Regorafenib impairs mitochondrial functions, activates AMP-activated protein kinase, induces autophagy, and causes rat hepatocyte necrosis

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5 **autophagy, and causes rat hepatocyte necrosis**
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12 **Abstract**
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15 The tyrosine kinase inhibitor regorafenib was approved by regulatory agencies for cancer treatment, albeit
16 with strong warnings of severe hepatotoxicity included in the product label. The basis of this toxicity is
17 unknown; one possible mechanism, that of mitochondrial damage, was tested. In isolated rat liver
18 mitochondria, regorafenib directly uncoupled oxidative phosphorylation (OXPHOS) and promoted calcium
19 overload-induced swelling, which were respectively prevented by the recoupler 6-ketocholestanol (KC) and
20 the mitochondrial permeability transition (MPT) pore blocker cyclosporine A (CsA). In primary
21 hepatocytes, regorafenib uncoupled OXPHOS, disrupted mitochondrial inner membrane potential (MMP),
22 and decreased cellular ATP at 1 h, and triggered MPT at 3 h, which was followed by necrosis but not
23 apoptosis at 7 h and 24 h, all of which were abrogated by KC. The combination of the glycolysis enhancer
24 fructose plus the mitochondrial ATPase synthase inhibitor oligomycin A abolished regorafenib induced
25 necrosis at 7 h. This effect was not seen at 24 h nor with the fructose or oligomycin A separately. CsA in
26 combination with trifluoperazine, both MPT blockers, showed similar effects. Two compensatory
27 mechanisms, activation of AMP-activated protein kinase (AMPK) to ameliorate ATP shortage and
28 induction of autophagy to remove dysfunctional mitochondria, were found to be mobilized. Hepatocyte
29 necrosis was enhanced either by the AMPK inhibitor Compound C or the autophagy inhibitor chloroquine,
30 while autophagy inducer rapamycin was strongly cytoprotective. Remarkably, all toxic effects were
31 observed at clinically-relevant concentrations of 2.5 to 15 μ M. These data suggest that uncoupling of
32 OXPHOS and the resulting ATP shortage and MPT induction are the key mechanisms for regorafenib
33 induced hepatocyte injury, and AMPK activation and autophagy induction serve as pro-survival pathways
34 against such toxicity.
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56 **Keywords:** regorafenib, hepatotoxicity, mitochondrion, rat, hepatocyte, necrosis
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1. Introduction

Regorafenib is a broad spectrum tyrosine kinase inhibitor (Davis et al. 2013). It was recently approved for treatment of metastatic colorectal cancer and advanced gastrointestinal stromal tumors by the U.S. Food and Drug Administration (FDA) (FDA 2012a), the Health Canada (Health-Canada 2013), and the European Medicines Agency (EMA) (EMA 2013). Based on clinical trial data, these regulatory agencies have called attention to liver toxicity as one of the most serious adverse effects of regorafenib (EMA 2013; FDA 2012a; Health-Canada 2013). A black box warning, the strongest warning about a drug's adverse effect issued by the FDA, has been included in the U.S. product labeling of regorafenib to highlight its liver risks. Similarly, the product monograph of regorafenib endorsed by Health Canada also contains black-boxed "serious warnings and precautions" about liver injuries, and the EMA requires that a "black inverted triangle" be displayed in the package leaflet to indicate that such risk is under "additional monitoring". As clinical trials are ongoing to examine if regorafenib provides an alternative treatment for hepatocellular carcinoma (Bruix et al. 2013), its clinical usage is expected to increase significantly, as are the risks of liver injury. Of note, studies conducted in preclinical test species identified the liver as a target organ of regorafenib toxicity in multiple animal species (mouse, rat and dog) (FDA 2012b), suggesting that animal based models may be used to understand the basis for regorafenib's clinical hepatotoxicity. However, the mechanism for regorafenib induced hepatotoxicity has not been explored to date, limiting the development of strategies to minimize liver risks.

Various mechanisms have been proposed as operative in drug hepatotoxicity. Among these, mitochondrial impairment appears to be commonly shared by many drugs and chemicals (Jones et al. 2010; Pessayre et al. 2012). Mitochondrial damage is usually considered as a drug's "off-target" effect contributing to adverse reactions, though recent evidence suggests that it may also be important for some drugs' pharmacological effects. For example, metformin, the first-line drug for type 2 diabetes, causes slight inhibition of mitochondrial respiratory complex I leading to downstream reactions responsible for the anti-diabetic and

possible anti-cancer effects (Bridges et al. 2014; Pernicova and Korbonits 2014). As for drug induced liver injury (DILI), it has been long recognized that acetaminophen overdose induced hepatotoxicity was associated with inhibition of mitochondrial respiration (Meyers et al. 1988). Most recently, it was suggested that a battery of *in vitro* mitochondrial assays may be predictive of a chemical's potential to induce DILI in humans (Porceddu et al. 2012), though the need for very high concentrations of drugs to trigger positive mitochondrial effects casts doubt on its *in vivo* relevance. This study examined if regorafenib causes mitochondrial dysfunction in isolated rat liver mitochondria and primary cultured hepatocytes. At concentrations comparable to the human therapeutic exposure of 8 μ M in the circulation (FDA 2012b), regorafenib uncoupled hepatic mitochondrial oxidative phosphorylation (OXPHOS), dissipated mitochondrial inner membrane potential (MMP), caused cellular adenosine triphosphate (ATP) shortage, and later induced mitochondrial permeability transition (MPT), all of which eventually led to necrosis but not apoptosis in primary rat hepatocytes. At the same time AMP-activated protein kinase (AMPK), the master regulator of energy homeostasis, and autophagy, a process to remove damaged mitochondria, were activated to promote hepatocyte survival. Thus the induction of liver mitochondrial dysfunction does indeed seem to be the basis for regorafenib-induced hepatotoxicity.

