Biomarkers of Flutamide-Bioactivation and Oxidative Stress In Vitro and In Vivo

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ABSTRACT

The nonsteroidal androgen-receptor antagonist flutamide is associated with hepatic injury. Oxidative stress and reactive metabolite formation are considered contributing factors to liver toxicity. Here we have used flutamide as a model drug to study the generation of reactive drug metabolites that undergo redox cycling to induce oxidative stress (OS) in vitro and in vivo. Lipid peroxidation (LPO) markers, as well as genes regulated by the redox-sensitive Nrf2 pathway, have been identified as surrogates for the characterization of OS. These markers and metabolism biomarkers for drug bioactivation have been investigated to characterize drug-induced hepatic damage. Rat hepatocytes and in vivo studies showed that several LPO markers, namely the isoprostanes 15R-PD2, dihydro keto PE2, and iPF2α-VI, as well as hydroxynonenal mercapturic acid metabolites, had increased significantly by 24 hours after flutamide treatment from 4.9 to 15.3-fold in hepatocytes and from 2.6 to 31.0-fold in rat plasma. Induction of mRNA expression levels for Nrf2-regulated genes was evident as well; with heme oxygenase 1, glutathione-S-transferase 1, and NAD(P)H dehydrogenase showing a 3.6-, 4.1-, and 1.9-fold increase in hepatocytes and 5.6-, 7.5-, and 94.1-fold in rat liver. All effects were observed at drug concentrations that did not show overt liver toxicity. Addition of an in situ hydro Peroxide-generating system to in vitro experiments demonstrated the formation of a reactive di-imine intermediate as the responsible metabolic pathway for the generation of OS. The dataset suggests that hepatic oxidative stress conditions can be mediated via metabolic activation and can be monitored with suitable biomarkers preceding the terminal damage.

Introduction

The nonsteroidal antiandrogen drug flutamide is used for treatment of progressed prostate carcinoma (Mutschler et al., 2001). Owing to its potential to induce liver injury, flutamide is marked with a black box warning. Case reports document elevated liver enzyme levels that were reversible in most cases but could have turned into fatal outcomes as well (Crowner et al., 1996; Cetin et al., 1999; Nakagawa et al., 1999). These effects are still considered to be under-reported (Osculati and Castiglioni, 2006), and the mechanism of toxicity so far has not been fully elucidated. However, some studies have demonstrated that bioactivation processes leading to redox cycling and GSH depletion are plausible mechanisms of flutamide toxicity (Kang et al., 2008; Wen et al., 2008).

To further evaluate the mechanism of flutamide-induced hepatotoxicity, it seemed reasonable, therefore, to further investigate metabolic liabilities involving reactive oxygen species (ROS) and oxidative stress.

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On the basis of this knowledge, we describe a validation study with flutamide for several noninvasive in vitro and in vivo biomarkers that composed a panel of complementary endpoints of oxidative stress, namely the integration of mRNA profiling in addition to small-molecule biomarker analysis. Isoprostanes and HNE metabolites as well as mRNA of six different Nrf2-regulated enzymes were assessed in rat hepatocytes and the corresponding rat in vivo models and then compared with established conventional parameters. The aim of these studies was to validate markers for oxidative stress that may also enable an early detection of drug-induced liver injury (DILI) and probe their clinical relevance by correlation between in vitro and in vivo results. A classification of the described markers was done by comparison with conventional cyto- and organ-toxicity markers. To increase the dynamic range of the ROS response in the in vitro system we used a horseradish peroxidase (HRP) system for the in situ generation of hydrogen peroxide. This technique was described previously by O’Brien’s group (Tafazoli et al., 2005), which showed alteration of cellular oxidative stress response for drugs that are prone to cause DILI. Particularly, drugs that carry structural moieties that may form quinone or quinone-imine metabolites are prone to undergo redox cycling. The stimulation of underlying inflammatory signals by hydrogen peroxide may lower the threshold for toxicity under these test conditions without affecting cell viability under control conditions.

