Effect of β-lactamase-catalyzed hydrolysis of cephalosporins on peroxynitrite-mediated nitration of serum albumin and cytochrome c

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The hydrolysis of β-lactam antibiotics by β-lactamases is one of the major bacterial defense systems. These enzymes generally hydrolyze a variety of antibiotics including the latest generation of cephalosporins, cephamycins and imipenem. In this paper, the effect of cephalosporins-based antibiotics on the peroxynitrite-mediated nitration of protein tyrosine is described. Although some of the antibiotics have weak inhibitory effect on the nitration reactions in the absence of β-lactamase, they exhibit very strong inhibition in the presence of β-lactamase. This is due to the elimination of heterocyclic thiol/thione moieties from cephalosporins by β-lactamase-mediated hydrolysis. After the elimination, the thiols/thiones effectively scavenge peroxynitrite, leading to the inhibition of the nitration reactions.

Antibiotics based on β-lactams such as pencillins, cephalosporins, penems are widely used for various bacterial infections.[1-3] However, bacteria produce β-lactamases that effectively hydrolyze the β-lactam ring of several antibiotics, leading to a drug resistance. This antibiotic resistance restricts the broad range clinical applications of these drugs. The active site of β-lactamases contains either serine residue (serine-β-lactamases) or zinc ions (metallo-β-lactamases, mβL). Mechanistic studies on the hydrolysis of β-lactams by various β-lactamases have shown that either the metal activated hydroxyl group in the mβL or the activated serine residue in serine-β-lactamases attacks at the highly strained four-membered β-lactam ring, resulting in opening of the

β-lactam ring (Scheme 1). Consequently, the drug becomes inactive towards the inhibition of cell wall synthesis of gram positive bacteria.[3-12]

Recently, we have shown that the thiols/thiones eliminated from the antibiotics by β-lactamase-mediated hydrolysis of some of cephalosporins exhibit antithyroid activity.[13] The hydrolysis of the β-lactam ring in such antibiotics results in the formation of thiols, which in turn can undergo a tautomerism to produce the corresponding thiones. For example, the hydrolysis of cephalosporins having a thio-tetrazole side chain leads to the formation of MTT in its thione form (Scheme 2). The rate of thiol-thione tautomerism depends on the nature of heterocycle. As MTT is structurally very similar to
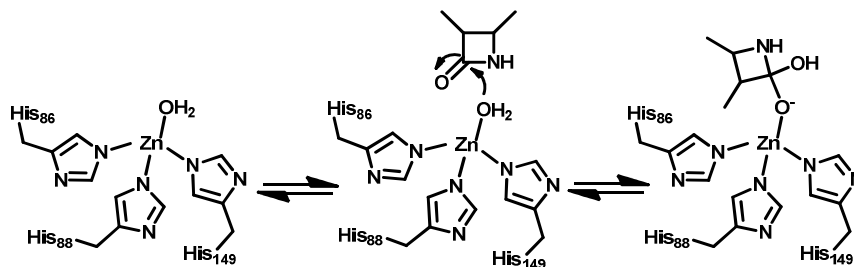
Hydrolysis of β-lactam ring by metallo-β-lactamases

Scheme 1
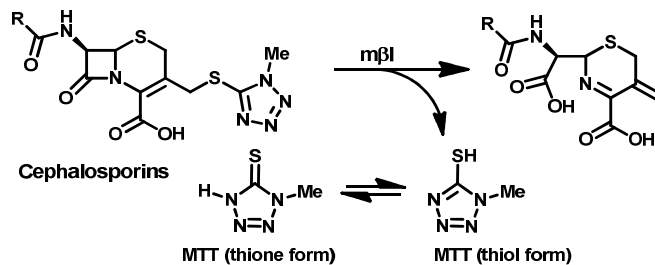
β-Lactamase-mediated elimination of heterocyclic thiol from cephalosporins and thiol-thione tautomerism[13]

Scheme 2

that of the most commonly used antithyroid drug methimazole, MTT strongly inhibits the peroxidase-catalyzed iodination of tyrosine. The irreversible inhibition is mainly due to the interaction of the MTT with the heme center of peroxidase.

