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Ex vivo Effects of Sorafenib and Regorafenib on Murine Hepatocytes

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Abstract

Sorafenib and regorafenib are structurally-related small-molecular-weight inhibitors of cellular kinases. Regorafenib has a Boxed Warning stating: "Severe and sometimes fatal hepatotoxicity has been observed in clinical trials", while sorafenib is considered less hepatotoxic. This *ex vivo* study assessed the effects of sorafenib (2.5 and 50 μM) and regorafenib (5.0 and 50 μM) on liver structure, ultrastructure, cellular respiration (mitochondrial O_2 consumption), ATP, caspase activity, urea synthesis, and glutathione. Liver fragments from Taylor Outbred mice were incubated in Krebs-Henseleit buffer (continuously gassed with 95% O_2:5% CO_2) with and without the drugs for 3 to 4 h. The presence of sorafenib or regorafenib had insignificant effects on liver structure, cellular respiration, ATP, caspase-3 activity, urea synthesis, and glutathione. At 3 h, liver histology with and without 2.5 μM sorafenib or 5.0 μM regorafenib was similar. Liver histology with 50 μM sorafenib was slightly worse than untreated tissue at 3 h, showing single hepatocyte necrosis and cellular disintegration. With 50 μM regorafenib, the histology was closely mirroring untreated tissue at 3 h. Similarly, caspase-3, caspase-9, cytochrome c, BAX and annexin A2 immunostains showed no significant drug effects at 4 h (2.5 μM sorafenib or 5.0 μM regorafenib). Electron microscopy revealed a more prominent loss of rough endoplasmic reticulum (rER) integrity with regorafenib treatment compared with sorafenib treatment. Thus, derangements in the rER were more prominent with regorafenib. Otherwise, the studied hepatic surrogate biomarkers did not distinguish between the two compounds.

Keywords: Mitochondria; Respiration; Caspases; Apoptosis; Liver tissue; Murine; Sorafenib; Regorafenib

Abbreviations

ER: Endoplasmic Reticulum; Rer: Rough endoplasmic reticulum; ROS: Reactive O_2 species; GSG: Glutathione; Pd phosphor: Pd(II) complex of *meso*-tetra-(4-sulfonatophenyl)-tetrabenzoporphyrin; mBBr: Monobromobimane; zVAD-fmk: *N*-benzyloxycarbonyl-val-ala-asp(O-methyl)-fluoromethylketone; Ac-DEVD-AMC: *N*-acetyl-asp-glu-val-asp-7-amino-4-methylcoumarin; AMC: Amino-4-methylcoumarin; TFA: Trifluoroacetic Acid; DTNB: 5,5'-dithio-bis(2-nitrobenzoic acid)]; KH buffer: Krebs-Henseleit buffer; EM: Electron Microscopy; MSA: Methanesulfonic Acid

Introduction

The use of medications is often limited by "off target" adverse events that frequently involve the mitochondria [1]. Drug development, thus, requires screening the candidate compounds for potential mitochondrial disturbances. Hepatic failure associated with the nucleoside reverse transcriptase inhibitors, for example, is due to a mitochondrial toxicity, which includes inhibition of mitochondrial DNA polymerases [2]. The "mitochondrial cell death pathway" is another example, which involves leakage of cytochrome c from the mitochondrial intermembrane to the cytosol. In combination with the apoptotic protease activating factor-1, cytochrome c triggers caspase cascades (cysteine, aspartate-specific proteases) [3]. Biomarkers for these events include decreased cellular respiration (mitochondrial O_2 consumption), reduced cellular ATP synthesis, and generation of reactive O_2 species (ROS; commonly associated with depletion of cellular glutathione, GSH) [4,5]. These changes are often associated with altered cellular and mitochondrial structures and ultrastructures.

Sorafenib (a biaryl urea) and regorafenib (fluoro-sorafenib) are novel anticancer drugs. These structurally-related, small molecular weight inhibitors of cellular kinases [e.g., vascular endothelial growth factor receptor 2 (VEGFR-2), platelet-derived growth factor receptor (PDGFR), rapidly accelerated fibrosarcoma (Raf) kinase, and Fms-like tyrosine kinase-3 (FLT3)] are known to induce various levels of liver injuries. Regorafenib has a Boxed Warning, stating: "Severe and sometimes fatal hepatotoxicity has been observed in clinical trials" [6]. The hepatotoxicity of sorafenib, on the other hand, is less pronounced [7]. Sorafenib is an approved treatment for renal cell carcinoma and hepatocellular carcinoma and regorafenib is an approved treatment for colorectal cancer.

The cytotoxicities of sorafenib and regorafenib include alterations in multiple signaling pathways, mitochondrial disturbances, execution of apoptosis (via caspase dependent and independent pathways), induction of endoplasmic reticulum (ER) stress, inhibition of protein synthesis, generation of ROS and depletion of cellular GSH. These mechanisms are mainly studied in malignant cells [8-15]. Thus, it is

unclear whether these modes of action are applicable to normal tissue (e.g., the liver). Furthermore, it is unknown whether the fatal hepatotoxicity of regorafenib is mediated through the same mechanisms. This study addressed some of these issues by testing liver fragments from Taylor Outbred mice, using highly-sensitive structural and functional biomarkers [4,5].

An *in vitro* liver preparation based on the work of Berry and others [16] was recently developed to study the effects of various drugs on hepatocyte bioenergetics (the biochemical processes involved in cellular energy metabolism and conversion) [4,5]. Exposure of liver fragments to 8 μM dactinomycin for 60 min was used as a positive control for induction of apoptosis [4], confirming the analytical system could detect hepatotoxicities. The same methodology was employed here to investigate whether regorafenib hepatotoxicity could be identified *in vitro*.

Experimental Section

Reagents

Sorafenib [*m.w.* 464.8; 4[4-({[4-chloro-3-(trifluoromethyl)phenyl]carbamoyl}amino)phenoxy] N-methylpyridine2carboxamide]andregorafenib[*m.w.* 482.8;4[4({[4chloro3(trifluoromethyl)phenyl]carbamoyl}amino)-3-fluorophenoxy]-N-methylpyridine-2-carboxamide] were purchased from Selleck Chemicals (Houston, TX, USA). Pd(II) complex of meso-tetra-(4-sulfonatophenyl)-tetrabenzoporphyrin (Pd phosphor) was purchased from Porphyrin Products (Logan, UT). Monobromobimane (mBBr, *m.w.*=271.1) was purchased from Molecular Probes (Eugene, Oregon).A lyophilized powder of the pan-caspase inhibitor N-benzyloxycarbonyl-val-ala-asp(O-methyl)-fluoromethylketone (zVAD-fmk, *m.w.*=467.5) was purchased from Calbiochem (La Jolla, CA). The caspase-3 substrate Ac-DEVD-AMC (N-acetyl-asp-glu-val-asp-7-amino-4-methylcoumarin; *m.w.*=675.64) was purchased from Axxora LLC (San Diego, CA).Complete® protease inhibitor cocktail was purchased from Roche Applied Science (Indianapolis, IN).Rabbit anti-cleaved caspase-3 antibody, rabbit anti-BAX antibody (#D2E11) and rabbit anti-annexin antibody (#D11G2) were purchased from Cell Signaling Technology (Boston, MA, USA).Rabbit anti-cytochrome c antibody [(H-104): sc-7159] was purchased from Santa Cruz Biotechnology, Inc. (Texas, USA). Rabbit anti-caspases-9 antibody (ab52299) was purchased from Abcam (Cambridge, MA, USA). Glucose, DTNB [5,5'-dithio-bis(2-nitrobenzoic acid)], GSH (*m.w.*=307.43), HPLC-grade methanol, dichloromethane, trifluoroacetic acid (TFA), methanesulfonic acid (MSA), MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] and remaining reagents were purchased from Sigma-Aldrich (St. Louis, MO).

