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Mechanisms of mitochondrial toxicity of the kinase inhibitors ponatinib, regorafenib and sorafenib in human hepatic HepG2 cells

Franziska Paech[a,b], Cécile Mingard[a,b], David Grünig[a,b], Vanessa F. Abegg[a,b], Jamal Bouitbir[a,b,c], Stephan Krähenbühl[a,b,c,*]

[a] Division of Clinical Pharmacology & Toxicology, University Hospital, Basel, Switzerland
[b] Department of Biomedicine, University of Basel, Switzerland
[c] Swiss Centre for Applied Human Toxicology, Switzerland

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ABSTRACT

Previous studies have shown that certain kinase inhibitors are mitochondrial toxicants. In the current investigation, we determined the mechanisms of mitochondrial impairment by the kinase inhibitors ponatinib, regorafenib, and sorafenib in more detail. In HepG2 cells cultured in galactose and exposed for 24 h, all three kinase inhibitors investigated depleted the cellular ATP pools at lower concentrations than cytotoxicity occurred, compatible with mitochondrial toxicity. The kinase inhibitors impaired the activity of different complexes of the respiratory chain in HepG2 cells exposed to the toxicants for 24 h and in isolated mouse liver mitochondria exposed acutely. As a consequence, they increased mitochondrial production of ROS in HepG2 cells in a time- and concentration-dependent fashion and decreased the mitochondrial membrane potential concentration-dependently. In HepG2 cells exposed for 24 h, they induced mitochondrial fragmentation, lysosome content and mitophagy as well as mitochondrial release of cytochrome c, leading to apoptosis and/or necrosis. In conclusion, the kinase inhibitors ponatinib, regorafenib, and sorafenib impaired the function of the respiratory chain, which was associated with increased ROS production and a drop in the mitochondrial membrane potential. Despite activation of defense measures such as mitochondrial fission and mitophagy, some cells were liquidated concentration-dependently by apoptosis or necrosis. Mitochondrial dysfunction may represent a toxicological mechanism of hepatotoxicity associated with certain kinase inhibitors.

1. Introduction

Tyrosine kinases are important enzymes involved in a variety of biological processes, including cell proliferation, survival, and differentiation (Shah et al., 2013; Shchemelinin et al., 2006). Due to their role in cell proliferation, expression of defective tyrosine kinases is involved in tumor initiation and progression (Shchemelinin et al., 2006) and dysregulation of tyrosine kinase expression can be associated with cancer development (Levitzki and Gazit, 1995). This role of tyrosine kinases in cancerogenesis has led to the development of a new class of anticancer drugs, the tyrosine kinase inhibitors (Krause and Van Etten, 2005). Most of them inhibit more than one kinase and are called multikinase inhibitors (MKIs).

Compared to classical cytotoxic agents, the hepatotoxicity of MKIs is generally less pronounced, but MKIs are associated for instance with toxicity of the skin, the intestine, the heart, and the liver (Breccia and Alimena, 2013). Hepatotoxicity has been reported for several MKIs, including ponatinib, regorafenib, and sorafenib (Josephs et al., 2013; Shah et al., 2013; Spraggs et al., 2013). Clinical trials indicated a low grade elevation in alanine aminotransferase (ALT) and/or aspartate aminotransferase (AST) in 25–30% and a high grade elevation (> 5 times upper limit of normal) in approximately 2% of patients treated with MKIs (Shah et al., 2013). A recent meta-analysis including more than 18,000 patients demonstrated an elevated risk of hepatotoxicity associated with tyrosine kinase inhibitors (Ghatalia et al., 2015) with an incidence of hepatic failure of 0.8%. Fatalities are fortunately rare, but have been reported for several MKIs including pazopanib (Klempner et al., 2012) and regorafenib (Mir et al., 2016).

