

Dedicated to my parents, Augustine and Rosemary Nti-Addae.

Acknowledgements

My sincere appreciation goes to my parents, Augustine and Rosemary Nti-Addae for their relentless support and loving care through out my life, and making my education a success. In addition I send my appreciation to my brothers, Yaw Appiah Nti-Addae and Akwasi Kay Nti-Addae for being my support away from home and always being there for me when ever I needed them.

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Finally, my appreciation goes to my Uncle, Kojo Nsiah Amanquah, without whom my move to the USA to continue my education would have not been possible.

The synthesis, physicochemical characterization and evaluation of sulfenamide derivatives of antimicrobial oxazolidinones as prodrugs are described in this dissertation.

Synthesis of sulfenamide derivatives described in this work involves the use of thiophthalimide intermediates. These thiophthalimide intermediates allow for a clean one step reaction, which requires less purification steps resulting in higher yields of sulfenamide products compared to a previously described synthetic route that incorporated the use of unstable sulfuryl chloride intermediates.

The sulfenamide prodrugs undergo chemical conversion through the hydrolytic cleavage of the N-S bond during aqueous stability studies to release the parent drug molecule. However, insufficient aqueous stability characteristics may pose potential problems for future development of sulfenamide prodrugs as ready to use liquid formulations.

The stability of the sulfenamide prodrugs was studied in the presence of small molecule thiols with varying thiol pKa. These studies showed that the thiolate ion was the species responsible for the nucleophilic attack on the sulfur atom of the N-S bond, leading to the cleavage of the bond to release the parent molecule and a mixed disulfide. Reactions of the sulfenamide prodrugs with thiol containing proteins such as human serum albumin and PRL-1 also resulted in the nucleophilic cleavage of the sulfenamide bond to release the parent molecule. The reactions of the sulfenamide prodrugs with small molecule thiols and thiol containing proteins led to the conclusion that *in vivo* conversion will occur via the nucleophilic attack of thiolate species.

Attempts to study the permeability of the sulfenamide derivatives were hindered by the rapid conversion of the sulfenamide prodrugs to the parent molecule in the transport study setup. This rapid conversion is believed to be caused by the presence of thiol containing proteins on the surface of the Caco-2 cell monolayer and also within the cells. However, analysis of the initial permeability data shows that the sulfenamide prodrugs are contributing to the slight improvement of the permeability of the parent molecule.

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Appendix A: Hemenway J, Nti-Addae KW, Guarino VR, Stella Valentino J 2007. Preparation, characterization and in vivo conversion of new water-soluble sulfenamide prodrugs of carbamazepine. *Bioorg Med Chem Lett.* 17:6629-6632.

Appendix B: Stella VJ, Nti-Addae KW 2007. Prodrug strategies to overcome poor water solubility. *Adv Drug Del Rev* 59:677-694.

Chapter 1

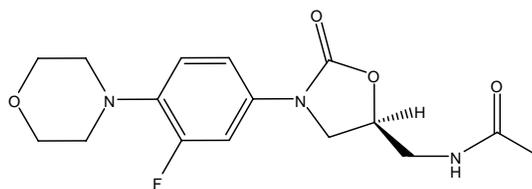
Application of sulfenamides as potential prodrugs to improve the physicochemical properties of amide containing drug molecules

1. Introduction and Background

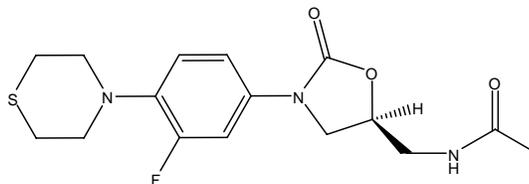
1.1. Statement of goals

The overall objective of this dissertation is to develop sulfenamide derivatives of antibacterial oxazolidinones and to evaluate their uses as prodrugs. It is our hypothesis that these sulfenamide prodrugs will revert to their parent drug in the presence of cysteine or glutathione *in vivo*. Specifically, this work will focus on the development of sulfenamide prodrugs of linezolid (Zyvox[®]) and its thiomorpholinyl analogues; U100480 and U101603 (Figure 1.1). Our goal is to synthesize and characterize sulfenamide prodrug models of these oxazolidinones to alter their physicochemical properties to improve delivery characteristics such as solubility and lipophilicity.

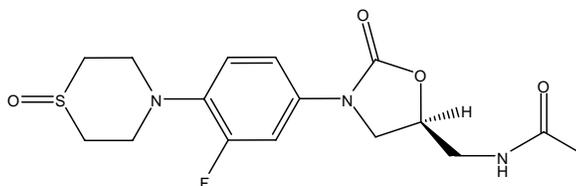
Oxazolidinones are a new class of antimicrobial agents which exhibit their activity by inhibiting protein synthesis in the bacteria, thus inhibiting bacterial growth and development.¹⁻⁴ This unique mode of action is believed to be the factor responsible for the inability of the bacteria to develop cross-resistance.^{3,5} In 1996, scientists at Pharmacia identified a number of oxazolidinones including U-100592 (Eperzolid), U-100766 (Linezolid) and its thiomorpholinyl analogues; U-100480 and U101603.⁶⁻⁸ Linezolid was eventually marketed as Zyvox[®] by Pfizer but the remaining oxazolidinones were dropped from development due to poor bioavailability resulting from both poor solubility and permeability across the intestinal cell wall.⁹



(*S*)-*N*-((3-(3-fluoro-4-morpholinophenyl)-2-oxooxazolidin-5-yl)methyl)acetamide
(Linezolid)



(*S*)-*N*-((3-(3-fluoro-4-(4-thiomorpholinyl)phenyl)-2-oxo-5-oxazolidinyl)methyl)acetamide
(U100480)



(*S*)-*N*-((3-(3-fluoro-4-oxothiomorpholinophenyl)-2-oxo-5-oxazolidinyl)methyl)acetamide
(U101603)

Figure 1.1: Structure of Linezolid and its thiomorpholinyl analogues.

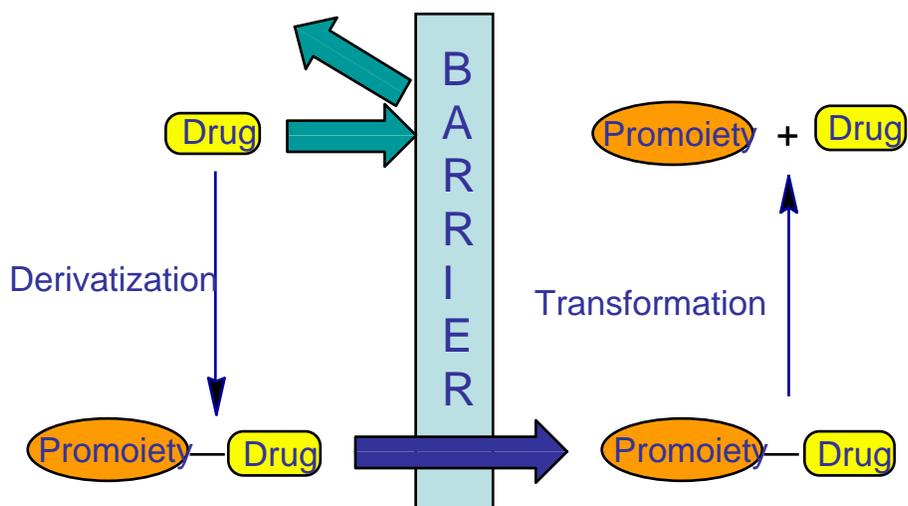
This dissertation focuses on applying sulfenamides as a new prodrug approach to improving the physicochemical characteristics of novel anti-microbial oxazolidinones that contain an amide functional group. The -NH proton of the amide group is a key hydrogen bond donor, which when removed, results in changes or disruptions in physical properties such as the crystal lattice packing, melting point and solubility behavior. By replacing the -NH proton with an appropriate promoiety, it is possible to selectively modify the physicochemical properties of the parent molecule (eg. solubility, lipophilicity, etc.). Additionally, this dissertation aims at understanding the mechanism of breakdown of the

sulfenamide prodrugs *in vitro* and *in vivo* to release the parent molecule and the sulfenyl moiety. It has been shown that the sulfenamide bond will be cleaved by nucleophilic attack of free sulfhydryls or thiol species such as cysteine and glutathione *in vitro*. The studies to understand the mechanism of breakdown of the sulfenamides will be followed by studies evaluating the stability of the prodrugs in the presence thiol containing proteins. Finally, this dissertation will shed more light on the use of sulfenamides in improving the permeability characteristics of oxazolidinones across cell monolayers.

1.2. Prodrugs

Prodrugs have been defined as pharmacologically inactive derivatives of drug molecules or drug candidates that undergo enzymatic or chemical transformations to release the active drug molecule upon being administered (Scheme 1.1).¹⁰⁻¹⁶ Prodrugs are used as an approach to overcome pharmaceutical, pharmacokinetic or pharmacodynamic barriers that prevent the effective administration of drug candidates. These barriers may include:

- poor aqueous solubility
- poor chemical stability
- poor dissolution rates
- insufficient oral absorption due to poor permeability across the intestinal cell wall
- low lipophilicity
- high presystemic metabolism
- and high toxicity



Scheme 1.1: Illustration of prodrug concept

All of these barriers result in poor drug performance such as low oral bioavailability of the drug candidates, thus preventing the drugs from attaining effective pharmaceutical or pharmacological effects. The idea behind prodrugs involves the attachment of a promoiety to the parent molecule or chemically modifying the parent molecule to alter the property of interest to overcome the barrier preventing the effective delivery of the drug. Once the prodrug has overcome the barrier, it is designed to undergo enzymatic or chemical transformation to release the active drug and a non-toxic promoiety.^{10-14,17,18}

The term “pro-drug” was first used by Albert in the late 1950’s, but the technique of using inactive derivatives to improve the physicochemical properties of drug candidates existed long before Albert.^{16,19} Albert described prodrugs as “therapeutic agents which are inactivate *per se* but transformed into one or more active metabolites.” Over the years there has been significant development of numerous prodrug techniques including the development of prodrugs that contain a promoiety and prodrugs that do not contain a promoiety; also know as bioprecursors or modular prodrugs. These bioprecursors or modular prodrugs do not have

a promoiety covalently attached to the parent molecule; however, they are a modification of the parent compound that undergoes biotransformation to form a pharmacologically active agent.^{19,20}

1.3. -NH acids (amides) and their prodrugs

Guarino and Stella recently defined -NH acids as compounds containing the -NH functionality adjacent to a carbonyl carbon and include compounds such as amides, carbamates, ureas, and imides (Figure 1.2).²¹ The -NH functionality in compounds provides a number of advantages for the preparation of prodrugs. First, the removal of the -NH proton removes a key hydrogen bond donor. This leads to the disruption in properties such as crystal lattice energy, often lowering the melting point, causing an increase in aqueous and lipid solubility, and improving dissolution rates.^{21,22} A remarkable example is the high melting weakly acidic drug, phenytoin (**1.1**) (Figure 1.3).

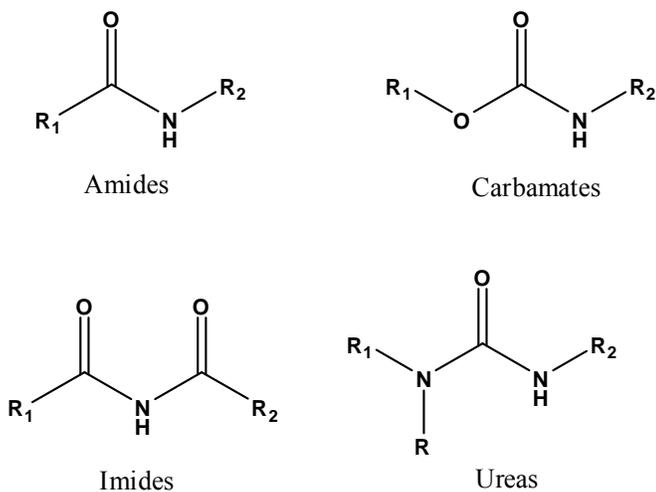


Figure 1.2: Generic structures of some -NH Acids

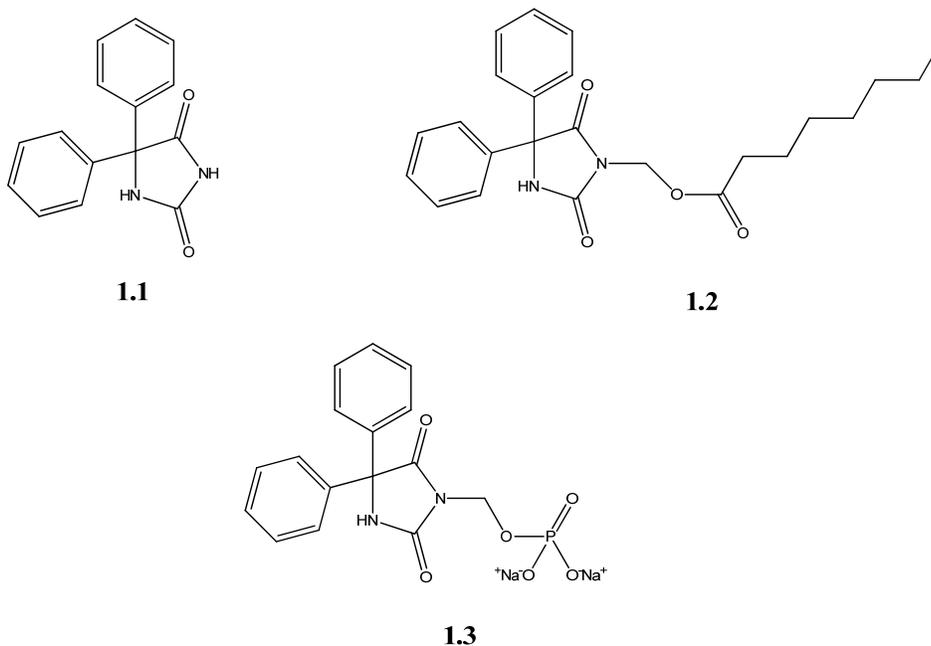


Figure 1.3: Phenytoin (**1.1**, melting point ~ 293 °C, aqueous solubility ~ 25 $\mu\text{g/mL}$), octanoyloxymethyl phenytoin prodrug (**1.2**), and phosphonoxymethyl phenytoin prodrug (**1.3**, aqueous solubility > 50 $\mu\text{g/mL}$)

Phenytoin has very poor aqueous solubility but when derivatized by removing one of its -NH protons and replacing it with an octanoyloxymethyl moiety, the crystal lattice is disrupted leading to a decreased melting point of the octanoyloxymethyl phenytoin prodrug (**1.2**) and an increased aqueous solubility and dissolution rates (Figure 1.3).²³⁻²⁸ The removal of the -NH proton also allows for the covalent attachment of a carefully selected moiety that modifies the physicochemical property posing as a barrier to the effective delivery of the parent drug. For example, in order to improve the aqueous solubility of the poorly soluble phenytoin, the ionizable phosphonoxymethyl moiety is attached to greatly enhance the aqueous solubility to give **1.3** (Figure 1.3).²³⁻²⁶ Similarly, if the goal is to improve the permeability characteristic due to low lipid solubility, a lipophilic moiety or a

hydrophobic promoiety can be attached to increase the lipophilicity of the parent molecule, thus allowing for improved permeability across the intestinal cell wall.

Previously, various approaches have been successfully applied to the improvement of the physicochemical or drug-like properties of drugs and drug candidates containing the N-H functional group. Some of these approaches include the N-acyl, N-hydroxymethyl, N-acyloxymethyl, N-phosphoryloxymethyl and the N-Mannich base prodrugs, all of which have their advantages and disadvantages.

1.4. Advantages and disadvantages of some -NH acid prodrugs

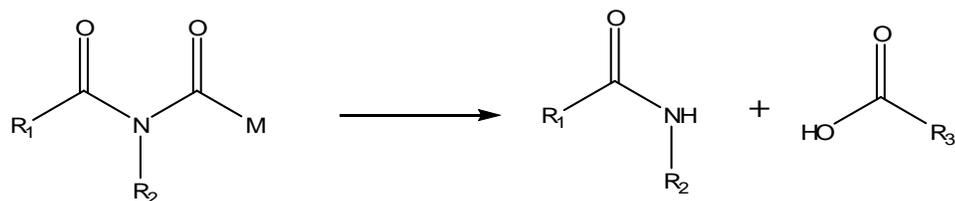
1.4.1. N-acyl prodrugs

N-acyl prodrugs can be grouped into three groups which provide varying stability characteristics depending on the moiety attached to the -NH acid.

1. N-alkylcarbonyl or N-arylcarbonyl prodrugs (Amide formation)
2. N-alkoycarbonyl derivatives (carbamate formation)
3. N-carbamoyl derivatives (urea formation)

All three categories generally degrade to release the parent -NH acid (Scheme 1.2.).²¹ One of the major problems associated with N-acyl prodrugs is their ability to degrade through multiple pathways such as the hydrolysis of the carbonyl, depending on the electron withdrawing abilities of the acyl moiety.^{21,29} Unfortunately not all the pathways lead to the release of the parent -NH acids, resulting in reported variations in aqueous stabilities.³⁰⁻³³

Despite the stability issues with N-acyl prodrugs, they have been reported to be effective in improving both aqueous and lipid solubilities.^{34,35}



M= R₃ N-alkylcarbonyl or N-arylcarbonyl (Amide Formation)

= OR₃ N-alkoxycarbonyl (Carbamate formation)

= NR₃R₄ N-carbonyl (Urea Formation)

Scheme 1.2. Bioconversion of a generic N-acyl prodrug.

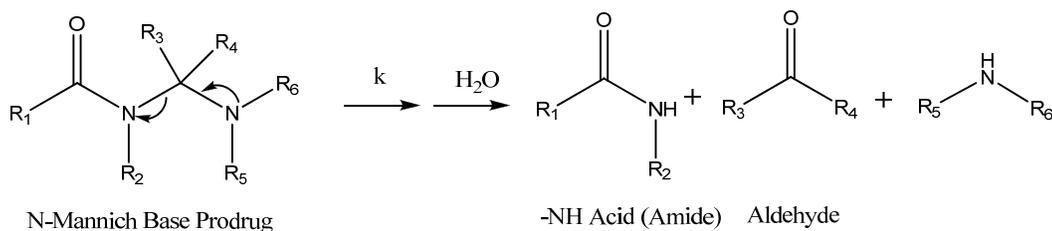
1.4.2. N-Mannich base prodrugs

N-Mannich base prodrugs as exhibited in Scheme 1.3 can be applied to both -NH acids and amines, and undergo bioconversion to release the parent -NH acid or amine and an aldehyde.²¹ The good stability in the protonated forms allows for formulations at acidic pH values. Mannich bases have been found to be very stable when protonated and undergo pseudo first order kinetic degradation with no effects from buffer species, ionic strength and plasma enzymes.^{36,37} Bundgaard and Johansen reported that the stability of the deprotonated form of N-Mannich base prodrugs is generally negatively affected by a number of factors. These include:

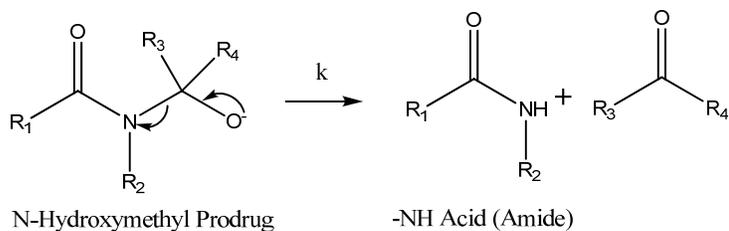
1. An increase in the steric bulk of the amine substituent
2. An increase in the amine basicity
3. and an increase in the NH acid acidity.

N-Mannich base prodrug approach is not an effective approach for improving imide type -NH acids. This is a result of the relatively high acidity of the parent molecules resulting in rapid degradation of the prodrugs under aqueous conditions.³⁸

N-Mannich base prodrugs were found to be effective in enhancing the solubility of secondary amines but not primary amines, due to possible intramolecular hydrogen bonding between the amine proton and the carbonyl oxygen of the N-Mannich base prodrugs on primary amines, hence preventing the solvation of polar groups of the prodrug molecule.^{36,37} Dissolution rates on the other hand are greatly improved for N-Mannich base salts compared to the free base forms.



Scheme 1.3- Bioconversion of a generic N-Mannich base prodrug



Scheme 1.4. Bioconversion of a generic N-hydroxymethyl prodrug

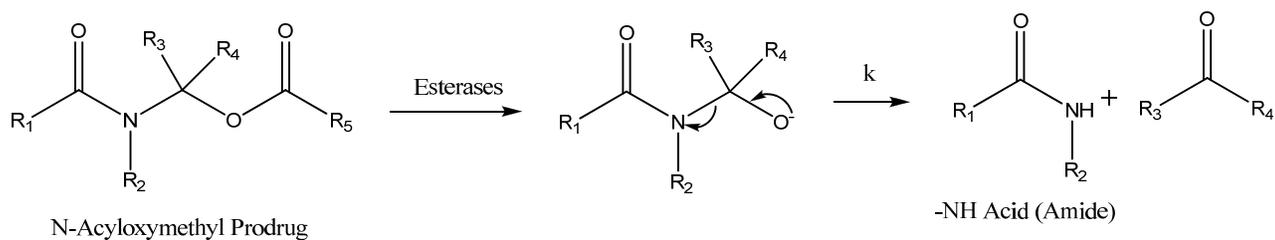
1.4.3. N-hydroxymethyl prodrugs

N-hydroxymethyl prodrugs similar to the N-Mannich base prodrugs undergo base catalyzed degradation to release the parent -NH acid (Scheme 1.4).²¹ Aqueous degradation of N-hydroxymethyl prodrugs show no dependency on buffer species, plasma enzymes or ionic strength; however, degradation is dependent on hydroxide ion concentration at pH 4 up to about pH 10.^{39,40} Stability decreases with an increase in the acidity of the parent molecule.

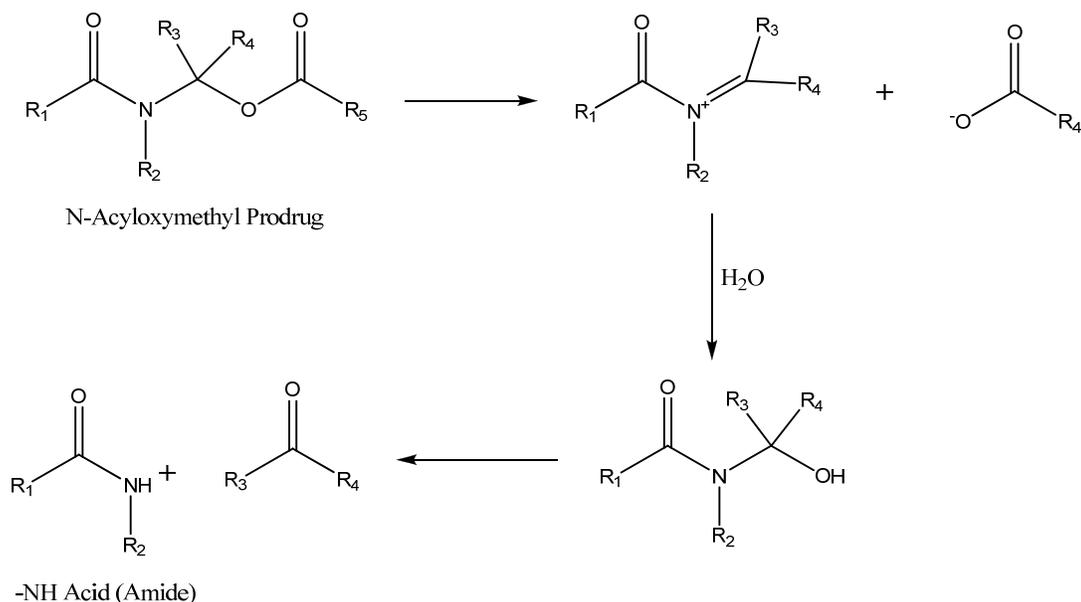
Aqueous solubilities and dissolution rates of N-hydroxymethyl prodrugs have been found to be greater than the solubilities and dissolution rates of their parent molecules by three to five fold.³⁹ These improvements are as a result in the disruption of the crystal lattice and an increase in the solvation of the hydroxymethyl promoeity.⁴¹ The N-hydroxymethyl prodrugs have also been found to be stable to proteolytic enzymes.⁴²⁻⁴⁴

1.4.4. N-acyloxymethyl prodrugs

N-acyloxymethyl prodrugs are degraded by esterases to release the N-hydroxymethyl intermediate, which then undergoes further degradation to release the parent -NH acid.²¹ However, this degradation in aqueous solutions can be either through the basic degradation, typically in imide-type -NH acids (Scheme 1.5.); or via an alternative route, typically found in amide-type -NH acids, as shown in Scheme 1.6. The degradation of the N-acyloxymethyl prodrugs of amide-type -NH acids via the alternative route compromises their aqueous stability, thus making them inefficient for use with imide-type -NH acids. One problem associated with N-acyloxymethyl prodrugs is the formation of the N-hydroxymethyl intermediates, which can turn to be very stable, there by prolonging the conversion times. Similar to the N-hydroxymethyl prodrugs, N-acyloxymethyl prodrugs have been found to be efficient in improving both lipid and aqueous solubility of -NH acids.^{31,33,45}



Scheme 1.5. Bioconversion of a generic N-Acyloxymethyl prodrug



Scheme 1.6. Alternative degradation mechanism for N-Acyloxymethyl prodrugs

1.5. Sulfenamides

Sulfenamides, also known as sulfenic acid amides, are compounds containing a trivalent nitrogen covalently linked to a bivalent sulfur by a single bond (Figure 1.4).⁴⁶ These compounds are derived from sulfenic acids, which are “sulfur acids” with a valence state of $+2$ (RS-OH). With the exception of a few sulfenic acids, most of the sulfenic acids have been found to be very unstable and readily react to form various degradation products. Consequently, few sulfenic acids such as the anthraquinone-1-sulfenic acids have been successfully isolated and studied. However they have been found to be key intermediates to a variety of organic compounds including sulfenamides.

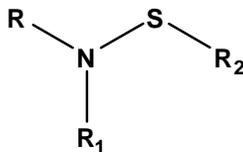
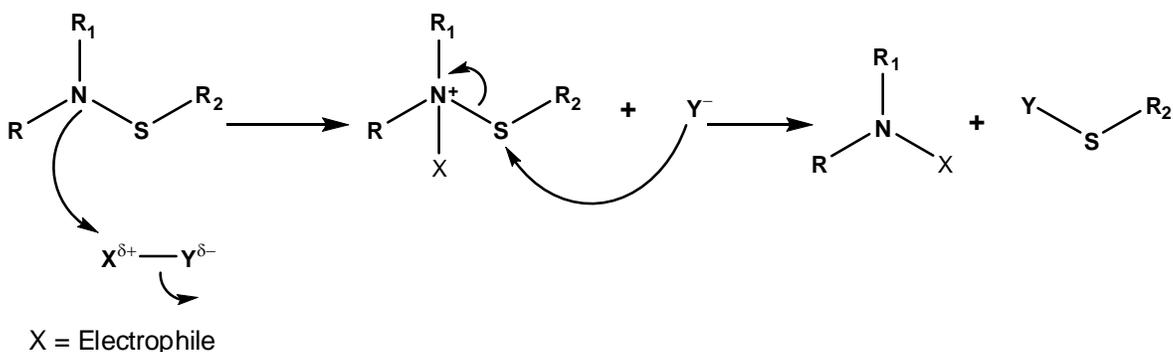


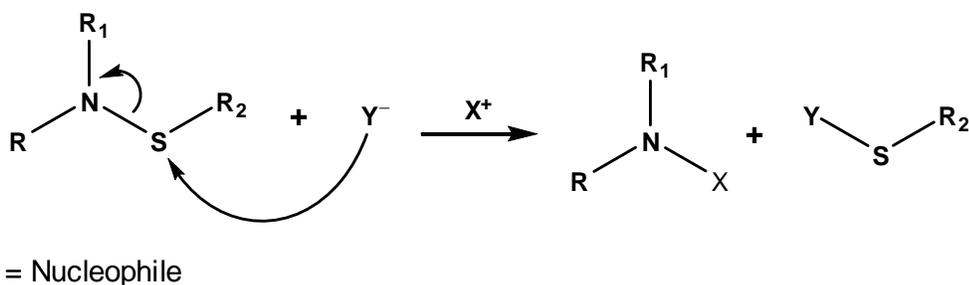
Figure 1.4: Generic structure of sulfenamides

1.6. Chemistry of sulfenamides

Sulfenic acid amides, or sulfenamides, were first discovered in early 20th century by Zincke during studies on the reactions of sulfenic acid chlorides with nitrogen containing compounds.^{47,48} Over the years, the chemistry of sulfenamides has been explored and found to have unique characteristics that allows for their numerous uses. The N-S bond has lone pairs of electrons present on both the sulfur and the nitrogen, but differences in electronegativity between the two atoms results in bond polarization, which results in a labile N-S bond that can be cleaved by both nucleophiles and electrophiles.^{49,50} Sulfenamides are therefore said to have two reaction centers; the sulfur, having a variable valence, is susceptible to nucleophilic attack and the nitrogen is susceptible to electrophilic attack.⁵¹ This makes the sulfenamide bond labile and allows for their use as sulfonyl transfer agents in the synthesis of disulfides, trisulfides and other sulfur containing molecules, and also as protective groups in peptide synthesis.⁵¹ The reaction of sulfenamides with electrophiles is thought to occur via the coordination of the electrophile activating the N-S bond and allowing for the displacement of the nitrogen moiety by nucleophilic attack on the sulfur to (Scheme 1.7).^{50,52} On the other hand reactions with nucleophiles involve the attack of the nucleophile on the more electropositive sulfur species to displace the nitrogen moiety (Scheme 1.8).



Scheme 1.7. Mechanism for the electrophilic cleavage of the sulfenamide bond.



Scheme 1.8. Mechanism for the nucleophilic cleavage of the sulfenamide bond.

1.7. Applications in the chemical and pharmaceutical industry

1.7.1. Applications in organic synthesis.

Since their discovery in the early 1900's, sulfenamides have been used in numerous industrial applications. Traditionally sulfenamides have been used as sulfonyl transfer agents in the synthesis of sulfides by sulfonylation of -CH acids or their salts, and also by the addition of sulfenamides across C-C double bond of unsaturated compounds.^{51,53,54} The synthesis of disulfides and trisulfides is also one of the common uses of sulfenamides in organic synthesis. Methods such as thermolysis, thiolysis, and hydrolysis of sulfenamides all lead to the formation of both symmetrical and unsymmetrical disulfides and trisulfides with reasonably good yields.⁵⁵⁻⁵⁸ Recently, there has been an increased interest in new applications of sulfenamides in synthetic organic chemistry. Further development has shown that

sulfenamides can be used as intermediates in the synthesis of other sulfenamides.^{59,60} Sulfenamides have also been used as transfer agents in the synthesis of chiral amines and amino acids and as a source of aminyl, amidyl and thioaminyl radicals.⁶¹⁻⁷⁰ The labile sulfenamide bond has also allowed for the use of sulfenamides as protective groups in peptide synthesis, allowing for the easy removal of the protective sulfenyl moiety once the peptide of interest has been successfully synthesized, purified and isolated.⁷¹

1.7.2. Applications in the agricultural industry.

Sulfenamides were first reported to have biological activity in 1944 when they were reported to have high activity towards disease causing fungi.⁷² Over the years, various research teams have explored the use of sulfenamides as effective fungicides in the treatment of fungal diseases that affect agricultural crops such as apples, oranges and pears.^{55,73-76} N-(trichloromethyl)thiophthalimide also known as Kapthan (Figure 1.5), has been reported as one of the most effective and valuable fungicides developed over the years. It is commercially available for the treatment of apple and pear scab, fungicide spots on fruit tree leaves, and diseases of grape vines including mildew, antracosis and red rot.⁴⁶ Also it is used in the treatment of phitophthora infection of potatoes and tomatoes.⁷³ Other analogues of Kapthan also have exhibited effective activity towards a variety of fungi. Euparen (figure 1.5) and its sulfenylurea derivatives also have been developed as fungicides and pesticides. In addition to activity towards fungi, other developed sulfenamides, including the pyridinyl and the nitrobenzyl sulfenamides, have been used as effective insecticides in the eradication on unwanted insects that destroy agricultural crops.