Materials and Methods

Chemicals. Williams’ medium E, dimethyl sulfoxide (DMSO) p.a., formic acid, insulin, streptomycin, penicillin, hydrocortisone, β-(n+)-glucose, peroxidase type VI from horseradish, glucose oxidase type II from Aspergillus were obtained from MilliporeSigma (St. Louis, MO). Glutamine and gentamycin were purchased from Life Technologies/Invitrogen (Lucerne, Switzerland) and acetone triethylammonium liquid chromatography–mass spectroscopy grade from Fisher Scientific (Wohlen, Switzerland). Water of chromatography grade was obtained from MilliporeSigma (Wohlen, Switzerland). Medium (see above) supplemented with 10% fetal calf serum was exchanged generating system (HRP system). Incubation was performed at 37°C with and without supplementation by 10 mM glucose, 0.05 IU/ml glucose oxidase, and 0.5 μM horseradish peroxidase in situ hydrogen peroxide–generating system (HRP system). Incubation was performed at 37°C in a humidified atmosphere (5% CO₂, 95% air) and stopped after 30, 60, and 180 minutes by adding 1 volume of ice-cold acetoneitrile. After centrifugation of precipitated samples at 5000g and 8°C for 10 minutes, the supernatant was diluted 1:1 with water containing 0.1% formic acid and directly used for liquid chromatography–tandem mass spectrometry (LC-MS/MS) analysis.

The instrumental setup consisted of an Acquity UPLC (Waters, Dublin, Ireland) equipped with an Atlantis HSS C18 column (1.8 μm, 2.1 × 50 mm; Waters) at 50°C. Ten microliters of sample were injected and chromatographically separated by gradient elution with a mobile phase consisting of water containing 0.5% formic acid/acetonitrile (eluent A; 95/5, v/v), and acetonitrile containing 0.1% formic acid (eluent B). The gradient started with a total flow of 0.500 ml/min at 100% A, which was kept for 1.0 minutes. Eluent B was then increased to 16% within 2.5 minutes and further to 100% in another 2.5 minutes. The system was kept at 100% B for 0.2 minutes and then switched back to 100% A within 0.05 minutes. From 6.25 minutes until the end of the run at 10 minutes the system was re-equilibrated with 100% eluent A.

MS Detection was done with a TripleTOF 5600+ mass spectrometer (AB Sciex, Warrington, UK) using a generic data-dependent acquisition mode with positive electrospray ionization. Source parameters were set to 25 (curtain gas), 45 (gas 1), 60 (gas 2), 5500 (ionspray voltage), 500 (temperature), and 80 eV (declustering potential). For the time-of-flight mass spectrometry scan, a collision energy of 10 eV and an accumulation time of 80 milliseconds was applied for a scan range from m/z 10 to m/z 1200. Eight information-dependent MS² scans were acquired using a collision energy of 40 ± 20 eV for the scan range from m/z 50 to m/z 1200 with an accumulation time of 50 milliseconds for ions with an intensity higher than 450 cps with a mass tolerance of 25 ppm. The resulting cycle time was 530 milliseconds.

Animal Study. Animals studies were carried out in accordance to Swiss animal welfare law and the Guide for the Care and Use of Laboratory Animals published by the National Institutes of Health. The animal test facility is fully accredited by the Association for Assessment and Accreditation of Laboratory Animal Care International. Twelve male Fischer 344 rats (249–269 g) were obtained from Charles River (Sulzfeld, Germany) and maintained at 23°C on a 12-hour light/12-hour dark cycle with food and water ad libitum. Two days prior to treatment animals were adjusted to metabolic cages. For the experiment, animals were divided into four groups (n = 3) and administered a single dose of flutamide (500 mg/kg body weight) as a microsuspension in sodium chloride/gelatin (treated group: groups 1 and 2) or vehicle alone by oral gavage (control: groups 3 and 4). Urine samples were collected on dry ice from −48 to −24 hours and from 0 to 24 hours (postdose) and stored at −20°C until analysis. Animals were sacrificed after 3 hours (groups 1 and 3) and 24 hours of treatment (groups 2 and 4) by CO₂ asphyxiation followed by cervical dislocation. Total blood was collected on EDTA, plasma prepared, and stored at −20°C.

Sample Work Up. Plasma samples were precipitated with 2 volumes of ethanol containing internal standard (PD2-d₄) at a concentration of 0.63 ng/ml to reach a final concentration of 0.42 ng/ml. Urine samples were diluted with 9 volumes of acetoneitrile/water containing 0.2% formic acid [1:1 containing internal standard (PD2-d₄) at a concentration of 0.47 ng/ml to reach a final concentration of 0.42 ng/ml]. To detect prostaglandin levels and 4-hydroxy-2(E)-enone (HNE) derivatives in hepatocyte incubation, the latter were quenched at the end of indicated treatment periods with 1 volume acetoneitrile containing internal standard (PD2-d₄) at a concentration of 0.84 ng/ml to reach a final concentration of 0.42 ng/ml. Approximatively 1 g of liver tissue was homogenized with 3 volumes of water at 5500 rpm for 40 seconds using a Lysing Kit (Precellys/Bertin Technologies, Montigny, France). One milliliter of the homogenate was then precipitated with 1 volume ice-cold acetoneitrile containing internal standard (PD2-d₄).

