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Mitochondria-targeted metformins: anti-tumour and redox signalling mechanisms

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Reports suggest that metformin exerts anti-cancer effects in diabetic individuals with pancreatic cancer. Thus, metformin is currently being repurposed as a potential drug in cancer treatment. Studies indicate that potent metformin analogues are required in cancer treatment because of the low bioavailability of metformin in humans at conventional antidiabetic doses. We proposed that improved mitochondrial targeting of metformin by attaching a positively charged lipophilic triphenylphosphonium group will result in a new class of mitochondria-targeted metformin analogues with significantly enhanced anti-tumour potential. Using this approach, we synthesized various mitochondria-targeted metformin analogues with different alkyl chain lengths. Results indicate that the antiproliferative effects increased with increasing alkyl chain lengths (100-fold to 1000-fold). The lead compound, mito-metformin$_{10}$, potently inhibited mitochondrial respiration through inhibition of complex I, stimulation of superoxide and hydrogen peroxide formation and activation of AMPK. When used in combination with ionizing radiation, mito-metformin$_{10}$ acted as a radiosensitizer of pancreatic cancer cells. Because of the 1000-fold-higher potency of mitochondria-targeted metformin$_{10}$, therapeutically effective plasma concentrations likely can be achieved in cancer patients.

1. Repurposing metformin in cancer treatment: an old drug with a new potential

Approved for antidiabetic treatment in 1995, metformin has become the most prescribed antidiabetic drug in the world [1]. Metformin is widely used by patients with type 2 diabetes mellitus, who take several grams of it daily to decrease blood glucose levels. Metformin is relatively safe, with minimal side effects. Metformin causes minimal lactic acidosis, a side effect of phenformin—a related pharmaceutical, and accumulates only in normal tissues expressing organic cation transporters. Metformin is not metabolized—it enters the body and is excreted out unchanged in the urine [2]. However, its efficacy is attributed to the many metabolic pathways it induces or alters in the body, as reviewed elsewhere [3–6]. It is assumed that mitochondria are the major target of metformin, leading to inhibition of mitochondrial respiration, AMPK activation and bioenergetic reprogramming [6–8].

Epidemiological studies have recently shown an association between metformin use and decreased incidence of cancer [9–11]. In particular, diabetic patients taking metformin show a decreased incidence of pancreatic cancer, stimulating a flurry of research activity on the potential anti-tumour effects of metformin. Meta-analyses of the results of epidemiological studies indicated that patients taking metformin display approximately 30% reduced overall cancer incidence as well as cancer mortality [10]. However, after adjusting for body/mass index and time-related biases, the chemoprotective effects of

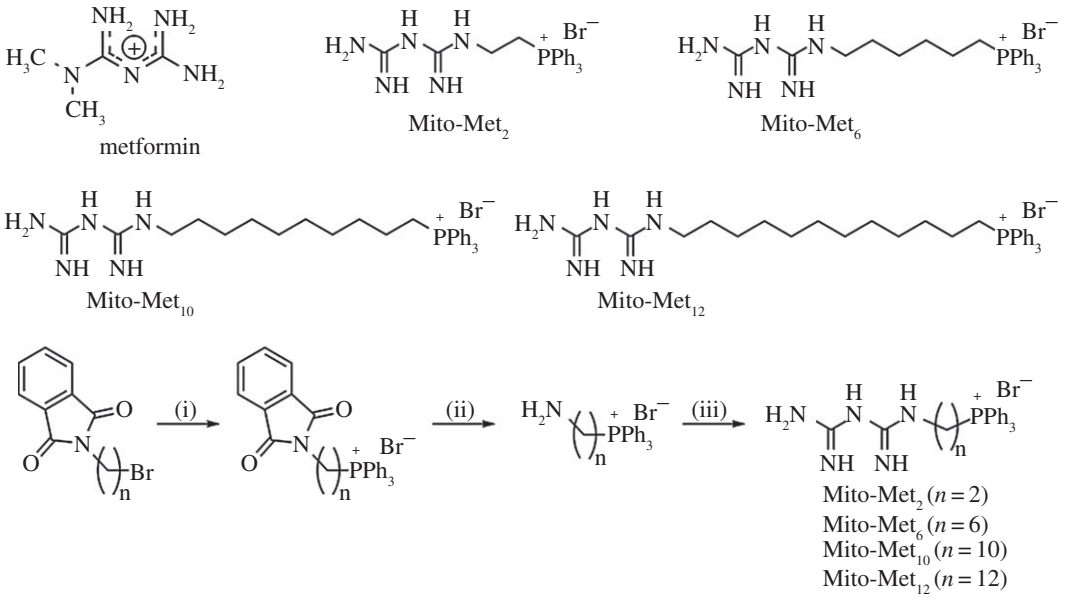

Figure 1. Chemical structures of metformin and its mitochondria-targeted analogues and the synthetic pathway for the mito-metformins. Adapted from [23].

