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Myotoxicity of Gemfibrozil in Cynomolgus Monkey Model and Its Relationship to Pharmacokinetic Properties

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Abstract

Fibrate drugs are PPAR α agonists prescribed for the treatment of dyslipidemia. Severe myotoxicity has been reported associated with their use albeit at a low frequency, especially for gemfibrozil. Few studies have investigated the mechanism of fibrate-induced myotoxicity *in vivo*. Considering the apparent species-related differences in PPAR α agonist-induced hepatotoxicity, we studied the myotoxicity of gemfibrozil in a Cynomolgus monkey model and explored the relationship between myotoxicity and pharmacokinetics. Six Cynomolgus monkeys were dosed with gemfibrozil twice daily at 600 mg/kg/day for the first two periods (P1 and P2, 8 days and 9 days respectively) and 300 mg/kg/day for the third period (P3, 14 days). Creatine kinase and myoglobin were measured, together with hepatotoxicity and nephrotoxicity markers. Behavioral responses were recorded for indication of toxicity. Pharmacokinetics was carried out following the 16th dosage of P1 and 17th dosage of P2 when myotoxicity was identified. Multivariable data analysis was employed to explore the relationship between pharmacokinetic parameters and myotoxicity markers. Consequently, myotoxicity occurred in monkey #2 (M2) and M6 in P1, M3 and M4 in P2, M3 and M6 in P3. Data analysis showed T80-150 (sustained time above the given concentration) contributed for myotoxicity discriminance and correlated with myotoxicity risk. This study revealed Cynomolgus monkey may be a good animal model for myotoxicity evaluation with sensitivity, reproducibility and similarities to humans. More interestingly they exhibited a much higher incidence of myotoxicity than that of human. Sustained high drug concentration plays an important role for the occurrence of myotoxicity. This may suggest an influence of drug transport and metabolism on myotoxicity.

Keywords

Gemfibrozil; Myotoxicity; Cynomolgus monkey; Animal model; Pharmacokinetics

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Introduction

Fibrates such as gemfibrozil, fenofibrate, and clofibrate are a group of agonists for the nuclear receptor peroxisome proliferator-activated receptor α (PPAR α) that regulates genes encoding enzymes involved in peroxisomal and mitochondrial fatty acid oxidation and glucose metabolism (Peters *et al.*, 2005). PPAR α heterodimerizes with the retinoic X receptor and then binds to the promoter of genes containing direct repeat 1 peroxisome proliferator response elements. Fibrates thus reduce hepatic lipids and serum triglycerides that are associated with increased risk of atherosclerosis (Barter *et al.*, 2007). Fibrates have been used for treatment of hypertriglyceridemia and mixed hyperlipidemia. However, fibrates as well as statins, another class of commonly used lipid lowering agent, are known to cause myotoxicity (Graham *et al.*, 2004). The incidence of myotoxicity associated with fibrates as monotherapy was estimated to be a 5.5-fold higher than that of statins. Coadministration of fibrates and statins can increase risk by additional 2-fold increased risk (Staffa *et al.*, 2002; Graham *et al.*, 2004). Gemfibrozil may caused much higher risk as monotherapy than other fibrates (Graham *et al.*, 2004) and accounted most for deaths caused by combination of cerivastatin and fibrates (Staffa *et al.*, 2002).

Mechanistic investigations of the myotoxicity caused by statins and fibrates have been focused on the former that came to the market in the 1990's when safety evaluation attracted more attention. Myotoxicity studies of simvastatin, pravastatin, lovastatin, and cerivastatin have been carried out in rats, mice, and rabbits (Sonoda *et al.*, 1994; Nakahara *et al.*, 1998; Schaefer *et al.*, 2004; Hanai *et al.*, 2007) with diverse results. For the myotoxicity of fibrates, limited investigations have been performed despite their long clinical history. Myotoxicity of fenofibrate in the rat was reported, but endpoints for myotoxicity or detailed results were not provided (Plasterer *et al.*, 2007). Recent study in rat by pathological analysis indicated a susceptibility difference for skeletal muscle caused by clofibrate and cerivastatin (Schaefer *et al.*, 2004; Okada *et al.*, 2007). In homogenates of rat skeletal muscle, fibrates induce mitochondrial dysfunction where fenofibrate predominantly acts by inhibition of complex I of the respiratory chain (Brunmair *et al.*, 2004). Studies in rat identified a PPAR α activation signature as fiber-type-selective and no off-target pathways were found that may drive adverse effects (De Souza *et al.*, 2006). In rat skeletal muscle cultures, gemfibrozil and bezafibrate increased the percentage of apoptosis at micromolar concentrations. It was proposed that PPAR α agonism partly mediates the toxicity response to PPAR α agonists and that PPAR α agonists and statins cause myotoxicity through independent pathways (Johnson *et al.*, 2005).

