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Inhibition of myeloperoxidase by salicylhydroxamic acid

Bernard DAVIES and Steven W. EDWARDS*
Department of Biochemistry, University of Liverpool, P.O. Box 147, Liverpool L69 3BX, U.K.

Salicylhydroxamic acid inhibited the luminol-dependent chemiluminescence of human neutrophils stimulated by phorbol 12-myristate 13-acetate or the chemotactic peptide *N*-formylmethionyl-leucyl-phenylalanine (fMet-Leu-Phe). This compound had no inhibitory effect on the kinetics of $O_2^{\cdot-}$ generation or O_2 uptake during the respiratory burst, but inhibited both the peroxidative activity of purified myeloperoxidase and the chemiluminescence generated by a cell-free myeloperoxidase/H_2O_2 system. The concentration of salicylhydroxamic acid necessary for complete inhibition of myeloperoxidase activity was 30–50 μM (I_{50} values of 3–5 μM) compared with the non-specific inhibitor NaN_3, which exhibited maximal inhibition at 100–200 μM (I_{50} values of 30–50 μM). Whereas taurine inhibited the luminol chemiluminescence of an H_2O_2/HOCl system by HOCl scavenging, this compound had little effect on myeloperoxidase/H_2O_2-dependent luminol chemiluminescence; in contrast, 10 μM-salicylhydroxamic acid did not quench HOCl significantly but greatly diminished myeloperoxidase/H_2O_2-dependent luminol chemiluminescence, indicating that its effects on myeloperoxidase chemiluminescence were largely due to peroxidase inhibition rather than non-specific HOCl scavenging. Salicylhydroxamic acid prevented the formation of myeloperoxidase Compound II, but only at low H_2O_2 concentrations, suggesting that it may compete for the H_2O_2-binding site on the enzyme. These data suggest that salicylhydroxamic acid may be used as a potent inhibitor to delineate the function of myeloperoxidase in neutrophil-mediated inflammatory events.

INTRODUCTION

Myeloperoxidase is a haemoprotein located within the azurophilic granules of granulocytes and constitutes up to 5% of the total cellular protein of mature neutrophils [1]. A wide range of enzymic activities have been attributed to myeloperoxidase, including peroxidase, catalase, oxidase, superoxide dismutase, DNA-binding, chlorination and bactericidal activities [2–7], and thus this protein plays an important role in the O_2-dependent processes of phagocytes. During phagocytosis it is discharged into the phagocytic vesicle by the process of degranulation, where it reacts with H_2O_2 (and possibly $O_2^{\cdot-}$) generated during the respiratory burst to form HOCl and related compounds. The potency of the myeloperoxidase/H_2O_2/halide system in bactericidal, fungicidal and tumoricidal activities is well documented, and since it may be actively secreted from neutrophils its extracellular activity may also play an important role as an inflammatory mediator.

In view of the crucial and diverse role played by myeloperoxidase in host protection, the discovery that patients with deficiencies in this enzyme have few, if any, clinical symptoms appears paradoxical [8–13]. Myeloperoxidase deficiencies are described as 'complete' or 'partial', but the latter is a somewhat arbitrary and imprecise definition since neutrophils with 'partial' deficiencies contain 22–60% of the normal activity of this enzyme [12,14]. In view of the fact that this enzyme is present in such high concentrations in normal neutrophils, even neutrophils with a substantial 'partial' deficiency may still contain sufficient amounts of this enzyme for full catalytic activity. Detection of myeloperoxidase deficiencies has been revolutionized in recent years by the introduction of automated flow cytochemistry [12,14], but, particularly in cases of 'complete' deficiencies, the extent of the deficiency must be quantified by the measurement of catalytic activity in neutrophil extracts. Indeed, in several reports of 'complete' deficiency the enzymic activity ranged from 5 to 25% of normal values [10,12], although contributions from eosinophil contamination must be critically assessed. However, it is generally accepted that patients with myeloperoxidase deficiencies do not usually suffer from recurrent bacterial infections, although the ability of these neutrophils to kill bacteria such as *Staphylococcus aureus* and *Serratia marcescens in vitro* was substantially diminished [9,14]. These patients do, however, suffer from candidal infections, and a correlation between myeloperoxidase deficiencies and leukaemia, preleukaemia and neoplasms in these patients has been reported by several investigators ([14–16] and references cited in ref. [8]).

