Inhibition of neutrophil oxidant secretion by D-penicillamine: scavenging of H₂O₂ and HOCl

Martin J Ledson, Roger C Bucknall, Steven W Edwards

Abstract
D-Penicillamine inhibited oxidant secretion from human neutrophils after activation by the chemotactic peptide N-formyl-methionyl-leucyl-phenylalanine (fMet-Leu-Phe), as assessed by luminal dependent chemiluminescence. In contrast, this drug had little effect on either intracellular oxidant production or lucigenin dependent chemiluminescence activated by the same agonist. The drug was shown to scavenge both H₂O₂ and HOCl in a cell free luminal chemiluminescence system, though its ability to scavenge HOCl was greater than that for H₂O₂. Both these oxidants could oxidise the drug, but again HOCl was more potent than H₂O₂. When D-penicillamine was oxidised by exposure to H₂O₂ it could no longer serve as a scavenger of secreted oxidants from neutrophils. These data suggest that in vivo the preferential scavenging of HOCl may be important under pathological conditions where secreted myeloperoxidase may be functional.

D-Penicillamine is a slow acting drug used in the treatment of rheumatoid arthritis, but despite its widespread use its biological mode of action remains obscure. Several mechanisms have been proposed to explain its efficacy, including effects of collagen metabolism, immunopathological effects in the presence of copper, superoxide dismutase activity, and ability to scavenge H₂O₂ and HOCl, both of which are products of activated phagocytes. Furthermore, a more direct inhibitory effect of this drug on the activity of the neutrophil enzyme myeloperoxidase has been proposed as it can promote the formation of compound III, which is inactive in the generation of HOCl.

In view of the fact that neutrophils within inflamed joints of patients with rheumatoid arthritis have been activated to secrete reactive oxidants and myeloperoxidase, one explanation for the therapeutic activity of D-penicillamine may reside in its ability to scavenge the neutrophil products H₂O₂ and HOCl within such joints. However, only modest inhibitory effects of this drug on neutrophil dependent oxidant production have been described. For example, after stimulation of neutrophils by phorbol myristate acetate, D-penicillamine at 0·1 mM only inhibited the activated chemiluminescence by 20%, and when opsonised zymosan was used as the stimulus the drug was reported to enhance the chemiluminescence response. These observations thus seem to contradict the view that D-penicillamine is an effective oxidant scavenger.

We have previously shown that activated neutrophils generate reactive oxidants both intra- and extracellularly, and that these two activities are dependent upon the nature of the stimulus used. For example, the chemotactic peptide N-formyl-methionyl-leucyl-phenylalanine (fMet-Leu-Phe) stimulates a rapid burst of oxidant secretion which is followed by a second phase of internally generated oxidants, whereas phagocytic stimuli, such as immune complexes or opsonised zymosan, activate oxidant production which is largely intracellular. Furthermore, the extent of myeloperoxidase secretion (and hence ability to constitute an extracellular myeloperoxidase-H₂O₂ system) is also dependent upon the nature of the neutrophil activating factor. Thus one explanation for the reports of an apparently low ability of D-penicillamine to scavenge neutrophil derived oxidants is that the experimental conditions used largely activated intracellular oxidant production, which may be inaccessible to the drug. This work aimed at establishing if D-penicillamine scavenged extracellularly released neutrophil oxidants (as these are likely to be of importance in inflammatory joint disease) and measuring the relative affinities of the drug for H₂O₂ and HOCl to determine its effects on oxidant production, dependent and independent of myeloperoxidase.

Materials and methods
ISOLATION AND PREPARATION OF NEUTROPHILS
Neutrophils were prepared from 20 ml heparinised venous blood from healthy volunteers using M-PRM (Flow Laboratories). After purification they were suspended in a Krebs/HEPES buffer containing (mmol/l): NaCl 120; KCl 4·8; KH₂PO₄ 1·2; CaCl₂ 1·3; MgSO₄, 1·2; HEPES (N-2-hydroxyethylpiperazine-N’-2-ethanesulphonic acid) 25 (pH 7·4); 0·1% bovine serum albumin. Cells were counted after a suitable dilution in the above buffer with a Fuchs–Rosenthal haemocytometer slide and used with four hours of preparation.

