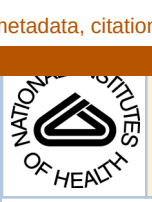

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Reversion of Sulfenamide Prodrugs in the Presence of Free Thiol Containing Proteins

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Abstract

The purpose of this work was to study the reaction kinetics between two model sulfenamide prodrugs of linezolid, N-(phenylthio)linezolid and N-((2-ethoxycarbonyl)ethylthio)linezolid, with free thiol containing proteins; human serum albumin (HSA); a constitutively active mutant of the protein tyrosine phosphatase PRL-1, PRL-1-C170-171S, a model protein; and diluted fresh human plasma. The reaction was followed by HPLC, both for the loss of prodrug and appearance of linezolid, and at different pH values with molar excess of the proteins relative to the prodrugs. Pseudo first-order kinetics were observed. Consistent with earlier findings for the reaction between similar sulfenamides and small molecule thiols, the reaction kinetics appeared to be consistent with thiolate attack at the sulfenamide bond to release the parent drug. The proteins reacted significantly slower on a molar basis than their small molecule counterparts. It appears that proteins such as HSA may play a role in the *in vivo* conversion of sulfenamide prodrugs to their parent drug.

Keywords

prodrugs; sulfenamides; bioreversion; thiols; HSA

INTRODUCTION

The overall goal of this work was to determine the possible role that proteins with a free thiol group might play in the reversion kinetics of sulfenamide model prodrugs of linezolid, N-(phenylthio)linezolid (**1**) and N-((2ethoxycarbonyl)ethylthio)linezolid (**2**) (Figure 1). Several previous reports established that sulfenamide prodrugs readily revert in the presence of small molecule thiols such as glutathione and cysteine to release the parent drug and a mixed disulfide.[1–5] *In vitro* and *in vivo* studies established that the reaction was practically instantaneous *in vivo,* and with glutathione and other thiols at physiological conditions *in vitro*.[3,5] Free thiol groups also exist on proteins. The present work attempts to measure the reversion kinetics of **1** and **2** in the presence of commercial human serum albumin (HSA), diluted fresh human plasma, and a free thiol-rich model protein tyrosine phosphatase, PRL-1-C170-171S, with a thiolate rich anion at physiological pH.[6] Scheme 1 describes the proposed reaction.

Human serum albumin (HSA) has 35 cysteine residues, 34 of which are involved in disulfide bonds and one free thiol at position-34. The free Cys-34 is readily available for

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formation of disulfide bonds with other free thiol groups, such as glutathione *in vivo* and is often partially and variably oxidized during purification in commercial HSA.[7–11]

PRL-1 is a part of a subfamily of protein tyrosine phosphatases (PTPs) responsible for cellular signal transduction mediating cell growth, differentiation, and tumor invasion.[12–13] Our aim with the PRL-1-C170-171S mutant is to compare the reactivity of this protein to that of commercial HSA and fresh human plasma.

EXPERIMENTAL, MATERIAL AND METHODS

Solvents and chemicals

Acetonitrile, acetone, acetic acid, formic acid, phosphoric acid, sodium phosphate (mono and dibasic), sodium acetate, sodium formate, and sodium chloride were from Fisher Scientific®. Cysteine, Thioglo-1® and HSA were purchased from Sigma-Aldrich® Chemicals and used without further purification. A 25.0 mg/mL PRL-1-C170-171S protein solution in a 50 mM phosphate buffer solution (I = 0.15) at pH 6.5 was expressed, purified and confirmed to be active as previously described by Skinner *et al.*[14] Purity was confirmed to be greater than 98% by size exclusion chromatography and densitometry of SDS-PAGE gels stained with Coomassie. A fresh human plasma sample was donated by one of us (KNA). Water used in all experiments was freshly distilled and deionized in-house using a Corning® Mega-pure One Liter distillation apparatus.

