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Review

Using exomarkers to assess mitochondrial reactive species *in vivo* ☆,☆☆

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ABSTRACT

Background: The ability to measure the concentrations of small damaging and signalling molecules such as reactive oxygen species (ROS) *in vivo* is essential to understanding their biological roles. While a range of methods can be applied to *in vitro* systems, measuring the levels and relative changes in reactive species *in vivo* is challenging.
Scope of review: One approach towards achieving this goal is the use of exomarkers. In this, exogenous probe compounds are administered to the intact organism and are then transformed by the reactive molecules *in vivo* to produce a diagnostic exomarker. The exomarker and the precursor probe can be analysed *ex vivo* to infer the identity and amounts of the reactive species present *in vivo*. This is akin to the measurement of biomarkers produced by the interaction of reactive species with endogenous biomolecules.
Major conclusions and general significance: Our laboratories have developed mitochondria-targeted probes that generate exomarkers that can be analysed *ex vivo* by mass spectrometry to assess levels of reactive species within mitochondria *in vivo*. We have used one of these compounds, MitoB, to infer the levels of mitochondrial hydrogen peroxide within flies and mice. Here we describe the development of MitoB and expand on this example to discuss how better probes and exomarkers can be developed. This article is part of a Special Issue entitled Current methods to study reactive oxygen species - pros and cons and biophysics of membrane proteins. Guest Editor: Christine Winterbourn.

1. Introduction

Relatively short-lived, reactive molecules occur in many different contexts in biology [1,2]. Typical examples are the reactive oxygen species (ROS) superoxide and hydrogen peroxide [3,4], but there are many others including the reactive nitrogen species nitric oxide (NO) and peroxynitrite [5], degradation products arising from oxidative damage such as 4-hydroxynonenal (4-HNE) [6], and side products of metabolic processes, such as methylglyoxal from glycolysis [7]. These reactive species are of considerable interest because they can directly damage other biological molecules and disrupt cell function, and also they can act as signals to modulate biological processes [1,2,8,9]. Consequently many biologically important questions can only be addressed fully by measuring the steady state levels and changes in these reactive species *in vivo* [10,11].

Measurement of the concentrations of reactive molecules such as superoxide, hydrogen peroxide and NO in biological environments is inherently challenging due to their short lifetimes, diverse chemistries and the limited selectivity of detection systems [1,10,12]. It is possible to apply more selective approaches *in vitro* such as using spin trapping followed by electron paramagnetic resonance (EPR) spectroscopy to ensure that only radical species are detected, or by using catalase- or superoxide dismutase (SOD)-sensitive changes in the absorbance or fluorescence of detector molecules to provide robust and useful

Abbreviations: EPR, electron paramagnetic resonance; GFP, green fluorescent protein; 4-HNE, 4-hydroxynonenal; MitoB, 3-(dihydroxyboronyl)benzyltriphenylphosphonium bromide; MitoP, (3-hydroxybenzyl)triphenylphosphonium bromide; ROS, reactive oxygen species; SOD, superoxide dismutase; TPMP, methyltriphenylphosphonium; TPP, triphenylphosphonium cation

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information [12–14]. However, to address many biologically important questions it is often necessary to assess the level of the reactive species in context, and with these more complicated systems the detection methods are less selective and sensitive. In cell systems, general changes in the ROS can be detected by determining the oxidation of probes such as dichlorofluorescin to its fluorescent product dichlorofluorescein, although there are significant limitations to the use and interpretation of this probe [15]. The production of specific ROS such as superoxide can be inferred, with certain caveats, by determining the changes in fluorescence of probes such as hydroethidine [14] or MitoSOX [16], or for hydrogen peroxide by using boronic acid-conjugated fluorophores [17–19]. Another important approach is to utilise engineered proteins derived from green fluorescent protein (GFP) such as redox sensitive GFP (roGFP) or HyPer [20,21]. The fluorescent intensity of these probes responds to changes in endogenous redox couples or levels of hydrogen peroxide and often has the significant advantage that their fluorescence is ratiometric, thereby facilitating calibration, and that they can be directed to particular cell types and compartments [20–22]. These approaches work well and produce robust and useful information, provided artefactual effects are recognized and steps are taken to correct them [15,20,23]. However, extension of these approaches from cells in culture to intact living organisms is challenging. In some circumstances optical techniques can be used, for example in the optically accessible surface cell layers by the use of two photon microscopy [24], by the use of chemoselective bioluminescent probes [25], or in transparent organisms such as zebra fish embryos [26]. In general though, it is very difficult to measure directly the levels of small, reactive molecules within living organisms.