Since it has been proposed that the activities of other, myeloperoxidase-independent, bactericidal processes are increased to compensate for the absence of this enzyme in deficiencies [9,11], the precise role of myeloperoxidase in the function of normal neutrophils is far from clear. One of the major problems in assessing the function of this enzyme is that a specific chemical inhibitor does not exist. Many studies have used the general and non-specific haem inhibitor NaN_3, but this compound also inhibits other enzymes involved in neutrophil oxidative metabolism (such as catalase), quenches $^1O_2$ and $^{\cdot}OH$ [17] and its broad toxicity renders it useless for cytotoxicity studies. Although monospecific anti-(myeloperoxidase) serum can inhibit the extracellular activity of this enzyme [18,19], the role of myeloperoxidase in normal neutrophil function can only be critically assessed when a more specific neutrophil-permeable myelo-

* To whom correspondence should be addressed.

peroxidase inhibitor is available. In the present paper we report that salicylhydroxamic acid, an inhibitor of the alternative, cyanide-insensitive, oxidase of plant mitochondria [20] and some other redox enzymes [21], appears to fulfil this function.

EXPERIMENTAL

Isolation and preparation of neutrophils

Polymorphonuclear leucocytes (neutrophils) were isolated from heparinized venous blood from healthy volunteers by utilizing either a dextran/Ficoll sedimentation procedure [18] or Mono-Poly Resolving Medium (Flow Laboratories) as in ref. [22]. After purification cells were suspended in a medium containing 120 mM-NaCl, 4.8 mM-KCl, 1.2 mM-KH_2PO_4, 1.3 mM-$CaCl_2$, 1.2 mM-$MgSO_4$, 25 mM-Hepes buffer, pH 7.4, and 0.1% bovine serum albumin. Neutrophils (> 98% purity) were counted with the use of a Fuchs–Rosenthal haemocytometer slide and used within 4 h of preparation.

Analytical methods

Chemiluminescence measurements were made in 1 ml suspensions containing 10 μM-luminol [23] with an LKB Wallac 1250 luminometer. Neutrophil suspensions contained 10^6 cells/ml, and the chemiluminescence of a myeloperoxidase/H_2O_2 system *in vitro* utilized 10 munits of myeloperoxidase/ml and concentrations of H_2O_2 as described in the text. The chemiluminescence studies of an H_2O_2/HOCl system were performed essentially as described in ref. [24] with 25 μM-H_2O_2, 16 μM-NaOCl and 10 μM-luminol. $O_2^{\cdot-}$ generation was measured as the superoxide dismutase-inhibitable reduction of cytochrome *c* in a 1 ml assay mixture containing 5×10^5 neutrophils and 75 μM-cytochrome *c* [25] by the use of a Perkin–Elmer Lambda 5 spectrophotometer, and O_2 uptake was measured on 2 ml suspensions (1.5×10^6 cells/ml) by using a Clark-type oxygen electrode (Rank Brothers, Cambridge, U.K.). Human myeloperoxidase was purified from buffy coats as described in ref. [26] and had an A_{430}/A_{280} ratio of 0.74. Myeloperoxidase secretion from neutrophils in response to treatment with 1 μM-fMet-Leu-Phe plus 5 μg of cytochalasin B/ml was as described in ref. [27], and activity was assayed by the guaiacol method [28]. Absorption spectra of purified myeloperoxidase were obtained with a Perkin–Elmer Lambda 5 spectrophotometer.

Chemicals

fMet-Leu-Phe, PMA, cytochalasin B, luminol, Hepes, guaiacol and salicylhydroxamic acid were from Sigma Chemical Co. All other chemicals were of the highest purity available.

RESULTS AND DISCUSSION

Effect of salicylhydroxamic acid and azide on neutrophil function

The effect of increasing NaN_3 concentrations on fMet-Leu-Phe-plus-cytochalasin B-stimulated neutrophil chemiluminescence is shown in Fig. 1(*a*): as the concentration of azide in the suspension was increased, so the chemiluminescence response decreased, with maximal inhibition occurring at 200 μM and an I_{50} value (concentration of inhibitor necessary for 50% inhibition) of 38 μM. A similar concentration-dependent inhibition of fMet-Leu-Phe-plus-cytochalasin B-stimulated neutrophil chemiluminescence by salicylhydroxamic acid was observed (Fig. 1*b*), but the response was more inhibitor-sensitive, with maximal inhibition at 50 μM and an I_{50} value of 5 μM. Similar inhibition profiles of chemiluminescence were observed when neutrophils were stimulated with fMet-Leu-Phe alone or phorbol 12-myristate 13-acetate (Fig. 1*b*).