Sorafenib and regorafenib were dissolved in dimethyl sulfoxide (DMSO) and stored at -20oC. GSH was dissolved in dH_2O and stored at -80°C; its concentration was measured by Ellman's reagent [17]. GS-bimane derivative, sodium methane sulfonate, mBBr (0.1 M in acetonitrile) and DTNB (10 mM in 100 mM Tri-Cl, pH 8.0) solutions were prepared and stored as described [18]. GSH standard (10 μM) was used to generate a calibration curve, which was linear from 10 to 200 picomoles. zVAD-fmk (2.14 mM), Ac-DEVD-AMC solution (7.4 mM), Pd phosphor solution (2.5 mg/mL=2 mM), NaCN (1.0 M), glucose oxidase (10 mg/mL), and Complete® protease inhibitor cocktail were prepared and stored as described [4,5].

Mice

Taylor Outbred (9-10 weeks old) mice were housed at the animal facility in rooms maintained at 22°C, 60% humidity and 12-h light-dark cycles. The use of Taylor Outbred mice was simply due to availability. The mice had ad libitum access to standard rodent chow and filtered water. The study received approval from the Animal Ethics Committee-United Arab Emirates University - College of Medicine and Health Sciences.

Liver specimens

Mice were anesthetized by sevoflurane inhalation (10 μL/g). Liver specimens (20 to 30 mg each; giving an average radius of ~1.5 mm, sufficiently small to allow penetration of O_2 and nutrients throughout the piece) were collected by 4-mm skin biopsy punches (Miltex GmbH, Germany) and *immediately* immersed in ice-cold *modified* Krebs-Henseleit (KH) buffer (115 mMNaCl, 25 mM $NaHCO_3$, 1.23 mM NaH_2PO_4, 1.2 mM Na_2SO_4, 5.9 mM KCl, 1.0 mM EDTA, 1.18 mM $MgCl_2$, 10 mM glucose, and 0.5 μL/mL Complete® protease inhibitor cocktail, pH 7.5) gassed with 95% O_2: 5% CO_2 as previously described [4,5]. The samples were then incubated at 37oC in 50 mL in normal KH buffer (115 mM NaCl, 25 mM $NaHCO_3$, 1.23 mM NaH_2PO_4, 1.2 mM Na_2SO_4, 5.9 mMKCl, 1.25 mM $CaCl_2$, 1.18 mM $MgCl_2$, and 10 mM glucose, pH 7.5) supplemented with 0.5 μL/mL Complete® protease inhibitor cocktail and gassed with 95% O_2: 5% CO_2.

Each drug was tested at its therapeutic concentration and at 50 μM. In humans, the geometric mean peak plasma level (C_{max}) of sorafenib is about 2.5 μM and of regorafenib is about 5.2 μM [6,7]. The duration of drug exposure was 3 to 4 h. The drug vehicle dimethyl sulfoxide was added to the control experiments.

Specimens were also processed for histology, electron microscopy, respiration, ATP, caspase activity, and urea synthesis as previously described [4,5,19-22]. A brief description of these analytical methods is given below.

Histology

The tissue was fixed in 10% neutral formalin, dehydrated in increasing concentrations of ethanol, cleared with xylene and embedded in paraffin. Three-micrometer sections were prepared from paraffin blocks, stained with hematoxylin and eosin (H&E), and immunostained for caspase-3, caspase-9, cytochrome c, BAX, and annexin [4,5].

Electron microscopy (EM)

Samples were processed for electron microscopy as previously described [19]. The tissue was immersed at 25°C for 3 h in McDowell and Trump fixative. It was then rinsed with phosphate-buffered saline (PBS) and fixed with 1% osmium tetroxide for 1 h. The sample was washed with dH_2O, dehydrated in graded ethanol and propylene oxide. The mixture was infiltrated and embedded in agar-100 epoxy resin. Polymerization was allowed to occur at 65°C for 24 h. Semi-thin (1 μm) and ultra-thin (95 nm) sections were prepared using Reichert–Jung Ultracut Ultramicrotome (Leica Microsystems, Wetzlar, Germany). Semithin sections were stained on glass slides with 1% aqueous toluidine blue. Ultrathin sections were contrasted on 200-mesh copper grids with uranyl acetate; this step was followed by lead

citrate double stain. The grids were imaged on CM10 transmission electron microscope (Philips, Amsterdam, Netherlands).

Cellular respiration

The phosphorescence O_2 analyzer was used to monitor O_2 consumption by the liver fragments [4,5]. O_2 detection was performed with the Pd phosphor (absorption maximum=625 nm and phosphorescence maximum=800 nm).Samples were exposed to 600 per min light flashes from a pulsed light-emitting diode array (peak output, 625 nm). Emitted phosphorescent light was detected by the Hamamatsu photomultiplier tube after passing through 800 nm filter.

A program was developed using Microsoft Visual Basic 6, Microsoft Access Database 2007, and Universal Library components (Universal Library for Measurements Computing Devices), which allowed direct reading from the PCI-DAS 4020/12 I/O Board (PCI-DAS 4020/12 I/O Board) [20].

The phosphorescence decay rate (1/τ) was characterized by a single exponential. The values of 1/ were linear with dissolved O_2: 1/o=1/ + $k_q[O_2]$, 1/τ=the phosphorescence decay rate in the presence of O2, 1/ τo=the phosphorescence decay rate in the absence of O_2, and k_q=the second-order O_2 quenching rate constant in s^{-1}• μM^{-1} [21].

In the vials sealed from air, $[O_2]$ decreased linearly with time (zero-order kinetics). The rate of respiration (k, in μM O_2 min^{-1}) was the negative of the slope $d[O_2]/dt$. NaCN inhibited respiration, confirming O_2 was consumed in the mitochondrial respiratory chain.

