Modified metformin as a more potent anticancer drug: Mitochondrial inhibition, redox signaling, antiproliferative effects and future EPR studies

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Abstract

Metformin, one of the most widely prescribed antidiabetic drugs in the world is being repurposed as a potential drug in cancer treatment. Epidemiological studies suggest that metformin exerts anticancer effects in diabetic patients with pancreatic cancer. However, at typical antidiabetic doses the bioavailability of metformin is presumably too low to exert antitumor effects. Thus, more potent analogs of metformin are needed in order to increase its anticancer efficacy. To this end, a new class of mitochondria-targeted metformin analogs (or mito-metformins) containing a positively-charged lipophilic triphenylphosphonium group, was synthesized and tested for their antitumor efficacy in pancreatic cancer cells. Results indicate that the lead compound, mito-metformin$_{10}$, was nearly 1,000-fold more potent than metformin in inhibiting mitochondrial complex I activity, inducing reactive oxygen species (superoxide and hydrogen peroxide) that stimulate redox signaling mechanisms, including the activation of AMP kinase and inhibition of proliferation of pancreatic cancer cells. The potential use of the low-temperature EPR technique in assessing the role of mitochondrial complexes including complex I in tumor regression in response to metformin and mito-metformins in the *in vivo* setting is discussed.

Keywords

modified metformin; reactive oxygen species; redox signaling; mitochondrial metabolism; pancreatic cancer

Introduction

Metformin (Met, Figure 1) is one of the most prescribed antidiabetic drugs in the world.(1, 2) Metformin is a derivative of guanidine, an active component in the extracts from French lilac (*G. officinalis*), the plant that has been used for centuries to combat diabetes. A major advantage of metformin as a drug is that it is relatively safe and diabetic patients typically take daily several grams of the drug. Recently metformin is being repurposed as an antitumor drug due to epidemiological findings revealing an association between decreased incidence of pancreatic cancer and metformin use in diabetic individuals.(3, 4) However, at typical antidiabetic doses, the bioavailability of metformin and/or its uptake by cancer cells is probably too low to exert its antitumor effects in humans, although significant antitumor activity was reported in the rodent models, at comparable plasma concentrations.(5, 6) It appears that the antineoplastic activity of metformin in humans will require much higher concentrations of the drug in the serum than what is typically administered in diabetics treated with metformin.(5) Thus, it is important to develop new and improved metformin analogs with increased bioavailability, resulting in more potent antitumor effects, although these analogs may show increased toxicity for routine treatment of diabetes. We hypothesized that attaching the triphenylphosphonium cation (TPP$^+$) to metformin via an alkyl chain would greatly enhance its accumulation in mitochondria of cancer cells, leading to selective inhibition of proliferation of cancer cells.(7) Results obtained in this study substantiate the proposal that TPP$^+$-modified metformins are more cytostatic to pancreatic cancer cells.

Metformin does not undergo metabolism like many other drugs but it is excreted out unchanged in the urine.(8) However, it induces extensive changes in the metabolic pathways and mitochondrial energy metabolism.(9) Although multiple mechanisms were proposed for metformin anticancer effects, mitochondria still remain as the major target of metformin, leading to inhibition of cell respiration, AMPK activation, mTOR inhibition, and inhibition of cell proliferation.(10, 11)

Recent research implicates a role for mitochondrial complex I inhibition in the antitumor effects of metformin.(12) Interestingly, cancers with mutations in mitochondrial genes encoding proteins of the mitochondrial complex I of the electron transport chain are more susceptible to biguanides such as metformin.(13) Thus, cancers with oxidative phosphorylation deficits are likely to be more sensitive to biguanides than normal tissues and consequently, patients with complex I mutated cancer phenotype may be more sensitive to metformin treatment.(14) Tumor microenvironment was proposed to be another factor for increased metformin potency in some tumor phenotypes.(15) More recently, metformin was reported to activate T cell-mediated immune response against cancer cells.(15-17)

