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Toxicity assessments of nonsteroidal anti-inflammatory drugs in isolated mitochondria, rat hepatocytes, and zebrafish show good concordance across chemical classes

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ABSTRACT

To reduce costly late-stage compound attrition, there has been an increased focus on assessing compounds in *in vitro* assays that predict attributes of human safety liabilities, before preclinical *in vivo* studies are done. Relevant questions when choosing a panel of assays for predicting toxicity are (a) whether there is general concordance in the data among the assays, and (b) whether, in a retrospective analysis, the rank order of toxicity of compounds in the assays correlates with the known safety profile of the drugs in humans. The aim of our study was to answer these questions using nonsteroidal anti-inflammatory drugs (NSAIDs) as a test set since NSAIDs are generally associated with gastrointestinal injury, hepatotoxicity, and/or cardiovascular risk, with mitochondrial impairment and endoplasmic reticulum stress being possible contributing factors. Eleven NSAIDs, flufenamic acid, tolfenamic acid, mefenamic acid, diclofenac, meloxicam, sudoxicam, piroxicam, diflunisal, acetylsalicylic acid, nimesulide, and sulindac (and its two metabolites, sulindac sulfide and sulindac sulfone), were tested for their effects on (a) the respiration of rat liver mitochondria, (b) a panel of mechanistic endpoints in rat hepatocytes, and (c) the viability and organ morphology of zebrafish. We show good concordance for distinguishing among/between NSAID chemical classes in the observations among the three approaches. Furthermore, the assays were complementary and able to correctly identify "toxic" and "non-toxic" drugs in accordance with their human safety profile, with emphasis on hepatic and gastrointestinal safety. We recommend implementing our multi-assay approach in the drug discovery process to reduce compound attrition.

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Introduction

Recently, there has been an increased focus on the field of predictive toxicology (Gibb, 2008), not only as a measure to limit serious adverse effects associated with drugs, but also as a response to a more challenging drug development environment where the percentage of compound attrition due to safety-related reasons is at least 30% and the cost for launching a drug on the market is nearly $1 billion (Kola and Landis, 2004). The rapid growth of predictive toxicology has lead to the development of a variety of *in vitro* methods that are designed to predict toxicity, particularly hepatotoxicity since it is a major reason for late stage attrition (Greaves et al., 2004; Olson et al., 2000; Peters, 2005). For example, isolated organelle approaches that detect mitochondrial toxicity have been used as a predictor of liver injury (Dykens et al., 2007; Porceddu et al., 2012). At the cellular level, high specificity in predicting liver injury has been demonstrated using high content imaging approaches in hepatocytes (Xu et al., 2008). Concurrently, there has been an active interest in using alternative species such as zebrafish in early predictive *in vivo* toxicity studies (McGrath and Li, 2008; Sukardi et al., 2011). A recent review (Hill et al., 2012) demonstrated that high content imaging of human hepatocytes and phenotypic assessments of zebrafish liver morphology were complimentary and, when used in combination, enhanced detection of potent hepatotoxicants.

Relevant questions when choosing appropriate *in vitro* and *in vivo* assays for de-risking compounds before traditional preclinical *in vivo*

Abbreviations: NSAID, nonsteroidal anti-inflammatory drug; COX, cyclooxygenase; GI, gastrointestinal; ER, endoplasmic reticulum; NOEC, no observed effect concentration; LOEC, lowest observed effect concentration; ROR, reporting odds ratio; AERS, adverse event reporting system; UC_{50}, the concentration at which 50% uncoupling occurred in State 2 respiration; IC_{50}, the concentration at which 50% inhibition occurred in State 3 respiration; AC_{50}, half-maximal activity concentration.

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studies are done are whether the observations from the various assays show a general concordance and whether the rank order of toxicity of the drugs in the experimental setting correlates with the safety profile of these drugs in humans. Hence, our aim in this study was to investigate members of several chemical classes of nonsteroidal anti-inflammatory drugs (NSAIDs) by three experimental approaches, an organelle-based approach using mitochondria, a cell-based approach using rat hepatocytes, and an *in vivo* approach using zebrafish to determine (a) whether there was any concordance among the data from the three approaches and (b) whether there was any correlation between the observed rank order of toxicity of the drugs and their reported human safety profile.

NSAIDs are widely prescribed therapeutic agents that are often used long-term in the treatment of rheumatic and arthritic diseases (Aithal and Day, 2007; Rubenstein and Laine, 2004). They are inhibitors of cyclooxygenase 1/2 (COX-1/2) and are generally classified by their chemical structures: salicylates, oxicams, sulfonanilides, acid derivatives (propionic, acetic, and fenamic), and selective COX-2 inhibitors (Coxibs).

As a class, NSAIDs are associated with upper gastrointestinal (GI) injury ranging from dyspepsia, bleeding, and ulceration to perforation of the stomach or intestines (Rubenstein and Laine, 2004). Some NSAIDs are associated with idiosyncratic hepatotoxicity, with symptoms ranging from elevation of serum transaminases to hepatocellular or cholestatic injury and, occasionally, to fatal fulminant hepatitis (Aithal and Day, 2007; Rubenstein and Laine, 2004). The NSAIDs most strongly associated with hepatotoxicity are nimesulide (Traversa et al., 2003), diclofenac (Banks et al., 1995), and sulindac (Tarazi et al., 1993). Moreover, some NSAIDs have been withdrawn from the market because of hepatotoxicity as in the case of benoxaprofen (Duthie et al., 1982) and bromfenac (Hunter et al., 1999), and at least one NSAID, sudoxicam, was discontinued after clinical trials because it caused acute liver injury (Lewis, 1984). A third adverse effect associated with NSAIDs, particularly COX2 inhibitors, is a higher incidence of myocardial infarction and stroke, as highlighted by the post-market withdrawal of rofecoxib (Vioxx) and valdecoxib (Bextra) (Conaghan, 2012).