All samples were centrifuged and the supernatant was directly injected onto the LC-MS/MS system. Quantitation was achieved by calibration with dilution series of standard compounds against a deuterated internal standard in a concentration range from 20 up to a concentration of 50,000 pg/ml.

LC-MS/MS Analysis. The system consisted of a YMC-Pack ODS-AQ, 20 × 2.1 mm, 5-μm column (YMC Europe, Dislaken, Germany) as trapping column and an Atlantis T3, 100 × 2.1 mm, 3-μm column (Waters) connected to a column two-dimensional HPLC consisting of a Shimadzu LC AD vp binary pump.
system and an Agilent 1100 series LC pump connected to a triple quadrupole tandem mass spectrometer (4000 QTRAP; Sciex, Warrington, UK). The analytical setup has been described for prostaglandin detection as well as for HNE analysis (Volkel et al., 2005; Sicilia et al., 2008; Ecker, 2012). Briefly, 500-μl samples were injected to the system, analytes were enriched on the trapping column, and chromatographically eluted from the analytical column. MS detection (4000 QTRAP; Sciex) operated in negative electrospray ionization mode using selected reaction monitoring (SRM) analysis. Transitions and tuning parameters were defined individually for each analyte with the help of authentic standards as follows: m/z 351.1 to m/z 271.1 for 15R-PD₂, m/z 351.1 to m/z 315.0 for dihydroketo PDE₃, m/z 353.1 to m/z 114.9 for iPDE₂⁻VI, m/z 318.1 to m/z 189.0 for HNE-MA, and m/z 320.1 to m/z 190.1 for DHN-MA. Quantitation of prostaglandins was achieved by calibration with dilution series of standard compounds against deuterated internal standard. As an authentic standard was not available for DHN-MA, HNE derivatives were quantified relative to the internal standard according to published procedures (Volkel et al., 2005). For sensitivity reasons in vivo samples were measured on a TripleTOF 5600+ mass spectrometer (Sciex) by the analogous technique MRMHR.

**Biochemical Analysis.** Lactate dehydrogenase (LDH) in the supernatant was determined after 6 hours and 24 hours of treatment (150 μl supernatant) by a commercially available test kit (cat. no. 07502999) on an ADVIA 1650 autoanalyzer (Siemens Diagnostics, Erlangen, Germany). Intracellular ATP content was determined after 6 hours and 24 hours by means of a bioluminescence-based ATP assay kit (Roche Diagnostics, Rotkreuz, Switzerland) on a Victor plate reader (PerkinElmer, Schwarzenbach, Switzerland).

**Isolation of RNA from Rat Liver Tissue.** Medium was aspirated from the incubation wells, and cells were washed with DMEM without serum. Then, cell suspensions were collected and stored at −80°C until RNA isolation was performed. Total RNA was extracted from the cell matrix by means of silica-membrane purification with spin columns (Qiagen, Hombrechtkon, Switzerland). RNA yield was quantified with a Nanodrop 1000 (Thermo Scientific, Wilmington, DE).

**Isolation of RNA from Rat Liver Tissue.** A piece of RNA-later-processed organ tissue (~30 mg) was cut and homogenized in RLT buffer in a FastRNA tube (cat. no. 6913-100; Qbiogene, Carlsbad, CA). After centrifugation of the lysate an aliquot of the supernatant was used for RNA extraction as described previously.