ATP content

Liver fragments were homogenized in ice-cold 2% trichloroacetic acid and neutralized with 100 mM Tris-acetate, 2 mM ethylenediaminetetraacetic acid (pH 7.75). The supernatants were stored at -20°C until analysis. ATP was measured using the Enliten ATP Assay System (Bioluminescence Detection Kit, Promega, Madison, WI). The luminescence reaction contained 2.5 μL of the acid-soluble supernatant and 25 μL of the luciferin/luciferase reagent. The luminescence intensity was measured at 25°C using the Glomax Luminometer (Promega, Madison, WI) [4,5].

Intracellular caspase activity

Liver specimens were incubated at 37°C in oxygenated KH buffer containing 37 μM Ac-DEVD-AMC with and without 32 μM zVAD-fmk (final volume, 0.5 mL). The tissue was disrupted by vigorous homogenization. The supernatants were centrifuged (16,300g for 90 min) through a Microcentrifuge Filter (*m.w.* limit=10,000 Dalton, Sigma©), separated on HPLC, and analyzed for the free fluorogenic AMC moiety [4,5].

Urea synthesis

Liver specimens were incubated at 37°C in 50 ml KH buffer (gassed with 95% O_2: 5% CO_2) for 3 h. Specimens were placed in 1.0 mL KH buffer supplemented with 10 mM NH_4Cl and 2.5 mM ornithine. The reactions were continued at 37°C for 50 min. The solutions were analyzed for urea as previously described [22].

Cellular GSH

Cellular GSH was labeled with mBBr in a 0.5 mL reaction containing the liver specimen, 10 mMTris-MSA (pH 8.0) and 1.0 mM mBBr. The mixture was incubated at 25°C in the dark for 15 min. The labeling was quenched with 100 μL of 70% perchloric acid. The solution was diluted with 10 mM Tris-MSA and the tissue was disrupted by homogenization. The supernatant was collected by centrifugation (13,000xg at 4°C for 10 min) and stored at -20°C until HPLC analysis [18].

HPLC

Reversed-phase HPLC system (Waters, Milford, MA, U.S.A.) was used. Ultrasphere IP column, 4.6×250 mm (Beckman, Fullerton, CA, U.S.A.) was operated at 25°C at 1.0 mL/min. For GSH detection, the analysis was performed as previously described [18]. Solvent A was 0.1% (v/v) TFA/water and solvent B was HPLC-grade methanol. The gradient was: 0 min, 10% B; 5 min 10% B; 13 min, 100% B; 15 min, 10% B; 20 min, re-inject. The excitation and emission wavelengths were 390 nm and 480 nm, respectively. Injection volume was 50 μL.

For AMC (amino-4-methylcoumarin) detection, the excitation wavelength was 380 nm and the emission wavelength 460 nm. Solvents A and B were HPLC-grade methanol:dH_2O 1:1 (isocratic). The run time was 15 min [4,5].

Statistical analysis

Data were analyzed on SPSS statistical package (version 19), using the nonparametric (2 independent samples) Mann-Whitney test.

Results

Histology

Figure 1A shows representative H&E assessments of liver fragments incubated with and without 2.5 μM sorafenib and 5.0 μM regorafenib for 3 h. Histology was similar in the three specimens, revealing mild cellular disintegrations in the forms of cytoplasmic ballooning, vacuolar degeneration and micro-steatosis (Figure 1A). Another experiment (Figure 1B) shows representative H&E images with and without 50 μM sorafenib or 50 μM regorafenib at 0 h and at 3 h. Cytoplasmic vacuolization (processing artifact) and early cytoplasmic ballooning were noted at 0 h; otherwise, the nuclear details, cell membrane integrity and hepatic architecture were preserved. Inflammation, apoptosis, necrosis and cholestasis were absent. At 3 h (untreated), hepatic architecture was relatively preserved. Hepatocyte ballooning, vacuolar degeneration, cell membrane disintegration, early nuclear disintegration and spotty necrosis were more evident. Thus, there were structural changes associated with *in vitro* incubations without addition of drugs.

With sorafenib, the histology was slightly worse than untreated tissue at 3 h, showing more single hepatocyte necrosis and cellular disintegration. The hepatic architecture, however, was preserved and inflammation and cholestasis were absent. With regorafenib, the histology was closely mirroring untreated tissue at 3 h (Figure 1B). Histological changes, thus, were not clearly noticeable at 3 h.

