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ORIGINAL PAPER

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 1000-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 adenosinemonophosphate kinase and inhibition of proliferation of pancreatic cancer cells. The potential use of the low-temperature electron paramagnetic resonance 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

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Introduction

Metformin (Met, Fig. 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

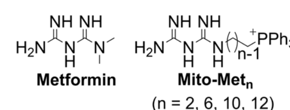

Fig. 1 Chemical structures of metformin and mito-metformin analogs

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, adenosine monophosphate (AMP)-activated protein kinase (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, Fig. 1), thereby generating a new class of metformin analogs with enhanced antitumor potential. Results show that the mito-metformin analogs are much more potent (100–1000-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 Fig. 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 1000-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 mM concentration of metformin, or 0.5–1 μM 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*. 1000-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 (Fig. 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 (Fig. 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

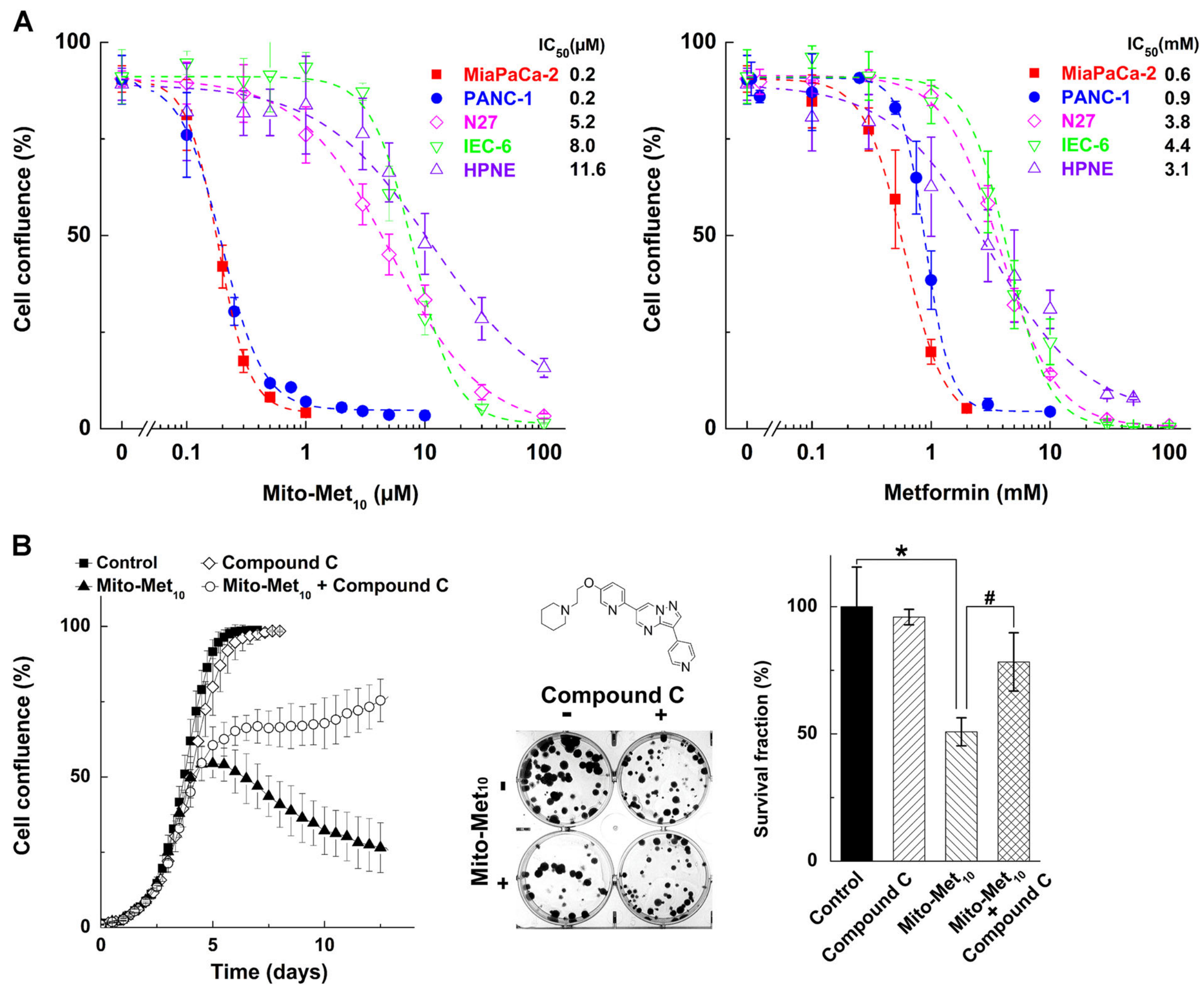

Fig. 2 Effect of metformin and mito-metformin$_{10}$ on PDAC proliferation. **a** Titration of pancreatic cancer (MiaPaCa-2 and PANC-1) and non-tumorigenic (N27, IEC-6 and HPNE) cells with Mito-Met$_{10}$ (*left*) and Met (*right*). **b** Effect of Mito-Met$_{10}$ on colony formation in MiaPaCa-2 cells and the rescue effects of AMPK inhibitor, compound C [7]

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 (Fig. 3).

Mito-Metformin-Mediated Stimulation of Reactive Oxygen Species (ROS) 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 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 non-cancerous human pancreatic epithelial renin-expressing (HPNE) cells (Fig. 4).

We used the probe *o*-MitoPhB$(OH)_2$ to detect H_2O_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 (Fig. 5). Although this product can be formed from either H_2O_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_2O_2 was responsible for oxidation of *o*-MitoPhB$(OH)_2$ to MitoPhOH [29–31].

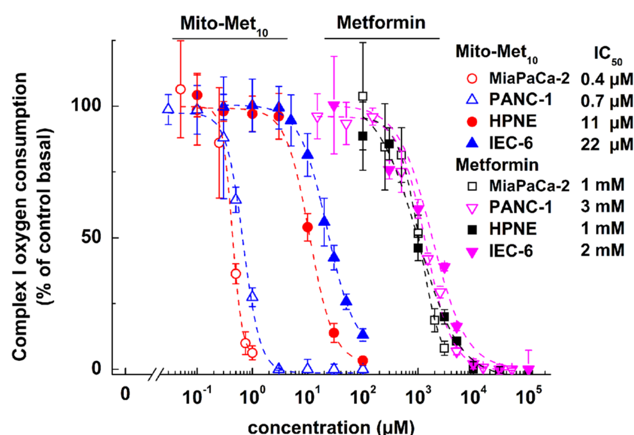

Fig. 3 Effects of metformin and mito-metformin$_{10}$ on mitochondrial complex I activity in pancreatic cancer (MiaPaCa-2 and PANC-1) and non-tumorigenic (HPNE and IEC-6) cells [7]

Mito-metformin$_{10}$ activated AMPK phosphorylation at a 1000-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

