Mechanism of horseradish peroxidase inactivation by benzhydrazide: a critical evaluation of arylhydrazides as peroxidase inhibitors

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Many compounds are oxidized by haem enzymes, such as peroxidases and cytochromes P450, to highly reactive intermediates that function as enzyme inactivators. To evaluate the potential of arylhydrazides as selective metabolically activated peroxidase inhibitors, the mechanism of HRP (horseradish peroxidase isoenzyme C) inhibition by BZH (benzhydrazide) was investigated in detail. No oxygen consumption was detected in BZH solutions at pH 7.0–12.0, but addition of HRP resulted in significant O₂ uptake above pH 8.0, indicating that the enzyme catalyses BZH oxidation. Addition of H₂O₂ to HRP plus BZH activates the latter as an inhibitor. This involves the three-electron oxidation of BZH in one-electron steps by the peroxidase catalytic intermediates, Compounds I and II, to produce a benzoyl radical that covalently alters the active site and inhibits peroxidase activity. Alternatively, the benzoyl radical could be produced by di-imide (NH=NH) elimination from the BZH radical. Production of Compound III (oxyperoxidase) followed by p-670 (*m/z* = 583, biliverdin-like derivative) was observed for HRP incubated with excess H₂O₂, and the addition of BZH resulted in an increase in the rate of p-670 production. BZH is an inefficient inhibitor of HRP with a *K*_i of 80 μM, an apparent inactivation rate constant (*k*_{inact}) of 0.035 min^{−1}, and an IC₅₀ of 1.0 mM. This prompted the investigation of HRP inactivation by a series of related arylhydrazides with known binding affinities for HRP. The hydrazide with the highest affinity (2-naphthoic hydrazide; *K*_d = 5.2 μM) was also found to be the most effective inhibitor with *K*_i, *k*_{inact} and IC₅₀ values of 14 μM, 0.14 min^{−1} and 35 μM, respectively.

Key words: hydrazide, inactivation, phenylhydrazine.

INTRODUCTION

Arylhydrazines are oxidized by haem peroxidases to highly reactive radical species that can be incorporated at the active site [1]. Covalent haem modification by hydrazines (R-NH-NH₂) has been employed as a method for probing active-site topology in a number of peroxidases [1–7]. Hydrazides (R-CO-NH-NH₂) are related compounds, and are known to be peroxidase inhibitors [8–13]. Several hydrazine and hydrazide derivatives have been used therapeutically, including isoniazid [INH (isonicotinic hydrazide) tuberculosis antibiotic], iproniazid (tranquillizer), isocarboxazid, mebanazine, phenelzine (monoamine oxidase inhibitors), hydralazine (antihypertensive) and PHZ (phenylhydrazine; control of polycythemia vera). However, many of these compounds are no longer in use as drugs due to the severe side effects resulting from their bioactivation, which include haemolysis, liver damage, lupus erythematosus and base-pair mutation [14–18].

Hydrazines and hydrazides are metabolically activated inhibitors. Following enzymic activation, metabolically activated inhibitors are released from the active site and can rebind the same enzyme or a different biomolecule, thereby leading to inactivation [19]. Their unwanted side effects may be due to low specificity of either the prodrug or the activated form, resulting in activation by, or inactivation of, non-target enzymes. Specificity depends on both the binding affinity and the reactivity of the activated inhibitor. HRP (horseradish peroxidase isoenzyme C) is efficiently inactivated by PHZ [2], but since PHZ undergoes

auto-oxidation at pH 7 to produce superoxide [20], it is unsuitable as a therapeutic [11]. In contrast, auto-oxidation of INH occurs at a much lower rate at pH 7 [11]. Following activation by peroxidase/catalase (KatG) of *Mycobacterium tuberculosis* [21], activated INH reacts with the cofactor of enoyl reductase to form an inhibitor that has been identified as isonicotinic-acyl-nicotinamide adenine dinucleotide [21,22]. Despite its severe side effects, including autoimmune syndromes (e.g. lupus, erythematous-like syndrome, rheumatic syndrome) and allergies, INH is still the primary drug worldwide for the treatment of tuberculosis [23].

The side effects observed for INH serve to illustrate the problems associated with the use of metabolically activated hydrazides as drugs. An investigation of the structural factors that control the efficiency of peroxidase inhibition by arylhydrazides is clearly necessary to assess their potential as therapeutic agents or as tools to define the activities of specific peroxidases *in vivo*. For example, a selective inhibitor would be of use in elucidating the role of MPO (myeloperoxidase) in inflammatory tissue damage. Although MPO-deficient mice have provided valuable information on the role of MPO in host defence and protection from atherosclerosis *in vivo*, extrapolation of these results from the mouse model to humans is complicated [24].

