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CEPHALOSPORINS ARE SCAVENGERS OF HYPOCHLOROUS ACID

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Abstract—Potential scavenging properties of cephalosporins (i.e. cefamandole, cefotaxime and ceftriaxone) towards hypochlorous acid (HOCl) as well as the antibacterial activity of control and HOCl-reacted antibiotics were investigated. We found that these drugs, at therapeutically relevant concentrations, are indeed scavengers of HOCl, with ceftriaxone showing the highest anti-HOCl capacity. However, the efficiency of cephalosporins in protecting biological molecules is also related to the chemical identity of such molecules. Indeed, the polyenoic compound β-carotene is much better protected than the thiol compound GSH against HOCl attack. Moreover, the drugs do not appear to form chloramine derivatives as a result of their reaction with HOCl, and they inhibit taurine–chloramine formation. After HOCl challenge, the antibacterial activity of cefamandole, cefotaxime and ceftriaxone (tested against the standard strain *Escherichia coli* ATCC 25922) is approx. 8-, 5- and 4-fold lower, respectively, than that of the HOCl-unreacted antibiotics. The depression of the antibacterial activity of cephalosporins appears inversely related to their HOCl scavenging capacity, suggesting that the drug antioxidant groups may protect the β-lactam ring against HOCl attack. In conclusion, physiological biomolecules are protected by cephalosporins against HOCl-driven oxidative injury with varying efficiency, this antioxidant defence being a consequence of a direct drug scavenging capacity towards HOCl. The interaction of cephalosporins with HOCl, however, results in a depression of their antibacterial activity.

Key words: cephalosporins; hypochlorous acid; inflammation; oxidative stress; antioxidant

Neutrophil-derived pro-oxidants are crucial in bacterial killing, but also do considerable tissue damage at inflammation sites because of their biological reactivity [1, 2]. Among the oxidant species generated by neutrophils, hypochlorous acid (HOCl§) can be regarded as the most toxic and abundant [1, 2]. Indeed, HOCl is a strong oxidant which readily reacts with various biochemical groups, including thiols and thioethers [1–3]. Some antibiotics widely used in clinical medicine, such as cephalosporins, possess thioether groups [4] which could react with and scavenge HOCl. This may result in biomolecule antioxidant protection during inflammatory processes. In such a context, other antibiotics, such as tetracycline and rifampicin, have been reported to protect α1-antiproteinase from HOCl-induced oxidative inactivation at therapeutically achievable drug concentrations [5]. Even though previous studies have shown that cephalosporins (i.e. cephalexin) do not antagonize the oxygen-derived free radicals superoxide anion, hydrogen peroxide and hydroxyl radical [6], the antioxidant capacity of cephalosporins towards HOCl has not yet been fully investigated. Moreover, no data are currently available concerning the possibility that the interaction of cephalosporins with HOCl could result in some changes in their antibacterial activity.

The present study was designed to investigate potential scavenging properties of cephalosporins towards HOCl, and the antibacterial activity of cephalosporins after their interaction with HOCl.

MATERIALS AND METHODS

Reagents were from Sigma Chemical Co. (St Louis, MO, USA) unless otherwise indicated. Cefamandole, cefotaxime and ceftriaxone were dissolved in pyrogen-free, bidistilled water (also used for buffer preparation), and used at blood therapeutically relevant concentrations of 18, 26, 50 and 100 μg/mL [4].