The synthesis of the two model, sulfenamide prodrugs, **1** and **2**, was described previously,[4] and reaction conditions and method of analyses were as previously described for the reaction with small molecule thiols.[5]

Determination of the free thiol concentration of HSA

L-cysteine was used as a standard in a slightly modified procedure outlined by Covalent® (Corvallis, OR) in determining the free thiol concentration present in human serum albumin purchased from Sigma-Aldrich®. Stock solutions of L-cysteine and HSA were prepared in distilled water to a concentration of 0.051 mM. A solution of ethanethiol, as a standard, was also prepared in acetonitrile to a final concentration of 0.051mM. Thioglo-1® was dissolved diluted in DMSO to give a final concentration of 0.253mM. Fifty microliters of L-cysteine or HSA were added to 0.5mL, 1.0 mL, 2.0 mL, 3.0mL, and 4.0mL solutions of 50mM phosphate buffer solutions at pH 7.4. Fifty microliter aliquots of the Thioglo-1® solution were added to each sample to give a 5:1 molar ratio of Thioglo-1® to L-cysteine or HSA. The samples were kept at 25°C and analyzed by spectrophotometer at an excitation of 360 nm and an emission of 500 nm.

Degradation of 1 in the presence of HSA

Commercial human serum albumin (HSA) solutions were prepared to a final concentration of 40 μM in 50 mM buffer solutions (I = 0.15) at pH 5.0, 6.0, 7.0, 7.4 and 7.5. **1** was added to give a final concentration of 4.5 μM. Aliquots of the samples (100 μL) were placed in micro centrifuge tubes and kept in a water bath at 25°C. At appropriate time points, 100 μL of a 1.0 mM N-methyl maleimide solution in acetonitrile was added to the 100 μL samples in the micro centrifuge tubes to quench the reaction and also precipitate protein from the solution. Previous controls showing excess N-methyl maleimide confirmed quantitative quenching of the reaction between thiols and sulfenamides.[5] The samples were vortexed for 15 seconds and centrifuged at 13,000 rpm for 5 minutes. The supernatant was analyzed by HPLC.

Degradation of 1 in the presence of PRL-1 C170-171S Mutant

PRL-1-C170-171S protein solutions were prepared as per HSA, to a final concentration of 12.4 μM. PRL-1-C170-171S was shown to only four cysteine residues; hence, at a concentration of 12.4 μM, the concentration of free thiol present was assumed to be 49.6 μM and the reaction conditions at pH 5.5, 6.0, 6.5, 7.0, 7.4 and 7.5. Kinetics and analyses were carried out as described for HSA.

Degradation of 1 and 2 in the presence of Human Plasma

Literature reports indicate human plasma contains 0.6 mM albumin.[15] Fresh human plasma (HP) solutions were diluted to obtain a nominal final albumin concentrations of 40 μM in 50 mM phosphate buffer solution (I = 0.15) at pH 7.4. **1** or **2** were added to give a final concentration of 4.4 μM. Samples were quenched, handled and analyzed as per the HSA experiments.

RESULTS AND DISCUSSION

Reversion of 1 in the presence of HSA and PRL-1-C170-171S

The addition of **1** to a 0.6 mM sample of commercial HSA at pH 7.4 and freshly harvested fresh HSA resulted in the immediate quantitative generation of the parent linezolid molecule. At lower HSA concentration (40 μM) the reaction was slower and hence one is able to follow the reaction by quenching followed by HPLC analysis. Due to the molar excess of free thiol proteins, pseudo first-order kinetics was observed for the loss of the prodrug at all pH values studied.

A pH-rate profile for both HSA and PRL-1-C170-171S can be seen in Figure 2. Unlike what was observed in the case with small molecule thiols and discussed in an earlier paper, the pH dependency was more complex.[5] That is, the reaction of the sulfenamide with the small molecule thiols showed a pH dependency that was consistent with the reaction of the thiolate anion specie with substrate sulfenamides where a semi-log plot of k_{obs} versus pH of solution showed a linear plot with a slope of approximately one. An apparent first order dependency on base is expected at pH values less than the pKa of the thiol group.[5] The same plots for HSA and PRL-1-C170-171S do not exactly show this pH behavior. For HSA the slope between experimental points changes form a slope of less than unity at lower pH values to greater than unity between the last two pH values.