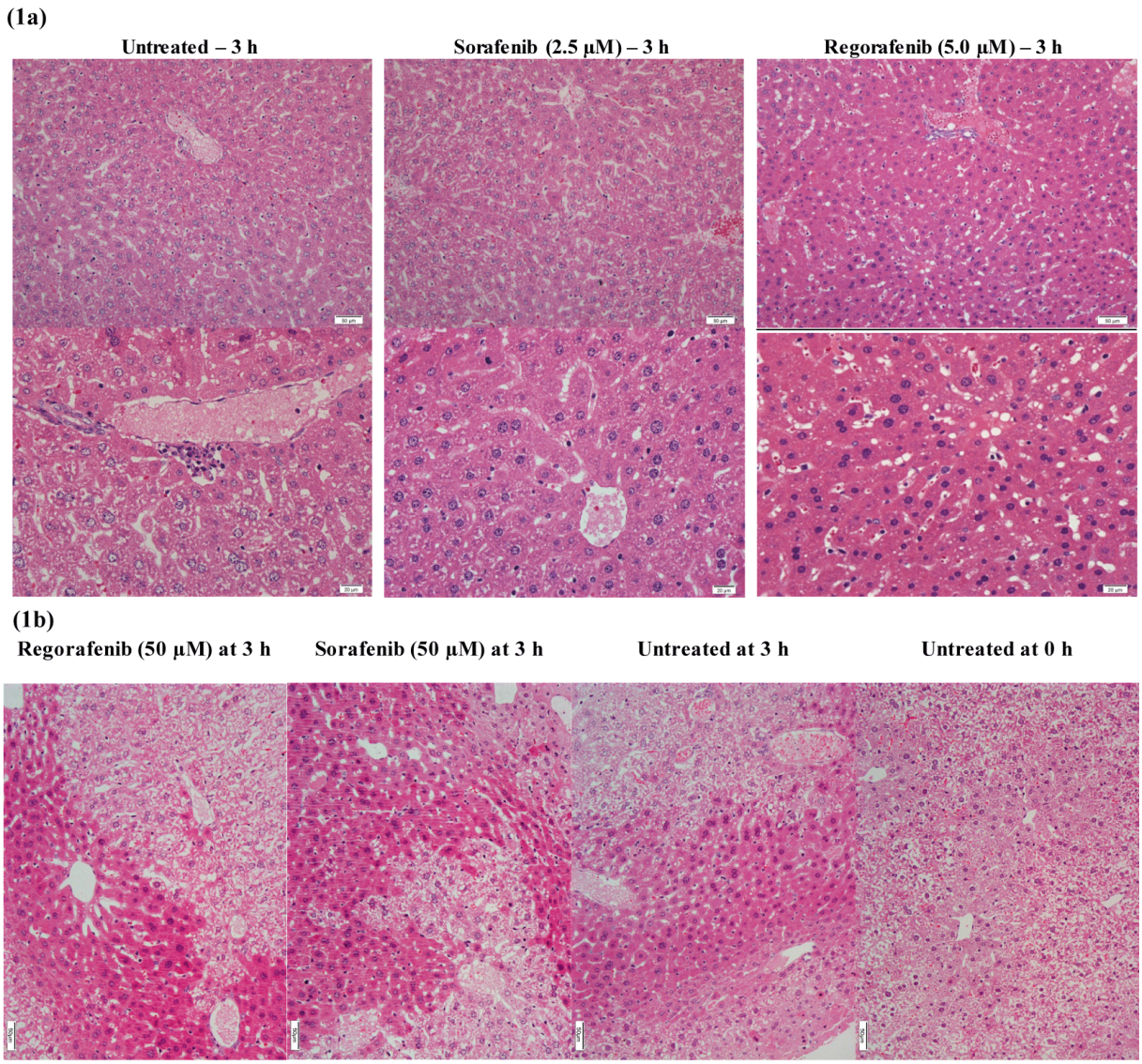

Figure 1: Liver histology. Panel A (H&E staining, 20x and 40x): Untreated at 3 h, 2.5 µM sorafenibat 3 h and 5.0 µM regorafenib at 3 h. **Panel B** (H&E staining, 20x): Untreated at 0 h, untreated at 3 h, 50 µM sorafenib at 3 h and 50 µM regorafenib at 3 h.

Liver fragments were incubated at 37oC in 50 mL KH buffer (continuously gassed with 95% O2: 5% CO2) with and without 2.5 µM sorafenib or 5.0 µM regorafenib for 4 h. The specimens were then processed for immunostaining with several apoptosis biomarkers. At 0 h (untreated liver fragment), the caspase-3 stain was negative. At 4 h, the caspase-3 stain in both untreated and treated specimens was 1%, mostly localized to Kupffer cells (Figure 1S, Supplementary Material). Cytoplasmic cytochrome c stain of the untreated liver fragment at 0 h was negative; at 4 h, the cytoplasmic staining was more intense than the treated tissue. Sorafenib treatment demonstrated a slightly more intense cytoplasmic positivity than regorafenib treatment (Figure 2S, Supplementary Material). BAX immunostain of the untreated liver fragment at 0 h was undetectable. BAX expression increased at 4 h in untreated and treated liver fragments (Figure 3S, Supplementary Material). Annexin A2 immunostain of the untreated liver fragment at 0 h was undetectable. The expression of annexin A2 increased at 4 h in untreated more than treated liver fragments (Figure 4S, Supplementary Material). The intensity of caspase-9 immunostain of untreated liver fragment at 0 h and 4 h and treated liver fragments at 4 h was zero (Figure 5S, Supplementary Material).

Electron microscopy

Since histological findings were not prominent at 3 h, EM studies were performed at 4 h. Representative images of liver fragments at 0 h and at 4 h with and without 2.5 µM sorafenib or 5.0 µM regorafenib are shown in Figure 2A. The hepatocyte architecture was preserved at 0 h. For untreated tissue at 4 h, the hepatocyte showed minimal distension of the mitochondria and minimal disintegration of the rER. For tissue treated with sorafenib, the mitochondrial swelling and the

rER disintegration were slightly more than in untreated tissue at 4 h. For tissue treated with regorafenib, the rER changes were evidently more prominent (Figure 2A). Thus, derangements in the rER were more prominent with a therapeutic dosing of regorafenib.

Figure 2B shows representative images of liver fragments at 0 h and at 3 h with and without 50 μM sorafenib or 50 μM regorafenib. For untreated tissue at 0 h, the hepatocyte showed intact mitochondria and rER. For untreated tissue at 3 h, the hepatocytes demonstrated minimal mitochondrial swelling and relatively preserved rER with attached ribosomes. For tissue treated with sorafenib, the hepatocyte showed mild swelling of the mitochondria and relatively preserved rER. The focal minimal detachment of ribosomes from the rER was similar to untreated tissue at 3 h. For tissue treated with regorafenib, the hepatocyte showed mild swelling (distension) of the mitochondria and disruption of the rER with focal detachments of ribosomes. Thus, exposure to high-dose regorafenib produces more noticeable rER derangements.

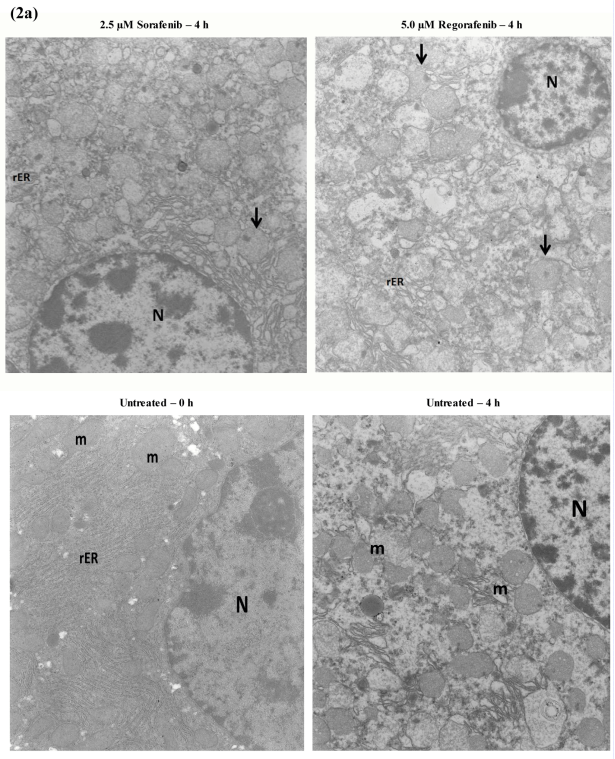

Figure 2: Hepatocyte ultrastructure. Panel A: Untreated tissue at 0 h, showing a hepatocyte with preserved architecture. Note hepatocyte nucleus (N), numerous intact mitochondria (m) and rER; magnification =98,000. Untreated tissue at 4 h, showing a hepatocyte with minimal distension of the mitochondria (m) and minimal disintegration of the rER; magnification =7,000. Tissue treated with 2.5 μM sorafenibat 4 h, showing a hepatocyte with mild distension of the mitochondria (arrow) with amorphous densities. Early loss of integrity of rER was also noted; magnification =7,000. Tissue treated with 5.0 μM regorafenib at 4 h, showing a hepatocyte with mild distension of the mitochondria (arrows) with amorphous densities. The rER changes were more evident; magnification =7,000.

