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A hydrogen peroxide electrode assay to measure thiol peroxidase activity for organoselenium and organotellurium drugs

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ABSTRACT

Molecular mimics of the enzyme glutathione peroxidase (GPx) are increasingly being evaluated as redox active drugs. Their molecular mechanism of action parallels that of the native enzyme; however, a major distinction is that GPx mimics can use alternative thiol substrates to glutathione. This generic thiol peroxidase activity implies that it is necessary to assess a GPx mimic's recognition of a range of cellular thiols in order to determine its potential therapeutic effects. We report an electrochemical assay that, by measuring the rate of decrease of the peroxide substrate, allows the activity of GPx mimics to be directly compared against an array of thiols. The derived pseudo zero-order rate constants, k_{obs}, for representative GPx mimics range between 0 and 6.6 min^{-1} and can vary by more than an order of magnitude depending on the thiol electron donor. An additional advantage of the assay is that it enables synergistic interactions between GPx mimics and cellular proteins to be evaluated. Here we report that glutathione disulfide reductase, which is commonly used to evaluate GPx mimic activity, recognizes the GPx mimic ebselen as a substrate, increasing its apparent k_{obs}. Therefore, reports relying on glutathione disulfide reductase to evaluate GPx mimic activity may exaggerate drug antioxidant action.

Drugs that mimic the action of the selenoenzyme glutathione peroxidase (GPx)[1] are currently being investigated as potential therapeutic treatments for diseases related to oxidative stress [1–9]. The GPx enzyme neutralizes the toxic or signaling effects of hydrogen and lipid peroxides by catalyzing their reduction to water and lipid alcohols, respectively [10]. This redox reaction uses the cellular thiol glutathione (GSH) as a cosubstrate, with oxidation of GSH to glutathione disulfide (GSSG) balancing the reduction of the peroxide species (Fig. 1). Drugs can be designed to replicate this catalytic activity, with an organoselenium or organotellurium group mimicking the function of the selenocysteine residue of GPx [3–5]. In the presence of substrates, these drugs then undergo a redox cycle analogous to that of GPx. Interestingly, unlike for GPx, it is not essential for these drugs to contain selenium in the selenol oxidation state, and a range of structurally diverse organoselenium species exhibit GPx-like catalysis (Fig. 2).

The first organoselenium agent to be examined for potential GPx activity was ebselen (Ebs, 2-phenyl-1,2-benzisoselenazol-3[2H]-one, originally known as PZ51), which has shown considerable promise as an antioxidant drug [11]. In humans, Ebs has been examined as a potential therapeutic for stroke, where it has progressed through phase II clinical trials [12] and currently entered phase III. Concurrently, a range of Ebs analogues are being evaluated as therapies for oxidative stress-related diseases [1–7].

In addition to Ebs, several other organoselenium drugs have been examined as therapeutics. In particular, the selenide DPS (diphenylselenide), the diselenide DPDS (diphenyldiselenide) [13], and the selenium species selenocyanate [14] and selenomethionine [15] have been investigated as potential anticancer drugs. In addition to their ability to use GSH, this drug class typically displays low substrate specificity and can accept a range of thiols. Hence, it is more accurate to consider organoselenium and organotellurium agents as thiol peroxidase catalysts rather than GPx mimics [16].

By acting as thiol peroxidases, organoselenium drugs can catalyze the oxidation of a range of cellular thiols. Of key importance are proteins, which can be structurally modified via the oxidation of their cysteine residues. Hence, in addition to their antioxidant function, these drugs have also been characterized as modulators of protein activity, with specific targets including zinc finger dependent transcription factors [17], the ryanodine receptor [18], divalent metal transporters [19], the zinc storage protein metallothionein [20], and the inflammatory enzyme lipoxygenase [21].

Due to this diverse range of cellular targets, to predict the outcome of organoselenium drug administration, it is desirable to

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[1] *Abbreviations used:* GPx, glutathione peroxidase; GSH, glutathione; GSSG, glutathione disulfide; Ebs, ebselen; DPS, diphenylselenide; DPDS, diphenyldiselenide; GDR, glutathione disulfide reductase; DTT, dithiothreitol; EDTA, ethylenediaminetetraacetic acid; DMSO, dimethylsulfoxide; DETAPAC, diethylenetriaminepentaacetic acid; H_2O_2, hydrogen peroxide.

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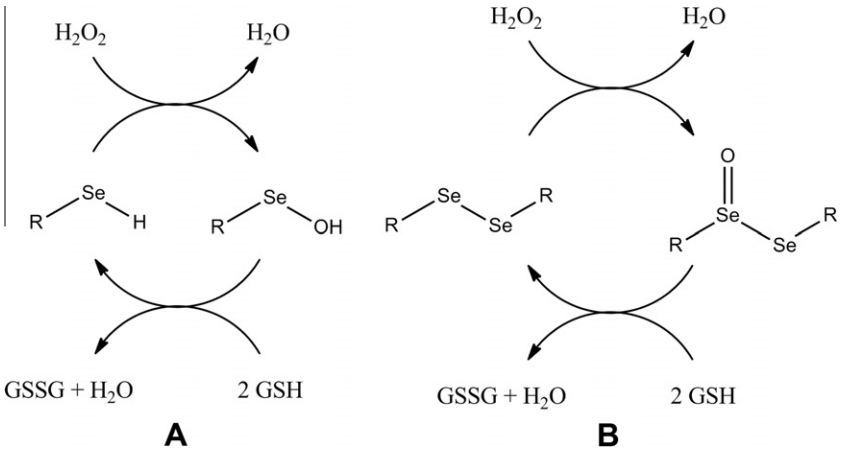

Fig.1. Catalytic cycle of GPx for selenols (A) and diselenides (B). R = variable chemical group.