Evaluation of HOCl scavenging properties of cephalosporins and of possible drug chloramine formation. It is known that HOCl induces β-carotene bleaching [7–9], which can be counteracted by HOCl scavengers [8, 9]. Reaction mixtures (1.0 mL) contained 1.3 μM β-carotene in 20 mM potassium phosphate buffer, pH 7.4, with and without various cephalosporin concentrations. HOCl was added at a 'physiologically' relevant final concentration of 70 μM [1, 2, 5, 8, 9] to start the reaction, followed by 20 min incubation at 25°; the β-carotene-related absorbance values at 451 nm (A_{451}) were then recorded spectrophotometrically against appropriate

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§ Abbreviations: HOCl, hypochlorous acid; GSH, reduced glutathione; DTNB, 5,5′-dithio-bis(2-nitrobenzoic acid); TauNHCl, taurine–chloramine; MIC, minimal inhibitory concentration.

drug-containing blanks to assess specific pharmacological effects. The concentrations of sodium hypochlorite-derived HOCl were calculated using a molar extinction coefficient of $100\,M^{-1}cm^{-1}$ at 235 nm [10].

Potential HOCl scavenging effects of cephalosporins were also evaluated by investigating their ability to inhibit the oxidation of another biologically relevant molecule (GSH) induced by HOCl. Moreover, this test provides information concerning the possibility that cephalosporins could form with HOCl oxidizing chloramine derivatives. Indeed, chloramines can oxidize thiols [11]; thus, if cephalosporins formed chloramines after incubation with HOCl, GSH oxidation should be favoured and not antagonized. Thus, in a first set of experiments, 70 μM HOCl and cephalosporins were pre-incubated for 15 min at 25° before the addition of 135 μM GSH. In a second set, similar to the β-carotene test, GSH, drugs and HOCl were incubated simultaneously to compare the reaction rate of HOCl with physiological thiols with that of drug functional groups. In both cases, reaction mixtures were then incubated for 15 min at 25°; GSH content was estimated spectrophotometrically at 412 nm with 300 μM of the thiol colorimetric detector DTNB, as previously reported [12], using appropriate drug-containing blanks.

The interaction of the amino compound taurine with HOCl forms TauNHCl which, differently from taurine and HOCl, absorbs maximally at approx. 252 nm [11, 13]. Thus, we also investigated whether cephalosporins could inhibit formation of TauNHCl as a result of direct HOCl scavenging activity. After incubation of 70 μM HOCl alone or of 70 μM HOCl with cephalosporins in 50 mM potassium phosphate buffer, pH 7.4 for 15 min at 25°, taurine was added at 3 mM final concentration, followed by further incubation for 10 min at 25° [13]. TauNHCl-related absorbance values at 252 nm (A_{252}) were then recorded spectrophotometrically, using blanks formed by HOCl and drugs in buffer (3 mM taurine did not virtually absorb at 252 nm).

To qualitatively characterize possible chloramine formation due to drug-HOCl interaction [11], an analysis of the spectral properties of 70 μM HOCl alone or of 70 μM HOCl incubated for 15 min at 25° with each cephalosporin in 50 mM potassium phosphate buffer, pH 7.4, was carried out on a double-beam Varian DMS 200 spectrophotometer in the ultraviolet region (310–230 nm). Suitable antibiotic-containing blanks were used in this test.

Antibacterial activity of control and HOCl-reacted cephalosporins. To evaluate whether the antibacterial activity of cephalosporins changed after their interaction with HOCl, 104 μg/mL of each drug was incubated for 15 min at 25° with 280 μM HOCl in 10 mM potassium phosphate buffer, pH 7.4. Possible residual amounts of HOCl (which could have interfered with the test due to their strong bactericidal effects [2]) were then neutralized with 350 μM thiourea [11], and scalar antibiotic concentrations were used to assess antibacterial activity towards the standard strain *Escherichia coli* ATCC 25922. In this regard, the MIC of each drug was determined in duplicate by a standard broth microdilution method, according to the recommendations of the National Committee for Clinical Laboratory Standards [14], using a Mueller Hinton broth (Unipath Ltd, Basingstoke, U.K.). MIC is the lowest antibiotic concentration allowing no visible bacteria growth after incubation for 24 hr at 37°. Drug dilutions were carried out with a multichannel pipette delivering 100 μL per well, and 100 μL inocula with a density of approx. 10^5 cfu/mL were suspended in each well. For control experiments, only HOCl was omitted.

