



HAL
open science

An old medicine as a new drug to prevent mitochondrial complex I from producing oxygen radicals

Dominique Detaille, Philippe Pasdois, Audrey Sémont, Pierre Dos Santos,
Philippe Diolez

► To cite this version:

Dominique Detaille, Philippe Pasdois, Audrey Sémont, Pierre Dos Santos, Philippe Diolez. An old medicine as a new drug to prevent mitochondrial complex I from producing oxygen radicals. PLoS ONE, 2019, 14 (5), pp.e0216385. 10.1371/journal.pone.0216385 . inserm-02196657

HAL Id: inserm-02196657

<https://inserm.hal.science/inserm-02196657>

Submitted on 29 Jul 2019

HAL is a multi-disciplinary open access archive for the deposit and dissemination of scientific research documents, whether they are published or not. The documents may come from teaching and research institutions in France or abroad, or from public or private research centers.

L'archive ouverte pluridisciplinaire **HAL**, est destinée au dépôt et à la diffusion de documents scientifiques de niveau recherche, publiés ou non, émanant des établissements d'enseignement et de recherche français ou étrangers, des laboratoires publics ou privés.

RESEARCH ARTICLE

An old medicine as a new drug to prevent mitochondrial complex I from producing oxygen radicals

Dominique Detaille^{1,2,3}, Philippe Pasdois^{1,2,3}, Audrey Sémont^{1,2,3}, Pierre Dos Santos^{1,2,3,4}, Philippe Diolez^{1,2,3*}

1 IHU Liryc, L'institut de rythmologie et modélisation cardiaque, Fondation Bordeaux Université, Pessac-Bordeaux, France, **2** Université de Bordeaux, Bordeaux, France, **3** INSERM, Centre de recherche Cardio-Thoracique de Bordeaux, Bordeaux, France, **4** Centre Hospitalo-Universitaire de Bordeaux (CHU), Pôle Cardio-thoracique, Pessac, France

* philippe.diolez@u-bordeaux.fr



OPEN ACCESS

Citation: Detaille D, Pasdois P, Sémont A, Dos Santos P, Diolez P (2019) An old medicine as a new drug to prevent mitochondrial complex I from producing oxygen radicals. PLoS ONE 14(5): e0216385. <https://doi.org/10.1371/journal.pone.0216385>

Editor: Edward J. Lesnefsky, Virginia Commonwealth University, UNITED STATES

Received: June 18, 2018

Accepted: April 21, 2019

Published: May 2, 2019

Copyright: © 2019 Detaille et al. This is an open access article distributed under the terms of the [Creative Commons Attribution License](https://creativecommons.org/licenses/by/4.0/), which permits unrestricted use, distribution, and reproduction in any medium, provided the original author and source are credited.

Data Availability Statement: All relevant data are within the paper and its Supporting Information files.

Funding: This work was supported by the Centre National de la Recherche Scientifique (P.D. salary), the French Government as part of the 'investments for the future' program managed by the National Research Agency (ANR), Grant reference ANR-10-IAHU-04, and the grant MITOCARD ANR-17-CE11-0041 (P.D.). The authors thanks the company OP2drugs (Pessac, France) for providing the

Abstract

Findings

Here, we demonstrate that OP2113 (5-(4-Methoxyphenyl)-3H-1,2-dithiole-3-thione, CAS 532-11-6), synthesized and used as a drug since 1696, does not act as an unspecific antioxidant molecule (i.e., as a radical scavenger) but unexpectedly decreases mitochondrial reactive oxygen species (ROS/H₂O₂) production by acting as a specific inhibitor of ROS production at the I_Q site of complex I of the mitochondrial respiratory chain. Studies performed on isolated rat heart mitochondria also showed that OP2113 does not affect oxidative phosphorylation driven by complex I or complex II substrates. We assessed the effect of OP2113 on an infarct model of *ex vivo* rat heart in which mitochondrial ROS production is highly involved and showed that OP2113 protects heart tissue as well as the recovery of heart contractile activity.

Conclusion / Significance

This work represents the first demonstration of a drug authorized for use in humans that can prevent mitochondria from producing ROS/H₂O₂. OP2113 therefore appears to be a member of the new class of mitochondrial ROS blockers (S1QELs) and could protect mitochondrial function in numerous diseases in which ROS-induced mitochondrial dysfunction occurs. These applications include but are not limited to aging, Parkinson's and Alzheimer's diseases, cardiac atrial fibrillation, and ischemia-reperfusion injury.

Introduction

The free radical theory of aging suggests that free radical-induced damage to cellular structures is a crucial event in aging [1]; however, clinical trials on antioxidant supplementation in

anethol trithione (OP2113) compound. The funding sources had no role in the study design or the analysis and interpretation of data or in the decision to submit the article for publication.

Competing interests: We report here that D.D. and P.D. are listed as inventors on pending and issued patent applications related to the molecule OP2113 described in this work (USA US62/215,215; France 15 184 217.6; France EP 17 159 691.9). P.D. is one of the founders of the OP2 drugs company. The funding sources had no role in the study design or the analysis and interpretation of data or in the decision to submit the article for publication. This does not alter our adherence to PLOS ONE policies on sharing data and materials.

Abbreviations: ATR, atractyloside; BSA, bovine serum albumin; CCCP, Carbonylcyanure m-chlorophénylhydrazone; DMSO, dimethyl sulfoxide; HRP, Horseradish peroxidase; LVDP, Left ventricular developed pressure; mGPDH, mitochondrial glycerol-3-P dehydrogenase; RET, reverse electron transfer; ROS, reactive oxygen species; RPP, rate-pressure product; S1QELs, Suppressors of complex 1 site Q electron leak; TTC, Triphenyl tetrazolium chloride.

various populations have not successfully demonstrated an anti-aging effect [2]. Current explanations include the lack of selectivity of available antioxidants for the various sources of oxygen radicals and the poor distribution of antioxidants to mitochondria, which are now believed to be both the primary sources of reactive oxygen species (ROS) and primary targets of ROS-induced damage [3]. Indeed, mitochondrial dysfunction that occurs due to accumulation of oxidative damage [4] is implicated in the pathogenesis of virtually all human age-related diseases [5, 6], including cardiovascular and neurodegenerative diseases, cancer, and diabetes [7–12], as well as ischemia-reperfusion injury [13].

Given the key role of age-dependent mitochondrial deterioration in aging [4], there is currently a great interest in approaches to protect mitochondria from ROS-mediated damage. Mitochondria are not only a major source of ROS but also particularly susceptible to oxidative damage. Consequently, mitochondria accumulate oxidative damage with age that contribute to mitochondrial dysfunction [4]. Cells and even organelles possess several protection pathways against this ROS-mediated damage given that local protection is fundamental to circumvent the high reactivity of ROS. Therefore, mitochondria appear as the main victims of their own ROS production, and evidence suggests that the best mitochondrial protection will be obtained from inside mitochondria. This conclusion has driven several potential therapeutic strategies to improve mitochondrial function in aging and pathologies. Antioxidants designed for accumulation by mitochondria *in vivo* have been developed [2, 14] and are currently being thoroughly tested for mitochondrial protection [15–17]. Given that functional mitochondria are characterized by a very high proton gradient, mainly represented by a negative-internal membrane potential gradient [18], lipophilic cationic compounds accumulate inside the mitochondrial matrix as they may cross the lipid bilayer barrier given the electrical gradient. Therefore, mitochondria-targeted antioxidants are essentially cationic lipophilic drugs combined with a quinone moiety with radical scavenging properties. The growing interest in ROS production associated with diseases has elicited numerous clinical trials that have also demonstrated that uncontained ROS reduction in cells is deleterious, and it appears that an adequate balance of ROS production is necessary for correct cell function [2]. As a consequence, there is also a growing interest in the selective inhibition of ROS production of mitochondrial origin that would not affect cellular signalization involving either mitochondrial [19] or cytosolic ROS production [20, 21]. Conditions of high ROS production in mitochondria are now better characterized [7, 22–24], and it appears that ROS may be produced at multiple sites of the respiratory chain in mitochondria. Maximal ROS production occurs under conditions of high reduction of electron transporters, chiefly quinones, and high membrane potential values. Paradoxically, these conditions are satisfied when mitochondrial oxidative phosphorylation is low (low cellular ATP turnover) [25, 26] or under low oxygen conditions (hypoxia, inhibition of terminal oxidase) [13].

The molecule OP2113 (Anetholtrithion, or 5-(4-methoxyphenyl)dithiole-3-thione—CAS number 532-11-6) has been marketed in many countries and used in human therapy in certain countries including France, Germany, and China for its choleric and sialogogic properties. Anetholtrithion also exhibits chemoprotective effects against cancer and various kinds of toxicity caused by some drugs and xenobiotics [27]. These chemoprotective effects appear to be mainly due to its antioxidant properties [28–30]. The most typical indications for which anetholtrithion is currently used include increasing salivary secretion in patients experiencing dry mouth. It is also indicated as an adjunctive therapy for cholecystitis, gallstone, indigestion, and acute/chronic hepatitis (see DrugBank database [31]). Anetholtrithion and derivatives have also been tested for their properties as H₂S donors and therapeutic effects [27, 32–34].

However, until now, no precise mechanism of action has been described for this molecule. Considering the high lipophilicity of OP2113, which represents a promising characteristic for

mitochondrial targeting, we investigated the effect of OP2113 on mitochondrial ROS/H₂O₂ production. Here we show that OP2113 decreases ROS/H₂O₂ production by isolated rat heart mitochondria. Interestingly, it does not act as an unspecific antioxidant molecule (*i.e.* as a radical scavenger), but as a direct specific inhibitor of ROS production at site I_Q of complex I of the mitochondrial respiratory chain, without impairing electron transfer.

Results

OP2113 does not inhibit mitochondrial oxidative phosphorylation

Using the classical oxygraph method, we first verified directly that the OP2113 compound did not affect oxidative phosphorylation or mitochondrial integrity using mitochondria isolated from rat heart. The substrate combination that fed electrons to the entire respiratory chain (see legend to Fig 1) was chosen given that it most closely resembles *in vivo* conditions where metabolism and Krebs cycle are active inside mitochondria and both NADH (complex I) and succinate (complex II) are oxidized by the respiratory chain. No statistically significant differences were observed regarding the presence of OP2113 for the large range of concentrations tested here (Fig 1), demonstrating that OP2113 has no effect on mitochondrial oxidative phosphorylation, respiratory chain activity, ATP synthesis, or mitochondrial inner membrane integrity (leak rate, in green) under these conditions. Overall, these results confirm the absence of any harmful effect of OP2113 on mitochondrial energetics under these conditions.

