syndrome (Pelsers et al., 2005). In these cases, Fabp3 has been shown to be a sensitive and rapid biomarker for myocardial injury. The sensitivity of Fabp3 is attributed to its high concentration in heart tissue. The rapidity of its response is attributed to the small size of the protein, which facilitates its release from damaged tissue. Serological Fabp3 has also been used to assess SKM injury in athletes (Sorichter et al., 1998), so the possibility exists that Fabp3 might be a useful biomarker of compound-mediated SKM necrosis in toxicology studies. With this possibility in mind, we investigated the diagnostic and predictive potential of Fabp3 as a candidate biomarker of SKM necrosis. Tissue distribution, plasma kinetics during SKM necrosis, and predictivity of serological Fabp3 for SKM necrosis detected by histology in rats in short duration (lead optimization) toxicology studies were determined for Fabp3 and compared with established biomarkers of SKM injury.

**MATERIALS AND METHODS**

**Collection and Homogenization of Tissues from Naïve Rats**

Soleus, biceps femoris, diaphragm, gastrocnemius (red, white, and mixed fiber samples collected as described by Armstrong and Phelps, 1984), left cardiac ventricle, kidney (cortex, outer medulla, and cross-section including both), and liver samples were collected from five/six Sprague–Dawley rats (10–11 weeks old, males ~350 g; females ~240 g, Charles River Laboratories, Wilmington, MA). Rats were acclimated for ~1 week to caging, feeding, and watering conditions. Tissues were collected within 10 min after euthanasia, placed in tissue cassettes, frozen in liquid nitrogen, and stored at −80°C until analysis.

In preliminary tissue cell lysis experiments, we compared a Tris-based buffer, pH 7.4 and a detergent-containing tissue protein extraction reagent (T-PER, Pierce cat # 78510, reported by the manufacturer to utilize a proprietary detergent in 25 mM bicine, 150 mM sodium chloride, pH 7.6), both with additional protease inhibitors. The Tris buffer released a greater amount of Fabp3 from muscle tissues (data not shown) and was used for all subsequent experiments. Tissues were homogenized in the Tris buffer (1 ml of 50mM Tris buffer, pH 7.4 per ~100 mg tissue) using Lysing Matrix D tubes and a FastPrep shaker (Qiobgene, Irvine, CA). Complete homogenization was achieved after three to five cycles of shaking on setting 5.5 for 20 s each cycle. Samples were placed on ice for 5 min between cycles. Homogenized samples were then centrifuged at 6000 × g then 18,000 × g (10 min at 4°C) and soluble fractions (supernatant) collected. Total protein was determined for each tissue supernatant sample (Bradford assay, Pierce Cat# 23236).

**Fabp3 Immunodepletion from Tissue Homogenates**

**Column preparation.** Chicken anti-rat Fabp3 antibody (Life Diagnostics Cat# 10092) was coupled to Affi-Gel 10 beads (BioRad Cat# 153-6009) at 5 mg antibody/ml of beads. Antibody was prepared for coupling by exchanging storage buffer with coupling buffer (50 mM [4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid]/200mM KCl, pH 7.6) by dialysis (Pierce Slide-A-Lyzer Dialysis Cassette, Cat# 66415). Approximately, 200 μl of Affi-Gel 10 beads was placed in a centrifugal filter tube (Amicon Low-binding Durapore polystyrene fluoride membrane (0.45 μm), Cat# UFC4 OHV 25) and washed three times with coupling buffer. One milligram of antibody was added and the solution incubated on a shaker at 2–8°C for 4 h. Twenty microliters of 1M ethanolamine–HCl, pH 7.6 per 200 μl of gel was added to immobilize antibodies bound to beads and to inactivate open binding sites on beads then solution incubated on a shaker at 2–8°C for 1 h. Beads were washed 3× with ice cold binding buffer (10 mM Tris, pH 7.5) then stored in binding buffer at 2–8°C until used.

**Fabp3 immunodepletion from supernatants.** Columns were centrifuged to remove binding buffer then 200 μl of tissue homogenate supernatant was added to the column and incubated on a shaker at 2–8°C for 2 h. The column was centrifuged and immunodepleted supernatant was collected. Columns were regenerated by washing three times with ice-cold Elution buffer (Pierce Immunopure IgG Elution Buffer Cat# 21004) followed by three times wash with ice cold binding buffer prior to additional cycles of immunodepletion. Aliquots of supernatant were collected after each cycle of immunodepletion. These aliquots were diluted and analyzed by enzyme-linked absorbent assay (ELISA) to quantify Fabp3. Supernatants were determined to be sufficiently depleted of Fabp3 when optical density values were similar to those for dilution buffer alone, and did not decrease with additional cycles of immunodepletion.

**Quantitation of Biomarker Concentrations in Tissues, Serum, and Plasma**

Analyses were conducted using a commercial Fabp3 ELISA assay (Cat# HK403, HyCult Biotechnology, Uden, The Netherlands). Standard curves were made by spiking Fabp3 standard (Cat# 7936, Life Diagnostics, Inc, West Chester, PA) into tissue-matched Fabp3-immunodepleted tissue supernatant. The purity of the Fabp3 standard was determined by sodium dodecyl sulfate polyacrylamide gel electrophoresis and the concentration was assessed by quantitative amino acid analysis. Dilutions of tissue homogenates were conducted in order to assure that Fabp3 signals were in the range of the assay for each tissue. Plates were read using a Spectramax Plus 384 spectrophotometer (Molecular Devices, Sunnyvale, CA). Fabp3 concentrations in plasma and serum were determined following 1:1 dilution with the supplied dilution buffer according to manufacturer’s protocol. The same Fabp3 standard as referenced above (with confirmed purity and concentration) was used to assay plasma and serum samples.