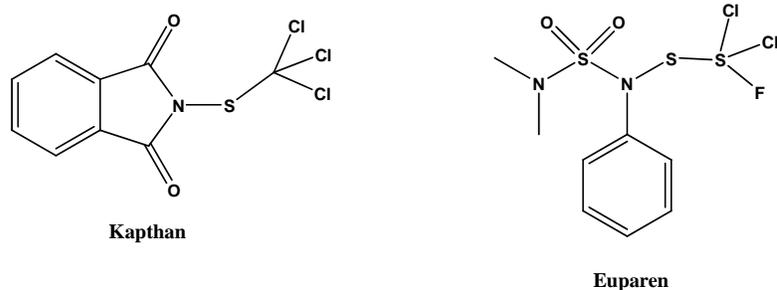


Figure 1.5: Some well known sulfenamides that are used as fungicides in the agricultural industry.

1.7.3. Other applications of sulfenamides.

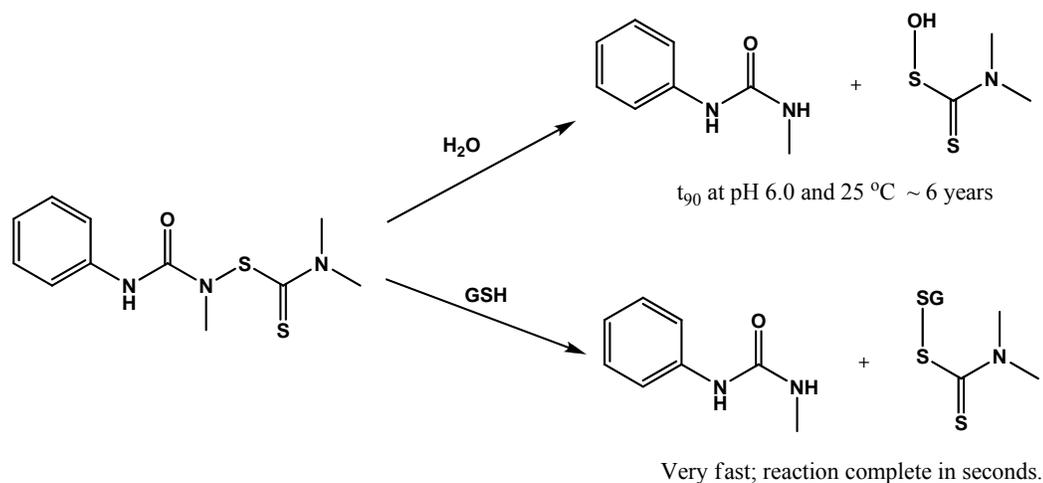
Another major application of sulfenamides occurs in the rubber industry during the vulcanization process. Sulfenamides readily undergo homolysis leading to the release of sulfenyl radicals that actively participate in the vulcanization of the rubber.⁶³ Also sulfenamides serve as a source of sulfur as a cross-linking agent in the vulcanization process of both natural and synthetic rubber.⁵⁰ The sulfenamides provide sources of sulfur that have improved heat resistant and strongly retarded the onset of vulcanization, increasing the safety of the vulcanization process; a process which produces rubber products with strong resistance to aging and with good elasticity and tensile strength.^{46,51,77} Sulfenamides also have found use in polymer chemistry as they are used to introduce or transfer anti-oxidative groups onto polymers to protect the polymers from oxidative degradation.^{46,51}

1.8. Sulfenamide Prodrugs

1.8.1. Origin of sulfenamide prodrug idea.

Recently, Stella *et al* have investigated the use of sulfenamides as potential prodrugs.^{21,78-80} The idea originated during the study and stability characterizations of phenylurea dithiocarbamate (PTC) at the University of Kansas. PTC is an anti-cancer agent

that contains a sulfenamide bond linking the phenyl urea moiety to the thiocarbamate moiety (Scheme 1.9). During aqueous stability characterization in buffered solutions, PTC was found to have a maximum stability at pH 6.0 and 25°C with an estimated shelf life of approximately six years. The hydrolysis of PTC in aqueous solutions occurred with the cleavage of the sulfenamide bond to release *N*-methyl phenyl urea and a mixture of products believed to result from the dithiocarbamate moiety. A study of PTC in the presence of excess free thiol containing compounds such as cysteine and glutathione resulted in a rapid cleavage (within a few seconds) of sulfenamide bond to release *N*-methyl phenyl urea (Scheme 1.9).⁸¹



Scheme 1.9. Breakdown of PTC in aqueous solutions and in the presence of glutathione

There are numerous free thiols present *in vivo*, with the most abundant free thiol being glutathione. Glutathione (GSH) a tripeptide thiol containing glutamic acid, L-cysteine, and glycine and has been defined as an important antioxidant, antitoxin and an essential cofactor to antioxidation enzymes.⁸²⁻⁸⁵ GSH has been found to have an electron donating ability due to its free thiol (-SH) group from the cysteine residue. Thus, it acts as a nucleophilic scavenger of free radicals and is referred to as a redox buffer in most living organisms, especially in aerobic organisms. GSH is the most abundant non-protein thiol

present *in vivo* in most organisms and exists readily in the reduced form with concentrations 0.1 to 10 mM in most tissues. In humans, glutathione exists predominantly in the liver and in red blood cells.⁸³

Other free thiols *in vivo* include thiol proteins such as albumin, which is the most abundant *in vivo* protein thiol. In humans, albumin concentrations can reach as high as 0.6 mM. Albumin contains 35 cysteine residues, 34 of which are involved in disulfide linkages, leaving one cysteine residue (Cys-34) with a free -SH group.⁸⁶ Also proteins such as integrin α -4, myosin heavy chain (non-muscle type A), myosin light-chain alkali (non-muscle isoform), and β -actin; all of which are found in the gut of humans contain free thiols that can serve as nucleophiles in reaction with a sulfenamide bond when introduced into the gastrointestinal track.⁸⁷ Hemoglobin which is found in red blood cells is also a thiol protein and together with albumin found in plasma will potentially serve as a source of free thiols to cleave the N-S bond in sulfenamides when introduced into whole blood.^{87,88}

The presence of *in vivo* free small molecule and protein thiols indicates a readily available source of nucleophiles for the cleavage of the N-S bonds in sulfenamides to release the parent drugs or molecule. The idea of using sulfenamides as potential prodrugs was originated in an attempt to improve the physicochemical properties such as solubility, lipophilicity and permeability of drug molecules or drug candidates containing -NH acid groups. The hypothesis being that free thiols *in vivo* will readily cleave the N-S bond to release the active parent drug or molecule.

1.8.2. Previously studied sulfenamide prodrugs.

Recently, Guarino and Stella reported their evaluation of model sulfenamides as potential prodrugs.^{21,78,79} Guarino was successful at synthesizing sulfenamide derivatives of

benzamide, phthalimide and carbamazepine and other -NH acids in an attempt to prove the concept of improving solubility and lipophilicity of -NH acids (Figure 1.6). This report, to our knowledge, was the first showing the application of sulfenamides as potential prodrugs.^{78,79} A study of the benzamide-cysteine derivative, 2-amino-3-(benzamidothio)propanoate (Figure 1.6), under aqueous conditions showed significant stability and hydrolytic degradation under to release the parent benzamide as the major product. At 25°C in 25 mM phosphate buffer solutions, the highest stability was found to occur at pH 6.0 with an estimated shelf life (t_{90}) of 6.0 years (Figure 1.7).

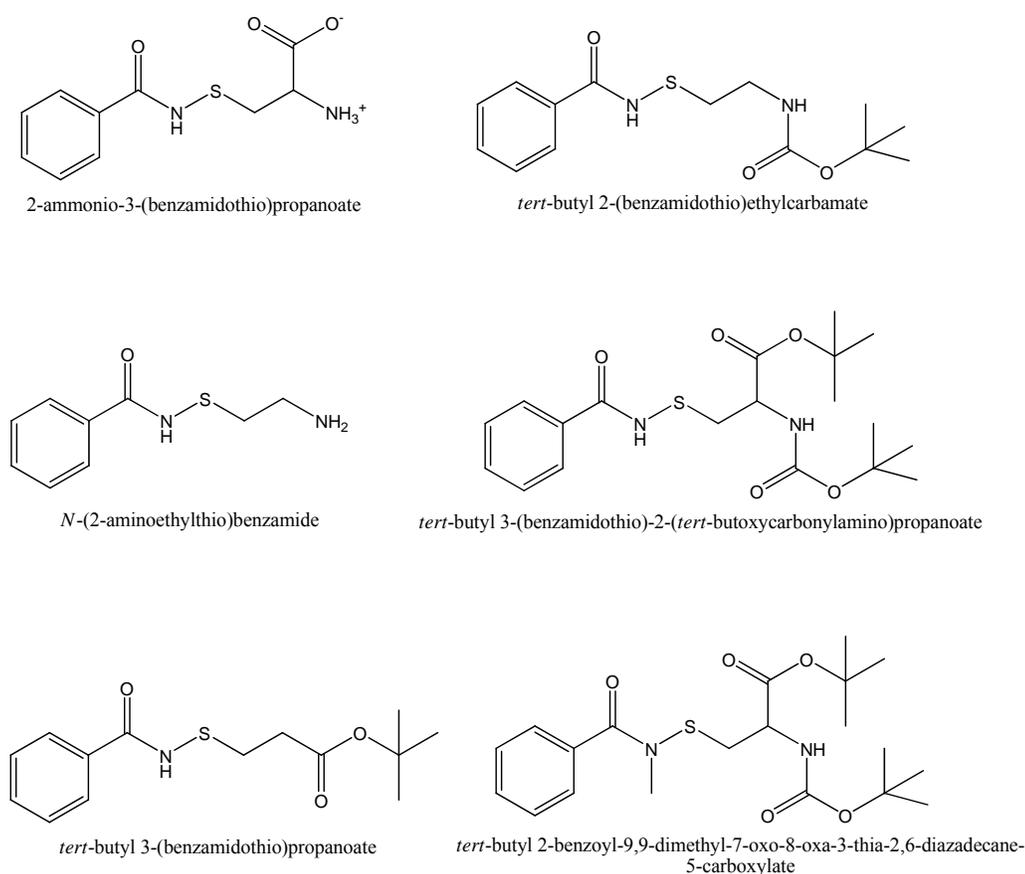


Figure 1.6: Model sulfenamide prodrugs of benzamide synthesized in attempts to improve aqueous and lipid solubility of benzamide

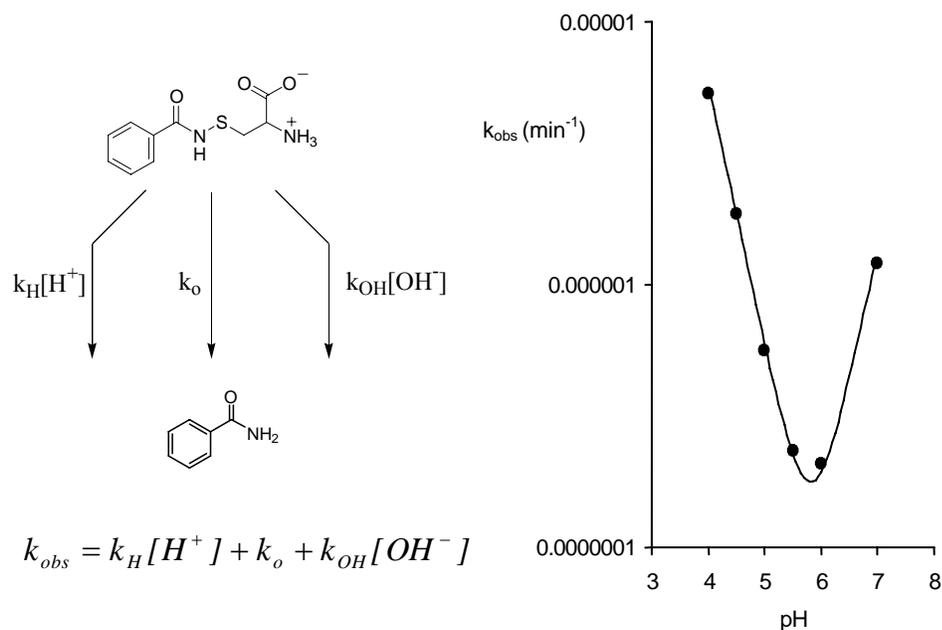
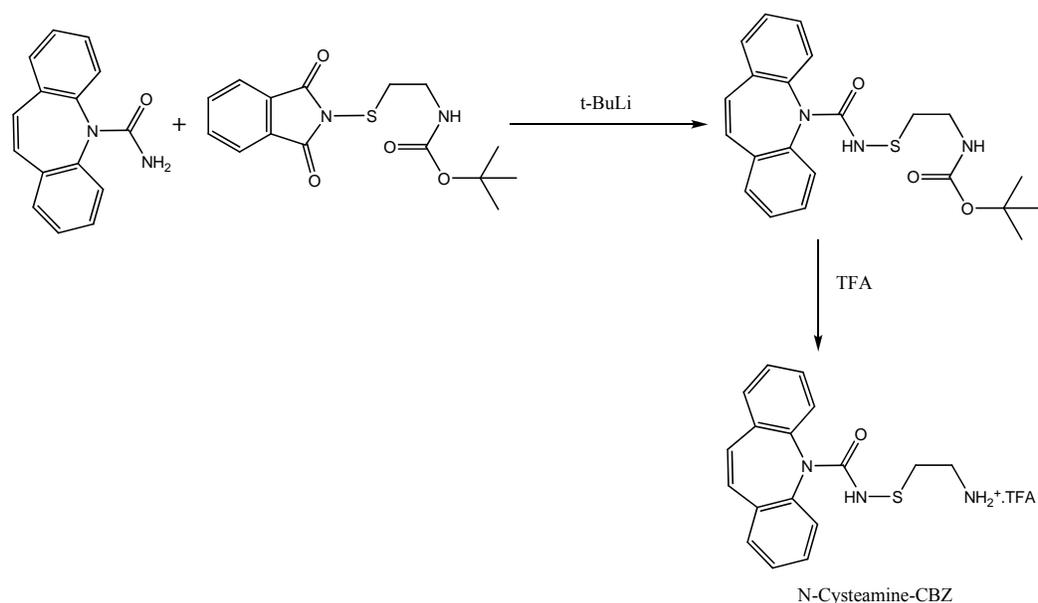


Figure 1.7: Aqueous stability of benzamide derivative using cysteine as a promoieity.

Maximum stability occurred at pH 6.0 with estimated half life of 6.33 years.^{78,79}

In a continuation of the work that was begun by Guarino, Hemenway *et al.*, also recently reported the synthesis and evaluation of a soluble carbamazepine derivative, using cysteamine as a promoieity (N-cysteamine-carbamazepine, Scheme 1.10).⁹⁰ Carbamazepine (CBZ) has very favorable activity and is effective in the treatment of general epileptic seizures. In comparison to other anticonvulsants such as phenytoin, phenobarbital and primidone, it is less sedating and shows lower cognitive impairments.⁹¹ However, due to the poor aqueous solubility of carbamazepine, high concentrations of co-solvents and surfactants were used for its parenteral formulations, all of which showed various levels of toxicities, resulting in the limited clinical use of carbamazepine in its injectable form.⁹²⁻⁹⁶

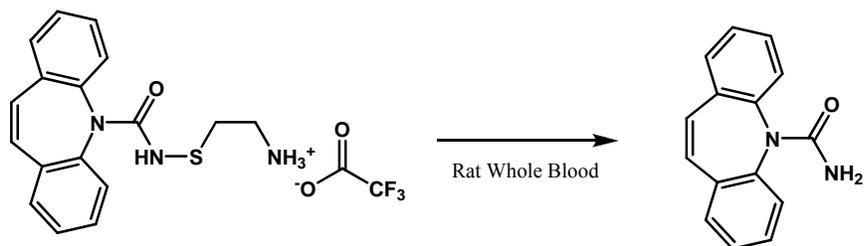


Scheme 1.10. Synthesis of N-cysteamine-CBZ from carbamazepine and *tert*-butyl 2-(1,3-dioxisoindolin-2-ylthio)ethylcarbamate.⁸⁹

N-cysteamine-CBZ, was synthesized by attaching the water soluble cystamine promoiety onto the amide (urea) functionality to form the sulfenamide bond, hence improving the aqueous solubility of the entire molecule. Aqueous stability studies conducted on N-cysteamine-CBZ in 25 mM isotonic buffer solutions at 25°C showed the prodrug to be fairly stable with maximum stability occurring at pH 4.0 with a half life of 5.7 years and a shelf life of 320 days. Temperature dependent studies estimated a shelf life of 75 years at 4°C, indicating that a refrigerated ready-to-use injectable formulation of N-cysteamine-CBZ could be possible.^{89,90}

In the presence of a 10 fold excess cysteine in 25mM isotonic buffer solution at pH 7.4, N-cysteamine-CBZ degraded in a matter of minutes to release the parent carbamazepine molecule. N-cysteamine-CBZ was administered to adult male rats and the plasma levels of CBZ observed showed an immediate spike in levels of CBZ with an AUC of 964 $\mu\text{g min/mL}$

for CBZ from N-cysteamine-CBZ and 959 $\mu\text{g min/mL}$ for CBZ from control (Scheme 1.11 and Figure 1.8).⁸⁹



Scheme 1.11. N-cysteamine-CBZ in the presence of rat whole blood rapidly converted (in minutes) to quantitative amounts of CBZ.⁸⁹

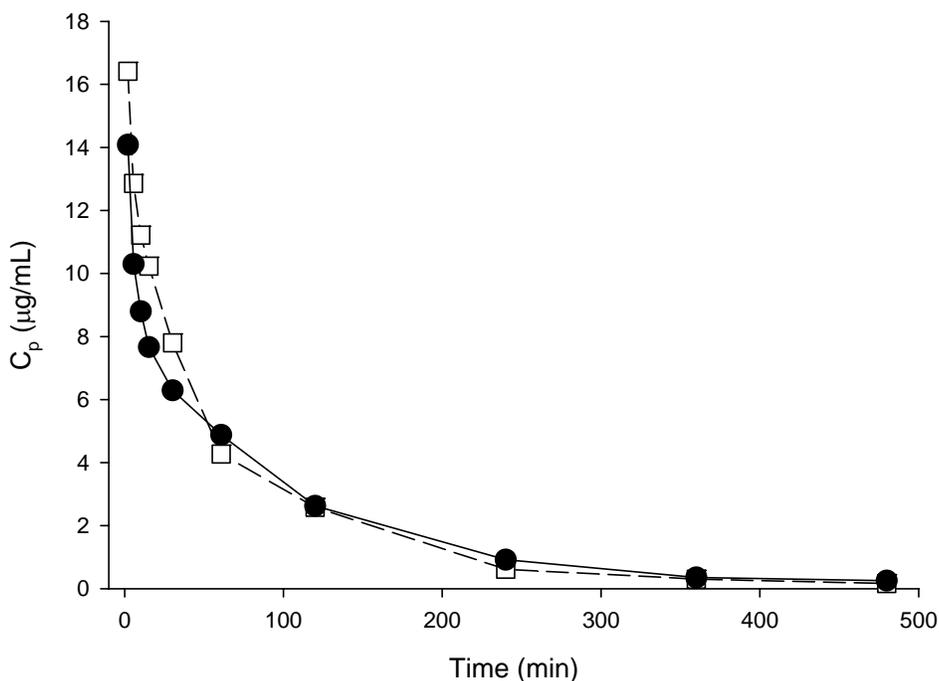


Figure 1.8. Plasma concentration of CBZ versus time profile following crossover IV administration of N-cysteamine-CBZ (●) and CBZ control (□) in rats.⁸⁹

1.9. Summary and Specific Aims

This dissertation is a continuation of work began by Guarino and Hemenway in the evaluation of sulfenamides as potential prodrugs. Our overall objective is to develop and study water soluble and permeable prodrugs of the novel oxazolidinones, Linezolid (Zyvox[®])

and its thiomorpholinyl analogues U100480 and U101603. To accomplish this goal and to contribute to work already done, this dissertation will focus on the following specific aims:

Specific Aim 1: Synthesize sulfenamide derivatives of Linezolid, U100480 and U101603 for evaluation as prodrugs.

Specific Aim 2: Determine the chemical stability of these derivatives to assess their potential to be formulated for parenteral and/or oral use.

Specific Aim 3: Determine the rate and degradation mechanism in the presence of free thiol containing compounds such as cysteine and glutathione.

Specific aim 4: Determine the breakdown of the sulfenamide prodrugs in the presence of free thiol containing proteins.

Specific aim 5: Determine and develop a model for the permeability characteristics of the prodrugs in Caco 2 cell monolayers.

The research presented in this dissertation is broken down into five remaining chapters. Chapter 2 reports the improved synthesis and the characterization of sulfenamide prodrugs from novel antibacterial oxazolidinones. Chapter 3 reports the *in vitro* conversion and the mechanism of breakdown of sulfenamides prodrugs in the presence of sulfhydryl compounds. Chapter 4 reports the *in vitro* conversion of sulfenamide prodrugs in thiol containing proteins. Chapter 5 reports the determination of the permeability characteristics of sulfenamide prodrugs in Caco-2 cell monolayers. Finally, Chapter 6 will contain an overall summary, conclusions drawn from this investigation, and suggestions for future research on this topic.

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Chapter 2

**Synthesis and physicochemical characterization of sulfenamide prodrugs from novel
antibacterial oxazolidinones.**

2.1. Introduction

2.1.1. Overall objective

The overall objective of this chapter is to effectively synthesize and characterize sulfenamide prodrugs from novel antimicrobial oxazolidinones. The purpose is to provide support for the application of sulfenamide prodrugs to improve the physicochemical properties of the “amide type” -NH acid drugs. The term “amide-type” will be used to refer to -NH acids whose active nitrogen, the nitrogen to be derivatized, is adjacent to just one carbonyl functional group. This chapter focuses on the synthesis of model sulfenamide derivatives from antimicrobial oxazolidinones, which contain an amide group adjacent to their oxazolidinyl ring. In addition to developing an effective synthetic route for making the prodrugs, it is our goal to study the aqueous stability characteristics of the prodrugs. This chapter also aims at understanding the degradation mechanism of the prodrugs under aqueous conditions.

2.1.2. Sulfenamides: Definition and Background.

Sulfenamides are defined as compounds containing a single bond between trivalent nitrogen and a bivalent sulfur atom.^{1,2} For the purposes of this dissertation, this single covalent bond will be referred to as the sulfenamide bond (N-S bond). They are generally considered to be derivatives of sulfenic acids but can also be readily synthesized from -NH acids including amides, imides, carbamates and ureas, by reactions with sulfonyl halides, disulfides, arylsulfenates, thiosulfenates and thiocyanates.²⁻⁵

Depending on the degree of substitution occurring on the nitrogen atom, sulfenamides can be grouped into three major categories; monosulfenamides, disulfenamides and trisulfenamides (Figure 2.1).² Unlike most organic molecules, all three types of sulfenamides have both the sulfur and the nitrogen atoms acting as reaction centers, allowing

for the cleavage of the bond by either nucleophiles or electrophiles. The electrophilic or nucleophilic cleavage permits for the ready removal of the sulfenyl moiety, making the sulfenamide bond fairly labile. This property of sulfenamides causes them to be useful in organic and peptide synthesis, where sulfenyl moiety is used as a protecting group that is readily cleaved once the peptide synthesis is complete.^{4,6} The labile sulfenamide bond also makes sulfenamides very effective sulfenyl and aminyl transfer agents in the synthesis of organic molecules, due in part to the stable sulfenyl and aminyl intermediates that are generated once the sulfenamide bond is cleaved.

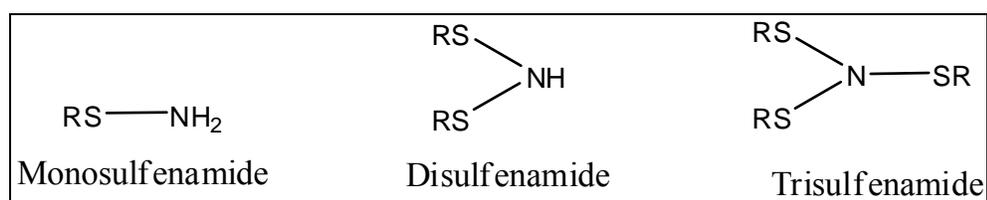


Figure 2.1: Three major categories of sulfenamides.

The chemistry occurring at the sulfenamide bond is as a result of a number of interactions between the sulfur and the nitrogen bond. The sulfur and the nitrogen atoms both have lone pairs of electrons, but due to p-d pi bonding occurring at the sulfenamide bond, nitrogen donates its pair of electrons to the sulfur atom. This results in a significant difference in the electronegativity of the two atoms, making the sulfur atom more susceptible to nucleophilic attack whereas the nitrogen atom undergoes electrophilic attack.^{2-4,6-8} This chemistry has allowed the use of sulfenamides in numerous industrial applications as mentioned in Chapter 1, Section 1.7; however, few literature reports the use of sulfenamides as prodrugs.

2.1.3. Oxazolidinones; Definition and background

Antibacterial oxazolidinones were first introduced in 1987 by DuPont researchers when they reported the synthesis two oxazolidinones; (S)-N-[(3-(4- acetylphenyl) -2-oxo -5-oxazolidinyl)methyl] acetamide (DuP 721) and (S)-N-[(3-(4-(methylsulfinyl) phenyl)-2-oxo-5-oxazolidinyl)methyl] acetamide (DuP 105) (Figure 2.2).⁹ DuP 721 and DuP 105 expressed potent *in vitro* and *in vivo* activity towards a variety of gram positive bacteria such as multi-resistant *Staphylococcus aureus* (MRSA), methicillin-resistant *Staphylococcus epidermidis* (MRSE) and *Mycobacterium tuberculosis*.¹⁰⁻¹³

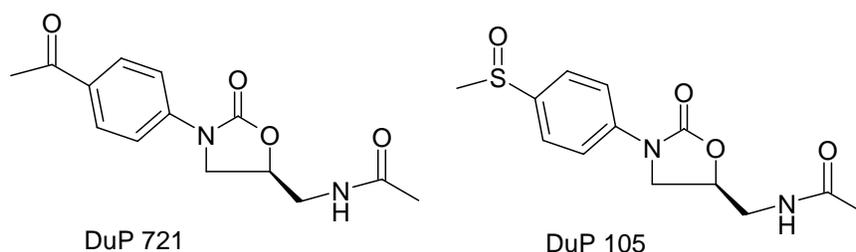


Figure 2.2: New novel oxazolidinone antimicrobial agents developed in 1987 by DuPont scientists.⁹

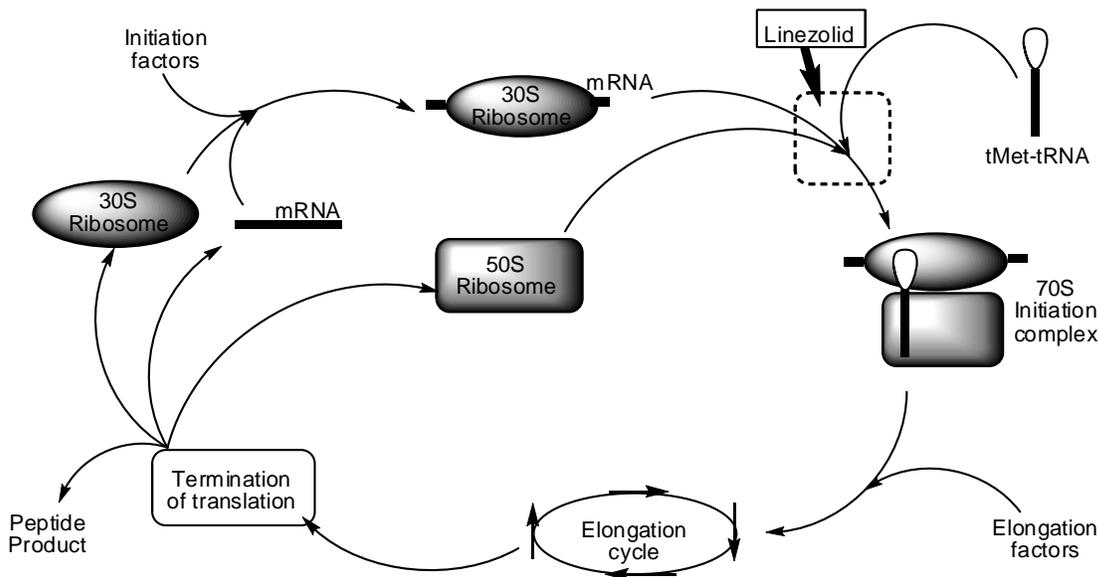


Figure 2.3: Protein synthesis inhibition by oxazolidinones.¹⁴

Further studies of oxazolidinones indicated that oxazolidinones presented the ability to withstand development of cross-resistance from the various bacterial strains that were tested. The ability to withstand the development of cross-resistance was found to occur due to a unique mode of action of the oxazolidinones on the bacteria., a mode of action that involved the inhibition of protein synthesis in the bacteria; thus preventing the bacterial growth and development.^{12,14-17} As illustrated in figure 2.3, oxazolidinones inhibit the formation of the tRNA^{fMet}-mRNA-70S complex in the translation step thus preventing successful protein synthesis process from occurring.^{14,17-20}

Despite the unique mode of action by the oxazolidinones discovered, DuPont was forced to abandon the development of DuP 721 and DuP 105 due to severe toxicity levels observed in their animal studies.²⁰ However, DuPont's discovery sparked a new interest in the development of oxazolidinones and their uses as antibacterial agents. In 1996, scientist at Pharmacia reported the synthesis of analogues to DuP 721, namely; U-100592 (Eperizolid), U-100766 (Linezolid) and U-100480.^{11,21,22}

Eperizolid and linezolid, produced identical MIC values, antibacterial spectrum, ED₅₀ values and pharmacokinetic behavior from *in vivo* studies. Phase 1 human studies showed that eperizolid had a significantly reduced oral bioavailability compared to linezolid, and as a result eperizolid was dropped from further development studies. Linezolid was later marketed as Zyvox[®] and is the first antibacterial agent of this new class of synthetic compounds.

Poor aqueous solubility resulted in U-100480 being dropped out of development. Attempts to improve on the solubility of U-100480 led to the synthesis of U-10163; however, poor intestinal permeability resulted in poor oral bioavailability of U-10163. Our ultimate goal in this research is to improve the bioavailability by improving the solubility and

permeability of the oxazolidinones using sulfenamide prodrug derivatives. This chapter will specifically focus on the synthesis and stability characterization of model sulfenamide derivatives of Linezolid and its analogues; U-100480 and U-101603.