In living systems, nitric oxide (NO) syntheses provide the biological precursors for the nitrating agents in various ways. These nitrating agents such as peroxynitrite (PN) are responsible for the protein tyrosine nitration. PN is generally produced via a diffusion controlled reaction between nitric oxide and superoxide (O_2^-) radical.[14-18] PN induces DNA damage and initiates lipid peroxidation in biomembranes or low density lipoproteins.[10,11] It is also known that PN can alter the function of many enzymes by nitrating the critical tyrosine residues, for example, in manganese superoxide dismutase.[19-24] The biological protein nitration is associated with more than 50 diseases including ocular inflammation, shock, cancer and neurological diseases like Alzheimer's and Parkinson's disease.[25-27] In this paper, we show for the first time that the heterocyclic thiols/thiones eliminated from certain cephalosporins by β-lactamase effectively inhibit the peroxynitrite-mediated nitration of proteins such as bovine serum albumin and cytochrome c.

Materials and Methods

Bovine serum albumin (BSA), penicillinase from *Bacillus cereus,* ceftriaxone, cephaperazine, cefazolin and cefametazole were purchased from Sigma-Aldrich Chemical Co. Cytochrome c from equine heart (oxidized form) was purchased from Calbiochem. The heterocyclic thione, 1-(dimethylamino)ethyl)-1*H*-tetrazole-5-thiol (DMETT) was obtained from Sigma-Aldrich and 1-methyl-1*H*-tetrazole-5-thiol (MTT), 2-methyl-1,2,4-triazine-5,6-dione-3-thiol (MTDT) and 2-methyl-1,3,4-thiadiazole-2-thiol (MDT) were

purchased from Alfa-Aesar. 6-*n*-Propyl-2-thouracil (PTU) and 2-mercapto-1-methylimidazole (MMI) was obtained from TCI (Tokyo Kasei, Japan). All the reactions were performed at the room temperature, unless mentioned.

Peroxynitrite synthesis

Synthesis of peroxynitrite was carried out by following the literature reports[28,29] with minor modifications. In a typical experiment, 5.7 mL of 30 % H_2O_2 (~8.8 *M*) was diluted to 50 mL with water, cooled to 4 ºC in an ice-water mixture and added to 30 mL (5 *N*) of NaOH and 5 mL of DTPA (0.04 m*M*) in NaOH (0.05 *N*) with gentle mixing. This mixture was diluted to a total volume of 100 mL. The resulting solution contained 0.5 *M* H_2O_2 in the *p*H range 12.5 – 13.0. This H_2O_2 solution was treated with an equimolar amount of isoamylnitrite (0.05 *M* or 6.7 mL) and stirred vigorously for 3-4 h at room temperature. The progress of reaction was monitored by taking aliquots at interval of 15-30 min and measuring the concentration of peroxynitrite by UV/vis spectrophotometer at 302 nm. After obtaining the maximum yield of peroxynitrite, isoamyl alcohol and isoamyl nitrite were removed by washing with dichloromethane, chloroform and hexane. The unreacted H_2O_2 was eliminated by passing the aqueous phase through a column filled with 25 g granular MnO_2. The concentration of the stock solution was determined after 500 times dilution with 0.1 *N* NaOH and then assayed for peroxynitrite ($\varepsilon = 1670$ M^{-1} cm^{-1}) by UV/vis spectrometric method at 302 nm.

Nitration of BSA

Bovine serum albumin (BSA) (0.12 m*M*), cephalosporins (1.0 m*M* by assuming 25 % hydrolysis by penicillinase), heterocycles (0.25 m*M*) and 50.0 µL (1.0 mg/mL, minimum of 10 units) of penicillinase were incubated in 100 m*M* sodium phosphate buffer at

*p*H 7.0 for 12 h at 20 °C. The reaction mixture was treated with 2.2 µL peroxynitrite (0.99 m*M*) for 30 min. The protein mixture was denatured by boiling at 100 °C for 5 min in the presence of sample loading dye. The above experiments were performed in the presence and absence of penicillinase. Finally, the denatured protein was subjected to SDS-PAGE for the estimation of tyrosine nitration by Western blotting method.