Statistics. Data were calculated as means ± SD of six different experiments. Drug antioxidant effects were evaluated by the one-way analysis of variance plus the Student–Newman–Keuls test [15]. $P < 0.05$ was considered as statistically significant.

RESULTS

HOCl scavenging properties of cephalosporins

As shown in Fig. 1, cephalosporins significantly protected β-carotene against HOCl-induced bleaching. At 18 and 26 μg/mL concentrations in particular, ceftriaxone was slightly more effective than cefotaxime, and both were significantly more effective then cefamandole. From 50 μg/mL on, however, significant differences between cephalosporins were not detected, indicating that at this concentration the reaction rate of cephalosporins with HOCl is high enough to afford total β-carotene protection.

A similar trend was observed when GSH was used as the oxidizable substrate and pre-incubating cephalosporins with HOCl (Table 1). These results further show drug scavenging properties on HOCl, and indicate that oxidizing chloramine by-products are apparently not generated from antibiotic–HOCl interaction. Indeed, as clearly evident at the highest drug concentration, GSH oxidation was fully prevented and chlorine species-mediated GSH oxidation was not observed. Moreover, the spectral study showed the disappearance of HOCl after its interaction with cephalosporins, as well as the absence of formation of compounds capable of absorbing in the UV region (Fig. 2). These findings demonstrate, besides a direct drug scavenging action towards HOCl, no generation of chloramine derivatives, which arise from the reaction of HOCl with NH_2 groups and absorb maximally at approx. 252 nm [11, 13]. In this regard, the absence of chloramine formation after interaction of HOCl with not only cefamandole (a NH_2-lacking drug, Fig. 3), but also with the NH_2-containing antibiotics cefotaxime and ceftriaxone (Fig. 3), indicates that the reaction rate of HOCl with NH_2 drug groups is always lower than that with other specific pharmacological groups, such as the thioethers [3]. Thus, cephalosporins preferentially scavenge HOCl without drug chloramine formation. Cephalosporins also inhibited TauNHCl production. Indeed, as expected, incubation of HOCl with taurine generated TauNHCl, which resulted in 0.027 ± 0.003 A_{252}. Notably, absorbance value at 252 nm became undetectable in the presence of 50 μg/mL of each antibiotic, demonstrating an effective drug scavenging activity towards HOCl with inhibition of TauNHCl formation.