metformin lost their statistical significance, suggesting that the actual effect is smaller than previously reported [10,11]. Other lipophilic analogues of metformin, such as phenformin, have increased bioavailability and exhibit more potent anti-tumour effects [12]. However, phenformin was discontinued more than 40 years ago in the USA because of enhanced incidence of acidosis (10- to 20-fold more in patients with renal dysfunction) [13,14].

A recent report indicates that cancers with mutations in mitochondrial genes encoding proteins of complex I of the mitochondrial electron transport chain are more susceptible to biguanides such as metformin [15]. Thus, pharmacological inhibition of mitochondrial energy metabolism compromised by mutations in complex I may further exacerbate mitochondrial energy deficits in cancer cells treated with metformin and analogues [15]. It was proposed that patients whose cancer harbours complex I mutations will be more sensitive to metformin. Additionally, it has been demonstrated that the availability of bioenergetic substrates may determine the sensitivity of cancer cells to metformin, implicating the tumour microenvironment as an additional factor to consider [15,16].

2. Mitochondria-targeted cations inhibit tumour proliferation

We and others have shown that mitochondria-targeted cationic compounds induce antiproliferative and/or cytotoxic effects in tumour cells without markedly affecting non-cancerous cells [17–19]. The selectivity has been attributed to an enhanced uptake of delocalized lipophilic cations by cancer cells owing to increased mitochondrial membrane potential [20]. A triphenylphosphonium cationic moiety (TPP^+) tethered to a nitroxide, quinone or a chromanol group via an aliphatic linker side chain formed a new class of mitochondria-targeted cations that potently decreased the proliferation of various cancer cells [17–19]. Selective cytostatic and cytotoxic effects of TPP^+-containing compounds in tumour cells when compared with normal cells could be attributed to enhanced uptake and retention in tumour mitochondria [18,21,22]. We hypothesized that attaching a positively charged lipophilic substituent will improve the mitochondrial targeting of metformin (i.e. mito-metformin), thus leading to a new class of compounds with improved anti-tumour potential.

3. Mitochondria-targeted metformins, inhibition of pancreatic ductal adenocarcinoma cell proliferation and cellular uptake

The base metformin is hydrophilic and weakly cationic (figure 1). We synthesized and characterized several metformin analogues (e.g. Mito-Met_2, Mito-Met_6, Mito-Met_{10} and Mito-Met_{12}) conjugated to a TPP^+ moiety via an alkyl linker chain (figure 1). We compared the relative antiproliferative potencies of mito-metformin homologues, phenformin and metformin in normal and pancreatic cancer cells. As shown in figure 2, Mito-Met_{10} more potently inhibited the colony formation of pancreatic ductal adenocarcinoma cells (PDACs) when compared with metformin. Furthermore, Mito-Met analogues are much more potent than metformin in inhibiting human pancreatic carcinoma (MiaPaCa-2) cell proliferation. These findings are consistent with their relative potencies to inhibit complex I-mediated cell respiration [23]. The cellular uptake of Mito-Met analogues increased as a function of increasing the carbon–carbon side chain length, and the most potent analogue, Mito-Met_{10}, was 100-fold more potent than phenformin [23].

