Experimental and Toxicologic Pathology xxx (2015) xxx–xxx

Contents lists available at ScienceDirect

Experimental and Toxicologic Pathology

journal homepage: www.elsevier.de/etp

Biomarker evaluation of skeletal muscle toxicity following clofibrate administration in rats

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ARTICLE INFO

Article history:
Received 26 January 2016
Accepted 2 March 2016

Keywords:
Rat
Clofibrate
Skeletal muscle biomarker
Gene expression

ABSTRACT

The use of sensitive biomarkers to monitor skeletal muscle toxicity in preclinical toxicity studies is important for the risk assessment in humans during the development of a novel compound. Skeletal muscle toxicity in Sprague Dawley Rats was induced with clofibrate at different dose levels for 7 days to compare standard clinical pathology assays with novel skeletal muscle and cardiac muscle biomarkers, gene expression and histopathological changes. The standard clinical pathology assays aspartate aminotransferase (AST), alanine aminotransferase (ALT), and creatine kinase (CK) enzyme activity were compared to novel biomarkers fatty acid binding protein 3 (Fabp3), myosin light chain 3 (Myl3), muscular isoform of CK immunoreactivity (three isoforms CK_{BB}, CK_{MM}, CK_{MB}), parvalbumin (Prv), skeletal troponin I (sTnI), cardiac troponin T (cTnT), cardiac troponin I (cTnI), CK_{MM}, and myoglobin (Myo). The biomarker elevations were correlated to histopathological findings detected in several muscles and gene expression changes. Clofibrate predominantly induced skeletal muscle toxicity of type I fibers of low magnitude. Useful biomarkers for skeletal muscle toxicity were AST, Fabp3, Myl3, (CK_{MB}) and sTnI. Measurements of CK enzyme activity by a standard clinical assay were not useful for monitoring clofibrate-induced skeletal muscle toxicity in the rat at the doses used in this study.

1. Introduction

A biological marker or biomarker is defined as a characteristic that is objectively measured and evaluated as an indicator of normal biological processes, pathogenic processes, or pharmacologic responses to a therapeutic intervention (Biomarkers Definitions Working Group, Atkinson et al., 2001). Correlation of biomarker measurements to histopathological changes induced by these toxicants is important to determine the specificity and sensitivity of these biomarkers. The FDA expert working group defined several criteria, which should be complied for useful biomarkers. An ideal biomarker has a high predictive and diagnostic value and a low rate of false-negative or false-positive results. A useful biomarker should be specific, sensitive, predictive, robust, bridging preclinical and clinical trials and non- or minimal-invasive and accessible (Wallace et al., 2004).

The identification of sensitive safety biomarkers in preclinical toxicity studies to monitor toxicological effects in human subjects during clinical studies becomes more and more indispensable. The identification and qualification of sensitive biomarkers to monitor skeletal muscle toxicity is an important factor for the clinical development if skeletal muscle toxicity is detected pre-clinically.

ALT and AST are indicators of hepatocellular injury, but both, especially AST are also present in skeletal and cardiac muscle. CK is present in all types of muscle, in the brain and nerves. CK catalyzes the conversion of creatine phosphate to creatine and adenosine triphosphate (ATP) in order to provide an energy source for active muscles (Jackson, 2007). Fatty acid binding protein 3 (Fabp 3) modulates the fatty acid uptake in muscle cells (skeletal muscle and heart) but also in other cells (liver, brain, small intestine). Pritt et al. (2008) showed that Fabp3 in combination with other skeletal muscle biomarkers was predictive for skeletal muscle necrosis. Myosin, a protein of muscle tissue, formes contractile bundles with actin filaments in the cytoplasm of myofibers. Each myosin molecule consists of six polypeptide chains: two identical heavy

Abbreviations: ALT, alanine aminotransferase; AST, aspartate aminotransferase; BID, "bis in die" (latin) means twice daily; CK, and creatine kinase; CK, creatine kinase; cTnI, cardiac troponin I; cTnT, cardiac troponin T; DD, dosing day; ELISA, enzyme-linked immunosorbent assay; FABP3, fatty acid binding protein 3; HE, hematoxylin and eosin; HMG-Co A, β-hydroxy-β-methylglutaryl coenzyme A; IHC, immunohistochemistry; Myl3, myosin light chain 3; Myo, myoglobin; Prv, parvalbumin; QD, "quaque die" means once daily; Rpm, Revolutions per minute; RNA, ribonucleic acid; sTnI, skeletal troponin I; V., Vena.

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http://dx.doi.org/10.1016/j.etp.2016.03.001

chains and two pairs of light chains (Alberts et al., 1989). Myl 3 is the myosin light chain 3 of cardiac and type I skeletal muscle fibers (http://www.ncbi.nlm.nih.gov/gene/4634). Skeletal troponin is one major accessory protein involved in Ca^{2+} regulation in vertebrate skeletal muscle. It is a complex of three polypeptides, troponin T, I and C (named for their *T*ropomyosin-binding, *I*nhibitory and *C*alcium-binding activities). Troponin I binds to actin (Alberts et al., 1989). The cardiac troponins have similar composition to the skeletal troponins in general, but they are nearly absolute myocardial tissue specific (Wallace et al., 2004). Prv is a calcium binding protein involved in the contraction and relaxation cycle of type II muscle fibers and is mainly found in skeletal muscles (Huber and Pette, 1996). Myo is an abundant heme-containing oxygen carrier expressed predominantly in cardiac and type I skeletal muscle fibers (Sorichter et al., 1999). Theory behind for utility of these enzymes and proteins as biomarkers is of course that they leak out into the serum in case of skeletal muscle fiber damage.