ANALYTICAL METHODS
Chemiluminescence
Neutrophils were suspended in buffer containing either 10 μmol/l luminol (5-amino-2,3-dihydro-1,4-phthalazinedione) or 25 μmol/l lucigenin (bis-N-methylacridinium nitrate) at 10⁶ cells/ml, in a total volume of 1 ml. After the addition of 1 μmol/l fMet-Leu-Phe (final concentration) chemiluminescence was measured with an LKB Wallac 1250 luminometer.
Effect and at were minute responses (b) of period presence buffer containing penicillamine on either fMet-Leu-Phe measured. was from luminol, lucigenin, fMet-Leu-Phe measured. Absorption meter. of superoxide dismutase. agents were superoxide generation. When neutrophil suspensions which requires the response, is peroxidase, was initial, rapid (within one minute) peak is shown as Figure 1. Effect of d-penicillamine on neutrophil chemiluminescence. Neutrophils (10⁶/ml) were suspended in buffer containing either (A) 10 μmol/l luminol or (B) 25 μmol/l lucigenin at 37°C in (b) the presence and (a) the absence of 10⁻⁴ mol/l d-penicillamine. After a two minute preincubation period fMet-Leu-Phe was added (final concentration 1 μmol/l) and the chemiluminescence responses measured.

Figure 2. Effect of d-penicillamine on intra- and extracellular oxidant production. Neutrophils (10⁶/ml) were suspended in buffer containing 10 μmol/l luminol at 37°C in the presence and absence of d-penicillamine at the concentrations indicated. After a two minute preincubation period fMet-Leu-Phe (final concentration 1 μmol/l) was added and the chemiluminescence responses measured. Extracellular oxidant secretion was measured as the peak response seen at one minute after stimulation, whereas intracellular production was measured as the peak response at three to five minutes. Values presented are the mean response (with standard deviations) of four determinations on different preparations of neutrophils and shown as percentage inhibition of the responses noted in the absence of drug.
scavenged O₂ either generated in a cell free xanthine/xanthine oxidase system or else secreted from activated neutrophils, by determining its effects on cytochrome c reduction were frustrated by the fact that the drug itself rapidly reduced the cytochrome c. The ability of the drug to scavenge H₂O₂ and HOCl was determined using a cell free chemiluminescence system. The system is based on the fact that when HOCl is added to mixtures of H₂O₂ and luminol a rapid chemiluminescence burst is initiated. Preincubation of the H₂O₂/luminol mixture with D-penicillamine reduced the chemiluminescence response initiated by HOCl by about 50% (table): doubling the H₂O₂ concentration restored activity, indicating that (a) the drug itself had no effect on the assay and (b) that it could reduce the chemiluminescence response by scavenging the H₂O₂. When the drug was added to the HOCl solution before its addition to the H₂O₂/luminol mixture, however, the inhibitory effect was much greater. Again, doubling the HOCl concentration restored activity, but merely doubling the H₂O₂ concentration did not restore activity to the HOCl/D-penicillamine mixture. These results indicate that D-penicillamine can scavenge both oxidants, but that its ability to scavenge HOCl is much greater than its ability to scavenge H₂O₂.

It seemed likely that when D-penicillamine scavenged these oxidants it might itself have become oxidised during the reaction. Absorption spectra of the native drug were recorded between 190 and 250 nm in the presence and absence of a chemical reductant (sodium dithionite) and oxidant (ammonium persulphate). These spectra indicated that the native drug was in a fully reduced state and that when oxidised its absorption maximum at 200 nm was lost (figs 3 (a) and (b)). Sequential additions of known concentrations of either H₂O₂ or HOCl were then added to samples of the native (reduced) drug and absorption spectra recorded. Both oxidants oxidised the drug as indicated by the decrease in absorbance at 200 nm (figs 3 (c) and (d)). On a molar basis, however, HOCl oxidised the drug more potently than did H₂O₂ (fig 4), being about twice as effective. This indicates that D-penicillamine is a more potent scavenger of HOCl than of H₂O₂, confirming the data shown in the table.