2. Materials and methods

2.1. Reagents and chemicals

Regorafenib with a purity of 99.62 % was obtained from Selleck Chemicals (Houston, TX; catalog number S1178; batch number S117804). Calcein acetoxymethyl ester (Calcein AM) and 5,5',6,6'-tetrachloro-1,1',3,3'-tetraethylbenzimidazolocarbo-cyanine iodide (JC-1) were from Life Technologies Corporation (Grand Island, NY). CellTiter-Glo Luminescent Cell Viability Assay kit (for measuring cellular ATP levels) and Caspase-Glo® 3/7 Assay Systems (for measuring caspase 3/7 activity) were from Promega (Madison, WI). AMPK Activator III (DHPO) and Compound C (CAS 866405-64-3) were from EMD Millipore (Billerica, MA). All other chemicals were from Sigma-Aldrich (St. Louis, MO).

2.2. Animal care

Male Sprague-Dawley rats weighing 250 to 400 g were obtained from the U.S. Food and Drug Administration National Center for Toxicological Research (NCTR) breeding colonies. Animal care and experimental procedures were approved by the NCTR Institutional Animal Care and Use Committee in accordance with the National Institutes of Health (NIH) "Guide for the Care and Use of Laboratory Animals."

2.3. Mitochondria isolation and drug treatment

Rat liver mitochondria were isolated using a published procedure (Frezza et al. 2007). Only mitochondria with a respiratory control ratio (RCR) >3 were used and all experiments were completed within 5 h after isolation. The protein concentration was adjusted to 0.5 mg/ml using the respiration buffer (Frezza et al. 2007). Regorafenib was dissolved in DMSO and added to final concentrations of 0, 1, 2.5, 5, and 10 μ M. The final DMSO concentration was 0.1%. After 3 min incubation oxygen consumption was measured as described below. In specified cases, mitochondria were treated for 3 min with 2 μ g/ml oligomycin A prior to addition of regorafenib, and after 3 min incubation 200 μ M 6-ketocholestanol (KC) was added to observe the recoupling effect (Starkov et al. 1997).

2.4. Hepatocyte culture and drug treatment

Primary hepatocytes were cultured in William's E medium which contains 10 mM D-glucose as detailed previously (Shi et al. 2011). Briefly, rats were anesthetized by sodium pentobarbital and the liver was perfused *in situ* with a buffer containing 137 mM NaCl, 5.4 mM KCl, 0.5 mM KH_2PO_4 , 0.6 mM Na_2PO_4 , 25 mM NaHCO_3 , 5 mM glucose, 5 μ g/ml bovine insulin, 50 unit/ml penicillin and 50 μ g/ml streptomycin for about 8 min. Then 2 mM CaCl_2 , 0.03% collagenase (Sigma catalog number C5138), and 0.01% soybean trypsin inhibitor was added to the perfusion buffer and the liver was perfused for additional 5 min. The digested liver was gently homogenized to release the cells and an isotonic Percoll® solution (pH7.3) was used to purify the hepatocytes (Shi et al. 2011). Of note, our preliminary data show that hepatocytes from male and female rats had the similar response to regorafenib. Therefore only male rats were used in subsequent experiments. The cells were allowed to attach overnight before drug treatment. Regorafenib was added to culture medium at final concentrations of 0, 2.5, 5, 7.5, 10 and 15 μ M. The final DMSO

concentration was 0.1%. In specified cases, cells were treated with regorafenib in the presence of either cyclosporine A (CsA; 0.5 μ M) plus trifluoperazine (TFP; 2.5 μ M) (Imberti et al. 1993; Reid et al. 2005), or fructose (15 mM), or fructose plus oligomycin A (0.6 ng/ml) (Nieminen et al. 1994). To inhibit autophagy, cells were treated with 15 μ M chloroquine (CQ). To induce autophagy, 5 μ M rapamycin was used (Ni et al. 2012). Compound C (10 μ M) was used to inhibit AMPK activation (Saber et al. 2014). A previously published procedure (Sunakawa et al. 2013) was modified to collect cells for measuring oxygen consumption. Briefly, hepatocytes seeded in 100 $\times$ 20 mm dishes were washed by PBS and detached by trypsinization. Cells were re-suspended in respiration buffer at 2×10^6 cell/ml and 0.0075% digitonin was added for 3 min to permeabilize the cell membrane (Sunakawa et al. 2013). The stock solution of 7.5% digitonin in water was prepared by heating at 95 $^{\circ}$ C for 5 min immediately before use.

2.5. Mitochondrial oxygen consumption

Clark type oxygen electrodes were used to measure oxygen consumption. Two Oxytherm systems from the Hansatech Instruments Ltd were used. Each mitochondrial sample (1 mg/ml, 2 ml) or preparation of permeabilized hepatocytes (2×10^6 cell/ml, 2 ml) was evenly divided, with one half used to measure glutamate-malate driven respiration and the other succinate driven respiration; the two measurements for the same sample were thus taken simultaneously. The succinate concentration was 5 mM, and the glutamate/malate concentrations were 5 mM and 2.5 mM, respectively, while for measuring oligomycin A-inhibited respiration, the concentrations of these substrates were decreased to 0.25-fold the original level so that the oxygen in the reaction tubes would not be exhausted too quickly. The ADP concentration was 100 μ M and 300 μ M for mitochondria and permeabilized hepatocytes, respectively. The maximal oxygen consumption rate after addition of ADP was assigned as state 3 respiration. When the added ADP is converted to ATP, the oxygen consumption rate decreases significantly to a slower rate, which was assigned as state 4 respiration. The RCR was calculated by dividing the state 3 respiration rate by the state 4 respiration rate. The well-established uncoupler carbonyl cyanide m-chlorophenyl hydrazone (CCCP) was used as a control.

