Modulation of mitochondrial respiratory function and ROS production by novel benzopyran analogues

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Abstract: A substantial body of evidence indicates that pharmacological activation of mitochondrial ATP-sensitive potassium channels (mKATP) in the heart is protective in conditions associated with ischemia/reperfusion injury. Several mechanisms have been postulated to be responsible for cardioprotection, including the modulation of mitochondrial respiratory function. The aim of the present study was to characterize the dose-dependent effects of novel synthetic benzopyran analogues, derived from a BMS-191095, a selective mKATP opener, on mitochondrial respiration and reactive oxygen species (ROS) production in isolated rat heart mitochondria. Mitochondrial respiratory function was assessed by high-resolution respirometry, and H2O2 production was measured by the Amplex Red fluorescence assay. Four compounds, namely KL-1487, KL-1492, KL-1495, and KL-1507, applied in increasing concentrations (50, 75, 100, and 150 μmol/L, respectively) were investigated. When added in the last two concentrations, all compounds significantly increased State 2 and 4 respiratory rates, an effect that was not abolished by 5-hydroxydecanoate (5-HD, 100 μmol/L), the classic mKATP inhibitor. The highest concentration also elicited an important decrease of the oxidative phosphorylation in a K+ independent manner. Both concentrations of 100 and 150 μmol/L for KL-1487, KL-1492, and KL-1495, and the concentration of 150 μmol/L for KL-1507, respectively, mitigated the mitochondrial H2O2 release. In isolated rat heart mitochondria, the novel benzopyran analogues act as protonophoric uncouplers of oxidative phosphorylation and decrease the generation of reactive oxygen species in a dose-dependent manner.

Key words: rat heart mitochondria, benzopyran analogues, protonophores, uncoupling, hydrogen peroxide.

Introduction

In the past decades mitochondria have emerged as major contributors to the pathogenesis of myocardial ischemia/reperfusion (I/R) injury as well as important therapeutic targets in cardioprotection (Camara et al. 2011; Di Lisa et al. 2007). The mitochondrial ATP-sensitive potassium channel (mKATP) is one of the putative structures at the inner mitochondrial membrane that has been extensively studied in several experimental models as a major target that can be modulated by pharmacological agents and conditioning strategies to protect the heart against the deleterious effects of reoxygenation/reperfusion (critically reviewed by Hanley and Daut (2005)). The channel functions as potassium uniporter, allowing the ions intake into the matrix and is inhibited by ATP, ADP, and fatty acids (Cardoso et al. 2010; Garlid and...
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Halestrap 2012). Despite the fact that the first reconstitution of the mKATP protein isolated from bovine heart in liposomes had been reported more than two decades ago (Paucek et al. 1992), the molecular structure of the channel still remains inconclusive; therefore, the role in cardioprotection and, even their existence, has been recently questioned (Garlid and Halestrap 2012).

Several research groups performed over the years a thorough functional characterization using the pharmacological approach, i.e., application of channel openers (agonists) or blockers (antagonists) and provided evidence for the role of mKATP in the regulation of mitochondrial matrix volume, calcium uptake, respiration, and reactive oxygen species (ROS) generation (reviewed in Ardehali and O’Rourke (2005) and Nishida et al. (2010)). It has also been reported that potassium channel openers act as uncoupling protonophores and thus contribute to cardioprotection (Holmuhamedow et al. 2004).

The role of mKATP in preventing the oxidative stress is particularly important during the posts ischemic reperfusion, when mitochondria are both sources and targets of the oxidative burst (Di Lisa 2001; Turrens 2003). The contribution of channel’s opening to ROS production is controversial, since both a decrease (Ferranti et al. 2003; Vanden Hoek et al. 2000) and an increase in mitochondrial ROS in the presence of mKATP agonists (Forbes et al. 2001; Krenz et al. 2002; Liu et al. 1998; Pain et al. 2000) have been reported in the literature.