Significant species differences exist for fibrates both pharmacodynamically and pharmacokinetically. Fibrates do not cause a rodent-type peroxisome proliferation and carcinogenesis response in either primate liver or cultured hepatocytes (Sausen *et al.*, 1995). Administration of gemfibrozil at 300 mg/kg/day to monkeys resulted in a 3-5 fold increase in peroxisome number and a 1-3-fold increase in peroxisome volume. However, equal dosage in rats resulted in a 7-fold increase in peroxisome number and over a 20-fold increase in peroxisome volume (Gray and de la Iglesia, 1984). This indicates the different refractory nature of primate and rodent livers to gemfibrozil that is similar to results with other fibrates (Hoivik *et al.*, 2004). Pharmacokinetically, many studies showed species difference of gemfibrozil in the excretion of metabolites, as well as bioavailability and elimination half-life (Okerholm *et al.*, 1976; Dix *et al.*, 1999). Rats excreted 25% of an oral dose of gemfibrozil in urine and nearly 50% in feces, and cumulative urinary and fecal excretion of gemfibrozil after a single oral dose were dose dependent. In contrast, humans excreted approximately 66% of an oral dose in urine and only 6% in feces. Pharmacokinetic profile of gemfibrozil in rhesus monkeys showed more similarity to humans than that of other species (Okerholm *et al.*, 1976). Additionally, PPAR α expression was found to be greatest in liver in rodents but in skeletal muscle for humans, and a species related responsiveness to PPAR activators between rats and human was

reported (Mukherjee *et al.*, 1994; Palmer *et al.*, 1998). By using a closer genetic relationship with humans and their resemblances, non human primates such as monkeys may be good animal models for toxicity studies.

Complicated drug-drug interactions caused by gemfibrozil have been recently elucidated (Shitara *et al.*, 2003). Intake of cerivastatin by hepatocyte transporter organic anion-transporting polypeptides 2 is inhibited by gemfibrozil and its metabolite. Gemfibrozil most potently inhibits cytochrome P450 2C9, and gemfibrozil glucuronide was found to be a mechanism-dependent inhibitor of P450 2C8. For UDP-glucuronosyltransferases (UGT), gemfibrozil can intensively inhibit UGT 1A1 and UGT 1A3 that are largely responsible for the glucuronidation and elimination of statins (Ogilvie *et al.*, 2006). Clinical studies indicated Gemfibrozil greatly increased area under concentration-time curve ($AUC_{0-\infty}$), peak concentration (C_{max}) or elimination half-life ($T_{1/2}$) of simvastatin, pravastatin, and cerivastatin (more than a 5 fold increased and maximum concentration for cerivastatin). These pharmacokinetic interactions may partly explain the high incidence of myopathy observed with the combination of fibrates and statins, but the role of pharmacodynamic modification cannot be excluded (Backman *et al.*, 2002).

The complexity to induce myotoxicity of fibrates is still poorly understood. A primate model of myotoxicity would yield more significant clinical relevance regarding the species difference in response to fibrates. In the present study, the ability of gemfibrozil, which produces the highest rate of myotoxicity among fibrates, was investigated to induce myotoxicity in Cynomolgus monkeys.

Materials and methods

Chemicals and reagents

Gemfibrozil was obtained from Hunan Qianjin Xiangjiang Pharmaceutical Co. Ltd. Sodium carboxymethylcellulose (CMC-Na) was purchased from Shanghai RichJoint Chemical Reagents Co.Ltd. Bezafibrate was purchased from Sigma-Aldrich. High performance liquid chromatography (HPLC) grade solvents (acetonitrile, ethanol) were purchased from Sigma-Aldrich Co.Ltd. Biochemical analysis kits of alanine aminotransferase (ALT), aspartate aminotransferase (AST), lactate dehydrogenase (LDH), creatinine (CRE), blood urea nitrogen (BUN) and creatinase (CK) were purchased from Shanghai Kehua Bio-engineering Co.Ltd. Myoglobin (MYO) analysis kit and standard of Trucal Myoglobin were purchased from DiaSys Diagnostic Systems GmbH.

Animals and treatments

Three male (M1, 2, 3) and three female (M4, 5, 6) Cynomolgus monkeys (*Macaca fascicularis*), 4-5 years old and weighing about 3-7 kg, were obtained from South-China Primate Research & Development Center[SCXK (Guangdong Province) 2004-001020066025]. All work was performed in accordance with Public Health Service policies, the Animal Welfare Act, and the Laboratory Animal Committee (LAC) of GIBH (Guangzhou Institute of Biomedicine and Health, Chinese Academy of Sciences) Policy on the Humane Care and Use of Vertebrate Animals.