Activities of ALT, AST, and CK in serum and tissue supernatants were determined using standard methods on a Hitachi 917 Automatic Analyzer (Roche Diagnostics Corporation, Indianapolis, IN). Samples were not immunodepleted for determination of ALT, AST, or CK activities. Serum concentrations of cardiac troponin I (cTnI) were determined using an Immulite Immunoanalyzer (Diagnostic Products Corporation, Los Angeles, CA) according to the manufacturer’s protocol.

**Quantitation of Compound Concentrations in Plasma and Binding Affinity for Receptor**

Compound concentrations in plasma were determined by liquid chromatography tandem mass spectrometry using an Applied Biosystems/MDS Sciex API 4000 triple quadrupole mass spectrometer equipped with a TurbolonSpray interface, and operated in negative ion mode. Selected reaction monitoring transitions with precursor and product ions specific for the analyte and internal standard were monitored for quantitation purposes. The analytes were chromatographically separated using a ThermoHypersil Biosil C18 2 × 20 mm 5 μm Javelin HPLC column. The plasma concentrations were calculated using an eight-point, 1–5000 ng/ml calibration curve, with accuracies for the back-calculated responses that were between 91.4% and 111.8% of the nominal concentration. Data were acquired and processed with Applied Biosystems/ MDS Sciex Analyst software. Binding affinity of the compound at the PPAR-α receptor was previously determined using the ABCD binding assay described by Brooks et al. (2001).

**In Vivo Experiments**

All animal procedures were performed according to protocols approved by the Eli Lilly Animal Care and Use Committee. Following phlebotomy, all rats were euthanized by cervical dislocation under anesthesia or by asphyxiation using CO2. To minimize volumes of blood collected at each time point, both Fabp3 and compound concentrations were measured from the same plasma
PPAR-α agonist experimental design. Female Sprague–Dawley rats (17–18 weeks, Charles River Laboratories, Wilmington, MA) had femoral artery cannulas implanted 3 days prior to study start to facilitate repeated blood collections. A concurrent control group was included to assess possible surgery-related SKM injury. Rats received four daily gavage doses of a PPAR-α agonist at 0 or 70 mg/kg. Serial blood samples were collected from the cannula into tubes containing ethylenediaminetetraacetic acid prior to first dose and at 4, 8, and 24 h postdose after two, three, and four daily doses. Plasma was analyzed for Fabp3 and compound concentrations. SKMs (soleus, biceps femoris, gastrocnemius, and diaphragm) were collected from all rats 24 h after the fourth dose and examined microscopically using hematoxylin and eosin (H&E) staining for evidence of injury.

Lead optimization toxicology studies. During this stage of drug development, assessments are made of a drug candidate’s ability to cause toxicity in short duration studies (e.g., four daily doses). Dosing levels, the number of daily doses, determination of plasma kinetics and sample collection parameters were tailored for each program based on expected target organ toxicities for the drug class under investigation and were not optimized for biomarker investigation. Compounds under investigation included investigational compounds from several drug classes, such as G protein–coupled receptors (G-PCRs), nuclear receptor agonists, and an 3-hydroxy-3-methyl-glutaryl Coenzyme A reductase inhibitor. The 19 candidate drug screening studies included in Table 2 had a similar study design. Male or Female Sprague–Dawley rats (7–8 weeks, Charles River Laboratories, Wilmington, MA) or Fischer-344 rats (7–8 weeks, Taconic Farms, Inc, Germantown, NY) were administered four or five daily doses of test compound. Two exceptions were the commercially available model compounds known to cause SKM injury. 2,3,5,6-Tetramethyl-1,4-phenyleneamine (TMPD) (Catalog #523755-10G, Sigma-Aldrich, St Louis, MO) was administered as a single dose in 10% Acacia and 0.05% Dow Corning Antifoam 1510-US in purified water, and Cerivastatin (Catalog #SRP02030c, Sequoia Research Products, Berkshire, UK) was administered as 4, 7, or 14 daily doses in phosphate buffered saline, pH 7.4 (Table 3). At approximately 24 h after the final dose, blood was collected for determination of biomarkers and tissues were collected for routine histopathology evaluation by H&E staining.

Histopathology
Tissues were fixed in 10% neutral buffered formalin for up to 24 h then trimmed and transferred to 70% dehydrant prior to processing. After fixation, hearts were sectioned longitudinally and SKMs were sectioned to provide longitudinal and cross-section profiles of myofibers. Tissues were processed routinely for paraffin embedding, sectioned at 5 μm, and stained with hematoxylin and eosin. The extent of tissue necrosis was classified as follows: minimal < slight < moderate < marked < severe.

Immunohistochemistry to Label Fabp3 in SKM
Immunohistochemistry for Fabp3 was performed using a Dako autostainer on formalin-fixed paraffin embedded sections (4 μm) placed on positively charged slides. Antigen retrieval used borate buffer (Mallinckrodt Baker, Phillipsburg, NJ) incubating at 98°C for 10 min. Endogenous peroxidase and nonspecific protein blocking steps were performed with routine methods. Sections were incubated with Fabp3 mouse anti-human monoclonal antibody (HyCell Biotechnology, Uden, The Netherlands) at dilution of 1:100. Biotinylated secondary anti-mouse IgG (Vector Laboratories, Burlingame, CA) was applied. Immunolabeling was visualized with avidin–biotin, Vectorstain Elite ABC Kit (Vector Laboratories) followed by diaminobenzidine chromagen (DAB) (DakoCytohhemat, Carpinteria, CA) and sections were briefly counterstained with hematoxylin.