Since it was not possible to measure more precisely the effect of OP2113 on the specific activity of complex I due to spectrophotometric interferences, we have been advised to assay by polarography the effect of OP2113 on rotenone-sensitive NADH oxidase activity by broken (frozen-thawed) mitochondria (S1 File), assimilated to complex I [36]. Interestingly, this activity turned out to be almost 30 times higher than the activity of oxidative phosphorylation driven by complex I substrates presented in Fig 1 (respectively 3500 versus 130 nmol O₂/min/mg prot). Due to this huge NADH oxidase activity as compared to complex I oxidative phosphorylation, we had to use a much lower protein content in the assay, while keeping the same drug to protein ratio. We could detect an inhibition (about 8%) of NADH oxidase activity on broken mitochondria starting at the equivalent of 20 μM (200 nmol OP2113 / mg of mitochondrial protein) and increasing to 45% for 80 μM (800 nmol OP2113 / mg of mitochondrial protein). However, due to the difference in activity, even at the higher OP2113 concentration the activity of rotenone-sensitive NADH oxidase is still 15 times higher than complex I driven oxidative phosphorylation rate. These results explain the total absence of effect of OP2113, even at very high concentration (80 μM), on complex I-driven oxidative phosphorylation by intact heart mitochondria.

OP2113 specifically inhibits mitochondrial superoxide/H₂O₂ production

We further tested the effects of OP2113 on ROS production by mitochondria under various conditions. As previously stated, mitochondrial ROS production is highly dependent on mitochondrial bioenergetic state, and maximal production occurs under conditions of high reduction of electron transporters and high membrane potential. These conditions are fulfilled in the presence of ATR (inhibition of ATP/ADP translocator; green bars, Fig 1B to 1D). Under these conditions ROS are produced at different sites of the respiratory chain [7, 24]. The main sites of production are located at complex I and III, where large changes in potential energy of electrons occur [5, 22, 25]. These complexes also allow proton pumping [18]. Based on the work of M.D Brand and colleagues [21, 24, 25], we designed a series of inhibitor titrations to decipher OP2113 action on ROS production by the mitochondrial respiratory chain under

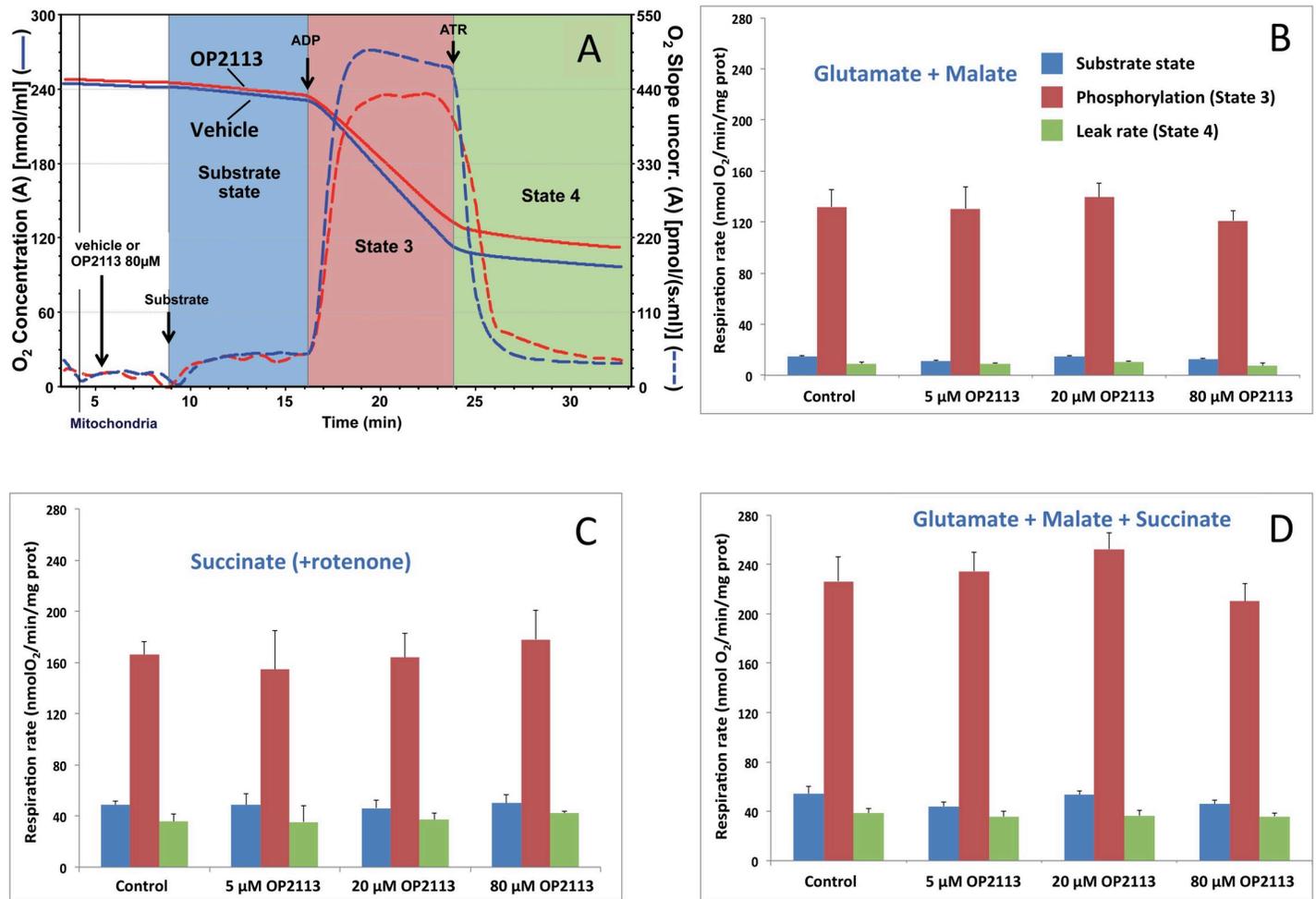


Fig 1. The lack of an effect of OP2113 on mitochondrial oxidative phosphorylation. Panel A: This diagram illustrates a classical respiration assay and presents O₂ concentration (solid lines) and O₂ consumption slope (dotted lines) of rat heart mitochondria after a short incubation in the presence of the vehicle (blue lines) or 80 μM OP2113 (red lines). Respiratory substrates (glutamate + malate in this assay) were added, triggering the onset of oxygen consumption (substrate state, blue background) and then phosphorylation was promoted by the addition of 1 mM ADP in order to obtain the maximal oxidative phosphorylation rate (State 3, red background) [4, 35]. Finally, atractyloside (ATR), which inhibits the ADP/ATP translocator, was added to yield the mitochondrial leak rate (green background) under non phosphorylating conditions (State 4). Panels B to D: The three energetic states were studied in the presence of increasing concentrations of OP2113 (5, 20, and 80 μM), and with mitochondria oxidizing different respiratory substrate combinations: glutamate + malate (B), which feeds electrons to complex I; succinate in the presence of rotenone (C), supplying electrons to complex II; and glutamate + malate + succinate (D) feeding electrons to both complexes I & II. Results are based on 3 independent experiments. No significant differences in mitochondrial respiration rates were noted after the addition of OP2113.

<https://doi.org/10.1371/journal.pone.0216385.g001>

conditions of maximal ROS production. The schematic representation of these experiments and of their rationale is presented in Fig 2.

Fig 3 presents the effect of increasing OP2113 concentrations (from 5 to 80 μM) on ROS/H₂O₂ production measured under the different conditions depicted in Fig 2. Results chiefly show that once rotenone has been added to the assay, ROS/H₂O₂ production becomes insensitive to OP2113 even at high concentrations, irrespective of the site involved. Thus, the only condition under which OP2113 is active in our assay is the condition where ROS are produced at the level of complex I (site I_Q sensitive to rotenone). These results demonstrate that OP2113 significantly decreases ROS/H₂O₂ production (by approximately 80%) under conditions where complex I is the main producer (before addition of rotenone), while no significant effect was observed under all other conditions (after addition of rotenone).

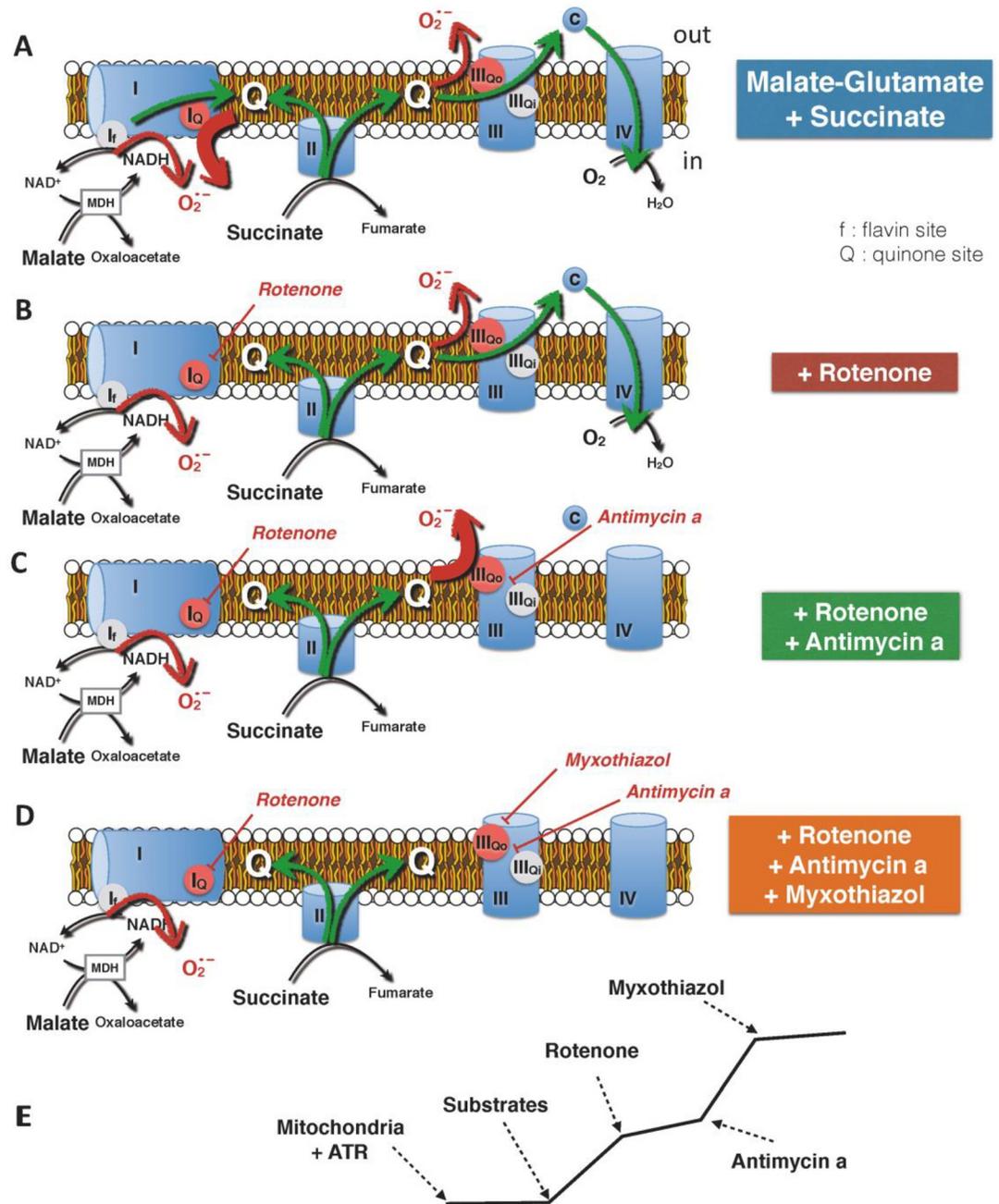


Fig 2. Main sites of oxygen radical production by isolated mitochondria. This scheme gives background information regarding the potential sites for ROS production, using different mitochondrial-targeted drugs in a protocol specifically designed for a study of the effect of a compound on mitochondrial ROS/H₂O₂ production. When mitochondria are energized by a combination of complex I (malate-glutamate) and complex II (succinate) substrates and in the absence of specific inhibitors of the complexes, ROS production is considered as mainly derived from reverse electron transport (RET) at site I_Q (A). Of note, ROS produced by complex I, either at site I_Q (quinone site) or at site I_f (flavin site), are delivered to the inner-(matrix-) side of the inner mitochondrial membrane. In the presence of rotenone, a specific inhibitor of complex I which blocks RET, ROS production is thought to occur predominantly at site III_{QO}, possibly with residual production at site I_f (B) [24]. If complex III is inhibited as it is the case in the presence of antimycin a, the reduced to oxidized quinone ratio increases due to complex II activity and triggers an increase in ROS production, essentially at site III_{QO} (C). Finally, myxothiazol (inhibitor of complex III site III_{QO}) is supposed to block complex III ROS production, and the remaining production is usually ascribed to the flavin site of complex I for which there is no known inhibitor (D)[25]. However, due to matrix antioxidant machinery, the possibility that some ROS/H₂O₂ produced in the matrix may escape to the measurement has been suggested from experiments carried out with submitochondrial particles [37]. A typical recording of ROS production kinetics by mitochondria during the designed inhibitor sequence is presented in E.