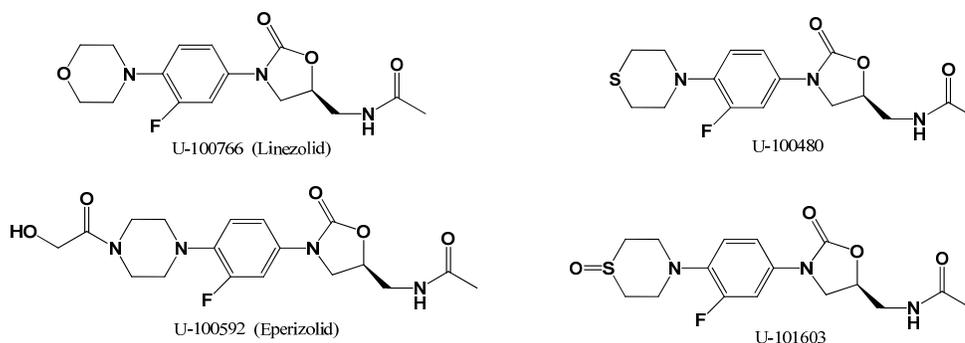


Figure 2.4: Novel Anti-bacterial oxazolidinones developed by Pharmacia® that showed significant activity towards gram-positive and gram-negative bacterial strains.

2.2. Materials and Methods

2.2.1. Solvents, Chemicals and Instruments

All organic solvents including hexanes, ethyl acetate, dichloromethane, 1,2-dichloroethane, tetra-hydrofuran (THF), tetra-chloromethane, chloroform, and toluene were purchased from Fischer Scientific (Pittsburg, PA) and used without further purification unless otherwise stated. Sodium bicarbonate, sodium chloride, hydrochloric acid (12N), trifluoroacetic acid (TFA), sulfuric acid (36 N), acetic acid and formic acid were also purchased from Fischer Scientific. Sodium hydride, sulfuryl chloride, phthalimide, potassium phthalimide, N-chlorophthalimide, N-bromophthalimide, N-(phenylthio)phthalimide, triethylamine, pyridine, *n*-butyllithium, tert-butyllithium, 3,4-difluoronitrobenzene, thiomorpholine, benzyl chloroformate, raney nickel, R-(-)-glycidyl butyrate, ammonium chloride, methane sulfonyl chloride, sodium azide, triphenyl-phosphine, Di-boc-cystamine,

acetic anhydride, phenyl disulfide, ethyl disulfide, dimethyl formaldehyde, benzophenone, chloroform-*d* (CDCl₃), deuterium oxide (DMSO-*d*₆), and bromine were purchased from Sigma-Aldrich Chemical Co. (St. Louis, MO). 3-mercapto propionic acid ethyl ester and 3-mercapto propionic acid isopropyl ester were purchased from TCI Chemicals (Wellesley, WI).

All reactions were carried out under inert atmosphere under argon unless otherwise stated. Glassware, syringes and needles used for all the reactions were washed with water and acetone before drying in 150°C oven overnight. The glassware was cooled under argon prior to use while syringes and needles were cooled in a desiccator prior to use. Anhydrous dichloromethane was freshly distilled over calcium hydride; whereas THF was distilled over sodium metal and benzophenone prior to use.

Flash chromatography used in purification processes required silica gel purchased from Fisher Scientific or alumina base gel purchased from Sigma-Aldrich. Linezolid was extracted with H₂O/CH₂Cl₂ from Zyvox[®] purchased from the local pharmacy.

All ¹H NMR and ¹³C NMR were carried out using a 400 MHz Bruker NMR instrument, and mass spectral analyses were carried out on a Micromass Quatro Micro Tandem Quadrupole Mass Spectrometer.

2.2.2. Synthesis and characterization of oxazolidinones

2.2.2.1. General

Oxazolidinones used in all studies were synthesized using slight modifications to previously described procedures.²¹⁻²⁷ 3-(3-fluoro-4-thiomorpholinophenyl)-5-(hydroxymethyl)oxazolidin-2-one (**2.1**) was synthesized as described by Barbachyn *et al* to a 83 % yield white crystalline solid.²¹

2.2.2.2. (R)-[3-[3-Fluoro-4-(4-thiomorpholinyl)phenyl]-2-oxo-5

oxazolidinyl)methyl methanesulfonate (2.2)

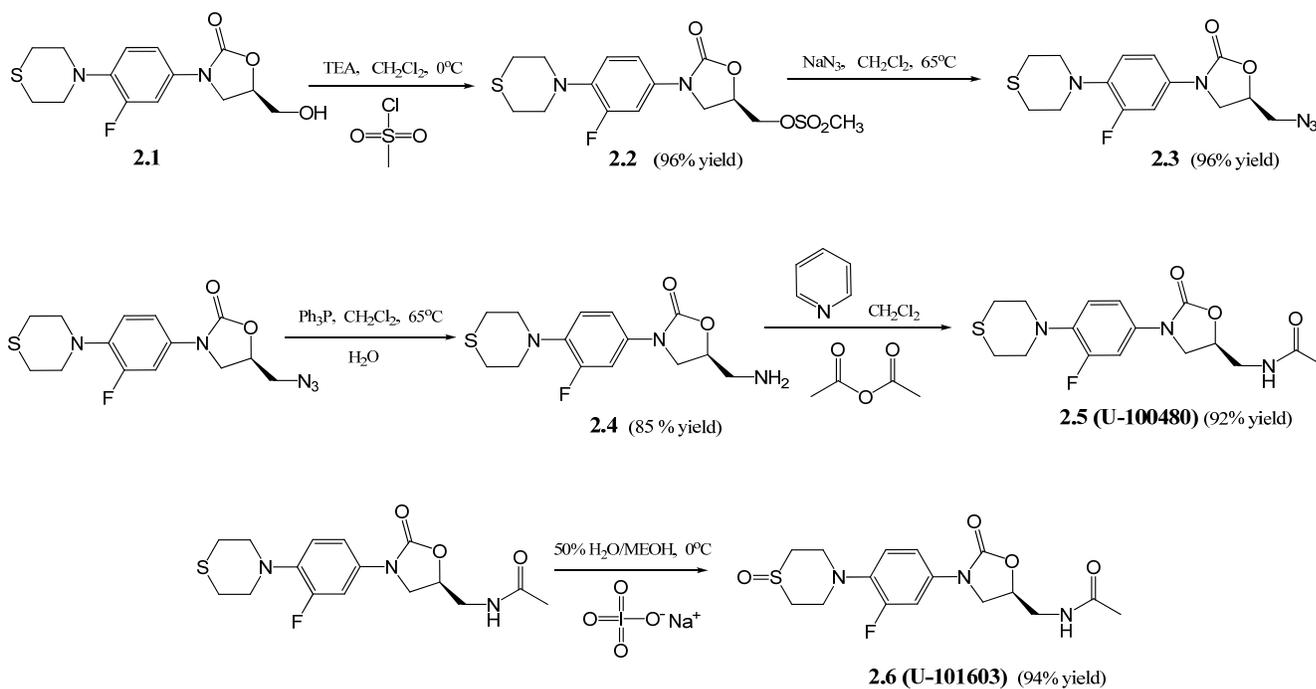
Triethylamine (TEA, 1.70 mL, 12.04 mmol) was slowly added (drop wise) to a solution of **2.1** (2.5 g, 8.0 mmol) in dry methylene chloride (CH₂Cl₂) at 0°C while stirring under argon. Methanesulfonyl chloride (0.65 ml, 8.38 mmol) was then added drop wise and the reaction mixture and stirred at 0°C for approximately three hours. The reaction was checked for completion with thin layer chromatography (TLC, ethyl acetate/hexane). The mixture was then transferred into a separatory funnel with CH₂Cl₂ washings, washed twice with 100 mL of deionized water (diH₂O), 100 mL of saturated sodium bicarbonate (NaHCO₃) and 100 mL of brine. The organic layer was dried over sodium sulfate (Na₂SO₄) and dried under vacuum to give an off white solid. The crude solid was purified by silica gel chromatography (ethyl acetate/hexane) to give a white powder (96 % yield) ¹H NMR (400 MHz, Chloroform-*d*) δ ppm 2.76 - 2.85 (m, 4 H) 3.11 (s, 3 H) 3.23 - 3.34 (m, 4 H) 3.92 (dd, *J*=9.06, 6.14 Hz, 1 H) 4.12 (t, *J*=9.21 Hz, 1 H) 4.38 - 4.54 (m, 2 H) 4.87 - 4.97 (m, 1 H) 6.97 (t, *J*=9.06 Hz, 1 H) 7.11 (ddd, *J*=8.77, 2.63, 1.17 Hz, 1 H) 7.37 - 7.48 (m, 1H)

2.2.2.3. (R)-5-(azidomethyl)-3-(3-fluoro-4-thiomorpholinophenyl)

oxazolidin-2-one (2.3)

Sodium azide (2.50 g, 38.5 mmol) was added to a solution of **2.2** (3.0 g, 7.7mmol) in dry CH₂Cl₂ at ambient temperature and placed under argon. The reaction mixture was then warmed to 65°C and stirred at this temperature for approximately 10 hours. The reaction was checked for completion with TLC (ethylacetate/ hexane) and the mixture transferred into a separatory funnel with CH₂Cl₂ washings, washed with diH₂O (2 X 100 ml) and brine

(100ml). The organic layer was dried over Na₂SO₄ and the solvent removed under vacuum to give a tan solid (96% yield). ¹HNMR (400 MHz, Chloroform-*d*) δ ppm 2.77 - 2.85 (m, 4 H) 3.25 - 3.33 (m, 4 H) 3.56 - 3.63 (m, 1 H) 3.67 - 3.74 (m, 1 H) 3.83 (dd, *J*=9.06, 6.14 Hz, 1 H) 4.06 (t, *J*=8.92 Hz, 1 H) 4.74 - 4.82 (m, 1 H) 6.93 - 7.01 (m, 1 H) 7.10 - 7.15 (m, 1 H) 7.44 (dd, *J*=14.03, 2.63 Hz, 1 H)



Scheme 2.1: Synthesis of antimicrobial oxazolidinones adopted from Barbachyn *et al.*²¹

2.2.2.4. (S)-5-(aminomethyl)-3-(3-fluoro-4-thiomorpholinophenyl) oxazolidin-2-one. (2.4)

Triphenylphosphine (1.1 eq, 1.63g, 6.20 mmol) was added to a solution of **2.3** (1.0 eq, 1.90g 5.64mmol) in 20 mL dry THF, placed under argon and stirred at ambient temperature for two hours of stirring. Deionized water (0.132 mL) was added and the reaction

mixture heated to 40°C, stirred for 15 hrs, allowed to cool to room temperature and diluted with 100ml of 1N HCl. The mixture was transferred into a separatory funnel and extracted with ethyl acetate (2 X 100 ml). The aqueous layer was made alkaline with 1N NaOH and extract with 1,2-dichloroethane (2 X 100 mL). The organic layer was washed with diH₂O and brine, dried over Na₂SO₄ and vacuum dried to give a crystalline off white solid (85% yield).
1H NMR (400 MHz, Chloroform-*d*) δ ppm 2.77 - 2.87 (m, 4 H) 2.94 - 3.02 (m, 1 H) 3.12 (dd, *J*=13.52, 3.92 Hz, 1 H) 3.24 - 3.34 (m, 4 H) 3.82 (t, *J*=7.58 Hz, 1 H) 3.97 - 4.06 (m, 1 H) 4.68 (d, *J*=6.57 Hz, 1 H) 6.91 - 7.00 (m, 1 H) 7.11 - 7.18 (m, 1 H) 7.41 - 7.51 (m, 1 H) 7.53 - 7.63 (m, 1 H) 7.64 - 7.77 (m, 1 H)

2.2.2.5. *N*-((3-(3-fluoro-4-thiomorpholinophenyl)-2-oxooxazolidin-5-yl)methyl)acetamide (U-100480) (2.5)

Twenty microliters of dry CH₂Cl₂ was added to **2.4** (1.0 eq, 6.12 mmol, 1.90 g) and placed under argon. Pyridine (2.0 eq, 1.0 mL, 1.24 mmol) was then slowly added (drop wise) while stirring, followed by acetic anhydride (2.0 eq, 1.2 mL, 1.24 mmol). The reaction mixture was stirred overnight at ambient temperature after which 100 mL of 1N HCl was added and transferred to a separating funnel. The aqueous layer was made alkaline with saturated NaHCO₃ and extracted with CH₂Cl₂. The organic layer was then washed with diH₂O and brine, dried over Na₂SO₄ and vacuum dried to yield a white powder (92% yield).²¹
Mp 182 to 184°C. 1H NMR (400 MHz, Chloroform-*d*) δ ppm 2.03 (s, 3 H) 2.79 - 2.87 (m, 4 H) 3.28 - 3.34 (m, 4 H) 3.58 - 3.66 (m, 1 H) 3.67 - 3.78 (m, 2 H) 4.02 (t, *J*=8.97 Hz, 1 H) 4.77 (tt, *J*=8.97, 3.28 Hz, 1 H) 6.15 (t, *J*=5.81 Hz, 1 H) 6.99 (t, *J*=8.46 Hz, 1 H) 7.05 - 7.11 (m, 1 H) 7.43 (dd, *J*=14.02, 2.40 Hz, 1 H)

2.2.2.6. (S)-N-((3-(3-fluoro-4-(1-oxothiomorpholin-4-yl)phenyl)-2-oxo-5-oxazolidinyl) methyl)acetamide (U101603) (2.6)

Half a gram of **2.5** (1.42 mmol) was added to a solution of sodium metaperiodate (1.1 eq, 0.334g, 1.56 mmol) in a 1:1 solution of diH₂O/MeOH and at 0°C. The reaction was stirred for three hours and placed in the refrigerator for approximately four days. The mixture was then transferred into a separatory funnel with 100 mL of diH₂O and washed with CH₂Cl₂, dried over Na₂SO₄ and the organic solvent removed under vacuum to yield a white crystalline solid (93.5% yield). ¹H NMR (400 MHz, Chloroform-*d*) δ ppm 2.03 (s, 3 H) 2.96 - 3.02 (m, 4 H) 3.23 - 3.30 (m, 2 H) 3.57 - 3.65 (m, 1 H) 3.68 - 3.79 (m, 4 H) 4.04 (t, *J*=9.06 Hz, 1 H) 4.74 - 4.82 (m, 1 H) 5.94 (t, *J*=4.97 Hz, 1 H) 7.01 - 7.12 (m, 2 H) 7.50 (dd, *J*=14.03, 2.34 Hz, 1 H)

2.2.3. Synthesis and characterization of sulfenamides

2.2.3.1. Ethylthiophthalimide. (2.7)

Ethyldisulfide (5.0 ml, 4.12 mmol) was added to a suspension of N-chloro phthalimide (1.5 g, 8.24 mmol) in 25mL of dry CH₂Cl₂ and stirred at ambient temperature for approximately five hours. The mixture was vacuum dried and the resulting residue was purified by silica gel chromatography (40% ethyl acetate/hexane) to yield an oil (31% yield). ¹H NMR (400 MHz, Chloroform-*d*) δ ppm 1.28 (t, *J*=7.31 Hz, 3 H) 2.85 - 2.99 (m, 2 H) 7.77 - 7.81 (m, 2 H) 7.92 - 7.95 (m, 2 H)

2.2.3.2. Ethyl 3-(1,3-dioxoisindolin-2-ylthio)propanoate. (2.8)

The procedure for the synthesis of thiophthalimides has been previously reported.^{28,29} N-chlorophthalimide (2.0 g, 11.0 mmol, 1.1 eq.) and 3-mercapto propionic acid ethyl ester

(1.34 g, 10 mmol, 1 eq.) were added to a flask containing 20 mL of dry CH₂Cl₂, placed under argon and cooled to 0°C. Triethylamine (11 mmol, 1.53 mL, 1.1 eq.) was then added drop wise and the reaction mixture stirred for 45 minutes at ambient temperature. The reaction was checked with TLC (ethyl acetate/hexane) for completion and transferred to a separatory funnel with CH₂Cl₂ washings. The mixture was washed three times with 100 mL of diH₂O, dried over Na₂SO₄ and the organic solvent removed under vacuum to yield a light brown solid. (32.2% yield) ¹H NMR (400 MHz, DMSO-*d*₆) δ ppm 1.11 (t, *J*=7.07 Hz, 3 H) 2.69 (t, *J*=6.69 Hz, 2 H) 3.03 (t, *J*=6.69 Hz, 2 H) 3.93 (q, *J*=7.07 Hz, 2 H) 7.87 - 7.90 (m, 2 H) 7.91 - 7.94 (m, 2 H)

2.2.3.3. (R)-N-((3-(3-fluoro-4-morpholinophenyl)-2-oxo oxazolidin-5-yl)methyl)-N-(phenylthio)acetamide. (2.9)

Tert-butyllithium (1.1 eq, 1.63 mmol) was added drop-wise to a solution of linezolid (0.5 g, 1.48 mmol) in dry CH₂Cl₂ at -78°C and stirred for 30 minutes. A solution of N-(phenylthio)phthalimide (0.42 g, 1.63 mmol, 1.1 eq) in dry CH₂Cl₂ was added drop-wise to the reaction mixture, stirred for an hour. The reaction was warmed to room temperature and stirred for another 3 hours. The mixture was transferred into a separatory funnel and washed with H₂O and brine, dried over Na₂SO₄ and dried under vacuum to give a yellow oil. The oil was purified with silica gel chromatography (5% MeOH/CH₂Cl₂) to give a 33.7% yield of an off white solid. 33.7 % yield as a white solid. ¹H NMR (400 MHz, Chloroform-*d*) δ ppm 2.41 (s, 3 H) 3.01 - 3.08 (m, 4 H) 3.75 (dd, *J*=9.06, 6.14 Hz, 1 H) 3.83 - 3.90 (m, 4 H) 3.94 (dd, *J*=14.03, 6.72 Hz, 1 H) 4.03 (t, *J*=8.92 Hz, 1 H) 4.07 - 4.15 (m, 1 H) 4.88 - 4.97 (m, 1 H) 6.93 (t, *J*=9.06 Hz, 1 H) 7.07 - 7.16 (m, 3 H) 7.24 - 7.30 (m, 1 H) 7.36 - 7.46 (m, 3 H)

2.2.3.4. (R)-N-((3-(3-fluoro-4-thiomorpholinophenyl)-2-oxooxazolidin-5-yl)methyl)-N-(phenylthio)acetamide. (2.10)

Synthesis was as described above for **2.9** above using **2.5** (0.64 g, 1.80 mmol, 1 equiv) instead of linezolid. Purification by silica gel chromatography (5% MeOH/CH₂Cl₂) gave an off white solid (50% yield). ¹H NMR (400 MHz, Chloroform-*d*) δ ppm 2.41 (s, 3 H) 3.01 - 3.08 (m, 4 H) 3.75 (dd, *J*=9.06, 6.14 Hz, 1 H) 3.83 - 3.90 (m, 4 H) 3.94 (dd, *J*=14.03, 6.72 Hz, 1 H) 4.03 (t, *J*=8.92 Hz, 1 H) 4.07 - 4.15 (m, 1 H) 4.88 - 4.97 (m, 1 H) 6.93 (t, *J*=9.06 Hz, 1 H) 7.07 - 7.16 (m, 3 H) 7.24 - 7.30 (m, 1 H) 7.36 - 7.46 (m, 3 H)

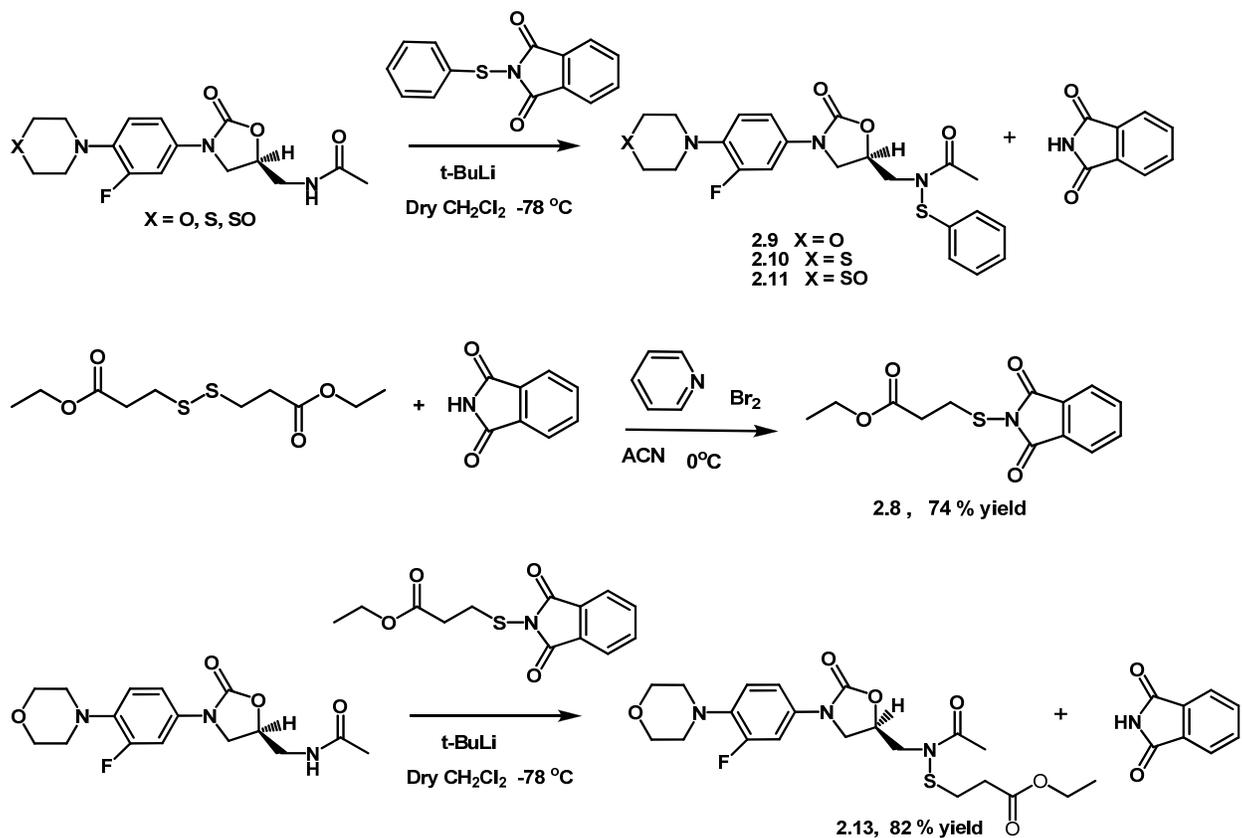
2.2.3.5. (R)-N-((3-(3-fluoro-4-(1-oxothiomorpholin-4-yl)phenyl)-2-oxooxazolidin-5-yl)methyl)-N-(phenylthio)acetamide. (2.11)

Synthesis was as described above for **2.9** above using **2.6** (0.30 g, 0.813 mmol, 1 equiv) instead of linezolid. Purification by silica gel chromatography (5% MeOH/CH₂Cl₂) gave an off white solid (70% yield). ¹H NMR (400 MHz, Chloroform-*d*) δ ppm 2.41 (s, 3 H) 3.01 - 3.08 (m, 4 H) 3.75 (dd, *J*=9.06, 6.14 Hz, 1 H) 3.83 - 3.90 (m, 4 H) 3.94 (dd, *J*=14.03, 6.72 Hz, 1 H) 4.03 (t, *J*=8.92 Hz, 1 H) 4.07 - 4.15 (m, 1 H) 4.98 - 5.12 (m, 1 H) 6.93 (t, *J*=9.06 Hz, 1 H) 7.07 - 7.16 (m, 3 H) 7.24 - 7.30 (m, 1 H) 7.56 - 7.86 (m, 3 H)

2.2.3.6. (R)-N-(ethylthio)-N-((3-(3-fluoro-4-morpholinophenyl)-2-oxooxazolidin-5-yl)methyl)acetamide. (2.12)

Tert-butyl lithium (0.80 mmol, 1.1 eq.) was added drop wise to a solution of linezolid (0.26 g, 0.77 mmol) in dry CH₂Cl₂ at -78°C and stirred for 30 minutes. A solution of ethylthiophthalimide (0.17 g, 0.80 mmol, 1.1 eq) in dry CH₂Cl₂ was then added drop wise to the reaction mixture and stirred at -78°C for 30 minutes, allowed to warm to room

temperature and stirred for another 2hrs. The mixture was transferred into a separatory funnel, extracted with diH₂O and brine, dried over Na₂SO₄, and vacuum dried to give a brown solid (50% yield). ¹H NMR (400 MHz, Chloroform-*d*) δ ppm 1.31 – 1.38 (m, 3H) 2.56 – 2.63 (m, 2H) 3.01 - 3.08 (m, 4 H) 3.83 - 3.90 (m, 4 H) 3.94 (dd, *J*=14.03, 6.72 Hz, 1 H) 4.03 (t, *J*=8.92 Hz, 1 H) 7.30 - 7.46 (m, 3 H)



Scheme 2.2: Synthesis of sulfenamide prodrugs of oxazolidinones

2.2.3.7. *Ethyl 3-(N-((3-(3-fluoro-4-morpholinophenyl)-2-oxo*

oxazolidin-5-yl)methyl)acetamidothio)propanoate. (2.13)

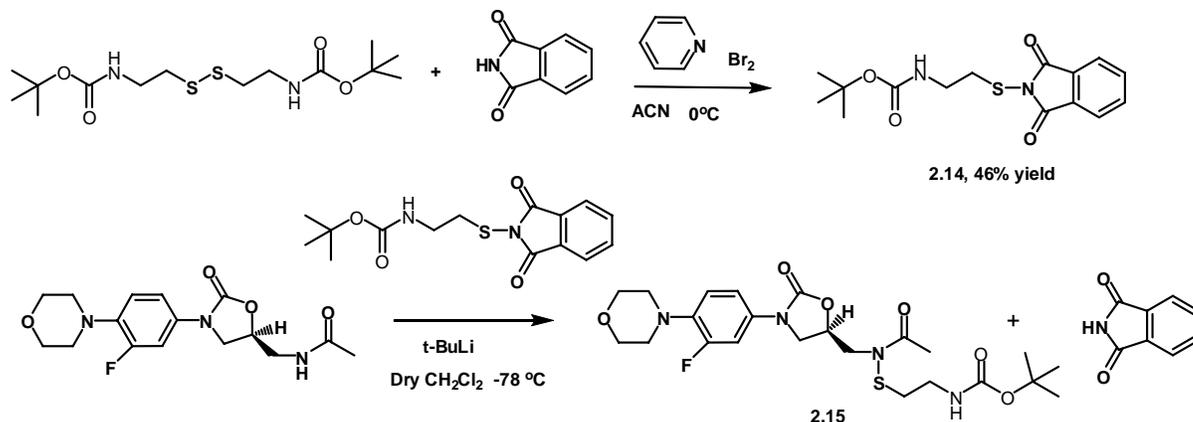
Ethyl 3-(1,3-dioxoisindolin-2-ylthiol) propanoate (**2.8**, 0.54 mmol, 0.15 g) in flask was dissolved with 2 mL of dry CH₂Cl₂ and cooled to -78°C. Linezolid (0.54mmol, 0.18g) was dissolved in 5 mL of dry CH₂Cl₂ in another flask under argon and cooled to -78°C. *Tert*-butyllithium was then added drop wise to the linezolid solution, stirred for 10 minutes, followed by the addition of the cooled solution of **8**. The reaction mixture was stirred at -78°C for 30 minutes and then at room temperature for an additional 30 minutes. Toluene (15 mL) was added to the reaction mixture and the resulting precipitates filtered off. The filtrate was then dried under vacuum to give a crude solid, which was then dissolved in 20 mL of ethyl acetate, filtered and vacuum dry to give a brown solid (82.3% yield). ¹H NMR (400 MHz, Chloroform-*d*) δ ppm 1.27 – 1.29 (t, 3H) 2.04 (s, 3H) 2.54 (t, 2H) 3.01 - 3.08 (m, 4 H) 3.75 (dd, *J*=9.06, 6.14 Hz, 1 H) 3.83 - 3.90 (m, 4 H) 3.94 (dd, *J*=14.03, 6.72 Hz, 1 H) 4.03 (t, *J*=8.92 Hz, 1 H) 4.07 - 4.13 (m, 1 H) 4.88 - 4.97 (m, 1 H) 6.72 (t, *J*=9.06 Hz, 1 H) 7.30 - 7.46 (m, 3 H)

2.2.3.8. *Attempted Synthesis of (R)-tert-butyl 4-(N-((3-(3-fluoro-4-*

morpholinophenyl)-2-oxooxazolidin-5-yl)methyl)acetamidothio)butanoate (2.15)

The synthesis of *Tert*-butyl 2-(1,3-dioxoisindolin-2-ylthio)ethyl carbonate (**2.14**) from *tert*-butyl 2,2-disulfanediybis(ethane-2,1-diyl)dicarbamate was previously reported using the procedure as outlined for **2.8** to a 46% yield.^{28,29} Attempts to synthesize **2.15** from linezolid and **2.14** in the presence of *tert*-butyl lithium as indicated in scheme 2.3 did not yield any products. Analysis of the reaction with TLC shown only linezolid, phthalamide

and *tert*-butyl 2,2-disulfanediyldis(ethane-2,1-diyl)dicarbamate present. ^1H NMR ^{13}C NMR confirmed that no oxazolidinones sulfenamide products were formed.



Scheme 2.3: Synthesis of sulfenamide prodrugs of oxazolidinones

2.2.4. Determining aqueous stability of sulfenamide prodrugs models.

2.2.4.1. Solvents and chemicals

Acetonitrile, acetone, hydrochloric acid (12N), acetic acid, formic acid, phosphoric acid, trifluoroacetic acid (TFA), sodium phosphate (mono and dibasic), sodium acetate, sodium formate, and sodium chloride were purchased from Fisher Scientific. Water used in all experiments was freshly distilled and deionized in house using Corning[®] Mega-pure One Liter distillation apparatus. Buffer solutions (table 2.1) were prepared to 50 mM concentrations and adjusted to ionic strength of 0.15 M with sodium chloride for pH values greater than 3.0. At pH values under 3.0, various concentrations of hydrochloric acid were used. The pH values of buffer solutions were measured with a Fischer Scientific Accumet Research AR15 pH meter with standard buffers of pH 4.0, 7.0 and 10.0 purchased from Fisher Scientific. Prior to use, all buffer solutions were filtered through 0.22 μm syringe filters, purchased from Fisher Scientific. All stability samples were kept in Blue M[®] Stabil-Term ovens at 50, 60 and 70°C.

2.2.4.2. *Aqueous stability sample preparation*

Stock solutions of all prodrugs studied were freshly prepared in acetonitrile to approximately 1.5 mM concentrations, prior to use. 100 μ L of stock prodrug solutions were then added to 10 mL of freshly filtered buffer solutions at each pH. Aliquots (300 μ L) of sample mixture were added to labeled amber ampoules, sealed with an open flame and placed in ovens at 50, 60 and 70 °C. The samples were analyzed at appropriate time points with HPLC.

pH range	Buffer System used
1.1 – 2.0	HCl
3.0 – 4.5	Formic Acid/ Sodium Formate
5.0 – 7.5	Sodium Phosphate (NaH_2PO_4 / Na_2HPO_4)
8.0 – 9.0	Tris HCl / Tris base

Table 2.1: Buffers used in aqueous stability studies of sulfenamide prodrugs.