4. Mitochondrial complex I inhibition

Recent studies have shown that inhibiting mitochondrial complex I in cancer cells causes a decrease in cell proliferation [5,16,23]. Metformin's inhibitory effects on tumourigenesis and cancer progression were attributed, in part, to its ability to inhibit mitochondrial complex I [5,6,16,24]. Reports also suggest that metformin-mediated inhibition of mitochondrial complex I is reversible, whereas rotenone, a classical complex I inhibitor, is an irreversible inhibitor [7]. Rotenone is relatively non-tissue selective and is highly toxic to both cancer

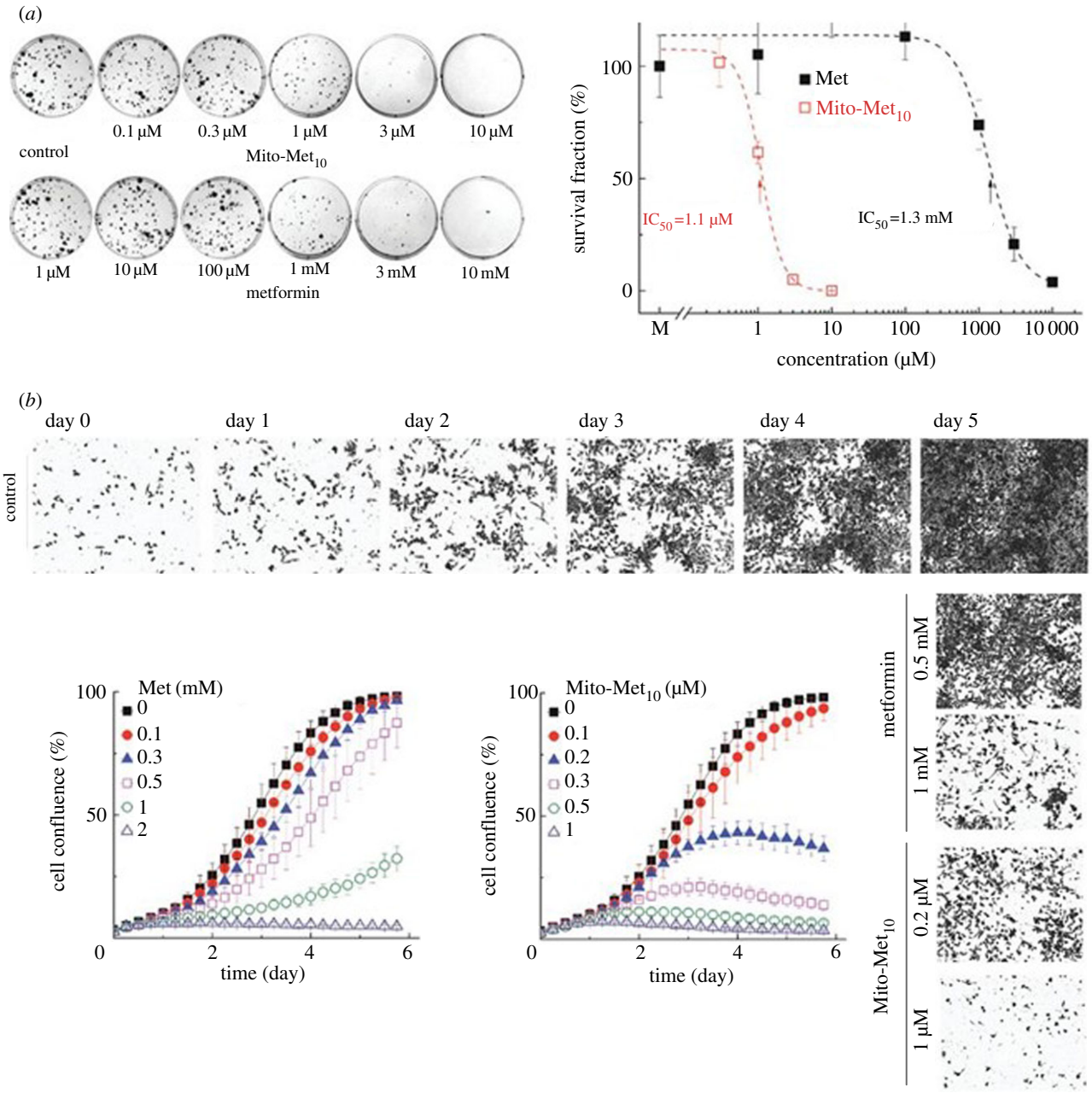

Figure 2. Effects of Mito-Met_{10} and metformin on MiaPaCa-2 (*a*) cell colony formation and (*b*) cell proliferation. Adapted from [23]. (Online version in colour.)

cells and non-cancerous cells. Thus, not all agents inhibiting mitochondrial complex I are equally cytotoxic. Furthermore, rotenone uptake into mitochondria is not dependent on mitochondrial membrane potential, limiting its effectiveness as an anti-cancer therapy.