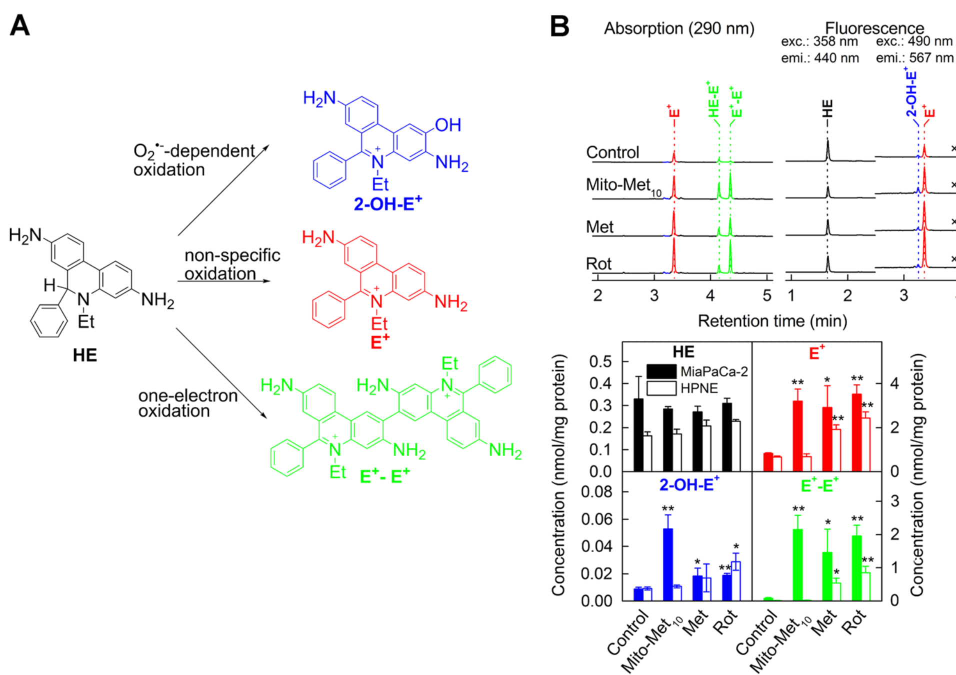

Fig. 4 Intracellular superoxide stimulation by mito-metformin$_{10}$. **a** Chemical scheme of superoxide-dependent and independent pathways of oxidation of hydroethidine (HE) probe. **b** Effect of Met (1 mM, 24 h), Mito-Met$_{10}$ (1 μM, 24 h) or rotenone (ROT, 1 μM, 1 h) on HE oxidation in MiaPaCa-2 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]

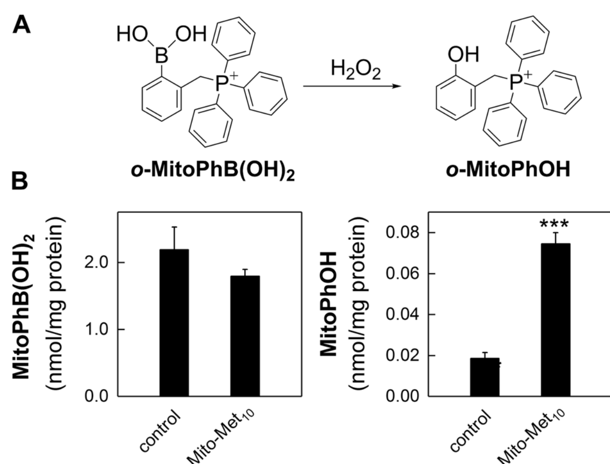

Fig. 5 Hydrogen peroxide formation induced by mito-metformin$_{10}$ in PDACs. **a** Chemical scheme of H_2O_2-dependent oxidation of the *o*-MitoPhB(OH)$_2$ probe. **b** Intracellular levels of *o*-MitoPhB(OH)$_2$ (*left panel*) and *o*-MitoPhOH (*right panel*) in MiaPaCa-2 pancreatic cancer cells treated for 24 h with Mito-Met$_{10}$

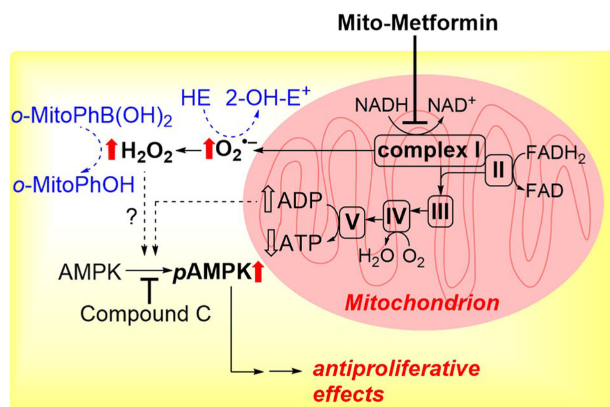

Fig. 6 Proposed signaling pathway induced by mito-metformin$_{10}$ in PDACs. *Block arrows* indicate the changes observed upon cell treatment with Mito-Met$_{10}$

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 $[2Fe\text{-}2S]^+$ 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 $[3Fe\text{-}4S]^+$ 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.

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Compliance with Ethical Standards

Conflict of Interest The authors declare that they have no competing interests.