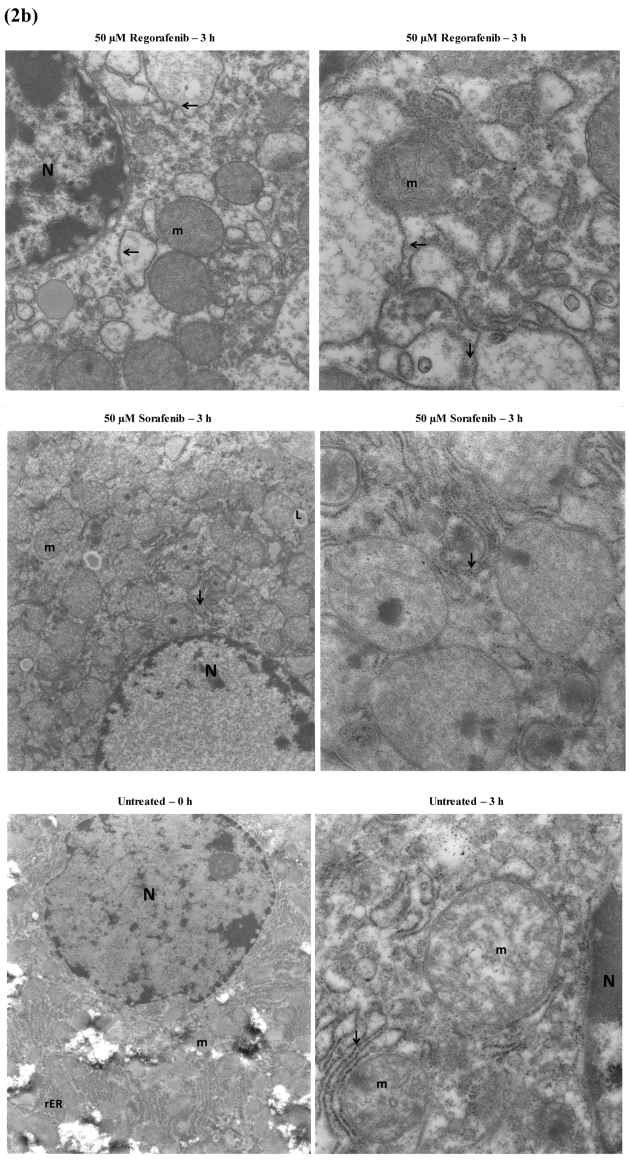

Figure 2B: Untreated tissue at 0 h, showing a hepatocyte with preserved architecture; magnification =7,000. The white areas represented an artifact from specimen collection or preparation. Untreated tissue at 3 h, showing a hepatocyte with relatively preserved cellular architecture. Note hepatocyte nucleus (N), mitochondria (m) with minimal swelling, relatively preserved rER (arrow) with attached ribosomes; magnification =27,500. Tissue treated with 50 μM sorafenib at 3 h, showing a hepatocyte with swollen mitochondria and relatively preserved rER. The focal minimal detachment of ribosomes (arrows) from rER was similar to untreated liver at 3 h; magnification =27,500.Tissue treated with 50 μM regorafenib at 3 h,showing a hepatocyte with mildly distended mitochondria (m), significant disruption of the rER and focal detachments of the ribosomes (arrows); magnification =14,000.

Cellular respiration

Five separate experiments, each involving five mice, were performed in KH buffer for each compound. Representative O2 runs are shown in Figure 3, and a summary of all results is shown in Table 1. The rate of respiration (mean ± SD, in μM O_2 min^{-1} mg^{-1}) without addition was 0.21 ± 0.05, with the addition of 2.5 or 50 μM sorafenib was 0.19 ± 0.01 (p=0.797), and with the addition of 5.0 or 50 μM regorafenib was 0.19 ± 0.02 (p=0.606).Thus, the drugs had no noticeable effects on hepatocyte respiration.

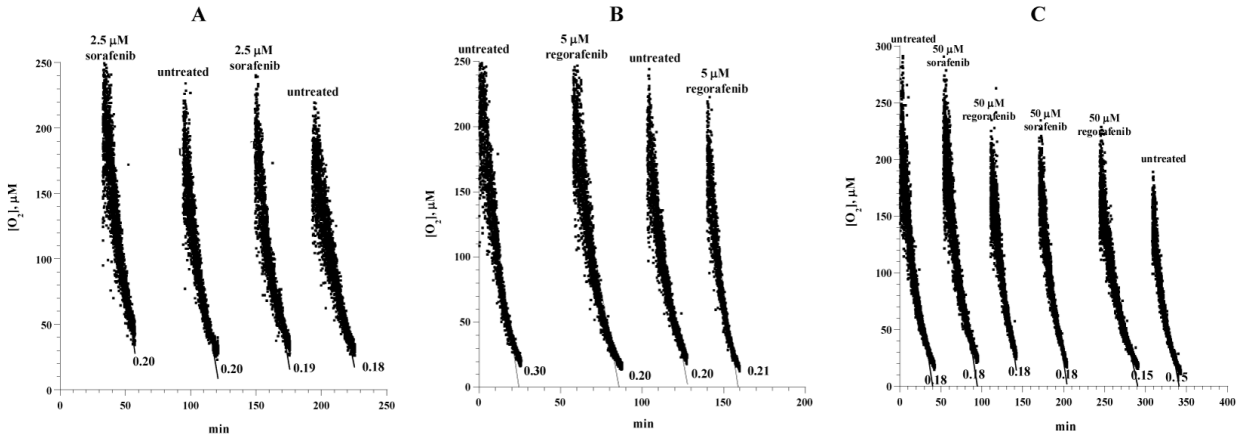

Figure 3: Hepatocyte respiration. Liver specimens were incubated at 37°C in 50 mL KH buffer (continuously gassed with 95% O_2: 5% CO_2) with and without designated concentrations of sorafenib or regorafenib. At specific time periods, samples were washed with KH buffer and processed for O_2 measurements at 37°C. Representative runs are shown. The lines are linear fits. The rate of respiration (k, μM O_2 min^{-1}) was set as the negative of the slope of [O_2] *vs.t.* The values of k_c (μM O_2 min^{-1} mg^{-1}) are shown at the bottom of each run. **Panel A:** Liver cellular respiration with and without 2.5 μM sorafenib. **Panel B:** Liver cellular respiration with and without 5 μM regorafenib. **Panel C:** Liver cellular respiration with and without 50 μM sorafenib or 50 μM regorafenib.

The same experiments were repeated in RPMI medium. The conditions were no addition, 2.5 and 5.0 μM sorafenib and 5.0 and 50 μM regorafenib. The incubation time was 0 to 300 min. The results are in Supplementary Material (Figure 6S). For t=0 min, the values of k_c were 0.41 ± 0.08 (n=4). For the sorafenib experiments and $0 < t \leq 300$ min, the values of k_c for untreated specimens were 0.25 ± 0.05 (n=8) and for treated specimens 0.29 ± 0.05 (n=8), p=0.130. For the regorafenib experiments and $0 < t \leq 300$ min, the values of k_c for untreated specimens were 0.25 ± 0.09 (n=8) and for treated specimens 0.24 ± 0.08 (n=8), p=0.867. Thus, the rate of respiration was slightly higher in RPMI medium than in KH buffer (Table 1).