Data Analysis
Data from lead optimization toxicology studies were initially analyzed by comparing the mean biomarker concentration or activity in each treatment group to the mean for the concurrent (intrasudy) control group. For each treatment group, a fold-increase from control mean was calculated for Fabp3, AST, ALT, and CK (Table 2). Concordance between biomarker increases and the prevalence of SKM necrosis was then examined. Treatments were ordered according to the incidence of SKM necrosis observed for rats in each group. (A rat was considered positive for SKM necrosis if at least one SKM had necrosis of any severity grade detected microscopically.) Treatments were then divided into categories of 80–100% incidence, 20–66% incidence, and 0% incidence of SKM necrosis. Histopathology is unlikely to detect all instances of SKM necrosis, however, so it is possible that some groups in the 0% incidence category actually had a low incidence of SKM necrosis that went undetected. It is also possible that one or more biomarkers could be premonitory, such that the biomarker concentration could increase in the absence of detectable SKM necrosis. We therefore divided the 0% incidence category into one category with Fabp3 increases > 2-fold above control and another category with Fabp3 increases < 2-fold above control and marked with an asterisk treatment groups in which there were detectable SKM necrosis in groups administered a higher dose of the same compound. The twofold cutoff has often been used as a sufficiently large increase to be of interest, and identification of groups of animals treated with compounds that caused detectable SKM necrosis at a higher dose are precisely those treatment groups where one should be able to detect a change in biomarker concentration that could be considered premonitory.

Individual animal data for biomarkers and SKM necrosis (Fig. 4) were analyzed by three methods: comparison of biomarker concentrations/activities to the severity of necrosis observed (Figs. 4A–D), comparison of biomarker concentrations/activities to the presence or absence of necrosis independent of severity (Figs. 4E–H), and last, by calculating the percent of rats with and without SKM necrosis at biomarker concentrations/activities across the observed range (Fig. 4I–L) to make initial assessments of positive and negative cutoff values.

Statistical analysis performed at the treatment group level is shown using tables constructed to compare biomarker concentrations/activities to histological observations of necrosis (Table 4). A treatment group was considered positive for histological findings if any of the rats in that group had necrosis detected microscopically in any of the SKM observed. Otherwise the histological finding for that group was considered negative for SKM necrosis. For each biomarker, ANOVA was performed separately for each drug candidate in each study. Treatment levels were the only factors included in the model with analysis being performed using the PROC MIXED (SAS Institute, Inc., 2004) procedure on SAS. Each treatment level was compared with the control by one-sided t-tests examining whether the mean biomarker concentration/activity for that treatment level was greater than that of the concurrent control group. If the P-value of the t-test was less than 0.05, the biomarker test was considered positive for indicating SKM injury. For all other cases, the test was considered negative.

The four biomarkers of SKM necrosis were compared individually and in combination using seven performance statistics (Table 4). Equations for calculating performance statistics assume histopathology findings are the gold standard by which biomarkers are assessed. SKM necrosis may be occurring in some animals but not detected by histology, and therefore, we may be underestimating the diagnostic power of these biomarkers. Concordance is the proportion of instances that were correctly reported [concordance = (TP + TN)/(TP + TN + FP + FN)]. Positive predictive value (PPV) is the proportion of treatment groups with positive biomarker test results that were correctly diagnosed [PPV = TP/(TP + FP), where TP = true positives and FP = false positives]. Negative predictive value (NPV) is the proportion of treatment groups with negative biomarker test results that were correctly diagnosed [NPV = TN/(TN + FN), where TN = true negatives and FN = false negatives]. Specificity is the proportion of TNs among all treatment groups with no SKM necrosis [specificity = TN/(TN + FP)]. Sensitivity is the proportion of TPs among all treatment groups that had SKM necrosis [sensitivity = TP/(TP + FN)]. False positive rate (FPR) is the proportion of negative instances that were erroneously reported as positive [FPR = FP/(FP + TN)]. False negative rate (FNR) is the proportion of positive instances that were erroneously reported as negative [FNR = FN/(FN + TP)].
RESULTS

Characterization of Fabp3 ELISA Assay

The sensitivity and precision of the commercial Fabp3 ELISA assay for detection of Fabp3 in rat serum was determined by recoveries of rat Fabp3 standard spiked into serum from untreated Sprague–Dawley rats. The lower level of quantitation (LLOQ) for the assay for serum was 1 ng/ml and the coefficient of variation (CV) was < 10%. For Fabp3-immunodepleted tissue homogenates, the LLOQ for recovery of spiked Fabp3 standard was 2 ng/ml and CV < 11%. Specificity of the Fabp3 ELISA assay was established in two ways. First, immunodepletion of tissue homogenates using an anti-Fabp3 antibody decreased the signal for Fabp3 concentration to background levels caused by buffer alone. And second, high concordance was observed between the ELISA assay and a mass-spectrometry-based analytical method for serum from naïve rats and from rats having SKM necrosis graded at various severities (Zhen et al., 2007). The Fabp3 ELISA assay was therefore considered a reliable assay to measure Fabp3 protein concentrations in serum and in supernatants of tissue homogenates.