References

1. Bailey, C., & Day, C. (2004). Metformin: Its botanical background. *Practical Diabetes International*, *21*, 115–117.

2. Thomas, I., & Gregg, B. (2017). Metformin; A review of its history and future: From lilac to longevity. *Pediatric Diabetes*, *18*, 10–16.
3. Evans, J. M., Donnelly, L. A., Emslie-Smith, A. M., Alessi, D. R., & Morris, A. D. (2005). Metformin and reduced risk of cancer in diabetic patients. *BMJ (Clinical Research Ed.)*, *330*, 1304–1305.
4. Heckman-Stoddard, B. M., Gandini, S., Puntoni, M., Dunn, B. K., Decensi, A., & Szabo, E. (2016). Repurposing old drugs to chemoprevention: The case of metformin. *Seminars in Oncology*, *43*, 123–133.
5. Kordes, S., Pollak, M. N., Zwinderman, A. H., Mathot, R. A., Weterman, M. J., Beeker, A., Punt, C. J., Richel, D. J., & Wilmink, J. W. (2015). Metformin in patients with advanced pancreatic cancer: A double-blind, randomised, placebo-controlled phase 2 trial. *The Lancet Oncology*, *16*, 839–847.
6. Chandel, N. S., Avizonis, D., Reczek, C. R., Weinberg, S. E., Menz, S., Neuhaus, R., Christian, S., Haegebarth, A., Algire, C., & Pollak, M. (2016). Are metformin doses used in murine cancer models clinically relevant? *Cell Metabolism*, *23*, 569–570.
7. Cheng, G., Zielonka, J., Ouari, O., Lopez, M., McAllister, D., Boyle, K., Barrios, C. S., Weber, J. J., Johnson, B. D., Hardy, M., Dwinell, M. B., & Kalyanaraman, B. (2016). Mitochondria-targeted analogues of metformin exhibit enhanced anti-proliferative and radiosensitizing effects in pancreatic cancer cells. *Cancer Research*, *76*, 3904–3915.
8. Graham, G. G., Punt, J., Arora, M., Day, R. O., Doogue, M. P., Duong, J. K., Furlong, T. J., Greenfield, J. R., Greenup, L. C., Kirkpatrick, C. M., Ray, J. E., Timmins, P., & Williams, K. M. (2011). Clinical pharmacokinetics of metformin. *Clinical Pharmacokinetics*, *50*, 81–98.
9. Foretz, M., Guigas, B., Bertrand, L., Pollak, M., & Viollet, B. (2014). Metformin: From mechanisms of action to therapies. *Cell Metabolism*, *20*, 953–966.
10. Bridges, H., Jones, A., Pollak, M., & Hirst, J. (2014). Effects of metformin and other biguanides on oxidative phosphorylation in mitochondria. *The Biochemical Journal*, *462*, 475–487.
11. Liu, X., Romero, I. L., Litchfield, L. M., Lengyel, E., & Locasale, J. W. (2016). Metformin targets central carbon metabolism and reveals mitochondrial requirements in human cancers. *Cell Metabolism*, *24*, 728–739.
12. Wheaton, W. W., Weinberg, S. E., Hamanaka, R. B., Soberanes, S., Sullivan, L. B., Anso, E., Glasauer, A., Dufour, E., Mutlu, G. M., Budigner, G. S., & Chandel, N. S. (2014). Metformin inhibits mitochondrial complex I of cancer cells to reduce tumorigenesis. *eLife*, *3*, e02242.
13. Birsoy, K., Possemato, R., Lorbeer, F. K., Bayraktar, E. C., Thiru, P., Yucel, B., Wang, T., Chen, W. W., Clish, C. B., & Sabatini, D. M. (2014). Metabolic determinants of cancer cell sensitivity to glucose limitation and biguanides. *Nature*, *508*, 108–112.
14. Pollak, M. (2014). Overcoming drug development bottlenecks with repurposing: Repurposing biguanides to target energy metabolism for cancer treatment. *Nature Medicine*, *20*, 591–593.
15. Eikawa, S., Nishida, M., Mizukami, S., Yamazaki, C., Nakayama, E., & Udono, H. (2015). Immune-mediated antitumor effect by type 2 diabetes drug, metformin. *Proceedings of the National Academy of Sciences*, *112*, 1809–1814.
16. Webb, T. J., Carey, G. B., East, J. E., Sun, W., Bollino, D. R., Kimball, A. S., Brutkiewicz, R. R., & Flajnik, M. (2016). Alterations in cellular metabolism modulate CD1d-mediated NKT-cell responses. *Pathogens and Disease*, *74*, ftw055–ftw055.
17. Delmastro-Greenwood, M. M., & Piganelli, J. D. (2013). Changing the energy of an immune response. *American Journal of Clinical and Experimental Immunology*, *2*, 30–54.