Effect of salicylhydroxamic acid on oxidant generation and myeloperoxidase activity

Since luminol-dependent chemiluminescence largely measures the activity of the myeloperoxidase/H_2O_2 system [23,29], the effect of salicylhydroxamic acid observed in Fig. 1 may be due to either (*a*) inhibition of oxidant generation or alternatively (*b*) inhibition of myeloperoxidase. These alternatives were therefore tested.

As shown in Fig. 2(*a*), 10 μM-salicylhydroxamic acid did not inhibit, but slightly enhanced, $O_2^{\cdot-}$ generation by

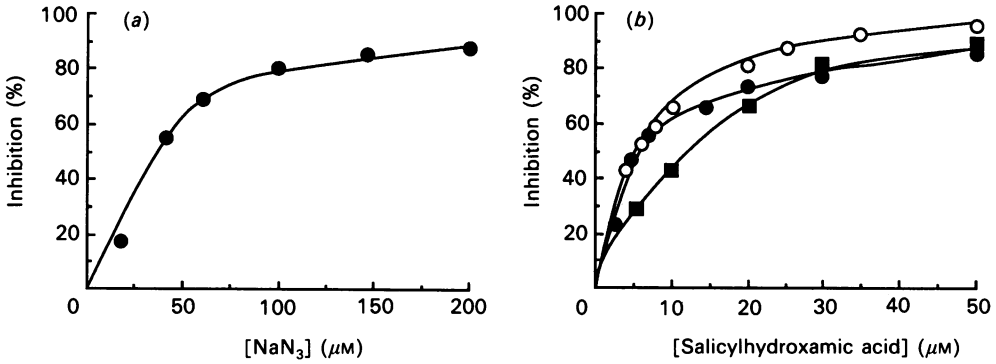

Fig. 1. Effects of azide and salicylhydroxamic acid on neutrophil chemiluminescence

Neutrophils were suspended in buffer at 10^6 cells/ml (total volume 1 ml) containing 10 μM-luminol at 37 °C in the absence or in the presence of different concentrations of NaN_3 or salicylhydroxamic acid as indicated. Chemiluminescence was stimulated by the addition of (final concentrations) 0.1 μg of phorbol 12-myristate 13-acetate/ml (●), 1 μM-fMet-Leu-Phe (○) or 1 μM-fMet-Leu-Phe plus 5 μg of cytochalasin B/ml (■). (*a*) Effect of azide; (*b*) effect of salicylhydroxamic acid. Maximal (uninhibited) rates of chemiluminescence were as follows: phorbol 12-myristate 13-acetate, 35 mV; fMet-Leu-Phe, 40 mV; fMet-Leu-Phe plus cytochalasin B, 105 mV.