We have recently shown that attaching a positively-charged lipophilic triphenylphosphonium group to a nitroxide, quinone, or a phenolic group *via* an aliphatic linker chain vastly enhanced their ability to target mitochondria, and that such mitochondria-targeted compounds (e.g., Mito-CP, Mito-Q and Mito-Vitamin-E) significantly induced antiproliferative and/or cytotoxic effects in tumor cells without markedly affecting noncancerous cells.(18-23) Similarly, we hypothesized that attaching a positively-charged

lipophilic substituent will improve mitochondrial targeting of metformin (e.g., mito-metformins, Figure 1), thereby generating a new class of metformin analogs with enhanced antitumor potential. Results show that the mito-metformin analogs are much more potent (100 to 1,000-fold higher) than metformin in inhibiting human pancreatic adenocarcinoma cells (PDACs).(7)

Antiproliferative effects of metformin and mito-metformin$_{10}$

The effect of mito-metformin$_{10}$ and metformin on MiaPaCa-2 cells was investigated by monitoring colony formation and cell proliferation. As shown in Figure 2, in order to inhibit proliferation of pancreatic cancer cells, metformin was used at millimolar levels, whereas mito-metformin$_{10}$ was used at micromolar concentrations. The IC$_{50}$ values for inhibition of proliferation of human pancreatic cancer cells (MiaPaCa-2 and PANC-1) were more than 1,000-fold lower for Mito-Met$_{10}$ (0.2 μM), as compared to Met (0.6-0.9 mM). MiaPaCa-2 cells which were treated with 0.5 or 1 millimolar concentration of metformin, or 0.5 to 1 micromolar concentration of mito-metformin, showed the same effects in colony formation and cell proliferation experiments. (7) The IC$_{50}$ values for inhibiting 50% of colony formation for metformin and mito-metformin$_{10}$ were calculated to be 1.3 mM and 1.1 μM, respectively. Thus, mito-metformin$_{10}$ is *ca.* 1,000-fold more effective than metformin. The colony forming ability of the pancreatic cancers cells, while decreased by treatment with Mito-Met$_{10}$, could be rescued be cell pre-treatment with compound C, an inhibitor of AMPK protein (Figure 2B). This indicates that AMPK signaling pathway is involved in the antiproliferative effects of Mito-Met$_{10}$. Interestingly, Mito-Met$_{10}$ showed a good selectivity towards pancreatic cancer cells, as compared to non-tumorigenic cells (Figure 2A), implicating a wider margin of therapeutic index for its use *in vivo*.

Inhibition of pancreatic cancer cell respiration: complex I involvement

Metformin is weakly cationic, and weakly targets mitochondria. Metformin's antitumor effects were attributed in part to its ability to inhibit mitochondrial complex I-dependent cell respiration and ATP generation.(7, 12) In contrast to rotenone, a non-selective inhibitor of complex I in both normal and cancer cells, metformin reversibly and more selectively inhibits mitochondrial complex I in cancer cells.(12) As mito-metformin$_{10}$ selectively accumulates into mitochondria, we compared the relative inhibitory effects of metformin and mito-metformin$_{10}$ on complex I activity in MiaPaCa-2 cells. The real time monitoring of oxygen consumption rate (OCR) was used as a measure of mitochondrial function in intact cells. The mitochondrial respiration or OCR was monitored in MiaPaCa-2 cells treated with metformin and mito-metformin$_{10}$ using the Seahorse XF extracellular flux analyzer.(7) Figure 3 shows the OCR measurement of mitochondrial complex I activity of MiaPaCa-2 cells pretreated for 24 h with metformin or mito-metformin$_{10}$. The IC$_{50}$ values for inhibition of mitochondrial complex I for metformin and mito-metformin$_{10}$ were determined to be ca. 1.1 mM and 0.4 μM. These results show that the complex I inhibition may be mechanistically related to the antiproliferative effects of these compounds. Again, Mito-Met$_{10}$ exhibited selectivity towards cancer cells in inhibiting mitochondrial complex I (Figure 3).