18. Cheng, G., Zielonka, J., McAllister, D., Hardy, M., Ouari, O., Joseph, J., Dwinell, M. B., & Kalyanaraman, B. (2015). Antiproliferative effects of mitochondria-targeted cationic antioxidants and analogs: Role of mitochondrial bioenergetics and energy-sensing mechanism. *Cancer Letters*, *365*, 96–106.
19. Cheng, G., Zielonka, J., Dranka, B. P., McAllister, D., Mackinnon, Jr., A. C., Joseph, J., & Kalyanaraman, B. (2012). Mitochondria-targeted drugs synergize with 2-deoxyglucose to trigger breast cancer cell death. *Cancer Research*, *72*, 2634–2644.
20. Cheng, G., Zielonka, J., McAllister, D., Tsai, S., Dwinell, M. B., & Kalyanaraman, B. (2014). Profiling and targeting of cellular bioenergetics: Inhibition of pancreatic cancer cell proliferation. *British Journal of Cancer*, *111*, 85–93.
21. Cheng, G., Zielonka, J., McAllister, D. M., Mackinnon, Jr., A. C., Joseph, J., Dwinell, M. B., & Kalyanaraman, B. (2013). Mitochondria-targeted vitamin E analogs inhibit breast cancer cell energy metabolism and promote cell death. *BMC Cancer*, *13*, 285.
22. Dickey, J. S., Gonzalez, Y., Aryal, B., Mog, S., Nakamura, A. J., Redon, C. E., Baxa, U., Rosen, E., Cheng, G., Zielonka, J., Parekh, P., Mason, K. P., Joseph, J., Kalyanaraman, B., Bonner, W., Herman, E., Shacter, E., & Rao, V. A. (2013). Mito-tempol and dexrazoxane exhibit cardioprotective and chemotherapeutic effects through specific protein oxidation and autophagy in a syngeneic breast tumor preclinical model. *PLoS ONE*, *8*, e70575.
23. Rao, V. A., Klein, S. R., Bonar, S. J., Zielonka, J., Mizuno, N., Dickey, J. S., Keller, P. W., Joseph, J., Kalyanaraman, B., & Shacter, E. (2010). The antioxidant transcription factor Nrf2 negatively regulates autophagy and growth arrest induced by the anticancer redox agent mitoquinone. *The Journal of Biological Chemistry*, *285*, 34447–34459.
24. Zhao, H., Joseph, J., Fales, H. M., Sokoloski, E. A., Levine, R. L., Vasquez-Vivar, J., & Kalyanaraman, B. (2005). Detection and characterization of the product of hydroethidine and intracellular superoxide by HPLC and limitations of fluorescence. *Proceedings of the National Academy of Sciences of the United States of America*, *102*, 5727–5732.
25. Zielonka, J., & Kalyanaraman, B. (2010). Hydroethidine- and MitoSOX-derived red fluorescence is not a reliable indicator of intracellular superoxide formation: Another inconvenient truth. *Free Radical Biology & Medicine*, *48*, 983–1001.
26. Zielonka, J., Vasquez-Vivar, J., & Kalyanaraman, B. (2008). Detection of 2-hydroxyethidium in cellular systems: A unique marker product of superoxide and hydroethidine. *Nature Protocols*, *3*, 8–21.
27. Zielonka, J., Hardy, M., & Kalyanaraman, B. (2009). HPLC study of oxidation products of hydroethidine in chemical and biological systems: Ramifications in superoxide measurements. *Free Radical Biology and Medicine*, *46*, 329–338.
28. Zielonka, J., Srinivasan, S., Hardy, M., Ouari, O., Lopez, M., Vasquez-Vivar, J., Avadhani, N. G., & Kalyanaraman, B. (2008). Cytochrome c-mediated oxidation of hydroethidine and mito-hydroethidine in mitochondria: Identification of homo- and heterodimers. *Free Radical Biology and Medicine*, *44*, 835–846.
29. Sikora, A., Zielonka, J., Adamus, J., Debski, D., Dybala-Defratyka, A., Michalowski, B., Joseph, J., Hartley, R. C., Murphy, M. P., & Kalyanaraman, B. (2013). Reaction between peroxynitrite and triphenylphosphonium-substituted arylboronic acid isomers: Identification of diagnostic marker products and biological implications. *Chemical Research in Toxicology*, *26*, 856–867.
30. Zielonka, J., Sikora, A., Adamus, J., & Kalyanaraman, B. (2015). Detection and differentiation between peroxynitrite and hydroperoxides using mitochondria-targeted arylboronic acid. *Methods in Molecular Biology*, *1264*, 171–181.
31. Zielonka, J., Zielonka, M., VerPlank, L., Cheng, G., Hardy, M., Ouari, O., Ayhan, M. M., Podsiadly, R., Sikora, A., Lambeth, J. D., & Kalyanaraman, B. (2016). Mitigation of NADPH oxidase 2 activity as a strategy to inhibit peroxynitrite formation. *The Journal of Biological chemistry*, *291*, 7029–7044.