Tissue Concentrations of Fabp3 Protein and AST, CK, and ALT Activities in Rat and the Relationship with Predominant SKM Fiber Types

<table>
<thead>
<tr>
<th>Fiber type, %abc</th>
<th>AST activity/mg TP (SD)</th>
<th>CK activity/mg TP (SD)</th>
<th>ALT activity/mg TP (SD)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Soleus</td>
<td>9.57 (1.50)</td>
<td>4.81 (0.73)</td>
<td>79.8 (15.96)</td>
</tr>
<tr>
<td>Diaphragm</td>
<td>15.1 (4.23)</td>
<td>10.5 (2.06)</td>
<td>86.0 (18.84)</td>
</tr>
<tr>
<td>Gastrocnemius, redd</td>
<td>5.69 (1.03)</td>
<td>6.10 (0.77)</td>
<td>115 (21.69)</td>
</tr>
<tr>
<td>Gastrocnemius, mixedd</td>
<td>1.10 (0.70)</td>
<td>2.85 (1.09)</td>
<td>204 (44.18)</td>
</tr>
<tr>
<td>Gastrocnemius, whitee</td>
<td>0.48 (0.13)</td>
<td>2.45 (0.71)</td>
<td>244 (86.55)</td>
</tr>
<tr>
<td>Biceps femoris</td>
<td>1.21 (0.53)</td>
<td>2.55 (0.60)</td>
<td>239 (22.03)</td>
</tr>
<tr>
<td>Left cardiac ventricle</td>
<td>9.68 (1.43)</td>
<td>8.47 (0.61)</td>
<td>21.5 (3.97)</td>
</tr>
<tr>
<td>Kidney, cortex</td>
<td>0.47 (0.12)</td>
<td>1.41 (0.26)</td>
<td>0.63 (0.13)</td>
</tr>
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<td>0.63 (0.13)</td>
</tr>
<tr>
<td>Liver</td>
<td>0.0069 (0.0018)</td>
<td>2.37 (0.59)</td>
<td>0.14 (0.04)</td>
</tr>
</tbody>
</table>

TABLE 1

Tissue Concentrations of Fabp3 Protein and AST, CK, and ALT Activities in Rat and the Relationship with Predominant SKM Fiber Types

*Koerker et al. (1990).
*dGastrocnemius sections containing predominately red, white, or mixed fibers were collected from locations outlined in Armstrong and Phelps (1984).

Bold faced values indicate the predominant fiber type for each skeletal muscle.

Oxidative (SO) fibers based on published data (Armstrong and Phelps, 1984; Koerker et al., 1990; Vork et al., 1991). SKMs composed primarily of SO fibers have the highest concentration of Fabp3. Fabp3 concentration is lower in muscles containing a high percentage of fast-twitch oxidative/glycolytic (FOG) fibers and still lower in muscles predominantly composed of fast-twitch glycolytic (FG) fibers. Left ventricle cardiac tissue contained Fabp3 at a concentration similar to SO fiber-rich SKMs. Kidney Fabp3 concentrations were similar to those found in FG fiber-rich muscles. Liver contained a Fabp3 concentration 70-fold lower than the SKM with the lowest Fabp3 concentration.

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Tissue Concentrations of Fabp3 Protein and AST, ALT, and CK Activities

Fabp3 protein concentrations were measured, as well as ALT, AST, and CK enzyme activities, in tissue homogenates from SKMs, cardiac muscle, liver, and kidney from naïve Sprague–Dawley rats. In Table 1, skeletal muscle groups are listed in order of highest to lowest percentage of slow-twitch oxidative (SO) fibers based on published data (Armstrong and Phelps, 1984; Koerker et al., 1990; Vork et al., 1991). SKMs composed primarily of SO fibers have the highest concentration of Fabp3. Fabp3 concentration is lower in muscles containing a high percentage of fast-twitch oxidative/glycolytic (FOG) fibers and still lower in muscles predominantly composed of fast-twitch glycolytic (FG) fibers. Left ventricle cardiac tissue contained Fabp3 at a concentration similar to SO fiber-rich SKMs. Kidney Fabp3 concentrations were similar to those found in FG fiber-rich muscles. Liver contained a Fabp3 concentration 70-fold lower than the SKM with the lowest Fabp3 concentration. AST and ALT activities in the muscles sampled have a distribution similar to Fabp3. SKM composed primarily of SO or FOG fibers and the left cardiac ventricle had higher (up to threefold) AST and ALT activities than muscles composed primarily of FG fibers. CK activity was higher (up to threefold) in the FG compared with SO and FOG fiber-rich muscles and was at least threefold lower in left cardiac ventricle compared with all SKMs sampled.

Gastrocnemius muscle has distinct compartments, each containing a different composition of muscle fiber types (Armstrong and Phelps, 1984) that are recognizable macroscopically by differences in their color: SO fiber-rich region being darkest red, FG fiber-rich region being white, and FOG fiber-rich region being pink (mixed). Fabp3 protein concentrations and AST and ALT activities in gastrocnemius were greatest in the SO (type I) fiber-rich region, followed by FOG (mixed), then FG (type II) regions, whereas CK activity was lowest in SO (type I) fiber regions.

The total pool of each biomarker in individual tissues (Fig. 1) was also considered. These values were derived from the...
total mass of each tissue as well as the biomarker concentration (mg of Fabp3 or activity of AST/ALT/CK per gram of wet weight tissue—data not shown). For example, soleus and diaphragm have two of the highest Fabp3 concentrations of all SKMs sampled (Table 1). However, by considering the large difference in mass between these two muscles, it is apparent that diaphragm holds a much larger pool of Fabp3 than the soleus, and can potentially release a much greater amount into circulation (Fig. 1).

In contrast with Fabp3 and CK, the total pool of AST and ALT is much greater in liver, followed by kidney, then muscle tissue. This tissue distribution makes AST and ALT less muscle-specific than Fabp3 and CK.