The oxygen consumption rate (OCR) in intact cells was measured as a readout of mitochondrial function (figure 3), and the complex I activity was determined by monitoring oxygen consumption in permeabilized cells in the presence of complex I substrates, using the Seahorse XF extracellular flux analyser (Agilent Technologies, Santa Clara, CA) [25]. Mitochondrial respiration was monitored in MiaPaCa-2 cells treated with varying concentrations of metformin and Mito-Met with different side chain lengths. Results indicate that the extent of OCR inhibition was dependent on the alkyl chain (linker) length with the mitochondrial inhibitory efficacy increasing in the order: metformin < Mito-Met_2 < Mito-Met_6 < Mito-Met_{10} (figure 3). The IC_{50} value for inhibition of mitochondrial complex I determined for metformin was 1.1 mM when compared with 0.4 μM observed for Mito-Met_{10} [23]. Interestingly, the complex I inhibitory activity of Mito-Met_{10} is much stronger in pancreatic cancer cells (MiaPaCa-2 and PANC-1, $IC_{50} < 1$ μM) than in non-tumourigenic cells (HPNE, IEC-6, $IC_{50} > 10$ μM), possibly owing to differential uptake of the compound [23].

5. Enhanced formation of superoxide and hydrogen peroxide induced by mito-metformin

One of the consequences of complex I inhibition is enhanced cellular generation of redox species (superoxide radical anion, hydrogen peroxide (H_2O_2) and haem-derived oxidants). To the best of our knowledge, mitochondria-derived superoxide has never been unambiguously determined in cancer cells. This is due, in part, to artefacts associated with probes typically used for cellular superoxide and the lack of determination of specific products formed from reactions of probes

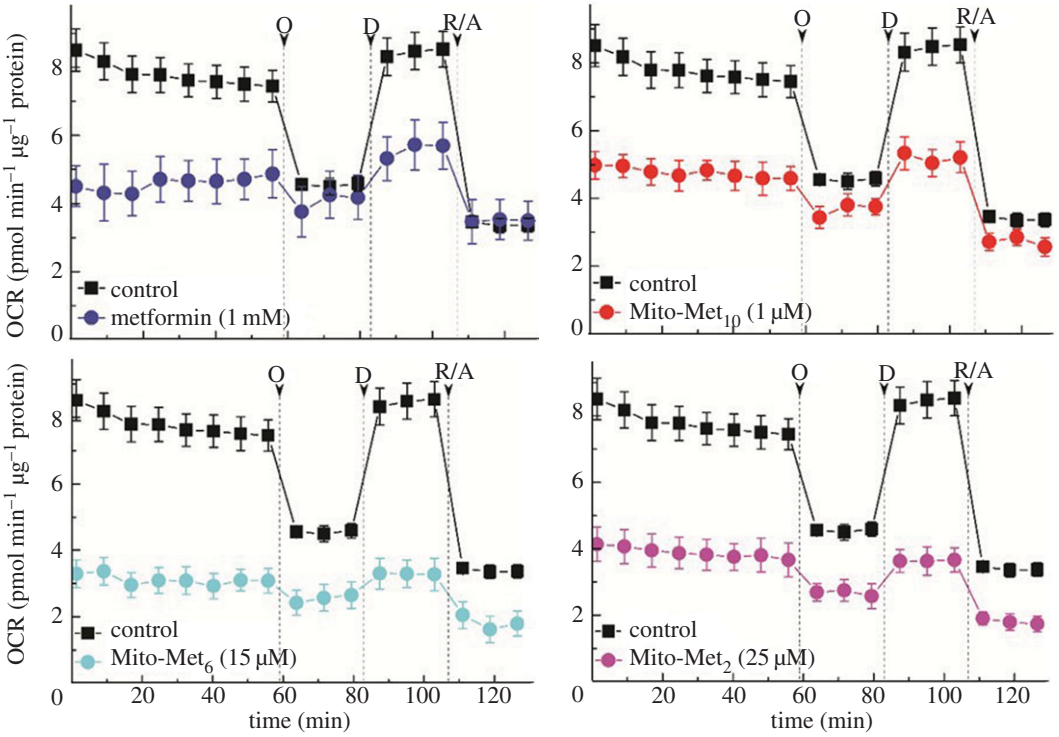

Figure 3. Effect of metformin and its mitochondria-targeted analogues on mitochondrial respiration in intact MiaPaCa-2 cells, as measured by real-time monitoring of the oxygen consumption rates in cell pretreated for 24 h with the compounds. O, oligomycin; D, 2,4-dinitrophenol; R/A, rotenone + antimycin A. Adapted from [23]. (Online version in colour.)

