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S-Nitrosothiols as Novel, Reversible Inhibitors of Human Rhinovirus 3C Protease

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Abstract—Human rhinovirus (HRV) 3C protease was inactivated by a series of *S*-nitrosothiols. These compounds exhibited different inhibitory activities in a time- and concentration-dependent manner with second-order rate constants (k_{inact}/K_I) ranging from 131 to 5360 $M^{-1}\,min^{-1}$. The inactive enzyme could be re-activated by DTT, GSH and ascorbate, which indicated the inactivation mechanism was through an *S*-transnitrosylation process. © 2000 Elsevier Science Ltd. All rights reserved.

As the major cause of the common cold, HRVs are a group of plus-strand RNA viruses belonging to the picornavirus family. To date, over one hundred HRV serotypes have been identified.[1] Similar to other picornaviruses, the genome of HRV contains a single open reading frame that can be translated into a 220 kDa viral polyprotein precursor in infected cells.[2,3] Subsequent processing of this polyprotein precursor is achieved by two virally encoded proteases, designated 2A and 3C.[2,3] Since the generation of mature viral proteins and functional enzymes is essential for viral replication and life cycle, both HRV 2A and 3C protease have been viewed as important targets for antiviral intervention. Specifically for HRV 3C protease, evidence has accumulated that this enzyme is not only a key enzyme but also an RNA binding protein essential for both viral protein maturation and viral replication.[3] In addition, this enzyme may play a role in regulation of host cell function.[4] Structural analysis has demonstrated that HRV 3C protease belongs to a novel class of cysteine proteases that has an overall trypsin-like serine protease fold but contains a cysteine residue as the active site nucleophile.[5] Due to its essential roles in viral infection and unique protein structure (distinct from other known cellular proteases) HRV 3C protease has been widely recognized as an attractive target for development of antiviral compounds.

Nitric oxide (NO) is a newly discovered biological messenger that plays important roles in physiological and pathophysiological conditions such as septic shock, inflammation, and endothelium-dependent vasorelaxation.[6,7] The interactions of NO with sulfhydryl-containing molecules and enzymes have gained considerable importance.[8,9] It has been demonstrated that many cysteine containing enzymes such as papain[10–12] and protein tyrosine phosphatase[13–15] could be efficently inactivated by NO or NO donors. Since HRV 3C contains an essential cysteine residue, this enzyme should be susceptible to NO donors. *S*-Nitrosothiols are a class of important widely used NO donors. Furthermore, it has been suggested that the actions of the endothelium-derived relaxing factor more closely resemble a low molecular weight nitrosothiol rather than NO itself.[16] In this report, we investigated the influence of *S*-nitrosothiols on HRV 3C protease. Our results shows *S*-nitrosothiols are efficient inhibitors and the inhibition leads to the formation of *S*–NO bond in the enzyme.

SNAP, GSNO, and *S*-nitrosocaptopril were used in our bioassay, because they are the most typical and stable *S*-nitrosothiols and have been widely used in the biological research of nitric oxide. Glucose-SNAP is a novel class of NO donor synthesized in this group.[17] The sugar unit of those compounds enhances water solubility, cell penetration, drug-receptor interaction and influences the dose–response relationships. A *S*-nitroso heptapeptide (EALFQCG-SNO) was specifically designed and synthesized for HRV 3C protease, because the peptide sequence Glu-Ala-Leu-Phe-Gln is a good substrate for this enzyme.[18,19] Hopefully this sequence will enhance the binding of the inhibitor to the enzyme. The structures of these inactivators are shown in Figure 1.[31]

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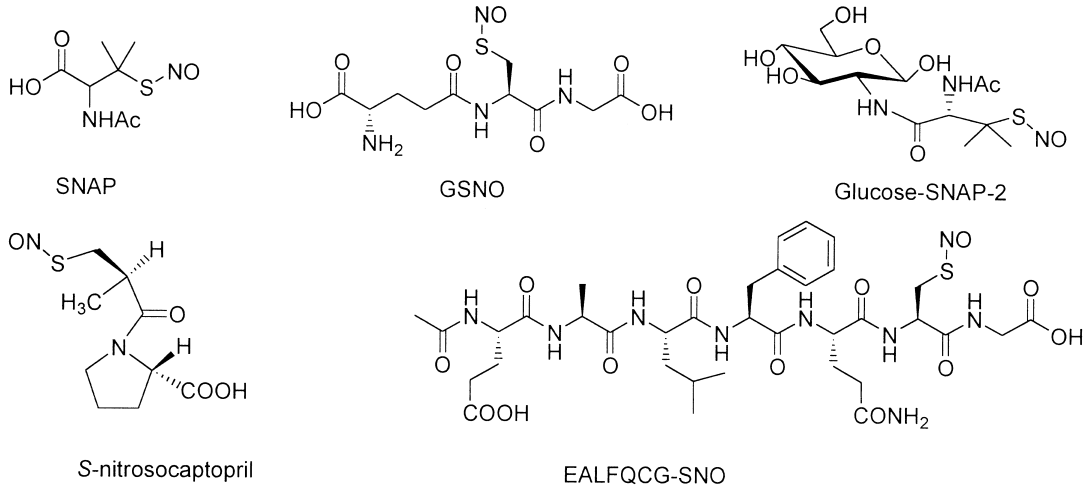

Figure 1. The structures of *S*-nitrosothiols used in this study.