Kinetics of Fabp3 in Plasma and SKM Histopathology

Fabp3 concentrations in plasma (pFabp3) were investigated following repeated daily oral administration of a PPAR-α agonist drug candidate. pFabp3 concentration (Fig. 2A) and compound concentration in plasma (Fig. 2B) were measured, and tissue samples of four SKMs (soleus, biceps femoris, gastrocnemius, and diaphragm) were evaluated for histopathology.

Acute SKM necrosis was characterized by scattered individual hypereosinophilic and swollen myocytes with hyalinized-to-fragmented sarcoplasm. Subacute necrosis was characterized by fragmented fibers intensely infiltrated by macrophages. Incidence of muscle necrosis occurred most commonly in soleus (five of five rats) and gastrocnemius muscles (four of five rats); lesion severity was greatest in soleus. Significant muscle lesions were not identified histologically in diaphragm or biceps femoris. The histologic features and distribution of affected myocytes in this biomarker study were consistent with those described in an earlier toxicology study (data not shown) of rats given the same doses of the same compound.

pFabp3 increased in individual compound-treated rats beginning after two doses (the earliest postdose time point measured) compared with pretreatment pFabp3 concentrations in the same rats. The magnitude of the pFabp3 concentration peaks increased following each dose administration, such that all compound-treated rats showed clear increases in pFabp3 at one or more sample times compared with predose concentrations (Fig. 2A). pFabp3 generally peaked between 4 and 24 h after dosing on each day. Compound concentrations in plasma peaked at 4–8 h after each dose and then declined (Fig. 2B).

Concordance between Serological Biomarkers and SKM Histopathology

Analyses at the individual animal level. The diagnostic potential of serological biomarkers of SKM toxicity was assessed by monitoring Fabp3 in short duration rat preclinical studies designed to evaluate the safety of 27 different drug candidates. In each study, serum Fabp3, AST, CK, and ALT, as well as morphologic changes in SKM, heart, liver, and kidney were determined 24 h after administration of the last daily oral dose.
SKM necrosis, often with macrophage infiltration, was readily discernable. A representative micrograph of SKM necrosis observed in rats from these studies is presented (Fig. 3). The microscopic changes observed with conventional brightfield examination were considered typical for necrosis. The earliest stages of muscle injury consisted of subtle changes, such as hyalinization, characterized by slightly rounded fibers with more darkly eosinophilic and homogeneous sarcoplasm with loss of detail, that were difficult to distinguish from similar artifactual changes related to tissue handling or fixation. More established features of necrosis were characterized by fragmentation of the coagulated fiber and macrophage infiltration into the injured myocyte (Fig. 3B). Features suggestive of apoptosis were not recognized, therefore additional procedures to evaluate apoptosis, such as TUNEL labeling or cleaved caspase 3 immunohistochemistry, were not pursued.

To better understand the relationship between increased serological Fabp3 concentrations and morphological changes in SKM of compound-treated rats, we used immunohistochemistry to investigate Fabp3 protein in muscle fibers in a number of rats. There was evidence for depletion of Fabp3 from individual injured muscle fibers adjacent to uninjured muscle fibers that had no apparent decrease in Fabp3 staining (Fig. 3C). Decreased levels of immunodetectable Fabp3 were also observed in areas containing apparently uninjured muscle fibers, which may be attributed to non-toxicologic biological variation in protein content or to artifactual fiber damage sustained during tissue processing. Nevertheless, the observations of focal depletion from necrotic fibers do suggest that Fabp3 increases in serum can be connected to decreased Fabp3 concentrations in compound-damaged muscle fibers.
Serum biomarkers measured at approximately 24 h after the last daily dose are presented relative to the severity of compound-mediated SKM necrosis observed for individual rats (Figs. 4A–D). These data show a clear increase in serum Fabp3 concentration with increasing severity of SKM necrosis (Fig. 4A). Serum AST activity also increases with increasing severity of SKM necrosis (Fig. 4B), but to a lesser magnitude than Fabp3, particularly in animals with minimal SKM necrosis. Serum CK and ALT activities also increase due to SKM necrosis (Figs. 4C and 4D), but to a lesser magnitude and primarily in animals with more severe SKM necrosis.

The possibility that one or more biomarkers would be premonitory of SKM necrosis was also considered. Based on experiments involving exercised-induced or mechanical injury to cells (McNeil and Ito, 1990; McNeil and Khakee, 1992), we suspect that minimal exposure to some drugs may cause cell membrane disruptions that are capable of leaking biomarkers but represent reversible injury that may not progress to myocyte necrosis likely to be detected by routine histology. Serum biomarker concentrations in individual rats administered compounds that did not cause SKM necrosis at a low dose were plotted separately if a higher dose of the same compound or additional dose administrations did cause SKM necrosis in other rats (histogram labeled no observed SKM necrosis/low dose of a compound that caused SKM necrosis at higher dose or more doses in Figs. 4A–D). Of the four biomarkers, only Fabp3 concentration was increased in these rats compared with rats dosed with compounds that did not cause SKM necrosis at any dose or exposure duration. This observation suggests that Fabp3 might be premonitory of histologically detectable SKM injury.

Serum biomarker concentrations/activities are also presented according to whether individual rats had histological evidence of SKM necrosis or not, independent of the severity of necrosis (Figs. 4E–H). Serum Fabp3 concentrations, AST activity, and ALT activity were greater in individual rats for which there was histological evidence of SKM necrosis compared with those rats for which there was no such evidence; in contrast, serum CK activity showed no difference between the groups. As with the previous analysis (Figs. 4A–D), the rank order of sensitivity of individual serum biomarkers for predicting SKM necrosis at the 24-h sampling time remained Fabp3 > AST > ALT > CK.