Since HRP is an archetypical peroxidase its inactivation by BZH (benzhydrazide), the simplest arylhydrazide, was investigated in detail. Additionally, HRP inactivation by a series of monosubstituted arylhydrazides (Figure 1) was examined. IC₅₀ as well as *K*_i and *k*_{inact} values were determined to identify the factors

Abbreviations used: BZH, benzhydrazide; -NH₂-BZH, -aminobenzhydrazide; HRP, horseradish peroxidase isoenzyme C; Compound I, two-electron-oxidized intermediate formed when 1 molar equivalent of H₂O₂ reacts with HRP(Fe^{III}); Compound II, species formed on one-electron reduction of Compound I; Compound III, HRP(Fe^{IV}O₂) (oxyperoxidase); INH, isonicotinic hydrazide; MPO, myeloperoxidase; 2-NZH, 2-naphthoic hydrazide; PHZ, phenylhydrazine.

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Figure 1 Structures of arylhydrazides and PHZ used in this study

NZH, naphthoichydrazide; NICH, nicotinic hydrazide.

necessary for the effective inhibition of HRPC. The results are compared with those reported for arylhydrazide inhibition of MPO [8–10].

MATERIALS AND METHODS

Lyophilized grade I HRPC was obtained from Roche Molecular Biochemicals. All hydrazides were purchased from Lancaster Synthesis (Easgate, Lancs., U.K.) with the exception of 3-hydroxybenzhydrazide and 4-hydroxybenzhydrazide, which were from Aldrich. PHZ was from Sigma. All reagents were of the highest quality available and were used without further purification.

Determination of K_i and k_{inact} values for inhibition of HRPC

HRPC (10 nM) was preincubated at 25 °C with 1 mM H₂O₂ and 0–10 mM hydrazide (0–5 mM for BZH) in 100 mM sodium phosphate buffer, pH 7.0. Aliquots were taken and tested for activity at time points between 0.5 and 120 min. The assay solution (200 µl) contained 5 mM guaiacol, the chromogenic donor substrate, and 1 mM H₂O₂ in 100 mM sodium phosphate buffer, pH 7.0, and the final HRPC concentration was 1 nM. HRPC activity at 25 °C was measured spectrophotometrically for 1 min at 450 nm with a SpectraMax 190 (Molecular Devices) microtitre plate reader. The percent remaining activity (ratio of initial velocity of guaiacol oxidation by treated and untreated HRPC) was plotted against preincubation time, and pseudo-first-order rate constants for HRPC inactivation (k_{obs} , min^{−1}) at each hydrazide concentration were calculated from least-squares fits to:

$$y = a + b \exp(-k_{\text{obs}}t) \quad (1)$$

where y , a and b represent HRPC activity at time t , $t = \infty$ (minimum activity) and $t = 0$ (maximum activity) respectively. The least-squares fit of eqn (2) to plots of k_{obs} against hydrazide concentration $[I]$ yielded values for K_i and k_{inact} .

$$k_{\text{obs}} = \frac{k_{\text{inact}}[I]}{K_i + [I]} \quad (2)$$

Determination of IC_{50} values for HRPC inhibition

HRPC (10 nM) was preincubated at 25 °C in 100 mM sodium phosphate, pH 7.0, containing 1 mM H₂O₂ and 0.001–50 mM hydrazide for 1 min prior to assaying activity. Guaiacol-oxidizing activity was assayed as described above, and the percent remaining activity was plotted against hydrazide concentration $[I]$ to generate titration curves that were fit to:

$$y = \frac{(a - b)}{1 + ([I]/IC_{50})^c} + b \quad (3)$$

where y is the percentage remaining activity, a is activity at $[I] = 0$, b is activity at $[I] \rightarrow \infty$, and c is the slope when $[I] = IC_{50}$ [25].

CO trapping of HRPC(Fe^{II})

Aliquots (2 ml) of 100 mM buffer (sodium phosphate at pH 7.0, 8.0 and 12.0; Taps at pH 9.0; Caps at pH 10.0) were de-aerated and saturated with CO in 3-ml cuvettes fitted with septa. HRPC(Fe^{III}) was added to 5.5 µM and the cuvettes were flushed with CO for a further 5 min at 25 °C prior to the addition of BZH (25 mM) from a CO-saturated stock solution. Spectra were recorded at 30-s intervals for a period of 30 min, and compared with a HRPC(Fe^{II}CO) reference prepared by addition of 10 µl of a saturated dithionite solution to 2 ml of 5.5 µM HRPC(Fe^{III}) saturated with CO.

O₂ and H₂O₂ concentration measurements

Solutions of 2.5 µM HRPC in 100 mM buffer (as detailed in the previous section) were prepared and aliquots transferred to a micro oxygen chamber (reaction volume 0.6 ml; Instech Laboratories, model SYS600) coupled to a dual oxygen electrode amplifier (Instech Laboratories, model 203). BZH was added to 25 mM after 1 min and the O₂ concentration was monitored for 30 min.