with superoxide. This problem has been overcome because of the intense research efforts devoted to understanding the reaction mechanisms and kinetics of fluorescent probes and reactive oxygen species (ROS) [26–28]. We used the cell-permeable probe, hydroethidine (HE), to detect superoxide. As shown in figure 4, Mito-Met$_{10}$ treatment of MiaPaCa-2 cells increased the formation of 2-hydroxyethidium (2-OH-E$^+$), a specific marker product of the HE/superoxide reaction. In addition, a marked increase in ethidium and diethidium was observed (figure 4*b*), suggesting that the Mito-Met$_{10}$ interaction with mitochondrial proteins induces generation of one-electron oxidant(s). Under similar conditions, Mito-Met$_{10}$ did not stimulate $O_2^{\bullet -}$ (superoxide) formation in control human pancreatic epithelial nestin-expressing (HPNE) cells, used as a normal cell control for MiaPaCa-2 cells. This result is consistent with the lack of inhibition of mitochondrial complex I in HPNE cells under these conditions [23]. However, at higher concentrations, Mito-Met$_{10}$ treatment led to inhibition of mitochondrial function and increased formation of 2-OH-E$^+$ in HPNE cells [23].

To detect H_2O_2 generated in Mito-Met$_{10}$ treated cells, we used the probe *o*-MitoPhB(OH)$_2$ [26,29–31]. The reason for using *o*-MitoPhB(OH)$_2$ instead of *m*-MitoPhB(OH)$_2$ (known also as the MitoB probe [29]) is that, whereas both probes react stoichiometrically with H_2O_2 to form the respective phenolic product (Mito-PhOH), only the *ortho*-substituted probe will react with peroxynitrite (ONOO$^-$) to form ONOO$^-$-specific cyclo-*o*-MitoPh [26] and *o*-MitoPhNO$_2$ [30,31] products (figure 5). Failure to detect those peroxynitrite-specific products means that ONOO$^-$ was not the active oxidant responsible for oxidation of *o*-MitoPhB(OH)$_2$ to *o*-MitoPhOH. Results showed that Mito-Met$_{10}$ treatment of MiaPaCa-2 cells in the presence of *o*-MitoPhB(OH)$_2$ leads to enhanced formation of *o*-MitoPhOH, indicative of H_2O_2, ONOO$^-$ or hypochlorous acid (HOCl) formation. However, the lack of formation of peroxynitrite- or hypochlorous-specific products accompanying *o*-MitoPhB(OH)$_2$ oxidation [26] suggests that neither ONOO$^-$ nor HOCl is responsible for oxidation of *o*-MitoPhB(OH)$_2$ to *o*-MitoPhOH (not shown).

6. Proposed signalling pathway

We showed that Mito-Met$_{10}$ activated adenosine monophosphate (AMP)-activated protein kinase (AMPK) phosphorylation at a 1000-fold lower concentration than metformin (not shown) [23]. AMPK, a master regulator of cellular energy homeostasis, is typically activated by intracellular AMP. Under conditions wherein intracellular adenosine triphosphate (ATP) levels decrease along with an increase in AMP (enhanced AMP/ATP ratio), AMPK is activated via phosphorylation of its threonine-172 residue [32]. We proposed that Mito-Met$_{10}$ likely exerts antiproliferative effects in PDACs via targeting the energy-sensing bioenergetics pathway (figure 6). H_2O_2, generated in mitochondria through dismutation of superoxide formed from complex I inhibition by Mito-Met$_{10}$, likely leads to AMPK phosphorylation [33,34], resulting in antiproliferative effects in cancer cells. It is conceivable that Mito-Met$_{10}$-induced increases in both intracellular AMP and H_2O_2 contribute to AMPK activation, leading to inhibition of cell proliferation (figure 6).

7. Metformin, mito-metformin and radiosensitization

Previously, mitochondria-targeted nitroxides have been shown to enhance radiosensitivity of neuroblastoma cells [35]. Metformin also increased the radiosensitivity of pancreatic cancer cells and inhibited tumour growth [36,37].