**Quantitative Real-Time Polymerase Chain Reaction.** A total of 500 ng of RNA was used to generate single-stranded cDNA using a commercially available cDNA synthesis kit (Transcriptor First Strand cDNA Synthesis Kit; Roche Diagnostics, Rotkreuz, Switzerland). The cDNA obtained was used as template for several PCRs along with appropriate specific primers and Taqman Hot-Start FastStart PCR Master Mix (LightCycler 480 Probes Master; Roche Diagnostics). The generation of double-stranded amplification products on a 384-multiwell plate was monitored by a LightCycler 480 instrument (Roche Diagnostics). All primer/probe mixes were from Roche Diagnostics with the assay ID 504934 [carboxylesterase 1 (CES1)], 504937 [glutamate-cysteine ligase, catalytic subunit (GCLC)], 502674 [glutathione-S-transferase α1 (GSTa1)], 503103 [glutathione-S-transferase π1 (GSTπ1)], 502688 [heme oxygenase (decycling 1) (HMox1)], 502689 [NAD(P)H dehydrogenase, quinone 1 (NQO1)], 503799 [glyceraldehyde-3-phosphate dehydrogenase (GAPDH)], 500152 [actin, beta (ACTB)] and 502309 [TATA box binding protein (TBP)]. Time course of the amplification cycle was as follows: An initial preincubation occurred for 10 minutes at 95°C followed by 45 amplification cycles at 95°C for 10 seconds, 60°C for 30 seconds, and 72°C for 2 seconds. In the end, cooling at 40°C was done for 60 seconds. Relative expression of genes with respect to the control cells was determined by normalization to the amount of GAPDH RNA.

**Determination of Liver Enzyme Levels from Plasma Samples.** Levels of liver integrity markers were determined in 3- and 24-hour plasma samples with enzymatic assay kits on an ADVIA 1650 autoanalyzer (Siemens Diagnostics, Erlangen, Germany). The respective lactate hydrogenase (LDH), alanine transaminase (ALT), and aspartate dehydrogenase (AST) test kits were from Siemens Diagnostics; the glutamate dehydrogenase (GLDH) test kit was from Roche Diagnostics; and sorbose dehydrogenase (SDH), from Sekisui Diagnostics (Dusseldorf, Germany).

**Determination of Creatinine Levels from Urine Samples.** The concentration of creatinine in the collected urine samples was determined with a creatinine assay kit (Siemens) on an ADVIA 1650 autoanalyzer.

**Statistical Analysis.** Statistical significance defined as P value of <0.05 was confirmed by t test analysis using the software GraphPad Prism 5.03.

**Results**

**Cytotoxicity Markers.** Biochemical status of cells upon treatment was assessed by determination of ATP content as an indicator of mitochondrial activity and LDH release as marker for membrane integrity (Table 1). When the control cells were supplemented with the hydrogen peroxide–generating system in situ, no increase in cytotoxicity markers could be observed. This indicates that HRP supplementation does not negatively impact cell viability in absence of the drug. In the HRP system, a transient decrease in ATP production after 6 hours of treatment with a high flutamide concentration was observed. In the HRP-free system, a similar effect was not evident after 24 hours. However, a trend for LDH was evident in the respective 24-hour incubation. On the basis of these data, an effect on cell viability with the high concentration treatment can be supposed.

**Biotransformation of Flutamide in Hepatocytes with and without HRP.** To evaluate the impact of the HRP system on biotransformation pathways, metabolite formation was compared from rat hepatocyte suspensions. The rate of flutamide biotransformation in general was not altered and the same metabolites were identified under both conditions. Under HRP treatment one metabolic pathway was enhanced, however. The reactive di-imine metabolite, whose structural identity was confirmed by the MS fragmentation pattern (Scheme 1, Fig. 1) was assigned as Flu-G2, increased and led to a significantly increased concentration of its derived glutathione adduct (Fig. 2).

**Lipid Peroxidation Products as Markers for Oxidative Stress.** Hepatocyte oxidative stress response to flutamide treatment was further assessed by a panel of small-molecule biomarkers composed of isoprostanes, prostaglandins, and hydroxynonanal metabolites, namely mercaptopurine acid conjugates. Different analytes revealed a time- as well as concentration-dependent response to flutamide treatment: As depicted in Fig. 3A, a flutamide concentration of 100 μM led to an increase of prostaglandin levels for all analytes significantly different from control cells after 6 and 24 hours of treatment. For the lower concentration (50 μM flutamide treatment) only small effects were observed after 6 hours but biomarker levels were significantly increased after 24 hours. For instance, the concentration of the isoprostane isomer 15R-PD₂ changed from 6.29 ± 0.82 ng/ml in the 24-hour control condition by 3.1-fold to 19.3 ± 6.1 ng/ml after 24 hours of treatment with a high flutamide concentration was observed. However, a trend for LDH was evident in the respective 24-hour incubation. On the basis of these data, an effect on cell viability with the high concentration treatment can be supposed.