The exact mechanism of MKI-induced hepatotoxicity has not been completely elucidated; however, it may be related to mitochondrial

Abbreviations: MKI, multikinase inhibitor; ALT, alanine aminotransferase; AST, aspartate aminotransferase; DRP1, dynamin-related protein 1; FIS1, mitochondrial fission 1; ROS, reactive oxygen species; DMSO, dimethylsulfoxide; AK, adenylate kinase; PBS, phosphate buffered saline; $\Delta\psi_m$, mitochondrial membrane potential; TMRM, tetramethylrhodamine methyl ester; FCCP, carbonyl cyanide-4-(trifluoromethoxy)phenylhydrazone; SEM, standard error of the mean; SOD, superoxide dismutase; LC3, Microtubule-associated protein light chain 3

* Corresponding author at: Clinical Pharmacology & Toxicology, University Hospital, 4031 Basel, Switzerland.
E-mail address: stephan.kraehenbuehl@usb.ch (S. Krähenbühl).

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damage. Recent publications suggest hepatic mitochondrial toxicity for dasatinib (Xue et al., 2012), lapatenib (Eno et al., 2016), and regorafenib (Weng et al., 2015; Zhang et al., 2017), and sorafenib and pazopanib (Zhang et al., 2017). Our group has shown recently in HepG2 cells that exposure to imatinib or sunitinib reduced the mitochondrial membrane potential, and was associated with impaired oxygen consumption and mitochondrial oxidative stress (Paech et al., 2017a). In a second recent publication, we reported that regorafenib, ponatinib and sorafenib inhibited mitochondrial oxidative metabolism and glycolysis, and induced apoptosis and/or necrosis of HepG2 cells at concentrations reachable in humans (Mingard et al., 2017). Importantly, MKIs cannot only damage hepatic mitochondria, but have also been described to be toxic for cardiac mitochondria, supporting the notion that MKIs are mitochondrial toxicants (Kerkela et al., 2006; Will et al., 2008).

Mitochondria are dynamic organelles, which have different possibilities to react after a toxic insult. A decrease in the mitochondrial membrane potential is associated with mitochondrial fission, with the aim to separate the defective and the functioning parts of the mitochondria (Westermann, 2010). Fission is initiated by recruitment of the dynamin-related protein 1 (DRP1) to the outer mitochondrial membrane by mitochondrial fission 1 (FIS1) (Palmer et al., 2011). After fission, the mitochondrial network has a fragmented appearance and the defective mitochondrial fragments can undergo mitophagy, which can be regarded as a protective process to remove damaged mitochondria (Ding and Yin, 2012). If the toxic insult is too pronounced, mitochondrial repair by fission and mitophagy is impossible and cells either undergo necrosis when the cellular ATP level is low or apoptosis, which is ATP-dependent. The induction of cell death can be initiated by release of cytochrome *c* out of damaged mitochondria into the cytoplasm (Green and Reed, 1998).

Based on these considerations, the aim of the current study was to investigate in more detail the mechanisms underlying the mitochondrial toxicity of ponatinib, regorafenib, and sorafenib in HepG2 cells. We determined the cellular ATP content and mitochondrial reactive oxygen species (ROS) production using a medium containing galactose, since HepG2 cells grown in galactose generate ATP mainly in mitochondria and are sensitive to mitochondrial toxicants (Brecht et al., 2017; Kamalian et al., 2015). In addition, we also determined the effects of the toxicants on the activity of the individual enzyme complexes of the mitochondrial electron transport chain and on the consequences resulting from impaired activity of these enzyme complexes, in particular on mitochondrial fission, mitophagy, and cell death.

2. Materials and methods

2.1. Chemicals

Ponatinib, regorafenib, and sorafenib were purchased from Sequoia research products (Pangbourne, UK). We prepared stock solutions in dimethylsulfoxide (DMSO) and stored them at −20 °C. All other chemicals were supplied by Sigma-Aldrich (Buchs, Switzerland), except where indicated.

2.2. HepG2 cell culture

The human hepatocellular carcinoma cell line HepG2 was obtained from the American type culture collection (ATCC, Manassas, VA, USA). HepG2 cells were cultured under two different conditions, low glucose and galactose.