2.2.4.3. *HPLC conditions*

The samples for all aqueous stability studies were analyzed on a Hewlett Packard® 1050 LC system consisting of a solvent delivery system with a quaternary pump, a UV-spectrophotometer detector, a 21 bottle auto sampler fitted with a 25 μ L needle, and a 100 μ L sample loop. The HPLC system was controlled by Chemstations® analytical software. A C¹⁸ ODS Hypercil® (15 cm x 4.6mm i.d. 5 μ m) HPLC column was used for all separations. Sample volumes of 20 μ L were injected with a flow rate of 1 mL/min and UV detection at 258 nm for all prodrugs studied. Samples were run for a total of 13 minutes with a gradient of mobile phases A (95% water, 5% acetonitrile and 0.1% formic acid) and B (95% acetonitrile, 5% water, 0.1% formic acid) as indicated in table 2.2. A calibration curve for

each compound was prepared prior to stability studies by analyzing varying concentrations of prodrugs and Linezolid in acetonitrile.

Time (mins)	Mobile Phase A (%)	Mobile Phase B (%)
0.0	75	25
8.0	10	90
10.0	10	90
12.0	75	25
15.0	75	25

Table 2.2: Gradient for HPLC analysis in aqueous stability studies.

2.2.4.4. Determination of degradation products by LCMS

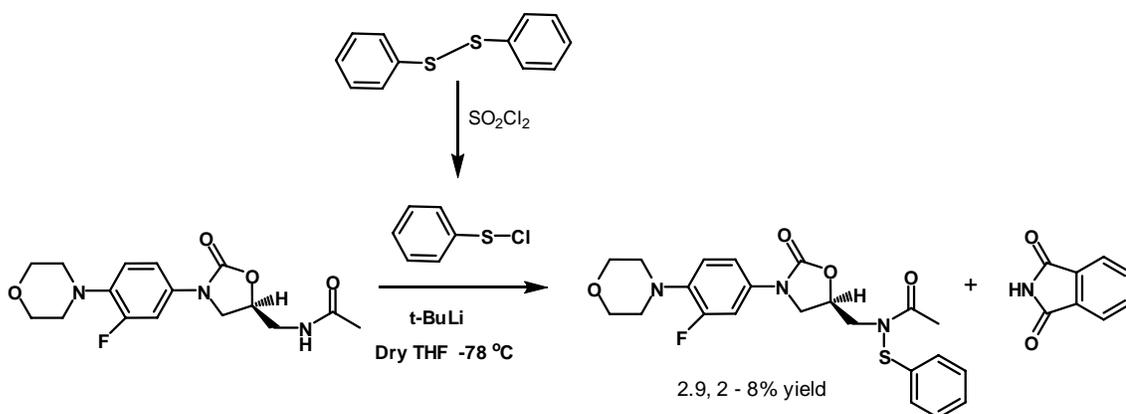
LC/MS separations were carried out on a Waters Alliance 2690 HPLC system interfaced with an electrospray ionization (ESI) attached to a Micromass Quatro Micro Tandem Quadrupole mass spectrometer. A C¹⁸ ODS Hypercil (15 cm x 4.6mm i.d. 5 µm) column was used for all separations. 20 µL of samples were injected and run at a flow rate of 1.0 mL/min, with a splitter fitted between the MS and the HPLC, allowing only 200 µL to flow into the MS. The gradient indicated in table 2.2 was also used in the LC/MS studies.

2.3. Results and Discussion

2.3.1. Synthetic results

Sulfenamide prodrug derivatives were successfully synthesized from the oxazolidinones using thiophthalimides intermediates to yields of 30 to 82%. Previous literature reported the synthesis of the sulfenamide derivatives through the reaction of a sulfenyl chloride derivative with the oxazolidinones in the presence of a base (Scheme 2.4).

This sulfenyl chloride synthetic route resulted in yields of 2 to 8%, and in some cases resulted in no products being formed. The poor yields were attributed to the unstable nature of sulfenyl chlorides, which readily reacted in solution to form disulfides; thus, preventing the transfer of the sulfenyl moiety onto the amide group of the oxazolidinones. In attempts to improve the yields in the sulfenyl chloride synthetic route, the reaction was carried out *in situ* without the isolation of the sulfenyl chlorides. However, there appeared to be interference from other reaction species in solution, and as a result there was no way of determining the actual sulfenyl chloride concentration formed in solution. All of these factors contributed to the very poor yields observed in the reactions using sulfenyl chlorides as intermediates.

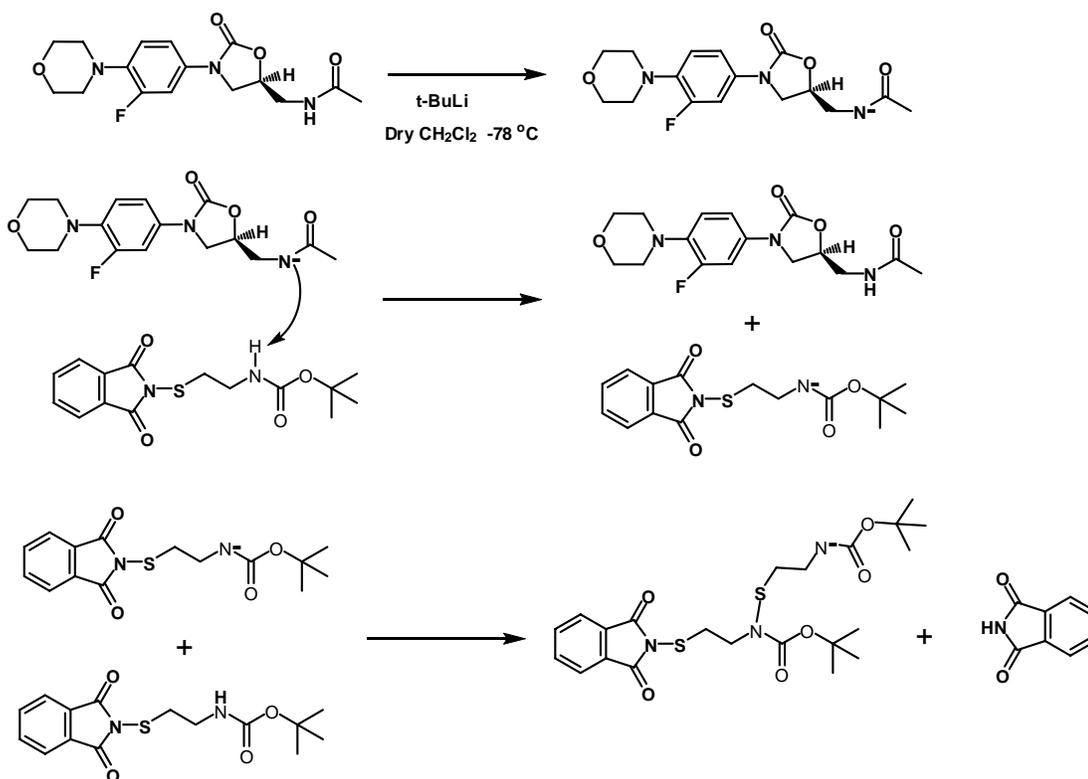


Scheme 2.4: Previous synthetic route reported for the synthesis of sulfenamide derivatives of oxazolidinones.³⁰

The thiophthalimides, on the other hand, have been found to be stable and superior sulfenyl transfer agents. Thiophthalimides can be readily isolated, purified and characterized before use in the reactions with the oxazolidinones in the presence of a strong non-nucleophilic base to form the sulfenamides of interest. This is a one-step reaction resulting in the improved yields. The reactions with thiophthalimides are clean reactions making isolation

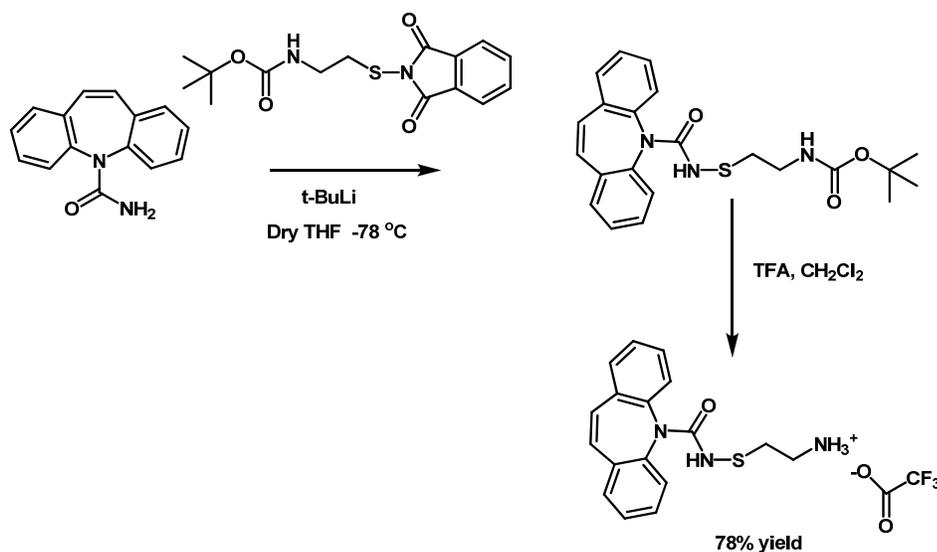
and purification of the products fairly easy without the degradation of the sulfenamide products.

Attempts to synthesize the sulfenamide derivative; **2.15**, using a protected cysteamine promoiety, did not yield any products. This was due to of the fact that the -NH proton of the amide functional group in the oxazolidinones has a pKa value lower than the pKa value of the -NH proton in **2.14**. As a result the deprotonated oxazolidinones attacked the -NH proton in the **2.14**, causing the quenching of the reaction (Scheme 2.5).



Scheme 2.5: Attempted synthesis of **2.15**. Differences in pKa of the -NH protons of linezolid and the thiophthalimide intermediate leads to no reaction to form expected sulfenamide prodrug.

However, successful synthesis has been reported using **2.14** to transfer the protected cysteamine moiety onto carbamazepine as shown in Scheme 2.6. The cysteamine derivative of carbamazepine was reported to have greatly improved the aqueous solubility compared to the parent carbamazepine.



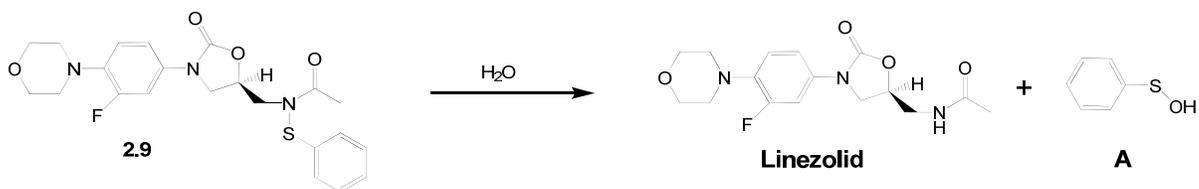
Scheme 2.6. Successful synthesis of cysteamine-CBZ from Boc-cysteamine-phthalimide (17) derivative.^{28,29}

2.3.2. Hydrolytic degradation of *N*-(phenylsulfenyl)linezolid (2.9) and *N*-(ethylsulfenyl) linezolid (2.12).

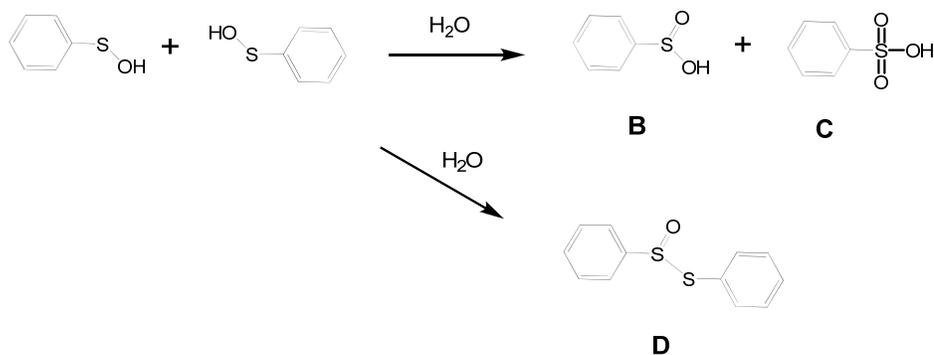
The aqueous stability studies of the sulfenamide prodrugs were studied in dilute aqueous solutions of 50 mM buffer concentrations with ionic strength adjusted to 0.15. Compound **2.9** was chosen as the candidate for aqueous stability studies based on the fact that the phenylthiol moiety is UV active and can be followed by both LCMS and HPLC analysis to help determine the degradation products formed as a result of the cleavage of the N-S bond in the sulfenamides. The S-phenyl moiety was expected to be a good leaving

group, hence **2.12** was also studied under the aqueous conditions to compare the effects of the straight chain promoiety, which was expected to not be as good of a leaving group as that of the S-phenyl promoiety.

The stability of **2.9** and **2.12** were monitored over a pH range of 1.0 to pH 7.5. At all pH values studied, **2.9** and **2.12** degraded hydrolytically to release linezolid as the major degradation product and a number of minor products. Scheme 2.7 and 2.8 shows a simplified degradation scheme accounting for the loss of prodrug and the generation of linezolid. An average of 75% of linezolid was recovered from aqueous stability studies as the major degradation product for all the prodrugs studied.



Scheme 2.7: Hydrolytic degradation of **2.9**.



Scheme 2.8: Minor hydrolytic products of the break down of **2.9**. The cleavage of the N-S bond releases a sulfenic acid which is very unstable in solution and immediately reacts to form a variety of minor prodrugs identifiable by LCMS.

LC/MS analysis confirmed the formation of Linezolid as the major degradation product as shown by the chromatogram in figure 2.5. The minor products generated were as a result of the production of sulfenic acids generated from the sulfenyl promoiety. Sulfenic acids have been known to be extremely unstable in aqueous solutions and immediately react to form other by products.³¹ The predicted minor products expected to be generated from the hydrolytic degradation of **2.9** include benzenesulfonic acid (**B**), benzenesulfonic acid (**C**), and S-phenyl benzenesulfonothioate (**D**) (See Scheme 2.6). However, the LCMS analysis only confirms the formation of the benzenesulfonic acid (**C**) as a minor product as shown in figures 2.6a and 2.6b. The LCMS data confirms our suspicion of the generation of sulfenic acid intermediates by the released promoiety.

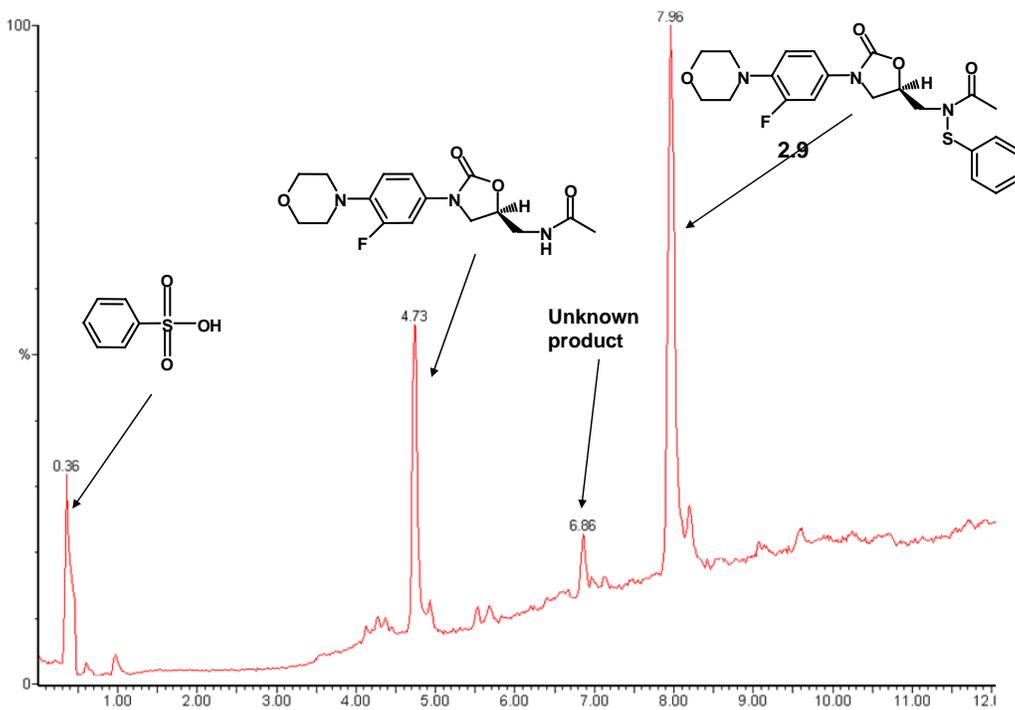


Figure 2.5: LCMS analysis of the degradation of **2.9** at low pH values (pH < 5.0)

HPLC analysis indicated that the prodrugs degraded through pseudo first order degradation kinetics. A logarithm plot of the prodrug concentrations versus time resulted in a linear correlation (Figure 2.7). From pseudo first order kinetics equation (Eq. 1), the slope of the curve was used to determine the observed rate constant (k_{obs}) for the aqueous degradation of the prodrugs in the pH range studied.

$$[A] = [A]_o \exp^{-k_{obs}t} \quad (1)$$

$$t_{1/2} = \frac{\ln(2)}{k_{obs}} \quad (2)$$

$$t_{90} = \frac{0.105}{k_{obs}} \quad (3)$$

$$k_{obs} = k_H [H^+] + k_O + k_{OH} [^-OH] \quad (4)$$

Where $[A]$ is the concentration of the prodrug remaining in solution, $[A]_o$ is the initial concentration of prodrug added, k_{obs} is the observed rate constant, $t_{1/2}$ is the half life of the prodrug in solution and t_{90} is the time at which 10% of the drug is degraded (shelf life). The k_H variables represent the rate constants for hydronium ion catalyzed hydrolysis. The k_o variables represent the rate constant for water-catalyzed or spontaneous hydrolysis. Lastly, the k_{OH} variables represent the rate constants for the hydroxide ion catalyzed hydrolysis.

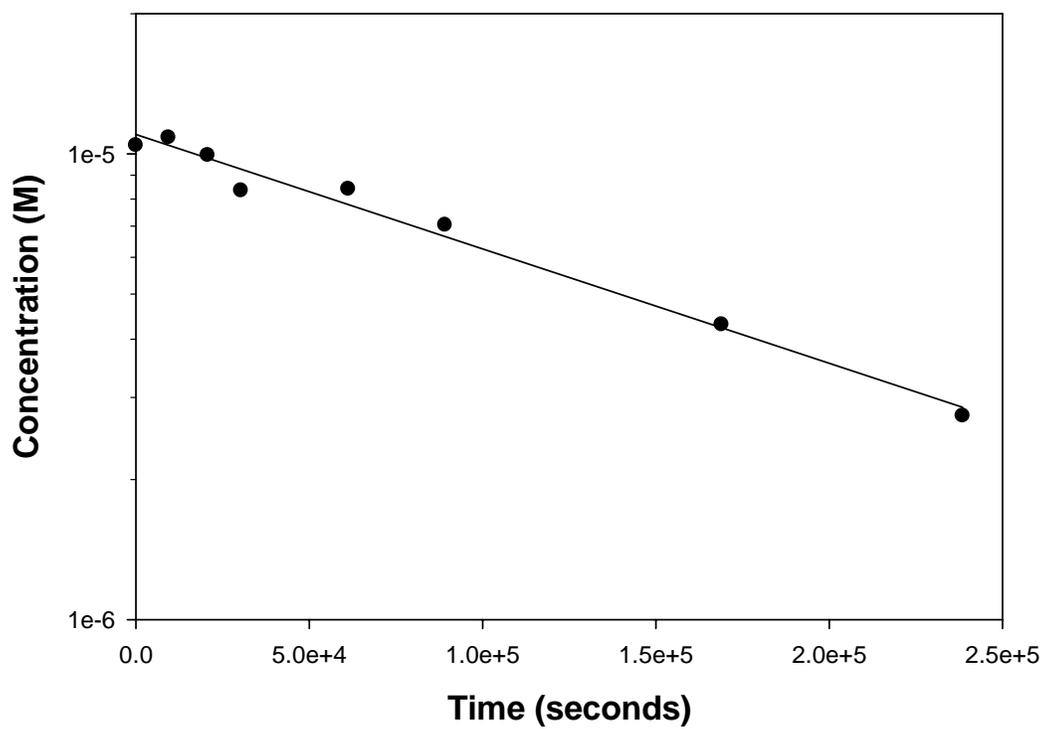


Figure 2.7: Pseudo first order degradation of **2.9** at pH 4.0 in 50 mM buffer concentration (I = 0.15 with NaCl) and at 60°C. $k_{\text{obs}} = 5.66 \times 10^{-6} \text{ M}^{-1}\text{sec}^{-1}$. $t_{1/2} = 34 \text{ hrs}$ (1.42 days).

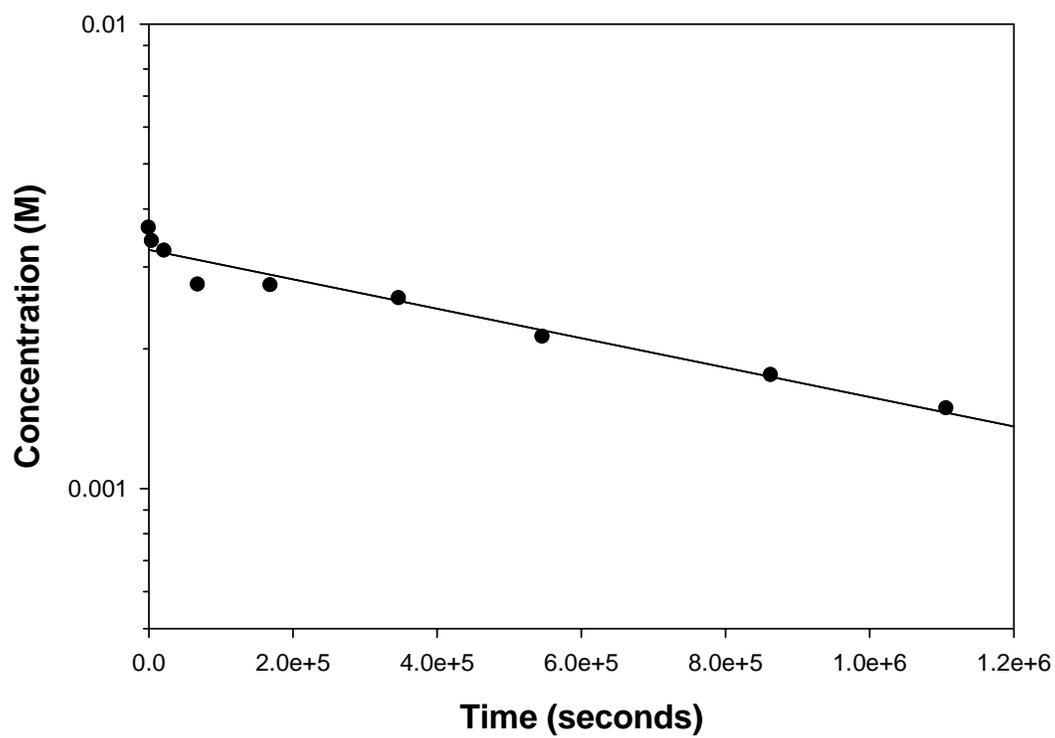


Figure 2.8: Pseudo first order degradation of **2.12** at pH 4.0 in 50 mM buffer concentration ($I = 0.15$ with NaCl) and at 60°C. $k_{\text{obs}} = 7.22 \times 10^{-7} \text{ M}^{-1}\text{sec}^{-1}$. $t_{1/2} = 266.6 \text{ hrs}$ (11.1 days).

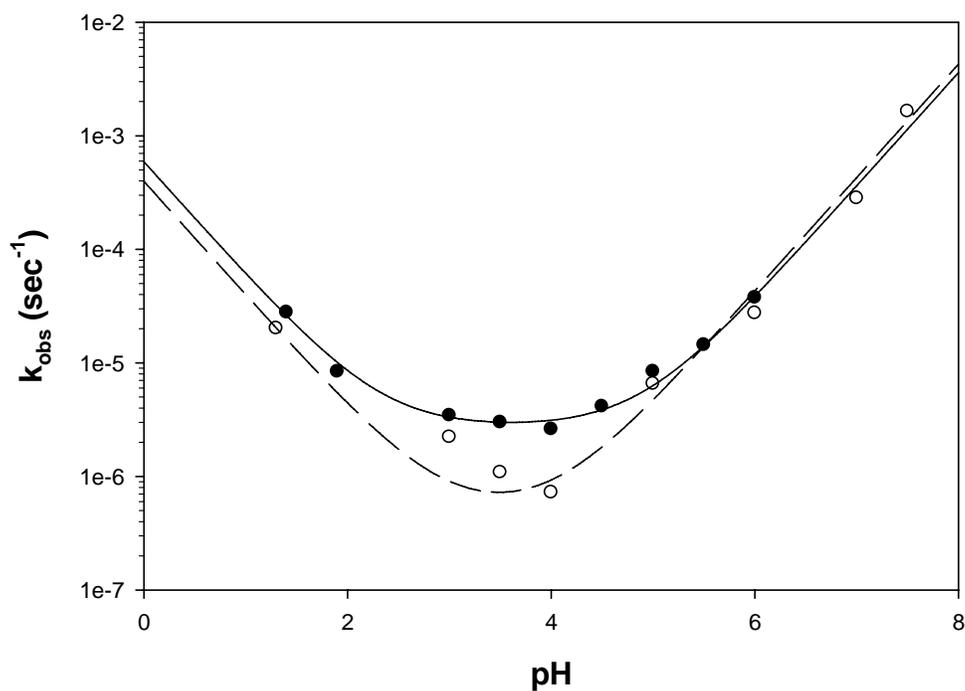


Figure 2.9: Plot of the observed rate constant (k_{obs}) for the loss of **2.9** (●) and **2.12** (○) as a function of pH at 60°C and in 50 mM buffer solutions ($I = 0.15\text{M}$ with NaCl). The lines (— **2.9**, -- **2.12**) represent the theoretical profiles generated from Eq. 4 with parameters $k_H = 8.33 \times 10^{-5} \text{ M}^{-1}\text{sec}^{-1}$, $k_o = 2.61 \times 10^{-6} \text{ M}^{-1}\text{sec}^{-1}$, and $k_{OH} = 1.11 \times 10^6 \text{ M}^{-1}\text{sec}^{-1}$ at 60°C for **2.9** and $k_H = 1.01 \times 10^{-4} \text{ M}^{-1}\text{sec}^{-1}$, $k_o = 7.22 \times 10^{-7} \text{ M}^{-1}\text{sec}^{-1}$, and $k_{OH} = 1.39 \times 10^4 \text{ M}^{-1}\text{sec}^{-1}$ at 60°C for **2.12**.

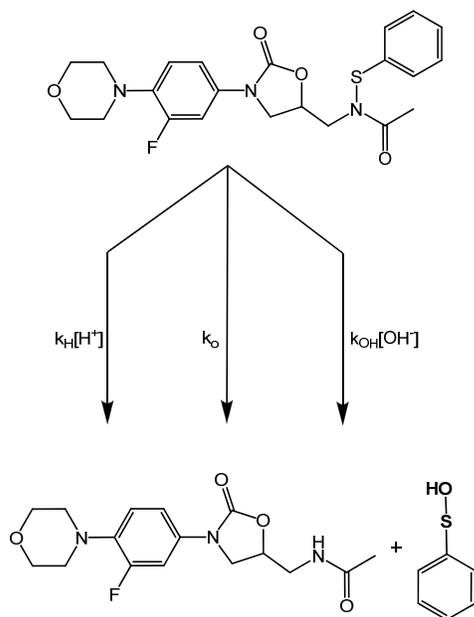


Figure 2.10: Macroscopic rate constants for the degradation of **2.9** under aqueous conditions

Stability is a useful factor in the development of drug candidates; however, it is also one of the limiting factors specifically when dealing with oral and parenteral formulations. Accordingly, adequate stability has to be determined under various conditions to conclude the optimal conditions necessary for the formulations. Figure 2.11 and 2.12 shows plots of the observed rate constant at maximum pH (pH 4.0) for both **2.9** and **2.12** respectively, at elevated temperatures of 50, 60 and 70°C. The results from these curves were applied to the Arrhenius equation (Eq. 2.5), and the activation energies calculated for each prodrug. The calculated activation energies were then used to estimate observed rate constants at 4, 25 and 37°C, where 4°C and 25°C are storage temperatures and 37°C is the physiological temperature of the human body. From Eq. 2 and Eq. 3 the half lives and shelf lives were then estimated to determine the stability characteristics at those temperatures as indicated in table 2.3.

Estimated aqueous stability for both **2.9** and **2.12** indicate inadequate stability under the aqueous conditions studied. Estimated shelf lives of half lives of 285 days and 1.35 years

at 4°C and pH 4.0 were estimated for the shelf lives in aqueous solutions. These values fell well below the standard two year shelf life required under FDA guidelines in solution dose forms. This insufficient aqueous stability observed presents limitations to the possible use of sulfenamides prodrugs of oxazolidinones for use in ready-to-use liquid formulations.

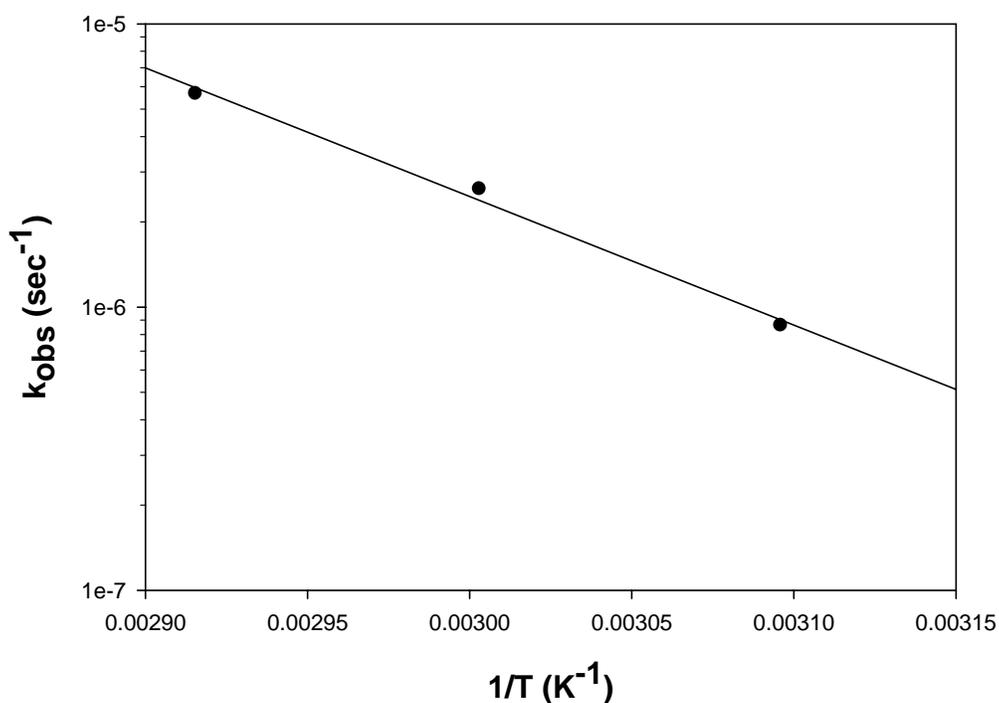


Figure 2.11: Arrhenius plot of temperature dependent study of **2.9** at pH 4.0 in 50 mM buffer solutions ($I = 0.15$), $E_a = 21.02 \text{ kcalmol}^{-1}$ (87.86 kJmol^{-1}), $\Delta H^\ddagger = 20.43 \text{ kcalmol}^{-1}$ (85.39 kJmol^{-1}).