**TABLE 1**

<table>
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<tr>
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<th>LDH release and ATP production in rat hepatocytes upon flutamide treatment with and without HRP system</th>
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<tbody>
<tr>
<td>Control</td>
<td>50 μM</td>
</tr>
<tr>
<td>LDH (kat/l) – HRP</td>
<td>6 h 0.85 ± 0.12</td>
</tr>
<tr>
<td>24 h</td>
<td>1.21 ± 0.26</td>
</tr>
<tr>
<td>LDH (kat/l) + HRP</td>
<td>6 h 0.66 ± 0.13</td>
</tr>
<tr>
<td>24 h</td>
<td>0.57 ± 0.28</td>
</tr>
<tr>
<td>ATP (pmol/well) – HRP</td>
<td>6 h 247 ± 61</td>
</tr>
<tr>
<td>24 h</td>
<td>160 ± 43</td>
</tr>
<tr>
<td>ATP (pmol/well) + HRP</td>
<td>6 h 333 ± 1</td>
</tr>
<tr>
<td>24 h</td>
<td>230 ± 11</td>
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24 hours upon both, 50 μM and 100 μM flutamide treatment. Even more, HNE-MA in cells treated with 100 μM flutamide changed by 5.5- and 4.8-fold compared with controls (6 and 24 hours, respectively).

mRNA Expression of Nrf2-Regulated Genes in Rat Hepatocytes. To investigate the cellular response to oxidative stress in more detail, mRNA expression levels of enzymes regulated by the Nrf2-Keap1 antioxidant response element pathway were determined: The transcript set consisted of carboxylesterase 1 (CES1), GCLC, glutathione-S-transferase α1 (GSTα1), glutathione-S-transferase π1 (GSTπ1), HMOX1, and NAD(P)H:quinone oxidoreductase 1 (NQO1).

As seen in Table 2 upregulation of the transcription was observed after 24 hours for GSTπ1, HMOX1, and NQO1 in cells treated with 100 μM of flutamide. For HMOX1, the antioxidant response was already initialized at the lower 50 μM dose. CES1, GCLC, and GSTα1 did not show significant change in mRNA expression levels at any of the investigated time points.

Response to Flutamide Administration in Fischer F344 Rats. For the in vivo biomarkers analysis, six male F344 rats were administered 500 mg/kg flutamide by gavage. A control group of six animals received 0.9% NaCl. Three hours and 24 hours postadministration three animals from each group were sacrificed. Plasma was collected on EDTA; liver was removed and placed in RNA later. Urine was collected from 2 days before treatment up to 24 hours post-treatment during three periods. Plasma levels of flutamide were determined to be 47.6 ± 11.4 μmol/l after 3 hours and 0.8 ± 0.5 μmol/l after 24 hours (Supplemental Table S1).

Liver Enzyme Levels. Several biochemical markers for hepatic damage were assessed: Levels of alanine amino transferase (ALT), aspartate amino transferase (AST), glutamate dehydrogenase, lactate dehydrogenase (LDH), and sorbitol dehydrogenase (SDH) in plasma did not show significant elevation in flutamide-treated animals compared with the control group (Table 3).

Scheme 1. Proposed mechanisms for the formation of primary metabolites of flutamide that may give rise to reactive species. Addition of H2O2 generating substrates leads to an increase in formation of a glutathione adduct (Flu-G2) deriving from oxidized M2.

Lipid Peroxidation Products as Markers for Oxidative Stress. As for in vitro experiments, prostanoids and hydroxynonenal derivatives were determined from rat plasma. Remarkably, 15R-PD2 and dihydro-keto PE2, two prostaglandin isomers that have shown a significant increase upon flutamide treatment in vitro also showed alterations in rats in vivo (Fig. 4, top): Twenty-four hours after compound administration the concentration of 15R-PD2 was increased from 80.3 ± 1.5 to 208.2 ± 1.1 pg/ml and that of dihydro-keto PD2 from 23.2 ± 3.4 to 267.0 ± 1.3 pg/ml in response to flutamide treatment. In contrast, concentrations of iPF2α-VI were below the limit of quantification.

As with the prostaglandin response, the lipid peroxidation–derived HNE metabolites HNE-MA and DHN-MA increased as a result of drug intake, even to a higher extent than for the prostaglandins (Fig. 5A): HNE-MA levels changed by 4.1 and 18.1-fold and DHN-MA by 6.1 and 30-fold after 3 and 24 hours, respectively. These results are consistent with the in vitro effect by which both analytes revealed an increase upon flutamide treatment in a time- and concentration-dependent manner.