H₂O₂ concentrations were determined using the Pierce perOXoquant kit. Aliquots of the BZH-containing solutions were mixed at a 1:10 ratio with a solution containing 250 µM ammonium ferrous sulphate, 25 mM H₂SO₄, 100 mM sorbitol and 125 µM Xylenol Orange. Following incubation at room temperature for 20 min the concentration of the resulting Xylenol Orange–Fe^{II} complex was determined by comparing the absorbance at 560 nm with a standard curve. The concentration of the H₂O₂ stock solution used to prepare the standard curve was determined spectrophotometrically ($\epsilon_{240} = 43.6 \text{ M}^{-1} \cdot \text{cm}^{-1}$) in water.

Isolation and characterization of modified haems from HRPC

A haem-extraction protocol described by Ator and Ortiz de Montellano [2] was used with minor modifications. Sufficient inhibitor ($I = 2\text{--}6 \text{ mM BZH}$ or $I = 3\text{--}8 \text{ mM PHZ}$ depending on the $I/\text{H}_2\text{O}_2$ ratio; see Figure 3E, below) and H₂O₂ (3–6 mM H₂O₂ depending on the $I/\text{H}_2\text{O}_2$ ratio; see Figure 3E, below) were added to 2.5-ml solutions of 20 µM HRPC to inhibit its guaiacol-oxidizing activity within 2 min. Following a 10-min incubation at room temperature the inactivated HRPC solutions were desalted by gel filtration on 9-ml G-25 columns (PD10; Amersham Biosciences). The protein-containing eluates (approx. 3.5 ml) were then acidified with 1 ml of glacial acetic acid saturated with NaCl, and the haems were extracted with three 3-ml portions

Figure 2 (A) Effect of BZH on the absorption spectrum of 2.5 μ M HRPC at pH 10.0 and (B) effect of pH on O₂ consumption by 25 mM BZH and 2.5 μ M HRPC

(A) Difference spectra (spectrum of HRPC + BZH minus spectrum of HRPC) are shown at 30 s (dashed line), 2 min (solid line) and 30 min (dotted line) following the addition of 25 mM BZH. (B) Effect of pH on O₂ consumption by 25 mM BZH and 2.5 μ M HRPC. BZH was added to the HRPC-containing solutions at the 1 min point and O₂ consumption was monitored at pH 7.0 (○), 8.0 (▽), pH 10.0 (△), and 12.0 (◇). O₂ consumption was also measured in a control solution containing only 25 mM BZH in 100 mM sodium phosphate, pH 12.0 (+).

of ethyl acetate. The combined organic layers were washed with water, evaporated to dryness under a stream of argon, and dissolved in 250 μ l of HPLC solvent A (methanol/water/acetic acid, 68:32:10 by vol.). Aliquots were separated by isocratic reversed-phase HPLC with solvent A on a Hewlett Packard 1090 HPLC with a Vydac C₁₈ column (1 mm $\times$ 250 mm) that was directly connected to the electrospray ionization source of a ThermoFinnigan SSQ 7000 single quadrupole mass spectrometer. The needle voltage was 4.5 kV, the capillary temperature was 210 °C, and online acquisition of mass spectra was performed at a rate of 5 s/scan.

RESULTS

Oxidation of BZH catalysed by HRPC

Under aerobic conditions at pH 10.0, HRPC is partially converted to a low-spin haem species in the presence of 25 mM BZH as evidenced by the presence of peaks at 543 and 577 nm in the difference spectra (Figure 2A, dashed line). Compound III [HRPC(Fe^{II}O₂), oxypoxidase] exhibits absorption maxima at these wavelengths [26]. Since the presence of 50 nM catalase did not eliminate Compound III formation (results not shown), it is not derived from the reaction of HRPC with H₂O₂ generated in solution. O₂ consumption is observed and increases with pH in solutions containing both HRPC and BZH (Figure 2B). However, in solutions containing BZH (e.g. pH 12.0 data, Figure 2B, +)

or HRPC alone the O₂ concentration remains constant. Approx. 50 % of HRPC is trapped as the Fe^{II}CO adduct (results not shown) following a 10-min incubation of the peroxidase with 25 mM BZH under saturating CO.

Kinetics of HRPC inactivation by BZH and H₂O₂

Inhibition of HRPC by BZH/H₂O₂ exhibits several characteristics common to both metabolically activated and mechanism-based inhibitors. These characteristics include: (i) time dependence as observed for HRPC inactivation by BZH/H₂O₂ in Figures 3(A) and 3(B), (ii) substrate protection as seen in the presence of equimolar guaiacol (Figure 3C) and (iii) covalent modification since the removal of low-molecular-mass species via gel filtration did not restore activity (results not shown). The rate of HRPC inactivation increased with enzyme concentration (Figure 3D), indicating that an activated form of BZH is released and rebound prior to inactivation. This distinguishes metabolically activated inhibitors from mechanism-based inhibitors, which are not released prior to enzyme inactivation. The observation that the inactivation efficiency of HRPC increases as the molar ratio of BZH/H₂O₂ decreases (0.5 > 1 > 2; Figure 3E) suggests that multiple oxidation steps are required to generate the activated inhibitor, and provides further evidence that BZH is a metabolically activated inhibitor. The rate of inactivation (k_{obs}) for a second aliquot of HRPC (100 nM) added to an incubation of 100 nM HRPC with 500 μ M BZH and 500 μ M H₂O₂, following complete inactivation (1 h), was identical to that of the first aliquot, indicating that the activated BZH species is labile.