Changes in reactive species *in vivo* are often inferred by measuring the accumulation of oxidative damage markers derived from the interaction of reactive species with protein, DNA or lipid [27–32]. While measurements of oxidative damage markers are very informative, a major limitation is that the actual amount of accumulation of a damage marker is determined not only by its rate of formation but also by the effectiveness of repair processes and defence mechanisms, all of which vary independently of each other [30,33]. Another possibility is to measure the expression levels of genes that respond to alterations in a reactive species, either as a damage response or as a redox signal [34,35]. However, again there are multiple levels of regulation and feedback making the link between the expression level of the particular gene and the amount of a specific reactive species tenuous. The current situation is that while changes in the levels of short-lived reactive molecules are often proposed to mediate damage and redox signals in a range of biological situations *in vivo*, we do not have the techniques available to test these hypotheses properly [10]. There is a critical unmet need for better measurements of the levels of reactive species *in vivo*.

One approach that can be used to assess the levels and identities of reactive species *in vivo* is by using *exo*genous *marker* molecules for which we have coined the term "exomarkers". This approach has many parallels with the use of biomarkers whereby changes in the levels of products, such as F_2-isoprostanes, from the interaction of reactive species with endogenous molecules are used to infer changes in reactive species *in vivo* [32]. However, the use of exomarkers differs in that an exogenous, artificial probe compound is administered to the organism (Fig. 1). Within the organism the probe is modified by reactive species to generate an exomarker product which is diagnostic of the reactive species, and which then can be assessed and used to infer levels of reactive species present *in vivo* (Fig. 1). Despite previously lacking a unifying name, the concept of administering exogenous probe molecules to a living organism in order to infer changes in reactive species *in vivo* by the measurement of exomarkers is not new. It has been used by a number of other groups in the past. For example, the selective reactivity of spin traps with free radicals and the subsequent analysis of the products by EPR spectroscopy [36–38], or by a combination of liquid chromatography and mass spectrometry [39,40] has been extensively exploited within experimental animals. Similarly,

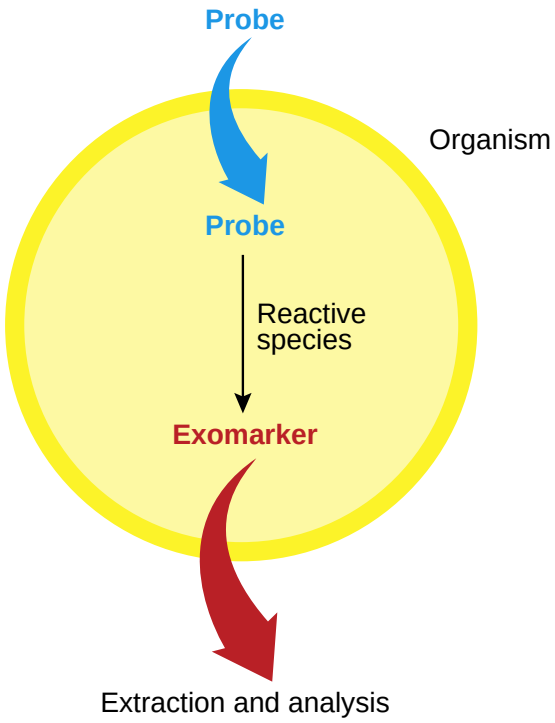

Fig. 1. The exomarker approach to assess levels of a reactive species *in vivo*. The probe is administered to the organism where it is converted to a diagnostic exomarker product by reaction with reactive species. The exomarker product is then extracted from the organism and analysed to infer the type and levels of reactive species that occur *in vivo*.

a range of chemical traps such as salicylate, DMSO, phenylalanine, 4-hydroxybenzoic acid and terephthalic acid have been used to infer the levels of hydroxyl radical production *in vivo* [12,41–47].

The ideal probe to generate an exomarker would have the following properties: non-toxic; does not alter endogenous metabolism; easily administered to the living organism; transports to a particular organ/tissue/cell type and localises within a single cell compartment; reacts selectively and efficiently with the target reactive species, with no side reactions, to produce a stable product; the reaction to generate the exomarker does not significantly alter the levels of the reactive species to be assessed; both the diagnostic exomarker and the probe are easily assessed at very low levels, ideally in urine or plasma. It is also important that the probe is synthetically accessible and chemically stable, and so an ideal probe is the simplest structure that fulfils all the requirements. While these are demanding criteria, many exomarker approaches will still be useful if they meet only some of them.