32. Hardie, D. G., & Ashford, M. L. (2014). AMPK: Regulating energy balance at the cellular and whole body levels. *Physiology (Bethesda, Md.)*, *29*, 99–107.
33. Mackenzie, R. M., Salt, I. P., Miller, W. H., Logan, A., Ibrahim, H. A., Degasperi, A., Dymott, J. A., Hamilton, C. A., Murphy, M. P., Delles, C., & Dominiczak, A. F. (2013). Mitochondrial reactive oxygen species enhance AMP-activated protein kinase activation in the endothelium of patients with coronary artery disease and diabetes. *Clinical Science* 124, 403–411.
34. Quintero, M., Colombo, S. L., Godfrey, A., & Moncada, S. (2006). Mitochondria as signaling organelles in the vascular endothelium. *Proceedings of the National Academy of Sciences of the United States of America*, *103*, 5379–5384.
35. Boukalova, S., Stursa, J., Werner, L., Ezrova, Z., Cerny, J., Bezawork-Geleta, A., Pecinova, A., Dong, L., Drahota, Z., & Neuzil, J. (2016). Mitochondrial targeting of metformin enhances its activity against pancreatic cancer. *Molecular Cancer Therapeutics*, *15*, 2875–2886.
36. Orme-Johnson, N. R., Hansen, R. E., & Beinert, H. (1974). Electron paramagnetic resonance-detectable electron acceptors in beef heart mitochondria. Reduced diphosphopyridine nucleotide ubiquinone reductase segment of the electron transfer system. *The Journal of Biological Chemistry*, *249*, 1922–1927.
37. Chandran, K., Aggarwal, D., Migrino, R. Q., Joseph, J., McAllister, D., Konorev, E. A., Antholine, W. E., Zielonka, J., Srinivasan, S., Avadhani, N. G., & Kalyanaraman, B. (2009). Doxorubicin inactivates myocardial cytochrome c oxidase in rats: Cardioprotection by Mito-Q. *Biophysical Journal*, *96*, 1388–1398.
38. Bennett, B., Helbling, D., Meng, H., Jarzembowski, J., Geurts, A. M., Friederich, M. W., Van Hove, J. L. K., Lawlor, M. W., & Dimmock, D. P. (2016). Potentially diagnostic electron paramagnetic resonance spectra elucidate the underlying mechanism of mitochondrial dysfunction in the deoxyguanosine kinase deficient rat model of a genetic mitochondrial DNA depletion syndrome. *Free Radical Biology and Medicine*, *92*, 141–151.
39. Ghosh, A., Chandran, K., Kalivendi, S. V., Joseph, J., Antholine, W. E., Hillard, C. J., Kanthasamy, A., Kanthasamy, A., & Kalyanaraman, B. (2010). Neuroprotection by a mitochondria-targeted drug in a Parkinson's disease model. *Free Radical Biology & Medicine*, *49*, 1674–1684.
40. Sobotta, M. C., Liou, W., Stöcker, S., Talwar, D., Oehler, M., Ruppert, T., Scharf, A. N. D., & Dick, T. P. (2015). Peroxiredoxin-2 and STAT3 form a redox relay for H2O2 signaling. *Nature Chemical Biology*, *11*, 64–70.
41. Babot, M., Birch, A., Labarbuta, P., & Galkin, A. (2014). Characterisation of the active/de-active transition of mitochondrial complex I. *Biochimica et Biophysica Acta (BBA) - Bioenergetics*, *1837*, 1083–1092.