Prostaglandin levels from liver tissue samples showed a different time course upon treatment (Fig. 4, bottom). The two isomers 15R-PD2 and dihydro-keto PE2 demonstrated a transient concentration increase after 3 hours of treatment which was followed by a significant decrease after 24 hours.

mRNA Expression of Nrf2-Regulated Genes in Rat Liver Tissue. Flutamide-induced gene expression changes in the liver for the same proteins as in the in vitro hepatocyte study were investigated in rats: Under in vivo conditions cellular response was expressed via the matching mRNA (Table 4). GSTπ1 and HMOX1 showed 7.5- and 5.6-fold induction, respectively. NQO1 exhibited the highest upregulation indicated by a 7.0-fold change from control after 3 hours and even by 94-fold 24 hours after flutamide treatment.

Effects of Flutamide on Oxidative Stress Markers in Rat Urine Samples. As for rat plasma, basal levels of prostaglandin as well as hydroxynonenal metabolites were detected in rat urine samples.
For better comparability all results were normalized to urine creatinine, which revealed a slight drop (2.7-fold) in treated animals, suggesting an impact on renal function under flutamide treatment. No statistically significant change upon flutamide treatment was observed for prostaglandins (data not shown). However, a statistically significant response upon drug administration was evident for HNE-MA and DHN-MA: HNE-MA increased by 4.6-fold and DHN by 7.3-fold compared with the predose urine, and therefore both metabolites seem to be attractive accessible noninvasive biomarkers (Fig. 5B).

**Discussion**

The purpose of this study was to elucidate the relationship between flutamide bioactivation in vitro and in vivo and the induction of hepatic...
oxidative stress. The temporal relationship between individual biomarker responses may suggest early noninvasive surrogates for the development of flutamide-induced liver injury. The focus of this study was the analysis of specific lipid peroxidation products and changes in gene expression as a consequence of flutamide bioactivation. These biomarkers were compared with conventional cytotoxicity markers aiming to validate biomarkers in vitro as well as in vivo experiments.

Flutamide causes DILI in rare case and is reported to induce oxidative stress as one contributing factor. Previous studies have revealed disruption of mitochondrial functions and glucose metabolism upon flutamide treatment (Kashimshetty et al., 2009; Choucha Snouber et al., 2013). Bioactivation of flutamide has been studied in the past, suggesting that the reduction of the compound’s nitro residue and subsequent formation of an iminoquinone moiety leading to redox cycling may be one possible mechanism leading to toxicity. Wen and coworkers (2008) had identified the enzyme NADPH:cytochrome P450 reductase as responsible for the first reduction step. Oxidative pathways have been studied by Kang et al. (2008), who reported the formation of reactive metabolites leading to glutathione adduct formation via CYP1A2 and CYP3A4. However, a causal relation between those findings and the induction of liver toxicity was not shown.

Generally, the induction of hepatotoxicity in rodent models with flutamide is challenging (Matsuzaki et al., 2006; Kashimshetty et al., 2009); application of the relevant human dose of 250–500 mg per day does not translate into significant effects in rodents. Therefore, a relatively high dose of 500 mg/kg was administered in this study. This dose, however, does not cause overt hepatotoxicity and has been applied by other investigators (McMillian et al., 2004; Coe et al., 2006; Higuchi et al., 2012). Drug plasma concentrations 3 hours after administration in rats were between 35.2 and 57.6 µmol/l (Supplemental Table 1). Assuming that these concentrations are relatively close to the maximum plasma concentration (C_max), matching concentrations of 50 and 100 µM were chosen for the corresponding in vitro experiments.

Likewise, established reference markers for liver damage, ATP, LDH (cellular assays), and activities of different aminotransferases (animal study) were analyzed. In addition to terminal endpoints, we also included monitoring of key biochemical parameters such as cell signaling that can point toward the mechanism by which a chemical exerts toxicity.

Here we adopted a sensitive in vitro tool applied to primary hepatocytes via in situ hydrogen peroxide–generating system originally reported by the O’Brien group (Tafazoli et al., 2005). These conditions significantly increased the magnitude of cellular oxidative stress response as indicated by the larger dynamic range of the analyzed biomarkers. This system is particular to drugs carrying structural motifs that may form quinone, quinone-imine, or di-imine metabolites prone to redox cycling. The system was validated by comparison with an analogous HRP-free experiment to ensure the absence of intrinsic cytotoxic effects. Therefore, all subsequent in vitro experiments were conducted using the HRP system. Complementary metabolite identification (MetID) experiments revealed that formation of the reactive di-imine species increased under these test conditions as indicated by the detection of its glutathione adduct.