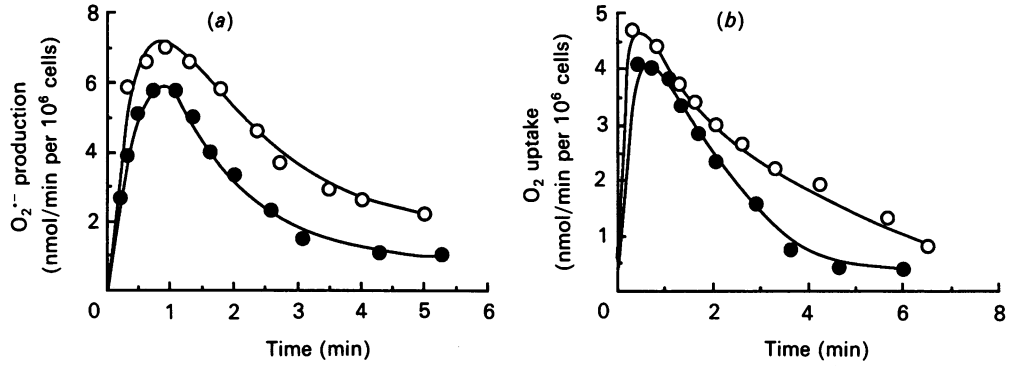

Fig. 2. Effect of salicylhydroxamic acid on respiratory-burst activity

In (*a*) neutrophils were suspended at 5×10^5 cells/ml (total volume 1 ml) in buffer containing 75 μM-cytochrome *c* in the absence (●) or in the presence (○) of 10 μM-salicylhydroxamic acid. In (*b*) neutrophils were suspended at 1.5×10^6 cells/ml (total volume 2 ml) in an oxygen-electrode chamber in the absence (●) or in the presence (○) of 10 μM-salicylhydroxamic acid. At time zero suspensions were stimulated by the addition of 1 μM-fMet-Leu-Phe plus 5 μg of cytochalasin B/ml.

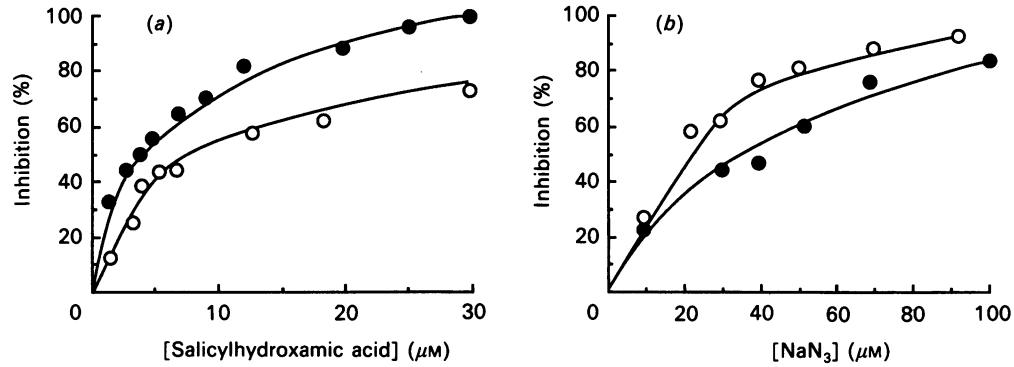

Fig. 3. Effects of salicylhydroxamic acid and azide on myeloperoxidase activity

Purified myeloperoxidase (10 munits; ●) or myeloperoxidase secreted from neutrophils after stimulation with 1 μM-fMet-Leu-Phe plus 5 μg of cytochalasin B/ml (○) was incubated in the absence or in the presence of salicylhydroxamic acid (*a*) or NaN_3 (*b*) for 5 min before the measurement of peroxidase activity by the formation of tetraguaiacol in an assay mixture containing 0.13 mM-H_2O_2 and 0.34 mM-guaiacol at pH 7.0.

Table 1. Effects of taurine and salicylhydroxamic acid on chemiluminescence

Luminol-dependent chemiluminescence of an H_2O_2/HOCl or a myeloperoxidase/H_2O_2 system was performed as described in the Experimental section. The inhibitory effects of taurine and salicylhydroxamic acid on these chemiluminescence activities are presented as means ± S.D.

Compound	Concentration (μM)	Inhibition of H_2O_2/HOCl chemiluminescence (%)	Inhibition of myeloperoxidase chemiluminescence (%)
Taurine	250	87.6 ± 6.0 ($n = 6$)	12.1 ± 3.6 ($n = 5$)
Salicylhydroxamic acid	10	12.6 ± 4.1 ($n = 6$)	86.5 ± 4.2 ($n = 5$)
	50	98.8 ± 1.6 ($n = 6$)	100 ($n = 6$)

activated neutrophils, and had little effect on O_2 uptake during the respiratory burst (Fig. 2*b*). These findings are consistent with salicylhydroxamic acid acting as an inhibitor of myeloperoxidase, since the activity of this enzyme has been shown to regulate $O_2^{\cdot-}$ generation in activated neutrophils [18]. The direct effect of salicylhydroxamic acid on myeloperoxidase was therefore investigated, by measuring its effects on the activity of the purified enzyme. Fig. 3 shows that the peroxidative activity of the purified enzyme was completely inhibited