The relationships between serum biomarker concentrations and prevalence of SKM necrosis are presented (Fig. 4I–L). Relatively low values of Fabp3 and AST are consistent with low prevalence of rats having SKM necrosis. This is revealed by the red line, which shows a high percent of rats without SKM necrosis occurring at low biomarker concentrations. High values for Fabp3 and AST are consistent with high prevalence of rats having SKM necrosis. This is revealed by the blue line, which shows a high percent of rats with SKM necrosis at higher biomarker concentrations. High values for Fabp3 and AST are consistent with high prevalence of rats having SKM necrosis. This is revealed by the blue line, which shows a high percent of rats with SKM necrosis at higher biomarker concentrations, eventually climbing to 100% as Fabp3, AST, and ALT values increase. A single animal with an Fabp3 concentration close to 100 ng/ml that did not have observed SKM necrosis was excluded from all plots in Figure 4. This animal also had an AST value 19-fold higher than the concurrent control group mean. The other four animals in the same treatment group had SKM necrosis with severities described as slight, moderate, and marked. It is unlikely that this animal was truly free of SKM necrosis, but rather this may represent the occasional animal for which the histology sampling paradigm did not locate the morphologic change. Aside from this individual, 100% of rats with ≥ 26 ng/ml Fabp3 in serum had observed SKM necrosis.

Overlapping distributions of all four serum biomarker concentration/activities were observed between rats with and without SKM necrosis. This made prediction of SKM injury in individual rats challenging, and indicated that statistical comparisons are most appropriate at the treatment group level.

**Descriptive statistics at the treatment group level.** A treatment group in these studies consisted of three to ten rats...
In both cases, Fabp3/AST performed better than did CK/AST. Tissue specific considerations for the combined use of these biomarkers. A combination of CK/AST (Table 4B–C). Two different applications were considered for the combined use of these biomarkers. In both cases, Fabp3/AST performed better than did CK/AST.

We also evaluated the use of Fabp3/AST in combination as predictors of SKM necrosis compared with the traditional combination of CK/AST (Table 4B–C). Two different applications were considered for the combined use of these biomarkers. In both cases, Fabp3/AST performed better than did CK/AST.

Predictivity at the treatment group level in short duration toxicology studies. Calculations of concordance, PPV, NPV, specificity, sensitivity, FPR, and FNR were made at the treatment group level for Fabp3, AST, CK, and ALT (Table 4). Fabp3 performed better than these traditional markers by all measures, with the exception of CK having higher specificity and lower FPR. These apparent advantages of CK, however, were overshadowed by its low sensitivity and high FNR (Table 4A).

Under the first application, it was most important that positive predictions be correct (Table 4B). Therefore, both biomarkers were required to be significantly greater than controls in order to make a prediction that SKM necrosis had occurred. If one or both biomarkers were not significantly increased, the prediction was that SKM necrosis had not occurred. This approach would remove from development those drug candidates with greatest probability of causing SKM necrosis and allow all others to proceed for further testing. Under these conditions, the FPR for Fabp3/AST was very low (<2% for Fabp3/AST), PPV increased from 73% (CK/AST) to 94% (Fabp3/AST), specificity increased from 95% to 98%, sensitivity increased from 28% to 55%, NPV increased from 71% to 81%, and concordance increased from 71% (CK/AST) to 83% (Fabp3/AST).

Under the second application, it was most important that negative predictions be correct (Table 4C). Therefore, both biomarkers were required to be not significantly different from controls to make a prediction that SKM necrosis had not occurred. Under these conditions, the FNR was lower for Fabp3/AST (21%) than for CK/AST (35%). NPV and sensitivity were also improved using Fabp3/AST compared with CK/AST. This approach would be useful when one wanted assurance from the Toxicology biomarkers that the compound brought forward for development had the lowest probability of causing SKM necrosis.

Considerations of Tissue Specificity in the Interpretation of Fabp3 Concentrations

cTnI concentrations were determined for rats on most studies as a project-related parameter used in addition to heart histopathology to monitor cardiac injury. Microscopic evidence of heart muscle necrosis was observed in only 2 of 81 treatment groups (Table 2, treatment groups 14 and 25). Treatment 14 was the only treatment group to have cTnI increased more than 2.8-fold higher than the concurrent control group mean. Because heart is a significant source of Fabp3 (Table 1, Fig. 1, Crisman et al., 1987; Vork et al., 1991) and of AST, ALT, and CK (Table 1, Fig. 1, Hoffmann et al., 1999), the absence of heart muscle necrosis enabled us to “rule out” heart injury as the source of increased biomarkers in serum. Fabp3 is also present within renal tubular epithelium, especially tubules within the medullary inner stripe in the rat (Kimura et al., 1991), and so kidney is also a possible source of Fabp3 in serum (Table 1, Fig. 1, Crisman et al., 1987; Maatman et al., 1992; Storch and Thumser, 2000) as well as AST and ALT (Table 1). Minimal renal tubular necrosis was observed in only 3 of 81 treatment groups (Table 2, treatment groups 39 and 72 [primarily medullary inner stripe tubules] and 47 [primarily cortical tubules]). None of these groups had Fabp3 concentrations increased more than 2.9-fold higher than the respective concurrent control group so kidney injury was not considered to be a significant source of increased serological Fabp3 in these groups. Because of the minimal grade in these few rats
FIG. 4.  (A–D) Box plots of log, base 2, transformed biomarker values for the categories: NO = no observed SKM necrosis; LD = low dose of a compound that caused SKM necrosis at higher dose or more doses; Severity: MI = minimal < SL = slight < MO = moderate < MA = marked. Boxes contain the middle 50% of the data (interquartile range). Bold line in box represents median value. Whiskers indicate the minimum and maximum data values, unless outliers are
and because appropriate time point evaluation of serum Fabp3 concentrations in rats with renal tubular injury have not been performed, a possible association of renal injury and serum Fabp3 changes remain to be fully characterized. It should be noted that injured renal tubular epithelium would likely release Fabp3 into urine rather than into serum. Microscopic evidence of liver necrosis was observed in several groups (Table 2, treatment groups 8, 9, 20, 21, 23, 24, 26, 28, 30, 34, 37, 41, 61, and 64). Although these groups did have increased ALT (1.2–30×) and AST (1.2–20.3×) activity compared with concurrent control groups, the Fabp3 concentration in liver tissue as well as the total pool of Fabp3 in the liver (Table 1, Fig. 1) is too small to make a significant contribution to the Fabp3 concentrations measured in serum.