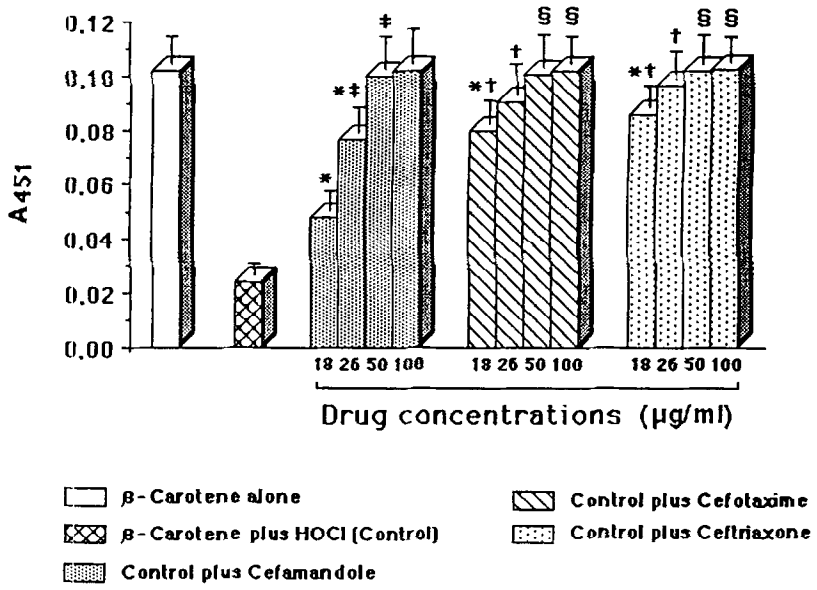


Fig. 1. Effects of cephalosporins on HOCl-induced β-carotene bleaching. β-Carotene (1.3 μM) and HOCl (70 μM) were incubated simultaneously with stated cephalosporin concentrations for 20 min at 25° in 20 mM potassium phosphate buffer, pH 7.4. Bars represent the β-carotene-related absorbance values detected spectrophotometrically at 451 nm (A_{451}), and they express means ± SD of six different experiments (see Materials and Methods section for detailed methodological explanations). The values detected with all drug concentrations are significantly different ($P < 0.05$) from those of the control; *, $P < 0.05$ versus β-carotene alone; †, $P < 0.05$ versus cefamandole; ‡, $P < 0.05$ versus the values that precede; §, $P < 0.05$ versus 18 μg/mL (one-way analysis of variance followed by the Student–Newman–Keuls test).

Table 1. Cephalosporin effects on HOCl-mediated GSH oxidation (GSH alone, 1.732 ± 0.060; control (GSH plus HOCl), 1.196 ± 0.077)

	Drug concentrations (μg/mL)			
	18	26	50	100
Cephalosporin-HOCl pre-incubated before addition of GSH				
Cefamandole	1.356 ± 0.081*	1.460 ± 0.083*‡	1.645 ± 0.079‡	1.724 ± 0.082
Cefotaxime	1.579 ± 0.060†	1.640 ± 0.071†	1.696 ± 0.063§	1.730 ± 0.055§
Ceftriaxone	1.594 ± 0.065†	1.665 ± 0.068†	1.702 ± 0.054§	1.731 ± 0.050§
GSH-cephalosporin-HOCl incubated simultaneously				
Cefamandole	1.199 ± 0.077	1.213 ± 0.079	1.229 ± 0.073	1.243 ± 0.069
Cefotaxime	1.206 ± 0.071	1.222 ± 0.076	1.241 ± 0.066	1.288 ± 0.061
Ceftriaxone	1.212 ± 0.064	1.229 ± 0.063	1.249 ± 0.057	1.302 ± 0.052‖

Oxidation of 135 μM GSH was induced by 15 min incubation at 25° with 70 μM HOCl in 50 mM phosphate buffer, pH 7.4, pre-incubating (15 min, 25°) stated drug concentrations with HOCl before addition of GSH, or incubating GSH, cephalosporins and HOCl simultaneously. GSH content was estimated via its reaction with 300 μM DTNB; the resulting absorbance values at 412 nm are reported in the Table. Results are means ± SD of six different experiments. In the experiments in which cephalosporins were pre-incubated with HOCl before addition of GSH, the values detected with all drug concentrations were significantly different ($P < 0.05$) from control values; *, $P < 0.05$ versus GSH alone; †, $P < 0.05$ versus cefamandole; ‡, $P < 0.05$ versus the values that precede; §, $P < 0.05$ versus 18 μg/mL (one-way analysis of variance followed by the Student–Newman–Keuls test). In the experiments in which GSH, cephalosporins and HOCl were incubated simultaneously, only the values obtained with 100 μg/mL of ceftriaxone were significantly higher than control values. (‖, $P < 0.05$), and significant differences among various drug concentrations were not observed (one-way analysis of variance followed by the Student–Newman–Keuls test).