Nitration of cytochrome c

Cytochrome c (0.2 m*M*), cephalosporins (0.5 m*M* by assuming 25 % hydrolysis by penicillinase), heterocycles (0.5 m*M*) and 6.25 µL (1.0 mg/mL, minimum of 10 units) of penicillinase were incubated for 12 h at 20 °C. The reaction mixture was then treated with 1.75 µL peroxynitrite (0.99 m*M*) for 30 min. The protein mixture was denatured by boiling at 100 °C for 5 min in the presence of sample loading dye. The above experiments were performed in the presence and absence of penicillinase as described for the BSA nitration. Finally, the denatured protein was subjected to SDS-PAGE for the estimation of tyrosine nitration by Western blotting method.

Determination of tyrosine nitration by Western blotting

The denatured proteins were subjected to SDS-PAGE by using 15 % and 10 % polyacrylamide for BSA and cytochrome c respectively, with 6 % stacking gel. The gel was allowed to run in a buffer having *p*H 8.3. After proper separation of proteins on the gel, the proteins were transferred to PVDF membranes. Non-specific binding sites were blocked with 5 % non-fat skimmed milk in PBST (0.1 % Tween-20) for 1 h. The nitrated protein was probed with rabbit polyclonal primary antibody against nitrotyrosine (1:20 000 dilutions) in blocking solution

for 1 h and washed with PBST (3×15 min) to remove the primary antibody residues. The resulting membrane was incubated with horseradish peroxidase conjugated donkey polyclonal anti-rabbit IgG secondary antibody (1:20 000 dilutions) for 1 h. The membrane was washed three times with PBST solution and then the immune reactive protein was detected by Luminol generated chemiluminiscence (ECL, Amersham). The tyrosine nitration was quantified by Quantity One software by using the Bio-Rad Chemidoc XRS system, and protein tyrosine nitration reported as % control activity.

Results and Discussion

In this study, we have used the cephalosporin-based antibiotics, cefmetazole, cefaperazone, ceftriazone and cefazolin that contain heterocyclic thiol moieties in the side chain (Fig. 1). Upon treatment with β-lactamase, these compounds are expected to eliminate the heterocyclic thiols attached to the side chain. While cefmetazole and cefaperazone would eliminate a thio-tetrazole moiety (MTT), ceftriaxone and cefazolin are expected to eliminate MTDT and MDT, respectively (Fig. 2).

Inhibition of peroxynitrite mediated nitration of BSA

As the structures of heterocycles derived from cephalosporins are very similar to that of MMI and PTU (Fig. 2), the PN-scavenging activity of these compounds may play an important role in the antioxidant activity of the antibiotics. Therefore, we have studied the PN-mediated tyrosine nitration of bovine serum albumin.[30] The tyrosine nitration was studied by SDS-PAGE and western blotting analysis using antibody against 3-nitro-L-tyrosine. The cephalosporins and the corresponding heterocyclic
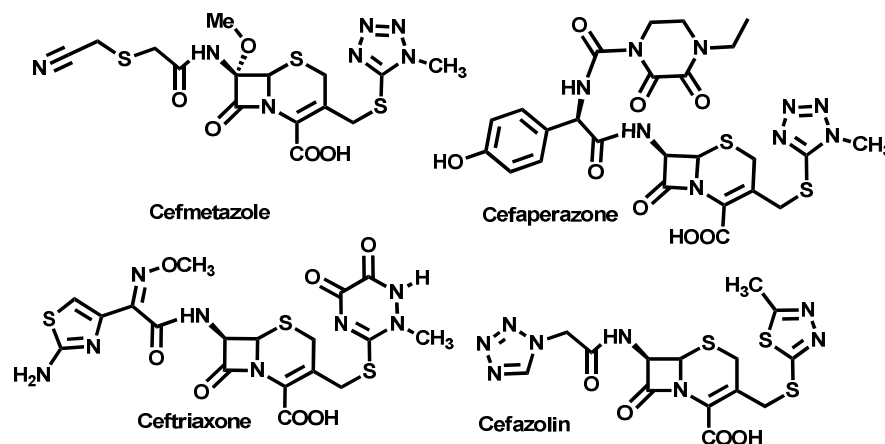