The enzyme activity was measured spectrophotometrically at 405 nm with an UV–vis spectrophotometer using chromogenic substrate EALFQ-pNA (0.25 mM) in PBS buffer.[20] Incubation of the enzyme with each of these *S*-nitrosothiols resulted in a time- and concentration- dependent loss of enzymatic activity. One typical inactivation process using SNAP is shown in Figure 2. The figure shows that the inactivation reactions obey apparent first-order kinetics. The apparent first-order inactivation constants (k_{obsd}) can be calculated by plotting the residual activity versus time and fitting a linear equation. The replots of k_{obsd} as a function of *S*-nitrosothiol concentration gave a Kitz–Wilson plot[10,21] (Fig. 3), indicating that the inactivation of HRV 3C protease by *S*-nitrosothiols was a bimolecular process. The calculated second-order rate constants for each *S*-nitrosothiol inactivation the enzyme are shown in Table 1.

The kinetic results presented in Table 1 indicated that *S*-nitroso compounds were moderate inactivators of HRV 3C protease. The second-order rate constants (k_i/K_I) for inactivation of the enzyme by these compounds ranged from 131 to 5360 $M^{-1}\,min^{-1}$. The intrinsic reactivity of the nitrosothiol moiety toward the enzyme differed in these five compounds, of which the most potent was EALFQCG-SNO. This result was consistent with the previous study on the peptidyl Michael inhibitors of the enzyme,[19] which demonstrated the longer peptide residues, the better potency. It suggested that the peptide sequence in this inhibitor contributed significantly to the stabilization of the inactivator–enzyme complex. By comparing the kinetic parameters of SNAP and Glucose-SNAP-2, it appeared that the introduction of sugar fragment increased the inhibitory potency of SNAP.

It is well known that the activities of many cysteine-containing enzymes are modulated by NO induced mechanism.[22] The present dogma for the mechanism of inactivation of critical thiol-containing enzyme by NO or NO donors is the formation of an *S*-nitroso adduct,[23,24]

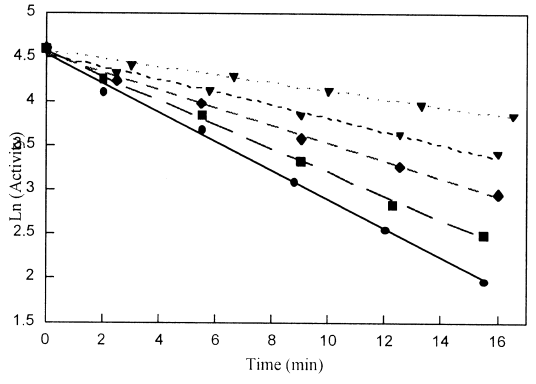

Figure 2. Time course of inactivation of HRV 3C protease (0.6 mg/mL) by SNAP. SNAP concentration: (●) 4 mM; (■) 2 mM; (◆) 1 mM; (▼) 0.8 mM; (▲) 0.4 mM.

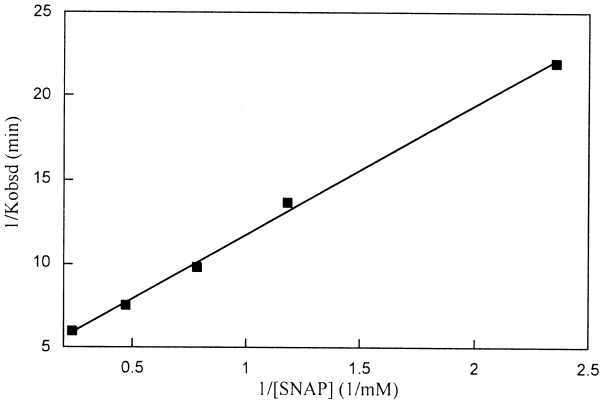

Figure 3. Kitz–Wilson plot for the inhibition of HRV 3C protease with SNAP.

Table 1. Kinetic parameters for the inactivation of HRV14 3C protease by *S*-nitrosothiols

Inhibitor	k_{inact} (min^{-1})	K_I (mM)	k_{inact}/K_I ($M^{-1}\,min^{-1}$)
SNAP	0.243	1.86	131
GSNO	0.237	0.36	656
Glucose-SNAP-2	0.982	5.04	195
S-NO Captopril	0.457	2.11	217
EALFQCG-SNO	0.107	0.02	5360

which could be reducible to the free thiol by DTT with the recovery of enzyme activity. However, several recent reports have shown that, if an *S*-nitrosothiol was used as NO donor, it would not only produce the transnitrosated product, but also give rise to mixed disulfides in many cases.[12,25,26] Both modifications would lead to the inactivation of cysteine containing enzymes that could be reversed with DTT. Thus, the enzyme's reactivation by DTT should not be taken along as evidence for the formation of *S*–NO bond. Several papers have shown that ascorbate could promote NO release from *S*-nitroso protein and produce the free thiol.[27–29] Furthermore, our previous study has demonstrated that ascorbate could be used as an efficient probe to identify the formation of *S*–NO and –S–S– bonds in the protein.[12] If the modification of the free thiol group in the enzyme was to form *S*-NO product, the addition of ascorbate could reduce the *S*-nitroso species to free thiol and the activity of enzyme would be recovered. If the modification of enzyme was to form a disulfide, the enzyme activity could not be recovered by the addition of ascorbate. Then, ascorbate together with DTT and GSH were used in this study to find out the mechanism of HRV 3C protease inhibited by *S*-nitrosothiols.