A number of possible explanations for the HSA data include; first, a change in pH of solution can result in a change in the conformational structure of the albumin molecule, thus changing the accessibility of the free thiol at Cys-34 to the prodrug. Second, in addition to altering accessibility, a change in conformation may lead to a change in the residues neighboring or surrounding the free thiol Cys-34 residue, thus significantly affecting any ionizable groups surrounding the free Cys-34 residue and affecting factors such as the apparent dissociation constant (K_a) of the thiol. Thus, changes in the fraction of the thiolate form of the Cys-34 thiol will significantly change its reactivity. The prodrug may also be capable of binding to HSA. If the prodrugs are partially or strongly bound to HSA at a site(s) removed from Cys-34 this could also affect the reactivity of the prodrugs with HSA. The pH of solution could alter the binding capacity of the prodrugs to HSA. Thus, while there are many possible explanations for the pH dependency seen in figure 2, it is likely that more than one cause is operative.

The determination of the free thiol concentration in the commercial indicated that only 30 to 45% of the thiol actually existed as a free thiol per mole of HSA, meaning that approximately 55 to 70% of the Cys-34 was oxidized. These values are consistent with

recent reports in which HSA from different commercial sources were tested for the free thiol concentrations.[16]

The pH-rate profile for the reaction of **1** with PRL-1-C170-171S showed a linear correlation with a positive slope (Figure 2). However, unlike small molecule thiols, which showed a slope of unity, the slope in this case was 0.52. Based on structural studies performed using solution NMR, minor perturbations in structure occur over the pH range used here (unpublished data), but conformational fluctuations promote formation of an internal disulfide bond between C49 and C104, which would decrease the number of free thiols while one of the PRL-1-C170-171S thiols may be fully ionized over the pH-range studied.[14]

Degradation of **1** in the presence of HSA and PRL-1-C170-171S resulted in a half-lives ($t_{1/2(HSA)}$ and $t_{1/2(PRL\text{-}1M)}$) of 6.7 and 6.8 minutes at pH 7.4, respectively. A comparison of the reactivity observed in HSA and in PRL-1 with the reactivity observed in cysteine and glutathione indicate a significantly higher reactivity for small molecule thiols at pH 6.0 and 7.4 (Table 1).

Degradation of sulfenamide prodrugs in the presence of human plasma

In the presence of diluted human plasma (HP, nominal albumin concentrations of 40μM), **1** and **2** degraded faster than in the presence of commercial HSA (40μM). For example, **1** at pH 7.4 had a half life of 3.7 minutes ($k_{obs(HP)} = 0.0032\ sec^{-1}$) in diluted human plasma, compared to 6.8 minutes ($k_{obs(HSA)} = 0.0017\ sec^{-1}$) in commercial HSA. Similarly, **2** had half-lives of 7.7 minutes ($k_{obs(HP)} = 0.0015\ sec^{-1}$) and 27.6 ($k_{obs(HSA)} = 0.00042\ sec^{-1}$) minutes, respectively. The higher reactivity of **1** is due to the phenylthiol promoiety being a much better leaving group, lower pKa value of 6.51 versus 9.7, compared to the 3-mercaptopropanoate ethyl ester promoiety of **2**.[5] These studies were carried out at the same nominal HSA molar concentration. However, as stated earlier, approximately 30 to 45% of the Cys-34 in commercially available HSA actually exists in its free thiol form. Thus, the lower reactivity is probably due to the differing states of thiol oxidation between commercial HSA and HSA in fresh plasma. If one accounts for the lower free thiol concentration in the HSA solution, differences in reactivity between the commercial HSA and diluted human plasma appear negligible.

On a molar basis, small molecule thiols cleave the sulfenamide bonds more rapidly than free thiol containing proteins (Table 1). Nevertheless, these observations suggest that albumin and other free thiol containing proteins could play some role in the *in vivo* conversion of the sulfenamide prodrugs to their parent drugs.