Here we describe our experience to date with one class of exomarker designed to report on mitochondrial ROS *in vivo* [11,48], discuss the strengths and weaknesses of this approach and consider how it may be extended and developed.

2. Assessing mitochondrial hydrogen peroxide *in vivo* with MitoB

In our laboratories one focus is to develop an understanding of the role of mitochondrial ROS such as hydrogen peroxide in biological damage and redox signalling [49–51]. The levels of mitochondrial hydrogen peroxide *in vivo* were not known, therefore to address this unmet need we developed an exomarker approach using a mitochondria-targeted mass spectrometric probe called MitoB [11,48]. MitoB (Fig. 2A) is comprised of the lipophilic cationic triphenylphosphonium (TPP) moiety linked to an arylboronic acid [11]. The TPP moiety has been used extensively to drive the uptake of a range of bioactive molecules into mitochondria *in vivo*, including antioxidants, probes and nitric oxide donors following oral, intravenous or intraperitoneal administration routes [52–55]. The lipophilic nature of the TPP moiety enables these molecules

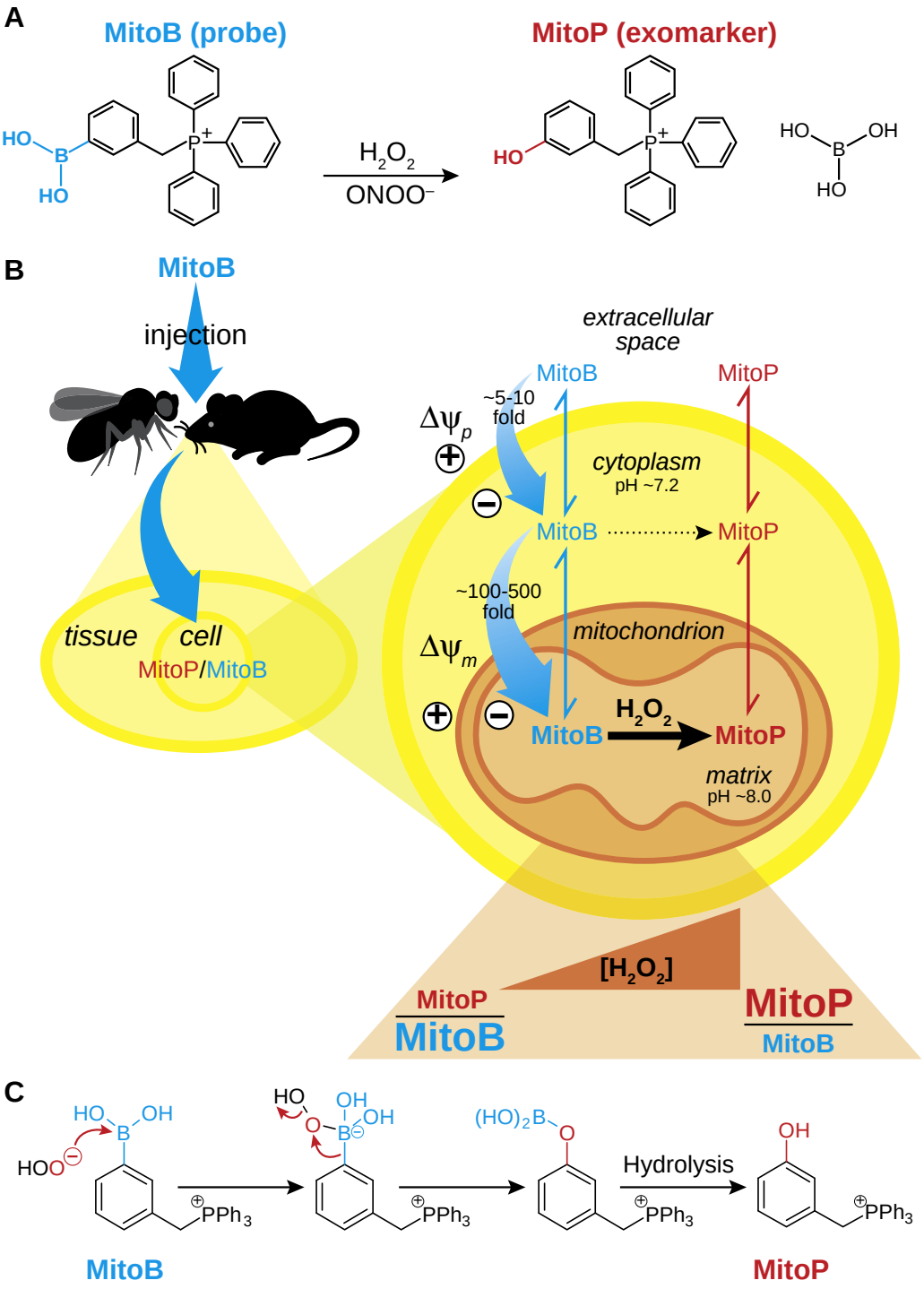

Fig. 2. Using MitoB to assess mitochondrial hydrogen peroxide formation *in vivo*. A: Reaction of MitoB with hydrogen peroxide or peroxynitrite converts the arylboronic component of MitoB to a phenol, MitoP. B: Administration of MitoB to an animal model such as *Drosophila* leads to the rapid accumulation of MitoB within cells and from there into mitochondria, driven by the plasma ($\Delta\psi_p$) and mitochondrial ($\Delta\psi_m$) membrane potentials. Consequently, MitoB is predominantly present within mitochondria *in vivo*. There MitoB will react with the local concentration of hydrogen peroxide to form MitoP causing the ratio of MitoP/MitoB to increase. Experimentally, MitoB is injected into the animal and after 3–6 h incubation *in vivo* the MitoP/MitoB ratio is determined by extracting MitoP and MitoB from the tissue and measuring them by liquid chromatography and tandem mass spectrometry. C: Mechanism of the reaction of hydrogen peroxide with MitoB.

to pass rapidly through biological membranes and accumulate within the cell driven by the plasma membrane potential, and then further accumulate within mitochondria driven by the large mitochondrial membrane potential (Fig. 2B) [53,56]. These lipophilic cations rapidly pass back and forth across biological membranes, thereby equilibrating with the local membrane potentials. The Nernst equation can adequately describe this uptake and the accumulation within cells and mitochondria is extensive (increasing ~10-fold for every 60 mV of membrane potential) with up to a thousand fold accumulation within mitochondria [56]. Consequently, attaching a small, non-polar moiety to a TPP molecule is an effective way of rapidly targeting the molecule to the mitochondrial compartment *in vivo* following many modes of administration [53,57].