Haem spectral changes during HRPC inactivation by H₂O₂ alone versus BZH/H₂O₂

Addition of 1 mM H₂O₂ to 2.5 μ M HRPC (1.5 nmol) in the absence of a donor substrate resulted in HRPC(Fe^{II}O₂) formation, which reached a maximum at approx. 3.5 min (Figure 4A, solid line) and decayed at longer times (Figure 4B, ○). After 30 min, 0.43 μ mol of H₂O₂ was consumed, 0.12 μ mol of O₂ had been produced, and the spectrum was dominated by a previously reported species [27] with a maximum at 670 nm (p-670, Figure 4A, dotted line; Figure 4B, ◆). In the presence of 25 mM BZH p-670 formation was accelerated (Figure 4C, ◆) and H₂O₂ consumption dropped as p-670 increased in intensity. After 30 min, only 0.1 μ mol of H₂O₂ was consumed and O₂ (0.05 μ mol) was also consumed, in contrast to the O₂ production observed in the absence of BZH. A second species with 820-nm absorption (p-820) was formed within the mixing time and decayed over 30 min (Figure 4C, △).

Isolation of modified haems resulting from HRPC inactivation by BZH and PHZ

The prosthetic group was extracted from untreated HRPC (control, Figure 5A), and HRPC inactivated at BZH/H₂O₂ molar ratios of 2 (Figure 5B), 1 (results not shown) and 0.5 (Figure 5C). The extracts were separated by reversed-phase HPLC and analysed by MS. The absorption spectra (Figure 6) of the main peaks in chromatogram B (BZH/H₂O₂ = 2) were identical to those of the corresponding peaks in chromatogram C (BZH/H₂O₂ = 0.5). Peak 1 (m/z 583) contained the p-670 prosthetic group as shown by its strong absorbance centred at 680 nm (Figure 6A). Peaks 2 (m/z 632), 4 (m/z 616, the main

Figure 3 Kinetics of HRPc inactivation by BZH/H₂O₂

(A) A subset of the data is shown for the inactivation of HRPc at 0–5 mM BZH (●, 0 mM; △, 0.020 mM; ×, 0.039 mM; ○, 0.078 mM; +, 0.625 mM; ▽, 2.5 mM; ◆, 5.0 mM). (B) Plot of k_{obs} against [BZH] fit to eqn (2). (C) Substrate protection of HRPc by guaiacol. HRPc (100 nM) was preincubated with 500 μM H₂O₂ only (△), or 500 μM BZH/H₂O₂ plus guaiacol (◆, 0 μM ; ○, 250 μM ; +, 500 μM), and assayed for guaiacol-oxidizing activity between 0.5 and 60 min. (D) Effect of HRPc concentration on its inactivation by incubation with 1 mM BZH plus 2 mM H₂O₂ for 0–20 min. (E) Effect of BZH/H₂O₂ ratio on HRPc inactivation. Aliquots of BZH and H₂O₂ were added incrementally to 20 μM HRPc at [BZH]/[H₂O₂] ratios of 2 (○), 1 (+) and 0.5 (◆) and the activity was measured 2 min after each addition.

peak in the control; Figure 5A) and 5 (m/z 720) exhibited similar absorption spectra (Figures 6B–6D). The spectra of peak 4 from BZH/H₂O₂-inactivated HRPc (Figure 6C) and untreated HRPc were identical, and were assigned to native haem. Based on their m/z values and the haem adducts previously observed for HRPc inactivation by PHZ [2], peaks 2 and 5 were assigned to hydroxyl- and benzoyl-haem, respectively. In contrast to peak 1, which increased in intensity with decreasing BZH/H₂O₂ ratios ($2 < 1 < 0.5$), peaks 2 and 5 were maximal in the BZH/H₂O₂ = 2 sample (Figure 5B). Peaks 3, 6 and 7 (Figure 5B) exhibited absorption spectra similar to native haem (Figure 6C) but were not identified by electrospray ionization MS. Peak 7 was also observed in the control extract (Figure 5A), which identifies it as an impurity in HRPc.

Haems were also extracted from HRPc exposed to PHZ/H₂O₂ at ratios of 2 and 0.5 (results not shown). These were identified as hydroxyl- (m/z 632), native (m/z 616) and phenyl-protoporphyrin IX haem (m/z 692), consistent with the previous report of 8-hydroxymethyl-haem and δ -meso-phenyl-haem formation in PHZ turnover by HRPc and H₂O₂ [2].