One of the confounding factors regarding the pharmacological approach in studies reporting on cardioprotective effects of mKATP openers is related to the lack of selectivity of the first developed agonists that were able to activate both the mitochondrial and sarcosomal ATP-sensitive channels (Hanley and Daut 2005). In the attempt to increase the selectivity versus mKATP, several benzopyran conjugates have been developed and tested in the past years (reviewed in Brechi et al. 2006). Among these compounds, BMS-191095 is a 4-((N-arylsulfonatophenyl)thio)benzopyran-2-one analogues of BMS–191095 that have been proposed to act as mKATP openers, on mitochondrial respiration and ROS release in isolated rat heart mitochondria.

Materials and methods

All experimental procedures were conducted in accordance with Directive 2010/63/EU and the corresponding Romanian law nr. 43/May 2014 regarding the protection of animals used for scientific purposes, respectively. The experimental protocol was approved by the Committee for Research Ethics of “Victor Babes” University for Medicine and Pharmacy of Timisoara, Romania.

Experiments were performed on Sprague Dawley (SD) adult female rats (4–6 months, n = 5–6/group). Animals were fed ad libitum and housed under standard conditions (constant temperature and humidity of 22.5 ± 2 °C and 55 ± 5%, 12 h light/dark cycle). Twenty-four hours prior to experiment, solid food was withdrawn with no limitation in water supply.

Rat heart mitochondria (RHM) isolation

Rats were anesthetized by the intraperitoneal administration of a mixture of ketamine (30 mg/kg) and xylazine (5 mg/kg). Hearts were rapidly excised and immersed in 20 ml. isolation medium (100 mmol/L sucrose, 50 mmol/L KCl, 20 mmol/L TES-2-[2-hydroxy-1,1-bis(hydroxymethyl)ethyl]aminoethanesulfonic acid, 1 mmol/L EDTA, 0.2% bovine serum albumin (BSA), pH 7.2 at 4 °C). Separate experiments were performed in mitochondria isolated in K⁺ free medium (220 mmol/L manitol, 70 mmol/L sucrose, 5 mmol/L N-[tris(hydroxymethyl)methyl]-2-aminoethanesulfonic acid (TES), 0.5 mmol/L EGTA, supplemented with 2 mg/mL bovine serum albumin (BSA), pH 7.4, adjusted with Trizma base; 2 °C). Ventricular tissue was manually triturated with surulisin (5 mg/g wet tissue), a non-specific protease, to release the interfibrillar mitochondria. The suspension was homogenised with a tissue homogenizer (Glas-Col 099C K5424 CE) for maximum 30 s. The final tissue homogenate obtained was processed at 4 °C using the differential centrifugation technique as previously described (Duicu et al. 2013). Mitochondrial protein concentration was determined using biuret method (Gornall et al. 1949).

High-resolution respirometry experiments

Oxygen consumption was measured at 37 °C by high-resolution respirometry (HRR) with the Oxygengraph-2k (Oroboros Instruments, Austria). RH (0.1 mg proteins/mL) were incubated in 2 ml of incubation medium (0.5 mmol/L EGTA, 3 mmol/L MgCl2·6H2O, 60 mmol/L K-lactobionate, 20 mmol/L taurine, 10 mmol/L KH2PO4, 20 mmol/L HEPES (4-(2-hydroxyethyl)-1-piperazineethanesulfonic) acid, 110 mmol/mL sucrose, 1 g/L BSA, essentially free fatty acid + 280 μmol/L catalase lyophilized powder, 2000–5000 units/mg protein, at pH 7.1, 37 °C). In separate experiments, mitochondrial respiratory rates were measured in KCl incubation medium (120 mmol/L KCl, 5 mmol/L KH2PO4, 5 mmol/L TES, and 1 mmol/L MgCl2, pH 7.4, adjusted with Trizma base at 37 °C) or in choline chloride medium (120 mmol/L choline chloride, 5 mmol/L NaH2PO4, 5 mmol/L TES, and 1 mmol/L MgCl2, pH 7.4, adjusted with Trizma base at 37 °C).