In MitoB an arylboronic acid moiety is attached to the TPP function [11], which reacts directly with the conjugate base of hydrogen

peroxide to form a phenol [58] (Fig. 2C). This selective chemistry was exploited by Chang who showed that the hydroxyl group in a fluorophore could be substituted with a boronate to generate a family of hydrogen peroxide-selective fluorescent probes [17–19,59]. These probes showed appreciable selectivity for hydrogen peroxide over other ROS and were a considerable improvement over other available fluorescent ROS probes because the reaction was non-enzymatic and more chemically selective [17–19] (Fig. 2C). The Kalyanaraman laboratory has subsequently shown that arylboronates also react very rapidly with peroxynitrite and hypohalous acids to generate the corresponding phenol [60,61]. This work indicated that the arylboronic acid moiety was a useful hydrogen peroxide-selective probe in a biological environment, provided that peroxynitrite and hypohalous acid production could be managed or estimated independently, and therefore it was incorporated into the design process for MitoB [11]. The mode of action of MitoB is shown in Fig. 2B: MitoB should accumulate rapidly within mitochondria *in vivo* and there be converted slowly and irreversibly to the phenol, MitoP, in direct proportion to the local hydrogen peroxide concentration [11]. Therefore measurements of the accumulation of the exomarker MitoP relative to the probe MitoB should be an indication of the average, local hydrogen peroxide concentration within mitochondria over the duration of the experiment.

Experiments are carried out by injecting MitoB into a living organism, for example into the thorax of *Drosophila* or intravenously into a mouse [11,48,62]. This results in rapid delivery of MitoB to the mitochondria within the tissues and the animal is then allowed to resume normal behaviour for 3 to 6 h. Over this period MitoB will be gradually lost from the organism by excretion, but in addition a proportion will be converted by reaction with hydrogen peroxide to MitoP. As the MitoB is essentially present only in mitochondria, the conversion is dependent on the concentrations of MitoB and hydrogen peroxide within the mitochondria. The uptake of MitoB and the retention of MitoP once formed are both dependent on membrane potential and both will be lost from the body at very similar rates [11]. Thus over time the ratio of MitoP/MitoB in a tissue will increase due to the MitoB to MitoP conversion, essentially integrating the average mitochondrial hydrogen peroxide concentration present over the duration of the experiment. By expressing the results as the ratio of MitoP/MitoB over time the assay corrects for changes in the uptake of MitoB into mitochondria in the tissue under consideration.