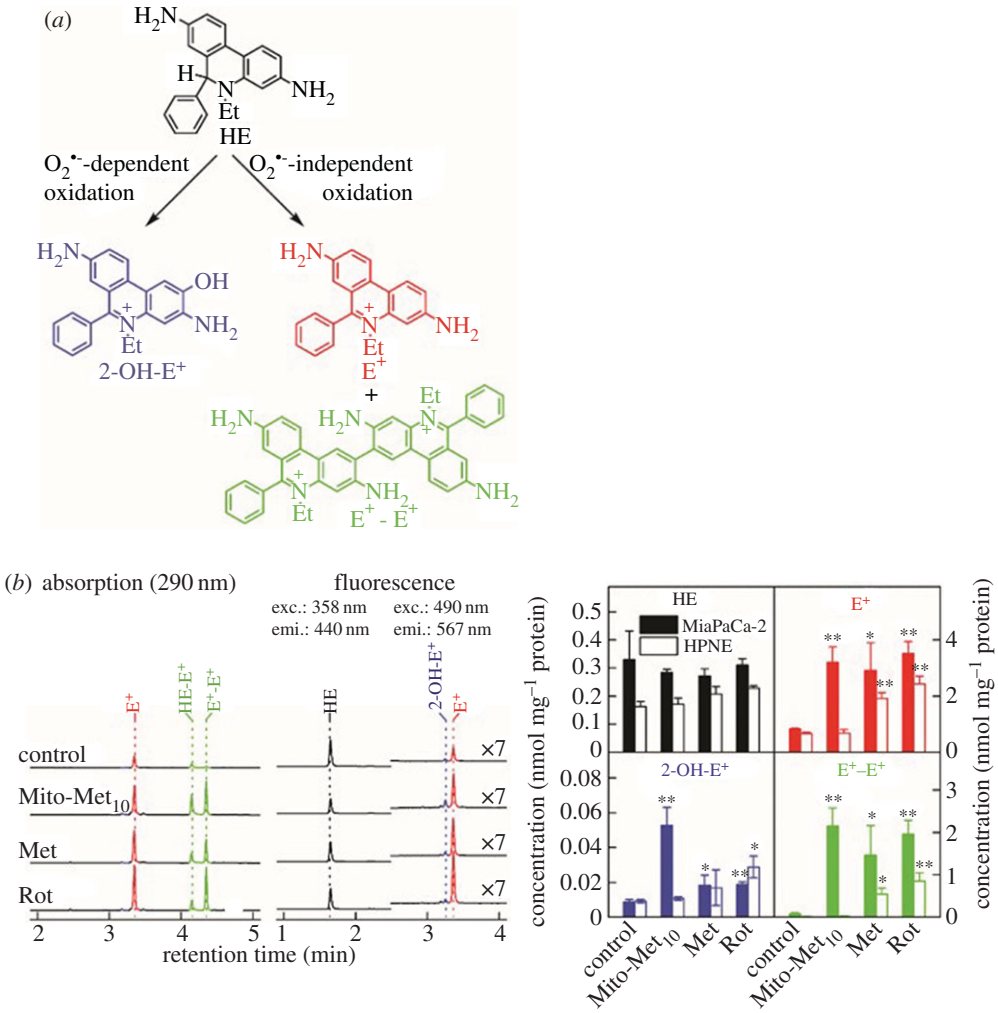

Figure 4. (*a*) Chemical structures of the specific oxidation products formed from hydroethidine (HE) probe; (*b*) intracellular oxidant formation induced by Mito-Met$_{10}$ (1 µM, 24 h), metformin (1 mM, 24 h) and rotenone (1 µM, 1 h), as measured by profiling the HE oxidation products upon 1 h incubation with the HE probe (10 µM). Adapted from [23]. (Online version in colour.)