Mito-metformin-mediated stimulation of reactive oxygen species and redox signaling

One of the consequences of inhibiting mitochondrial complex I is stimulation of one-electron reduction of oxygen to superoxide radical anion ($O_2^{\bullet-}$) and other oxidants (hydrogen peroxide and higher oxidation heme species) derived from superoxide.(7) As mito-metformin$_{10}$ treatment (at the concentration inhibiting proliferation) did not exert any cytotoxic effects, it is likely that reactive oxygen species (ROS) generated from mitochondria could be involved in redox signaling.(7) We used the cell-permeable probe, hydroethidine (HE), to detect superoxide by monitoring 2-OH-E$^+$, the specific marker product of the reaction between superoxide and HE.(24-26) A marked increase in 2-OH-E$^+$ formation was observed in mito-metformin$_{10}$-treated MiaPaCa-2 cells (Fig. 4). Evidence for other one-electron oxidation species formed from mito-metformin$_{10}$ interaction with mitochondrial proteins arose from studies demonstrating the formation of ethidium and a dimeric product of HE (E$^+$ and E$^+$-E$^+$, Fig. 4) in isolated mitochondria and upon incubation of HE with heme proteins.(27, 28) Under similar experimental conditions, mito-metformin$_{10}$ did not stimulate superoxide formation in noncancerous human pancreatic epithelial renin-expressing (HPNE) cells (Fig. 4).

We used the probe o-MitoPhB(OH)$_2$ to detect H$_2$O$_2$ generated in mito-metformin-treated cells.(29-31) Results show that mito-metformin$_{10}$ treatment of MiaPaCa-2 cells in the presence of o-MitoPhB(OH)$_2$ led to an increase in o-MitoPhOH formation (Figure 5). Although this product can be formed from either H$_2$O$_2$, peroxynitrite, or hypochlorous acid reaction with o-MitoPhB(OH)$_2$, the lack of detection of peroxynitrite- or hypochlorous acid-specific products in addition to o-MitoPhOH suggests that H$_2$O$_2$ was responsible for oxidation of o-MitoPhB(OH)$_2$ to MitoPhOH.(29-31)

Mito-metformin$_{10}$ activated adenosinemonophosphate (AMP)-activated protein kinase (AMPK) phosphorylation at a 1,000-fold lower concentration than metformin.(7) AMPK is a master regulator of cellular energy homeostasis and is activated *via* phosphorylation of its threonine-172 residue.(32) AMPK activation was linked to increased formation of hydrogen peroxide.(33, 34) Based on the results showing that mito-metformin$_{10}$ stimulated mitochondrial superoxide, hydrogen peroxide and AMPK activation, we proposed that mito-metformin$_{10}$-mediated antiproliferative effects are linked to inhibition of mitochondrial complex, enhanced ROS formation, and AMPK phosphorylation (Fig. 6). These observations have been recently confirmed by an independent study on antiproliferative effects of triphenylphosphonium-linked metformin derivatives against pancreatic cancer cells. (35)

Potential use of *ex vivo* low-temperature EPR in elucidating mitochondrial mechanism

Whereas the Seahorse technique can be used to measure the activities of mitochondrial complexes in cells, it is challenging to use this technique to measure mitochondrial activities in tissues. The low-temperature electron paramagnetic resonance (EPR) spectroscopy is

uniquely capable of providing a "snapshot" of mitochondrial status in intact tissues.(36) To our knowledge, the EPR technique has not been used to monitor changes in the redox status of mitochondrial iron-sulfur complexes in cancer cells. The effect of mito-metformin$_{10}$ and metformin on complex I activity in tumor tissues can be determined by performing EPR measurements at liquid helium temperatures. The signal at g=1.94 attributable to the reduced form of $[2Fe-2S]^+$ center of mitochondrial complex I is monitored.(36, 37) Inhibition of this signal is equated to inhibition of complex I. In a recent publication, Bennett *et al.* discussed the use of low-temperature EPR in mitochondrial diseases.(38)

ROS generated from inhibiting complex I will react with other iron sulfur-containing proteins (e.g., mitochondrial and cytosolic aconitases), and the levels of oxidatively-inactivated aconitases can be monitored at g=2.03 and 2.01 EPR signals due to the $[3Fe-4S]^+$ form of aconitase.(37, 39) Oxidatively-inactivated mitochondrial aconitase has a distinctly different EPR signal from oxidatively-inactivated cytosolic aconitase.(37, 39) Other signals from reduced and oxidized iron sulfur centers associated with downstream complexes I and II in the electron transport chain are also detectable using the low-temperature EPR. These experiments can be done in both preclinical and human tumor samples, and may provide additional mechanistic insights into response to therapy.