The amount of MitoP relative to MitoB in tissue samples was measured by liquid chromatography–tandem mass spectrometry (LC–MS/MS), normalised to deuterated internal standards [11]. This approach was chosen because the TPP cation has the further advantage of a fixed positive charge that greatly enhances the sensitivity of detection by mass spectrometry. Derivatization with a TPP moiety is widely used to enhance the sensitivity of detection by mass spectrometry [63,64]. To quantify the amounts of MitoB and MitoP in a tissue sample, the compounds are first extracted from the tissue, separated by reverse phase HPLC (RP-HPLC), introduced into a mass spectrometer and then the precursor ion is broken up and the fragment ions measured by tandem mass spectrometry [65,66]. These measurements are carried out relative to deuterated internal standards of MitoB and MitoP that are added to the biological sample prior to extraction and analysis in order to correct for both inefficiencies in extraction and in the LC–MS/MS assay [11]. MitoB incorporates the desirable features of a probe into a very simple structure, so that MitoB, MitoP and the deuterated standards are easily prepared [11].

The use of MitoB has been described in detail in previous publications [11,48,62]. It has been used to assess changes in the production of mitochondrial hydrogen peroxide in *Drosophila* with age [11]. In this work MitoB was injected into anesthetized flies that were allowed to recover and fly freely. Then, after 4 to 6 h, they were harvested and the MitoP/MitoB ratio determined. This approach allowed the average mitochondrial level of hydrogen peroxide to be assessed in free living flies for the first time and showed that there was an increase in average mitochondrial hydrogen peroxide concentration with age, and that this was decreased by enhanced physical activity [11]. MitoB was also applied to mammalian systems and it was found that an intravenous injection of MitoB into a mouse enabled sufficient MitoB to be taken up into the heart to assess changes in mitochondrial ROS production during ischemia–reperfusion injury [62]. Therefore MitoB can be used as a probe to assess mitochondrial hydrogen peroxide production *in vivo*, through generation of MitoP as an exomarker, and many other experimental models are currently being interrogated using MitoB. In the next section, we use MitoB as an example to discuss the strengths and weaknesses of the exomarker approach and suggest how it can be extended and improved.

3. Critical considerations for the development of exomarkers

3.1. Mode of probe delivery in vivo

In the development of MitoB we found that delivery as a single bolus by injection was preferable [11,48,62]. This enabled the probe to be delivered to the required location essentially at the time of administration and thereby enabled the accumulation of the exomarker product to be interpreted more easily (Fig. 3). If the mode of delivery was different, for example orally, the uptake into the tissue would be spread over a longer time, making the kinetics of accumulation of the exomarker derivative more difficult to assess. For mouse experiments, a single injection of MitoB led to rapid delivery to mitochondria within organs such as the heart [62]. This was due to the rapid clearance of TPP compounds from the circulation into cells and mitochondria [57]. There is a concern that a probe, or its exomarker derivative, may redistribute over time, however with TPP compounds the rapid initial uptake into organs due to their rapid passage through biological membranes and their accumulation within cells and mitochondria renders the steady-state levels in the circulation very low [57]. Furthermore, any TPP compounds that redistribute back into the plasma are removed by the kidneys and biliary excretion pathways [57,67,68]. Thus tissue redistribution is a minor concern for the TPP class of exomarker [57], but for other probes and exomarkers that are not actively accumulated secondary redistribution throughout the organism may affect the interpretation of the endpoint measurements. It may also be possible to deliver a probe to the desired location over a short time by other approaches, for example, it may be possible to achieve a step change in probe concentration in nematodes by soaking in concentrated liquid