<https://doi.org/10.1371/journal.pone.0216385.g002>

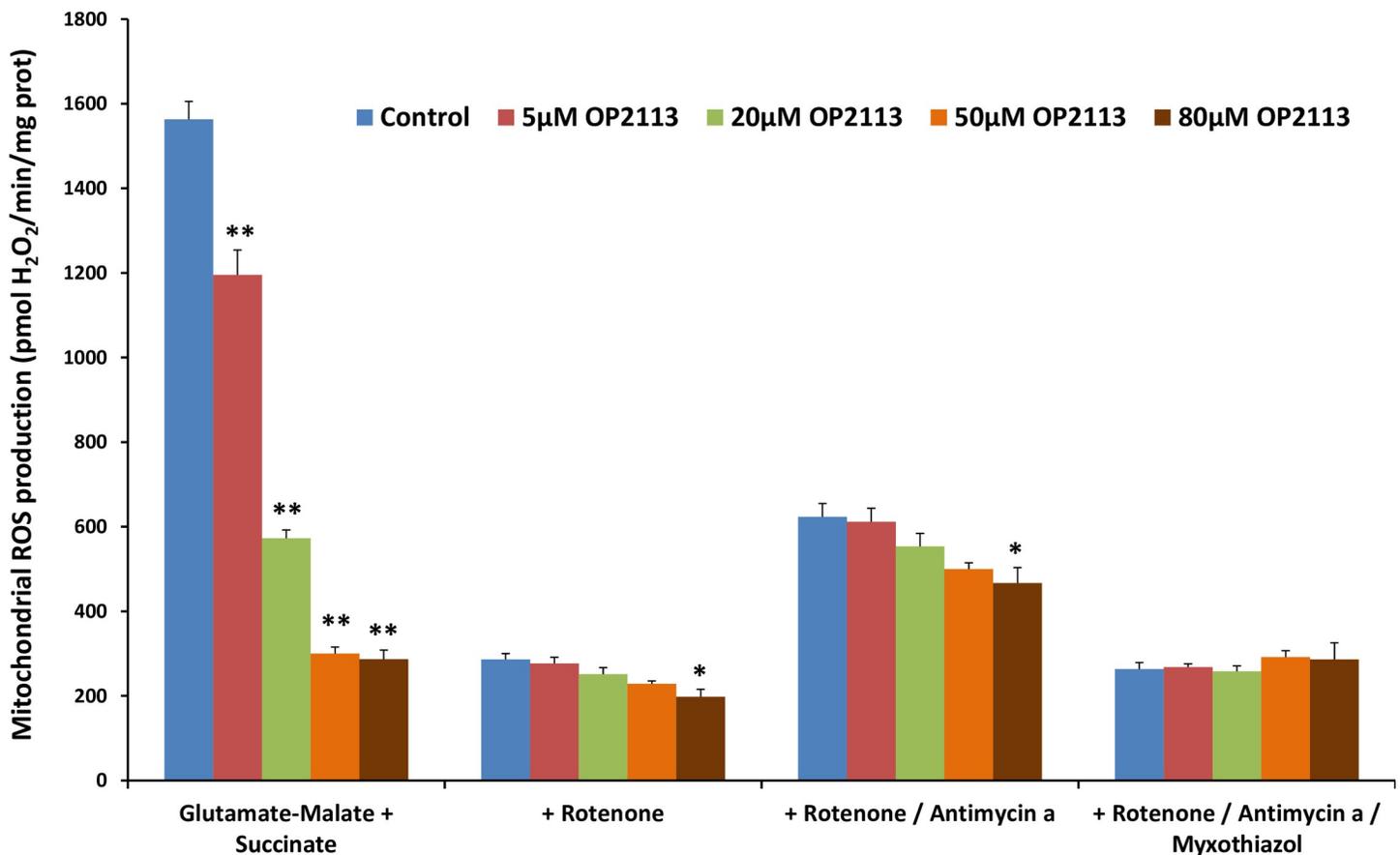


Fig 3. Effect of OP2113 on ROS/H₂O₂ production by isolated rat heart mitochondria. The rate of ROS/H₂O₂ production by isolated rat heart mitochondria respiring on glutamate + malate + succinate was measured under the different conditions described in Fig 2 in the presence of increasing concentrations of OP2113 (5 to 80 µM). In the absence of specific inhibitors of the complexes (see Fig 2A), ROS/H₂O₂ production is maximal and is mainly derived from reverse electron transport at site I_Q (see comments in Fig 2). Following blockade of reverse electron transport by addition of rotenone (1.5 µM) (Fig 2B), ROS/H₂O₂ production is reduced and occurs essentially at site III_{QO}. The subsequent addition of Antimycin a (2 µM) (Fig 2C), which blocks the transfer of electrons to oxygen, increases this ROS/H₂O₂ production. Finally, myxothiazol (0.2 µM) blocks ROS/H₂O₂ production at site III_{QO} (Fig 2D). Data are based on 4 independent experiments, each performed in duplicate. *P < 0.05, **P < 0.005 versus control group.

<https://doi.org/10.1371/journal.pone.0216385.g003>

Given that OP2113 surprisingly did not inhibit all mitochondrial ROS/H₂O₂ production, further investigations were conducted to obtain better insight into the action of the compound on the different mitochondrial sites based on the excellent pioneering work of MD Brand's group [21, 22, 24, 26, 38]. These experiments confirmed that OP2113 affects complex I ROS production, without any measurable effect on the other main sites of ROS/H₂O₂ production tested (Fig 4). These results allowed the calculation of a half maximal effective concentration (EC₅₀) of 10.2 ± 0.9 µM for OP2113 on the inhibition of ROS/H₂O₂ production by complex I in isolated rat heart mitochondria under our experimental conditions.

The specific effect of OP2113 on a unique site of mitochondrial ROS/H₂O₂ production is not only surprising but raises interesting questions about the mechanism of action of OP2113 on mitochondria. These results exclude the hypothesis that the therapeutic effects of OP2113 arise from a mere radical scavenger property as previously reported [28–30]. Indeed, we show in this study that OP2113 does not trap all the ROS produced by mitochondrial respiratory chain, independently of their site of production.

Although the mechanism of action requires further investigations, evidence is presented here that OP2113 directly interferes with mitochondrial complex I ROS production and

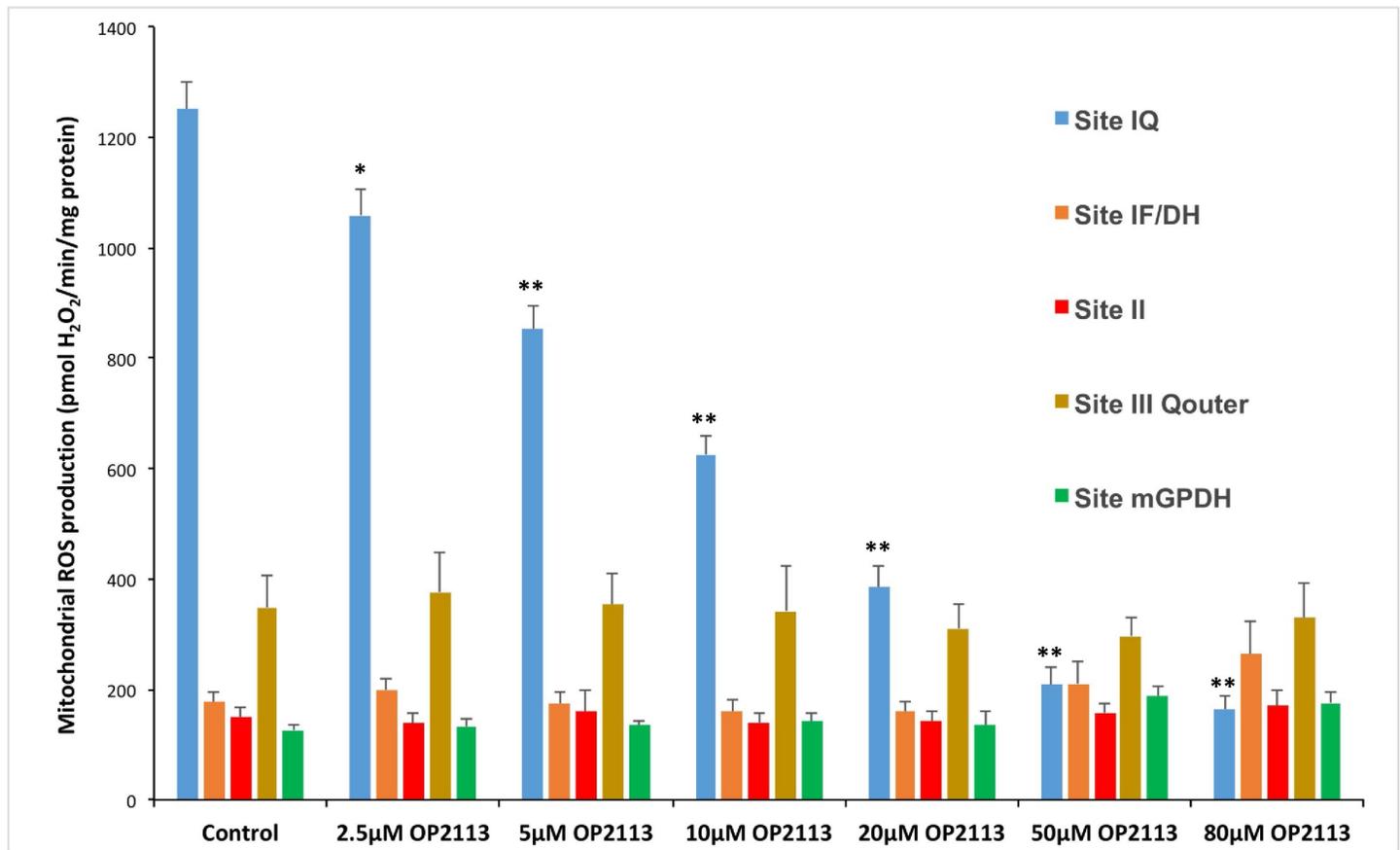


Fig 4. Specific effects of OP2113 on ROS/H₂O₂ production at different sites in isolated rat heart mitochondria. The rates of ROS/H₂O₂ production were measured under conditions specifically designed by MD brand's group for the identification of the different mitochondrial ROS production sites [21, 22, 24, 38] (see Methods section for details). The effects of increasing concentrations of OP2113 (from 2.5 to 80 µM) were tested for each condition of ROS/H₂O₂ production. Data are based on 3 to 5 independent experiments, each performed in duplicate or triplicate. *P < 0.01, **P < 0.005 versus control group.