The results using GSNO as inactivator are shown in Figure 4. The activity of HRV 3C protease dropped to around 7% after incubating the enzyme (0.6 mg/mL) with GSNO (0.5 mM) for 1 h (Fig. 4, 2). Then, the inactive enzyme (0.5 mg/mL) was incubated with DTT (5 mM), GSH (5 mM), or ascorbate (15 mM) respectively. Three hours later, the activity of enzyme was measured. As shown in Figure 4, the activity of HRV 3C protease could be totally recovered by DTT (100%, 3) and GSH (93%, 4). This result could be predicted since no matter the modification of the enzyme active thiol group was to form *S*–NO or disulfide bond, both DTT and GSH could reduce it. However, unlike our results in papain inhibition by *S*-nitrosothiols,[12] about 68% of the initial HRV 3C protease activity was recovered during the incubation with ascorbate (Fig. 4, 5). This result indicated that inhibition of this enzyme by *S*-nitrosothiol was due to the formation of an *S*-nitroso adduct. Since EALFQCG-SNO exhibited much higher potency than other *S*-nitrosothiols and Glu-Ala-Leu-Phe-Gln is a good substrate for this enzyme, our results also suggested the transnitrosation was occurred in the active site of the enzyme. Generally *S*-nitroso compounds are unstable species, this *S*-nitroso enzyme may partly decompose to form the disulfide compound. This should explain why DTT and GSH could recover the activity totally but ascorbate could not.

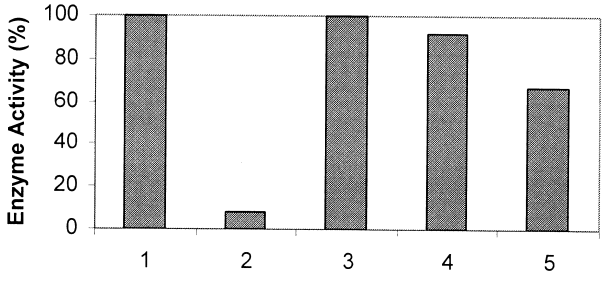

Figure 4. Reactivation of HRV 3C protease with DTT, GSH and ascorbate. 1 Control; 2 Inactive enzyme; 3 Inactive enzyme incubated with DTT (5 mM) for 3 h; 4 Inactive enzyme incubated with GSH (5 mM) for 3 h; 5 Inactive enzyme incubated with ascorbate (15 mM) for 3 h.

S-Nitrosothiols such as GSNO display several important biological effects including platelet deactivation, immunosuppression, relaxation of the vascular smooth muscle cells, and neurotransmission.[9] Micromolar concentrations of nitrosothiols have been detected in the plasma and in bronchial lavage fluid.[30] In this study, we demonstrate that *S*-nitrosothiols could serve as good inactivators to HRV 3C protease through a *S*-transnitrosylation process. Our results also show that the inhibition of HRV 3C protease is a transient phenomenon, because the inactivation of the viral enzyme can be reversed by DTT, GSH and ascorbate. Therefore, the reversible covalent modification of reactive protein thiols by *S*-nitrosothiols represents an interesting type of regulation of biological functions.

Acknowledgements

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31. SNAP, GSNO, and *S*-nitroso-captopril were prepared by the methods of Field et al., [32] Hart,[33] and Loscalzo et al.,[34] respectively. Glucose-SNAP-2 was synthesized according to our previous method.[17] Peptide EALFQCG was synthesized using solid-phase peptide synthesis techniques and Fmoc synthetic protocol. ^{13}C NMR (DMSO-d_6) 18.402, 22.148, 23.142, 23.653, 24.684, 28.140, 28.474, 30.840, 31.456, 32.079, 36.484, 41.320, 41.498, 48.744, 51.859, 52.504, 52.986, 54.099, 126.841, 128.636, 129.956, 138.329, 163.004, 170.010, 171.430, 171.600, 171.764, 171.912, 172.438, 172.616, 174.426, 174.656. FABMS calcd for $C_{35}H_{52}N_8O_{12}S$ 808.34, found 831.28 ($M+Na^+$), 847.28 ($M+K^+$). Then, the solution of this free thiol peptide (40 mg) in 5 mL DMF was added $NaNO_2$/HCl (1.01 equiv) at 0 °C. The mixture was stirred for 20 min. The solvent was removed in vacuum. The solid residue was washed with cold water and the red *S*-nitroso peptide was obtained (86%).
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