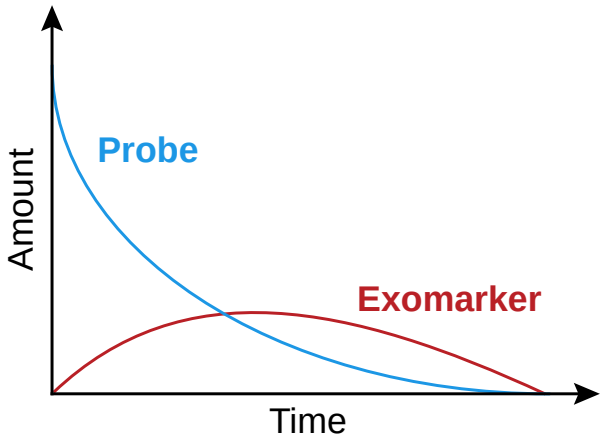

Fig. 3. Time course of generation of exomarkers. Here it can be seen that a bolus administration of a probe leads to an initial high concentration in the organism that is then lost over time by excretion. In contrast the exomarker is initially absent but its content increases over time due to the reaction of the probe with the target reactive species. Consequently the ratio of the exomarker to that of the initial probe increases over time.

incubation medium to allow oral absorption, followed by transfer to fresh incubation medium. Other modes of administration, for example by gavage or by continual steady state delivery in the drinking water or by infusion may also be useful in certain circumstances.

3.2. Location of the probe in vivo

The transformation of a probe to an exomarker is most informative and easily interpreted if it is located in a defined organ, cell type and intracellular location. The approximately thousand fold greater local concentration of MitoB within mitochondria, compared to the extracellular environment, also enhances selectivity [11]. This is because the rate of the reaction of MitoB with hydrogen peroxide is second order, so the high local concentration of MitoB will in itself greatly increase the selective reporting of mitochondrial reactive species, even if the hydrogen peroxide concentration is the same in the cytosol and mitochondrial compartments.

The use of TPP to target molecules such as MitoB to mitochondria is well established in isolated mitochondria, in cells and *in vivo* [53–56,69]. These findings are based on extensive studies showing that the uptake of a range of TPP compounds into isolated mitochondria and into mitochondria within cells occurs in response to the plasma and mitochondrial membrane potentials and is described adequately by the Nernst equation [53,54,56,69]. We have made a series of TPP compounds conjugated to thiol-reactive moieties which form stable, covalent attachments to protein thiols that can be detected using antibodies against the TPP moiety on western blots or by immunohistochemistry [70]. These compounds only label mitochondria in cells in culture [57,69,70], and also within mice [57] and flies [11]. Administration of radiolabelled TPP compounds to animals has shown distribution into tissues that is affected by mitochondrial membrane potential [57,71]. Furthermore, TPP conjugated to fluorescent moieties shows essentially complete spatial localisation within mitochondria in cells [16,17,72]. All these studies on a wide range of TPP compounds suggest that MitoB and MitoP will obey the Nernst equation and justify our assumption of their mitochondria-selective accumulation *in vivo* [11]. Consequently the whole organism or tissue can be assessed with confidence that any formation of the exomarker in non-mitochondrial locations is negligible [11]. A further point is that as the uptake into mitochondria of MitoP and MitoB driven by the mitochondrial and plasma membrane potentials is very similar [11], the accumulation of MitoP can be normalised to that of MitoB. This ability to assess the ratio of the probe and its exomarker product is a significant advantage in correcting for random alterations in uptake and distribution of the probe. For example, measuring the MitoP/MitoB ratio enables a change in MitoB uptake due to the mitochondrial membrane potential to be corrected for by a similar change in MitoP retention. However in situations where normalisation of the exomarker to the probe is not possible then factors that affect the uptake or retention of the probe may affect the production of the biomarker and must be considered.

A significant weakness in this strategy is that only the average mitochondrial content of reactive species over a time period within a tissue is assessed. This may become acute if changes in reactive species in a small number of cells within a tissue or organism are important, or a short-term release of reactive species is involved, as these types of changes would not be seen by the use of probes such as MitoB. Alternative approaches, such as cell specific targeting of proteins in conjunction with optical methods would have to be used in these situations [22]. Alternatively it may be possible to design a probe, which, upon interaction with the reactive species, is transformed into an exomarker that was retained within the cell or organelle enabling *ex vivo* separation prior to analysis. In principle, complementary targeting strategies could be used to assess other intracellular or tissue locations *in vivo* and their development would facilitate the development and usefulness of the exomarker approach.