### EFFECT OF OXIDISED D-PENICILLAMINE ON NEUTROPHIL OXIDANT PRODUCTION

If the proposed effect of D-penicillamine on

#### Effect of D-penicillamine on H₂O₂/HOCl dependent luminol chemiluminescence

<table>
<thead>
<tr>
<th>Luminal mixed with:</th>
<th>Reaction initiated by:</th>
<th>Chemiluminescence (% of control)†‡</th>
</tr>
</thead>
<tbody>
<tr>
<td>H₂O₂</td>
<td>HOCl</td>
<td>100 (9) (n=23)</td>
</tr>
<tr>
<td>H₂O₂+D-penicillamine</td>
<td>HOCl</td>
<td>47 (11) (n=9)*</td>
</tr>
<tr>
<td>2×H₂O₂+D-penicillamine</td>
<td>HOCl</td>
<td>100 (8) (n=12)**</td>
</tr>
<tr>
<td>HOCl</td>
<td>HOCl+D-penicillamine</td>
<td>8-8 (6) (n=12)**</td>
</tr>
<tr>
<td>H₂O₂</td>
<td>2×HOCl+D-penicillamine</td>
<td>105 (10) (n=5)</td>
</tr>
<tr>
<td>2×H₂O₂</td>
<td>HOCl+D-penicillamine</td>
<td>8 (5) (n=8)*†</td>
</tr>
</tbody>
</table>

H₂O₂ (8 μmol/l) was mixed with luminal (10 μmol/l) and the chemiluminescence generated upon injection of HOCl (8 μmol/l) was measured and taken as 100%. Where indicated, D-penicillamine (100 μmol/l) was added either to the luminol/H₂O₂ mixture or else to the HOCl solution and its effect on the chemiluminescence response measured.

*p<0.05, **p<0.001, using Student’s t test.  †‡x represents experiments in which the concentration of the indicated oxidant was doubled. †Number of determinations is given (n) together with the standard deviation.

![Figure 3](http://ard.bmj.com/ on June 26, 2017 - Published by group.bmj.com)  
**Figure 3**. Effects of oxidants on D-penicillamine absorption spectra. The absorption spectrum of D-penicillamine (2×10⁻³ mol/l) was recorded between 190 and 250 nm with a Perkin-Elmer lambda 5 spectrophotometer. The absorption spectrum of the native drug (scan a) was unchanged after addition of the reductant, sodium dithionite (data not shown). Spectra were then recorded after the addition of a few crystals of ammonium peroxysulphate (scan b), 9 mmol/l H₂O₂ (scan c), and 9 mmol/l HOCl (scan d).

![Figure 4](http://ard.bmj.com/ on June 26, 2017 - Published by group.bmj.com)  
**Figure 4**. Oxidation of D-penicillamine by H₂O₂ and HOCl. D-Penicillamine was incubated in the absence and presence of varying concentrations of either H₂O₂ (●) or HOCl (●), as described in the legend to fig 3. The oxidation of the drug was then assessed as the decrease in absorbance at 200 nm as a particular oxidant concentration and is expressed as a percentage of the untreated (native) drug absorbance.
neutrophil oxidant secretion was to scavenge extracellular oxidants (figs 1 and 2) and the drug itself became oxidised during this process (fig 4) then the oxidised drug should have little or no effect on neutrophil function. Thus a solution of D-penicillamine was mixed with just sufficient H$_2$O$_2$ for its complete oxidation, and its ability to scavenge neutrophil derived oxidants generated in response to fMet-Leu-Phe was determined. Whereas the native (reduced) drug inhibited oxidant secretion by over 60%, the oxidised form of the drug had no effect on oxidant secretion (fig 5). These data confirm that D-penicillamine scavenges secreted oxidants, thus reducing oxidant production by neutrophils, itself becoming oxidised (and non-functional) in the process.