The substrate-uncoupler-inhibitor-titration (SUIT) protocol, GMState2 + ADP/OXPHOS + c + OmyState4 + FCCPETS + (Amanox), was previously described (Duicu et al. 2013) and comprised the following steps: (i) addition of complex 1 respiratory substrates, 10 mmol/L glutamate (G) and 2 mmol/L malate (M), State 2 (basal respiration); (ii) addition of 5 mmol/L adenosine diphosphate (ADP) to assess the maximal oxidative phosphorylation capacity (OXPHOS), State 3 (active respiration); (iii) addition of 10 μmol/L cytochrome c (c) to check for the integrity of mitochondrial outer membrane; (iv) addition of 2 μg/mL oligomycin (Omy), an F1F0-ATP synthase inhibitor, to inhibit State 3 at the level of State 4; (v) successive titrations (0.5 μmol/L steps) with FCCP ([carbonyl cyanide p-(trifluoro-methoxy) phenyl-hydrazone) to obtain the uncoupled respiration and to measure the electron transport system capacity, ETS; (vi) inhibition of respiration with 2.5 μmol/L antimycin A (Ama) to measure the residual oxygen consumption, ROX state. Mitochondrial respiration was corrected for oxygen flux due to instrumental background and ROX. The respiratory control ratio (RCR) was calculated as the ratio OXPHOS/State 4.

Assessment of hydrogen peroxide (H2O2) production

Mitochondrial H2O2 release was measured using the Amplex Red (10 μmol/L) fluorescent marker (wavelengths: excitation at 530 nm and emission at 590 nm, Hitachi F-7000 spectrofluorimeter) as previously described (Duicu et al. 2013). RH (0.25 mg protein/mL) were incubated in 2 ml incubation buffer (250 mmol/L sucrose, 1 mmol/L EGTA, 1 mmol/L EDTA, 20 mmol/L Tris/Cl, and 1.5 mg/ml defatted BSA, pH 7.4, 37 °C), supplemented with CI-dependent substrates: G (5 mmol/L) and M (5 mmol/L). At the beginning of each measurement, the background fluorescence was quantified by adding known amounts of H2O2 to the incubation buffer, in the absence of mitochondria. Net fluorescence was then calculated by measuring the fluorescence variation in function of time, minus background, and H2O2 production was expressed in pmol H2O2/min/mg proteins.

Chemicals

The benzopyran analogues were synthesized by Kiss Lorand at the Institute of Pharmaceutical Chemistry, Faculty of Pharmacy, University of Szeged, Hungary. Calcium Green-5N and Amplex Red were purchased from Molecular Probes (Leiden, The Netherlands). The chemicals were of analytical grade and were purchased from Sigma-Aldrich (Switzerland) and Merck (Darmstadt, Germany). The reagents and substrates were from Sigma-Aldrich (Switzerland) and Merck (Darmstadt, Germany). The oligonucleotides were purchased from Integrated DNA Technologies (Coralville, Iowa). The peptides, TRH, and T3 were purchased at Sigma-Aldrich (Switzerland). The chemicals, including the fluorescent probes, inhibitors, and enzymes, were from Sigma-Aldrich (Switzerland) and Merck (Darmstadt, Germany). The reagents and substrates were from Sigma-Aldrich (Switzerland) and Merck (Darmstadt, Germany). The oligonucleotides were purchased from Integrated DNA Technologies (Coralville, Iowa). The peptides, TRH, and T3 were purchased at Sigma-Aldrich (Switzerland).

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Red were purchased from Invitrogen. All the other chemicals were from Sigma-Aldrich.

**Statistical analysis**

Data were expressed as means ± SEM. Data analysis used one-way ANOVA followed by a post-hoc Tukey’s multiple comparison test (GraphPadPrism v. 5.0 Software, SUA). The difference was considered statistically significant if \( p < 0.05 \).

**Results and discussion**

**High-resolution respirometry**

Four benzopyran derivatives, namely KL-1487, KL-1492, KL-1495, and KL-1507, in increasing concentrations (50, 75, 100, and 150 \( \mu \text{mol/L} \) respectively) were screened. Stock solutions of benzopyran analogues were made up in DMSO (60 mmol/L) and then serially diluted to give the above mentioned concentrations. The final DMSO concentration was constant (0.25%) throughout the experiments and did not influence the respiratory rates (data not shown).