Flutamide did not exhibit significant effects on the biochemical markers for direct cytotoxicity at concentrations of 50 and 100 µM. A transient decrease of ATP content after 6 hours was observed and a slight increase of LDH levels after 24 hours, but these alterations were not considered significant for a pronounced cytotoxic effect.

A similar trend on ATP content and LDH leakage was observed with 100 µM flutamide, also in the absence of the HRP system; it was concluded that the addition of HRP to the culture system only slightly
aggravates the slight intrinsic cytotoxic effect of flutamide. These results are in accordance with previous studies: Fau et al. (1994) observed significant changes for both measures only at 1 mM flutamide concentration in hepatocyte suspension. For a deficiency in oxygen consumption by 50%, more than 100 μM substrate was needed in plated HepG2 cells as investigated by Will’s group (Nadanaciva et al., 2012). They investigated K562 cells also, which revealed only slight changes in ATP content and membrane integrity at 100 μM flutamide (Swiss et al., 2013). In contrast, O’Brien’s group (Tafazoli et al., 2005) reported cytotoxicity in rat hepatocyte suspension when incubating with 75 μM flutamide. The different incubation conditions and higher concentrations of glucose oxidase (1 IU/ml versus 0.05 IU/ml) may explain the slightly deviating results (MacAllister et al., 2013).

Also in rats in vivo no elevations for the tested amino transferases were detected, indicating lack of overt hepatocellular damage at 1 day after administration of 500 mg/kg flutamide. The observed slight decrease for AST and LDH values upon treatment may be attributable to intergroup differences and remained constant over the time of the experiment.

The complementary investigations of oxidative stress showed that determination of isoprostanes as well as gene expression analysis were

| Plasma of Fischer F344 rats treated with 500 mg/kg flutamide was tested for liver function by changes in the concentration of amino transferases compared with control rats |
|-------|-------|-------|-------|-------|
|       | ALT μkat/l | AST μkat/l | GLDH μkat/l | LDH μkat/l | SDH μkat/l |
| Control 3 h | 0.98 ± 0.19 | 3.36 ± 0.55 | 0.28 ± 0.03 | 87.39 ± 26.3 | 0.23 ± 0.02 |
| Control 24 h | 1.42 ± 0.58 | 3.44 ± 2.01 | 0.15 ± 0.16 | 33.31 ± 35.6 | 0.16 ± 0.09 |
| 500 mg/kg 3 h | 0.97 ± 0.09 | 1.47 ± 0.01 | 0.16 ± 0.03 | 7.17 ± 3.09 | 0.16 ± 0.07 |
| 500 mg/kg 24 h | 0.48 ± 0.33 | 1.19 ± 0.47 | 0.25 ± 0.17 | 9.08 ± 2.84 | 0.09 ± 0.01 |

GLDH, glutamate dehydrogenase.

**Fig. 4.** Effect of flutamide (500 mg/kg) on prostaglandin concentrations in plasma and liver tissue of Fischer F344 rats: Values are means ± SD (n = 3). Significance is indicated by *(P < 0.05), ***(P < 0.01) and ***(P < 0.001).
much more sensitive: In vitro prostaglandin isomers as well as hydroxynonenal metabolites revealed a time- and dose-dependent course of an oxidative stress response, already showing significance after 6 hours of incubation and for 50 μM substrate concentration.

In accordance with our results, ROS formation upon treatment with flutamide has previously been reported after use of a hydrogen peroxide–generating substrate in rat hepatocytes (MacAllister et al., 2013). The magnitude of response as measured by the increase in a generic fluorescent marker for ROS was, however, less pronounced than the increase in individual and thus probably more sensitive and selective biomarkers in the present study.

In line with the in vitro studies, the in vivo results exhibited a similar pattern: The same prostaglandin isomers responded to flutamide treatment in vivo. In plasma, these markers showed significant increases compared with control animals after 24 hours. Hydroxynonenal metabolites were found to be even more markedly increased upon flutamide treatment. At 3 hours postadministration, the increase in plasma levels was already statistically significant and even further enhanced after 24 hours. Moreover, an increased renal excretion of these two markers was also evident compared with vehicle controls. These data are in line with data from previous investigation of the potential of HNE derivatives as biomarkers for oxidative stress. Most of those studies investigated HNE-derived thiol conjugates after induction of oxidative stress directly via free radicals from, e.g., carbon tetrachloride or iron nitrilo-triacetate (Kadiiska et al., 2005; Völkel et al., 2005) or under disease conditions that are associated with increase oxidative tissue injury such as Alzheimer’s disease (Dalle-Donne et al., 2006; Völkel et al., 2006).