**DISCUSSION**

**Biomarker Predictivity**

New serological biomarkers of SKM necrosis would be useful if they could improve upon the predictive power of existing biomarkers of SKM injury. We explored the tissue distribution of Fabp3 protein, the kinetics and amplitude of plasma concentrations of Fabp3 during compound-mediated SKM necrosis, and the focal depletion of immunodetectable Fabp3 protein from injured SKM fibers. We then compared the predictive power of Fabp3 with that of AST, CK, and ALT for SKM necrosis detected by histopathology in rat toxicology studies. We focused our assessment on widely-accepted statistical parameters of biomarker performance: concordance, PPV, NPV, specificity, sensitivity, FPR, and FNR. The data used for the comparisons came from short duration (4–14 days) rat toxicology studies involving 27 drug candidates that have high affinity for a range of drug targets that include G-PCRs, nuclear receptors, and kinases. The 27 compounds were tested at multiple doses in 81 treatment groups involving 585 rats, and showed clear separation between the biomarkers in terms of their predictive power.

Fabp3 had the greatest concordance, PPV, NPV, and sensitivity among the four biomarkers tested at 24 h after the final compound dose. AST was the next most valuable single predictor of SKM necrosis; CK and ALT were comparatively poor predictors of SKM necrosis at this 24-h blood collection time.

Consideration was given to the use of these biomarkers in combination. Predictions were improved by the combined use of Fabp3 and AST. PPV for the combined use of Fabp3 and AST increased to 94% when both biomarkers were used to predict SKM necrosis, compared with 71% and 66% for Fabp3 and AST alone, respectively (Table 4). This improvement in PPV only slightly diminished the NPV compared with Fabp3 and AST used alone. Therefore, if only a single serological biomarker of SKM necrosis were to be used under conditions similar to these studies, Fabp3 would be the most valuable. If two biomarkers of SKM necrosis were to be used, Fabp3 and AST would be the most valuable.

These experiments suggest Fabp3 is a useful biomarker for diagnosing SKM necrosis in rat toxicology studies, and that it can be used in combination with existing SKM toxicology biomarkers to improve our ability to predict SKM necrosis in rats. It will be important to get additional data beyond the relatively small group of drug targets and chemical structures reported here. In addition, we only investigated blood collected 24 h after the last dose of compound. No single blood collection time is ideal because compounds vary in their toxicokinetic/toxicodynamic relationships that lead to SKM injury, and because biomarkers possess different properties of release from injury and clearance from blood, such that there is no optimal time for measuring all biomarker responses. The standard blood collection time for serum chemistry biomarkers (e.g., AST, ALT, and CK) on our lead optimization rat studies is 24 h after the last daily dose—the same time as collection of tissues for microscopic evaluation. Instead of attempting to characterize the postdose timing of various treatment-induced injuries and comparing biomarkers in blood collected at each of their optimal postdose sample times, we chose to compare Fabp3 to established biomarkers and histology observations at the typical 24-h collection time. With this approach, we evaluated the predictivity of Fabp3 by the most convenient way to introduce Fabp3 within existing toxicology study protocols, not by its optimal potential for identifying SKM injury.

**Safety Biomarkers in Preclinical Drug Development**

Serological biomarkers are used to help assess the safety of compounds both in clinical trials and in nonclinical toxicology studies. The value of serological biomarkers differs significantly between these two settings, however, according to the availability and influence of histopathology data. In clinical trials, histopathology data are rarely available, serological biomarkers are routinely measured, and consequently the influence of serological biomarkers on clinical safety assessments is high. In toxicology studies, on the other hand, histopathology assessments of animal tissues are routinely conducted, and their influence on compound safety assessments usually supersedes that of serological biomarkers. What then is the rationale for developing additional biomarkers of toxicity for nonclinical applications?
<table>
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<tr>
<th>Treatment</th>
<th>Incidence of SKM necrosis (%)</th>
<th>Fold increase for Fabp3</th>
<th>Mean Fabp3 fold increase</th>
<th>Fold increase for AST</th>
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Treatments 1–15 caused high incidence of SKM necrosis (80–100%).

Treatments 16–28 caused lower incidence of SKM necrosis (20–66%).

Treatments 29–49 caused 0% incidence of SKM necrosis, Fabp3 increased ≥ twofold.

Treatments 50–81 caused 0% incidence of SKM necrosis, Fabp3 increased < twofold.
Serological biomarkers have important advantages that make them well-suited for assessing compound-related toxicities early in drug development. The data are quantitative and, as shown here, their predictive value can be assessed quantitatively. Repeated sampling can be made of test subjects, thereby increasing the value of animals used in drug development and analyses can be automated for relatively low cost and rapid turnaround time. In conventional toxicology studies, however, these advantages are lost to the lengthy timelines and expenses associated with histopathology assessments of tissues from the same animals from which the serological biomarker data were obtained.