**Discussion**

The data presented in this paper confirm that D-penicillamine is a potent scavenger of both H$_2$O$_2$ and HOCl, two products of the respiratory burst of activated neutrophils. As the relative concentrations of these two oxidants at inflammatory sites will depend on the levels of extracellular activity of the O$_2$/H$_2$O$_2$ generating NADPH oxidase and the activity of secreted myeloperoxidase, it is important to identify the potency of D-penicillamine in scavenging both H$_2$O$_2$ and HOCl. We have shown here that on a molar basis D-penicillamine is at least twice as efficient at scavenging HOCl than at scavenging H$_2$O$_2$. This may be of pathological importance as we have previously shown that although neutrophils isolated from the synovial fluid of patients with rheumatoid arthritis have been activated in vivo to secrete reactive oxidants, they have also been activated to secrete myeloperoxidase in these joints, and the myeloperoxidase is in a molecular form which indicates that it has reacted with H$_2$O$_2$. Furthermore, factor(s) present within synovial fluid can activate the myeloperoxidase-H$_2$O$_2$ system, and hence the ability of D-penicillamine to scavenge HOCl within the inflamed joint may be of more importance than its ability to detoxify H$_2$O$_2$.

Previous work has shown only a modest inhibitory effect of D-penicillamine on phorbol myristate acetate stimulated luminol chemiluminescence of neutrophils, and even a stimulatory effect on opsonised zymosan activated chemiluminescence, despite the fact that the drug efficiently scavenges oxidants in cell free systems. Neither of these experimental conditions, however, results in optimal secretion of reactive oxidants from neutrophils. In our study we used the chemotactic peptide fMet-Leu-Phe to stimulate neutrophils because this agonist activates clearly defined phases of oxidant secretion (detected within one minute of addition) followed by intracellular oxidant production. Under these conditions 10$^{-4}$ mol/l D-penicillamine inhibited oxidant secretion by over 70% (fig 1), compared with only 20% inhibition of phorbol myristate acetate stimulated luminol chemiluminescence. The drug had little effect on intracellular oxidant production and this phenomenon was not dependent on concentration. The biological function of such intracellular oxidant generation and the significance of the effects of D-penicillamine on this phenomenon are unknown.

Luminol chemiluminescence requires the activity of the myeloperoxidase–H$_2$O$_2$ system, whereas lucigenin is thought to measure only O$_2^\cdot$ or H$_2$O$_2$ secretion, or both, though the precise molecular species reacting with the latter lumigenic probe has not been unambiguously defined. Therefore, it is of considerable interest to note that although D-penicillamine scavenged extracellular luminol chemiluminescence, it had little effect on lucigenin chemiluminescence (fig 1). This implies that either lucigenin does not in fact measure O$_2^\cdot$ or H$_2$O$_2$, or else that the predominant effect of D-penicillamine in neutrophils is to scavenge a myeloperoxidase dependent product (that is, HOCl) rather than
Oxidant scavenging by D-penicillamine

H₂O₂. Therefore, we tested for the ability of D-penicillamine to scavenge both H₂O₂ and HOCl and found that it is a more potent scavenger of the latter oxidant.

The ability of D-penicillamine to scavenge H₂O₂ and HOCl resides in its thiol group. The drug becomes oxidised during its interaction with oxidants, and the oxidised form of the drug is incapable of interfering with the processes of neutrophil oxidant production. Although the serum concentration of this drug may be as high as 20 μmol/l, it’s effective concentration within joints, which may be exposed to high levels of neutrophil derived oxidants, will be reduced owing to its oxidation, and this may be of pathological importance. In view of the emerging evidence indicating that an extracellular myeloperoxidase–H₂O₂ system is operational in the rheumatoid joint, scavenging of HOCl (and to a lesser extent H₂O₂) may be one means by which the efficacy of this drug is explained.

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