2.6. Mitochondria swelling assay

A published procedure (Waldmeier et al. 2002) with only slight modifications was used to measure mitochondria swelling. Briefly, freshly isolated rat liver mitochondria (1 mg/ml protein) were re-suspended in a buffer containing 70 mM sucrose, 190 mM mannitol, 20 mM HEPES, 5 mM glutamate, 0.5 mM malate (pH 7.5), and then regorafenib was added to final concentrations of 1 to 15 μ M, with DMSO (0.1 %) used as a control. After 3 min, 1 mM K_2HPO_4 and 25 μ M $CaCl_2$ were added, and then the decrease in absorbance at 535 nm was immediately measured using a Synergy 2 Multi-Mode Microplate Reader every 2 min for 30 min. To prevent MPT, CsA (1 μ M) was added for 1 min prior to regorafenib treatment.

2.7. *MMP detection*

Hepatocytes cultured in 6-well plates were trypsinized and re-suspended at 0.6×10^6 cell/ml in culture medium containing 10 μ M JC-1. Cells were incubated at 37 °C for 15 min, with occasional shaking to ensure a good suspension. Cells were pelleted by centrifugation and re-suspended at 1.2×10^6 cell/ml in PBS. A small fraction of the cells (usually 20 μ l) was mounted on a microscope slide to observe fluorescence change using the FITC and the Texas Red channels, and 50 μ l cells were transferred into a 96-well black plate for the measurement of JC-1 fluorescence by a plate reader. The excitation wavelength was 488 nm, and the emission wavelengths were 530 nm and 590 nm. The fluorescence intensity ratio between 590 nm and 530 nm was calculated to reflect changes in MMP (Cervinkova et al. 2007).

2.8. *MPT detection*

Calcein AM with cobalt (II) chloride quenching was used for MPT detection. While calcein AM readily enters cells and universally stains all the mitochondrial, nuclear and cytosolic compartments, cobalt (II) chloride selectively quenches the fluorescence in the latter two subcellular locations, leaving the signal in mitochondria intact. When MPT occurs, the mitochondrion traps less calcein AM and cellular fluorescence is therefore decreased (Petronilli et al. 1999). A published procedure using calcein AM (Petronilli et al. 1999) was modified to monitor MPT in rat hepatocytes. Briefly, hepatocytes seeded in 6-well plates were trypsinized and re-suspended at 1×10^6 cell/ml in modified Hanks' Balanced Salt Solution (HBSS) containing 0.2 μ M calcein AM. Cells were incubated at 37 °C with occasional shaking for 10 min, and then 400 μ M cobalt (II) chloride was added. After 1 min, cells were pelleted by centrifugation and re-suspended

in PBS after washing with HBSS to remove excessive calcein AM and cobalt (II) chloride. All the processed samples were immediately analyzed on an Aria-III flow cytometer (BD Biosciences) using the 488 nm laser and FITC detection channel. The flow cytometry data were analyzed using FACS DIVA7 software (BD Biosciences). Mean immunofluorescence intensity (MFI) value was used to determine the average staining intensity of calcein AM.

2.9. ATP detection and lactate dehydrogenase (LDH) leakage assay

Cellular ATP levels were determined using a commercial kit as described previously (Sonko et al. 2011). The LDH leakage assay, whose results correlated well with the trypan blue exclusion method, was performed following the published procedure (Shi et al. 2011).

2.10. Apoptosis detection

Hepatocytes were grown in 6-well plates on 22 × 22 mm cover glasses coated with type I bovine collagen. Apoptotic cells were immediately detected by Nikon Eclipse Ti microscopy at the 405 nm channel following staining for 30 min in culture medium containing 8 µg/ml Hoechst 33258. Cells displaying nuclear condensation and chromatin fragmentation were considered as apoptotic. At least 500 cells were counted (Nagai et al. 2002). Apoptosis was also determined by measuring caspase 3/7 activity (Caspase-Glo® 3/7 Assay) following the manufacturer's protocol. Briefly, hepatocytes were seeded in coated 96-well white plates with a clear bottom, and incubated with regorafenib for 1 to 24 h. Cells were washed once with PBS, after which an equal volume of culture medium and caspase 3/7 substrates (50 µl for each) were added. After incubation at 37 °C for 30 min, cellular luminescence was measured to determine caspase 3/7 activity. The luminescence signal was normalized to DMSO (0.1%) treated cells. Diclofenac (400 µM) was used as a positive control to induce hepatocytes apoptosis (Gomez-Lechon et al. 2003).

2.11. Detection of AMPK activation and autophagy induction

The previously published procedures were modified to detect AMPK and autophagy activation (Ding and Yin 2009; Lim et al. 2012). Briefly, cells were lysed using an ice-cold buffer containing 50 mM Tris-HCl (pH 7.4), 50 mM sodium fluoride, 5 mM sodium pyrophosphate, 5 mM sodium orthovanadate, 1 % sodium dodecyl sulfate (SDS), 0.25 % sodium deoxycholate, 1 % Triton X-100, 1 mM phenylmethanesulfonyl

fluoride, 1 mM benzamidine, 5 µg/ml soybean trypsin inhibitor, 1:100 protease inhibitor cocktail (Sigma-Aldrich, St. Louis, MO; catalog number P8340), 2 mM ethylenediaminetetraacetic acid (EDTA) and 2 mM ethylene glycol tetraacetic acid (EGTA). The lysates were centrifuged at 20,000 g for 10 min and then the supernatant was used for Western blot. AMPK activation was detected using an antibody against phospho-AMPK alpha (threonine-172) (p-AMPK) (Cell Signaling Technology, Beverly, MA; catalog number 8359S) (Lim et al. 2012). An AMPK antibody (Cell Signaling Technology, Beverly, MA; catalog number 8359S) was also used to determine AMPK levels. The antibody against the microtubule-associated protein 1 light chain 3 (LC3-II) (Novus Biologicals, Littleton, CO; catalog number NB100-2220) was used to assess autophagy (Ding and Yin 2009). The same samples were also probed with a beta-actin antibody (Santa Cruz Biotechnology, Dallas, TX; catalog number sc-1616) for data normalization.