3.3. Selective reactivity and excretion of the probe and exomarker

A critical requirement for the efficacy of the exomarker approach is the selective alteration of the probe by the reactive molecule to be assessed (Fig. 4). There are a number of fates for a probe molecule: excretion, side reactions with other components within the biological matrix and reaction with the reactive species to generate the diagnostic exomarker product. The ideal is a probe with no side reactions that produces a single diagnostic exomarker product; furthermore the exomarker should be stable and not biotransformed to other molecules. These criteria seem to be met for MitoB in *Drosophila* where the MitoB reacts only with hydrogen peroxide to form MitoP. However the reaction of MitoB with peroxynitrite may affect the interpretation of MitoP accumulation under some conditions. There is also evidence that arylboronic acids can react with hypohalous acids such as hypochlorous acid to form a phenol [61], which may also affect the interpretation of the results. In addition, the rate of reaction of MitoB with hydrogen peroxide is pH-dependent [11], consequently it is important to ensure that alterations in the rate of accumulation of MitoP reflect changes in hydrogen peroxide concentration and not pH. When probing the level of a reactive species *in vivo* it is useful if the reaction between the probe and the reactive species to generate the exomarker is relatively slow as otherwise it would distort the levels of the reactive species *in vivo* and thereby modify biological function. It is important to note that this slow rate of reaction of a probe with a reactive species does not limit its usefulness even when there are many far faster endogenous degradation pathways for the reactive species. For example, the rate of reaction of MitoB with hydrogen peroxide is ~9 M^{-1} s^{-1} [11], which is negligible compared to that of peroxidases such as peroxiredoxin 3 where the rate is ~2 × 10^7 M^{-1} s^{-1} [73]. The probe is not in competition with the other potential sinks for the reactive species; instead it is reacting by a second order process with the target species and thus responds to its steady state concentration. This steady state will in turn be set by both the rates of formation and degradation of the reactive species and changes in these will alter the levels of the exomarker. Furthermore, a slow reaction rate between the probe and the reactive species ensures the generation of the exomarker which remains proportional to the reactive species' concentration, as the ratio of exomarker to probe remains low. The downside of the slow rate of reaction of the probe with the reactive species, such as in the case of MitoB, is that it may take a long time for accumulation of sufficient exomarker to reach the threshold for detection. Consequently, probes such as MitoB integrate the average level of the reactive species over a few hours. This facilitates assessing the levels of reactive species in free living animals under normal conditions as the compound can be administered under anaesthesia and the animal followed as required for a few hours. However, for this to work it is essential that the lifetime of the probe *in vivo* is sufficient to enable integration of the signal for this

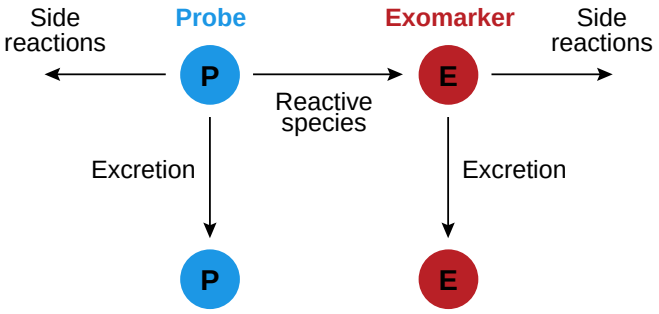

Fig. 4. Potential fates of a probe *in vivo*. Once administered *in vivo* the probe, P, can be excreted intact, undergo side reactions or react with the target reactive species to form the diagnostic exomarker E. Then E itself can be excreted, or further modified.

length of time. A corollary is that this approach requires very sensitive detection methods, hence the use of LC–MS/MS in conjunction with deuterated standards.

3.4. Sampling of the probe and exomarker

An important aspect of using the exomarker approach to assess reactive species within a living organism is to sample the accumulation of the diagnostic exomarker effectively. In the case of MitoB, this was done destructively by the extraction of the exomarker and its products from the whole organism or organ. While this approach has the advantage that different organs can be assessed independently, it would be useful to be able to assess the exomarker non-destructively, for example by analysing plasma or urine preferably in a dynamic mode. For MitoB the rapid clearance of TPP compounds from the plasma precludes plasma analysis. However, MitoB and MitoP are excreted into the urine following injection into mice [11,48]. While the relationships between the levels that accumulate in urine and concentrations of reactive species in mitochondria *in vivo* are unclear, future work assessing this link may facilitate the non-destructive analysis of mitochondrial ROS levels *in vivo*. This would make assessment of changes in mitochondrial ROS during the development of a pathology or treatment possible and might also enable this approach to be translated to human studies.