<https://doi.org/10.1371/journal.pone.0216385.g004>

selectively inhibits superoxide production from the ubiquinone-binding site of complex I (site I_Q) with no effect on superoxide production from other sites or on oxidative phosphorylation processes. As can be seen in Fig 4, the half maximal effective concentration of OP2113 inhibiting mitochondrial Complex I driven ROS production is equal to 10 µM. This concentration corresponds to 100 nmol /mg protein, an OP2113 quantity that is affecting neither the rotenone-sensitive NADH oxidase activity (see above) nor the oxidative phosphorylation in our experimental conditions (see Fig 1).

Another compound also inhibiting mitochondrial complex I ROS production, N-cyclohexyl-4-(4-nitrophenoxy) benzenesulfonamide, has very recently been described by Brand's group [7] along with other molecules [38, 39]. Similarly with OP2113, these chemicals (S1QELs) do not modify the activity of complex I-driven oxidative phosphorylation [38, 39].

The specificity of OP2113 for mitochondrial ROS production by complex I was further tested *in vitro* on the ROS/H₂O₂ production by NAD(P)H oxidase (S2 File). We did not observe any inhibition of resorufin production under these conditions, indicating that OP2113 does not interfere either with the ROS/H₂O₂ measurement system (amplex red) or by direct interaction with H₂O₂. These results also suggest that OP2113 may not inhibit ROS/H₂O₂ production by cytosolic NAD(P)H oxidases, which are likely the major non-mitochondrial ROS/

H₂O₂ producers in the cells. These results are in striking contrast with previous assertions on the putative effect of OP2113 as a radical scavenger [28–30].

OP2113 protects heart from ischemia-reperfusion injury (infarct model)

The role of mitochondrial ROS production in ischemia reperfusion injury is now heavily documented and complex I appears to play a central role, during both ischemia and reperfusion [40]. Recent works [13] have for instance demonstrated that the mechanism by which extensive ROS generation occurs at reperfusion involves reverse electron transport at mitochondrial complex I [38] is due to succinate accumulation during ischemia [13, 41], although we did question this mechanism in the context of ischemic preconditioning [42, 43]. It appears also that mitochondrial respiratory chain—and specifically complex I—damages occur during ischemia [40, 44], and that these damages were paralleled by further ROS production and infarct development. Indeed, previous works have shown that a ROS production sensitive to complex I inhibitors occurs during the ischemic phase that may be involved in the mechanisms of heart pharmacological protection [45–48]. Interestingly, ROS production sensitive to complex I inhibitors has been shown to be involved in the damages to complex I occurring during ischemia [44] which increase complex I capacity of ROS production [49] at reperfusion. Reversible ischemia-induced conformational change of complex I to a deactive form has also been shown [50].

Considering the crucial role of ROS from complex I in the complex mechanisms of heart ischemia-reperfusion and the effect of OP2113 on complex I driven ROS production, we appraised that protection of the infarcted heart from ischemia-reperfusion damage may represent a demonstrative experiment to test the effect of OP2113 on mitochondria in living tissues.

This hypothesis was assessed by investigating the cardioprotective effects of OP2113 pre-treatment of Langendorff-perfused rat hearts submitted to an ischemia-reperfusion protocol (see S3 File). Cardioprotection was assessed from the recovery of contractile performance during reperfusion (Fig 5) and quantification of infarct size by triphenyltetrazolium chloride (TTC) staining at the end of the reperfusion period (Fig 6). Fig 5 shows the time course of the rate-pressure product (RPP calculated as the product of left ventricular developed pressure by heart rate). After the stabilization period, the presence of ethanol in the vehicle during the infusion period caused a similar transitory decrease of the RPP in the two groups studied (Fig 5, orange background). Contraction was stopped during the 30 min ischemia and restart at reperfusion. Fig 5 shows that pre-treatment with OP2113 significantly improves the recovery of RPP at the end of the reperfusion period (34% vs 11% of baseline value in OP2113 and control conditions, respectively, $p < 0.05$).

The infarct size was analyzed by TTC staining and quantified at the end of the reperfusion period for a series of 6 experiments per condition (see typical photographs of heart slices in Fig 6 right panel). Results clearly demonstrate that OP2113 significantly decreased infarct size at the end of reperfusion by approximately 50% (Fig 6, left panel). This observation fits well with the better recovery of heart contractile performance of hearts treated with OP2113 as compared to controls (see Fig 5).

Discussion

When tested on isolated mitochondria from rat heart, OP2113 effectively decreases mitochondrial ROS/H₂O₂ production (in isolated mitochondria, H₂O₂ is produced from the reduction of superoxide anion by mitochondrial superoxide dismutase). Moreover, the results presented here clearly demonstrate that OP2113 presents a very strong selectivity towards the formation of ROS by site I_Q in complex I (Fig 1), which demonstrates that OP2113 does not simply

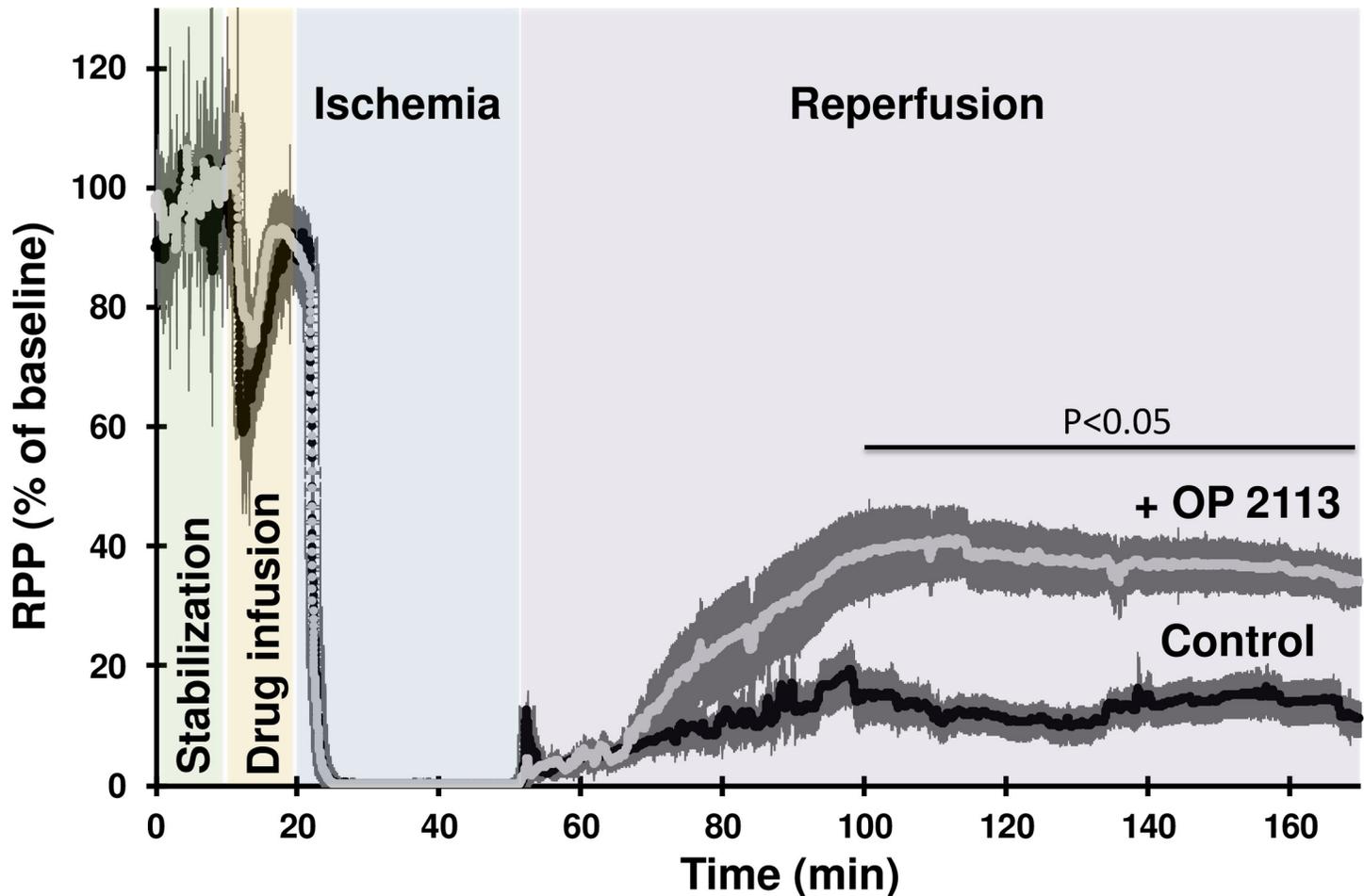


Fig 5. OP2113 improves the recovery of the contractile performance of Langendorff-perfused rat heart during the reperfusion phase following ischemia. This figure shows the time course of the rate-pressure product of two groups of isolated langendorff-perfused rat hearts ($n = 6$ in each group) during a protocol of ischemia-reperfusion. Rate Pressure Product (RPP), the product of the left ventricular developed pressure (mmHg/beat) by heart rate (beat/min) is used as an index of contractile performance and is expressed as % of baseline value measured at the end of the stabilization period (control 28932 ± 2467 mmHg/min, OP2113 31653 ± 4611 mmHg/min). Each heart was allowed to stabilize during 10 min (green background) before perfusion of vehicle (control group, black trace) or $10 \mu\text{M}$ OP2113 (+ OP2113 group, grey trace) during 10 more minutes (orange background). Hearts were then submitted to 30 min zero-flow ischemia (blue background) before 120 min of reperfusion in the absence of vehicle or OP2113 (purple background). For more details on the perfusion protocol see also the [Methods](#) section and [S3 File](#). Data are expressed as the mean \pm SEM for 6 independent experiments. The thickness of the line represents the error bars.