The mechanisms contributing to NSAID-induced GI and liver injury are not well understood but may involve mitochondrial dysfunction and endoplasmic reticulum (ER) stress. Accumulation of NSAIDs within cells of the gastrointestinal lining, with subsequent impairment of mitochondrial function, has been proposed to cause NSAID-induced GI injury (Somasundaram et al., 1997, 2000). In addition, induction of the ER stress response leading to mitochondria-mediated cell death has also been proposed as a major mechanism (Boelsterli et al., 2013).

In this study, we investigated NSAIDs that are predominately associated with GI injury and liver injury but not cardiac injury. Three fenamic acids (flufenamic, tolfenamic, and mefenamic acid), three oxicams (meloxicam, sudoxicam, piroxicam), two salicylates (aspirin and diflunisal), two acetic acid derivatives (diclofenac and sulindac plus its two metabolites) and the sulfonanilide, nimesulide, were investigated for their effects on (a) the respiration of rat liver mitochondria, (b) a panel of mechanistic endpoints, *via* high content imaging, in rat hepatocytes and (c) the viability and liver/GI morphology of zebrafish, with the goal of determining the discriminating power of all three assay platforms towards the prediction of human safety, with emphasis on liver and gastrointestinal injury.

Materials and methods

All chemicals, with the exception of sudoxicam, were purchased from Sigma-Aldrich (St. Louis, MO), Axxora LLC (San Diego, CA) or Toronto Research Chemicals (Toronto, Canada); sudoxicam was obtained from the Pfizer chemical bank (Groton, CT). The phosphorescent oxygen-sensitive probe, type A65N-1, was from Luxcel Biosciences (Cork, Ireland).

Animals

Care and maintenance of all animals were in accordance with the principles described in the Guide for Care and Use of Laboratory Animals (NIH Publication 85-23, 1985). Male Sprague–Dawley Rats (150–180 g) were purchased from Charles River (Wilmington, MA). The rats were housed in pairs in a controlled environment with constant temperature (21 ± 2 °C) and a 12 hour light/dark cycle. Food and water were available *ad libitum*. Animals were euthanized with an overdose of carbon dioxide. Organs were rapidly excised and placed into ice-cold mitochondrial isolation buffers (see below).

Wild-type adult zebrafish (*Danio rerio*) were obtained from Carolina Biological Supply Company (Burlington, NC) and cultivated internally. Zebrafish were held in colonies maintained in a re-circulating aquaria system designed by Pharmacal Research Laboratory (Naugatuck, CT). Water was controlled at a pH 7.35 (±0.65) and 28 (±1) °C. Lighting was set on a 14 h light:10 h dark cycle (light on at 06:30).

Measurement of respiration in isolated rat liver mitochondria

Liver mitochondria were isolated and oxygen consumption was monitored in 96-well plate format using a phosphorescent oxygen-sensitive probe as previously described (Hynes et al., 2006; Will et al., 2006). All drug concentrations are presented as nmol/mg of mitochondrial protein. After completion of fluorescence measurements, time profiles of fluorescence intensity in each well were analyzed using Magellan® (Tecan) and Excel® (Microsoft) software, to determine the rates of oxygen consumption based on the known relationship between probe fluorescence and oxygen concentration (Will et al., 2006). Rates of change of dissolved oxygen were subsequently determined from the slopes of these concentration profiles, over the initial 8 min.

Primary rat hepatocyte cell culture

Primary rat hepatocytes were prepared by standard two-step collagenase perfusion as described by Berry et al. (1991) with the following changes: an equal volume of a 2× Percoll buffer (74% Percoll, 20 mM fructose, 6 mM glycine, 25 mM HEPES, 50 mM sodium bicarbonate in 2× Krebs–Henseleit buffer containing 2.5 mM calcium chloride, pH 7.4) was added to the re-suspended hepatocytes for purification purposes. The cells were then centrifuged at 45 *g* for 8 min at 4 °C. The resulting supernatant was removed and the pellet was resuspended in 4 °C minimum essential medium containing 10% fetal calf serum. The hepatocytes were viewed under an inverted phase contrast microscope and evaluated for cellular morphology, viability, and density for plating purposes.

Treatment of rat hepatocytes with compounds

8000 cells per well were added to 384-well black-walled clear-bottom plates in Williams E medium. Following a 3 h incubation to allow for attachment and spreading, cells were treated with test compounds or DMSO (final concentration of 1% v/v) for the appropriate time. All endpoints were measured at 24 and 48 h except for the endpoints, glutathione (GSH) depletion (18 h), reactive oxygen species (ROS) (4 h), biliary flux assessment (1 h), cytokine-mediated cytotoxicity (48 h only), and a 5-day cytotoxicity. The bile canalicular staining and the 5-day cytotoxicity assay were performed on matrigel-overlaid cells. The compounds were evaluated over a 10-point dose response, using 2-fold dilutions starting at 1 mM.

Mechanistic endpoint determination in rat hepatocytes

The mechanistic endpoints, mitochondrial membrane potential, lysosomal mass, lipid content, GADD153 induction, cytochrome *c*

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