Fig. 1—Cephalosporins having heterocyclic thiol moieties in the side chains.
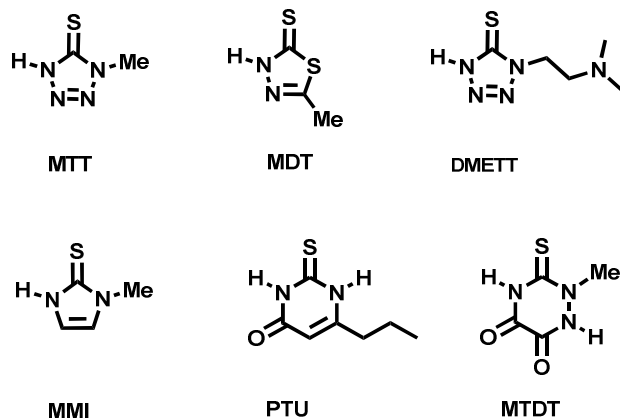
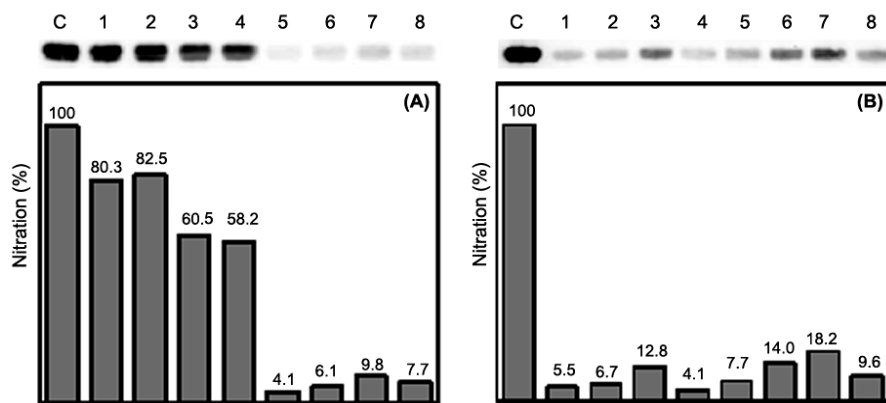Fig. 2—Structures of various heterocyclic thiones employed in this study.

Fig. 3—Western blots obtained for the inhibition of BSA nitration. [Cond.: 120 µM BSA, 1 mM cephalosporins or 250 µM heterocycles, 1.35 mM peroxynitrite in 100 mM phosphate buffer at pH 7.0. (A) cephalosporins in the absence of penicillinase; (B) cephalosporins in the presence of 0.5 units (50 µL of 1.0 mg/mL, minimum of 10 units) of penicillinase. Lane C: control; lanes 1-4: ceftriaxone, cefazolin, cefmetazole and cefaperazine, respectively; lanes 5-8: MTDT, MDT, MTT and DMETT, respectively].

thiones were used as inhibitors in the tyrosine nitration assay. As can be seen from Fig. 3, the inhibition of tyrosine nitration by the free thiones is much higher than that of the cephalosporins. The difference in the PN-scavenging activity of various heterocycles led us to investigate the effect of β-lactamase (penicillinase) on PN-mediated protein nitration inhibition. For this, the cephalosporins having heterocyclic side chains were added as inhibitors and incubated with β-lactamase (assuming 25 % hydrolysis) for 12 h at 20 °C. These experiments revealed that the cephalosporins treated with β-lactamase exhibited much better inhibition than the untreated antibiotics. Furthermore, the inhibitory activity of cephalosporins in the presence of β-lactamase was found to be almost similar to that of free heterocyclic thiones (Fig. 3B). These results

indicate that the heterocycles eliminated from cephalosporins by β-lactamase-mediated hydrolysis is responsible for the inhibition.