Cellular ATP

Four separate experiments, each involving four mice, were performed. Hepatocyte ATP ($pmol^{-1}mg^{-1}$) without addition was 56 ± 23 (n=5), with 2.5 or 50 μM sorafenib was 47 ± 42 (n=4, p=0.556) and with of 5.0 or 50 μM regorafenib was 41 ± 22 (p=0.556), Table 1. Thus, the drugs had no noticeable effects on hepatocyte ATP.

Intracellular caspase activity

The caspase-3 substrate analogue Ac-DEVD-AMC was used to measure hepatocyte caspase activity. Three separate experiments each involving three mice were performed. Representative HPLC runs are shown in Figure 4A-4D, and a summary of all of the results is shown in Table 1. Briefly, liver specimens were incubated with and without 50 μM sorafenib or regorafenib. At t=3 h, the specimens were transferred to the Ac-DEVD-AMC cleavage reaction in the presence and absence of zVAD-fmk. The tissue was then vigorously disrupted and the supernatants were separated on HPLC and analyzed for the released AMC moiety (retention time=4.6 min). zVAD-fmk inhibited the release of AMC, confirming Ac-DEVD-AMC was mainly cleaved by intracellular caspases (Figure 4). The AMC peak area (arbitrary unit $mg^{-1} \div 10^3$) in untreated sample at t=0 h was 72, and at t=3 h was 145. The AMC peak area in the sample treated with sorafenib was 81 and with regorafenib was 70 (Figure 4). In another experiment, the AMC peak area at t=3 h without treatment was 66, with 2.5 μM sorafenib was 65 and with 5.0 μM regorafenib was 30. Thus, the drugs had no noticeable effects on hepatocyte caspase activity. Consistently, the drugs had no significant effects on casapse-3, cytochrome c, BAX, caspase-9 and annexin A2 immunostains (Figure 1S-5S, Supplementary Material) and MTT assay (Table 1S, Supplementary Material).

Urea synthesis

Liver specimens were incubated as above with and without sorafenib (2.5 or 50 μM) or regorafenib (5.0 or 50 μM). At t=3 h, specimens were rinsed and incubated in 1.0 mL KH buffer supplemented with 10 mM NH_4Cl and 2.5 mM ornithine for 50 min. The solutions were then analyzed for urea. Four individual experiments involving four mice were performed for sorafenib and five individual experiments involving five mice were performed for regorafenib. The concentration of urea (mg/dL mg^{-1}) without addition was 0.16 ± 0.03, with sorafenib was 0.17 ± 0.03 (p=0.610), and with regorafenib was 0.17 ± 0.06 (p=0.429). The concentration of urea in specimens that were immediately placed KH-NH_4Cl-ornithine solution (t=0 h) was 0.25 ± 0.01 mg/dL mg^{-1} (Table 1). Thus, the drugs had no noticeable effects on hepatocyte urea synthesis.