2.12. Statistical analysis

One-way analysis of variance (ANOVA) followed by Dunnett's test were used to compare the means among groups using the Graph Pad Prism software (version 6; La Jolla, CA). A *p* value of less than 0.05 or 0.01 was considered to be of statistical significance.

3. Results

3.1. Regorafenib directly uncoupled liver mitochondrial respiration

Freshly isolated rat liver mitochondria were used to observe the direct effect of regorafenib on mitochondrial respiration. Fig. 1A shows that the glutamate/malate-driven state 4 respiration was significantly increased by regorafenib in a concentration-dependent manner, with a maximal increase of approximately 7-fold the base line level at 5 and 10 µM, and the state 3 respiration was also enhanced, though to a much less extent, that is, about 2-fold the base line level at 5 and 10 µM. The corresponding RCR began to decrease at 1 µM regorafenib and reached about 1 that is indicative of complete uncoupling at 2.5 to 10 µM. Very similar results were obtained with succinate-driven respiration (Fig. 1B), though in this case the maximal stimulation of the state 4 respiration was approximately 3-fold and the state 3 respiration was unaffected. The effects of regorafenib were similar to the classical uncoupler CCCP. These data provide initial evidence that regorafenib is an uncoupler of mitochondrial respiration.

To confirm that regorafenib is an uncoupler, oligomycin A was used to eliminate the possible confounding effect of ADP on oxygen consumption (Brand and Nicholls 2011). It was found that oligomycin A-inhibited respiration, whether it was driven by glutamate/malate (Fig. 1C) or succinate (Fig. 1D), was concentration-dependently enhanced by regorafenib, with a maximal stimulation of about 3-fold at 10 μ M. These data provide convincing evidence that regorafenib uncouples mitochondrial respiration.

To further characterize regorafenib-induced uncoupling, the well-established recoupler 6-ketocholestanol (KC) (Starkov et al. 1997) was used. Fig. 1C and 1D show that regorafenib-induced stimulation of oxygen consumption was significantly diminished, but not completely prevented, by 200 μ M KC, indicating that regorafenib uncouples mitochondrial respiration partially by mechanisms similar to classical uncouplers such as carbonyl cyanide-4-(trifluoromethoxy)phenylhydrazone (FCCP) (Starkov et al. 1997). KC was only effective against FCCP or CCCP induced uncoupling, but had no effects on fatty acids induced uncoupling, possibly due to the involvement of thermogenin and ATP/ADP-antiporter in the latter case (Starkov et al. 1997). Our data suggest that regorafenib may uncouple respiration by mechanisms involved in both FCCP and fatty acids induced uncoupling.

3.2. Regorafenib promoted calcium overload-induced mitochondrial swelling

A mitochondrial swelling assay, which may reflect MPT but does not directly measure it (Waldmeier et al. 2002), was used to test regorafenib effects on MPT in isolated mitochondria. Fig. 1E shows that regorafenib at 2.5 to 15 μ M, but not 1 μ M, remarkably enhanced calcium overload-induced mitochondrial swelling in a concentration-dependent fashion, and Fig. 1F shows that when mitochondria were pretreated with CsA, a cyclophilin D protein (CypD) binding drug (Waldmeier et al. 2002), the swelling triggered by regorafenib was completely prevented. These data indicate that regorafenib induces MPT when calcium homeostasis is disrupted.

3.3. Regorafenib uncoupled mitochondrial respiration in primary hepatocytes

To observe if regorafenib uncouples mitochondrial respiration in intact cells, rat hepatocytes were treated for 1 h and mitochondrial functions determined. Of note, hepatocyte death measured by LDH leakage (described later in Fig. 3) or caspase activation (data not shown) did not occur at this time point. Fig. 2

shows that both glutamate/malate (Fig. 2A) and succinate (Fig. 2B) driven respiration were significantly uncoupled, as demonstrated by a remarkably increase in the state 4 respiration and a sharp decrease in RCRs. In the meantime, the state 3 respiration was barely affected. A near complete uncoupling, as determined by an RCR of about 1, was observed at 15 μ M regorafenib. The stimulation of the state 4 respiration and the decrease in RCR was significantly antagonized by the recoupler KC, particularly in the 5 to 10 μ M groups, for which the uncoupling effect of regorafenib was almost completely abrogated. However, the uncoupling induced by 15 μ M regorafenib was only partially abolished by KC (Fig. 2A and 2B). These data demonstrate that regorafenib induces KC-preventable uncoupling in primary hepatocytes.

3.4. Regorafenib decreased MMP in primary hepatocytes

As uncouplers may disrupt MMP (Brand and Nicholls 2011), the effect of regorafenib on hepatocyte MMP was determined using JC-1 at 1 h. JC-1 forms aggregates (which show red fluorescence) in cells with normal MMP and exists as monomers (which show green fluorescence) when MMP is disrupted (Pernelle et al. 2011). Fig. 2C shows that hepatocytes treated with vehicle (DMSO) were stained predominantly red with almost no green signal, while regorafenib treated cells displayed a concentration-dependent decrease in red signal and a corresponding increase in green signal, indicating that MMP was depolarized. Again this detrimental effect was significantly ameliorated by the recoupler KC. The quantitative measurement of the red vs. green fluorescence ratio is presented in Fig. 2D, which confirmed that MMP was depolarized after regorafenib treatment, and the recoupler KC profoundly alleviated this effect. These data suggest that regorafenib disrupted MMP via its uncoupling effects.