Inhibition of peroxynitrite-mediated nitration of cytochrome c

Apoptosis is a genetically dictated cell suicide program, which is morphologically and biochemically distinct from the other forms of cell death.[31] During the apoptosis signaling, it is known that cytochrome c moves from mitochondria to cytosol and forms a complex with Apaf-1 (Apaf-1 serves as docking protein for cyt-c and caspase-9). In the presence of ATP or dATP, cytochrome c Apaf-1 complex binds to caspase-9, and therefore, initiates primary event in the caspase-cascade in the cell death program.[32] It has been shown that the continuous exposure of PN at low concentrations specifically nitrates at least three tyrosine residues, viz., Y48, Y67 and Y74 of
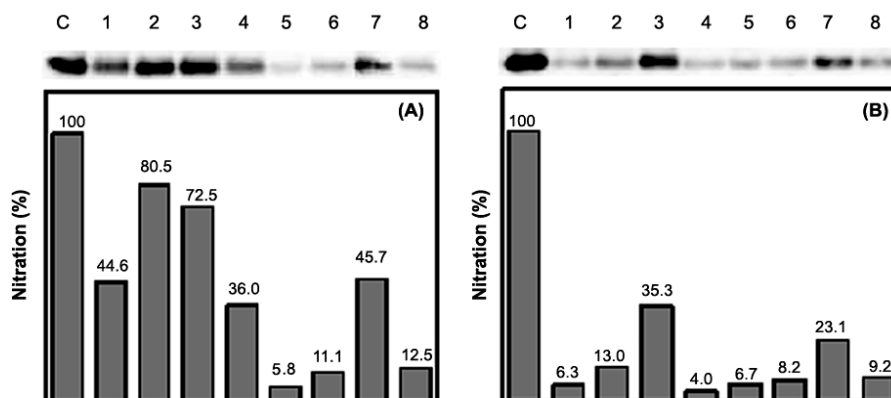
Fig. 4—Western blots obtained for the inhibition of cytochrome c nitration. [Cond.: 200 μM cytochrome c (oxidized form), 500 μM cephalosporins or heterocycles, 1.35 mM peroxynitrite in 100 mM phosphate buffer at pH 7.0. (A) cephalosporins in the absence of penicillinase; (B) cephalosporins in the presence of 62.5 milli units (6.25 µL of 1.0 mg/mL, minimum of 10 units) of penicillinase. Lane C: control; lanes 1-4: ceftriaxone, cefazolin, cefmetazole and cefaperazine, respectively; lanes 5-8: MTDT, MDT, MTT and DMETT, respectively].

the cytochrome c. The nitration of Y74 has been shown to be crucial in the attenuation of cyt-c binding to Apaf-1 protein.[33]

The effect of cephalosporin antibiotics on PN-mediated nitration of cytochrome c and its implications are useful in understanding the antibiotics action. It is known that cytochrome c is localized on the inner-membrane space side of the mitochondrial membrane and participates in mitochondrial electron transport.[34-36] Cytochrome c is a small globular protein that contains covalently bound heme and is located in an internal pocket formed by highly conserved amino acids. It is known that NO˙, $O_2^{˙-}$ and the reaction product, PN, can inhibit the mitochondrial electron transport via a reversible binding to cytochrome c oxidase or the inactivation of complexes I, II and ATPase, respectively.[37-42] It has also been shown that the PN formation and subsequent interactions with mitochondria result in tyrosine nitration.[43,44] Based on this information, we have performed the PN-mediated nitration of cytochrome c (oxidized) by following a method employed for the nitration of BSA (Fig. 4).

As it can be seen from Fig. 4, all the cephalosporins exhibited very weak inhibition of cytochrome c nitration in the absence of β-lactamase. However, when β-lactamase was added to the reaction mixture, a strong inhibition of the nitration was observed. In both BSA and cytochrome c nitration, cefmetazole exhibited a relatively weak inhibition. This is either due to the slow elimination of MTT moiety from cefmetazole upon treatment with β-lactamase or due to the relatively less inhibitory

potency of MMT thione. Further studies with isolated thiones indicate that the inhibitory effect of MTT on protein nitration is considerably lower than that of MDT, MTDT and DMETT (Figs 3 and 4).

Conclusions

We have shown for the first time that some of the cephalosporin-based antibiotics effectively inhibit peroxynitrite-mediated protein tyrosine nitration upon hydrolysis by β-lactamase. The inhibition is mainly due to the elimination of heterocyclic thiol/thione moieties from cephalosporins by the β-lactamase-mediated hydrolysis. After the elimination, the thiols/thiones effectively scavenge peroxynitrite, leading to the inhibition of the nitration reactions. This study suggests that the heterocyclic thiols/thiones present in the β-lactam antibiotics may have some beneficial effects in oxidative stress. As these thiols/thiones also irreversibly inhibit the peroxidase-catalyzed iodination of tyrosine, both these effects, i.e., peroxidase inhibition and antioxidant activity, must be taken into account while designing new antibiotics based on cephalosporins. The introduction of a substituent that exhibits weak peroxidase inhibition and strong antioxidant activity upon cleavage by the β-lactamase may lead to the development of better antibiotics.

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