In contrast to HNE conjugates, prostaglandin levels in urine were not altered as a consequence of flutamide administration. This finding indicates that the observed changes in plasma levels originate from hepatotoxicity and probably are not caused by renal damage. Analysis of liver tissue supports this suggestion; here, two prostaglandin isomers

**TABLE 4**

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<thead>
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<th>GSTπ1</th>
<th>HMOX1</th>
<th>NQO1</th>
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<tbody>
<tr>
<td></td>
<td>500 mg/kg</td>
<td></td>
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<tr>
<td><strong>GSTπ1</strong></td>
<td>3 h</td>
<td>1.0 ± 0.2</td>
<td>**</td>
</tr>
<tr>
<td></td>
<td>24 h</td>
<td>7.5 ± 0.8***</td>
<td>**</td>
</tr>
<tr>
<td><strong>HMOX1</strong></td>
<td>3 h</td>
<td>0.9 ± 0.5</td>
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<td></td>
<td>24 h</td>
<td>5.6 ± 1.1**</td>
<td>**</td>
</tr>
<tr>
<td><strong>NQO1</strong></td>
<td>3 h</td>
<td>7.0 ± 2.8*</td>
<td>**</td>
</tr>
<tr>
<td></td>
<td>24 h</td>
<td>94.1 ± 41.4*</td>
<td>**</td>
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</table>
that were altered in plasma were increased as well. However, the transient augmentation of 15R-PD2 and dihydro-keto PE2 reverted into a significant reduction 24 hours after treatment, most likely owing to a significant reduction 24 hours after treatment, most likely owing to

Gene expression analysis supported these findings. Even though a change in mRNA expression was not pronounced for all examined genes regulated via Nrf2, the affected genes were consistently affected in vivo and in vitro. CES1, GCLC, and GST change in mRNA expression was not pronounced for all examined

24 hours. This can be a result of differences in sensitivity toward Nrf2 activation or alternative dimerization partners of Nrf2 for different target genes, as recently discussed (Goldring et al., 2004). Induction of HMOX1 and NQO1 via Nrf2 induction has been explored in the past after treatment with sulforaphane and butylated hydroxyanisole (Nioi et al., 2003; Keum et al., 2006). Sharma et al. (2013) demonstrated induction of GSTτ1 and NQO1 by different P450-inducing xenobiotics, suggesting regulation of activating and detoxifying enzymes in rat liver. Researchers from Daiichi Sankyo investigated the effect of different drugs on Nrf2-regulated genes in human hepatocytes and found mainly effects for HMOX1, CES, NQO1, and UGT1A1 (Takakusa et al., 2008). Park et al. (Goldring et al., 2004) had identified increased nuclear Nrf2 protein levels upon treatment of CD1-mice with the hepatotoxic acetaminophen already at nontoxic doses and could also find subsequent functional changes of mRNA levels of HMOX1, GCLC, and mEH (microsomal epoxide hydrolase). This effect is not attributable to GSH depletion alone but rather electrophilic properties of the proposed toxin are necessary to activate the Nrf2 pathway.

In good accordance with our results, a study of gene expression signature by McMillian et al. (2004) demonstrated significant induction of mRNA levels for GSTτ1, HMOX1, and NQO1 in rat liver in response to administration of 500 mg/kg flutamide to rats. These results support causality and relevance of our findings.

Conclusion

This study demonstrates the interrelation of flutamide bioactivation and its consequence on cellular oxidative stress via redox cycling after reduction of its aromatic nitro group. We were able to assemble a panel of noninvasive biomarkers and experimental conditions suitable for the characterization of drug-induced effects in vitro as well as in vivo. Previous studies showed that higher doses and repeated administration lead to liver enzyme elevations as signs of overt liver damage. Here the presented markers not only have the ability of designating oxidative stress but can serve as early signals of developing liver injury before overt toxicity manifests. In general, they support elucidation of mechanistic liabilities for DILI by monitoring mechanism-specific biomarkers and by observing relevant physiologic and drug-specific metabolism pathways. A combination of metabolism and biomarker studies may help characterize hazard potentials for drug candidates and identify imbalance between activation and detoxification pathways.

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