Those monkeys were individually housed in suspended, stainless steel cages and were maintained under a standard 12h light/12h dark cycle with free access to water. Environmental controls for the animal rooms were set to maintain 18~29°C and a relative humidity of 30~70%. A certified primate pellet diet of a commercial source was provided twice daily about 2~3 h after dosing in the morning and before dosing in the afternoon respectively. Fruits were supplemented regularly for nutritional requirement as the common practice.

Three periods of experiments were designed. Dosages of the first two periods (P1 and P2) were 600mg/kg/day and of the third period (P3) was 300mg/kg/day. The dosages were given twice daily about 12h apart and at least six weeks of clearance was allowed between successive periods. Four days before the start of each period, vehicle of 0.5% CMC-Na (g/ml in purified water) was given by individualized volume. Monkeys were dosed with gemfibrozil by oral *gavage* and the dosing volume was accordingly recalculated when the body weight was newly determined. When vomiting happened within 2 hours after oral *gavage*, half the dosed volume was supplemented. For P1 and P2, when myotoxicity occurrence was indicated, dose treatment was stopped for the whole colony according to the protocol. For P3, dose period was lasted for 14 days which was approximately two-fold length of P1 and P2; however, when the myotoxicity occurred, the dose treatment for such individuals was stopped while others continued. This design avoided irreversible pathological changes and severe fatal rhabdomyolysis. Thus it helped us both monitor the myotoxicity occurrence and ensure the animal welfare of non human primate (NHP) animal model. Data collected in the three phases of studies included body weight, biochemistry status, behavioral responses, pharmacokinetic analysis, toxicology and pathology analysis.

Biochemical status monitoring

For biochemistry status monitoring, ALT, AST, LDH, CRE and BUN were selected as indicators of hepatotoxicity and nephrotoxicity of gemfibrozil. In addition to CK, which is used clinically as marker of myotoxicity of statins and fibrates, MYO was also determined as another specific endpoint of myotoxicity in this study. Thus, about 0.6ml blood was withdrawn from veins from anterior or hind limbs once daily for three days before the first dosing of gemfibrozil, once 2 or 3 days during the experiment and till the recovery of toxic effect after dosing finished for each period. The isolated serum after complete blood coagulation was transported at 0~8°C and analyzed as soon as possible by an automatic biochemistry analyzer (SHIMADZU CL-8000, Japan).

Phenotype and myotoxic behavioral response

The food-intake was closely observed and the body weight was determined every 3 days. Phenotypes of behavioral responses were intensively recorded for judgement of myotoxicity in addition to the above toxic parameters for myotoxicity, hepatotoxicity and nephrotoxicity.

Pharmacokinetics

Pharmacokinetics was carried out for the 16th and 17th dosage of P1 and P2 respectively when myotoxicity was found to have occurred. For pharmacokinetics of P1, 0.3ml blood was drawn at 0, 0.5, 1.5, 2, 3, 4, 7, 12h based on clinical half-life time. For P2, the blood samples were drawn at 0, 2, 4, 6, 8, 10, 13, 18, 24h which was adjusted according to the low elimination of P1 by its pharmacokinetic result. For P3, because the myotoxicity occurred at different time points, the trough concentrations instead of pharmacokinetics were analyzed for exposure evaluation. All the blood samples were immediately centrifuged at 4000rcf for 15min for collection of plasma and then kept at -80°C till analysis. The gemfibrozil concentrations in the plasma were determined by validated LC/MS/MS method with bezafibrate as an internal standard.

After addition of bezafibrate, gemfibrozil was extracted from 20 µl of plasma by protein precipitation with acetonitrile added to a final volume of 1ml. The samples were centrifuged at 16000rcf at 4°C for 30 min and 25 µl of the supernatant was further diluted to 1ml with methanol:water (50:50). An aliquot of 10µl of supernatant was subjected to LC/MS/MS analysis by use of an ABI3000 mass spectrometer (Applied Biosystems, USA) coupled with HPLC (SHIMADZU 10A Japan) and an MPS3C autosampler (GERSTEL, Germany) three of which were controlled by Analyst 1.4.2 workstation software. The separation was performed

on a CAPCELL PAK C18 column (5 μ m, 2.0mmID*50mm) at room temperature with the flow rate of 0.2ml/min. LC gradient was applied as the following: 50%B (methanol containing 0.1% formic acid) for 0.3min, then a linear gradient to 100%B to 1.5min and held for 1.5min, lastly dropped to 50%B within 0.3min for equilibration. The mass spectrometer was operated in negative ion mode using multiple reaction monitoring with transitions m/z 360.1/274.3 for bezafibrate and 249.1/121.1 for gemfibrozil. The turbo ion spray was maintained at 350°C with 4500V applied to the spray needle. Nitrogen was used as nebulizer gas, curtain gas and collision gas. All the raw data was processed using the Analyst software 1.4.

Histological Analysis

Tissues of liver, kidney and other organs of M2 who died accidentally during P3 were fixed in 10% phosphate-buffered formalin, embedded in paraffin, sectioned at 5 μ m, and stained with hematoxylin and eosin (HE). The sections were examined under light microscopy by a pathologist in a blind manner and pathological alterations were recorded.