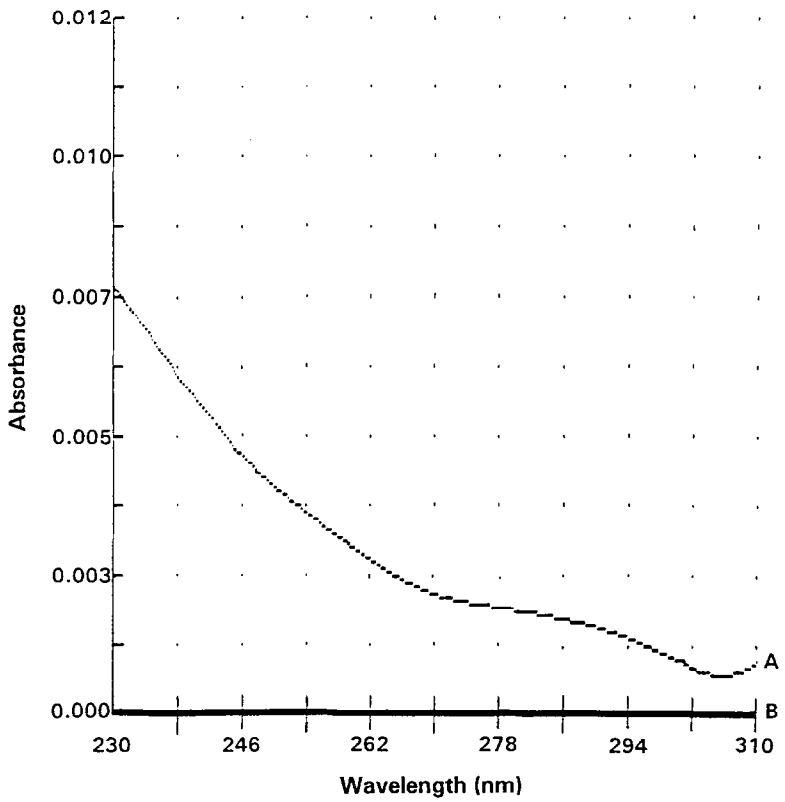


Fig. 2. Absorbance spectra of 70 μM HOCl alone (A), or in the presence of 50 μg/mL of cefamandole, cefotaxime or ceftriaxone (all three shown as B). Samples were incubated in phosphate buffer, for 15 min at 25° before being scanned in the UV wavelength range (scanning speed: 200 nm/min). Blanks were formed by cephalosporins in buffer. After interaction with cephalosporins, HOCl and its spectrum totally disappeared, and formation of 252 nm absorbing chloramine derivatives was not observed.

When GSH, antibiotics and HOCl were incubated simultaneously, a significant protection of GSH against HOCl-driven oxidation was observed only with 100 μg/mL of ceftriaxone, which resulted in approx. 9% inhibition of tripeptide oxidation (Table 1). At 100 μg/mL, cefotaxime and cefamandole gave about 7.5 and 4.5% inhibition of GSH oxidation, respectively (P = NS). In agreement with previous reports, these data are indicative of the high reactivity of HOCl towards thiol groups with respect to other functional groups, included thioethers [1–3, 11].

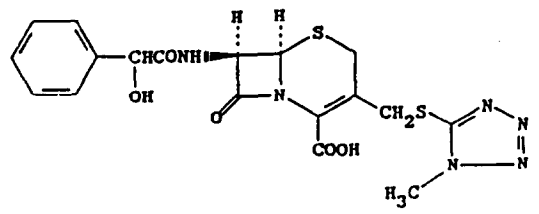
Antibacterial activity of control and HOCl-reacted cephalosporins

In comparison with the HOCl-unreacted antibiotics, cefamandole, cefotaxime and ceftriaxone, after HOCl interaction, underwent a decrease in their anti-bacterial activity approx. 8-, 5- and 4-fold, respectively (Table 2).