Temperature (°C)	k_{obs} (sec ⁻¹)	$t_{1/2}$ (days)	t_{90} (days)
4	4.25×10^{-9}	2724.00 (7.5 yrs)	285.94
25	6.26×10^{-8}	128.13	19.41
37	2.48×10^{-7}	32.34	4.90

Table 2.5: Estimated values from temperature dependent studies of **2.9** at pH 4.0.

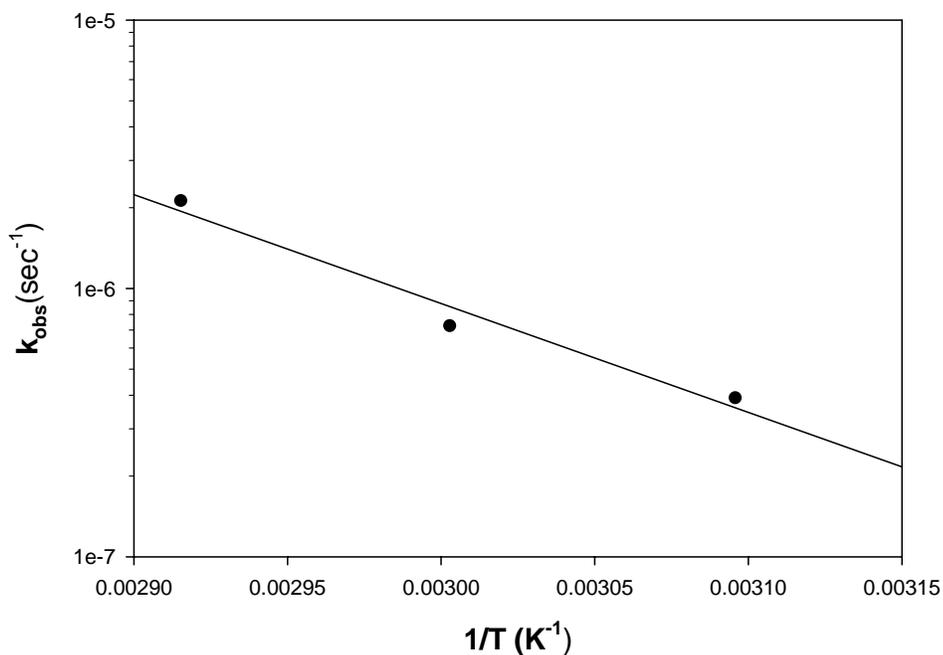


Figure 2.12: Arrhenius plot of temperature dependent study of **2.12** at pH 4.0 in 50 mM buffer solutions ($I = 0.15$), $E_a = 18.59 \text{ kcalmol}^{-1}$ (77.78 kJmol^{-1}).

Temperature (°C)	k_{obs} (sec ⁻¹)	$t_{1/2}$ (days)	t_{90} (days)
4	2.46×10^{-9}	3255.8 (8.92 yrs)	493 (1.35yrs)
25	2.66×10^{-8}	301.5	45.7
37	8.98×10^{-8}	89.3	13.5

Table 2.5: Estimated values from temperature dependent studies of **2.12**

2.4. Conclusions

Thiophthalimides have been effectively used as sulfenyl transfer agents to transfer the sulfenyl promoiety onto the parent drug molecules in the synthesis of model sulfenamide prodrugs of antimicrobial oxazolidinones. Yields obtained from the synthesis show significant increase in yields compared to a previously used synthetic route that used sulfenyl chloride intermediates.

The aqueous stability of the prodrug models indicate the hydrolytic degradation of the sulfenamide prodrugs via the cleavage of the N-S bond to release the parent molecule and a sulfenic acids which further reacts, due to their high aqueous instability, to generate sulfonamides as minor products. Estimated half lives and shelf lives of the sulfenamide prodrug models showed insufficient stability under the aqueous conditions studied. The low aqueous stability profile of the sulfenamide may pose a problem the sulfenamides be formulated as ready-to-use aqueous formulation.

In the next three chapters we will evaluate the degradation mechanism of the sulfenamide prodrugs in the presence of thiol containing compounds such as glutathione and cysteine and also evaluate the conversion of the prodrugs to the parent molecule in the presence of thiol containing proteins and human plasma. Finally we will evaluate the permeability characteristic of the sulfenamides in Chapter 5.

2.5. References

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Chapter 3

Degradation of sulfenamides in the presence of small molecule thiols

3.1. Introduction

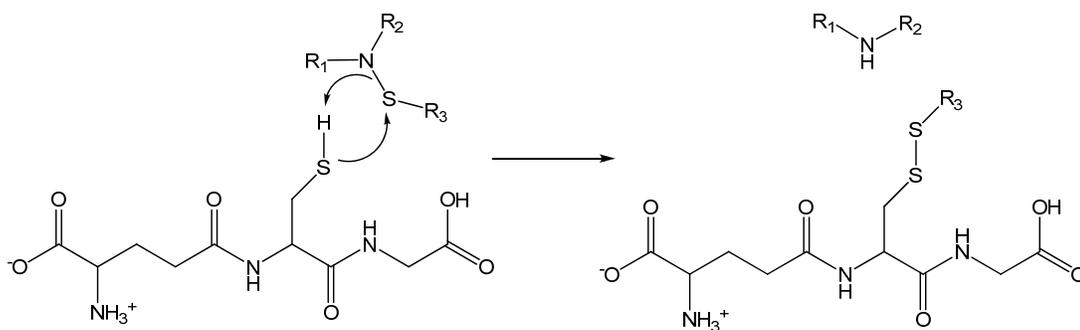
3.1.1. Overall Objective

The objective of the work to be presented in this chapter is to evaluate the reactivity of model sulfenamide prodrugs of oxazolidinones with thiol groups such as those found on cysteine and glutathione. The goal is to be able to determine the conversion mechanism of the sulfenamides prodrugs to the parent drug in the presence of these and other thiols. Once the mechanism has been determined it will be possible to predict the degradation products that can be formed from this breakdown. It is our hypothesis that the sulfenamide prodrugs will be cleaved by a nucleophilic attack of thiolate ions in solution to release the parent oxazolidinone, and a mixed disulfide of the sulfenyl moiety and the thiol molecule.

Previous studies have identified sulfenamide derivatives of benzamide and carbamazepine that are very chemically stable showing adequate aqueous stability.¹⁻⁴ In the presence of thiol containing compounds such as glutathione and cysteine, however, the sulfenamide bond readily cleaves in a matter of seconds to release the parent compound and the sulfenyl moiety. However, the mechanism of such reactions was not explored.

3.1.2. Reactivity of Sulfenamides

The reactivity of sulfenamides has been studied in previous reports.^{5,6} Evidence of bond polarization between the sulfur and nitrogen atoms results in sulfur atom donating its lone pair of electrons to the nitrogen atom. The sulfenamide bond therefore has two potential reaction centers; the electronegative nitrogen which is susceptible to electrophilic attack and the electropositive sulfur which is susceptible to nucleophilic attack.^{5,6}



Scheme 3.1: Degradation mechanism of sulfenamide prodrugs in the presence of sulfhydryl compounds such as glutathione.⁷

Under acidic conditions sulfenamide bonds have been found to be cleaved by electrophilic attack involving the coordination of the electrophile with the nitrogen atom followed by the a nucleophilic attack on the sulfur to break the N-S bond.⁸ This process has been used in peptide synthesis where sulfenamides have been used to transfer sulfenyl protecting groups into the peptides. Acidic conditions are used to remove protecting group once the peptide of interest has been synthesized.⁹ In the presence of nucleophiles, the attack occurs on the more electropositive sulfur atom. Nucleophilic substitutions in sulfenamides have been used in the synthesis of symmetric and asymmetric disulfides.¹⁰⁻¹²

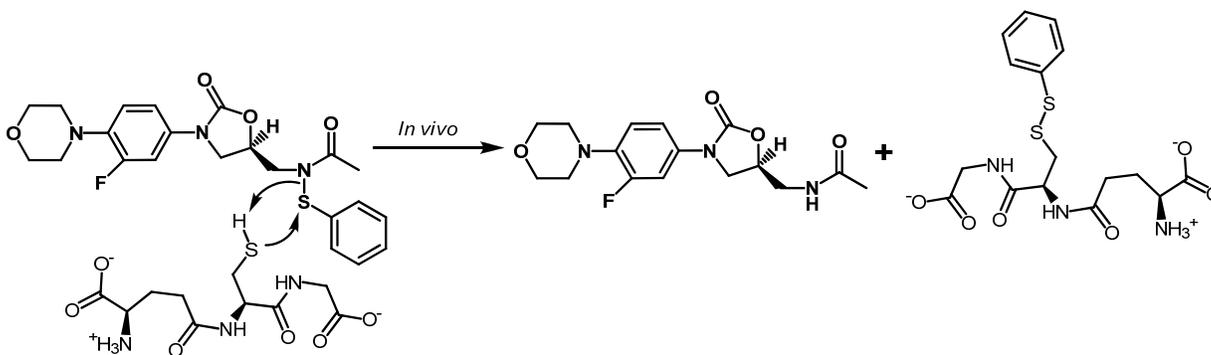
3.1.3. Proposed reaction of sulfenamides prodrugs with thiols

The origin of the use of sulfenamide prodrugs was based on the rapid degradation of PTC in the presence of excess cysteine and glutathione(see earlier discussion in Chapter 1). To further evaluate the break down of sulfenamide prodrugs, preliminary studies have been conducted on N-(phenylthio)linezolid in the presence of dog whole blood and the prodrug was found to rapidly degrade in a few seconds to release the parent oxazolidinone. Hemenway studied the *in vivo* conversion of carbamazepine derivatives by administering N-cysteamine-CBZ derivative into rats (see scheme 1.11 and figure 1.8 in Chapter 1). The

prodrug rapidly degraded *in vivo* releasing the parent carbamazepine which was comparable to CBZ control studies. All of these previous studies led to the proposal that the break down of the sulfenamide prodrugs *in vivo* occurred via nucleophilic attack of sulfhydryl molecules or free thiols, specifically glutathione which has been found to have high intracellular concentrations *in vivo*.

Literature reports and theoretical analysis of thiolate reactions with disulfides suggest two possible reaction mechanisms in disulfide exchange reactions; the nucleophilic attack of the thiolate at the sulfur atom by a concerted S_N2 mechanism or the by addition-elimination mechanism with the formation of an intermediate.¹³⁻¹⁸ The reaction of thiols with disulfides is expected to be very similar to the reaction of the thiols with the sulfenamides, thus, the reaction of the thiols to cleave the sulfenamide bond is expected to be either via a concerted S_N2 attack or an addition-elimination reaction.

Our goal in this chapter is to determine the mechanistic route of degradation of the prodrug in the presence of the thiols. Our specific aims include determining the reaction species in solution to determine the rate limiting step for the degradation of the sulfenamide prodrug in the presence of thiols and identifying the degradation products released or produced as a result of the cleavage of the sulfenamide bonds in the presence of the thiols.



Scheme 3.2: Proposed *in vivo* conversion of sulfenamide prodrugs in the presence of glutathione.

3.2. Experimental Materials and Methods

3.2.1. Solvents and chemicals

Acetonitrile, acetone, hydrochloric acid (12N), acetic acid, formic acid, phosphoric acid, sodium phosphate (mono and dibasic), sodium acetate, sodium formate, and sodium chloride were purchased from Fisher Scientific (Pittsburg, PA). N-(phenylthio)phthalimide, ethylthiol and thiophenol were purchased from Aldrich Chemical Company (Milwaukee, WI), 3-mercaptopropionic acid ethyl ester was purchased from TCI America (Wellesley Hills, MA). Cysteine and glutathione (reagent grade) were purchased from Sigma-Aldrich Chemicals (St. Louis, MO) and used without further purification. Water used in all experiments was freshly distilled and deionized in house using Corning® Mega-pure One Liter distillation apparatus.

Buffer solutions (50 mM) were prepared and adjusted to ionic strength of 0.15 M with sodium chloride for pH values above 3.0. For buffer solutions with pH values below 3.0, HCl solutions with ionic strength adjusted to 0.15 with sodium chloride were used. A Fischer Scientific Accumet Research AR15 pH meter was used to measure the pH values of buffer solutions after standardization with standard buffers of pH 4.0, 7.0 and 10.0. All buffer solutions were filtered through 0.22 μm syringe filters prior to use.

Thiol	pKa of thiol group*
Phenylthiol	6.51 ^{19,20}
Cysteine	8.3 ²¹
Glutathione	8.7 ^{22,23}
3-mercaptopropanoate ethyl ester	9.7
Ethylthiol	10.6 ^{24,25}

Table 3.1: Thiols used in studies showing varying pKa of the thiol group. The pKa values are values of all sulfhydryl compounds studied were obtained from the literature.

Biological and therapeutically relevant thiols have a pKa of 8.0 to 10.0 for -SH groups, as a result the selected thiols used were selected such that it covered a range of thiol pKa values of 6.5 to 10.6 (Table 3.1), thus accounting for pKa values of physiologically relevant thiols. Stock solutions of all prodrugs, ethylthiol, thiophenol and 3-mercaptopropionic acid ethyl ester were prepared in acetonitrile and solutions of cysteine and glutathione were prepared in distilled water. All stock solutions of the thiols were freshly prepared prior to use to minimize the oxidation of the thiols to their disulfides.

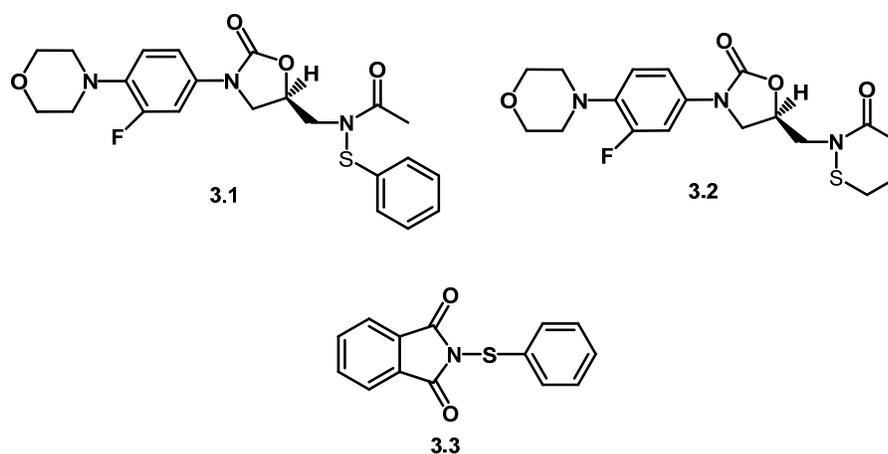


Figure 3.1: Model sulfenamide prodrugs studied. N-(phenylthio)linezolid (**3.1**), N-(ethylthio)linezolid (**3.2**), and N-(phenylthio)phthalimide (**3.3**)

3.2.2. Aqueous stability sample preparation

Glutathione and cysteine are slightly acidic compounds in solution owing to their normal ionic state.²⁶ As a result the pH of solutions changes slightly when there are dissolved in weak buffer solutions. To prevent this from happening 50mM buffer solutions were used for all experiments to help obtain adequate buffer capacity.^{27,28} Studies were carried out on the pH range of 3.0 to pH 7.5. Three prodrug models were used in all experiments; N-(phenylthio)linezolid (**3.1**), N-(ethylthio)linezolid (**3.2**) and N-(phenylthio)phthalimide (**3.3**)

(Figure 3.1). Stock solutions of all prodrugs studied were freshly prepared in acetonitrile to 1.0 mM concentrations, prior to use. One hundred microliters of stock prodrug solutions were added to 9.9 mL of freshly filtered buffer solutions at each pH to give a final concentration of 10.0 μ M. Stock solutions of the thiols were prepared to 10mM concentrations. One hundred microliters, 200 μ L and 300 μ L of the thiol solutions were added to the sample solutions to give a final concentration of 100 μ M, 200 μ M, and 300 μ M respectively. That is, a 10, 20 and 30 fold molar excess of thiol concentration compared to the prodrug concentration. The samples were kept in screw top vials and shaken in a water bath at 25°C. At the appropriate time points 100 μ L of the samples were added to 100 μ L of a 100 μ M N-methylmaleimide solution. N-methylmaleimide is a scavenger of thiols or sulfhydryls and was added to the sample solutions at each time point to quench the reaction and prevent further degradation of the prodrugs by the sulfhydryls while the samples were waiting to be analyzed on HPLC.

Two sets of control reactions were carried out. The first control was carried out to monitor the stability of the prodrugs in buffered solutions in the absence of the sulfhydryl compounds. The second control was carried out to monitor the stability of the prodrugs in the presence of 100 μ M N-methylmaleimide. The latter control was carried out to ensure that N-methylmaleimide was not contributing to the degradation of the sulfenamide prodrugs.

3.2.3. HPL conditions

All samples were analyzed on a Hewlett Packard[®] 1050 LC system consisting of a solvent delivery system with a quaternary pump, a UV-spectrophotometer detector, a 21 bottle auto sampler fitted with a 25 μ L needle and a 100 μ L sample loop. The HPLC system was controlled by Chemstations[®] analytical software. A C¹⁸ ODS Hypercil[®] (15 cm x 4.6 mm i.d. 5 μ m) HPLC column was used for all separations. Sample volumes of 20 μ L were

injected with a flow rate of 1 mL/min and run for a total of 13 minutes with a gradient of mobile phases A and B as indicated in table 2.2. Mobile phase A consisted of 95% water, 5% acetonitrile and 0.1% formic acid. Mobile phase B consisted of 95 % acetonitrile, 5 % water, 0.1 % formic acid. Detection was by UV at 254 nm for **3.1** and **3.2** and at 258 nm for **3.3**. A calibration curve for each compound was prepared prior to stability studies by analyzing varying concentrations of prodrugs and linezolid in acetonitrile.

Time (mins)	Mobile Phase A (%)	Mobile Phase B (%)
0.0	100	0
8.0	10	90
10.0	10	90
12.0	100	0
15.0	100	0

Table 3.2: Gradient for HPLC analysis in aqueous stability studies.

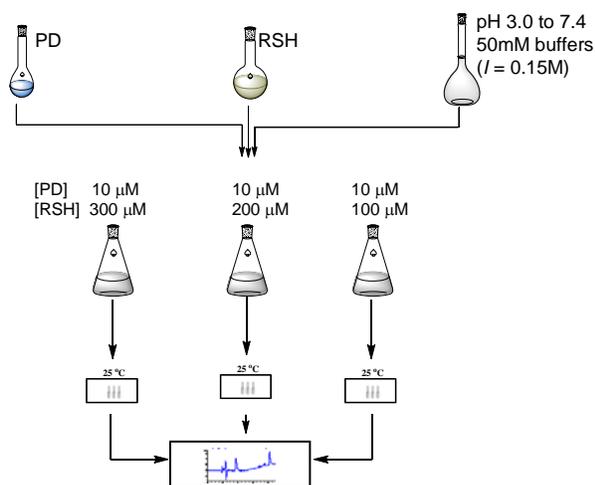


Figure 3.2: Experimental setup in the study of the sulfenamide prodrugs of oxazolidinones in the presence of small molecule thiols.

3.2.4. Determination of degradation products by LCMS

A solution of **3.1** was prepared to a concentration of 10 μM as described above in 50mM buffer solution at pH 4.0 in a scintillation vial. Solution pH of 4.0 was selected for the study as aqueous stability analysis as described in chapter 2 indicated that pH 4.0 was the most stable pH for the aqueous stability of **3.1** and **3.2**. Glutathione (100 μL of a 10mM solution) was added to the sample to give a final concentration of 100 μM . The sample was shaken in a water bath at 25°C for 24 hours and analyzed by LCMS. For the control, a similar sample was prepared without glutathione and kept at 25°C for 24 hours, followed by analysis by LCMS. All samples were also analyzed by HPLC to determine the concentration of prodrug remaining.

LCMS separation was carried out on a Waters Alliance 2690 HPLC system interfaced with electrospray ionization (ESI) attached to a Micromass Quatro Micro Tandem Quadrupole mass spectrometer. A C^{18} ODS Hypercil (5 cm x 4.6mm i.d. 5 μm) column was used for all separations. Twenty microliters of samples were injected and separated on the column at a flow rate of 1.0 mL/min. A splitter was fitted between the HPLC column and the electrospray to direct only 200 μL to go into the MS. The gradient indicated in table 2.2 was also used in the LCMS studies.

3.3. Results and discussion

3.3.1. LCMS analysis.

The control stability study of **3.1** in aqueous buffer solution at pH 4.0 and at 25°C showed very little or no degradation. This is consistent with aqueous stability analysis carried out as described in Chapter 2. The estimated shelf life (t_{90}) for the aqueous stability of **3.1** at pH 4.0 and 25°C is approximately 152 days, thus after 24 hours there is very little to no

degradation occurring. In contrast, the sample containing 10 fold molar excess of glutathione, after 24 hours there was no detectable prodrug presence in solution even at pH 4.0. LCMS analysis showed that the major products formed were the parent linezolid (MW = 337.35, retention time (t_r) = 3.59) and the glutathione-phenylthiol mixed disulfide (MW = 415.45, t_r = 3.42) as shown in Figures 3.3 through 3.5. It should be noted that HPLC analysis showed that approximately 75 to 80% of linezolid was recovered from the prodrug.

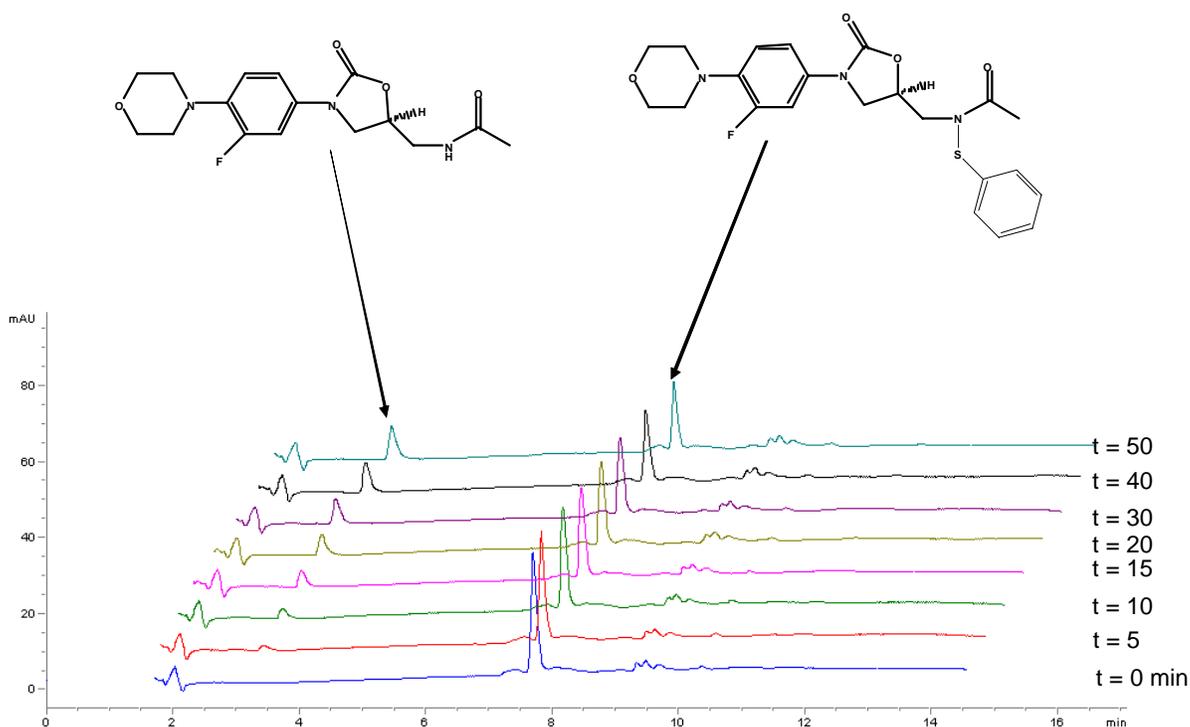


Figure 3.3: Chromatogram from the HPLC analysis of **3.1** in the presence of 10 fold molar excess of glutathione, showing the disappearance of prodrug **3.1** and the appearance of linezolid. Units for time is minutes

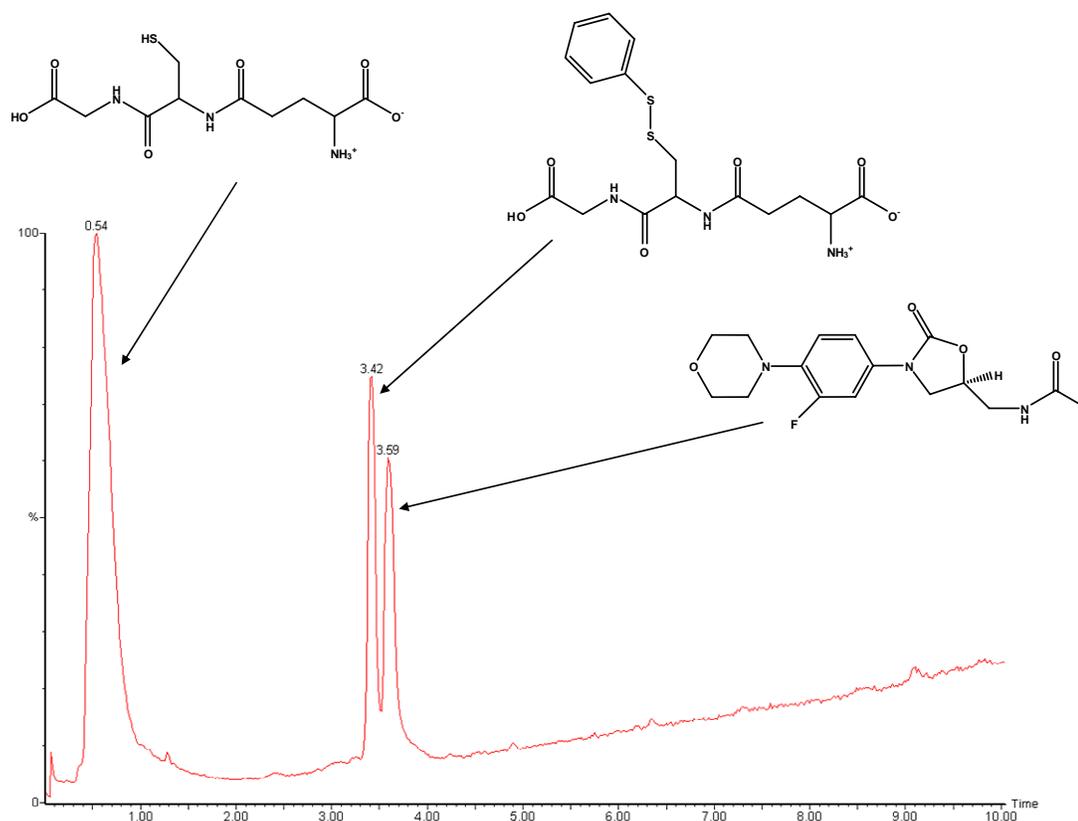


Figure 3.4: Chromatogram showing the degradation of **3.1** in the presence of 10 fold molar excess of glutathione. Sample was analyzed less than 5 minutes after the addition of the prodrug to the glutathione solution in 50mM buffer solution at pH 4.0. Chromatogram shows no prodrug present at the time of analysis and two major degradation products.

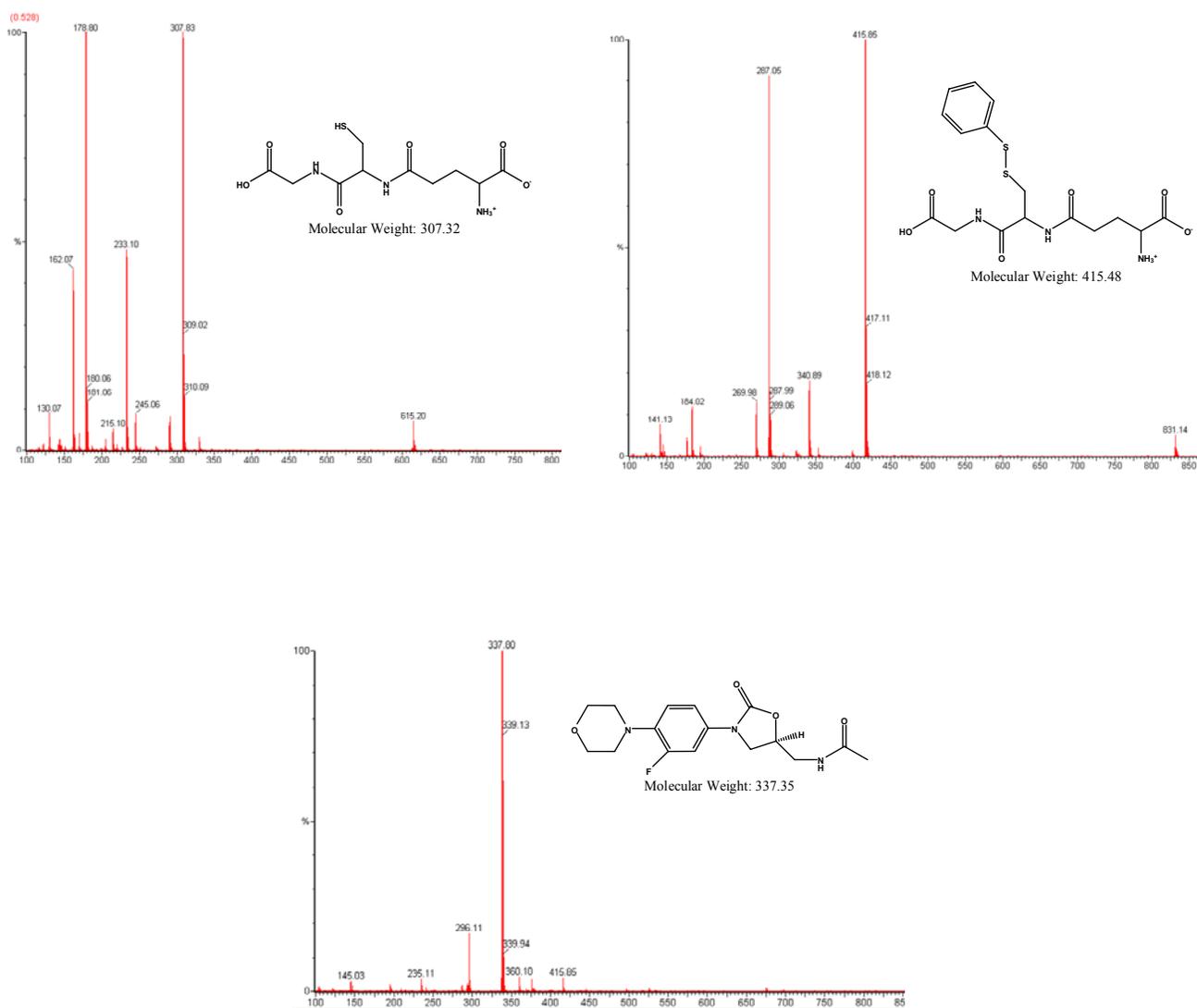


Figure 3.5: LCMS Analysis of the degradation of **3.1** in the presence of 10 fold molar excess of glutathione at pH 4.0 in 50 mM acetate buffer ($I = 0.15$) and at 25°C

3.3.2. Pseudo 1st order reactions of sulfenamide prodrug models in molar excess of thiols.

All the reactions carried out at pH greater than 6.5 were too fast to follow by HPLC. At pH 7.4, no prodrug was detected even after attempts to quench the reaction after less than five seconds following the addition of ten fold molar excess of the thiols to the buffered prodrug solutions. This suggested that the degradation of the prodrugs in the presence of molar excess of thiols was occurring instantaneously at physiological pH. HPLC analysis confirmed the formation of linezolid as the major product from the breakdown of **3.1** and **3.2**.