The major finding of this work is that we report a dose-dependent modulation of the mitochondrial respiratory function for all investigated compounds. Accordingly, a significant increase in respiratory State 4 (Table 1, Fig. 2) was recorded when the compounds were added in the highest concentrations (150 and 100 \( \mu \text{mol/L} \)). The maximum increase was found for KL-1492, while the lowest yet significant change was found in the case of KL-1507 (Table 1, Fig. 2).

However, when applied in the maximal concentration (150 \( \mu \text{mol/L} \)), all compounds induced a significant decrease in OXPHOS (State 3, ADP-stimulated respiration) vs. control (Ctrl), with the most important inhibition recorded for KL-1487 (Table 1, Fig. 3). These effects (increase in State 4 together with the decrease in OXPHOS) for 150 \( \mu \text{mol/L} \) resulted in a significant reduction of the respiratory index (OXPHOS/State 4) vs. Ctrl for all tested compounds (Table 1, Fig. 4).

As mentioned in the previous section, State 2 represents the basal respiration, whereas State 4 was pharmacologically induced.
with oligomycin (Omy), which inhibits the proton entry at the level of F$_0$-ATPase. Compounds able to stimulate respiratory rates in State 4 (and 2) in a dose-dependent fashion are known as uncouplers, because they disrupt (or uncouple) the link between substrate oxidation and ADP phosphorylation in ATP. Conversely, mitochondrial respiration that has been inhibited by inhibitors such as antimycin cannot be released by the uncouplers (Terada 1990).

Data presented in this study suggest that all four investigated compounds act as inhibitory uncouplers, showing an intrinsic uncoupling effect with increasing concentrations and inhibitory property when used in the highest doses. Indeed, starting from 50 μmol/L, these benzopyran analogues uncoupled the oxidative phosphorylation of mitochondria respiring on the NAD-dependent substrates glutamate-malate.

We further tested the effects of 5-hydroxydecanoate (5-HD, 100 μmol/L), which is known as putative selective mK$_{ATP}$ inhibitor. Our data clearly demonstrated that the uncoupling effect induced by all four benzopyran analogues was not abolished by 5-HD.

Fig. 2. State 4 respiratory rates in the presence of K-1487, KL-1492, KL-1495, and KL-1507. Results are expressed in pmol/(s·mL). Values are means ± SEM (*$p < 0.05$; **$p < 0.01$; ***$p < 0.001$ vs. Ctrl).

Fig. 3. OXPHOS respiratory rates in the presence of K-1487, KL-1492, KL-1495, and KL-1507. Results are expressed in pmol/(s·mL). Values are means ± SEM (**$p < 0.01$; ***$p < 0.001$ vs. Ctrl).
suggesting that the increase in CI-supported basal respiration was not related to the opening of mK<sub>ATP</sub> channels. Our findings agrees with the well-established effects of the mK<sub>ATP</sub> openers, diazoxide and pinacidil, that act as uncoupling protonophores, mainly when applied in high concentrations, in isolated rodent mitochondrial preparations (Drose et al. 2006; Hanley et al. 2002; Holmuhamedov et al. 2002; Korotkov et al. 2006; Kowaltowski et al. 2001) in a potassium channel-independent manner. Also, our data regarding the effect of 5-HD are in agreement with the previous reports showing that the mK<sub>ATP</sub> blocker had no effect on the activation of the basal mitochondrial respiration by diazoxide and pinacidil applied in similar concentrations (Kopustinskiene et al. 2010; Korotkov et al. 2006). Furthermore, all four compounds significantly decreased State 3 respiration as was earlier demonstrated for diazoxide (Kopustinskiene et al. 2002; Rousou et al. 2004) and pinacidil (Kopustinskiene et al. 2010).