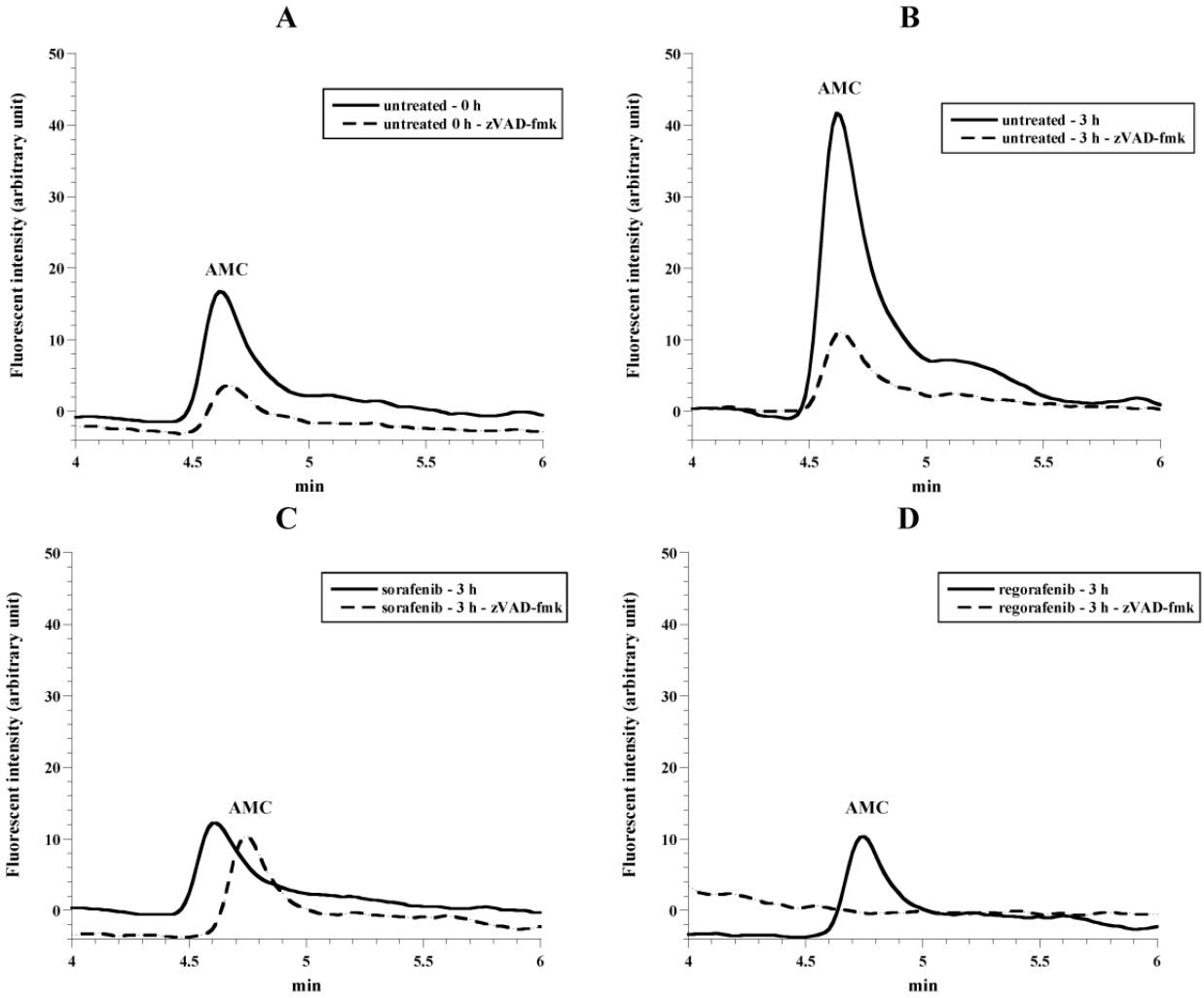

Figure 4: Hepatocyte caspase activity. Representative HPLC runs of liver tissue extracts showing intracellular caspase activity. Specimens were incubated at 37°C in 50 mL KH buffer (continuously gassed with 95% O_2: 5% CO_2) without additions or with the addition of 50 μM sorafenib or 50 μM regorafenib. At *t*=3 h, the specimens were transferred to the Ac-DEVD-AMC (casapse-3 substrate) reaction in the presence and absence of zVAD-fmk (pancaspase inhibitor). Tissues were then vigorously disrupted and the supernatants were separated on HPLC and analyzed for the AMC moiety (AMC retention time =4.6 min). **Panel A:** Intracellular caspase activity with and without zVAD-fmk at 0 h. **Panel B:** Intracellular caspase activity with and without zVAD-fmk at 3 h. **Panel C:** Intracellular caspase activity at 3 h in the presence of 50 μM sorafenib with and without zVAD-fmk. **Panel D:** Intracellular caspase activity at 3 h in the presence of 50 μM regorafenib with and without zVAD-fmk.

Cellular GSH

Liver specimens were incubated as above with and without sorafenib (50 μM) or regorafenib (50 μM). At *t*=180 min, the specimens were rinsed and transferred to the mBBr labeling reaction. Cellular GS-bimane was determined on HPLC as shown in Figure 5.Two individual experiments involving two mice were done. Cellular GSH (pmol mg^{-1}) without addition was 279 ± 9, with the addition of sorafenib was 384 ± 76, and with the addition of regorafenib was 312 ± 45. GSH content in specimens that were immediately placed in the mBBr reaction at *t*=0 min was 685 ± 129 pmol mg^{-1} (Table 1). Thus, the drugs had no noticeable effects on hepatocyte GSH.

Discussion

The Food and Drug Administration (FDA) approved the kinase inhibitor sorafenib for treatment of advanced renal cell (2005) and hepatocellular carcinomas (2007). Regorafenib, as approved in 2012 for colorectal cancer. Although these two compounds are closely related, their clinical adverse events differ. For example, regorafenib may produce fatal hepatotoxicity, a complication that has not been linked to sorafenib [6,7].

This study employed structural (hepatocyte histology and EM) and functional (hepatocyte respiration, ATP, caspase activity, and urea synthesis) surrogate biomarkers to investigate the toxic effects of sorafenib and regorafenib *in vitro*. The measured functional

biomarkers were similar in treated and untreated liver specimens (Table 1). The ultrastructural changes, most notably loss of rER integrity and detachment of ribosomes, were more evident with regorafenib than sorafenib (Figures 2A and B). At 0 h, the untreated liver specimen showed well preserved hepatocyte architecture. At 4 h, the hepatocytes demonstrated only *minimal* mitochondrial distension and rER disintegration. Sorafenib treatment (2.5 μM for 4 h) produced *mild* mitochondrial distension and rER disintegration. The rER changes were more noticeable with regorafenib treatment (2.5 μM for 4 h), Figure 2A. Higher sorafenib dose (50 μM for 3 h) showed only mitochondrial distension with relatively preserved rER. Significant disruption of the rER and focal detachments of the ribosomes were evident in the tissue treated with 50 μM regorafenib at 3 h (Figure 2B).

Biologic activities of sorafenib and regorafenib can be demonstrated *in vitro* within a few hours of exposure to 0.1-10 μM of the drugs [8-13]. The cytotoxicity is cell specific and includes alterations in multiple signaling pathways, execution of apoptosis, induction of ER stress, and inhibition of protein synthesis. At 5 to 50 μM, for example, sorafenib inhibited the proliferation of hepatocellular carcinoma cell lines; the degree of inhibition was dependent on pERK expression [10]. Exposure of human leukemia cells to 10 μM sorafenib produced cytotoxicity that involved inducing ER stress and generation of ROS [12]. At 3 to 20 μM, sorafenib induced apoptosis in melanoma cells in 4 h, mainly by nuclear translocation of the apoptosis-inducing factor [14]. In cell lines, apoptosis is induced via caspase dependent (e.g., caspase-2 and caspase-4 processing) and independent (e.g., nuclear translocation of the apoptosis-inducing factor) pathways.Regorafenib also inhibited the proliferation of human hepatocellular carcinoma cell lines, but the cells regrew after drug removal [13,15].

In contrast to these malignant cells, the findings here show high doses of sorafenib and regorafenib (50 μM) do not alter normal liver caspase-3 activity (Figure 4) or GSH content (Figure 5). The results also show hepatocyte bioenergetics (respiration and ATP content) following *in vitro* exposure to sorafenib or regorafenib for several hours is similar to that of untreated tissue (Figure 3 and Table 1). Consistently, hepatocyte urea synthesis is similar with and without the drugs (Table 1). By contrast, both compounds produce subtle derangements in hepatocyte ultrastructure (Figure 2). The rER changes, however, are more prominent with regorafenib, perhaps accounting for its potential hepatotoxicity (Figures 2A and 2B). Of note, the mitochondrial swelling is relatively similar in samples treated with sorafenib or regorafenib (Figures 2A and 2B).

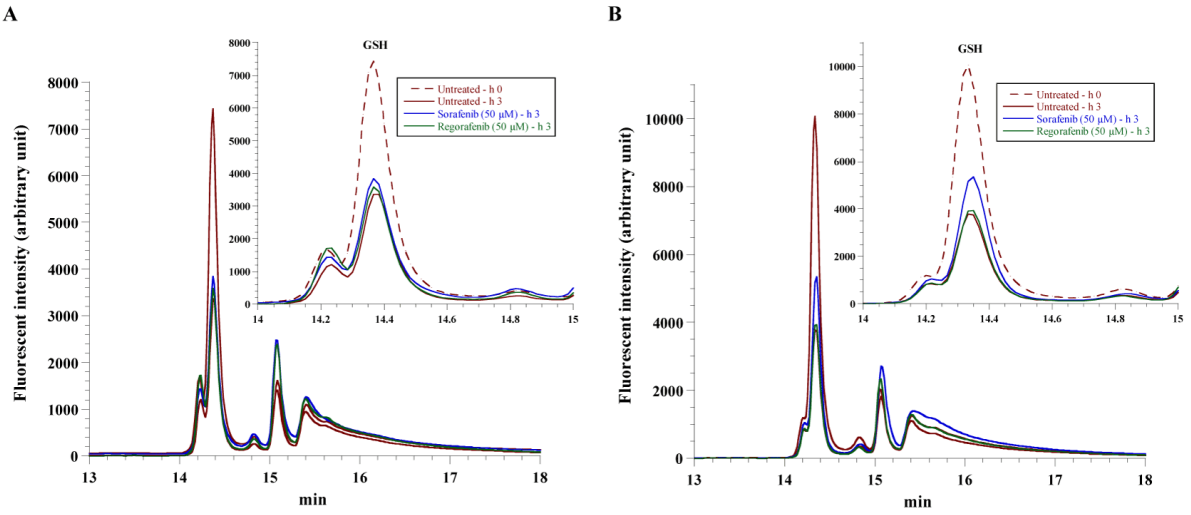

Figure 5: Hepatocyte GSH. HPLC runs of liver acid-soluble supernatants, showing the GS-bimane peaks with a retention time of 14.3 min. Specimens were incubated at 37°C without addition with 50 μM sorafenib or 50 μM regorafenib. At *t*=3 h, the specimens were transferred to the mBBr labeling reaction and processed and analyzed as described in Methods.