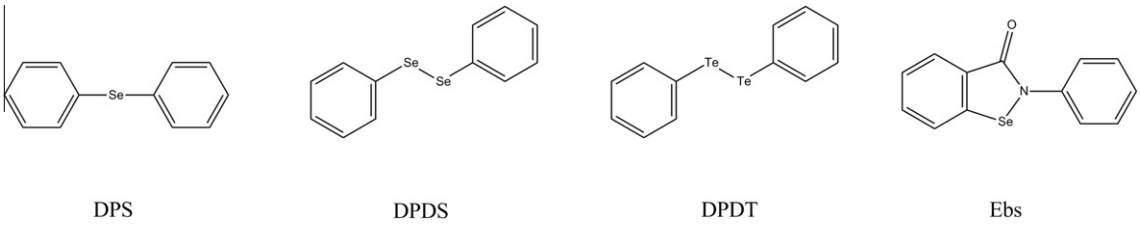

Fig.2. Structures of GPx mimics.

know the rates at which a drug will interact with the potential thiol substrates. Currently, there is no generic assay that can be readily applied to acquire this data. Most commonly used methodologies are indirect in that they do not measure the actual kinetics of thiol oxidation. Instead, the assays rely on related measurements, such as the subsequent action of the enzyme glutathione disulfide reductase (GDR) to reduce GSSG back to GSH. This coupled reaction uses NADPH as an electron donor, with the rate of formation of the spectroscopically detectable $NADP^+$ being assumed as equal to the rate of GSSG formation [11]. Although this GDR assay gives a good indication of GPx activity, it cannot be used to measure thiol peroxidase activity because alternative disulfides are not recognized by GDR. Therefore, to estimate thiol peroxidase activity, a range of assays that measure the rate of oxidation of one particular thiol substrate have been developed, such as the protein metallothionein [22] and the organic molecule benzenethiol [23].

All of these methodologies have similar limitations in that they measure the oxidation of only a single thiol substrate, whereas organoselenium drugs are known to interact with multiple targets. Therefore, we considered that it would be advantageous to develop an assay that could measure the rate at which an organoselenium drug would react with *any* thiol. We hypothesized that this could be achieved if, rather than measuring the rate at which the thiol substrate was oxidized, the assay probed the rate at which the peroxide substrate was reduced. This was achieved by employing a hydrogen peroxide selective electrode, which enabled the kinetics of hydrogen peroxide consumption to be followed in real time. This approach has two major advantages. First, the assay can derive the rate of oxidation of any thiol substrate. Second, the assay is not subject to potential artifacts generated by the drug interacting with other components of the assay.

This article describes the application of this peroxide electrode-based assay to measure the catalytic constants for selected organoselenium drugs with the representative thiol substrates GSH and dithiothreitol (DTT). We demonstrate that drug activity varies remarkably with the nature of the thiol substrate. We also show that previous reports using the GDR coupled assay have overestimated the GPx activity of Ebs because its redox activity is augmented by interactions with the enzyme.

Materials and methods

Chemicals and equipment

All reagents and drugs were obtained from Sigma–Aldrich (Auckland, New Zealand). Electrochemical measurements were performed in a sealed four-port chamber using a Free Radical Analyzer system (WPI, Sarasota, FL, USA) with a 2-mm-diameter hydrogen peroxide selective sensor electrode (ISO-HPO-2). This electrode is based on a Clark-type design that uses an internal platinum wire disk working electrode and an Ag/AgCl reference/counter electrode combination, which are separated from the assay buffer via a stainless steel sleeve with membrane cap [24]. The electrode was held at a voltage of +400 mV, which allowed the oxidation of hydrogen peroxide to be detected amperometrically ($H_2O_2 \rightarrow O_2 + 2H^+ + 2e^-$). The electrode does not display any interference from common buffer reagents or dissolved oxygen and has a response time of less than 5 s, which allowed for accurate rate determination. The electrode performance was highly reproducible (see Fig. 3B), and each membrane had a lifespan of approximately 4 to 6 weeks, after which it needed to be replaced. A hydrogen peroxide calibration curve was performed at the start of each assay to detect any deterioration in electrode performance (see below).

Hydrogen peroxide electrode calibration

The concentration of a hydrogen peroxide standard solution was accurately determined using $\varepsilon_{240} = 39.4\ M^{-1}\ cm^{-1}$. The hydrogen peroxide electrode was then calibrated using a range of hydrogen peroxide concentrations (0–1.5 mM) in a stirred buffer