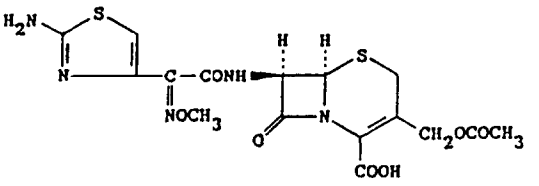
DISCUSSION

The present study shows that cephalosporins are scavengers of HOCl, and that they undergo a depression in their antibacterial activity after interaction with HOCl. In such a context, it should be noted that ceftriaxone has two thioether groups and one heterocyclic ring linked to the dihydrothiazine ring (Fig. 3). Even though cefotaxime also has two thioether groups, it lacks the heterocyclic ring (Fig. 3) while cefamadole has only one thioether group (Fig. 3). The number of thioether groups is important, since these can readily react with HOCl [1–3, 11]. The presence of heterocyclic rings may further favour drug reactivity towards HOCl-related electrophilic reactions [16]. These chemical features may explain why the anti-HOCl activity of ceftriaxone is higher than that of cefotaxime, which is in turn more active than cefamandole. On the other hand, after interaction with HOCl, cefamandole experiences a greater loss of antibacterial activity than cefotaxime, as does cefotaxime than ceftriaxone. It is conceivable, therefore, that it is firstly the number of thioether groups and then that of heterocyclic rings of cephalosporins which afford protection of physiologically relevant biomolecules and of the antibiotic β-lactam ring (which is responsible for the antibacterial activity [4]) against HOCl attack.

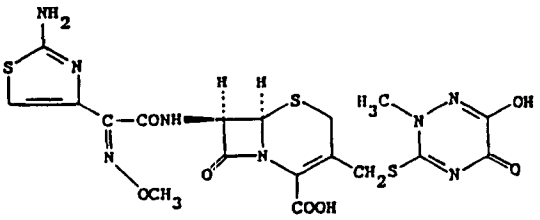
It is remarkable that cephalosporins can inhibit TauNHCl generation and that they do not form with HOCl chloramine derivatives, which may be



CEFAMANDOLE



CEFOTAXIME



CEFTRIAXONE

Fig. 3. Chemical structure of cephalosporins used in the study.

Table 2. Antibacterial activity of control and HOCl-reacted cephalosporins

	MIC* (μg/mL)
Cefamandole	3.2
Cefamandole plus HOCl	25.0
Cefotaxime	0.08
Cefotaxime plus HOCl	0.39
Ceftriaxone	0.1
Ceftriaxone plus HOCl	0.39

* Evaluated using the bacterial strain *Escherichia coli* ATCC 25922 (see Materials and Methods for detailed methodological explanations).

powerful oxidants even more toxic than HOCl itself [2, 11]. Thus, the interaction of cephalosporins with HOCl may result in tissue antioxidant protection in infective-inflammatory diseases, though further studies are needed to address this issue.

When HOCl is generated by neutrophils *in vivo*, both endogenous oxidizable molecules and any drug administered to the patient will be present simultaneously. Our data indicate that under these conditions cephalosporins probably do not readily protect thiols against HOCl, although other biomolecules, such as polyenoic compounds, should be strongly protected. However, our results show that when HOCl reacts with cephalosporins before interacting with GSH, tripeptide oxidation is markedly antagonized. In this context, it is known that cephalosporins can penetrate neutrophils [17]. These drugs, therefore, could scavenge some HOCl directly within neutrophils, thus decreasing the amount of releasable HOCl able to oxidize thiols at blood and tissue level. Our experiments also show that β-carotene is protected by cephalosporins even at 18 μg/mL (i.e. approx. 33 μM) drug concentration, whereas HOCl-induced GSH oxidation is significantly antagonized by ceftriaxone only at 100 μg/mL (i.e. 167 μM). This latter concentration especially may be difficult to maintain in some tissue, such as the lung, presenting purulent infections, making the administration of high antibiotic doses via an aerosolization route perhaps a wiser option [18].

In conclusion, cephalosporins, at therapeutically relevant concentrations, can scavenge HOCl, although physiological biomolecules are protected with different degrees of efficiency by these drugs against HOCl oxidant aggression. The interaction of cephalosporins with HOCl results in a depression of their antibacterial activity, which may decrease antibiotic effectiveness in the clinical setting of infective-inflammatory diseases.

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