Sorafenib and regorafenib are tested at their therapeutic concentrations (2.5 and 5 μM, respectively [6,7] and at a 10- to 20-fold higher than the therapeutic concentration (50 μM). The first objective of using 50 μM was to investigate potential concentration-dependent hepatotoxicity. Of note, a few of the fatal regorafenib-associated hepatotoxicity were in patients with dehydration, a complication that increased serum drug concentration. The second aim was to compensate for the relatively short drug exposure (3-4 h).

In contrast to previous toxicology studies that were performed on isolated hepatocytes [23], this study utilized viable liver fragments. Advantages of our approach include minimum tissue handling and avoiding extensive collagenase digestion required for single cell preparations. Successful liver fragment collection, however, requires rapid sampling of thin (<0.2 mm) slices, preferably <20 mg, while the liver is still perfused [24].The specimens should be immediately immersed in appropriate buffer supplemented with protease inhibitors.

Important limitation of this study is deterioration of the measured biomarkers with time in KH buffer (Table 1) and RPMI medium (Figure 6S, Supplementary Material). The biomarker values at *t*=0 (immediately after tissue collection) corresponded to the best possible results (Table 1). At $180 \leq t \leq 240$ min, hepatocyte respiration decreased by 30%, ATP decreased by 66%, caspase-3 activity increased by 47%, urea synthesis decreased by 36%, and GSH decreased by 59% (Table 1). This limitation prevented extending the incubation beyond 4-5 h.

	t=0 min	180 ≤ *t* ≤ 240 min		
	No addition	**No addition**	**Sorafenib**	**Regorafenib**
k_c [a] ($\mu M\ O_2\ min^{-1}\ mg^{-1}$)	0.30 ± 0.08 (n=4)	0.21 ± 0.05 (n=9)	0.19 ± 0.01 (n=5)	0.19 ± 0.02 (n=5)
ATP [b] ($pmol\ mg^{-1}$)	167	56 ± 23 (n=6)	47 ± 40 (n=4)	41 ± 22 (n=4)
AMC peak area [c] (arbitrary unit $mg^{-1} \div 10^3$)	71	104 ± 41 (n=4)	96 ± 41 (n=3)	47 ± 21 (n=3)
Urea synthesis [d] ($mg/dL\ mg^{-1}$)	0.25 ± 0.01 (n=6)	0.16 ± 0.03 (n=6)	0.17 ± 0.03 (n=4)	0.17 ± 0.06 (n=5)

[a]Five individual experiments involving five mice were done for each compound. The concentration of sorafenib was 2.5 μM in three experiments and 50 μM in two experiments. The concentration of regorafenib was 5 μM in three experiments and 50 μM in two experiments. The *in vitro* incubation with the drugs was up to 6 h. There was no statistical significance between the untreated and treated pairs.

[b]Four individual experiments involving four mice were done for each compound. The concentration of sorafenib was 2.5 μM in three experiments and 50 μM in one experiment. The concentration of regorafenib was 5 μM in three experiments and 50 μM in one experiment. The *in vitro* incubation with the drugs was 180 to 240 min. There was no statistical significance between the untreated and treated pairs (p>0.556).

[c]Three individual experiments involving three mice were done for each compound. The concentration of sorafenib was 2.5 μM in two experiments and 50 μM in one experiment (Figure 4). The concentration of regorafenib was 5 μM in two experiments and 50 μM in one experiment (Figure 4). The *in vitro* incubation with the drugs was 180 to 240 min. There was no statistical significance between the untreated and treated pairs (p>0.114).

[d]For sorafenib, four individual experiments involving four mice were done. The concentration of sorafenib was 2.5 μM in two experiments and 50 μM in two experiments. For regorafenib, five individual experiments involving five mice were done. The concentration of regorafenib was 5 μM in three experiments and 50 μM in two experiments. The *in vitro* incubation with the drugs was 180 min. There was no statistical significance between the untreated and treated pairs (p>0.429).

Values are mean ± SD

Table 1: Effects of sorafenib and regorafenib on liver tissue respiration, ATP content, caspase activity, and urea synthesis.

Intracellular caspase activity is measured on viable liver fragments, using Ac-DEVD-AMC. This substrate is cleaved by several caspases, including caspase-3 (k_{cat}/K_m=218,000 s^{-1}), caspase-7 (k_{cat}/K_m=37,000 s^{-1}), caspase-1/interleukin-1 converting enzyme (k_{cat}/K_m=30,000 s^{-1}), caspase-6 (k_{cat}/K_m=2,000 s^{-1}), and caspase-4 (k_{cat}/K_m=1,800 s^{-1}) [25]. Of note, ER stress triggers a specific cascade involving caspase-12, -9, and -3 in a cytochrome c-independent manner [26]. Consistently, caspase-3 labeling at 4 h was similar in treated and untreated specimens; the few caspase-3 positive cells were mostly localized to Kupffer cells (Figure 1S, Supplementary Material). Similarly, compared with untreated specimens, cytochrome c, BAX, caspase-9 and annexin A2 labeling showed no significant drug effects at 4 h (Figure 2S – 5S, Supplementary Material).

The concentrations used in this study were therapeutics and 10 to 20-fold higher than therapeutics. These drug levels produced structural and ultrastructural changes in the liver (Figures 1 and 2). It is unclear, however, whether the observed adverse effects were due to multikinase inhibition (e.g., VEGFR-2, PDGFR, Raf kinase, FLT3, Ret, and cKit) or "off" target effects. Further studies are needed to address this important issue.

In conclusion, this *in vitro* study shows murine hepatocyte bioenergetics, caspase-3 activity, urea synthesis and GSH are not significantly affected by sorafenib or regorafenib. Altered hepatocyte rER is more noticeable with regorafenib. Thus, these data demonstrate ultrastructural changes with regorafenib treatment, justifying its Boxed Warning of hepatotoxicity. The findings call for novel methods that allow early detection of regorafenib and sorafenib hepatotoxicities.

Author Contributions

The manuscript was written through contributions of all authors. All authors have given approval to the final version of the manuscript.

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Conflict-of-Interest

The authors declare that they have no conflict of interest.

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