Kinetics of HRPc inactivation by monosubstituted arylhydrazides plus H₂O₂

The 21 arylhydrazides listed in Table 1 exhibit similar kinetic parameters for the time-dependent inactivation of HRPc as BZH (Figure 3B). The measurement of enzyme activity by transfer of aliquots from preincubations to assay conditions resulted in the presence of 0–1 mM and 0–5 mM hydrazide in the activity assays for the determination of K_1 and k_{inact} , and IC_{50} values, respectively. However, the ability of guaiacol (5 mM in the assays) to provide complete protection against the inactivation of HRPc by an equimolar concentration of BZH (Figure 3C) demonstrates that competition between hydrazide and guaiacol for oxidation by HRPc in the assays is not a significant source of error.

DISCUSSION

Oxidation of BZH catalysed by HRPc

The proposed reactions involved in HRPc-catalysed O₂ consumption in BZH solutions are summarized in Scheme 1. In the

Figure 4 Haem spectral changes accompanying HRPc inactivation by H_2O_2 and BZH/ H_2O_2 in 100 mM phosphate buffer, pH 7.0

(A) Spectra of 2.5 μM HRPc (dashed line) and at 3.5 min (solid line) and 30 min (dotted line) following addition of 1 mM H_2O_2 . (B and C) Absorption intensities at 580 nm ($\circ$), 670 nm ($\blacklozenge$) and 820 nm ($\triangle$) monitored versus time for 2.5 μM HRPc plus 1 mM H_2O_2 in the absence (B) and presence (C) of 25 mM BZH.

model proposed for MPO-catalysed oxidation of 4- NH_2 -BZH (4-aminobenzhydrazide), the hydrazide undergoes free-metal-ion-catalysed oxidation to produce H_2O_2 , which is subsequently consumed in the MPO-catalysed oxidation of 4- NH_2 -BZH [10]. Free-metal-ion-catalysed oxidation has also been proposed for INH and PHZ [13,28,29] but no O_2 consumption was observed here in BZH solutions in the absence of HRPc (Figure 2B). O_2 consumption was observed in solutions containing both BZH and HRPc (Figure 2B). As proposed in Scheme 1, the BZH anion (see below) is oxidized by the ferric enzyme to give $\text{HRPc}(\text{Fe}^{\text{II}})$, which binds O_2 with a rate constant of $5.3 \times 10^4 \text{ M}^{-1} \cdot \text{s}^{-1}$ to form $\text{HRPc}(\text{Fe}^{\text{II}}\text{O}_2)$ [26]. Direct evidence for $\text{HRPc}(\text{Fe}^{\text{II}})$ formation is provided by the trapping of the $\text{Fe}^{\text{II}}\text{CO}$ adduct of HRPc under saturating CO. Also, the addition of catalase to HRPc/BZH solutions had no effect on the formation of $\text{HRPc}(\text{Fe}^{\text{II}}\text{O}_2)$, eliminating a role for H_2O_2 in this process.

$\text{HRPc}(\text{Fe}^{\text{II}}\text{O}_2)$ can reversibly eliminate O_2 or $\text{O}_2^{\cdot-}$ to form $\text{HRPc}(\text{Fe}^{\text{II}})$ or $\text{HRPc}(\text{Fe}^{\text{III}})$, respectively [30], or accept three electrons and release the bound O_2 as H_2O . Compounds I and II

Figure 5 HPLC analysis of the haems extracted from inactivated HRPc

Haem was extracted from (A) a control sample of untreated HRPc, and from HRPc inactivated with (B) a 2:1 ratio of BZH/ H_2O_2 or (C) a 1:2 ratio of BZH/ H_2O_2 .

are intermediates in the latter process, which regenerates resting $\text{HRPc}(\text{Fe}^{\text{III}})$ (Scheme 1). Thus the $\text{HRPc}(\text{Fe}^{\text{III}})$ – $\text{HRPc}(\text{Fe}^{\text{II}}\text{O}_2)$ cycling shown in Scheme 1 will result in BZH oxidation and O_2 consumption. O_2 will also be consumed by reaction with the $\text{BZH}^{\cdot-}$ radicals generated in Scheme 1 to yield either $\text{O}_2^{\cdot-}$ or peroxy radicals by adding O_2 [13]. Reaction of $\text{HRPc}(\text{Fe}^{\text{III}})$ with $\text{O}_2^{\cdot-}$ would allow further HRPc-catalysed oxidation of BZH and enhance O_2 consumption by increasing $\text{BZH}^{\cdot-}$ radical formation. The spectrum of the $\text{HRPc}(\text{Fe}^{\text{III}})$ –BZH binary complex was observed (Figure 2A) when anoxic conditions had been reached (e.g. > 30 min at pH 10; Figure 2B, $\triangle$). This binary ferric complex is stable in the absence of O_2 [31], which reveals that reduction of $\text{HRPc}(\text{Fe}^{\text{III}})$ by BZH is O_2 (or CO) driven.