3.5. Regorafenib triggered MPT in primary hepatocytes

As Fig. 1E and 1F suggest that regorafenib may directly interact with one of the mPTP component CypD in isolated mitochondria, we next examined if regorafenib induces MPT in primary hepatocytes. A representative flow cytometer measurement is shown in Fig. 2E, and the mean and SD of three independent experiments are shown Fig. 2F. Both figures show that the calcein AM fluorescence signal in mitochondria after 3 h started to decrease at the lowest dose of regorafenib (2.5 μ M), and the decrease became statistically significant at 5 to 15 μ M, indicating that regorafenib induced MPT in hepatocytes at higher

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4 concentrations. Notably, the MPT signal was only about 15% of the control in the 15 μ M regorafenib group.
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6 No changes were observed at 1 h or 2 h (data not shown) when significant uncoupling already commenced
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8 (Fig. 2A-B), indicating that MPT was a downstream effect of mitochondrial uncoupling. This notion was
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10 further strengthened by the observation that the recoupler KC remarkably prevented MPT (Fig. 2E and 2F).
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12 Specifically, the MPT induced by regorafenib doses of 2.5 to 7.5 μ M was almost completely prevented by
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14 KC. These data provide strong evidence that regorafenib induces MPT at a later time point via its initial
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16 uncoupling effects.
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19 *3.6. Regorafenib caused ATP shortage in primary hepatocytes*

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22 Severe mitochondrial uncoupling could cause cellular ATP shortage in hepatocytes (Masubuchi et al. 2000).
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24 Fig. 3A-D show that regorafenib indeed lowered cellular ATP in a time- and concentration-dependent
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26 manner. It can be seen that ATP began to decrease as early as 1 h when no LDH leakage was observed, and
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28 such decrease was completely prevented by the recoupler KC (Fig. 3A), suggesting that ATP shortage was
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30 due to the uncoupling effect of regorafenib. At 3 h, ATP levels continued to drop, and KC only partially
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32 prevented such decrease at 15 μ M regorafenib (Fig. 3B), which is in line with the finding in Fig. 2A-B that
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34 KC only partially prevented regorafenib's uncoupling effect at high concentrations. A similar trend was
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36 observed at 7 h and 24 h when LDH leakage became apparent (Fig. 3C-D). Notably, 15 μ M regorafenib
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38 reduced cellular ATP by about 80% at 7 h when only about 25% cells were dead as determined by LDH
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40 release (Fig. 3C). These data demonstrate that ATP shortage, which is largely due to uncoupling, is an early
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42 event prior to cell death.
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45 *3.7. Regorafenib induced necrosis but not apoptosis in hepatocytes*

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48 Significant mitochondrial impairments, particularly MPT, will eventually lead to hepatocyte death, which
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50 can be either apoptotic or necrotic (Malhi et al. 2010). Two methods were used to detect apoptosis, nuclear
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52 fragmentation/chromatin condensation and caspase 3/7 activity. As a positive control, the well-established
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54 apoptosis-inducing drug diclofenac (Gomez-Lechon et al. 2003) was tested in parallel with regorafenib. Fig
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56 3E shows that regorafenib did not induce nuclear condensation or chromatin fragmentation at 7 h, both of
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58 which were readily observed in diclofenac-treated cells, as indicated by arrows. The quantitative results of
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Fig. 3E were shown in Fig. 3F. Fig. 3G shows that regorafenib at 7 h also did not change caspase 3/7 activities, which were remarkably increased by diclofenac. These results demonstrate that regorafenib did not induce hepatocyte apoptosis at 7 h. Of note, a similar trend was obtained at 3 h and 24 h (data not shown).

Hepatocyte necrosis was quantified by LDH leakage, which reflects cell membrane integrity and is a well-accepted indicator of necrosis (Chan et al. 2013). Fig. 3A-B shows that no necrosis was observed at 1 h and 3 h. A moderate but statistically meaningful increase of LDH release was seen at 10 and 15 μ M regorafenib at 7 h (Fig. 3C), which was significantly enhanced at 24 h (Fig. 3D), when 15 μ M regorafenib caused almost 100% cell death, and 7.5 μ M regorafenib led to about 20% cell death, while the lowest concentration of 2.5 μ M elicited only a negligible increase of LDH that was not statistically significant (Fig. 3D). Interestingly, the recoupler KC remarkably reduced regorafenib induced LDH release (Fig. 3C and 3D). These data suggest that regorafenib causes time- and concentration- dependent hepatocyte necrosis that is largely due to the uncoupling of mitochondrial respiration.

3.8. Regorafenib induced necrosis was delayed by the glycolysis enhancer plus mitochondrial ATPase synthase inhibitor

As cellular ATP depletion occurred prior to hepatocyte necrosis and enhanced ATP levels are associated with higher hepatocyte viability (Fig. 3), the effects of the glycolysis enhancer fructose, which provides an alternative energy source to mitochondrial OXPHOS, and the mitochondrial ATPase synthase inhibitor oligomycin A, which prevents the enhanced hydrolysis of ATP induced by uncoupling (Imberti et al. 1993; Masubuchi et al. 2000), were investigated. Fig. 4A shows that fructose alone was not effective in preventing regorafenib induced LDH release, but its combination with oligomycin A almost completely abolished regorafenib toxicity at 7 h, indicating a causal relationship existed between uncoupling of OXPHOS and regorafenib induced hepatocyte death. Of note, oligomycin A alone was strongly toxic (data not shown). At 24 h (Fig. 4B), fructose or its combination with oligomycin A afforded no protection, likely because glycolysis alone failed to provide sufficient energy for survival or other pro-death pathways predominated.

3.9. Regorafenib induced necrosis was partially prevented by MPT blockers

As MPT may determine if cells will survive or die (Malhi et al. 2010), the effect of MPT blockers CsA and TFP (CsA/TFP) (Kon et al. 2004) on regorafenib induced necrosis was investigated. As shown in Fig. 4C, hepatocyte death induced by 15 μ M regorafenib was partially decreased by CsA/TFP at 7 h, indicating that MPT is causatively associated with regorafenib induced hepatocyte injury at the early stage. However, Fig. 4D shows that CsA/TFP afforded no protection at 24 h, likely because regorafenib triggered other pro-death pathways (such as profound ATP depletion as shown in Fig. 3) that cannot be antagonized by MPT blockage after a relatively longer period of time.