Data Analysis

For the body weight changes, paired-samples T test was used for comparison by gender respectively (SPSS 11.5 for Windows). The myotoxicity criteria were employed based on two times of increase of CK or MYO and obviously changed behavioral responses. When two or three of these above criteria were met for an individual monkey, myotoxicity was confirmed while clinical diagnosis of myotoxicity by approximately 10-fold increase of CK (McClure *et al.*, 2007). For hepatotoxicity and nephrotoxicity evaluations, when the marker values increased two times of the base line level, toxicity related increase was defined.

Pharmacokinetic parameters such elimination half-life ($T_{1/2}$), peak concentration (C_{max}) for each individual were estimated using DAS2.0 (Anhui Medical University of China, 2002). Exposure parameters, such as T_x (time length at which drug concentration above a given concentration x) and the corresponding area under concentration-time curve (AUC_x) were calculated by a self-developed program in MATLAB (MathWorks, version 6.5, 2002). Because the experimental sample was small and some of those parameters were not independent variables, linear regression or logistic analysis could not be used for data analysis. Thus the above pharmacokinetic parameters and any combination of them were imported into SIMCA-P+11 (Umetrics AB, Umea, Sweden) as X variables. Myotoxic markers CK and MYO were imported as Y variables. Projection to latent structures discriminant analysis (PLS-DA, a multiple regression method which can cope with multiple X and Y variables and can indicate those contributing and predictive X variables) was performed to explore the parameters for grouping the myotoxic and non-myotoxic clusters which may explain the risk of myotoxicity.

Results

Phenotype of gemfibrozil treated monkeys

For treated monkeys, serious anorexia occurred and their appetite for primate pellet was depressed by the gemfibrozil treatment. The average body weights decreased during the three periods of experiments. For the low dosage of P3, the average body weight (expressed as means $\pm$ SD) of male monkeys decreased by 14.8 $\pm$ 4% ($t=4.198$, $P=0.052$) and the female monkeys by 6.5 $\pm$ 0.5% ($t=35.501$, $P=0.001$). For the high dosage of P2, the body weight changed more than that for the low dosage (male: 12.37 $\pm$ 1.6%, $t=6.806$, $P=0.021$; female: 14.3 $\pm$ 3.9%, $t=8.193$, $P=0.015$). Additionally, occasional vomiting occurred for both dosage levels and this happened more frequently for males.

Myotoxicity of gemfibrozil treatment

During P1, CK of M2 began increasing more than 3 times after 4 days of dosing. While on the 4th day after CK levels increased, its MYO level increased approximately 8 times. For M6, the CK and MYO increased simultaneously after 8 days of dosing treatment. Their fold increase in M6 was about 2 to 3 times which was lower than that of M2 (Figure 1). During P2, the CK of M4 increased larger than 2-fold after 5 days of dosing and the increase of MYO occurred 3 days later. For M3, the severely changed behavioral responses occurred on the 8th day of treatment, and accordingly the blood sampling on this day was not successfully performed. Considering the approximately 4-fold increased CK and MYO detected 3 days after the 8-day treatment (Figure 2), their fold increase would probably be even higher on the 8th of dosing treatment when behavioral responses appeared. During the P3 stage, CK and MYO of M3 increased about 11 days after dosing treatment. The fold increase was both about 5 times of its baseline levels. As for M6, considering its obviously changed behavioral response and elevated MYO level 3 days after clearance, we presumed the occurrence of myotoxicity, though its CK levels remained unchanged all times (Figure 3).

When CK and/or MYO were abnormally increased, the monkeys showed behavioral response changes (data of representative pictures for the three periods not showed). The detailed behavioral changes and the detected peak fold increase were used for identification of occurrence of myotoxicity (Table 1).

According to the above increased biochemical markers and changes of behavioral response, myotoxicity was confirmed for M2 and M6 in P1, M3 and M4 in P2, M3 and M6 in P3. Thus the myotoxicity incidence rate was 2/6 for one period and 4/6 for three periods. When the myotoxicity occurred, the drug administration was stopped according to the protocol.

Pharmacokinetics and myotoxicity relationship

By LC/MS/MS analysis, concentration of gemfibrozil after 8 days of dosing reached an average of 200 mg/l during P1 and P2 (Figure 4 A and B). The high concentration fluctuated during 12 hours for P1 and 24 hours for P2 at the end of the two high dosage periods which is probably due to the saturated absorption, distribution, metabolism, and excretion (ADME) process. For P3, trough concentrations of d3, d7, d11 were analyzed and average concentration was calculated (Figure 4 C).