Conclusions

We have demonstrated that more potent analogs of metformin (a.k.a. mito-metformins) can be synthesized by attaching a mitochondria-targeting triphenylphosphonium group tethered to an alkyl side chain. Mito-metformin$_{10}$ was determined to be the most potent analog tested in inhibiting mitochondrial respiration, ROS activation, AMPK phosphorylation, and inhibition of proliferation in pancreatic cancer cells. We propose that AMPK activation induced by complex I-mediated ROS signaling is responsible for the antiproliferative effects. More recently, hydrogen peroxide was reported to induce redox signaling via a redox-relay involving peroxiredoxins and STAT3.(40) One of the major targets of mito-metformins is the mitochondrial complex I in the electron transport chain. The exact mechanism by which metformin analogs inhibit complex I is not yet ascertained. It has been reported that the occurrence of mitochondrial complex I A/D transition could regulate ROS production. (41)The D-form of the enzyme has been shown to generate more $O_2^{\bullet-}$ than the A-form, and irreversible locking of complex I in the D-form may contribute to more $O_2^{\bullet-}$ formation.(41) Establishing this mechanism in the *in vivo* preclinical pancreatic cancer xenografts may be accomplished using the low-temperature EPR technique. This *ex vivo* EPR technique may also be extrapolated to analysis of human pancreatic tumors.

Acknowledgments

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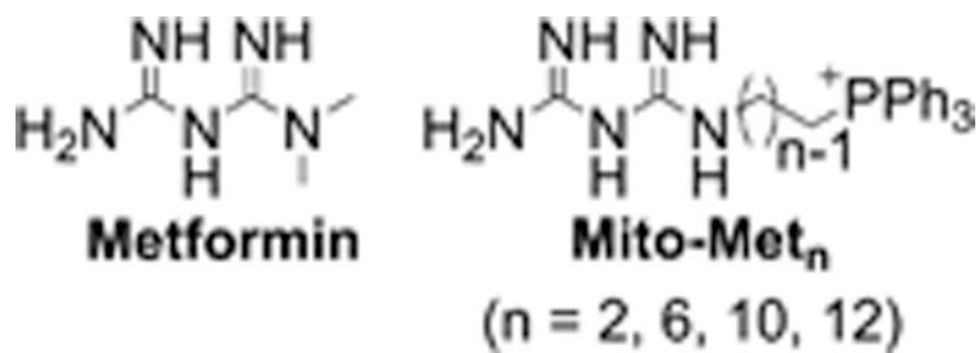
Figure 1.
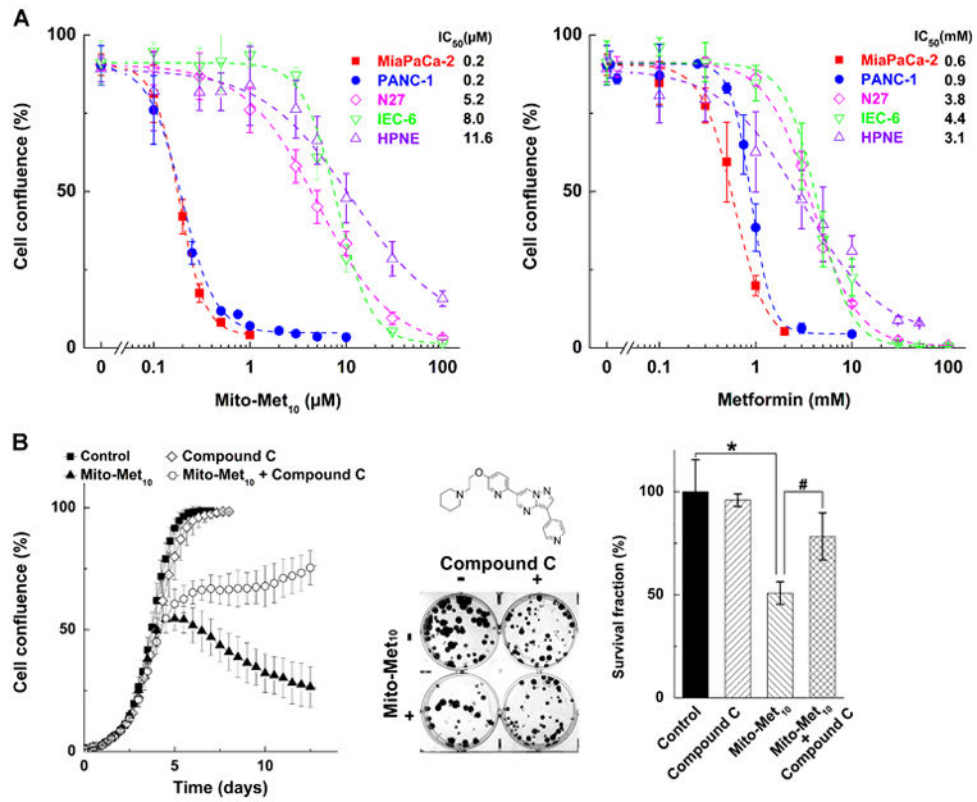Chemical structures of metformin and mito-metformin analogs.