Rates of $\text{HRPc}(\text{Fe}^{\text{II}}\text{CO})$ formation (results not shown) and O_2 consumption (Figure 2B) increase with pH, suggesting that the donor to $\text{HRPc}(\text{Fe}^{\text{III}})$ is BZH^- rather than its neutral form ($\text{pK}_a = 12.5$ [32]). Given that the reduction potential, E° , of $\text{HRPc}(\text{Fe}^{\text{III/II}})$ is more negative at higher pH [33], $\text{HRPc}(\text{Fe}^{\text{II}}\text{CO})$ formation would probably decrease with increasing pH if unionized BZH were the reductant. The negligible O_2 consumption at pH 7.0 (Figure 2B) shows that BZH oxidation and free-radical generation is minimal at physiological pH. 4- NH_2 -BZH oxidation by MPO in the absence of added H_2O_2 is also minimal at pH 7.0 and increases above this pH [10]. In contrast,

Figure 6 Absorption spectra of peaks (A) 1, (B) 2, (C) 4 and (D) 5 from chromatogram B in Figure 4

Table 1 k_{inact} , K_i , IC_{50} and K_d values for the inactivation of HRPc by arylhydrazides

K_i and k_{inact} determined as described in the Materials and methods section. Preincubation conditions for IC_{50} determinations were 10 nM HRPc, 1 mM H_2O_2 and $(1-5) \times 10^4 \mu\text{M}$ hydrazide in 100 mM sodium phosphate, pH 7.0, for 1 min at 25 °C. IC_{50} values are means from at least three experiments. MPO IC_{50} values are from [10] and K_d values are from [31]. -OH-BZH, -hydroxybenzhydrazide; -CH₃-BZH, -methylbenzhydrazide; -NH₂-BZH, -aminobenzhydrazide; -Cl-BZH, -chlorobenzhydrazide; -OH-NZH, -hydroxybenzhydrazide; -NO₂-BZH, -nitrobenzhydrazide; NICH, nicotinic hydrazide; 2-NZH, 2-naphthoic hydrazide.

Inhibitor	k_{inact} (min ⁻¹)	K_i (mM)	$\text{IC}_{50}^{\text{HRPC}}$ (mM)	$\text{IC}_{50}^{\text{MPO}}$ (mM)	K_d (mM)	K_i/K_d
2-NZH	0.14 ± 0.01	0.014 ± 0.006	0.0350 ± 0.0008		0.0052 ± 0.0001	2.7
3-CH ₃ -BZH	0.049 ± 0.006	0.7 ± 0.2	0.58 ± 0.03		0.108 ± 0.003	6.3
BZH	0.035 ± 0.003	0.08 ± 0.03	1.00 ± 0.05	0.042	0.120 ± 0.002	0.66
3-OH-BZH	0.073 ± 0.003	0.7 ± 0.1	0.8 ± 0.2		0.120 ± 1	5.6
4-CH ₃ -BZH	0.088 ± 0.004	0.45 ± 0.05	0.48 ± 0.03		0.143 ± 5	3.1
4-NH ₂ -BZH	0.45 ± 0.02	1.4 ± 0.2	2.2 ± 0.4	0.002	0.23 ± 0.02	6.1
3-NH ₂ -BZH	7 ± 2	60 ± 20	1.5 ± 0.1		0.25 ± 0.01	320
3-Cl-BZH	0.025 ± 0.003	1.4 ± 0.5	2.4 ± 0.1		0.27 ± 0.01	5.2
4-Cl-BZH	0.33 ± 0.01	6.0 ± 0.5	2.0 ± 0.2	0.25	0.30 ± 0.01	20
4-OH-BZH	0.41 ± 0.01	0.28 ± 0.03	0.52 ± 0.02	0.018	0.54 ± 0.02	0.52
2-OH-BZH		> 10	5.7 ± 0.2		0.63 ± 0.02	54
2-Cl-BZH	0.13 ± 0.02	7 ± 2	22 ± 1		2.05 ± 0.04	3.6
2-CH ₃ -BZH		> 10	11 ± 2		2.76 ± 0.03	13
4-NO ₂ -BZH		> 10	3.9 ± 0.3		3 ± 1	0.76
2-NH ₂ -BZH	0.47 ± 0.02	0.9 ± 0.1	1.1 ± 0.2		3.1 ± 0.2	0.27
INH	0.025 ± 0.002	1.4 ± 0.4	3.3 ± 0.2		3.36 ± 0.04	0.42
NICH	0.014 ± 0.003	0.6 ± 0.5	8.5 ± 0.1		4.6 ± 0.1	0.13
3-NO ₂ -BZH		> 10	5.9 ± 0.3		9.11 ± 0.05	0.20
2-NO ₂ -BZH	0.03 ± 0.02	9 ± 10	61 ± 4		90 ± 30	0.11
1-NZH	0.46 ± 0.01	0.94 ± 0.08	0.6 ± 0.1			
3-OH-2-NZH	0.05 ± 0.01	0.3 ± 0.2	0.25 ± 0.03			

PHZ undergoes both extensive auto-oxidation and haem-catalysed oxidation at physiological pH [20,29]. Auto-oxidation should not impede the development of hydrazides as potential therapeutics. However, recent results have demonstrated that the therapeutic effects of INH are due to its oxidation via the superoxide pathway rather than the peroxidase pathway of KatG [13,28]. Therefore, further work on hydrazide-dependent free radical generation

catalysed by different peroxidases is a necessary step in assessing the potential of these compounds as therapeutic agents.