After parameters were calculated by a MATLAB based program and pharmacokinetic software DAS, multivariate data analysis was performed. As the score plots shown in Figure 5A and C, the myotoxic individuals deviated from the cluster of non-myotoxic individuals and those contributing variables were identified in the corresponding left-down side in the loading plots (Figure 5 B and D). T values of 80-150 (the sustained time length of drug concentration from 80 to 150 mg/l) contributing for grouping clusters of the myotoxic and non-myotoxic individuals in P1 were identified. For P2, T values of 70-230 were identified (enlarged range probably because parameters of myotoxic 3 were missed due to the reason indicated above). By careful examination of the T values of 80-150 for both P1 and P2 and the myotoxicity rate of those monkeys during the three treatment periods (Table 2), it is evident that when the T value of 140-150 was above about 10h, the myotoxicity was almost certain (M2, 3, 4, 6 are the cases). T80-150 of M1 was lower for both two high dosage periods and myotoxicity never occurred. Thus, the exposure levels by a combination of exposure concentration and sustained time played a more important role in myotoxicity. The average trough concentrations of P3 which could most represent the overall exposure levels of P3 treatment (Figure 4 C) showed that exposure levels of myotoxic M2, 3, 4 and 6 were higher than that of M1.

Hepatotoxicity, Nephrotoxicity and Pathology

As determined by biochemistry auto-analyzer (SHIMADZU CL-8000), liver and kidney related toxicity markers such as ALT, AST, LDH, BUN and CRE were monitored and the incidence of toxic reaction recorded for the three periods of experimentation (data not showed). Dosage of 600mg/kg/day for 8 days caused high rate of hepatotoxicity (3/6 for P1 and 6/6 for P2) and nephrotoxicity (6/6 for both P1 and P2). When the dosage was lowered to 300 mg/kg/day in P3, the hepatotoxicity and nephrotoxicity incidences were 2/6 and 5/6 respectively.

As an unexpected result of this myotoxicity study, M2 unfortunately died during P3. Its hepatotoxicity biochemistry analysis history was recorded as Table 3. The ALT and AST were slightly elevated during P1 and P2 at the end of dosing treatment (about 8 days after dosing treatment). During P3, its ALT and AST increased about 50 and 12 times of the baseline level about 11 days after dosing treatment, though the dosage was only one half of P1 and P2 (Table 3). Pathology analysis by HE staining revealed it might die of severe hepatotoxicity. As newly developed pathological changes, extensive necrosis and adipose degeneration of hepatocytes was obvious and generous infiltration of inflammatory cell was found (Figure 6 A and B). Light microscopy exhibited lamellar calcification coming from obsolete pathological changes which were probably caused by treatment of P1 and P2 (Figure 6 A). For nephrotoxicity, some of renal tubules demonstrated features of degeneration and necrosis (Figure 6 C). Also protein cast and proteic exudatum were found in some renal glomeruli (Figure 6 D) and infiltration of lymphocyte in renal interstitium was indicated (data not showed).

Discussion

Myotoxicity of statins has been successfully induced in animal models such as rats, rabbits and mice (Sonoda *et al.*, 1994; Nakahara *et al.*, 1998; Schaefer *et al.*, 2004; Hanai *et al.*, 2007). However, with fibrates, another widely used and clinically important lipid-lowering agent, myotoxicity had been found infrequently as single agent or concomitantly when administered with statins (Graham *et al.*, 2004). Yet no appropriate animal model has been established for myotoxicity research and thus the mechanism of toxicity remains poorly understood (Okada *et al.*, 2007). Several reasons account for this slow-moving progress including the long but presumed safe clinical application, the relative rarity of myotoxicity and the absence of a good animal model. NHP, of which Rhesus and Cynomolgus are two representative species, enjoys a similar physiology, pathology and toxicology with humans and the application of NHP for drug development and evaluation has been considered necessary and imperative.

In the view of animal model, good clinical similarities of myotoxicity in Cynomolgus were demonstrated both biochemically and behaviorally in this study. Clinically a 10 times increase in CK is considered to be a critical marker of myotoxicity and patients usually present complaints of soreness and pains (McClure *et al.*, 2007). In this study, CK levels were actually increased by 2 to 15 times and MYO levels increased in a similar manner when myotoxicity was confirmed. Additionally, MYO levels of more than half of the myotoxic individuals increased as early as CK did and for the others at the same time with behavioral response which occurred later than the CK increase. In P3 in this study, myotoxicity occurred in M6 with MYO increased by more than 3 times and behavior response was obvious. But CK in M6 remained unchanged all the while. This is similar with those case reports where myotoxicity occurred with absence of CK increase which was associated with statins (Phillips *et al.*, 2002). It was also a warning sign that myotoxicity with normal CK may be also the case with fibrates. Thus, elevated CK is not always a sensitive marker for myotoxicity and MYO is helpful as another important marker in some cases. By intensive observation and detailed comparison, significant changes in behavioral response were noted when increased CK and/or MYO were found in this study. All the myotoxic monkeys showed behavioral response (Table 1) and those with normal CK and/or MYO did not. These changed phenotypes were probably results of

tenderness, weakness and limb suffering caused by gemfibrozil treatment which was similar with that of clinical response. These above results showed the value of using Cynomolgus monkey as an animal model for *in vivo* myotoxicity mechanistic investigations.