3.10. Regorafenib caused AMPK activation that might be cytoprotective

As ATP shortage usually leads to AMPK activation to counterbalance energy deficiency (Viollet et al. 2009), the next set of experiments was carried out to observe if regorafenib activates AMPK in hepatocytes. As shown in Fig. 5A and 5B, pAMPK levels showed no changes at 1 h, but were significantly enhanced at 3 h when no cell death occurred, and a further increase was seen at 7 h, with a maximal increase of about 3-fold the control level. These data demonstrate that regorafenib activated AMPK pathway in hepatocytes.

Chemical regulators of AMPK were then used to examine if AMPK activation contributes to hepatocyte necrosis. Fig. 5C shows that the AMPK inhibitor Compound C barely affected regorafenib induced LDH release at 7 h, but remarkably enhanced 7.5 and 10 μ M regorafenib induced cell death at 24 h (Fig. 5D), indicating that AMPK activation may serve as a pro-survival mechanism. Of note, the AMPK Activator III, DHPO, failed to reduce regorafenib induced hepatocyte necrosis (data not shown), very likely because the AMPK pathway was already maximally activated by regorafenib.

To observe if AMPK activation was triggered by the uncoupling effect of regorafenib, the recoupler KC was used. As shown in Fig. 5E and 5F, pAMPK levels at 7 h were significantly lower in regorafenib (5-10 μ M) plus KC treated hepatocytes than in regorafenib (5-10 μ M) alone treated cells, and KC alone showed no effects on pAMPK levels, suggesting that AMPK activation was very likely initiated by the uncoupling effect of regorafenib.

3.11. Regorafenib caused mild autophagy induction that alleviated cell death

One well-established downstream effect of AMPK activation is autophagy induction (Egan et al. 2011), and MPT may also promote autophagy (Rodriguez-Enriquez et al. 2004), which can be either beneficial or detrimental to hepatocytes (Apostolova et al. 2011; Ni et al. 2012; Saberi et al. 2014). The last experiment then examined if autophagy plays a role in regorafenib induced hepatocyte injury. Fig. 6A and 6B show that high concentrations of regorafenib caused a mild accumulation of LC3-II at 7 h, but not at 1 h or 3 h, suggesting that autophagy was only slightly enhanced at later stages. Fig. 5C shows that the autophagy inducer rapamycin remarkably reduced regorafenib cytotoxicity, and the autophagy inhibitor chloroquine significantly enhanced regorafenib toxicity, indicating that the mild induction of autophagy by regorafenib is likely a protective mechanism to maintain cell viability.

4. Discussion

This is the first study to explore the mechanism of regorafenib hepatotoxicity. A schematic diagram of major findings is presented in Fig. 7.

Hepatotoxic drugs may damage liver mitochondria in many different ways, which can be interrelated (Jones et al. 2010; Pessayre et al. 2012). Here it was found that regorafenib uncoupled OXPHOS, depolarized MMP, depleted ATP, and triggered MPT. Experiments with the recoupler KC established that uncoupling of OXPHOS is the initiating event leading to downstream effects. Time-dependent studies show that uncoupling of OXPHOS occurred prior to MPT induction in primary hepatocytes (Fig. 2), further indicating that MPT is a consequence of uncoupling. Of note, both regulated and un-regulated MPT have been reported for other drugs (Okuda et al. 2010), and here it is clearly demonstrated that regorafenib induced MPT was regulated by CsA, which opens up the possibility of pharmacologically manipulating MPT to alleviate regorafenib toxicity. Interestingly, the mode of regorafenib induced hepatocyte death was predominantly necrosis but not apoptosis, very likely due to the lack of sufficient cellular ATP, which is a consequence of uncoupling, to help execute apoptosis (Malhi et al. 2006). This is also in line with other uncouplers such as usnic acid causing necrosis but not apoptosis in primary hepatocytes (Han et al. 2004).

We found that the rate of state 3 respirations was increased by regorafenib in isolated mitochondria (Fig 1), but not in primary hepatocytes (Fig. 2). The likely reason is that the secondary effects of uncoupling, such