CONCLUSIONS

Small molecule thiols were shown to readily react with sulfenamide prodrugs to release the parent drug and a mixed disulfide.[5] Here, free thiol containing proteins (HSA and PRL-1-C170-171S) similarly react with sulfenamide to release the parent drug, albeit at a slower rate compared to that seen on a molar basis with small molecule thiols. Results obtained earlier from small molecule thiols showed the formation of a mixed disulfide between the nucleophilic thiol and the promoiety of the prodrug. One can assume that a mixed disulfide will be formed between the thiol proteins and the sulfenyl promoieties. This may lead to potential concerns in toxicity and immunogenicity. Further studies therefore need to be carried out to confirm the formation of mixed disulfides between the proteins and the sulfenyl promoieties and their possible toxic effects.

Acknowledgments

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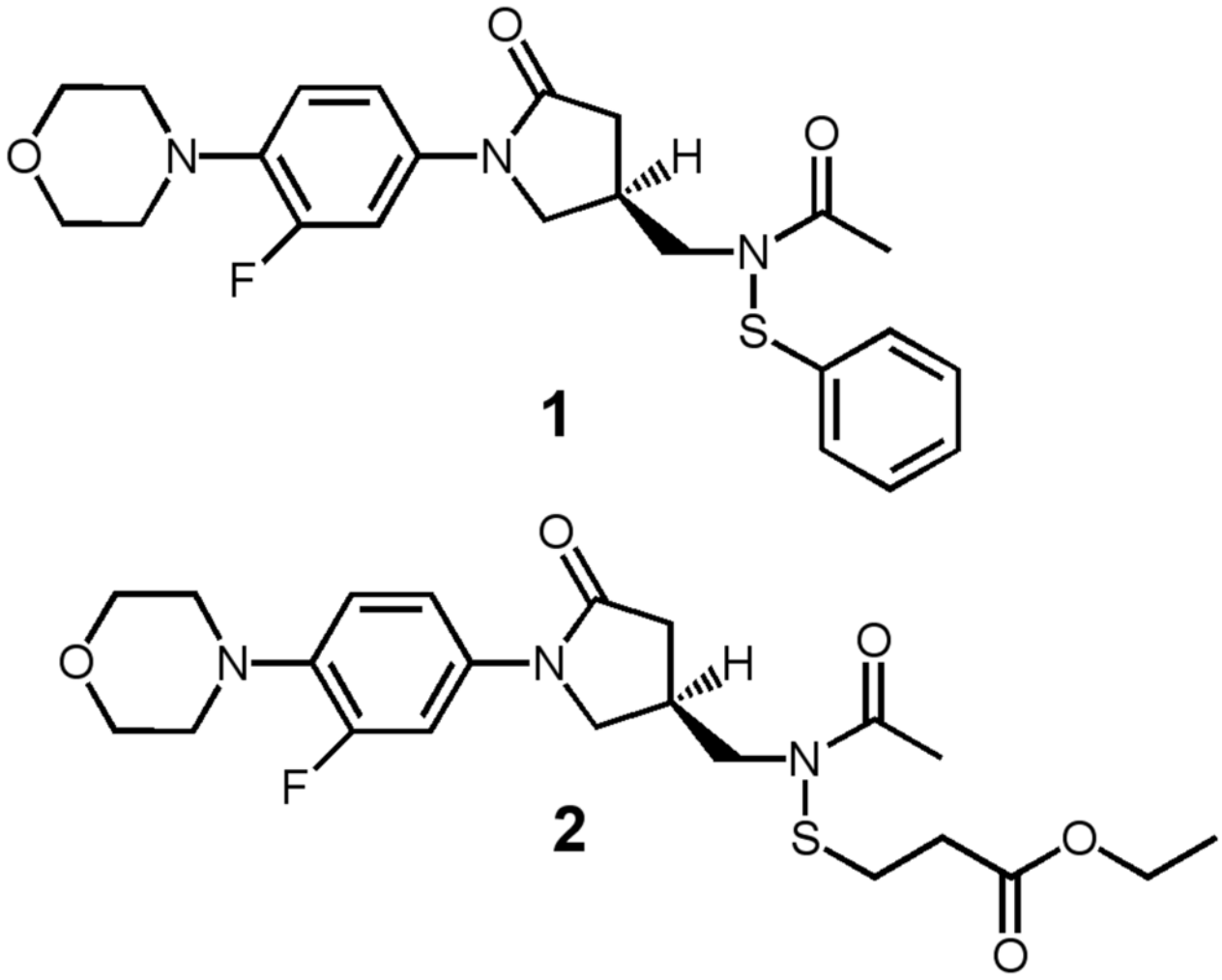