<https://doi.org/10.1371/journal.pone.0216385.g005>

interact with superoxide radicals but specifically prevents their formation by complex I. In that respect, OP2113 therefore appears as a member of the brand new class of oxidative stress protectants. Whereas antioxidants generally do not interfere directly with electron transport and scavenge ROS and/or H_2O_2 downstream from production and therefore can never fully suppress the effect of ROS [7], OP2113 may act differently by preventing ROS formation and thus being more active to protect mitochondria from their own ROS. Data presented here further demonstrate that OP2113 is a specific inhibitor of ROS formation at site I_Q of complex I of the mitochondrial respiratory chain. Further experiments are however required to ascertain whether OP2113 has no effect on other mitochondrial ROS production sites, but this does not preclude the above conclusions. Incidentally, the observed inhibition of the rotenone-sensitive NADH oxidase activity in broken mitochondria may reflect a different process, since it occurs at higher concentrations than ROS inhibition and without any effect on oxidative phosphorylation. Nonetheless, at this stage we cannot rule out that in our *ex vivo* experiments the

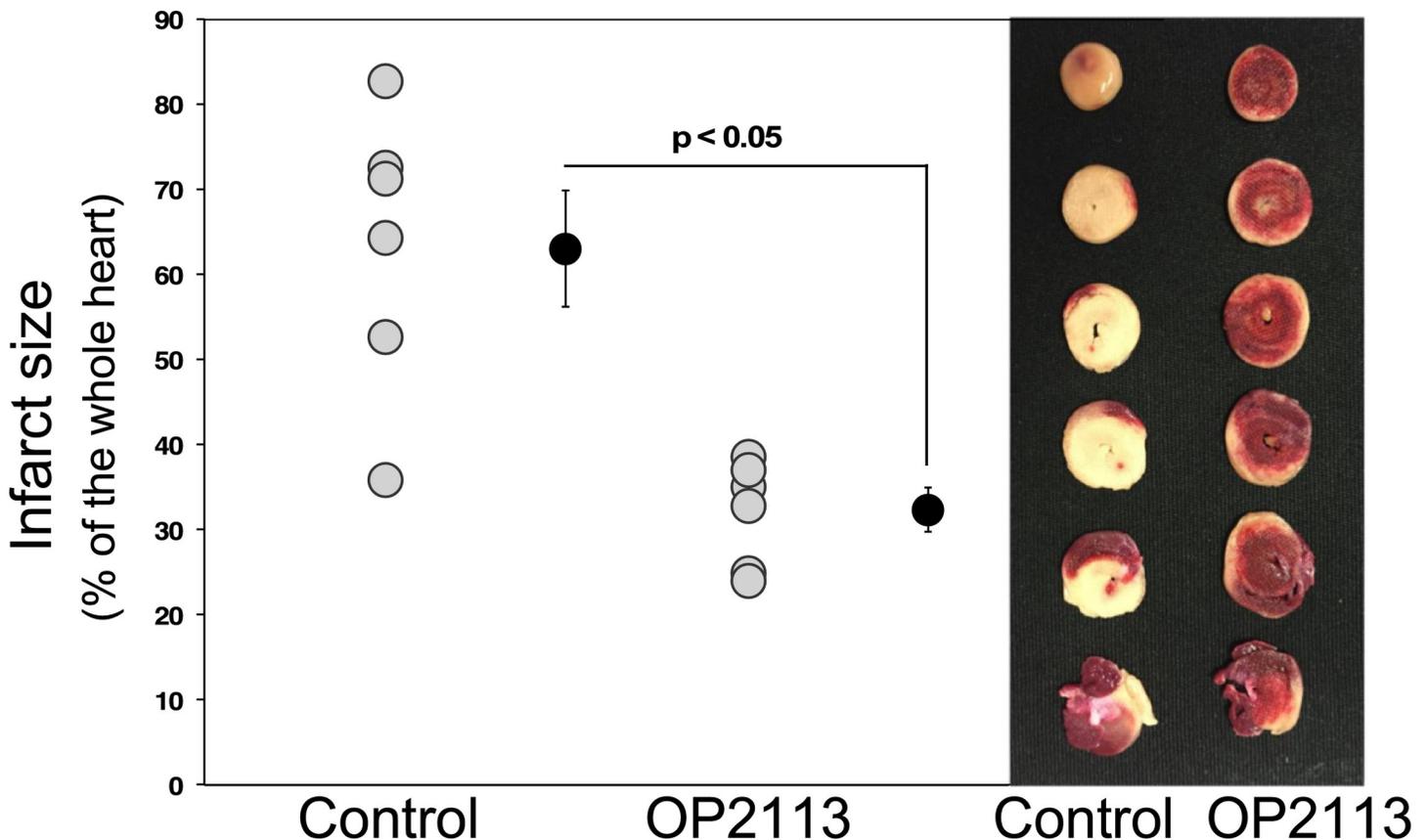


Fig 6. Effect of OP2113 on infarct size. Hearts were pre-treated (OP2113) or not (Control) with OP2113 before 30 min of ischemia followed by 120 min of reperfusion (see Fig 5). Left panel, grey dots represent individual experiments and black dots mean \pm SEM (see Methods, $n = 6$ independent experiments for each condition). Right panel, typical pictures obtained in the control and OP2113 groups following TTC staining. The tissues appearing in red are living tissues stained with TTC, whereas damaged tissue appears white.

<https://doi.org/10.1371/journal.pone.0216385.g006>

cardioprotective effect brought by OP2113 could be secondary to complex I inhibition during the ischemic phase, a cardioprotective strategy as shown by Lesnfsky and collaborators [44, 45, 51].

We also present evidence that OP2113 may interact only with mitochondria without affecting ROS formation in the cytosol and therefore would not affect intracellular signaling. Selective modulators of superoxide production from site I_Q would offer unique opportunities to probe the putative role of mitochondrial ROS production in normal and pathological processes [7] and during a life span [2]. OP2113's specificity towards mitochondrial ROS production ("bad" ROS) would preserve cytosolic ROS signaling ("good" ROS) and therefore appears as a very promising property that may circumvent the bias of the use of non-specific antioxidants in clinical trials.

Since OP2113 does not present a permanent positive charge, it may not accumulate in mitochondria due to the inner mitochondrial membrane potential difference. However, to our knowledge, all previous chemical and pharmacological data [31] confirm the high lipophilicity of the molecule and its large distribution in tissues. The observed cardioprotective effect of OP2113 against damages induced by ischemia-reperfusion strongly suggest that, at least when added before reperfusion, the drug reaches mitochondrial membranes.

As discussed previously, the mechanisms leading to ischemia-reperfusion injury are highly complex and ROS, especially from complex I, are involved during both ischemia and reperfusion

[40]. At this stage, we cannot effectively conclude if OP2113 acts by protecting complex I from his own ROS during the ischemic period [44–49] or by inhibiting reverse electron transfer and consequent ROS production at the onset of reperfusion [13, 38, 41]. Furthermore, as underlined in the introduction, besides these new specific properties, we must also consider the role of OP2113 as a potential H₂S donor [27, 32–34] in the isolated heart ischemia-reperfusion experiments. Indeed H₂S is now considered as a signaling molecule with potential therapeutic applications [52–54], including ischemia-reperfusion and cardiac pathologies [55]. H₂S at low concentration may be oxidized by mammalian mitochondria [56] and protect mitochondria during the ischemic phase [40] and reperfusion [54, 57]. These mechanisms may also be involved in heart protection by OP2113 during ischemia-reperfusion (Fig 6).

In summary, OP2113 acts upstream from ROS production, therefore insuring increased protection compared with standard antioxidants. OP2113 acts specifically on mitochondrial ROS production to ensure mitochondrial protection, and this action is crucial for numerous pathologies, especially cardiac diseases. OP2113 does not seem to interfere with cell signaling ("good" ROS) (Fig 7). OP2113 acts specifically on site I_Q in complex I, which is the main mitochondrial site and may be implicated in important diseases, including Parkinson's and cardiac arrhythmias.

To conclude, OP2113's properties may represent a break-through in the search for specific modulators of ROS/H₂O₂ production in cells. This is a timely and important discovery given that OP2113 has a great advantage over newly discovered molecules as it is currently authorized for use in humans [31] and may therefore be rapidly included in clinical trials [58–60]. OP2113 may represent the first medicinal member of a new class of "protectants" that specifically prevent ROS production inside mitochondria and may therefore be used for mitochondrial protection during various oxidative stresses, therefore preventing diseases with minimal effects on crucial cellular ROS signaling.

Methods

Animal procedures and ethics statement

All experiments described adhered to the guidelines in the National and European Research Council Guide for the care and use of laboratory animals. P. Dioloz has a valid license to conduct experiments on animals from the Service Vétérinaire de la Santé et de la Protection Animale of the Ministère de l'agriculture et de la Forêt, France (03/17/1999, license number 3308010). All procedures conformed to the UK Animals (Scientific Procedures) Act 1986 and the Guide for the Care and Use of Laboratory Animals published by the National Institutes of Health (NIH Publication No. 85–23, revised 1996).

Materials

All the chemicals were reagent grade and purchased from Sigma Chemical (St. Louis, MO), except for sucrose and NADH oxidase (Merck, Darmstadt, Germany). OP2113 [anethol-trithion, 5-(4-Methoxyphenyl)-3H-1,2-dithiole-3-thione, CAS 532-11-6] was a gift from the private company OP2 drugs (Pessac, France). A 15 mM stock solution of OP2113 was prepared in DMSO and stored away from light at 0°C for only few days. For heart perfusion a daily prepared solution was used.

Isolation of mitochondria

Male Wistar rats (250–325 g; obtained from Janvier Labs, Le Genest-Saint-Isle, France) were anesthetized using 3% isoflurane, heparinized and euthanized by a lethal intra-peritoneal injection of pentobarbital (130 mg/kg), and the heart was quickly removed and washed in cold