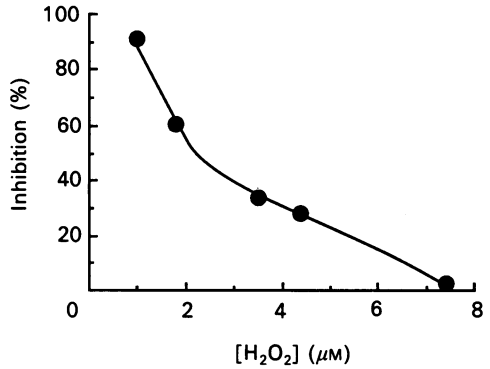

Fig. 4. Effect of H_2O_2 concentration on inhibition of myeloperoxidase chemiluminescence by salicylhydroxamic acid

Purified myeloperoxidase (10 munits) was incubated in phosphate buffer containing 10 μM-luminol in the presence or in the absence of 10 μM-salicylhydroxamic acid. Chemiluminescence was initiated by the addition of various concentrations of H_2O_2 as indicated, and the inhibitory effect of salicylhydroxamic acid on this response was calculated.

by 30 μM-salicylhydroxamic acid (I_{50} value of 4 μM), compared with the effect of NaN_3, which exhibited an I_{50} value of 25 μM (Fig. 3*b*).

Inhibition of myeloperoxidase/H_2O_2 and H_2O_2/HOCl chemiluminescence

Fig. 3 clearly shows that salicylhydroxamic acid is a potent inhibitor of the peroxidative activity of myeloperoxidase. Similarly, this compound also inhibited the luminol chemiluminescence of a cell-free myeloperoxidase/H_2O_2 system, resulting in over 85% inhibition of the response at a concentration of 10 μM (Table 1). However, photon emission by this enzymic system may result from one or two sources, namely (*a*) a peroxidase-dependent oxidation of luminol or (*b*) the co-oxidation of luminol by H_2O_2 and HOCl [24,30]. Taurine, a scavenger of HOCl [24], inhibited H_2O_2/HOCl/luminol chemiluminescence by over 85% at a concentration of 250 μM but had little effect at this concentration on luminol chemiluminescence of the myeloperoxidase/H_2O_2 system (Table 1). In contrast, 10 μM-salicylhydroxamic acid had little effect on H_2O_2/HOCl chemiluminescence (12%), but inhibited myeloperoxidase/H_2O_2 chemiluminescence at this concentration by over 85%. Higher concentrations of salicylhydroxamic acid (50 μM) inhibited H_2O_2/HOCl chemiluminescence, which may be attributed to HOCl (rather than H_2O_2) scavenging, since it had no effect on catalase activity. These results together strongly suggest that the inhibition of the myeloperoxidase/H_2O_2/luminol chemiluminescence by salicylhydroxamic acid is due to its effects as a peroxidase inhibitor, and that HOCl is not the major source of photon emission in this system since taurine had little effect.

Effect of H_2O_2 concentration on inhibition of myeloperoxidase by salicylhydroxamic acid

Purified myeloperoxidase was incubated in the presence or in the absence of 10 μM-salicylhydroxamic acid (together with 10 μM-luminol) and the chemiluminescence response was measured after the additions of various concentrations of H_2O_2 (Fig. 4). At an H_2O_2 concentration of 1 μM the chemiluminescence response was inhibited by over 90%, but as the H_2O_2 con-

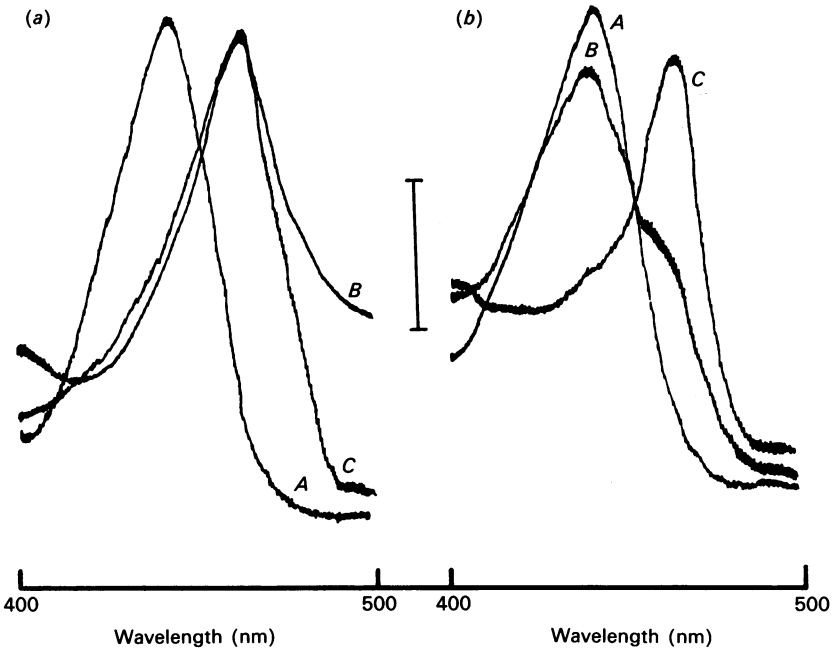

Fig. 5. Effect of salicylhydroxamic acid on the absorption characteristics of myeloperoxidase