In an early study where male Cynomolgus monkeys were given fenofibrate for up to 15 days, the magnitude of increase in the peroxisome and mitochondria numbers did not parallel the magnitude of the dose-dependent increase in exposure (Hoivik *et al.*, 2004). However, there were no treatment-related increases in chemical markers of hepatotoxicity and no report of myotoxicity related information. Similarly in our another metabolomic study, when the Cynomolgus monkeys were dosed with fenofibrate (2500 mg/kg/day) for 12 days, liver and kidney related toxicity markers such as ALT, AST, CRE, BUN, LDH were normal in comparison with baseline levels (data not shown), and the appetite and body weight did not change during the treatment period. In term of myotoxicity, CK and MYO detected in that study did not increase and no behavioral responses were observed. Thus, the Cynomolgus monkey resembles the human response in term of sensitivity for hepatotoxicity and myotoxicity where gemfibrozil causes higher risk than fenofibrate. These were in accordance with clinical report that gemfibrozil accounted for most of clinical myotoxicity caused by fibrates (Graham *et al.*, 2004). By comparison with reported studies in rats where 750mg/kg/day of clofibrate and 300mg/kg/day fenofibrate (30 and 60 times of clinical dosage) were dosed, the periods needed for myopathy were about 15 to 30 days (Okada *et al.*, 2007; Plasterer *et al.*, 2007). Contrastingly in this study in P3, 300mg/kg/day was about 15 times of clinical dosage and it took 11 days for occurrence of myotoxicity. Also it can not be overlooked that no biochemical or behavioral endpoints were detailed reported in those study of myotoxicity with rodent. Thus the differences of these three fibrates in rodent and NHP for myotoxicity study were probably due to both sensitivity of two species and myotoxic potential of agents. This strongly supports the serviceability of NHP as an animal model for risk assessment of myotoxicity potential.

It has been difficult to define whether genetic background plays a key role or not considering the extremely low incidence of myotoxicity caused by fibrates clinically. Also a polymorphism is usually considered to have at least a frequency of 1% in the population. In this study, the high incidence of myotoxicity (2/6 in P1, P2 and P3; totally 4/6 for three periods) in this Cynomolgus colony showed its probability of independence upon a genetic polymorphism besides the good reproducibility of the myotoxic response. The non-individual selectivity of myotoxicity for the three periods (m2 and m6 in P1, m3 and m4 in P2, m3 and m6 in P3) indicated that the occurrence of myotoxicity was affected by toxicity tolerance and/or physical conditions. These facts not only suggest that genetic background does not play a decisive roll in the occurrence of myotoxicity, but also demonstrate the good sensitivity and reproducibility of Cynomolgus monkey as an animal model for mechanistic study of myotoxicity caused by gemfibrozil.

In clinical study, trough concentrations of gemfibrozil at steady state were 1.2-1.5 mg/L and the C_{max} was 25.6 mg/L (Schneck *et al.*, 2004) and the range was 22-49 mg/L (Kyrklund *et al.*, 2003). In this study in P1 and P2, the average concentrations were approximately 250mg/L which was about 10 times typical clinical exposure levels in humans (base on C_{max}). The concentration did not decrease for up to 12-24h suggesting that saturated ADME process occurred when the dosage was high. This could partly justify why a higher dosage of 600mg/kg/day did not introduce higher incidence of myotoxicity than that of 300 mg/kg/day. It was believable that exposure base on AUC in this study was much more than 10 times of clinical treatment. Additionally in this study in P3, lower dosage of gemfibrozil took a longer time to induce myotoxicity (11 days of P3 verse 8 days of P1 and P2), and clinically fibrates caused myotoxicity usually more than 100 days after treatment, indicating some dependence upon dosage. By PLS-DA, T80-150 (especially T140-150) correlated well with the risk of occurrence of myotoxicity as indicated in Figure 5 and Table 2. Thus, combined exposure level

of drug concentration and sustained time plays an important role for the occurrence of myotoxicity. This can be partly supported by the shorter time needed for myotoxicity for higher dosages and longer time needed for lower dosage in this study. This indicates the risk importance of combined exposure levels and the time range of the levels, but this does not mean that whenever the parameter is met, myotoxicity will take place. M5 was an example case for this, where myotoxicity never occurred although its T values and average concentration were high in the three treatment periods.