HepG2 cells under low glucose conditions were cultured in Dulbecco's Modified Eagle Medium (DMEM containing 5.55 mM (1 g/l) glucose, 4 mM L-glutamine, and 1 mM pyruvate from Invitrogen, Basel, Switzerland) supplemented with 10% (v/v) heat-inactivated fetal bovine serum (FBS), 2 mM GlutaMax, 10 mM HEPES buffer, 10 mM non-essential amino acids, 100 units/ml penicillin, and 100 µg/ml streptomycin.

HepG2 cells under galactose conditions were first cultured in low glucose medium. On the day of the experiment, HepG2 cells were centrifuged, the supernatant was removed and cells were resuspended in galactose medium (Dulbecco's Modified Eagle Medium (DMEM, no glucose, 4 mM L-glutamine) from Invitrogen (Basel, Switzerland) supplemented with 10% (v/v) heat-inactivated FBS, 10 mM galactose, 2 mM L-glutamine, 10 mM HEPES buffer, 1 mM sodium pyruvate, 100 units/ml penicillin, and 100 µg/ml streptomycin) for 4 h or 24 h before starting the treatment (Swiss and Will, 2011). The FBS was dialyzed through a Slide-A-Lyzer Dialysis Flask (Thermo Scientific, Reinach, Switzerland) to remove glucose according to the manufacturer's protocol. All experiments were performed under glucose medium except the release of adenylate kinase, the intracellular ATP content and the mitochondrial superoxide accumulation, which were performed under galactose medium.

All cells were kept at 37 °C in a humidified 5% CO_2 cell culture incubator and passaged using trypsin. The cell number was determined using a Neubauer hemacytometer and viability was checked using the trypan blue exclusion method.

2.3. Isolation of mouse liver mitochondria

The experiments were performed in accordance with the institutional guidelines for the care and use of laboratory animals. Male C57BL/6J mice (n = 3, age 7–10 weeks) were purchased from Charles River Laboratories (Sutzfeld, Germany) and housed in a standard facility with 12 h light–dark cycles and controlled temperature (21–22 °C). The mice were fed a standard pellet chow and water *ad libitum*.

Liver mitochondria were isolated by differential centrifugation as described before (Hoppel et al., 1979). The mitochondrial protein content was determined using the Pierce BCA protein assay kit from Merck (Zug, Switzerland).

2.4. Membrane toxicity

Membrane toxicity was assessed by using the ToxiLight assay from Lonza (Basel, Switzerland) according to the manufacturer's protocol. This assay measures the release of adenylate kinase (AK) in the medium, which reflects the plasma membrane's integrity. HepG2 cells in galactose conditions were grown in a 96-well plate (25′000 cells/well) and exposed to different concentrations of MKIs (5–50 µM) for 24 h. The plasma concentrations of ponatinib, regorafenib and sorafenib are in the range of 0.1, 2.5 and 5 to 10 µM, respectively (Herbrink et al., 2016; Huynh et al., 2017; Sunakawa et al., 2014). Since the liver concentration of MKIs can be considerably higher than the plasma concentrations (Lau et al., 2015), it can be assumed that at least the lowest concentrations used can be reached in the liver *in vivo*. The negative control was 0.1% DMSO and the positive control was 0.5% Triton-X. After incubation, 20 µL supernatant of each well was transferred to a new 96-well plate. Then, 50 µL of assay buffer was added to each well. After incubation in the dark for 5 min, the luminescence was measured using a Tecan M200 Pro Infinity plate reader (Männedorf, Switzerland). All data were normalized to positive control incubations containing 0.5% Triton X (set at 100% cell lysis).

2.5. Intracellular ATP content

The intracellular ATP content was measured using the CellTiter-Glo kit from Promega (Wallisellen, Switzerland) according to the manufacturer's protocol. HepG2 cells in galactose conditions were grown in a 96-well plate (25,000 cells/well) and exposed to different concentrations of MKI (5–50 µM) for 24 h. The negative control was 0.1% DMSO and the positive control was 0.5% Triton-X. After treatment, medium was removed in order to have 50 µL remaining in each well and afterwards 50 µL of assay buffer was added to each well. After incubation in