Figure 2.
Effect of metformin and mito-metformin$_{10}$ on PDAC proliferation. (A) Titration of pancreatic cancer (MiaPaCa-2 and PANC-1) and non-tumorigenic (N27, IEC-6 and HPNE) cells with Mito-Met$_{10}$ (left panel) and Met (right panel). (B) Effect of Mito-Met$_{10}$ on colony formation in MiaPaCa-2 cells and the rescue effects of AMPK inhibitor, compound C.(7)
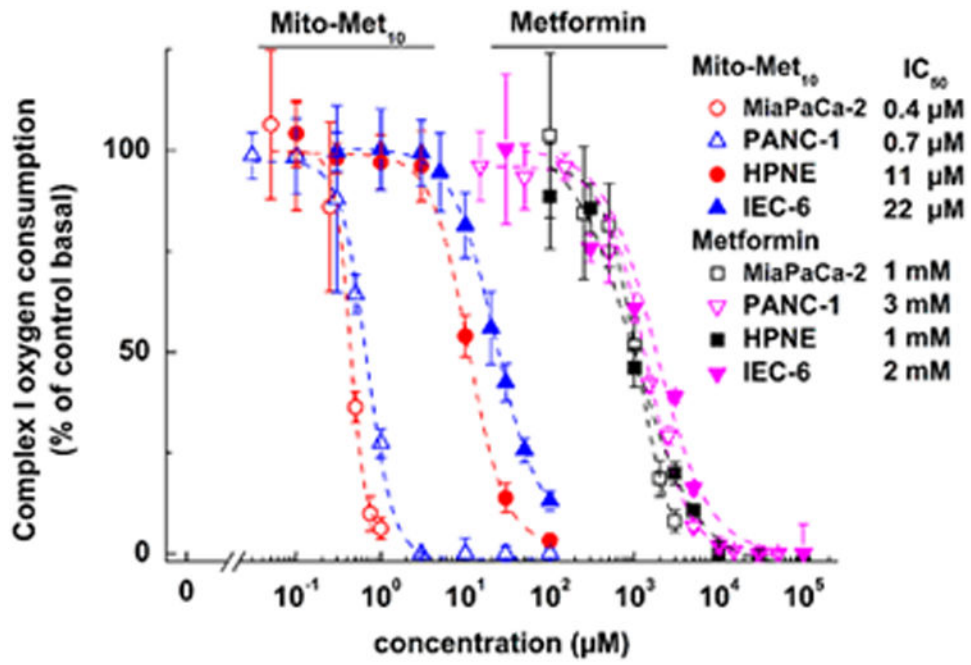
Figure 3.
Effects of metformin and mito-metformin$_{10}$ on mitochondrial complex I activity in
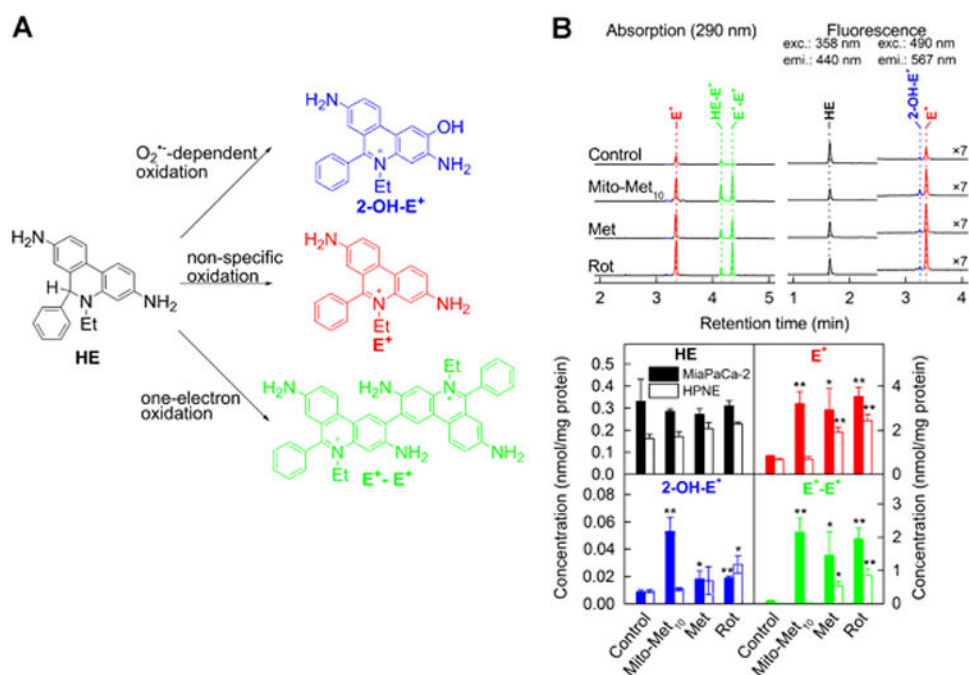pancreatic cancer (MiaPaCa-2 and PANC-1) and non-tumorigenic (HPNE and IEC-6) cells.
(7)