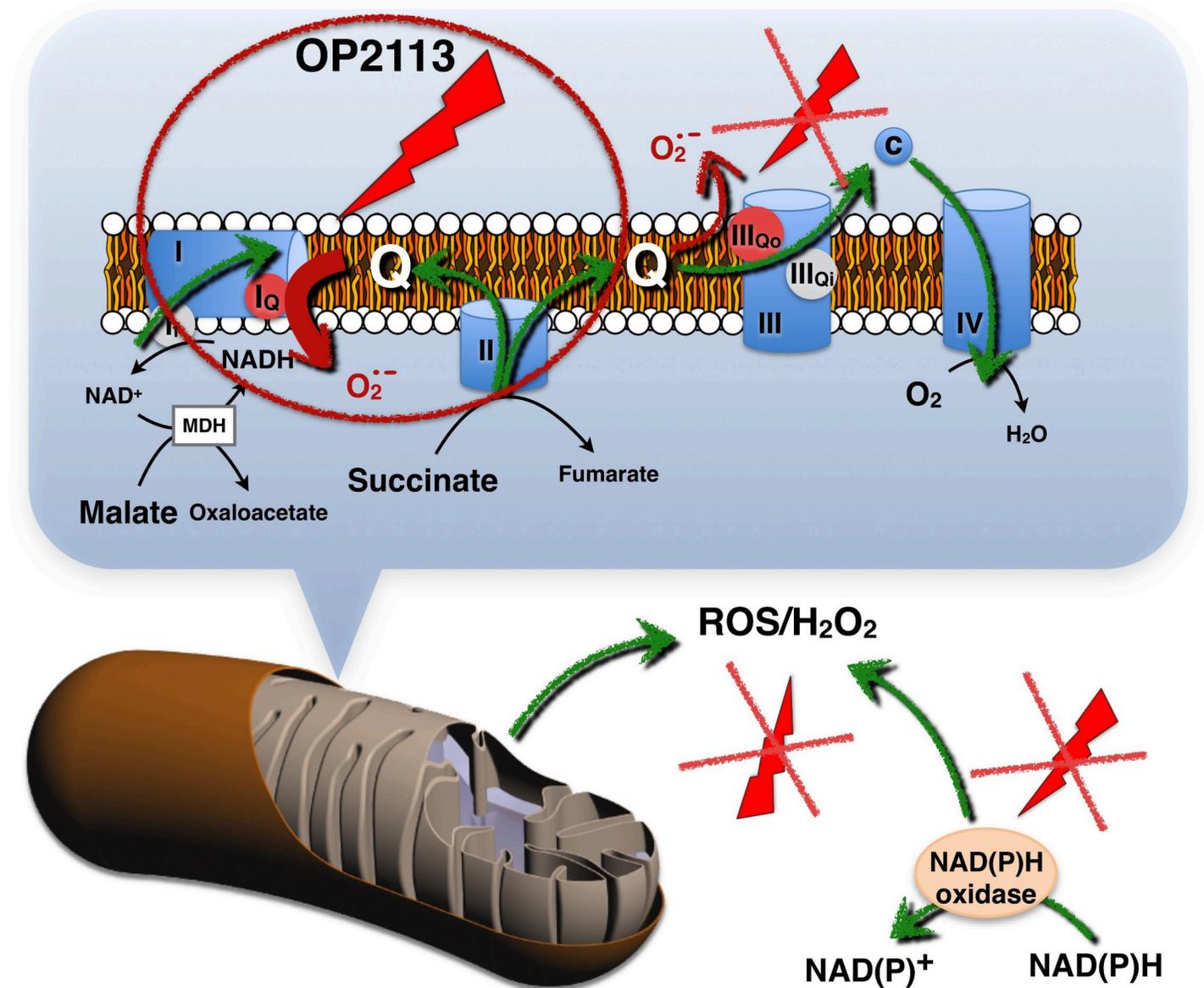


Fig 7. Scheme presenting the specific effects of OP2113 on ROS production by complex I.

<https://doi.org/10.1371/journal.pone.0216385.g007>

isolation medium containing 100 mM sucrose, 180 mM KCl, 50 mM Tris, 5 mM MgCl₂, 10 mM EDTA, and 0.1% (w/v) defatted BSA (pH 7.2).

Isolation of heart mitochondria was performed in a cold chamber. Before homogenization, hearts (approximately 1.5 g) were minced with scissors and treated for 5 min in 5 ml of the same medium supplemented with protease (2 mg of bacterial proteinase type XXIV per ml of isolation buffer) with stirring. The tissue suspension was poured into a 50-ml glass Potter homogenizer, diluted with 20 ml of isolation buffer, and then homogenized for 3 min using a motorized Teflon pestle. The homogenate was filtered through bolting cloth (Sefar Nitex) to remove debris and centrifuged at 8,000 g for 10 min. The resulting pellet was rinsed with 5 ml of isolation buffer, resuspended in 25 ml of the same buffer, and then subjected to low speed centrifugation (400 g) for 8 min. The resulting supernatant was centrifuged twice at 7,000 g for

15 min to yield a washed mitochondrial pellet that was gently resuspended in 150 μ l of isolation buffer. Protein concentration was determined by the Bradford method (Sigma, kit # B6916) using BSA as a standard. Mitochondria were kept on ice at a final concentration of 40–50 mg/ml for less than 5 hours.

Mitochondrial respiration

Oxygen consumption rates of heart mitochondria (0.1 mg/ml) incubated in the absence or presence of OP2113 at increasing doses (from 0 to 80 μ M final concentration) were recorded polarographically under constant stirring at 25°C using a high resolution oximeter (Oxygraph-2K, Oroboros Instruments, Austria). The respiration medium consisted of 140 mM sucrose, 100 mM KCl, 1 mM EGTA, 20 mM MgCl₂, 10 mM KH₂PO₄, and 1 g/L (w/v) BSA essentially fatty acid free (pH 7.2). Oxidative phosphorylation has been carried out using various substrate combinations: Glutamate (5 mM)/malate (2.5 mM) as complex I substrates, succinate (5 mM in the presence of 1.5 μ M rotenone) as complex II substrates, and the combination Glutamate + Malate + Succinate.

Mitochondrial ROS/H₂O₂ production

Rates of ROS/H₂O₂ production from heart mitochondria were assessed through the oxidation of the colorless, non-fluorescent indicator Amplex Red in the presence of exogenous horseradish peroxidase (HRP, EC 1.11.1.7, Sigma). H₂O₂ reacts with Amplex Red in a 1:1 stoichiometry, yielding the fluorescent compound resorufin (excitation: 560 nm; emission: 585 nm), which is stable once formed. Fluorescence was measured continuously with a spectrofluorometer equipped with temperature control and stirring (SAFAS Xenius, Monaco).

Isolated mitochondria (0.1 mg/ml) were incubated in the same experimental buffer as previously described supplemented with 15 μ M Amplex Red and 10 μ g/ml HRP. Glutamate (5 mM)/malate (2.5 mM) together with succinate (5 mM) were used as complex I and complex II substrates, respectively. Experiments were conducted under non-phosphorylating conditions in the presence of 15 μ M atractyloside (inhibitor of adenine nucleotide translocator), *i.e.*, under conditions where the mitochondrial membrane potential is maximal. Afterwards, rotenone (1.5 μ M), antimycin A (2 μ M), and myxothiazol (0.2 μ M) were sequentially added to inhibit the redox centers within the electron transfer chain (see Fig 2), namely sites I_Q, I_F (with rotenone), III_{Q_i} (with antimycin A) and III_{Q_o} (with myxothiazol). The assay was finally calibrated with known amounts of H₂O₂ (steps of 300 nM) in the presence of all relevant compounds, including OP2113. The control test of the absence of effect of OP2113 on the amplex red assay itself and NAD(P)H oxidase ROS/H₂O₂ production was performed in the absence of cardiac mitochondria and the presence of NAD(P)H oxidase (EC 1.6.3.3, 1 mU/ml) and NADH (150 μ M) solutions.

The measurement of the rates of ROS/H₂O₂ production from major separate mitochondrial sites was performed as described by MD Brand's group [21, 24]. Sites of mitochondrial superoxide/H₂O₂ production were targeted individually using distinct combinations of mitochondrial substrates and inhibitors [21, 24] designed to generate maximal rates of ROS/H₂O₂ production predominantly from a single site within the respiratory chain. The sites of production targeted and the solutions used to drive H₂O₂ production were as follows: site I_Q with 5 mM succinate; site I_F/DH with 5 mM glutamate + 2.5 mM malate and 4 μ M rotenone; site III_{Q_o} with 5 mM succinate, 4 μ M rotenone and 2.5 μ M antimycin A; site II_F with 15 μ M palmitoylcarnitine, 2.5 μ M antimycin A and 2 μ M myxothiazol; and mitochondrial glycerol-3-phosphate dehydrogenase (mGPDH) with 25 mM glycerolphosphate, 4 μ M rotenone, 2.5 μ M antimycin A, 1 mM malonate and 2 μ M myxothiazol.

The final effect of the OP2113 compound was scaled to positive controls included in each assay. In addition, 4 μ M CCCP, 20 mM aspartate, 2 μ M myxothiazol, and 10 mM malonate were used as positive controls for site I_Q, I_F, III_{Qo} and II_F, respectively [21, 24]. No positive control is available in the case of mGPDH.

Results were analyzed using one-way analysis of variance, followed by Bonferroni's test to check for significant differences, using Statview Software. Significance was accepted at $P < 0.05$.

Heart perfusion

Male Wistar rats (250–300 g) were anesthetized by 3% isoflurane, heparinized and euthanized by a lethal IP injection of pentobarbital (130 mg/kg). Hearts (~0.95 g fresh weight) were rapidly harvested and placed into ice-cold Krebs-Henseleit buffer containing (in mmol/L): NaCl 118, NaHCO₃ 25, KCl 4.8, KH₂PO₄ 1.2, MgSO₄ 1.2, glucose 11 and CaCl₂ 1.8. The solution was gassed with 95% O₂/5% CO₂ at 37°C (pH 7.4). Langendorff heart perfusions were performed as described previously [61], and isometric contractile performance (rate-pressure product (RPP)) was assessed from continuous monitoring of the left ventricular developed pressure (LVDP) via a balloon placed in the left ventricle and connected to a pressure transducer (RPP (mmHg/min) = LVDP (mmHg/beat) x heart rate (beat/min)) [61]. Hearts were perfused in a constant flow mode (12 ml/min) during 10 min for stabilization followed by 10 min treatment with the vehicle (Control; final concentrations: 0.83% ethanol + 0.07% Dimethyl-Sulfoxide) or 10 μ M OP2113 solution in the same vehicle. Global normothermic ischemia was induced by halting perfusion flow for 30 min while immersing the heart in perfusion buffer thermostabilized at 37°C. Then, hearts were reperfused for 2 hours.

Assessment of infarct size

At the end of the 2-hour reperfusion period, hearts were stained with triphenyltetrazolium chloride (TTC). Hearts were stained by perfusion for 7 min at 12 ml/min with a 1% (w/v) TTC solution. Hearts were then detached from the cannula and incubated for 4 min at 37°C before being sliced perpendicularly to the longitudinal axis into 6 slices. The slices were then treated in 4% (w/v) formalin solution overnight at 4°C and weighed before both sides of each slice were photographed. The surface of the necrotic and at risk areas of each side were determined for each photograph by planimetry (AlphaEase v5.5). Infarct size was expressed as the percentage of the total cross-sectional area of the heart given that the total heart was subjected to ischemia under our conditions. Data in supporting information [S3 File](#).

Statistical analysis

Data from 6 independent heart perfusions are expressed as the means \pm SEM. As the number in each group was less than 20, the distribution was considered non-normal. Consequently, a non-parametric Mann-Whitney test (SPSS statistics 17.0) was performed for comparisons between the control and OP2113 groups. The results were considered statistically significant if the p -value was less than 0.05.

Supporting information

S1 File. Effect of OP2113 on mitochondrial NADH oxidase activity. After freeze-thaw treatment, rat heart mitochondria were used to assess mitochondrial rotenone-sensitive NADH oxidase activity by polarography as described in the supplementary Materials and Methods. Panel A: Typical polarographic trace showing the rotenone-sensitive NADH oxidase activity

and the effect of the addition of increasing quantity of OP2113 from 50 to 800 nmol / mg mitochondrial protein on oxygen consumption. Panel B: Bar graph representing the mean oxygen consumption expressed in nmol O₂ / min / mg mitochondrial protein. Rotenone addition completely stop oxygen consumption suggesting that the activity is mainly supported by the mitochondrial complex I. Data are presented as means ± SD. 4 independent mitochondrial preparation were used for the assay and for each mitochondrial batch the assay was realized in quadruplicate. High quantity of OP2113 inhibit partly the mitochondrial rotenone-sensitive NADH oxidase activity.