To investigate whether the decrease of State 3 respiratory rate can be assigned to the mK<sub>ATP</sub> opening, we recapitulated the experiments on mitochondria isolated in free potassium media, and we measured the respiratory rates in potassium chloride and choline chloride medium (without K<sup>+</sup>). Since the effect of all four compounds at 150 μmol/L on State 3 respiration remained unchanged (Fig. 5) no relation with the K<sup>+</sup> flux into the matrix can be affirmed. This observation is in line with the results reported for pinacidil in the same experimental model by Toleikis’ group (Kopustinskiene et al. 2010), who found a K<sup>+</sup>-independent decrease in State 3 and, also in uncoupled respiration, in the presence of complex I respiratory substrates.

It has to be mentioned that the most investigated benzopyrane derivate, BMS 191095, was reported to exert cytoprotective effect against a calcium ionophore-induced injury in a skeletal muscle cell line C2C12 (Malinska et al. 2010); the compound promoted cellular survival despite an impaired mitochondrial function, as shown by a decrease in State 3 respiration. Interestingly, cytoprotection against calcium overload elicited by the specific mK<sub>ATP</sub> opener was not reversed in this model by 5-HD, the channel inhibitor applied in high concentration (500 μmol/L). Whether this might be related to the previously reported inhibition of respiration by high concentrations (100 and 300 μmol/L) of 5-HD is not known (Lim et al. 2002). Moreover, the beneficial neuroprotective effects of BMS 191095 against cerebral ischemia could be reversed only when 5-HD was applied in very high (10 and 20 mmol/L) concentrations (Mayanagi et al. 2007). However, it cannot be ruled out in these models that, as shown for diazoxide, the ability of 5-HD to reverse protection elicited by channel openers is related to its metabolic effects and does not result from mK<sub>ATP</sub> inhibition (Hanley et al. 2005).

Collectively, our data show a significant uncoupling effect of these novel benzopyran analogues at 100 and 150 μmol/L concentration and the inhibition of mitochondrial respiration when the compounds were applied in the highest concentration (150 μmol/L) in isolated rat heart mitochondria. These effects are not related to the activation of mK<sub>ATP</sub> channels, but they are rather suggestive for a protonophoric action, as reported for the prototype mK<sub>ATP</sub> openers, diazoxide, in the pioneering studies of

### Table 2. HRR studies for CI-supported respiration in the presence of 5-hydroxydecanoic acid (5-HD).

<table>
<thead>
<tr>
<th>OXPHOS State 4</th>
<th>RCR</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ctrl</td>
<td>711.8±87.2</td>
</tr>
<tr>
<td>KL-1487</td>
<td>183.6±13.5*</td>
</tr>
<tr>
<td>KL-1487 + 5-HD</td>
<td>185.8±17.03*</td>
</tr>
<tr>
<td>Ctrl</td>
<td>589.5±43.65</td>
</tr>
<tr>
<td>KL-1492</td>
<td>250.8±39.81*</td>
</tr>
<tr>
<td>KL-1492 + 5-HD</td>
<td>242.6±27.3*</td>
</tr>
<tr>
<td>Ctrl</td>
<td>243.2±23.72*</td>
</tr>
<tr>
<td>KL-1495</td>
<td>656.4±49.04</td>
</tr>
<tr>
<td>KL-1495 + 5-HD</td>
<td>341.2±32.6*</td>
</tr>
</tbody>
</table>

**Note:** Data are expressed in pmol/(s·mL). Values are means±SEM (*p < 0.05 vs. Ctrl.).
Portenhauser et al. (1971) and more recently, for diazoxide and pinacidil (Holmuhamedov et al. 2004; Kowaltowski et al. 2001). However, several issues that remain to be clarified can be regarded as limitations of the present study. First, assessing the effect of BSA on the uncoupling effect is worthy, since it has been reported that the uncoupling effect of diazoxide was significantly depressed by BSA in the incubation medium (Kopustinskiene et al. 2010). Furthermore, since the uncoupling effect can also be determined by the opening of the mitochondrial permeability transition pore (mPTP), the effect of a mPTP desensitizer (e.g., cyclosporine A) on the increased State 4 respiratory rates will be assessed.