Skeletal muscles of rats can be divided into slow-twitch (type I fiber) and fast-twitch (type II) fiber types, according to the expression of their particular myosin heavy chain (MHC) isoform (Schiaffino and Reggiani, 1994). In comparison to humans, the rat has a relatively low proportion of type I fibers in all muscles described in the literature (Staron et al., 1999; Eng et al., 2008), whereas human muscles contain type I and type II muscle fibers to a more similar distribution in most muscles. Deeper portions have a higher percentage of type I fibers whereas more superficial muscles or portions of muscles have a higher amount of type II muscle fibers (Dahmane et al., 2005).

The slow isoform (type I fiber) is MHCI and the three fast isoforms (type II fibers) are MHCIIa, MHCIId, and MHCIIb. In some reports, MHCIId has also been referred to as MHCIIx (Schiaffino et al., 1989; De Nardi et al., 1993). Most fibers express one MHC isoform only, but single fibers may also contain two MHC isoforms (hybrid fibers) (Staron and Pette 1986; Staron et al., 1999). In this investigation, we only focused on the differentiation of type I and type II muscle fibers without distinguishing between the type II subtypes or hybrid fibers. Reports indicated that fibrates in general induce skeletal muscle toxicity of type I skeletal myofibers (De Souza et al., 2006). Generally in the rat, this fiber type is represented by the soleus muscle consisting predominantly of type I skeletal muscle fibers (more than 80%) (Novák et al., 2010), but other muscles of the hindlimb have locations where type I muscle fibers are present (Eng et al., 2008).

Several drugs are on the market, which are known to cause skeletal muscle toxicity, for example lipid lowering drugs (e.g. fibrates and statins). Clofibrate which is an agonists of the peroxisome proliferator-activated receptors alpha (PPAR-α) is used for the treatment of hyperglyceridemia and also causes a favourable increase of high density cholesterol (Chapman, 2003). Although generally well tolerated, the most important complications with clofibrate are myopathies, ranging from myalgia, muscle weakness, muscle tenderness and muscle cramps to destruction of skeletal muscle (Rush et al., 1986). Standard clinical chemistry analysis often shows significant increases in CK and AST (SGOT) activities in humans (Rush et al., 1986; Le Quintrec and Le Quintrec, 1991). The pathomechanism of clofibrate-induced myopathy is still poorly understood. Teräväinen et al. (1977) and Okada et al. (2007) reported about morphologic and ultrastructural changes of clofibrate-induced myopathy in rats. The fiber type selectivity of fenofibrate to affect type I muscle fibers was shown to be consistent with increased fatty acid uptake and beta-oxidation, which represent the major clinical benefits of hypolipemic compounds (De Souza et al., 2006).

Because of the relatively low representation of type I fibers in the rat, clofibrate was chosen for induction of skeletal muscle toxicity of low magnitude.

The goal of this investigation was to evaluate a set of skeletal muscle biomarkers (mentioned above) in rats with induced skeletal muscle toxicity of low magnitude on protein and gene expression level with morphologic and immunohistochemical changes as well as the fiber type predeliction of clofibrate-induced myopathy in the rat.

2. Materials and methods

2.1. Animal husbandry

Twenty (experiment 1) and ten (experiment 2) male Sprague Dawley (Crl:CD(SD)) rats approximately eight weeks old were obtained from Charles River Laboratories (Sulzfeld, Germany). Following physical examination by a veterinarian, the rats were allowed to acclimate for seven days before the first dosing.

All rats were individually housed in U-Temp™ Type III cages (Tecniplast S.p.A. Buguggiate, Italy), under barrier conditions with bedding (dust-free softwood shavings; Ssniff Spezialdiäten GmbH, Soest, Germany) containing chewing wood (Ssniff Spezialdiäten GmbH) as enrichment under controlled conditions (temperature 22 ± 2 °C, humidity 55 ± 10%, LD (light-dark) 12:12). The rats had *ad libitum* access to a standard rodent diet (R/M-H, pellets® Rodent Diet, Ssniff Spezialdiäten GmbH) and to autoclaved tap water. Food was removed overnight for 18 h prior to blood withdrawal (2–3 h prior to the dark phase). The rats were moved to clean cages once per week. All rats were observed at least once daily for general condition. Body weights were measured upon arrival and on the day of terminal blood withdrawal.

Rats were housed, treated, and sacrificed complying with the regulations of Germany and the European Union, including the German Animal Welfare Act that implements the European Council Directive 86/609/EEC. AbbVies's laboratory animal programs in general as well as the local facility where the experiments of this study were conducted were accredited by the Association for Assessment and Accreditation of Laboratory Animal Care International (AAALAC) during the conduct of the study and all experiments were conducted according to the standards put forth in the National Research Council's *Guide for the Care and Use of Laboratory Animals* and were approved by the government of Rhineland-Palatinate.

2.2. Test material

Clofibrate (purity ≥98.8%) was obtained from TCI Europe N.V. (Zwijndrecht, Belgium) and was formulated for dosing as suspension in miglyol-812. The formulations were prepared weekly and stored at room temperature (RT) protected from light.

2.3. Treatment

In experiment 1, the rats were randomly allocated to four groups with five animals each group and were treated over a period of at least seven days via gavage. The control group received the vehicle (miglyol-812) and three dose groups received clofibrate at doses of 150 mg/kg QD, 150 mg/kg BID, and 500 mg/kg QD. In experiment 2, rats were randomly allocated to two groups with five animals each group. The control group received the vehicle (miglyol-812) and the dose group received clofibrate at a dose of 500 mg/kg QD over a period of at least seven days via gavage. The animals were fasted over night before scheduled necropsies.

Please cite this article in press as: K. Bodié, et al., Biomarker evaluation of skeletal muscle toxicity following clofibrate administration in rats, Exp Toxicol Pathol (2016), http://dx.doi.org/10.1016/j.etp.2016.03.001