Figure 1.
Structures of linezolid prodrug models used in the study of sulfenamide stability in the presence of molar excess of thiol proteins. In the reactions with thiol proteins only **1** was used and both prodrugs were used in the reactions with diluted fresh human plasma.

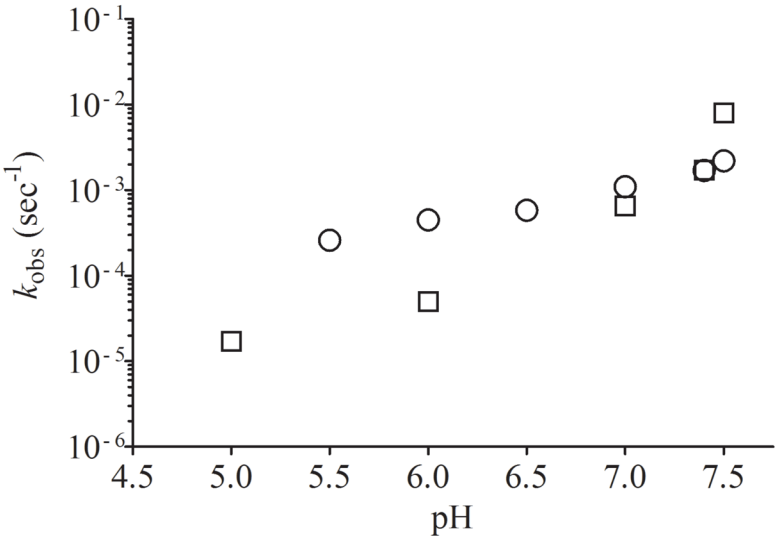
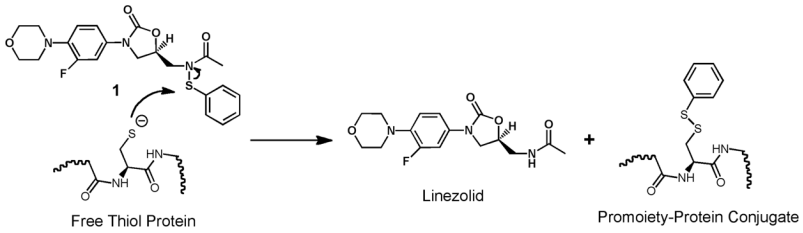


Figure 2.

pH dependency (pH-rate profile) for the reaction of **1** in the presence of a nominal 10 fold molar excess (assuming one free thiol per mole of HSA) of human serum albumin (HSA, □) in 50 mM buffer solutions (I=0.15) at 25°C. Free thiol analysis of the commercial HSA used in this study indicated that only 30 to 45% of the thiol actually existed as a free, non-oxidized thiol per mole of HSA. Also plotted is the reaction of **1** in the presence of 2.5-fold molar excess, or 10 fold excess in total thiols, of PRL-1-C170-171S (○) assuming four free thiols per mole of PRL-1 C170-171S protein.



Scheme 1.
Proposed mechanism for the reaction of **1** with cysteine thiol groups on a protein.

Table 1

A comparison of the half lives for the reversion of **1** in the presence of nominal 40 μM of thiols (cysteine and glutathione), and free thiol proteins at pH 6.0 and 7.4 in 50 mM phosphate buffer solutions.

Reacting Species	pH 6.0	pH 7.4
Cysteine	1.4 min	4.1 sec
GSH, glutathione	2.3 min	5.4 sec
HSA	233 min	6.7 min
PRL-1-C170-171S	25.1 min	6.8 min