(ZIP)

S2 File. Effect of OP2113 on the artificial H₂O₂-producing NAD(P)H oxidase system. The rates of H₂O₂ production were measured in the presence of NAD(P)H oxidase (1 mU/ml) and NADH (150 μM), and in the absence of heart mitochondria. Data are based on 3 independent experiments, each performed in duplicate. No significant effect of OP2113 on this experimental H₂O₂ production was noted.

(ZIP)

S3 File. Detailed information and pictures about ischemia/reperfusion in rat heart. Supporting data contain supplementary informations concerning the experiments on isolated rat heart ischemia and reperfusion. Raw data presents contractile activity (RPP), whole heart oxygen consumption (MVO₂) during the pre-schemic and post-ischemic (reperfusion) phases for all the experiments, as well as all data used for the determination of infarct size. Separate files describe the results of all the statistical analyses presented in Figs 5 and 6. Finally, supplementary figures present pre-ischemic RPP and MVO₂ and reperfusion phases (MVO₂ and RPP to MVO₂ ratio), as well as a graphic description of the protocols used in the study.

(ZIP)

Acknowledgments

The authors thank Pippa McKelvie-Sebileau of the LIRYC Institute for editorial assistance, Emma Abell for technical assistance and Y. Chatenet for 3D mitochondria drawing (Striking image).

Author Contributions

Conceptualization: Dominique Detaille, Philippe Diolez.

Data curation: Dominique Detaille, Philippe Pasdois, Audrey Sémont.

Formal analysis: Philippe Pasdois, Audrey Sémont, Philippe Diolez.

Funding acquisition: Pierre Dos Santos, Philippe Diolez.

Investigation: Dominique Detaille, Philippe Pasdois.

Methodology: Dominique Detaille, Philippe Pasdois, Audrey Sémont, Philippe Diolez.

Resources: Pierre Dos Santos.

Supervision: Philippe Diolez.

Validation: Dominique Detaille, Philippe Pasdois, Pierre Dos Santos, Philippe Diolez.

Writing – original draft: Dominique Detaille, Philippe Pasdois, Philippe Diolez.

Writing – review & editing: Dominique Detaille, Pierre Dos Santos, Philippe Diolez.

References

1. Harman D. The free radical theory of aging: effect of age on serum copper levels. *Journal of gerontology*. 1965 Apr; 20:151–3. PMID: [14284786](#).
2. Dai DF, Chiao YA, Marcinek DJ, Szeto HH, Rabinovitch PS. Mitochondrial oxidative stress in aging and healthspan. *Longevity & healthspan*. 2014; 3:6. <https://doi.org/10.1186/2046-2395-3-6> PMID: [24860647](#). Pubmed Central PMCID: 4013820.
3. Harman D. The biologic clock: the mitochondria? *Journal of the American Geriatrics Society*. 1972 Apr; 20(4):145–7. PMID: [5016631](#).
4. Gouspillou G, Bourdel-Marchasson I, Rouland R, Calmettes G, Biran M, Deschodt-Arsac V, et al. Mitochondrial energetics is impaired in vivo in aged skeletal muscle. *Aging cell*. 2014 Feb; 13(1):39–48. <https://doi.org/10.1111/acer.12147> PMID: [23919652](#).
5. Balaban RS, Nemoto S, Finkel T. Mitochondria, oxidants, and aging. *Cell*. 2005 Feb 25; 120(4):483–95. <https://doi.org/10.1016/j.cell.2005.02.001> PMID: [15734681](#).
6. Murphy MP. How mitochondria produce reactive oxygen species. *The Biochemical journal*. 2009 Jan 1; 417(1):1–13. <https://doi.org/10.1042/BJ20081386> PMID: [19061483](#). Pubmed Central PMCID: 2605959.
7. Orr AL, Ashok D, Sarantos MR, Shi T, Hughes RE, Brand MD. Inhibitors of ROS production by the ubiquinone-binding site of mitochondrial complex I identified by chemical screening. *Free radical biology & medicine*. 2013 Dec; 65:1047–59. <https://doi.org/10.1016/j.freeradbiomed.2013.08.170> PMID: [23994103](#).
8. Finkel T, Holbrook NJ. Oxidants, oxidative stress and the biology of ageing. *Nature*. 2000 Nov 9; 408(6809):239–47. <https://doi.org/10.1038/35041687> PMID: [11089981](#).
9. Lin MT, Beal MF. Mitochondrial dysfunction and oxidative stress in neurodegenerative diseases. *Nature*. 2006 Oct 19; 443(7113):787–95. <https://doi.org/10.1038/nature05292> PMID: [17051205](#).
10. Muller FL, Lustgarten MS, Jang Y, Richardson A, Van Remmen H. Trends in oxidative aging theories. *Free radical biology & medicine*. 2007 Aug 15; 43(4):477–503. <https://doi.org/10.1016/j.freeradbiomed.2007.03.034> PMID: [17640558](#).
11. Nunnari J, Suomalainen A. Mitochondria: in sickness and in health. *Cell*. 2012 Mar 16; 148(6):1145–59. <https://doi.org/10.1016/j.cell.2012.02.035> PMID: [22424226](#).
12. Van Gaal LF, Mertens IL, De Block CE. Mechanisms linking obesity with cardiovascular disease. *Nature*. 2006 Dec 14; 444(7121):875–80. <https://doi.org/10.1038/nature05487> PMID: [17167476](#).
13. Chouchani ET, Pell VR, Gaude E, Aksentijevic D, Sundier SY, Robb EL, et al. Ischaemic accumulation of succinate controls reperfusion injury through mitochondrial ROS. *Nature*. 2014 Nov 20; 515(7527):431–5. <https://doi.org/10.1038/nature13909> PMID: [25383517](#). Pubmed Central PMCID: 4255242.
14. Vysokikh MY, Antonenko YN, Lyamzaev KG, Rokitskaya TI, Skulachev VP. Methodology for use of mitochondria-targeted cations in the field of oxidative stress-related research. *Methods in molecular biology*. 2015; 1265:149–59. https://doi.org/10.1007/978-1-4939-2288-8_12 PMID: [25634274](#).
15. Smith RA, Murphy MP. Mitochondria-targeted antioxidants as therapies. *Discovery medicine*. 2011 Feb; 11(57):106–14. PMID: [21356165](#).
16. Reily C, Mitchell T, Chacko BK, Benavides G, Murphy MP, Darley-Usmar V. Mitochondrially targeted compounds and their impact on cellular bioenergetics. *Redox biology*. 2013; 1(1):86–93. <https://doi.org/10.1016/j.redox.2012.11.009> PMID: [23667828](#). Pubmed Central PMCID: 3647698.
17. Gruber J, Fong S, Chen CB, Yoong S, Pastorin G, Schaffer S, et al. Mitochondria-targeted antioxidants and metabolic modulators as pharmacological interventions to slow ageing. *Biotechnology advances*. 2013 Sep-Oct; 31(5):563–92. <https://doi.org/10.1016/j.biotechadv.2012.09.005> PMID: [23022622](#).
18. Nicholls DG, Ferguson S. *Bioenergetics 4*. Amsterdam: Elsevier; 2013.
19. Mailloux RJ. Teaching the fundamentals of electron transfer reactions in mitochondria and the production and detection of reactive oxygen species. *Redox biology*. 2015; 4:381–98. <https://doi.org/10.1016/j.redox.2015.02.001> PMID: [25744690](#). Pubmed Central PMCID: PMC4348434.
20. Finkel T. Signal transduction by reactive oxygen species. *The Journal of cell biology*. 2011 Jul 11; 194(1):7–15. <https://doi.org/10.1083/jcb.201102095> PMID: [21746850](#). Pubmed Central PMCID: 3135394.
21. Quinlan CL, Treberg JR, Pervoshchikova IV, Orr AL, Brand MD. Native rates of superoxide production from multiple sites in isolated mitochondria measured using endogenous reporters. *Free radical biology & medicine*. 2012 Nov 1; 53(9):1807–17. <https://doi.org/10.1016/j.freeradbiomed.2012.08.015> PMID: [22940066](#). Pubmed Central PMCID: 3472107.

22. Brand MD. The sites and topology of mitochondrial superoxide production. *Experimental gerontology*. 2010 Aug; 45(7–8):466–72. <https://doi.org/10.1016/j.exger.2010.01.003> PMID: 20064600. Pubmed Central PMCID: 2879443.
23. Brand MD. The role of mitochondria in longevity and healthspan. *Longevity & healthspan*. 2014; 3:7. <https://doi.org/10.1186/2046-2395-3-7> PMID: 24855560. Pubmed Central PMCID: 4030464.
24. Quinlan CL, Perevoshchikova IV, Hey-Mogensen M, Orr AL, Brand MD. Sites of reactive oxygen species generation by mitochondria oxidizing different substrates. *Redox biology*. 2013; 1:304–12. <https://doi.org/10.1016/j.redox.2013.04.005> PMID: 24024165. Pubmed Central PMCID: PMC3757699. Epub 2013/09/12. Eng.
25. Goncalves RL, Quinlan CL, Perevoshchikova IV, Hey-Mogensen M, Brand MD. Sites of superoxide and hydrogen peroxide production by muscle mitochondria assessed ex vivo under conditions mimicking rest and exercise. *The Journal of biological chemistry*. 2014 Nov 11; 290(1):209–27. <https://doi.org/10.1074/jbc.M114.619072> PMID: 25389297.
26. Wong HS, Dighe PA, Mezera V, Monternier PA, Brand MD. Production of superoxide and hydrogen peroxide from specific mitochondrial sites under different bioenergetic conditions. *The Journal of biological chemistry*. 2017 Aug 24; 292(41):16804–9. <https://doi.org/10.1074/jbc.R117.789271> PMID: 28842493.
27. Dulac M, Sassi A, Nagarathinan C, Christen MO, Dansette PM, Mansuy D, et al. Metabolism of Anethole Dithiolethione by Rat and Human Liver Microsomes: Formation of Various Products Deriving from Its O-Demethylation and S-Oxidation. Involvement of Cytochromes P450 and Flavin Monooxygenases in These Pathways. *Drug Metab Dispos*. 2018 Oct; 46(10):1390–5. <https://doi.org/10.1124/dmd.118.082545> PMID: 30018103.
28. Pouzaud F, Christen MO, Warnet JM, Rat P. [Anethole dithiolethione: an antioxidant agent against tenotoxicity induced by fluoroquinolones]. *Pathol Biol (Paris)*. 2004 Jul; 52(6):308–13. <https://doi.org/10.1016/j.patbio.2003.11.001> PMID: 15261372. L'anethole dithiolethione: un agent cytoprotecteur contre la tenotoxicite induite par les fluoroquinolones.
29. Christen MO. Anethole dithiolethione: biochemical considerations. *Methods Enzymol*. 1995; 252:316–23. PMID: 7476368.
30. Ben-Mahdi MH, Gozin A, Driss F, Andrieu V, Christen MO, Pasquier C. Anethole dithiolethione regulates oxidant-induced tyrosine kinase activation in endothelial cells. *Antioxid Redox Signal*. 2000 Winter; 2(4):789–99. <https://doi.org/10.1089/ars.2000.2.4-789> PMID: 11213483.
31. Wishart DS, Feunang YD, Guo AC, Lo EJ, Marcu A, Grant JR, et al. DrugBank 5.0: a major update to the DrugBank database for 2018. *Nucleic Acids Res*. 2018 Jan 4; 46(D1):D1074–D82. <https://doi.org/10.1093/nar/gkx1037> PMID: 29126136. Pubmed Central PMCID: PMC5753335.
32. Chen P, Luo Y, Hai L, Qian S, Wu Y. Design, synthesis, and pharmacological evaluation of the aqueous prodrugs of desmethyl anethole trithione with hepatoprotective activity. *Eur J Med Chem*. 2010 Jul; 45(7):3005–10. <https://doi.org/10.1016/j.ejmech.2010.03.029> PMID: 20392547.
33. Kashfi K, Olson KR. Biology and therapeutic potential of hydrogen sulfide and hydrogen sulfide-releasing chimeras. *Biochem Pharmacol*. 2013 Mar 1; 85(5):689–703. <https://doi.org/10.1016/j.bcp.2012.10.019> PMID: 23103569. Pubmed Central PMCID: PMC3566320.
34. Sparatore A, Santus G, Giustarini D, Rossi R, Del Soldato P. Therapeutic potential of new hydrogen sulfide-releasing hybrids. *Expert Rev Clin Pharmacol*. 2011 Jan; 4(1):109–21. <https://doi.org/10.1586/epc.10.122> PMID: 22115352.
35. Goupillou G, Rouland R, Calmettes G, Deschodt-Arsac V, Franconi JM, Bourdel-Marchasson I, et al. Accurate determination of the oxidative phosphorylation affinity for ADP in isolated mitochondria. *PLoS One*. 2011; 6(6):e20709. <https://doi.org/10.1371/journal.pone.0020709> PMID: 21694779. Pubmed Central PMCID: PMC3111431.
36. Hoppel CL, Kerr DS, Dahms B, Roessmann U. Deficiency of the reduced nicotinamide adenine dinucleotide dehydrogenase component of complex I of mitochondrial electron transport. Fatal infantile lactic acidosis and hypermetabolism with skeletal-cardiac myopathy and encephalopathy. *J Clin Invest*. 1987 Jul; 80(1):71–7. <https://doi.org/10.1172/JCI113066> PMID: 3110216. Pubmed Central PMCID: PMC442203.
37. Chen Q, Vazquez EJ, Moghaddas S, Hoppel CL, Lesnefsky EJ. Production of reactive oxygen species by mitochondria: central role of complex III. *The Journal of biological chemistry*. 2003 Sep 19; 278(38):36027–31. <https://doi.org/10.1074/jbc.M304854200> PMID: 12840017.
38. Brand MD, Goncalves RL, Orr AL, Vargas L, Gerencser AA, Borch Jensen M, et al. Suppressors of superoxide-H₂O₂ production at site IQ of mitochondrial complex I protect against stem cell hyperplasia and ischemia-reperfusion injury. *Cell Metab*. 2016 Oct 11; 24(4):582–92. <https://doi.org/10.1016/j.cmet.2016.08.012> PMID: 27667666. Pubmed Central PMCID: PMC5061631.