In an earlier toxicology study of gemfibrozil in Rhesus monkeys dosed at 300 mg/kg/day for three months, the animals showed sporadic anorexia and occasional salivation and vomiting. There was no body weight loss and no myotoxicity phenotype was ever reported. Gross and microscopic examination of tissues disclosed no drug-related lesions (Kurtz *et al.*, 1976). These were the key reason according to which we set the dosage of 600 and 300 mg/kg/day for the three consecutive periods. However in the third period of present study with Cynomolgus monkey of which the dosage was the same as the above study, the appetite was greatly decreased for most individuals and body weight loss was significant. Besides those myotoxic behavior responses, the markers for hepatotoxicity and nephrotoxicity increased after no more than 7 days of treatment. Thus it can be imagined chronic treatment could lead to potentially fatal hepatotoxicity and nephrotoxicity. This could be suggested by the toxicological changes of M2 in P3 that died of hepatotoxicity. It was the dosing duration which provoked severe fatal hepatotoxicities although some old pathological changes were presumably caused by treatment of P1 and P2. And also in P3, myotoxicity occurred in two monkeys as indicated above. These phenotypes, toxicological response and pathological changes all showed some difference from those reported in Rhesus monkeys. This indicates that Cynomolgus monkeys probably were more susceptible to gemfibrozil than Rhesus monkeys with respect to the toxic response. Another warning indication was that a dosage of 300 mg/kg/day in P3 was only about 15 times the clinical dosage based on body weight and that it could cause a high incidence of hepatotoxicity (2/6) and nephrotoxicity (5/6) in Cynomolgus. Thus the safety range or safety index of gemfibrozil should be paid more attention clinically.

The present study offers valuable clues that can be examined with additional studies even though the mechanism of gemfibrozil inducing myotoxicity remains unclear. The most important one is that exposure instead of genotype drove the occurrence of myotoxicity. It was probably the higher exposure that caused much higher incidence of myotoxicity than that of clinical dosage. Actually two-fold higher dosage of gemfibrozil in Sprague–Dawley rats caused no myotoxicity biochemically or pathologically, and the exposure level was lower than Cynomolgus monkeys reported in this study (unpublished data). Unknown factors not yet defined as well as species related difference in terms of pharmacodynamics and pharmacokinetics may contribute to this result. In cultured skeletal muscle cells, gemfibrozil caused interference of calcium homeostasis which may be the triggering event for myotoxicity (mechanism research ongoing).

In summary, the Cynomolgus monkey is a good animal model with good sensitivity, reproducibility and similarity to humans for mechanistic investigations of myotoxicity caused by gemfibrozil. Gemfibrozil at 300mg/kg/day can cause high rate of myotoxicity (2/6) and twice this dosage can shorten the time (about 8 days of treatment versus 11 days of lower dosage) needed for occurrence of myotoxicity. Myotoxicity of gemfibrozil in Cynomolgus showed the similarities with humans both biochemically and behaviorally. A combination of CK, MYO and behavioral phenotype can be used for judgement of occurrence of myotoxicity in this primate model. The high rate (2/6 for single period and 4/6 for three periods) of myotoxicity by gemfibrozil in the Cynomolgus monkey and the non-individual selectivity for three periods indicate that genotype may not play the key role for the occurrence of myotoxicity. T80-150 (especially T140-150) contributes to the deviation of myotoxic individuals from non-

myotoxic ones, showing that dosage related exposure levels of drug concentration and sustained time play a more important role for myotoxicity than other factors.

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Abbreviations

PPAR	peroxisome proliferator-activated receptor
P450	cytochrome P450
UGT	UDP-glucuronosyltransferases
AUC	area under concentration-time curve
C_{max}	peak concentration
T_{1/2}	

	elimination half-life
CMC-Na	sodium carboxymethylcellulose
HPLC	high performance liquid chromatography
ALT	alanine aminotransferase
AST	aspartate aminotransferase
CRE	creatinine
BUN	blood urea nitrogen
CK	Creatinkinase
MYO	myoglobin
NHP	non human primate
LC/MS/MS	high performance liquid chromatography-tandem mass spectrometry
HE	hematoxylin and eosin
PLS-DA	projection to latent structures discriminant analysis
ADME	absorption, distribution, metabolism, and excretion

FIG. 1.
Biomedical status of MYO and CK in P1 of experiments (B: days before dosing, D: days after dosing, C: days after clearance).

FIG. 2.

Biomedical status of MYO and CK in the P2 (B: days before dosing, D: days after dosing, C: days after clearance). CK and MYO of M3 on D8 were unexpectedly missed because no blood was withdrawn for monitoring due to the urgent need for salvage.

FIG. 3.

Biomedical status of MYO and CK in the P3 (B: days before dosing, D: days after dosing, C: days after clearance). CK of M6 kept unchanged although MYO increased more than 3 times. CK and MYO of M4 increased only 2-3 times without typical phenotype observed, thus the myotoxicity was only suspected and data was not specifically shown here.

FIG. 4.