However, there was a 75% to 80% recovery of the parent linezolid from the degradation of the sulfenamide prodrugs. This recovery was semi-qualitative and could be as a result of lower purity levels of the prodrugs samples that were used. Also the lower recovery could indicate the occurrence of a side reactions leading to the formation of minor products that were not detected by HPLC. Control reactions of linezolid in the presence of the thiols in buffered solutions did not show any reactions occurring within the time frame in which the reactions were run. The LCMS analyses showed the generation of a number of unknown peaks that were not accounted for.

The degradation of **3.3** in the presence of the thiols resulted in the formation of phthalimide as the major product with approximately 95% of phthalimide recovered from the breakdown.

The reactions of the sulfenamides in the presence of molar excess quantities of thiols exhibited pseudo first order kinetics; a log-linear correlation was observed for the disappearance of the prodrugs with time (Figure 3.6).

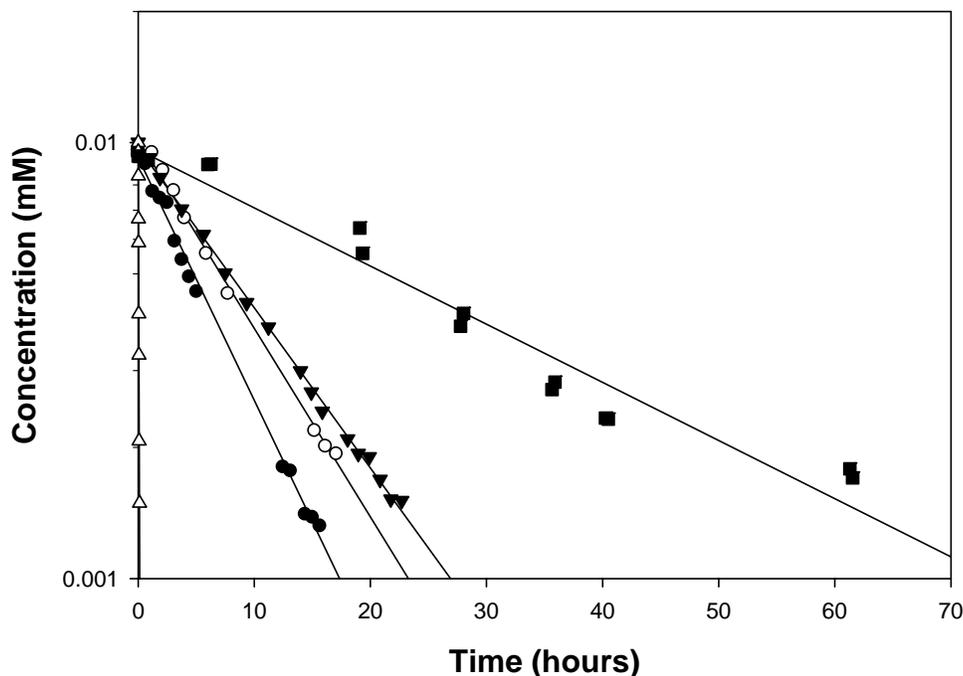


Figure 3.6: Pseudo first order degradation of **3.1** in the presence of 10 fold molar excess of thiols in 50mM buffer solutions at pH 4.0. Δ -Phenylthiol (thiol pKa = 6.5), \bullet -Cysteine (thiol pKa = 8.3), \circ -Glutathione (thiol pKa = 8.7), \blacktriangledown -3-mercaptopropanoic acid ethyl ester (thiol pKa = 9.7), \blacksquare -Ethylthiol (thiol pKa = 10.6).

A plot of the observed rate constant (k_{obs}) for compounds **3.1** and **3.2** versus the pK_a of the thiol group indicates a decreasing reaction rate as the pK_a of the thiol increases as shown in figure 3.7, signifying the fact that an increase in the acidity of the thiol group increases the reactivity of the thiol with the sulfenamide prodrugs. This may seem to be inconsistent with the proposed mechanism as the expectation that the thiolate is the reactive species in solution and if that were the case, an increase in the basicity of the thiol group should lead to an increase in the reactivity of the thiols with the sulfenamide prodrugs. However, the plot as shown in Figure 3.7

does not take into account the fraction of the thiol in its thiolate form since these reactions were carried out at only one fixed pH value (pH 4.0).

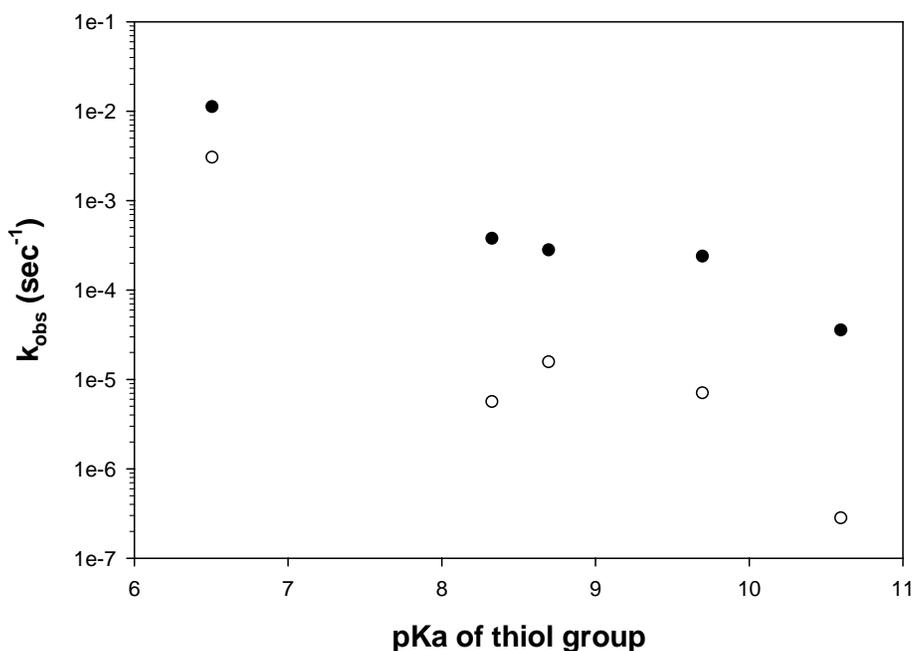
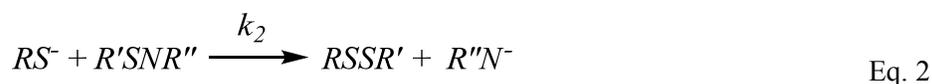


Figure 3.7: Semi-log plot of k_{obs} for the degradation of 3.1 (●) and 3.2 (○) versus the pK_a of the reactive thiol. The reactions were carried out in the presence of 10 fold molar excess of the thiols at pH 4.0 in 50 mM buffer solutions ($I = 0.15 \text{ M}$) and at 25°C .

To show consistency, the fraction of the thiol in the thiolate form has to be accounted for and in so doing, account for the second order rate constant. All the reactions carried out between the sulfenamides and the thiols are expected to be a second order reaction; however, since the reactions were carried out in the presence of molar excess thiol concentrations, the observations follow pseudo first order kinetics. Second order dependency reactions were carried out at varying pH and varying thiol concentration.

3.3.3. Kinetic Analysis of sulfenamides prodrug reactions with thiols.

Under the pseudo first order study conditions, the reaction of the sulfenamide prodrugs can be assumed to follow a three-step reaction, first is the fast ionization of the sulfhydryl compounds (RS^H) to the negatively charged thiolate species (RS^- , Eq. 1), second is the reaction of the thiolate species with the sulfenamides ($R'SNR''$, Eq. 2) and finally the fast reaction of the nitrogen moiety with water to form the parent drug molecule ($R''NH$, Eq. 3).



where K_a is the acid dissociation constant of the thiol and k_2 is the second order rate constant for the reaction between the thiolate anion and the sulfenamide. The rate of the overall reaction can be represented by the rate equation as indicated in Eq. 4, if one assumes that Eq. 1 and 3 above are instantaneous.

$$\begin{aligned} -\frac{d[R'SNR'']}{dt} &= k_2[RS^-][R'SNR''] \\ &= k_2 \cdot f_- \cdot [RS^H]_T [R'SNR''] \end{aligned} \quad \text{Eq. 4}$$

where f_- is the fraction of the thiol in the thiolate form in solution at any given pH. From Eq. 1 the fraction of thiolate in solution can be given as

$$f_- = \frac{[RS^-]}{[RSH]_T} = \frac{K_a}{K_a + [H^+]} = \frac{1}{1 + 10^{(pK_a - pH)}} \quad \text{Eq. 5}$$

Since pseudo first order kinetics were observed, Eq. 4 can be simplified to Eq. 6.

$$-\frac{d[R'SNR'']}{dt} = k_{obs} \cdot [R'SNR''] \quad \text{Eq. 6}$$

where k_{obs} is the observed pseudo first order rate constant at a fixed pH in the presence of molar excess of thiol. Assuming that the thiolate anion is the reacting species in solution then the observed rate constant can be expressed as

$$k_{obs} = k_2 \cdot [RS^-] = k_2 \cdot f_- \cdot [RSH]_T = k'_2 \cdot [RSH]_T \quad \text{Eq. 7}$$

k'_2 is defined by Eq. 8 and is a constant at any given pH value but will vary with pH since f_- is sensitive to changes in pH. $[RSH]_T$ is the total concentration of the thiol present in solution and is equal to the initial concentration of thiol added to the solution.

$$k'_2 = k_2 \cdot f_- = \frac{k_2}{1 + 10^{(pK_a - pH)}} = \frac{k_2 K_a}{[H^+] + K_a} \quad \text{Eq. 8}$$

From Eq. 8, the apparent second order rate constant (k'_2) can be determined for any given pH from plots of k_{obs} versus $[RSH]_T$. This apparent second order rate constant serves as a measure of the rate of the reaction of the sulfenamide prodrugs in the presence of the thiol species. It should be dependent on both the pK_a of the thiol species and also the pH of the solution of reaction media, if the thiolate is the reactive species.

Since the reactions at pH values greater than 6.5 were too fast to follow by HPLC, the reaction of the sulfenamide prodrug models with the thiol compounds were studied at lower pH values (3.0 to 6.0). The lowest -SH group pK_a of the thiols studied was that of phenylthiol. In this pH range it can be assumed that the concentration of hydrogen ion is much greater than the value

of the dissociation constants of the -SH groups. Thus Equation 5, describing the fraction of the thiolate in solutions, can be simplified to Eq. 9.

$$f_- \cong \frac{K_a}{[H^+]} \quad \text{Where } [H^+] > K_a \quad \text{Eq. 9}$$

Hence the observed rate constant equation changes to;

$$k_{obs} = k_2 \cdot [RS^-] = k_2 \cdot f_- \cdot [RSH]_T = k_2 \cdot [RSH]_T \cdot \frac{K_a}{[H^+]} \quad \text{Eq. 10}$$

$$\log(k_{obs}) = \log(k_2 [RSH]_T K_a) - \log([H^+]) \quad \text{Eq. 11}$$

$$k_{obs} = k_2 \cdot [RSH]_T \cdot \frac{K_a}{K_w} \cdot [^-OH] \quad \text{Eq. 12}$$

A semi-log plot of the k_{obs} versus the pH of solution should result in a linear correlation with a slope of unity for a fixed $[RSH]_T$ value as shown in figure 3.8. A slope of unity confirms that the reaction is apparent base catalyzed. Control reactions of the sulfenamide in buffered solutions in the absence of thiols resulted in no reaction, suggesting that the base catalyzing the reaction cannot be hydroxide ions. Thus it can be concluded that the thiolate ions are the species in solution resulting in the breakdown of the sulfenamide prodrug models to the parent drug. A zero order slope would have suggested that the reaction was between the sulfenamide and the thiol species rather than the thiolate species as illustrated in scheme 3.3.

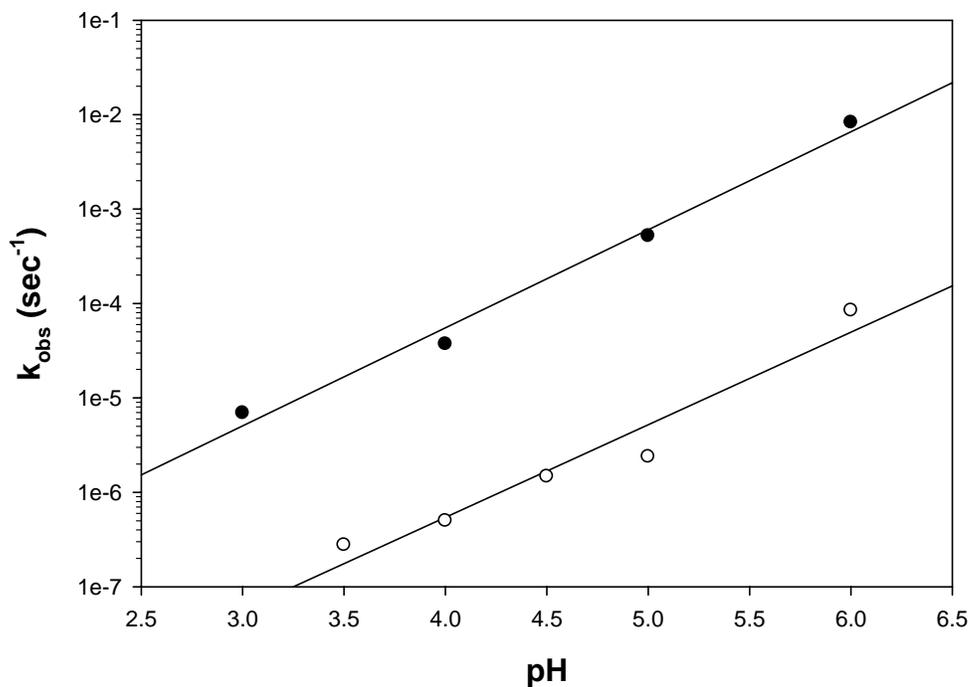
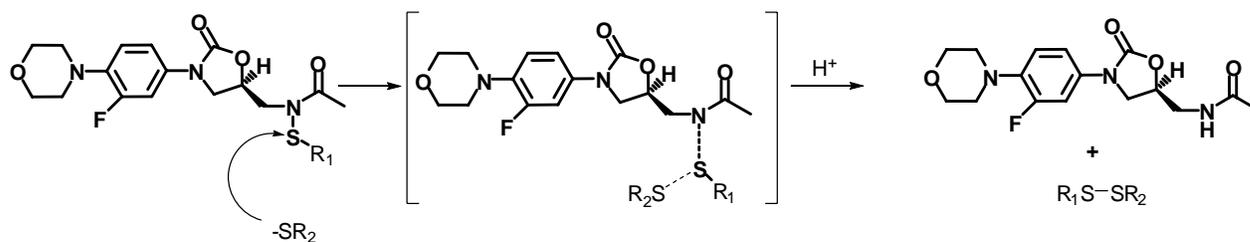


Figure 3.8: Plot of pH vs. k_{obs} for the degradation of sulfenamide prodrugs in 10 fold (100 μ M) excess of cysteine. (● — **3.1**, Slope = 1.04, ○ — **3.2**, Slope = 0.98) at a fixed $[RSH]_T$ value. The straight light is a linear fit of the data to Eq. 11.



Scheme 3.3: Confirmed degradation mechanism of sulfenamide prodrugs showing the rate determining step as the attack of the sulfur moiety by the thiolate ions in solution.

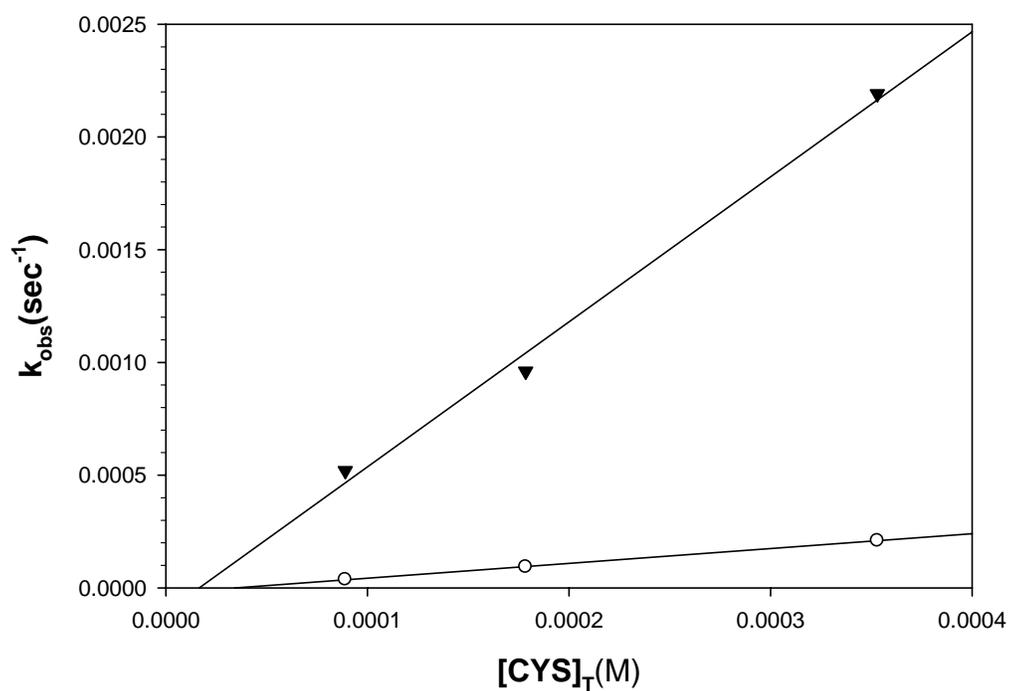


Figure 3.9: Degradation of **3.1** in excess cysteine at pH 4.0 (\circ) and pH 5.0 (\blacktriangledown), showing a plot of k_{obs} versus the total sulfhydryl concentration added. The straight lines are linear fits to the data sets

3.3.4. Mathematical determination of 2nd order rate constants

From Equation 10, a plot of the observed rate constant (k_{obs}) versus the total concentration the thiol added to the solutions ($[RSH]_T$) results in a linear plot with an intercept of zero, and a slope ($Slope_1$) (Figure 3.9), where $Slope_1$ is a product of k_2 and the fraction of thiolate ions in solution (f) hence $Slope_1$ is equal to k'_2 as in Eq 13.

$$Slope_1 = k'_2 = k_2 \frac{K_a}{[H^+]} \quad \text{Eq. 13}$$

Subsequently, a plot of $Slope_1$ (k'_2) versus the inverse of the hydrogen ion concentration ($1/[H^+]$) at the various pH gives another linear plot with a slope ($Slope_2$), where $Slope_2$ is defined by Eq. 14 as the product of the second order rate, k_2 , constant and the dissociation constant (K_a) of the -SH group of the thiol compounds (Figure 3.10).

$$Slope_2 = k_2 K_a \quad \text{Eq. 14}$$

Knowing the K_a values of the thiol groups allows one to estimate the k_2 value, the specific second order rate constant for the reaction of the sulfenamides with the specific thiolate. The estimated k_2 values, shown in Table 3.3, indicate that an increase in the pK_a results in an increase in the k_2 values. That is an increase in the basicity of the thiol group increase the ability of the thiolate species to attack the sulfur of the N-S bond, resulting in the break down of the prodrugs and leading to increases in the second order rate constants.

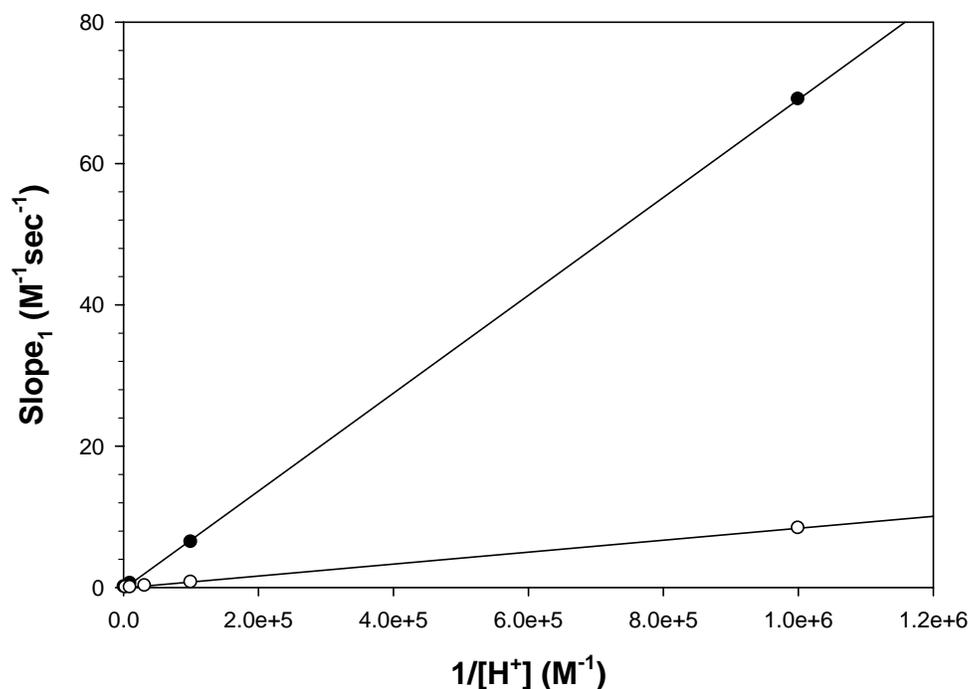


Figure 3.10: Plot of Slope₁ versus the inverse of the hydrogen ion concentration from Equation 12. (●-3.1, ○-3.2). The straight lines are a linear fit to the data sets

An extended Bronsted plot of the estimated k_2 values versus the pK_a of thiol groups with pK_a values above 8.0, show a linear correlation (Figure 3.11). If the reactions were not dependent on the thiolate species, no dependency would be observed in the Bronsted plots. The slopes obtained decreases in the order of **3.2>3.1>3.3** with values of 0.79, 0.67 and 0.56 respectively. This suggests that there is greater S-S bond formation for 3.2 than for 3.1 and for 3.1 than for 3.3. The degree of S-S bond formation is consistent with the leaving group ability of the promoity. The better the leaving group the less S-S bond is formed before the N-S bond is completely cleaved.

Thiol	pKa	3.1 $k_2 \times 10^5$ $M^{-1}sec^{-1}$	3.2 $k_2 \times 10^5$ $M^{-1}sec^{-1}$	3.3 $k_2 \times 10^6$ $M^{-1}sec^{-1}$
Phenyl thiol	6.5	0.32	0.13	
Cysteine	8.3	1.20	0.14	7.98
Glutathione	8.7	2.51	0.20	15.0
3-mercapto propanoic acid ethyl ester	9.7	15.0	1.60	50.1
Ethythiol	10.6	39.8	7.96	

Table 3.3: A collection of the k_2 values estimated for all the sulfenamides and the thiols studied.

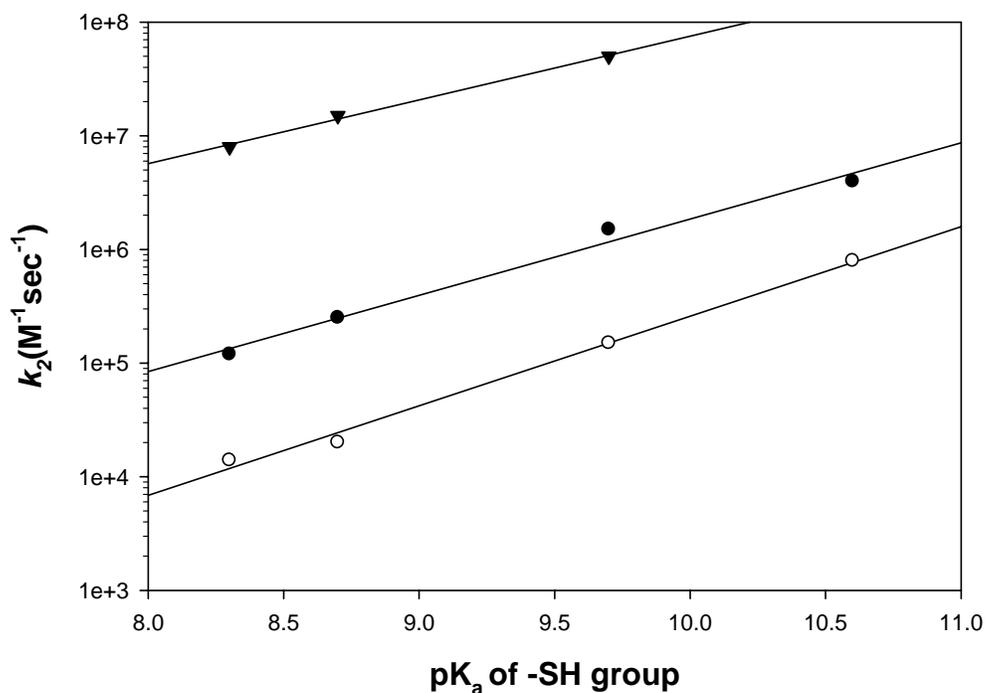


Figure 3.11: An Extended Bronsted plot of the second order rate constant (k_2) and the pK_a of the reacting thiol group. (\bullet -3.1, \circ -3.2, \blacktriangledown -3.3). The straight lines represent linear fit to the data points with slopes of 0.79, 0.68 and 0.56 respectively.

The sulfenamide, **3.2**, has an ethylthiol promoiety which is a short straight chain alkyl moiety and expected to be a poorer leaving group compared to the phenylthiol promoiety of **3.1**. As a result more energy is required to break the N-S bond in **3.2** compared to breaking the N-S bond in **3.1**, in which the phenylthiol is known to be a very good leaving group.

On the other hand **3.3** contains two very good leaving groups in the form of the phenylthiol promoiety and the phthalimide moiety itself, hence a much earlier transition state would be expected for the break down of **3.3**. The differences in the leaving group ability of the promoieties also account for the differences in the k_2 values. The better the leaving group the higher the k_2 values, hence the faster the rate of the cleavage of the N-S bond.

The reactions of **3.1** and **3.2** with phenylthiol resulted in estimated k_2 values higher than expected based on the pK_a value of the -SH group of phenylthiol (Table 3.3). When the values for the reactions with phenylthiol are included on the extended Bronsted plot of k_2 versus pK_a of the thiol groups, a deviation from the linear dependency is observed (Figure 3.12). A number of possible explanations can be given for the deviation observed.

The first, the phenylthiol is the only aromatic thiol that was used in the studies and it is likely that a similar transition state and reaction pathway is occurring. However, due to the electronic contribution from the aromatic rings a much higher intrinsic reaction is observed leading to higher k_2 values. Also the electronic contribution from the phenyl ring could lead to some differences in solvation that could impact the reactivity of the aromatic thiol. The solvation around the thiolate of the phenylthiol could be loosely packed as a result of the electronic dissociation in the phenyl ring. Loosely packed solvent molecules around the thiolate ions result in easy access of the sulfenamide to the thiolate species leading to higher reactivity. Highly

packed solvent molecules in the case of alkyl thiols results in higher energies required for desolvation, leading to lower reactivity.

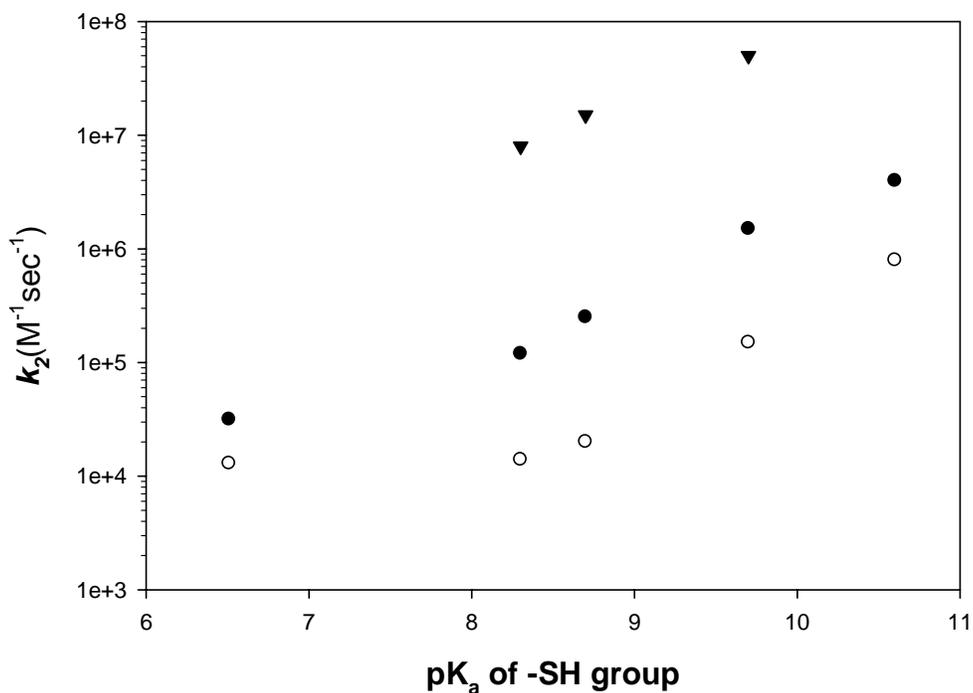


Figure 3.12: Relationship between the second order rate constant and the pKa of the thiol group of sulfhydryl compounds showing pK_a value lower than 8.0. (\blacktriangledown - 3.1, \bullet - 3.2, \circ - 3.3)

In addition to increases in the reactivity of the phenyl thiol with the N-S bond, the electronic contribution from the phenyl ring could lead to a change in the rate limiting step of the reaction accounting for the deviation from the observed linearity.

3.4. Conclusions

Results from the study of sulfenamide prodrug models in the presence of thiol compounds of varying -SH pK_a values have shown that the thiolate ions are the species involved in the breakdown of the sulfenamide prodrugs in the presence of the thiols. Determination of the second

order rate constants for the reactions between the sulfenamides and the thiols indicate that an increase in the basicity of the thiol group results in larger second order rate constants. However, the observed rate constants are dependent on both the pK_a of the reacting thiol group and the pH of the reaction.

At physiological pH, it is expected that any thiol containing species could provide a rapid conversion of the sulfenamide prodrugs to the parent drug. Glutathione is the most likely candidate, but other thiol groups, including thiols on proteins could breakdown the prodrugs. Chapter 4 will discuss work evaluating the degradation of the sulfenamide prodrug models in the presence of thiol containing proteins such as albumin. The goal is to determine the rate of conversion of the prodrugs to the parent drug in the presence of these macromolecules

3.5. References

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Chapter 4

Degradation of sulfenamide prodrugs in the presence of thiol containing proteins

4.1. Introduction

4.1.1. Overall objective

The overall goal of this chapter is to determine the rate of degradation of sulfenamide prodrugs in the presence of free thiol containing proteins and also in the presence of human plasma. The previous chapter established that the sulfenamide prodrugs readily degrade in the presence of small molecule thiols, such as glutathione, to release the parent drug (linezolid) and a mixed disulfide. Also, it was established that the reaction at physiological pH was practically instantaneous as a result of the higher concentrations of thiolate ions likely present *in vitro*. Glutathione is the most abundant small molecule free thiol present *in vivo*, however, many *in vivo* free thiols also exist on proteins. Our goal is to study the degradation of the sulfenamide prodrugs in the presence of thiol containing proteins and whole plasma, which contains numerous proteins including albumin, and to determine the rate of conversion. The study of the sulfenamide prodrugs in the presence of thiol containing proteins and in plasma will serve as a predictor for the rate of conversion of the prodrugs *in vivo*.