39. Brand MD. Mitochondrial generation of superoxide and hydrogen peroxide as the source of mitochondrial redox signaling. *Free radical biology & medicine*. 2016 Nov; 100:14–31. <https://doi.org/10.1016/j.freeradbiomed.2016.04.001> PMID: 27085844.
40. Lesnefsky EJ, Chen Q, Tandler B, Hoppel CL. Mitochondrial Dysfunction and Myocardial Ischemia-Reperfusion: Implications for Novel Therapies. *Annu Rev Pharmacol Toxicol*. 2017 Jan 6; 57:535–65. <https://doi.org/10.1146/annurev-pharmtox-010715-103335> PMID: 27860548.
41. Murphy MP. Understanding and preventing mitochondrial oxidative damage. *Biochem Soc Trans*. 2016 Oct 15; 44(5):1219–26. <https://doi.org/10.1042/BST20160108> PMID: 27911703. Pubmed Central PMCID: PMC5095902.
42. Andrienko T, Pasdois P, Rossbach A, Halestrap AP. Real-Time Fluorescence Measurements of ROS and [Ca²⁺] in Ischemic / Reperfused Rat Hearts: Detectable Increases Occur only after Mitochondrial Pore Opening and Are Attenuated by Ischemic Preconditioning. *PLoS One*. 2016; 11(12):e0167300. <https://doi.org/10.1371/journal.pone.0167300> PMID: 27907091. Pubmed Central PMCID: PMC5131916.
43. Andrienko TN, Pasdois P, Pereira GC, Ovens MJ, Halestrap AP. The role of succinate and ROS in reperfusion injury—A critical appraisal. *J Mol Cell Cardiol*. 2017 Sep; 110:1–14. <https://doi.org/10.1016/j.yjmcc.2017.06.016> PMID: 28689004. Pubmed Central PMCID: PMC5678286.
44. Chen Q, Camara AK, Stowe DF, Hoppel CL, Lesnefsky EJ. Modulation of electron transport protects cardiac mitochondria and decreases myocardial injury during ischemia and reperfusion. *Am J Physiol Cell Physiol*. 2007 Jan; 292(1):C137–47. <https://doi.org/10.1152/ajpcell.00270.2006> PMID: 16971498.
45. Aldakkak M, Stowe DF, Chen Q, Lesnefsky EJ, Camara AK. Inhibited mitochondrial respiration by amobarbital during cardiac ischaemia improves redox state and reduces matrix Ca²⁺ overload and ROS release. *Cardiovasc Res*. 2008 Jan 15; 77(2):406–15. <https://doi.org/10.1016/j.cardiores.2007.08.008> PMID: 17900548.
46. Kevin LG, Camara AK, Riess ML, Novalija E, Stowe DF. Ischemic preconditioning alters real-time measure of O₂ radicals in intact hearts with ischemia and reperfusion. *Am J Physiol Heart Circ Physiol*. 2003 Feb; 284(2):H566–74. <https://doi.org/10.1152/ajpheart.00711.2002> PMID: 12414448.
47. Riess ML, Camara AK, Kevin LG, An J, Stowe DF. Reduced reactive O₂ species formation and preserved mitochondrial NADH and [Ca²⁺] levels during short-term 17 degrees C ischemia in intact hearts. *Cardiovasc Res*. 2004 Feb 15; 61(3):580–90. <https://doi.org/10.1016/j.cardiores.2003.09.016> PMID: 14962488.
48. Vanden Hoek TL, Becker LB, Shao Z, Li C, Schumacker PT. Reactive oxygen species released from mitochondria during brief hypoxia induce preconditioning in cardiomyocytes. *The Journal of biological chemistry*. 1998 Jul 17; 273(29):18092–8. PMID: 9660766.
49. Chen Q, Moghaddas S, Hoppel CL, Lesnefsky EJ. Ischemic defects in the electron transport chain increase the production of reactive oxygen species from isolated rat heart mitochondria. *Am J Physiol Cell Physiol*. 2008 Feb; 294(2):C460–6. <https://doi.org/10.1152/ajpcell.00211.2007> PMID: 18077608.
50. Galkin A, Abramov AY, Frakich N, Duchon MR, Moncada S. Lack of oxygen deactivates mitochondrial complex I: implications for ischemic injury? *The Journal of biological chemistry*. 2009 Dec 25; 284(52):36055–61. <https://doi.org/10.1074/jbc.M109.054346> PMID: 19861410. Pubmed Central PMCID: PMC2794721.
51. Mohsin AA, Chen Q, Quan N, Rousselle T, Maceyka MW, Samidurai A, et al. Mitochondrial Complex I Inhibition by Metformin Limits Reperfusion Injury. *J Pharmacol Exp Ther*. 2019 Mar 7. <https://doi.org/10.1124/jpet.118.254300> PMID: 30846619.
52. Wallace JL, Wang R. Hydrogen sulfide-based therapeutics: exploiting a unique but ubiquitous gaso-transmitter. *Nat Rev Drug Discov*. 2015 May; 14(5):329–45. <https://doi.org/10.1038/nrd4433> PMID: 25849904.
53. Wu D, Hu Q, Zhu Y. Therapeutic application of hydrogen sulfide donors: the potential and challenges. *Front Med*. 2016 Mar; 10(1):18–27. <https://doi.org/10.1007/s11684-015-0427-6> PMID: 26597301. Epub 2015/11/26. eng.
54. Dyson A, Dal-Pizzol F, Sabbatini G, Lach AB, Galfo F, Dos Santos Cardoso J, et al. Ammonium tetrathiomolybdate following ischemia/reperfusion injury: Chemistry, pharmacology, and impact of a new class of sulfide donor in preclinical injury models. *PLoS Med*. 2017 Jul; 14(7):e1002310. <https://doi.org/10.1371/journal.pmed.1002310> PMID: 28678794. Pubmed Central PMCID: PMC5497958.
55. Zhang L, Wang Y, Li Y, Li L, Xu S, Feng X, et al. Hydrogen Sulfide (H₂S)-Releasing Compounds: Therapeutic Potential in Cardiovascular Diseases. *Front Pharmacol*. 2018; 9:1066. <https://doi.org/10.3389/fphar.2018.01066> PMID: 30298008. Pubmed Central PMCID: PMC6160695.
56. Abou-Hamdan A, Guedouari-Bounihi H, Lenoir V, Andriamihaja M, Blachier F, Bouillaud F. Oxidation of H₂S in mammalian cells and mitochondria. *Methods Enzymol*. 2015; 554:201–28. <https://doi.org/10.1016/bs.mie.2014.11.042> PMID: 25725524.

57. Nandi S, Ravindran S, Kurian GA. Role of endogenous hydrogen sulfide in cardiac mitochondrial preservation during ischemia reperfusion injury. *Biomed Pharmacother*. 2018 Jan; 97:271–9. <https://doi.org/10.1016/j.biopha.2017.10.118> PMID: 29091875.
58. Hamada T, Nakane T, Kimura T, Arisawa K, Yoneda K, Yamamoto T, et al. Treatment of xerostomia with the bile secretion-stimulating drug anethole trithione: a clinical trial. *Am J Med Sci*. 1999 Sep; 318(3):146–51. PMID: 10487404.
59. Lam S, MacAulay C, Le Riche JC, Dyachkova Y, Coldman A, Guillaud M, et al. A randomized phase IIb trial of anethole dithiolethione in smokers with bronchial dysplasia. *J Natl Cancer Inst*. 2002 Jul 3; 94(13):1001–9. PMID: 12096085.
60. Wang H, Liu ZG, Peng J, Lin H, Zhong JX, Hu JY. [The clinical therapeutic efficiency of anethol trithione on dry eye]. *Zhonghua Yan Ke Za Zhi*. 2009 Jun; 45(6):492–7. PMID: 19957670.
61. Garlid KD, Puddu PE, Pasdois P, Costa AD, Beauvoit B, Criniti A, et al. Inhibition of cardiac contractility by 5-hydroxydecanoate and tetraphenylphosphonium ion: a possible role of mitoKATP in response to inotropic stress. *Am J Physiol Heart Circ Physiol*. 2006 Jul; 291(1):H152–60. <https://doi.org/10.1152/ajpheart.01233.2005> PMID: 16473956.