Gemfibrozil concentration-time profile of 16th dosage of P1 and 17th dosage P2 (A and B) showed the high concentration fluctuated lightly during 12 hours for P1 and 24 hours for P2 at the end of the two high dosage periods which probably due to the saturated ADME process. Average trough concentrations of three days for P3 were monitored which represented the total exposure levels of those monkeys during the whole treatment period (C).

FIG. 5.

Score plots and loading plots of those calculated exposure variables and myotoxicity markers of CK and MYO by PLS-DA using SIMCA-P+11(P1, $R^2X(\text{cum})=0.902$ $R^2Y(\text{cum})=0.853$ $Q^2(\text{cum})=0.533$; P2, $R^2X(\text{cum})=0.978$ $R^2Y(\text{cum})=0.606$ $Q^2(\text{cum})=0.0353$). The myotoxic individuals deviated from the clusters of non-myotoxic individuals in score plots (A and C) and those contributing variables were identified in the corresponding left-down side in the loading plots (B, T80-150 for P1 and D, T70-230 for P2). Here R^2 was the fraction of Sum of Squares of all the variables explained by the first two components and Q^2 was the fraction of the total variation that can be predicted by a component determined by cross validation.

FIG. 6.

HE staining liver and kidney of M2 after death during P3. Extensive necrosis and adipose degeneration of hepatocytes was obvious and generous infiltration of inflammatory cell was found. Lamellar calcification coming from obsolete pathological changes was obvious (A and B). Some of renal tubules demonstrated features of degeneration and necrosis (D). Also protein cast and proteic exudatum was found in some renal glomeruli (C and D).

Indications of Myotoxicity in Cynomolgus Monkeys by Biochemical Markers and Behavioral Responses during Three Treatment Periods

Myotoxicity criteria	P1		P2		P3	
	M2	M6	M3	M4	M3	M6
CK fold increase	3.8	6.5	3.9	2.2	15.6	<2.0
MYO fold increase	8.8	3.5	4.1	3.2	6.1	3.4
Behavioral Responses						
Standing up inability	×		×		×	
Climbing inability	×	×	×	×	×	×
Limbs twisted	×		×	×	×	
Tenderness struggling				×		
Decreased Alertness	×		×		×	
Myotoxicity occurrence	Yes	Yes	Yes	Yes	Yes	Yes

Biochemical markers fold increase was calculated by comparison with the average base line level of the same monkey. The daily behavioral responses were recorded and the responses before and after the biochemical markers changed were compared. The changed responses were accompanied with the biochemical markers increased. The appearance of two or three of these indicated criteria in one monkey was considered that the myotoxicity occurred.

TABLE 2

Relationship of Myotoxicity Risk and T80-150 Values Contributing for Discrimination from P1 and P2 by Partial Least Squares-Discriminant Analysis

Monkey ID	1	2	3	4	5	6
T80(P1/P2)	10.52/11.72	12/13	12	10.55/13	12/13	10.9/13
T90(P1/P2)	10.38/11.03	12/13	12	9.84/12.98	12/13	10.87/13
T100(P1/P2)	10.25/10.34	12/13	12	9.11/12.68	12/13	10.84/13
T110(P1/P2)	10.12/9.88	12/12.95	12	7.26/12.39	12/12.71	10.81/13
T120(P1/P2)	9.99/9.52	12/12.5	12	5.42/12.06	11.95/12	10.79/13
T130(P1/P2)	9.87/9.16	12/12.04	12	3.57/11.65	11.86/11.29	10.76/13
T140(P1/P2)	9.74/8.8	12/11.58	11.99	3.32/11.23	11.5/10.57	10.73/13
T150(P1/P2)	9.62/8.43	11.8/11.12	11.93	2.97/10.82	10.55/9.95	9.4/12.84
Risk	0	1/3	2/3	1/3	0/3	2/3

T140 and T150 showed more correlation with myotoxicity risk. The risk was higher when the value was above about 10h indicating combination of exposure concentration and the sustained time are important for myotoxicity.

TABLE 3
Markers of Hepatotoxicity of M2 during the Three Periods of Experiments

Time	P1			P2			P3		
	ALT	AST	Time	ALT	AST	Time	ALT	AST	Time
B2	35.6	31	B2	38.6	46.6	B2	31.7	51.8	
B1	34.3	29.2	B1	36.3	104.9	B1	35.5	50.7	
D1	36.8	33	D1	49.1	84.7	D3	42.5	25	
D5	51.3	29.9	D5	33.4	55.4	D7	79.1	58.7	
D9	119.5	46.9	D8	71.6	59.9	D11	1561	624.5	
C3	67	30.1	C1	70.4	73.2	D13	2406	1648.1	
C11	55.4	36.2	C8	48.1	35.8	D14	316.8	1840.1	

Bx, Dx and Cx represent days before dosage, days after dosage and days after clearance.