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4 as the acidifying of cytosolic compartment and the impairment of functions of other organelles, may render
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6 the maximal respiration rate in cells “considerably less than” in isolated mitochondria (Brand and Nicholls
7
8 2011). Additional reasons may also include (1) isolated mitochondria were treated for only 3 min, while the
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10 hepatocytes were treated for 1 h (2) digitonin was included to measure respirations in hepatocytes but not
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12 isolated mitochondria and (3) regorafenib may undergo extensive metabolism in hepatocytes producing
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14 metabolites that inhibit respiration. To explore the role of biotransformation in regorafenib induced
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16 mitotoxicity and cytotoxicity may be an interesting future direction.
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19 Many drugs and chemicals have been recognized as uncouplers of liver mitochondrial respiration (Pessayre
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21 et al. 2012; Porceddu et al. 2012), but no therapeutic approaches abrogating this toxicity are currently
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23 available. Though fructose plus oligomycin A prevented uncoupler-induced cell death for a short period of
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25 time (Fig. 4A), they are of no clinical value because toxicity was unaffected at a longer treatment time (Fig.
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27 4B), and oligomycin A is highly toxic by itself. The findings that the recoupler KC significantly
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29 antagonized regorafenib’s uncoupling effect and therefore was protective against hepatocyte death is also
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31 of little clinical value, because the safety profiles of KC are unknown and we found that KC was strongly
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33 toxic to hepatocytes at higher concentrations of $\geq 200 \mu\text{M}$ (data not shown).
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36 Given that therapeutic agents to directly remedy uncoupling of OXPHOS are lacking, it becomes
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38 imperative to identify downstream pathways that contribute to regorafenib induced hepatocyte necrosis.
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40 Toward this end, we have found that MPT, AMPK, and autophagy were all associated with regorafenib
41
42 cytotoxicity. Importantly, it is shown that MPT blockers and autophagy inducers, though exerting their
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44 effects at different levels in the signaling cascade, were both effective in reducing regorafenib cytotoxicity.
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46 As these protective agents are approved drugs that are in current clinical use, they might have potential to
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48 alleviate regorafenib hepatotoxicity. However, the “off-target” effects of these agents, such as the
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50 nephrotoxicity of CsA, which was originally approved as an immunosuppressant (Horina et al. 1993), must
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52 be taken into considerations for this purpose.
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55 The mitotoxicity of regorafenib seems unlikely to be due to overall kinase inhibition, as several other
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57 kinase inhibitors have been shown to have no mitochondrial liability at a wide range of concentrations
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59 (Will et al. 2008). The fact that some approved kinase inhibitors are non-hepatotoxic (Shah et al. 2013)
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4 further indicates that regorafenib's hepatocyte toxicity is likely independent of overall kinase inhibition. On
5
6 the other hand, the observation that AMPK was activated in response to regorafenib's mitotoxicity may
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8 very well contribute to its anti-cancer effects, because pharmacologically induced AMPK activation is
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10 tumor suppressive in both animal models and clinical trials (Shackelford and Shaw 2009). This is
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12 reminiscent of the recent finding that the drug metformin has anti-cancer properties due to its activation of
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14 AMPK by impairing mitochondrial OXPHOS (Leone et al. 2014). Future investigation will examine if
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16 regorafenib's mitotoxicity is involved in its pharmacological effect.
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19 One interesting finding is that regorafenib caused mitotoxicity and subsequent cell death in hepatocytes at
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21 clinically-relevant concentrations. This is in contrast to the majority of previous reports on other drugs, for
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23 which detrimental mitochondrial effects were usually observed at exceptionally high drug concentrations,
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25 that is, up to ten-fold or even one hundred-fold of the average maximal blood concentration (C_{max}),
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27 leaving the *in vivo* relevance uncertain (Dyken and Will 2007; Porceddu et al. 2012). At the recommended
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29 oral dose of 160 mg per day, the average human C_{max} of regorafenib was 8 µM (FDA 2012b). At the
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31 human-equivalent dose, the average rat C_{max} of regorafenib was 10 µM (FDA 2012b). A high-fat meal or
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33 co-administration with a cytochrome P450 (CYPs) inhibitor increased the systemic exposure of regorafenib
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35 by 30-50% in humans (FDA 2012b). It is thus clear that the C_{max} in either rats or human could be as high
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37 as 12 to 15 µM. Here it was found that regorafenib impaired mitochondrial functions and caused
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39 hepatocyte necrosis at 5-15 µM, concentrations readily achieved under *in vivo* conditions in both animals
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41 and humans, indicating that these *in vitro* results are very likely of *in vivo* relevance.
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44 The finding of mitochondrial toxicity at *in vitro* regorafenib concentrations similar to clinically therapeutic
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46 blood concentrations begs the question as to why hepatotoxicity is not more frequently seen in preclinical
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48 studies or in patients treated with this drug. The C_{max} in clinical studies was reached about 3 h after
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50 regorafenib administration, after which regorafenib blood concentration began to decrease rapidly (60%
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52 C_{max} at 8 h) (FDA 2012b), indicating that on average hepatocyte exposures to significant drug
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54 concentrations were limited. Our data show no cell death following 7 h exposures to 7.5 µM regorafenib
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56 (though ATP was decreased by about 40%). Taking into consideration that the liver has very strong
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58 capacity to regenerate, our data are consistent with the observation that most patients and animals show no
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4 apparent hepatotoxicity. Thus, the infrequent but profound hepatotoxicity seen in clinical settings may
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6 reflect individual variations in regorafenib pharmacokinetics such that *in vivo* hepatocyte exposures indeed
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8 approach the hepatotoxic level observed in the current experiments.
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11 Our findings that regorafenib impairs mitochondrial functions may warrant further investigations to
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13 discover circulating biomarkers leaked from damaged liver mitochondria to better monitor regorafenib
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15 hepatotoxicity, as has been recently reported for acetaminophen overdose in humans (McGill et al. 2012). It
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17 may also warrant future structural modifications on regorafenib to reduce or eliminate mitotoxicity while
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19 retaining its pharmacological effects.
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22 This study also suggests that when regorafenib is co-administrated with drugs known to impair
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24 mitochondrial functions, particularly those that inhibit CYPs causing increased C_{max} (FDA 2012b), such
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26 as ketoconazole (Rodriguez and Acosta 1996), the likelihood of developing fatal liver injury could be
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28 increased. Of course, as regorafenib is used for cancer patients who have exhausted conventional treatment
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30 options, the liver risks, as well as the mitotoxicity reported here, shall be carefully weighed against its
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32 clinical benefits. However, as has been highlighted in a recent commentary, drug hepatotoxicity in patients
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34 with advanced cancers shall not be underestimated using a looser criterion (Senior 2013). New strategies to
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36 minimize regorafenib induced liver injury are of key importance and the present study provides useful
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38 insights into mechanisms that should be considered in such strategies.
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41 In summary, we demonstrated the regorafenib impaired mitochondrial functions, activated AMPK kinase,
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43 induced autophagy and caused rat hepatocytes necrosis at clinically relevant concentrations. Our study
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45 provides novel insights into the mechanisms of regorafenib induced hepatotoxicity.
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Figure legend