Figure 4.
Intracellular superoxide stimulation by mito-metformin$_{10}$. (A) Chemical scheme of superoxide-dependent and independent pathways of oxidation of hydroethidine (HE) probe. (B) Effect of Met (1 mM, 24 h), Mito-Met$_{10}$ (1 μM, 24 h) or rotenone (ROT, 1 μM, 1 h) on HE oxidation in MiaPaCa-2 pancreatic cancer cells. Upper panel shows the HPLC chromatograms and lower panels shows the quantitative analysis of intracellular levels of HE and its oxidation products.(7)
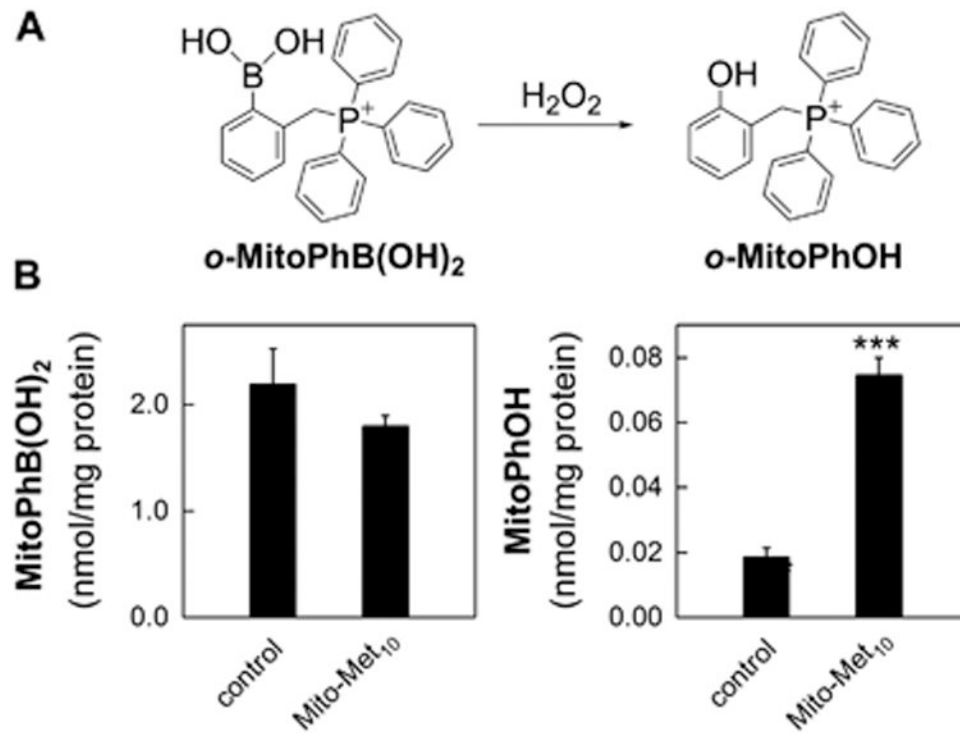
Figure 5.