HRPC inactivation by H_2O_2

Approx. 0.12 μmol of O_2 was produced during the consumption of 0.43 μmol of H_2O_2 (approx. 300 molar equivalents) by 1.5 nmol

Scheme 1 Mechanism of HRPC-catalysed O₂ consumption in BZH solutions

HRPC(Fe^{III}) oxidizes the BZH⁻ anion to give HRP(Fe^{III}) and the BZH• radical. O₂ reacts with the reduced peroxidase to give HRP(Fe^{II}O₂) (Compound III). Using BZH⁻ as a donor, reductive cleavage of the O–O bond of compound III yields Compound I, which is reduced by two equivalents of BZH⁻ in two single-electron steps to yield BZH• and HRP(Fe^{III}). The BZH• radicals react with O₂ to give O₂•⁻ and/or a peroxy radical. Compound III can also be formed by the reaction of O₂•⁻ with HRP(Fe^{III}). Definitions of Compounds I and II are given in the Discussion.

of HRPC over 30 min. This observation is consistent with previous reports that HRPC is able to consume H₂O₂ by a catalytic mechanism (2H₂O₂ → O₂ + 2H₂O) via the following reactions [34,35]:

where Compound I is the two-electron-oxidized intermediate formed when 1 molar equivalent of H₂O₂ reacts with HRP(Fe^{III}). The rate-limiting step in O₂ production is reaction (5), which has a rate constant of $5 \times 10^2 \text{ M}^{-1} \cdot \text{s}^{-1}$ [34]. Compound II (the species formed on one-electron reduction of Compound I) can also react with H₂O₂ (with a rate constant of $2.1 \text{ M}^{-1} \cdot \text{s}^{-1}$ [34]) to give Compound III [36,37]:

As discussed above, HRP(Fe^{II}O₂) can reversibly eliminate O₂ or O₂•⁻ (Scheme 1 [30]). Additionally, HRP(Fe^{II}O₂) decays irreversibly to p-670 (Figures 3A and 4B [27]), which contains a biliverdin-like (open-ring porphyrin lacking iron) prosthetic group (*m/z* 583). Formation of both Compound III (maximal by approx. 3.5 min) and p-670 (maximal by approx. 30 min) were observed here during the turnover of approx. 300 molar equivalents of H₂O₂ by HRPC (Figure 4C).

HRPC inactivation by BZH/H₂O₂

Inactivation of HRPC by BZH/H₂O₂ probably involves covalent modification of the enzyme since removal of low-molecular-mass species by gel filtration did not restore activity (results not shown). The correlation between HRPC concentration and the rate of enzyme inactivation (Figure 3D) suggests that release and rebinding of an activated BZH species occurs prior to inactivation, as expected for a metabolically activated inhibitor.

This is supported by the observations that inactivation is time-dependent (Figures 3A and 3B) and that equimolar guaiacol provided complete protection against inactivation (Figure 3C).

The isolation of hydroxyl-haem (*m/z* 632) and benzoyl-haem (*m/z* 720) adducts provides further evidence for covalent modification of HRPC by BZH/H₂O₂. Nonetheless, native haem was the predominant species isolated from BZH/H₂O₂-inactivated HRPC (Figure 5). The yield of hydroxyl-protoporphyrin IX haem was dramatically higher in HRPC samples treated with PHZ/H₂O₂ (results not shown) than with BZH/H₂O₂ (Figure 5). It is possible that HRPC inactivation by BZH/H₂O₂ is due predominantly to modification of the polypeptide rather than the haem, and a phenyl-protein adduct was detected (but not localized) in PHZ/H₂O₂-inactivated HRPC [2]. Modification of protein residues around the active site by benzoyl adduct formation would result in diminished access to reducing substrates but not to H₂O₂, thus accelerating the formation of Compound III and p-670, as observed in the presence of BZH/H₂O₂ (Figure 4C).

Based on their masses, absorption spectra (Figure 6), and the reported haem shielding by the polypeptide that directs reactants to the δ-meso-position [2,38], the modified haems extracted from BZH/H₂O₂-treated HRPC (Scheme 2, compounds 9 and 10) are assumed to be δ-meso-benzoyl-haem (*m/z* 720) and 8-hydroxymethyl-haem (*m/z* 632). The growth and decay of 825-nm absorption during the inactivation of HRPC by phenylethylhydrazine and H₂O₂ was attributed to the transient formation of an isoporphyrin cation following attack of the phenylethyl radical on the haem of Compound II [3]. The detection of transient 820-nm absorption (Figure 4C) indicates that a similar isoporphyrin cation intermediate (p-820) leads to benzoyl-haem formation (Scheme 2, compound 10) in the HRPC/BZH/H₂O₂ reaction. This transient cation is formed from compound 8 on donation of the unpaired electron to the haem iron (Scheme 2). No transient 820-nm absorption was detected in the HRPC/H₂O₂ reaction (Figure 4B), confirming that p-820 formation is hydrazide-dependent.