A more valuable point for implementation of serological biomarkers of toxicity may be earlier in the development process, such as in preclinical pharmacology studies during lead generation and early lead optimization. At this early stage of drug development, many compounds are being evaluated for a multiplicity of properties that, individually or in combination, influence the identification and advancement of new lead molecules. In vivo toxicology data, though valuable, are usually not collected at this early stage of drug development. In part this reflects the view that histopathology data are required to make decisions about the safety of a molecule in nonclinical experiments. However, incorporation of serological biomarkers of toxicity into in vivo pharmacology models helps the toxicologist identify target organs of toxicity and assess margins of safety in the same in vivo experiments as are used by the pharmacologist to assess target modulation and compound efficacy. We have found this approach to be useful in identifying, early in development, compounds with improved margin of safety, and deprioritizing compounds that would have inappropriately been advanced to the more expensive and more time-consuming toxicology studies. We have also increased the numbers of compounds for which we obtain toxicity data.

<table>
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<th>Treatment</th>
<th>Incidence of SKM necrosis (%)</th>
<th>Fold increase for Fabp3 fold increase</th>
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Note. Each of the 81 treatments represents a unique compound/dose level combination. All treatments were 4 or 5 doses except treatments #4, 10, 15, 65, 66 (1 dose), #25, 55, 58 (7 doses), and #9, 12, 28, 50, 59 (14 doses). SKM = SKM.

*Treatments that did not produce SKM necrosis but the same compound did produce SKM necrosis at higher dose level or with greater number of doses.*
by drug developers. This strategy maximizes the advantages of serological biomarkers at a point in drug development that measures risk and benefit in a common biological experiment.

Strength of the Biomarker Panel

The predictive power of a serological biomarker of toxicity is affected by many variables, including the tissue distribution of the biomarker, the nature of tissue injury (tissues injured, time course, severity, and mechanism), the magnitude and rate of release of the biomarker into the blood from the injured tissue, and the clearance of the biomarker from the blood. Additional variables that would be expected to affect biomarker predictive performance due to inability to detect the protein include structural modifications of the protein (e.g., oxidation of troponins—Horwitz et al., 1979) and interactions of the protein with other biological molecules (Katrukha et al., 1997).

Biomarker distribution can be a major determinant of the predictive value of a biomarker. Fabp3 is a cytosolic protein that binds fatty acids for cellular metabolism (Glatz et al., 2003; Storch and Thumser, 2000), and consequently is expressed at higher concentration in muscle groups where the primary metabolism is oxidation of fatty acids. Data from our laboratory (Table 1) and others (Armstrong and Phelps, 1984; Koerker et al., 1990; Vork et al., 1991) confirm that soleus and diaphragm muscles consist primarily of type I (SO) fibers and contain among the highest concentrations of Fabp3 per unit of total protein (Crisman et al., 1987; Vork et al., 1991). One would therefore expect that compounds that injure type 1 muscle fibers would cause increases in serological Fabp3.

PPAR-α agonists often cause SKM necrosis focused in type 1 muscle fibers, and caused significant increases in serological Fabp3 concentrations after just two oral doses (Fig. 2A). Administration of this and related compounds caused more microscopic lesions in soleus (primarily SO) than in biceps femoris (primarily FG). Similar observations of fiber type sensitivity have been made by other investigators for PPAR-α agonists (DeSouza et al., 2006). On the other hand, statins cause SKM necrosis predominantly in type 2 muscle fibers (Smith et al., 1991, Westwood et al., 2005). Therefore, one would expect SKM necrosis caused by statins to cause relatively small increases in serological Fabp3 concentrations compared with biomarker proteins expressed at higher concentrations in type 2 muscle fibers (e.g., parvalbumin or skeletal troponin I—Celio and Heizmann, 1982; Simpson et al., 2005).

Tissue distribution is an important, but insufficient, determinant of biomarker performance. CK was the most specific SKM biomarker based on tissue distribution, yet it was the least useful biomarker for predicting SKM necrosis in these studies. This may be due to the short half-life of CK in the blood of rats (Hoffmann et al., 1999) that is less suitable for detection with blood sampling 24 h after last compound administration in the toxicology studies.

### Table 3

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<th>Model compound</th>
<th>Dose (mg compound/kg body weight)</th>
<th>Number of administrations</th>
<th>Number of rats with SKM necrosis</th>
<th>Mean Fabp3 concentration in serum [range] (ng/ml)</th>
<th>Treatment ID in Table 2</th>
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<td>1.3 [1.0–3.0]</td>
<td>n/a</td>
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<tr>
<td>6</td>
<td>9 of 10</td>
<td>10</td>
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<td>61.7 [31.8–100.9]</td>
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<tr>
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<td>4</td>
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<tr>
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*100% early mortality of animals administered 3mg/kg in the 14-day group.
All four biomarkers measured in these studies occur at high concentration in one or more SKMs, but they also occur in other tissues. To the extent that they lack tissue specificity, they are more likely to have decreased predictive value as a diagnostic of SKM injury. Biomarkers that are both highly sensitive and highly specific are difficult to identify, therefore a panel of biomarkers is more likely than an individual biomarker to achieve the desired balance of sensitivity and specificity. We are currently evaluating additional markers that can complement those explored here as a way of being more predictive of injury to both type I (SO) and type II (FG) muscle fibers.

**ACKNOWLEDGMENTS**

The authors wish to thank Robert Jolly for his assistance in gathering data on binding affinity (Ki) at the PPAR-alpha receptor.

**REFERENCES**