Hydrogen peroxide formation induced by mito-metformin$_{10}$ in PDACs. (A) Chemical scheme of H_2O_2-dependent oxidation of the o-MitoPhB(OH)$_2$ probe. (B) Intracellular levels of o-MitoPhB(OH)$_2$ (left panel) and o-MitoPhOH (right panel) in MiaPaCa-2 pancreatic cancer cells treated for 24 h with Mito-Met$_{10}$.
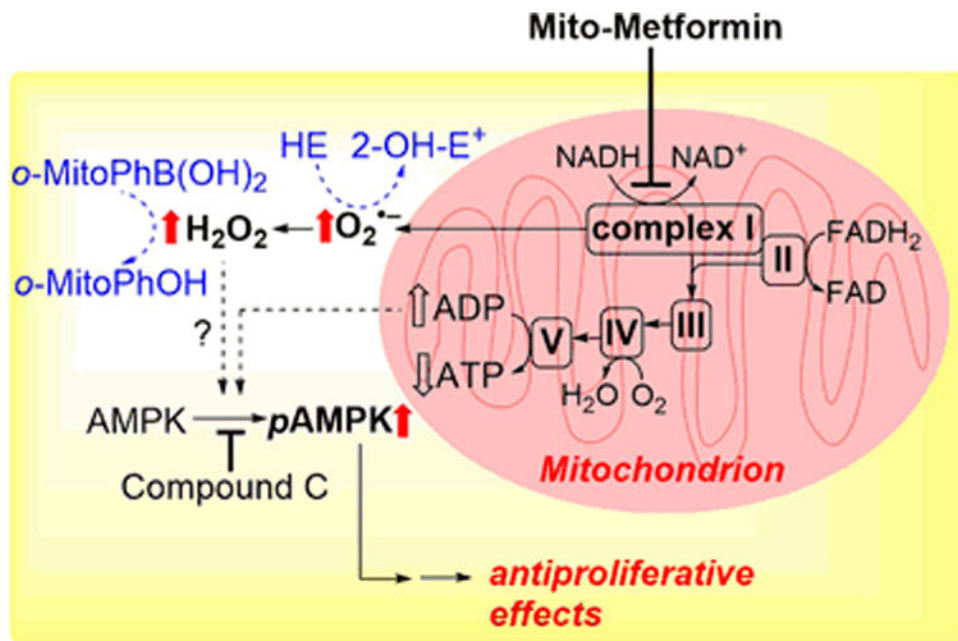
Figure 6.
Proposed signaling pathway induced by mito-metformin$_{10}$ in PDACs. Block arrows indicate the changes observed upon cell treatment with Mito-Met$_{10}$.