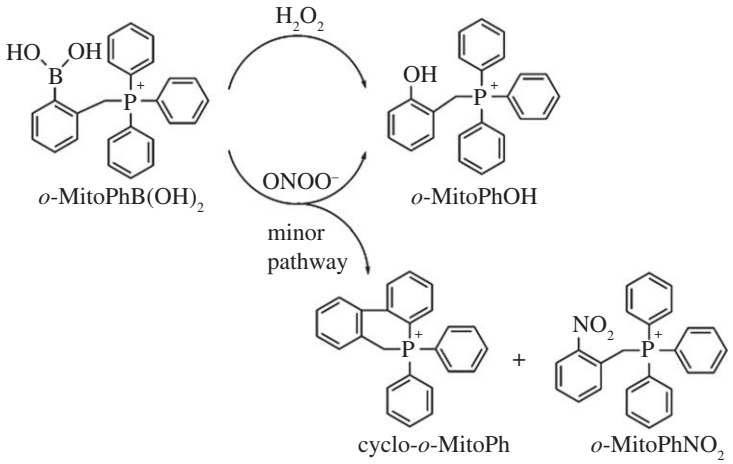

Figure 5. Chemical structures of products formed from hydrogen peroxide (H_2O_2) and peroxynitrite ($ONOO^-$)-induced oxidation of *o*-MitoPhB(OH)$_2$ probe.

The enhanced radiosensitivity of metformin was attributed to activation of the AMPK pathway and/or improved tumour oxygenation owing to inhibition of mitochondrial complex I and tumour cell respiration in irradiated tumours [36,37]. Studies suggest that decreasing oxygen consumption with pharmacological drugs is an effective route for increasing tumour oxygenation and radiosensitivity [38–40]. As presented previously (figure 3), Mito-Met$_{10}$ inhibited mitochondrial complex I activity and pancreatic cancer cell respiration at micromolar levels, a 1000-fold lower than metformin. Mito-Met$_{10}$ induced AMPK activation at a 1000-fold lower concentration when compared with metformin. As shown previously [23], Mito-Met$_{10}$ enhanced radiation sensitivity in PDAC at a 1000-fold lower concentration than metformin. This is a very significant finding that could have high clinical relevance as relatively non-toxic

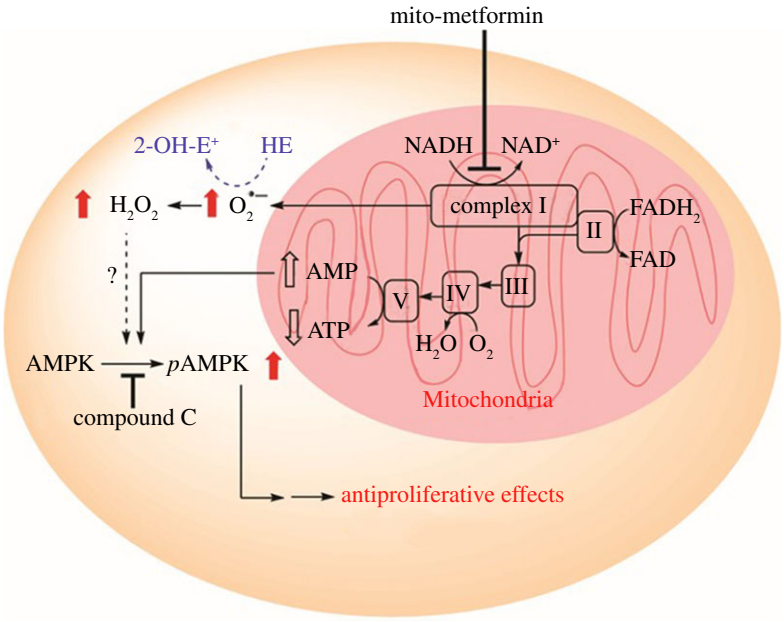

Figure 6. Proposed signalling pathway activated by metformin analogues. Adapted from [23]. (Online version in colour.)

mitochondria-targeted metformin analogues could be used in combination with radiotherapy in treating pancreatic cancer. Mito-Met$_{10}$ also decreased the growth of the pancreatic tumour in the mouse xenograft model *in vivo*, without any sign of toxicity under those conditions [23]. It is conceivable that administration of Mito-Met$_{10}$ exhibiting a 1000-fold-higher potency, when compared with metformin, would result in a therapeutically effective plasma concentration in cancer patients.

8. Conclusion

This study shows how mitochondrial targeting of metformin enhances its antiproliferative effects in pancreatic cancer cells. The lead compound, Mito-Met$_{10}$, potently inhibited mitochondrial respiration through inhibition of complex I activity, resulting in ROS-mediated stimulation of AMPK. Mito-Met$_{10}$ acts as a radiosensitizer in pancreatic cancer cells.

Authors' contributions. All authors prepared the manuscript and gave final approval for publication.

Competing interests. The authors have no competing interests.

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