Fig. 5. HRR studies for OXPHOS rates in choline chloride (ChoCl) medium vs. KCl medium. Results are expressed in pmol/(s·ml). Values are means ± SEM (*p < 0.05; **p < 0.01 vs. Ctrl).

Fig. 6. Assessment of H$_2$O$_2$ release in mitochondria respiring on glutamate/malate. Results are expressed in pmol/(mg prot·min). Values are means ± SEM (*p < 0.05; **p < 0.01; ***p < 0.001 vs. Ctrl).
Mitochondrial H$_2$O$_2$ production

In the second part of the study we addressed the effect of these four benzopyran compounds on ROS released by isolated mitochondria respiring in the presence of Cl substrates (glutamate/malate), using the Amplex Red assay. Our results show a significant decrease of mitochondrial H$_2$O$_2$ for the first three compounds (KL-1487, KL-1492, and KL-1495) when applied at 100 and 150 $\mu$mol/L (Fig. 6) and only in the presence of the highest concentration in the case of KL-1507, respectively (Fig. 6). Interestingly, the last compound KL-1507 showed a slight increase in ROS production when applied at 100 $\mu$mol/L, whereas the highest concentration elicited a significant decrease of H$_2$O$_2$ release (Fig. 6).

Our data confirm previous studies reporting on the ability of the m$_{K_{ATP}}$ openers to protect mitochondria function and structure by suppressing ROS generation during reoxygenation (Ferranti et al. 2003; Ozcan et al. 2002; Vanden Hoek et al. 2000).

In the past decade the interest of the scientific community largely moved towards the pharmacological modulation of the mitochondrial permeability transition pore (mPTP) as novel mitochondrial target for cardioprotection (recently reviewed in Bernardi and DiLisa (2015)), while studies addressing m$_{K_{ATP}}$ channels mainly attempted to elucidate its structure (Foster et al. 2012). Besides the unequivocal role of m$_{K_{ATP}}$ openers in reducing infarct size, an emerging research direction is represented by their role in preserving the electrical stability of the heart (reviewed in Muntean et al. 2015). Interestingly, a functional crosstalk between mPTP and m$_{K_{ATP}}$ that determined the arrhythmic vulnerability to oxidative stress has been recently reported (Xie et al. 2014).

The selective benzopyran derivate, BMS 191095, elicited both antinecrotic (Neckar et al. 2002) and antiarrhythmic (Fischbach et al. 2004) protection in the rodent heart subjected to I/R injury, being recognized as an useful tool for basic research (Grover and Atwal 2002). Accordingly, further studies addressing the role of novel benzopyran analogues in preventing the deleterious effects of reperfusion injury are warranted. Whether their above reported effects can be recapitulated in the settings of I/R injury remains to be demonstrated. Indeed, different behaviours may arise in pathological conditions, since as pointed out in a critical review by Brookes (2005), “uncoupling of mitochondria decreases ROS but uncoupling of inhibited mitochondria increases ROS”.

Nevertheless, m$_{K_{ATP}}$ and mPTP represent targets for pharmacological interventions that could be jointly applied to mitigate one of the major consequences of mitochondria dysfunction, namely the oxidative stress, and thus to provide better cardioprotection.

Conclusions

KL-1487, KL-1492, KL-1495, and KL-1507 are novel benzopyran analogues with protonophoric properties that modulate mitochondrial respiratory function and hydrogen peroxide release in isolated rat heart mitochondria respiring on physiological complex I substrates. High-resolution respirometry studies conducted in the presence of the pharmacological modulators of m$_{K_{ATP}}$ revealed the uncoupling effect and respiratory inhibition, respectively, in a K$^+$ independent manner. Moreover, when applied in the highest concentrations, all the investigated compounds decreased H$_2$O$_2$ release. Whether these effects can be associated with cardioprotection in the setting of ischemia/reperfusion injury remains to be demonstrated.

Acknowledgements

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