Fig. 1 Effects of regorafenib on mitochondrial respiration and calcium overload-induced swelling. Freshly isolated rat liver mitochondria (1 mg/ml protein) were treated by regorafenib (1, 2.5, 5 and 10 μ M) with 0.1 % DMSO as a control, and then oxygen consumption measured after 3 min. CCCP (0.3 μ M) was used as a control (A and B). In C and D, oligomycin A (2 μ g/ml) was added to the mitochondria prior to the addition of substrates (glutamate/malate or succinate), and then regorafenib was added, which was followed by 200 μ M KC, and oxygen consumption was measured 3 min after each treatment. No ADP was added for these experiments. In panel E and F, regorafenib was used at 1, 2.5, 5, 10 and 15 μ M. In panel E, 1 mM K_2HPO_4 and 25 μ M $CaCl_2$ were used to induce mitochondrial swelling before the absorbance at 535 nm was recorded for 30 min. In panel F, 1 μ M CsA was added for 1 min prior to regorafenib treatment. Data are mean $\pm$ SD, n=3. * $p<0.05$, compared to 0 μ M regorafenib (that is, 0.1% to 0.3% DMSO) treated mitochondria; # $p<0.05$, compared to the corresponding samples treated by KC.

Fig. 2 Effects of regorafenib on hepatocyte oxygen consumption, MMP and MPT. Primary rat hepatocytes were treated with regorafenib (0, 5, 7.5, 10, and 15 μ M) alone or together with KC (100 μ M) for 1 h, and then cells trypsinized to measure mitochondrial oxygen consumption (A and B). In Fig. C and D, hepatocytes were treated by regorafenib (0, 2.5, 5, 7.5, 10, and 15 μ M) with or without KC for 1 h, and then stained with 10 μ M JC-1 for 15 min. Cells were collected by trypsinization. A small fraction was used for microscopic observation (C), and 50 μ l cell suspensions were used to measure red and green fluorescence, whose ratio was presented in panel D. In panel E, hepatocytes treated for 3 h were stained with calcein AM to detect MPT using a flow cytometer, and the corresponding results from three experiments were presented in panel F. The quantitative data are mean $\pm$ SD, n=3. * $p<0.05$, compared to vehicle (0.2 % DMSO) treated hepatocytes; # $p<0.05$, compared to the corresponding samples treated by KC.

Fig. 3 Effects of regorafenib on hepatocyte cellular ATP levels, LDH release, apoptosis, and caspase activity. Primary rat hepatocytes were treated with regorafenib alone or together with KC for 1 to 24 h, and

cellular ATP and LDH were measured at 1, 3, 7 and 24 h (panel A to D). At 7 h, hepatocytes were stained by Hoechst 33258 to visualize nuclear changes that reflect apoptosis (panel E), whose quantitative data were shown in Fig. F. The hepatocyte apoptosis was also measured by caspase 3/7 activities (panel G). Diclofenac was used as a positive control for apoptosis detection. The quantitative data are mean $\pm$ SD, n=3. * p <0.05, compared to vehicle (0.2 % DMSO) treated hepatocytes; # p <0.05, compared to the corresponding samples treated by KC.

Fig. 4 Effects of fructose, oligomycin A, cyclosporine A (CsA), and trifluoperazine (TFP) on regorafenib induced hepatocyte death. Primary hepatocytes were treated with regorafenib in the absence or presence of fructose (15 mM), or fructose plus oligomycin A (0.6 ng/ml), or cyclosporine A (CsA; 0.5 μ M) plus trifluoperazine (TFP; 2.5 μ M), and LDH release was measured at 7 h and 24 h. The data are mean $\pm$ SD, n=3. * p <0.05, compared to vehicle (0.1% or 0.2 % DMSO) treated hepatocytes; # p <0.05, compared to the corresponding cells treated by regorafenib alone.

Fig. 5 AMPK activation and effects of Compound C in regorafenib treated hepatocytes. Primary rat hepatocytes were treated by regorafenib for 1 h to 7 h, and then AMPK activation was detected by Western blot (panel A). p-AMPK level was normalized to beta-actin and the quantitative data were presented in panel B. In panel C and D, LDH release was measured at 7 h and 24 h after hepatocytes were treated with regorafenib in the absence or presence of 10 μ M Compound C. The effect of KC on AMPK activation is shown in panel E and F. All quantitative data are mean $\pm$ SD, n=3. * p <0.05, compared to vehicle (0.2 % DMSO) treated hepatocytes; # p <0.05, compared to the corresponding cells treated by regorafenib alone.

Fig. 6 LC3-II accumulation and effects of autophagy modulators in regorafenib treated hepatocytes. Primary rat hepatocytes were treated by regorafenib for 1 h to 7 h, and then LC3-II levels were detected by Western blot (panel A). LC3-II levels were normalized to beta-actin and the quantitative data were presented in panel B. In panel C and D, LDH release was measured at 7 h and 24 h after hepatocytes were treated with regorafenib in the absence or presence of 5 μ M rapamycin (Rap) or 15 μ M chloroquine (CQ). The quantitative data are mean $\pm$ SD, n=3. * p <0.05, compared to vehicle (0.1 to 0.2 % DMSO) treated hepatocytes; # p <0.05, compared to the corresponding cells treated with regorafenib alone.

Fig. 7 A schematic diagram of major findings in the present study. 1. KC, 6-ketocholestanol, a recoupler; 2. C+T, cyclosporin A (CsA) and trifluoperazine (TFP), MPT blockers; 3. F+O, fructose and oligomycin A, a glycolysis enhancer and a mitochondrial ATPase synthase inhibitor, respectively; 4. CC, Compound C, an AMPK inhibitor; 5. CQ, chloroquine, an autophagy inhibitor; 6. RAP, rapamycin, an autophagy inducer. Red and green arrows indicate pro-death and pro-survival pathways, respectively.

Figure 1

Figure 2A-D

2E

2F

Figure 2E&F

Figure 3

Figure 4

A

B

C

D

E

F

A

B

C

D

Figure 6