4.1.2. Background

4.1.2.1. Thiol containing proteins and their significance

There are three main types of protein thiols found *in vivo*; thiols found at the active sites of enzymes and considered as essential to the functions of the enzymes, thiols exposed on the surface of proteins, and thiols that are buried in the complex structures of proteins.¹ Of these three types, the exposed thiols on the surfaces of proteins would seem to be of much interest as they include regulatory thiols that are primarily involved in maintenance of a balanced oxidation state *in vivo* to prevent oxidative damage. Some surface thiols are involved in redox signaling, both

inside and outside cells and tissues. However, free thiols exposed on the surfaces of proteins readily undergo modifications via the formation of disulfides or mixed disulfides with other free protein thiols and low molecular mass thiols such as glutathione (GSH).^{2,3} They are also known to undergo disulfide exchange with oxidized glutathione (GSSG) to form mixed disulfides. Reports have shown that the mixed disulfide (protein-glutathione) bond formation are readily reversible, but under oxidizing conditions, the bond can be maintained indefinitely.^{4,5}

Free protein thiols have also been found to react with drugs containing thiols to form mixed disulfides. Similar to the protein-glutathione mixed disulfide formations, most protein-drug disulfide formations have been found to be reversible *in vivo*, as a result of the interactions of glutathione and other endogenous protein thiols present. Protein-drug disulfide formations can sometimes improve on the pharmacokinetic properties of the drug, such as increased solubility in blood plasma, decreased toxicity and protection of the drug molecules from oxidation. Protein-drug interactions also serve as a means of transportation of the drug molecules via the circulatory system.⁶ However, they can also have negative implications, for instance an increase in the *in vivo* half life of the therapeutic agent may lead to increases in toxicities, and also an increase in toxicity can occur as a result of immunogenicity.

In the previous chapter, it was established that the degradation of the sulfenamide prodrugs in the presence of thiols occurs via the nucleophilic attack of thiolate ions in solution to cleave the N-S bond, thus releasing the parent oxazolidinone and a mixed disulfide. Based on the data obtained in Chapter 3, it can be hypothesized that the sulfenamide derivatives of oxazolidinones will be readily cleaved by proteins containing free thiols. Our goal therefore is to carry out *in vitro* studies on the sulfenamide prodrugs in the presence of albumin and other thiol containing proteins, such as PRL-1 (Phosphatase of regenerating liver) C170/171S mutant, to

determine if sulfenamides will be readily cleaved by thiols exposed on the surface of the protein and also thiols at the active site.

Human serum albumin (HSA, Figure 4.1) is a single chain protein, consisting of 585 amino acid residues with a molecular mass of 66,500 Da.⁷ HSA is known to be the most abundant protein in human plasma with an approximate concentration of 0.6 mM in healthy adult humans and serves as both a transport and depot protein for various endogenous and exogenous molecules including drug molecules. Structural determination of the HSA protein has shown that it contains 35 cysteine residues, 34 of which are involved in disulfide linkages, leaving one cysteine residue as a free thiol.^{6,8} The only free cysteine molecule in HSA is found in position 34 (Cys-34) and is found in a crevice on the surface of the first sub-domain of HSA, hence it is unable to participate in disulfide linkages or salt bridges.^{6,9,10} The free Cys-34 is readily available for formation of disulfide bonds with other free thiol groups, such as glutathione *in vivo*.

The formation of a disulfide with other thiols results in the free thiol being 'blocked' on exposure to oxygen. This occurs readily when HSA is handled during purification. Cys-34 can also readily undergo oxidation into sulfenic and /or sulfonate states. HSA with blocked or oxidized Cys-34 has been referred to as non-mercaptoalbumin (NHA) which accounts for approximately 10% of HSA *in vivo* and HSA with free thiols of Cys-34 is referred to as mercaptoalbumin (MHA). HSA is therefore a heterogeneous mixture of NHA and MHA.

Cys-34 has been found to be located in a pocket close to the surface of the HSA structure (see figure 4.1), hence is expected to be available for the nucleophilic attack of the sulfenamide bond. Our goal with the work on HSA is to establish that the surface exposed Cys-34 will readily cleave the sulfenamide bond of linezolid prodrugs.

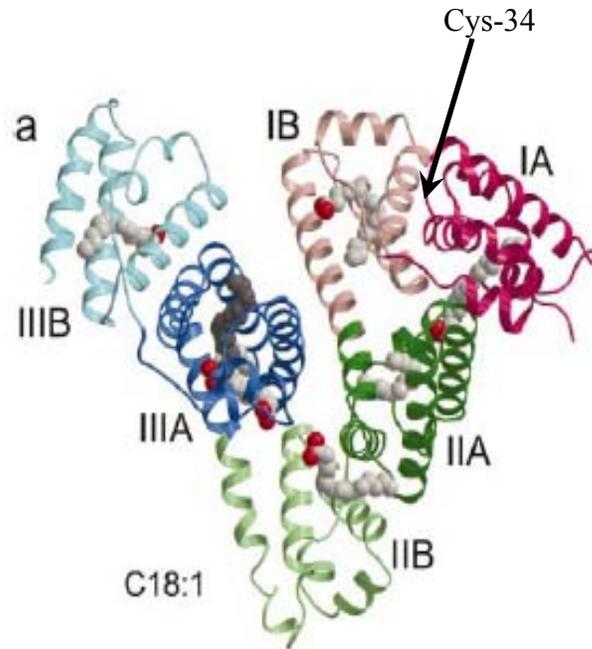


Figure 4.1: Crystal structure of the HSA-myristate-hemin complex from Zunszain *et al* (a) The protein secondary structure is shown schematically with the sub-domains color-coded as follows: IA, red; IB – light red; IIA, green; IIB – light-green; IIIA, blue; IIIB – light blue; The arrow points to the position of Cys-34.¹¹

PRL-1 (figure 4.2) is a part of a subfamily of protein tyrosine phosphatases (PTPs) responsible for cellular signal transduction mediating cell growth, differentiation, and tumor invasion.¹²⁻¹⁵ PRL-1 is a small C-terminal-prenylated protein that plays critical roles in targeting proteins into membranes in cells thus activating the cellular signaling process. Recent exploration of the structure of PRL-1 has determined that the protein contains six cysteine residues; Cys-49, Cys-98, Cys-99, Cys-104, Cys-170 and Cys-171. Cys-104 and Cys-49 are located in the active site of the protein and with C104 serving as the active nucleophile in the active site. Cys-170 and Cys-171 are found in the prenylated motif of the C-terminus where as Cys-98 and Cys-99 are

buried in the structure of the protein. Mass spectral analyses have shown that all six cysteine residues exist as free thiols but indicate that Cys-98 and Cys-99 are inaccessible due to the fact that they are buried in the structure.^{14,15} PRL-1 C170-171S, which was recently isolated and purified by Dr. J. Laurence and members of her lab group at the university of Kansas, has both Cys-170 and Cys-171 mutated to serine residues, leaving the active site cysteine residues (Cys-104 and Cys-49) as the only apparently accessible free thiols present in the mutant protein. Our aim with studies on PRL-1 C170-171S mutant is to establish that active site free thiols can also readily attack the sulfenamide bonds leading to the release of the parent oxazolidinone molecule.

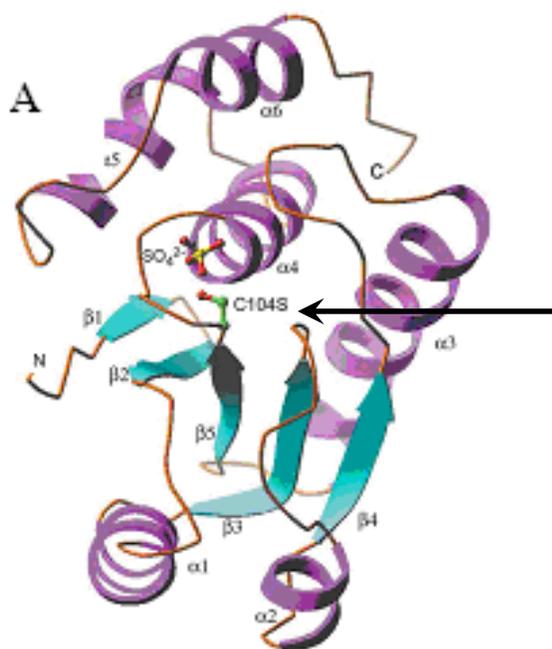


Figure 4.2: Ribbon diagram structure of PRL-1 borrowed from Jeong *et al*, showing secondary structural elements (helices, purple; strands, blue; loops, yellow). The catalytic Cys-104 (mutated to serine for crystallization, shown by the arrow) and the bound sulfate ion are shown as a ball-and-stick representation.¹⁵

4.2. Experimental, Material and Methods

4.2.1. 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 and HSA were purchased from Sigma-Aldrich[®] Chemicals and used without further purification. A 25.0 mg/mL PRL-1 C170-171S mutant solution in a 50 mM phosphate buffer solution (I=0.15) at pH 6.5 was obtained from the Laurence lab at the University of Kansas, Pharmaceutical Chemistry Department, and used without further purification. Human plasma samples (from Kwame W. Nti-Addae) were obtained from Watkins Memorial Health Center at the University of Kansas, Lawrence, KS. Water used in all experiments was freshly distilled and deionized in-house using a Corning[®] Mega-pure One Liter distillation apparatus.

Buffer solutions (50 mM) adjusted to ionic strength of 0.15 M with sodium chloride for pH values were prepared freshly prior to use. The pH values of buffer solutions were measured with a Fischer Scientific[®] Accumet Research AR15 pH meter with standard buffers of pH 4.0, 7.0 and 10.0 purchased from Fisher Scientific[®]. All buffer solutions were filtered through 0.22 μm syringe filters prior to use in studies. Stock solutions of all prodrugs were freshly prepared in acetonitrile. Analyses were carried out on a HP 1050 LC system operated with Agilent Chem Station[®] Software. A Zorbax C¹⁸ (5mm by 2.1 mm, 5 μm particle size) column was used for HPLC analysis. UV was observed at 258 nm. The gradient method mentioned in Chapter 3, table 3.1, was used for all HPLC analysis. The two prodrug models indicated in Figure 4.3 were used in the reactions with the thiol proteins and plasma

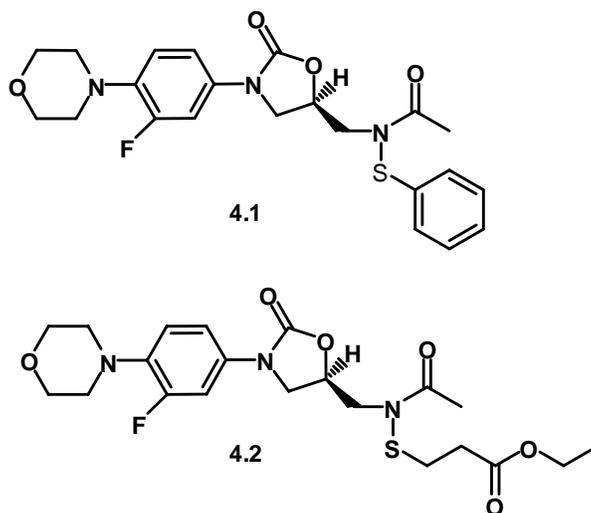


Figure 4.3: Structures of linezolid prodrug models used in the study of sulfenamide stability in the presence of molar excess of thiol proteins. In the reactions with thiol proteins only **4.1** was used and both prodrugs were used in the reactions with plasma.

4.2.2. Determination of the free thiol concentration in HSA.

L-cysteine was used as a standard in a slightly modified procedure outlined by Covalent[®] 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 was also prepared in acetonitrile to a final concentration of 0.051mM. Thioglo1[®] was dissolved diluted in DMSO to give a final concentration of 0.253mM. Fifty microliters of L-cysteine and 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 Thioglo1[®] solution was added to each sample to give a 5:1 molar ratio of Thioglo1[®] 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.

4.2.3. Degradation of 4.1 in the presence of HSA

Human serum albumins (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, 6.5, 7.0, 7.4 and 7.5. Compound **4.1** 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 in solution. The samples were vortexed for 15 seconds and centrifuged at 13,000 rpm for 5 minutes. The supernatant was analyzed by HPLC.

4.2.4. Degradation of 4.1 in the presence of PRL-1 C170-171S Mutant.

PRL-1 C170-171S mutant solutions were prepared in 50 mM phosphate buffer solutions at pH 5.5, 6.0, 6.5, 7.0, 7.4, and 7.5, to a final concentration of 12.4 μM . Theoretically, PRL-1 C170-171S mutant only contains four cysteine residues; hence at a concentration of 12.4 μM , the concentration of free thiol present was estimated to be 49.5 μM . Compound **4.1** was added to 2.0 mL solutions of PRL-1 C170-171S mutant at the various pH 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-methylmaleimide solution in acetonitrile was added to the 100 μL samples in the micro centrifuge tubes, vortexed for 15 seconds and centrifuged at 13000 rpm for 5 minutes. The supernatant was analyzed by HPLC.

4.2.5. Degradation of 4.1 and 4.2 in the presence of Human Plasma.

Literature reports indicate human plasma contains 0.6 mM albumin.¹⁶ Fresh human plasma 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. N-(phenylthio)linezolid (**4.1**) and N-((2ethoxycarbonyl)ethylthio)linezolid (**4.2**) were added to a final concentration of 4.4 μM . One hundred microliter aliquots of the samples 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-methylmaleimide solution in acetonitrile was added to the 100 μL samples in the micro centrifuge tubes to quench the reaction and also precipitate the protein in solution. The samples were vortexed for 15 seconds and centrifuged at 13,000 rpm for 5 minutes. The supernatant was analyzed by HPLC.

Controls were run using 40 μM HSA in 50 mM phosphate buffer solution ($I = 0.15$) at pH 7.4 instead of plasma solutions as reaction time in 100% plasma was instantaneous.

4.3. Results and Discussions

4.3.1. Degradation of 4.1 in the presence of HSA and PRL-1 C170-171S.

The addition of **4.1** to 0.6 mM HSA solution at pH 7.4 resulted in the immediate quantitative generation of the parent linezolid molecule. At lower albumin concentration (40 μM) the reaction was much slower and hence one is able to follow the reaction using HPLC analysis. The data showed that in the presence of a ten fold molar excess of HSA, first order kinetics were observed for the loss of the prodrug at pH 7.0 (Figure 4.4).

However, a semi-log plot of k_{obs} versus the pH of solution was not linear (Figure 4.5) unlike what was observed in the case with small molecule thiols as discussed in Chapter 3. That is, the reaction of the sulfenamide with the small molecule thiols showed a pH dependency and

was consistent with the reaction of the thiolate species with substrate sulfenamides. Thus a semi-log plot of k_{obs} versus pH of solution showed a linear plot with a slope of approximately unity (see chapter 3, section 3.3.3). The same plot with HSA clearly does not show this behavior.

A number of possible explanations include; first, albumin is a dynamic protein and changes conformation readily with changes in its environment. 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 prodrug to the free thiol at Cys-34. In addition to altering the accessibility, a change in conformation leads 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 dissociation constant (K_a) of the thiol. Changes in the reaction of thiolate form of the Cys-34 thiol will significantly change the fraction of thiolate species present in solution as discussed in the previous chapter, thus altering the reactivity of the thiol.

The determination of the free thiol concentration in the HSA purchased from Sigma-Aldrich with Thioglo1® 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. It was found that approximately 45 to 80 % of Cys-34 was oxidized in some form as a result of commercial isolation and purification processes.^{17,18} Thus, while a nominal 10 fold molar excess of the free thiol HSA was added, only an actual three to four fold molar excess of free thiol was actually present, surprisingly, apparent first order kinetics were still observed (See figure 4.4).

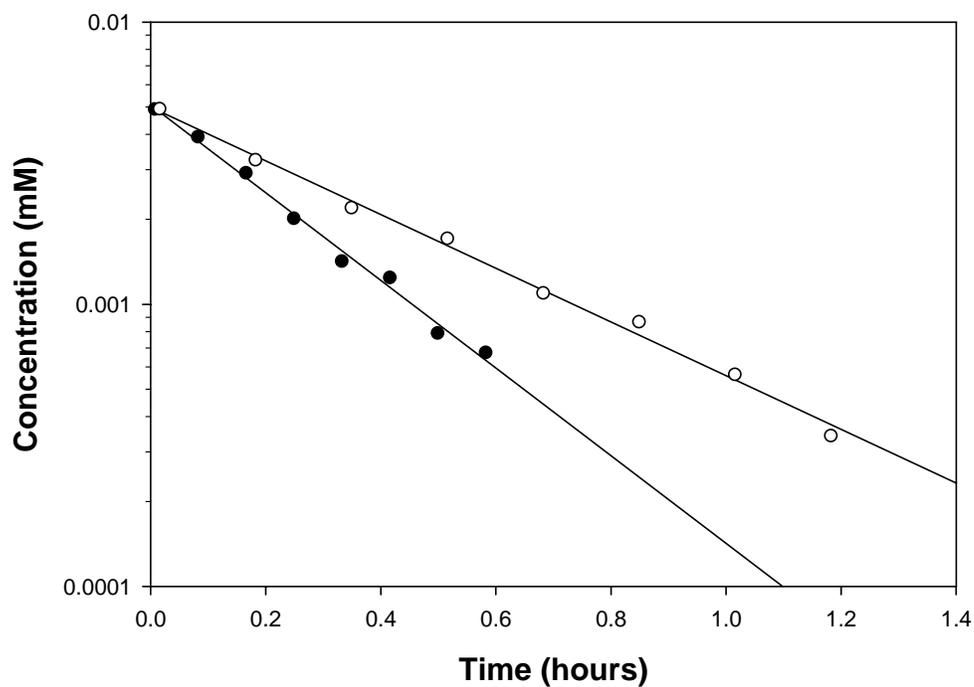


Figure 4.4: Semi-log plot for the degradation of **4.1** in the presence of a 10 fold molar excess HSA (○) and a 5 fold molar excess of PRL-1 C170-171S mutant (●) at pH 7.0 in 50mM buffer solutions ($I = 0.15$) at 25°C. These concentrations were used assuming there is one free thiol per mol of HSA and two free reactive thiols per mole of PRL-1 C170-171S mutant. PRL-1 C170-171S mutant actually contains 4 free thiols but two are buried in the structure and assumed to be inaccessible. Thus a total of 10 fold molar excess of free thiols in both cases.

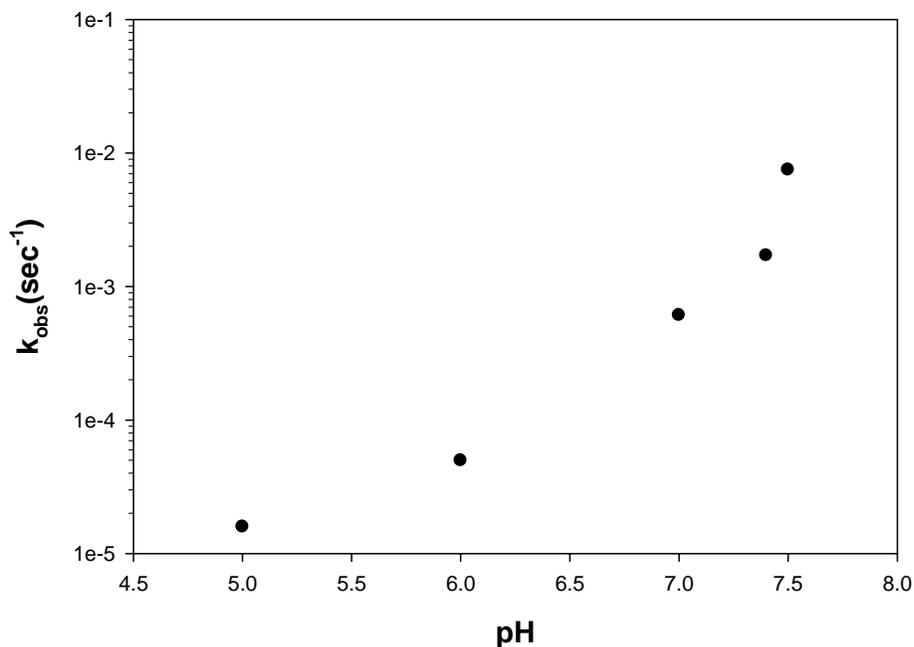


Figure 4.5: pH dependency of the reaction of **4.1** in the presence of a nominal 10 fold molar excess of human serum albumin (HSA) in 50mM buffer solutions (I=0.15) at 25°C, assuming one free thiol per mole of HSA.

Drugs and prodrugs are also capable of binding to HSA. If the sulfenamide prodrugs are partially or strongly bound to HSA at a site(s) removed from Cys-34 this could also affect the rate of reaction between the prodrugs and HSA. The reaction rate could also be affected by the pH of solution, which can also alter the binding capacity of the prodrugs to HSA. Thus, while there are many possible explanations to the observations seen in figure 4.5, it is likely that more than one cause is operative.

Similar to the degradation in HSA, the degradation in the presence of excess PRL-1 C170-171S mutant followed pseudo first order kinetics (see Figure 4.4). A semi-log plot of the observed rate constant (k_{obs}) versus the pH of solution showed a linear correlation with a positive

slope (Figure 4.6), consistent with nucleophilic attack of the thiolate to cleave the N-S bond. However, unlike for small molecule thiols which showed a slope of unity, the slope in this case was estimated to be 0.52. This deviation from expectation again can be attributed to conformational changes occurring as pH changes, leading to further changes in accessibility to the free thiols, neighboring functional groups, changes in the fraction of thiolate species in solutions, and other factors that lead to a decrease in expected reactivity.

The degradation in the presence of PRL-1 C170-171S mutant was however significantly faster than reactions in the presence of HSA at pH values below 7.0. However, at pH 7.4 the reactions showed very similar rates as shown by the estimated half lives in table 4.1. Degradation of **4.1** in the presence of ten-fold molar excess of HSA and PRL-1 C170-171S mutant resulted in a half lives ($t_{1/2(\text{HSA})}$ and $t_{1/2(\text{PRL-1M})}$) of 6.7 and 6.8 minutes respectively. A comparison of the reactivity observed in HSA and in PRL-1 with the reactivity observed in cysteine and glutathione (see Chapter 3) indicate a significantly higher reactivity observed in the small molecule thiols at pH 7.4.

4.3.2. Degradation of sulfenamide prodrugs in the presence of Human Plasma

In the presence of diluted human plasma (nominal albumin concentrations of 40 μM), **4.1** and **4.2** were observed to degrade faster than in the control reactions in the presence of HSA. The degradation of **4.1** in the presence of human plasma ($t_{1/2(\text{HP})}$) at pH 7.4 was gave an estimated half life of 3.7 minutes ($k_{\text{obs}(\text{HP})} = 0.00316 \text{ sec}^{-1}$), compared to a half life ($t_{1/2(\text{HSA})}$) of 6.8 minutes ($k_{\text{obs}(\text{HSA})} = 0.00171 \text{ sec}^{-1}$) in HSA (Figure 4.6). The degradation of **4.2** resulted in $t_{1/2(\text{HP})}$ and $t_{1/2(\text{HSA})}$ of 7.7 minutes ($k_{\text{obs}(\text{HP})} = 0.00149 \text{ sec}^{-1}$) and 27.6 ($k_{\text{obs}(\text{HSA})} = 0.00042 \text{ sec}^{-1}$) minutes respectively.

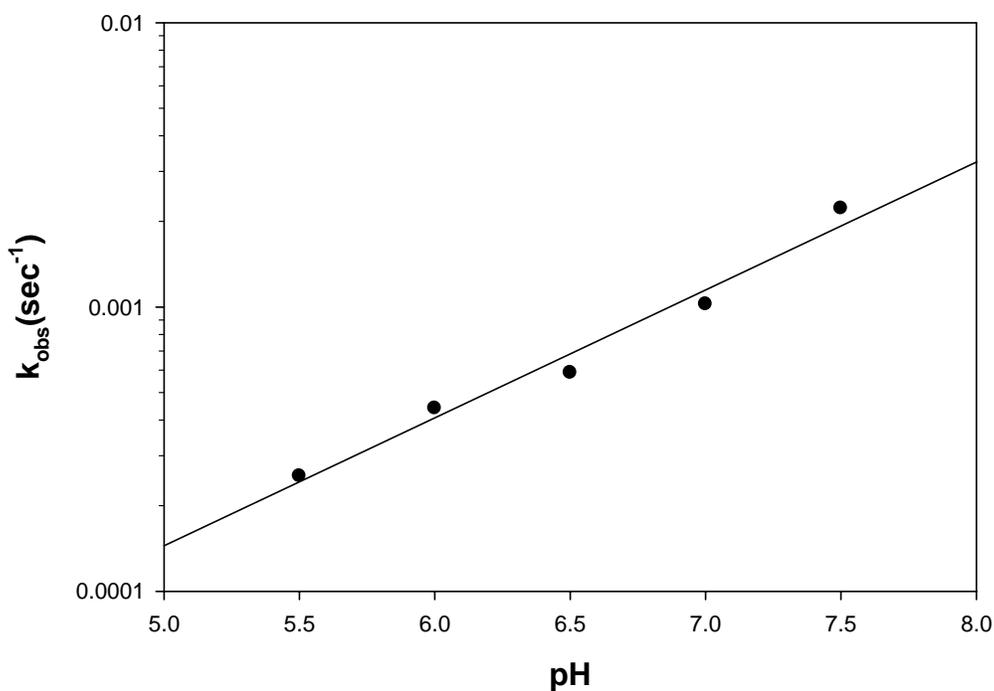


Figure 4.6: Stability of **4.1** in the presence of 5 fold molar excess PRL-1 C170-171S mutant in 50mM phosphate buffer solutions ($I = 0.15$) at 25°C, assuming 2 free thiols per mole of PRL-1 C170-171S mutant.

Reacting Species	$t_{1/2}$ (pH 6.0)	$t_{1/2}$ (pH 7.4)
Cys	1.40 mins	4.10 sec
GSH	2.30 mins	5.40 sec
HSA	233 mins	6.70 mins
PRL-1 C170-171S	25.1 mins	6.80 mins

Table 4.1: A comparison of the half lives of the reactivity of nominal 10 fold molar excess of thiols and thiol proteins with **4.1** at pH 6.0 and 7.4 in 50 mM phosphate buffer solutions.

Faster reaction rates were observed for the study of **4.1** due to the fact that the phenylthiol promoiety is a much better leaving group (see chapter 3) compared to the 3-mercaptopropanoate ethyl ester promoiety of **4.2**. 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 existed as free thiols and is consistent with the lower reaction rates observed in the HSA solutions. Also, human plasma may contain small molecule thiols such as glutathione, in addition to a collection of thiol containing proteins including albumin. Accounting for the low free thiol concentration in HSA solutions leads to insignificant differences in the reaction rates. For instance, in the case of **4.1**, assuming 100% of the Cys-34 existed as the free thiol form in the commercially available plasma, then the estimated half life would be approximately 2.02 to 3.03 minutes, and for **4.2** it is expected to be 8.25 to 12.38 minutes. These values are not significantly different from the values observed in the presence of diluted human plasma. These observations suggest that albumin will play a significant role *in vivo* in the conversion of the sulfenamide prodrugs to their parent drugs.

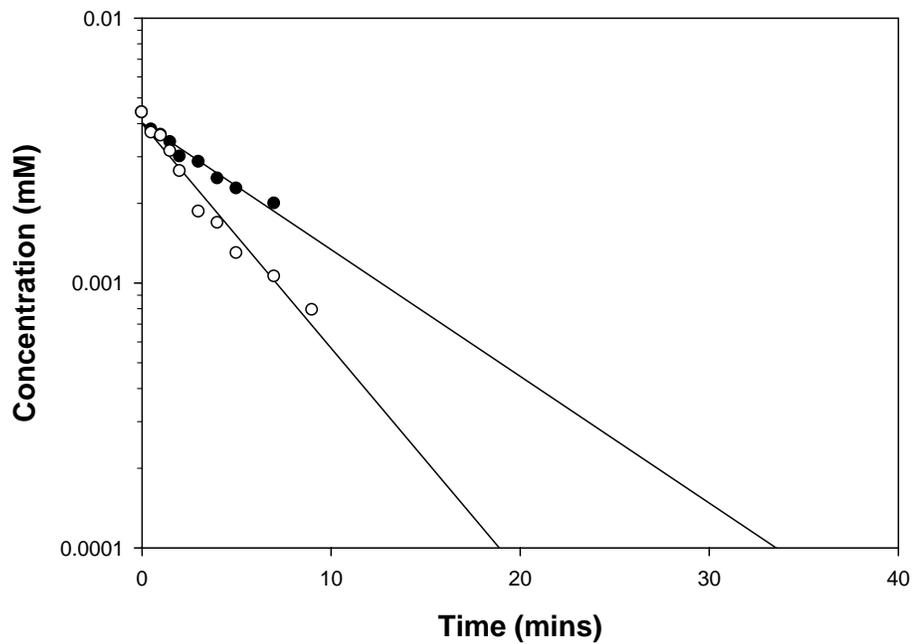


Figure 4.7: Semi log plot of the conversion of **4.1** to Linezolid in the presence of 40 μ M HSA (●) and Human plasma (○) diluted to a nominal albumin content of 40 μ M at pH 7.4 in 50mM phosphate buffer solutions ($I = 0.15$) and at 25°C

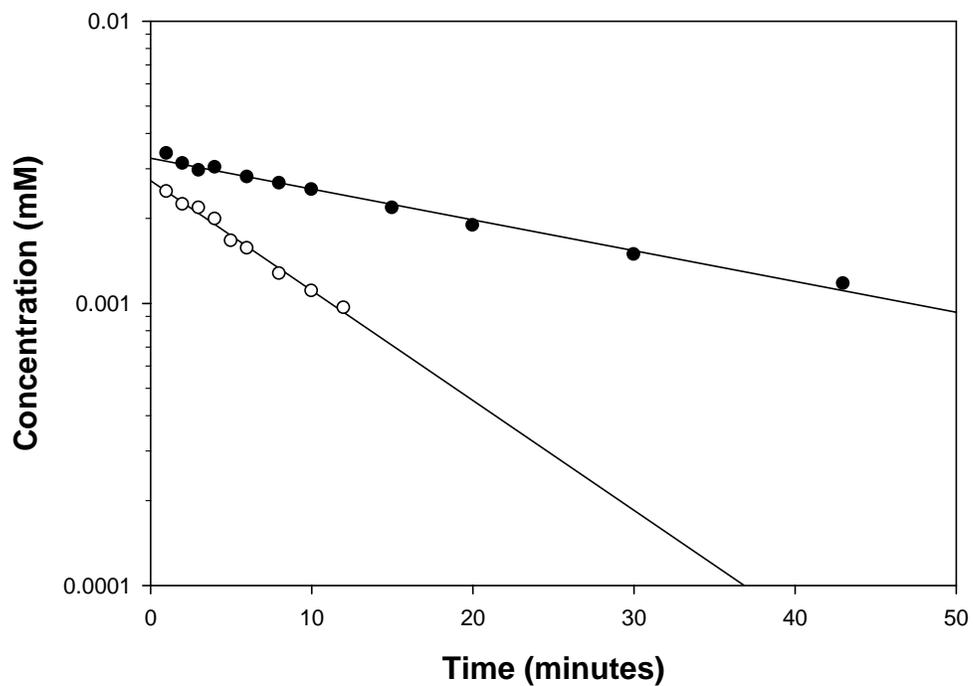


Figure 4.8: Degradation of **4.2** in the presence of HSA (●) and Human Plasma (○) diluted to a nominal albumin content of 40 μ M at pH 7.4 in 50mM phosphate buffer solutions ($I = 0.15$) and at 25°C

4.4. Conclusions.

Earlier in chapter 3, small molecule thiols were shown to readily react with sulfenamide prodrugs to release the parent drug and a mixed disulfide. Here it was shown that free thiol containing proteins (HSA and PRL-1 C170-171S Mutant) similarly react with sulfenamide prodrugs to release the parent drug. On a molar basis, small molecule thiols cleave the sulfenamide bonds more rapidly than thiol containing proteins, even when the protein (such as PRL-1) contains multiple free thiol groups.