Formation of hydroxyl-haem (compound 9) is also shown in Scheme 2 and is based on the mechanism proposed for HRPC inactivation by PHZ/H₂O₂ [2]. Abstraction of a hydrogen atom from the haem (compound 6) by the benzoyl radical (compound 5) results in a carbon-centred radical (compound 7) that is oxidized by the Fe^{IV}=O centre. Subsequent addition of water to the cation leads to the formation of compound 9. Water rather than O₂ was observed to be the source of the hydroxyl group in the HRPC/PHZ/H₂O₂ reaction [2].

Two mechanisms for benzoyl radical generation are considered (Scheme 2). Following mechanism 1, proposed for INH activation [39], one-electron oxidation of BZH by Compound I (or Compound II) yields the BZH• radical (compound 2) which eliminates NH=NH (di-imide) to produce the benzoyl radical (compound 5). In the second mechanism, based on that proposed for PHZ activation [2], BZH is oxidized in three single-electron steps by Compounds I/II to the diazene radical (compound 4). N₂ release from compound 4 gives the benzoyl radical (compound 5), which covalently modifies the peroxidase. Both mechanisms could be operative in HRPC inactivation by BZH/H₂O₂ for the following reasons. (i) Oxidation of BZH (compound 1) would compete with oxidation of the diazene (compound 3) or inactivation by the free benzoyl radical (compound 5) so that the efficiency of inactivation would decrease at higher BZH/H₂O₂ ratios as observed in Figure 3(E). (ii) The release and oxidation of BZH• (compound 2) or the benzoyl radical (compound 5) from the active site of HRPC, or the direct formation of compound 5 from compound 2 would result in O₂ consumption, which was observed during the reaction of HRPC with BZH/H₂O₂ (results not

Scheme 2 Mechanism of BZH oxidation and modification of the haem of HRPc (based on [2])

For details see the Discussion.

shown). In fact, the peroxy radical, formed by the addition of O_2 to the acyl radical, has been reported as a major product of INH oxidation by HRPc [13].

Kinetics of HRPc inactivation by monosubstituted arylhydrazides plus H_2O_2

Table 1 summarizes K_1 , k_{inact} and IC_{50} values for HRPc inhibition by the 21 hydrazides tested. These data demonstrate that the selected hydrazides are clearly not efficient inactivators of HRPc. Nonetheless, the kinetic parameters vary approx. 5000-fold (Table 1), indicating that the aromatic ring and its substituents are important determinants of efficiency and specificity. The variation in K_1 is

reflected in large part in the equilibrium K_d values [31] since the K_1/K_d ratios are approx. 0.1–10 with a few exceptions, most notably 3-NH₂-BZH (Table 1). None of the ring substituents decreased the K_d or K_1 values of the hydrazides in comparison with BZH (Figure 2). The relatively low K_1 value for 2-NZH (2-naphthoic hydrazide; 0.014 mM, Table 1) suggests that the diazene (compound 3) and/or the naphthoyl radical (compound 5) derived from 2-NZH bind HRPc more tightly than those of the other hydrazides, which would be consistent with the low K_d (5.2 μ M, Table 1) of 2-NZH. With the exception of 3-NO₂-BZH, the IC_{50} values for the *meta*- and *para*-substituted hydrazides vary by only 3-fold or less. Substituents in the *ortho*-position result in increased IC_{50} values (Table 1), which is likely to be due to the confined active site of HRPc [38].

The high IC_{50} and low k_{inact} values for HRPc inhibition by the aromatic hydrazides in Table 1 suggest that the design of an HRPc-selective hydrazide inhibitor will be challenging. Tight inhibitor binding is important since the additional phenyl ring of 2-NZH lowers the IC_{50} and K_d values by 28- and 23-fold, respectively, relative to BZH. Addition of polar ring substituents will be desirable for solvation of larger aromatic hydrazides and selectivity will be promoted by the placement of the ring substituents. For example, substituents *ortho* to the hydrazide group are expected to decrease HRPc selectivity (Table 1).

Comparison with MPO inhibition by 4-NH₂-BZH plus H_2O_2

The inhibition of MPO by aromatic hydrazides has also been investigated in detail [8–10]. These compounds are more effective inhibitors of MPO [10], and the $IC_{50}^{HRPC}/IC_{50}^{MPO}$ ratios range from 8 (4-chlorobenzhydrazide) to 1100 (4-NH₂-BZH). The high selectivity of 4-NH₂-BZH for MPO is encouraging since it suggests that the design of peroxidase-specific aromatic hydrazide inhibitors may be feasible. Also, the relatively low IC_{50} values for MPO [10] suggest that to design hydrazide inhibitors with high affinities should be possible. This would prevent modification of other biomolecules and free radical generation by release of activated intermediates from peroxidases. Thus further investigation of the inhibition of mammalian peroxidases by hydrazides should provide tools to better define the roles of these enzymes *in vivo*.

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