Purified myeloperoxidase (30 munits) was suspended in phosphate buffer containing 25 mM-NaCl, in the absence (*a*) or in the presence (*b*) of 10 μM-salicylhydroxamic acid. Spectra *A*, native (oxidized) myeloperoxidase; spectra *B*, plus 2.5 μM-H_2O_2; spectra *C*, plus 10 μM-H_2O_2. The slit width was 2 nm, and the scanning speed was 2 nm/s. The bar marker represents $\Delta A = 0.003$. All spectra were recorded against a phosphate buffer blank and baselines were automatically subtracted.

centration was progressively increased so the inhibitory effect diminished. Thus at an H_2O_2 concentration of 7.5 μM no inhibition of chemiluminescence by 10 μM-salicylhydroxamic acid was observed. In order to investigate the effects of salicylhydroxamic acid on myeloperoxidase inhibition, absorption spectra of the purified enzyme were recorded in the presence and in the absence of both H_2O_2 and inhibitor. As shown in Fig. 5, native myeloperoxidase exhibited an absorption maximum at 430 nm, characteristic of the oxidized form of the enzyme [31]. Upon the addition of 2 μM- or 10 μM-H_2O_2 this absorption maximum was replaced by a peak at 457 nm due to the formation of Complex II [32]. However, although 10 μM-salicylhydroxamic acid had no detectable effect on the absorption characteristics of the native (oxidized) enzyme, this concentration of inhibitor prevented Complex II formation upon the addition of 2 μM-H_2O_2, but Compound II formation still occurred after the addition of 10 μM-H_2O_2. Thus it appears that salicylhydroxamic acid may compete for the H_2O_2-binding site on the enzyme.

Salicylhydroxamic acid has been extensively used as an inhibitor of the alternative, cyanide-insensitive, oxidase of mitochondria of plants and other eukaryotes [20,33] and has no inhibitory effect on catalase, superoxide dismutase or cytochrome oxidase [21]. It has been previously shown to inhibit horseradish peroxidase [21] and here we now show that it acts as a potent inhibitor of myeloperoxidase. This compound effectively inhibited the activity of the purified enzyme (Fig. 3) and also the chemiluminescence responses generated by myeloperoxidase/H_2O_2 both in a cell-free system and in intact cells (Fig. 1 and Table 1). It may also act as a scavenger of HOCl (Table 1), but, since 10 μM-salicylhydroxamic acid did not quench HOCl but substantially inhibited myeloperoxidase/H_2O_2 chemiluminescence (in contrast with the effects of the HOCl scavenger taurine), its effects on myeloperoxidase chemiluminescence are largely due to its action as a peroxidase inhibitor rather than as a non-specific HOCl scavenger. It had little effect on the kinetics of $O_2^{\cdot-}$ generation and O_2 uptake, other than a slight enhancement of respiratory-burst activity that would be predicted since myeloperoxidase has been shown to regulate the duration of the respiratory burst [18]. Interestingly, salicylhydroxamic acid can inhibit both the intra- and extra-cellular portions of neutrophil chemiluminescence activated by phorbol 12-myristate 13-acetate and fMet-Leu-Phe [19], and experiments have shown that it freely permeates neutrophils, by completely inhibiting the intraphagosomal chemiluminescence of phagocytosing neutrophils (B. Davies & S. W. Edwards, unpublished work).

Preliminary experiments have shown that concentrations of salicylhydroxamic acid sufficient to inhibit myeloperoxidase completely (up to 50 μM) have no detectable effect on the growth and division of a number of target cells (B. Davies & S. W. Edwards, unpublished work). Thus it will now be possible to use this inhibitor to delineate the function of myeloperoxidase in bacterial and tumoricidal killing and also in other inflammatory conditions.

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