3.5. Analytic approaches to identify and quantify exomarkers

While there are a range of possible approaches for the *ex vivo* analysis of an exomarker, one constraint is that the probe compounds are administered for a few hours to animals and thus the amounts detected are small, necessitating very sensitive analytical approaches. The use of LC–MS/MS relative to internal stable isotope standards currently seems the most useful. Other approaches to analyse *ex vivo* samples, such as EPR spectroscopy, HPLC with electrochemical detection or gas chromatography may also be used for particular molecules under certain circumstances. The LC–MS/MS approach combines sensitive quantification and accurate identification with the possibility of relatively high throughput. One constraint is the requirement for the synthesis of stable isotope internal standards for identification and quantification.

Most approaches reported to date require knowledge of the identity of the reactive species, but it is likely that there are many similar compounds that are important *in vivo* but which are currently unsuspected due to the difficulties of analysis. Tandem mass spectrometry using precursor ion scanning in conjunction with broadly reactive probe traps that generate a range of distinct exomarkers may be one way to identify previously unknown reactive species, although this is not a high throughput approach. For example, consider a mitochondria-targeted TPP probe conjugated to a chemical trap E, chosen so that it was modified by a range of reactive species (Fig. 5). The probe would react *in vivo* to form a series of different exomarker products that can be analysed by tandem mass spectrometry in precursor ion scanning mode to pick up a distinctive fragment ion derived from the TPP moiety [11,48]. The mass of the parent ion of the exomarker containing the TPP moiety can thereby be determined and the nature of the reactive species inferred (Fig. 5).

4. Conclusion

Here we have outlined work to date on the mitochondria-targeted mass spectrometry exomarker approach. In this we have suggested that MitoB satisfies some of the criteria for an ideal probe to generate the exomarker MitoP. At the moment we and others are building on this work to generate further mitochondria-targeted probes to extend the exomarker approach to analyse other reactive molecules within mitochondria *in vivo*. The goal is to learn more about how changes in reactive species contribute to pathologies and normal biological function. The ability to target probes to mitochondria through the use of the TPP cation has facilitated this work. However, there are many other cellular compartments to target and the development of alternative approaches to direct probes to these locations is an important area for future development. The *ex vivo* analysis of exomarkers by mass spectrometry has many advantages, but one problem is that this methodology is inherently invasive and another is the time-consuming nature of the sample processing. The development of methods to assess changes in exomarkers less invasively and in real time would greatly enhance their usefulness.

The need to assess reactive species *in vivo* is clearly an important goal. The continued development of probes and exomarkers is one approach that shows promise to contributing to this unmet need. However, as with all assessments of reactive species it must be applied cautiously and the accuracy and robustness of the approach continually probed and reassessed by orthogonal methods.

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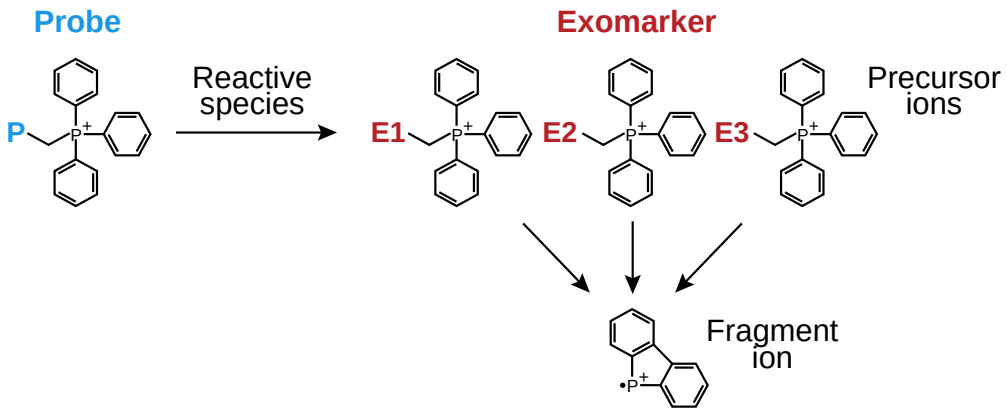

Fig. 5. Using a probe in conjunction with precursor ion scanning to identify unknown reactive species. Here a hypothetical mitochondria-targeted probe is shown that comprises the TPP moiety connected to the chemical trap P. *In vivo* the interaction with unknown reactive species converts P to a series of products (E1, E2 and E3). These can be assessed by tandem mass spectrometry with precursor ion scanning mode to identify the precursors of a distinctive fragment due to the TPP moiety and thus to characterise precursor ions by mass. From this, the nature of the unknown reactive species that interacted with P to generate E1, E2 and E3 can be inferred.

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