A reasonable explanation to the lower level of reactivity observed in the presence of proteins is that the substrates have less access to the protein thiols due to steric factors or that the fraction of thiol in the thiolate form is different in proteins than in the more 'exposed' thiolate groups in small molecules.

Since the sulfenamides might also bind to HSA and any protein, such binding would also lower the effective free fraction of substrate available to react with the protein thiol. If one accounts approximately for the fraction of thiol in its reduced form in commercially available HSA, HSA has a similar reactivity to freshly isolated human plasma diluted to the same appropriate molar HSA value. This suggests that HSA is the most likely reactive protein in plasma.

The studies with PRL-1 C170-171S mutant illustrate the fact that the reaction of free thiols on proteins is a result of the attack of the thiolate species, however due to complex structure and environment of thiols in proteins, one is unable to say why the pH dependency doesn't show the same dependency on free thiolate fraction seen with small molecule thiols.

Results obtained earlier from small molecule thiols suggest the formation of a mixed disulfide between the nucleophilic thiol and the promoiety of the prodrug. With this information

one can assume that a mixed disulfide will be formed between the thiol proteins and the sulfenyl promoieties. This may lead to some concerns in toxicity and immunogenicity, should sulfenamide prodrugs be administered into humans. Further studies therefore need to be carried out to determine the formation of mixed disulfides between the proteins and the sulfenyl promoieties and their possible toxic effects.

In the next chapter, the permeability of a sulfenamide prodrug through Caco-2 cell monolayers will be presented. On the surface of the enterocytes in the GI are embedded proteins, some containing free thiols groups capable of converting the sulfenamides to the parent drug and affecting the permeability characteristics of the sulfenamide prodrugs.

4.5. References

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Chapter 5

The Determination of the Permeability Characteristics of Sulfenamide Prodrugs of Antimicrobial Oxazolidinones Across Caco-2 Cells.

1.1. Introduction

1.1.1. Overall Objective

The overall objective of the work presented in this chapter is to evaluate the permeability characteristics of sulfenamide prodrugs of the antimicrobial oxazolidinone, linezolid. The work presented in this chapter outlines a permeability study using Caco-2 cell monolayers to model the permeability characteristics of the sulfenamide prodrugs.

1.1.2. Permeability

The passage of orally dosed drugs into the circulatory system from the intestinal lumen is a very important property that often determines the success of drugs intended for oral administration. As a result, a lot of time and effort is spent on permeability studies to evaluate the permeability of early development compounds before further development. One means of evaluating permeability is via Caco-2 monolayer transport studies. Caco-2 monolayer permeability studies were first introduced in the late 1980's and is currently recognized by the FDA as one of the leading methods in evaluating the permeability of compounds across the intestinal mucosa.¹ Caco-2 cells are of a colonic origin but differentiate into polarized, columnar cells that mimic the intestinal mucosa, exhibiting similar properties and features such as well developed microvilli, presence of brush border enzymes, and readily form normal transport epithelia when grown on plastic filters.²⁻⁷

Our goal of developing sulfenamides as prodrugs is to improve physicochemical properties, such as the permeability of drug molecules or candidates. Previous studies have shown that sulfenamides can be successfully used to improve solubility; however, studies to evaluate the permeability characteristics of sulfenamide prodrug models of linezolid in MDCK

cell monolayers was inconclusive, mainly due to the rapid degradation of the prodrugs in the experimental setup resulting in no detectable prodrug concentration in the receiver compartments of the apical (AP) to basolateral (BL) directional study and vice versa.⁸⁻¹⁰ A comparison of the data obtained from a linezolid control studies and the data of the linezolid generated from the permeability study of the sulfenamide prodrugs led to the tentative conclusion that the prodrugs were contributing to some extent to a slight increase in the permeability of linezolid.

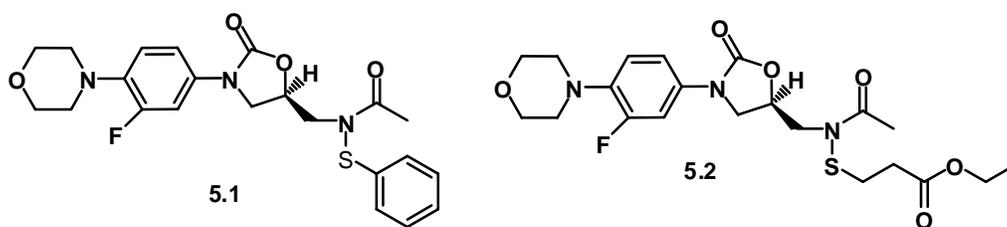


Figure 5.1: Structures of linezolid prodrug models used in the study caco-2 permeability studies.

In this chapter, we present a study of the permeability of the sulfenamide prodrug models indicated in figure 5.1, using Caco-2 cell monolayers that closely mimic the intestinal epithelia. The goal of this study is to determine a model that effectively represents the degree to which the prodrugs contribute to the permeability of parent molecule. Specifically our aim is to be able to develop a model that will clarify the contribution of the sulfenamide prodrugs to the permeability of the parent molecule or drug candidate.

1.2. Experimental Materials and Methods

1.2.1. Solvents and chemicals

Fetal bovine serum (heat-inactivated), buffered DMEM (High glucose with L-glutamine, 25mM HEPES buffer, pyridoxine hydrochloride, without sodium pyruvate), unbuffered DMEM,

Hank's balance salt solution (HBSS), Dulbecco's phosphate buffer solution, non-essential amino acid, trypsin (with 0.25% EDTA), and penicillin-streptomycin (10000 I.U. Pen/ 10000 µg/mL Strep) were purchased from Mediatech Inc. (Manassas, VA). Transwell filter plates (12 well, 0.3 µm pore size) and T-75 flasks were purchased from Fisher Scientific Inc. (Pittsburg, PA). Caco-2 cells were obtained from the Audus Lab at the University of Kansas (Lawrence, KS).

Analyses were carried out on an HP 1050 LC system operated with Agilent Chem Station® Software. A Zorbax C¹⁸ (5 mm by 2.1 mm, 5µm particle size) column was used for HPLC analysis. UV was observed at 258 nm and the method used is as described in Chapter 3 section 3.2.3.

1.2.2. Caco-2 Permeability Assay:

1.2.2.1. Media preparation.

A ten percent fetal bovine serum (FBS) DMEM caco-2 media was used for the culture of all caco-2 cells. To prepare 500 mL of the FBS-DMEM Caco-2 media solution, 50 mL of FBS (heat inactivated, 10 %) was filtered through 500 mL cellulose acetate filter with 5.5 mL non-essential amino acids (1%) and five microliters of 100 units of penicillin/100 µg/ml streptomycin (1%). A 190 mL aliquot of buffered DMEM was added, followed by 250 mL of unbuffered DMEM to give a final volume of 500 mL.

1.2.2.2. Cell culture.

A cryogenic tube containing caco-2 cells was placed in a water bath at 37°C to thaw the cells. After several minutes, the cryogenic tube containing the thawed cells was sterilized in 70% ethanol before pipetting cells into a T-75 flask containing 13 mL of caco-2 media. The cells were

grown in an incubator at 37°C in an atmosphere of 5% CO₂. The media was changed every other day and the cells were passed into a T-175 flask after the cells became 80% confluent (approximately 6 days). The media was aspirated from the cells, washed twice with a 10 mL PBS at 37°C, and treated with 10 mL of 0.25% trypsin containing 1 mM EDTA for 30 seconds. Most of the trypsin was aspirated leaving approximately 1 mL in the flask. The flask was then incubated for about five to eight minutes until cells detached from the surface of the flask. The trypsin was then deactivated with 10mL of Caco-2 media. One hundred microliters of the suspension was pipetted into an eppendorf tube and diluted with an equal amount of Trypan blue. The cells were then counted on a hemocytometer under a microscope and counted. After counting the cells the cells suspension in the T-75 flask was diluted to a cell count of 1.8 x 10⁵ cells/mL based on calculations from Eq. 1 to Eq. 3. The cells were then subcultured into 12 well transwell plates as indicated in figure 5.2 below.

$$\left[\begin{array}{c} \text{Average} \\ \text{Number of} \\ \text{Cells} \end{array} \right] \times 10^4 \times \left[\begin{array}{c} \text{Dilution} \\ \text{Factor} \end{array} \right] = \left[\begin{array}{c} \text{Cells/mL} \end{array} \right] \quad \text{Eq. 1}$$

$$\left[\begin{array}{c} 1.8 \times 10^5 \\ \text{cells/min} \end{array} \right] \times \left[\begin{array}{c} \text{Volume desired} \\ \text{for plating} \end{array} \right] \div \left[\begin{array}{c} \text{Cells/mL} \end{array} \right] = \left[\begin{array}{c} \text{Volume of cell} \\ \text{suspensions} \end{array} \right] \quad \text{Eq. 2}$$

$$\left[\begin{array}{c} \text{Volume added} \\ \text{to dilution} \end{array} \right] - \left[\begin{array}{c} \text{Volume of cell} \\ \text{suspensions} \end{array} \right] = \left[\begin{array}{c} \text{Volume of media added} \\ \text{to cell suspensions} \end{array} \right] \quad \text{Eq. 3}$$

1.2.2.3. *Sub culturing cells*

The cells were sub cultured every seven days at a density of 1.80 x 10⁵ cells/mL. Twelve millimeter transwell filters with a pore size of 3.0µm (12 well plates, figure 5.2) were used for all

sub culturing of cells. One microliter of Caco-2 media was added to the basolateral side followed by the addition of 0.5 mL of cells suspension to the apical side. The cells were then grown in an incubator at 37°C with a 5% CO₂ atmosphere for 21 days. The media was changed every other day for the first week and once every day for the remaining two weeks.

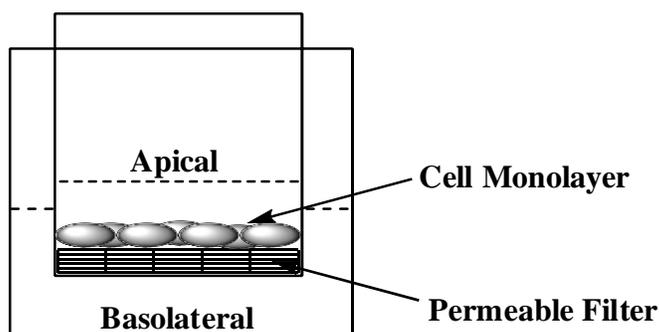


Figure 5.2: Transwell setup of Caco-2 cell monolayer permeability study. In the Apical (AP) to basolateral (BL) studies, the prodrug was added to the apical side and in the basolateral to apical, the prodrug was added to the basolateral side.

1.2.2.4. Permeability Experiments

All experiments were carried out in triplicate for both AP to BL and BL to AP directions at 40 μ M starting concentrations. The prodrugs were first dissolved in dimethyl sulfoxide and added to a 10 mL solution of HBSS to give a final concentration of 40 μ M. The percentage of DMSO in the final solution was 0.25%. The sample solution in HBSS was then filtered through 0.22 μ m syringe filters prior to use in the permeability studies. All caco-2 cell monolayers used were approximately 22 days old. The cells were washed three times with warm PBS and preconditioned with HBSS pH 7.4. For Apical-basolateral direction studies, HBSS on the apical side was aspirated and replaced with 0.5 mL of 40 μ M sample solution, and for basolateral-apical

direction, HBSS on basolateral side was replaced with 1.0 mL of 40 μ M sample solution. The plates were kept incubated at 37°C and placed on a plate shaker at 65 rpm. Samples of 100 μ L were removed from both the apical and basolateral sides at 5, 15, 25, 35, 45, 60, 75, 90, 120, 150 and 180 minutes. One hundred microliters of HBSS were added to the receiver compartment and a 100 μ L of 40 μ M sample solution was added to the donor compartment after samples were removed at each time point. Samples were diluted with 100 μ L of N-methylmaleimide solution (1.0 mg/mL) in micro centrifuge tubes, centrifuged at 13000 rpm for five minutes and analyzed by HPLC. Samples waiting to be analyzed by HPLC were kept in the refrigerator at 5°C. All experiments were conducted in an incubator at 37°C unless otherwise stated. Bidirectional studies were performed at the same age, passage number and date.

1.2.2.5. Control studies.

Control studies were carried out using 40 μ M linezolid for both the AP to BL and BL to AP direction. Also a control study was run using 40 μ M of the prodrugs in fresh HBSS solution at pH 7.4 and at 37°C. The latter control was run to determine the stability of the prodrugs in the absence of the cell monolayers.

1.3. Results and Discussions

1.3.1. Cell transport studies

A control study involving the AP to BL and BL to AP transport of linezolid, was carried out to determine the relative effects of the prodrugs on the permeability characteristics of the parent linezolid. The previous transport studies using MDCK cell monolayers were carried out prior to studies performed to understand the mechanistic breakdown of the sulfenamide prodrugs

in the presence of small molecule thiols and thiol containing proteins (discussed in chapters 3 and 4). As a result the MDCK cell transport study did not generate enough data points to be able to observe and account for the rapid degradation of the sulfenamide prodrugs prior to permeating the cells. The studies carried out in chapters 3 and 4 provided key information on the rate and mechanistic break down of the sulfenamide prodrugs and allowed for a more carefully designed permeability study using Caco-2 cell monolayers.

1.3.2. Linezolid

A plot of the concentration of linezolid in the receiver compartments from both the AP to BL and BL to AP studies with respect to time (Figure 5.3) resulted in linear fits with slopes of 1.88×10^{-8} M/min for the AP to BL study and 4.85×10^{-8} M/min for the BL to AP study. From Eq. 4 and the slopes of the plots, the apparent permeability (P_{app}) of linezolid in both directions was determined.

$$P_{app} = \frac{\left(\frac{dC_r}{dt}\right)(V_r)}{(C_d)_0(A)} \quad \text{Eq. 4}$$

Where P_{app} is the apparent permeability of the linezolid, V_r , A and $(C_d)_0$ corresponds to the volume of the receiver compartment, the surface area of the transwell permeable support and the initial concentration of linezolid added to the donor compartment respectively.

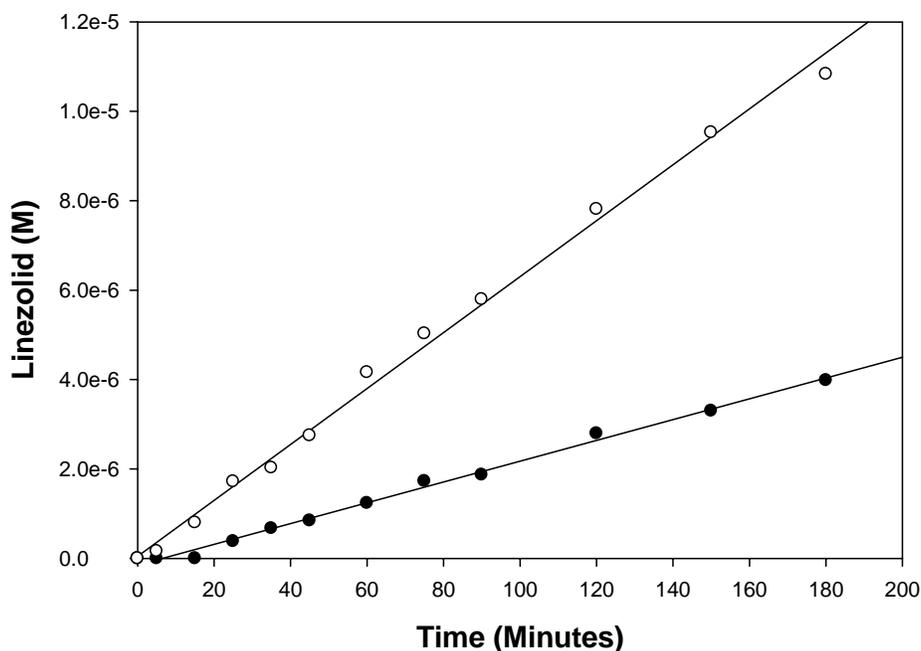


Figure 5.3: Plot of linezolid concentration with respect to time in the receiver compartment from caco-2 cell monolayer transport studies of linezolid. The straight line is a linear fit of the data for the AP to BL study (●) and the BL to AP study (○). Studies were carried out in HBSS at pH 7.4 and at 37°C

The apparent permeability of linezolid for the AP to BL direction ($P_{app, A-B}$) was found to be slightly lower at $6.9 (\pm 0.2) \times 10^{-6}$ cm/sec, than the apparent permeability for the BL to AP direction ($P_{app, B-A}$), which was $9.02 (\pm 0.2) \times 10^{-6}$ cm/sec. Both values obtained were slightly lower than previous values obtained in a transport study using MDCK cell monolayers. The previous study determined apparent permeability to be $8.96 (0.4) \times 10^{-6}$ cm/sec for the AP to BL direction and $1.06 (0.02) \times 10^{-5}$ cm/sec for the BL to AP direction.⁸ The slight differences can be attributed to differences associated with the MDCK and Caco-2 cell monolayers. A ratio of greater than one for the BL to AP versus the AP to BL study suggests an efflux of linezolid back

into the apical side. However, a ratio of approximately unity suggests that the permeability of linezolid does not involve an efflux mechanism.^{2,11,12}

1.3.3. Compound 5.1 and 5.2

When the permeability of compounds **5.1** and **5.2** were performed, neither **5.1** nor **5.2** were detected in the receiver compartments from the AP to BL and BL to AP directional transport studies. Control studies of the stability of **5.1** and **5.2** in HBSS buffer solutions at pH 7.4 and 37°C resulted in no observable loss of prodrugs after 180 minutes. The control study in addition to data obtained and explained in chapter 3 and 4, confirms the fact that the sulfenamide prodrugs were probably degrading in the permeability studies, in the presence of free thiol species probably present on the surface of the cells. The varying concentration of free thiol species on both the apical side versus the concentration on the basolateral side is responsible for differences in the observed degradation rates of the sulfenamide prodrugs on both the apical and basolateral sides. A higher concentration of free thiol species on the apical side results in much faster degradation reactions of the sulfenamide prodrugs occurring at the apical side compared to the basolateral side.

In the study using **5.1**, no prodrug was detected in the donor compartments after approximately 35 minutes (estimated $t_{1/2} = 8$ minutes) for the AP to BL study as shown in figure 5.4, and in the BL to AP direction, no prodrug was detected in the donor compartment after 120 minutes (estimated $t_{1/2} = 18$ minutes) as shown in figure 5.5.

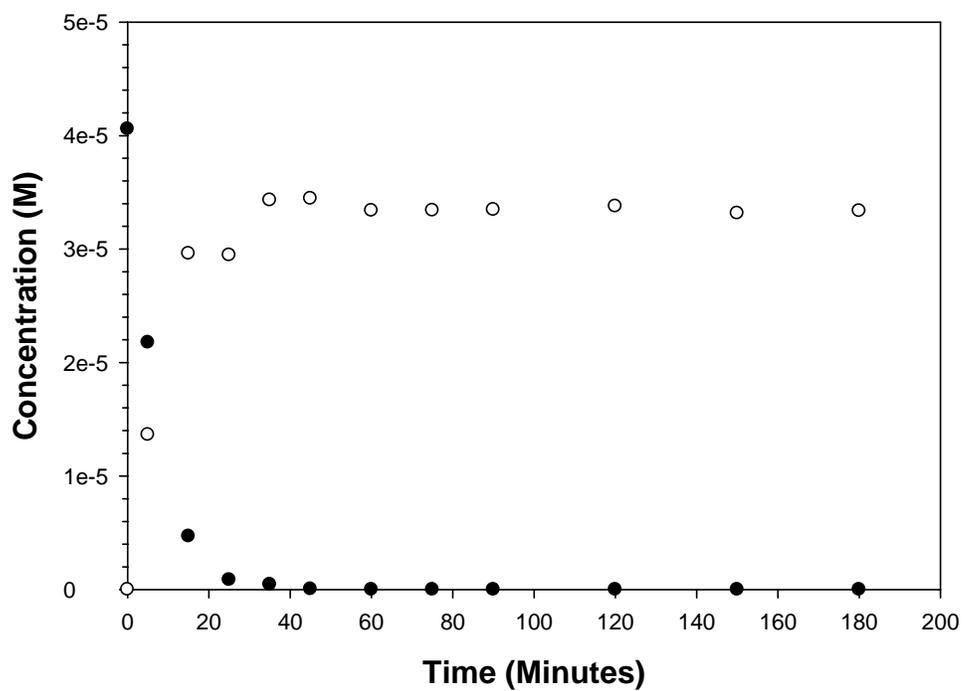


Figure 5.4: Plot of donor compartment concentrations of **5.1** (●) and linezolid (○) generated with respect to time in the AP to BL direction study of **5.1** in Caco-2 cell monolayers. Studies were carried out in HBSS at pH 7.4 and at 37°C

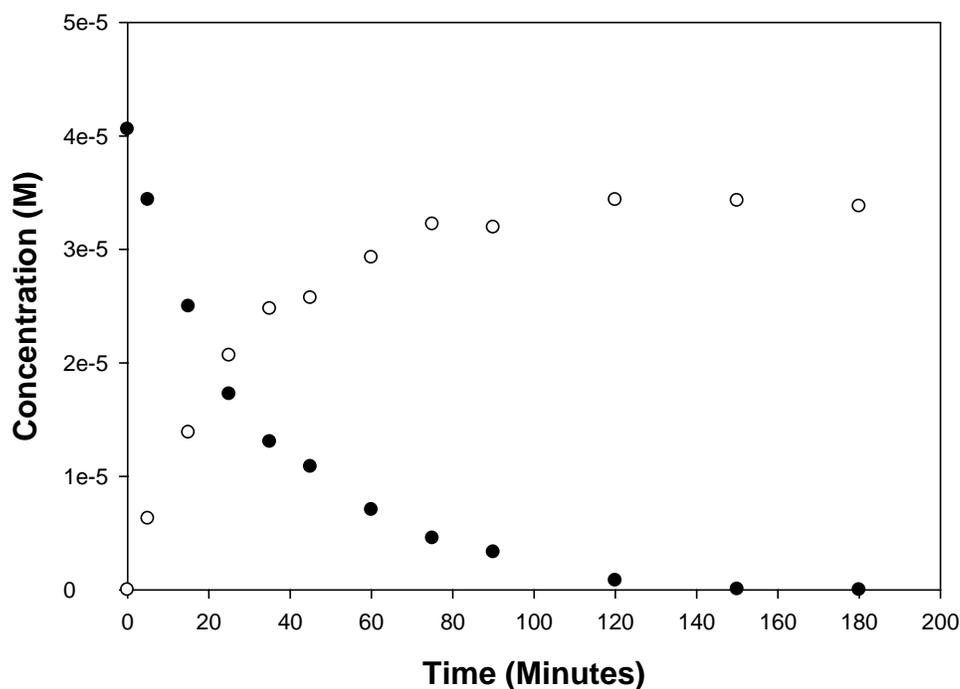


Figure 5.5: Plot of donor compartment concentrations of **5.1** (●) and linezolid (○) generated with respect to time in the BL to AP direction study of **5.1** in Caco-2 cell monolayers. Studies were carried out in HBSS at pH 7.4 and at 37°C

A plot of the concentration of the linezolid observed in the receiver compartments from the caco-2 cell permeability study with **5.1** results in a linear fit for the post 35 minute data for both the AP to BL and the BL to AP study. The slopes from the linear fits are used to estimate the apparent permeability (P_{app}) of the linezolid released from **5.1** using equation 4. The apparent permeability for linezolid released from **5.1** in the AP to BL study ($P_{app\ A-B}$) was estimated to be $7.56 (\pm 0.12) \times 10^{-6}$ cm/sec and the $P_{app\ B-A}$ for the BL to AP direction was estimated to be $8.98 (\pm 0.18) \times 10^{-6}$ cm/sec.

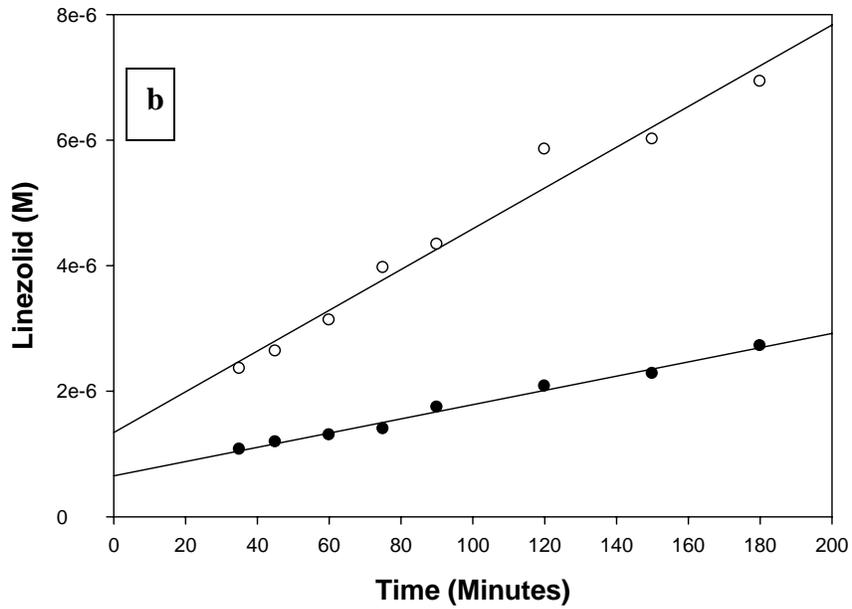
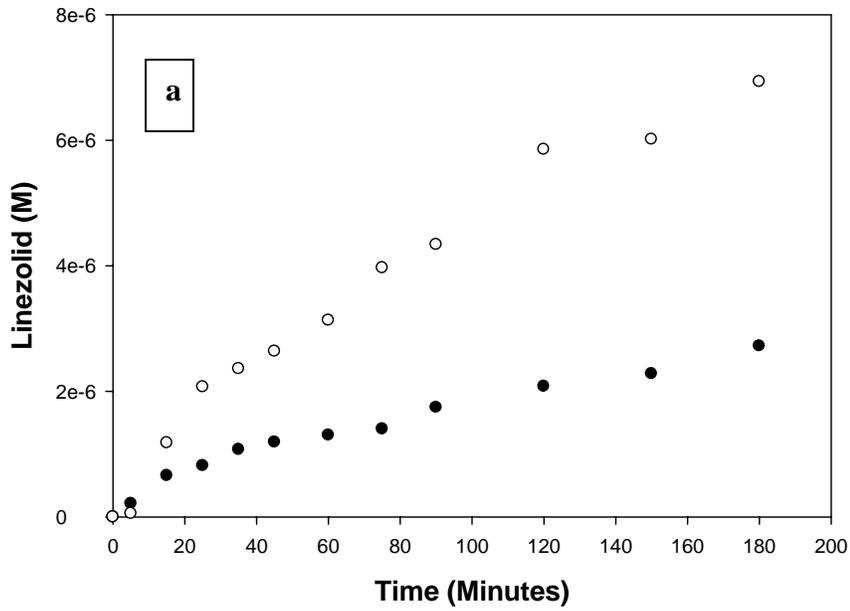


Figure 5.6 a & b: Plot of receiver compartment concentrations of linezolid with respect to time in the AP to BL direction (●) and the BL to AP (○) direction for the transport study of **5.1** at pH 7.4 and at 37°C.

A comparison of the P_{app} values estimated from the study of **5.1** and the P_{app} values estimated from the linezolid control studies show a slight improvement in the permeability of linezolid released from **5.1** in the AP to BL study, but no improvement in the permeability of linezolid released from **5.1** in the BL to AP study as shown in figure 5.7.

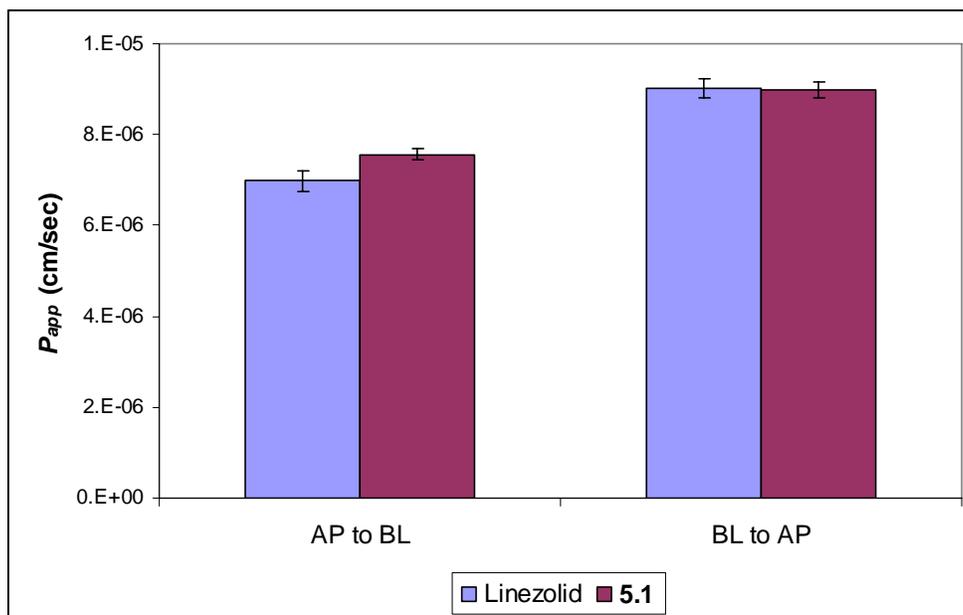


Figure 5.7: Comparison of the apparent permeability of linezolid from control study and linezolid released from the study of **5.1** in AP to BL and BL to AP study of **5.1**

From figure 5.4 and 5.5, there is no prodrug present in the donor compartments after 35 minutes for the AP to BL study and after 120 minutes for the BL to AP study. The final permeability at 180 minutes can be assumed to be as a result of the permeability of only linezolid. To determine if the prodrug is having any effect on the permeability of linezolid, the permeability values have to be estimated or calculated when there is still prodrug present in the donor compartment. For both AP to BL, there is still prodrug present in the first 35 minutes where as in

the BL to AP studies, there is prodrug still present in the first 120 minutes. To make an effective comparison, the P_{app} was estimated for the first 35 minutes using plots as shown in figure 5.8, for both AP to BL and BL to AP directions,.

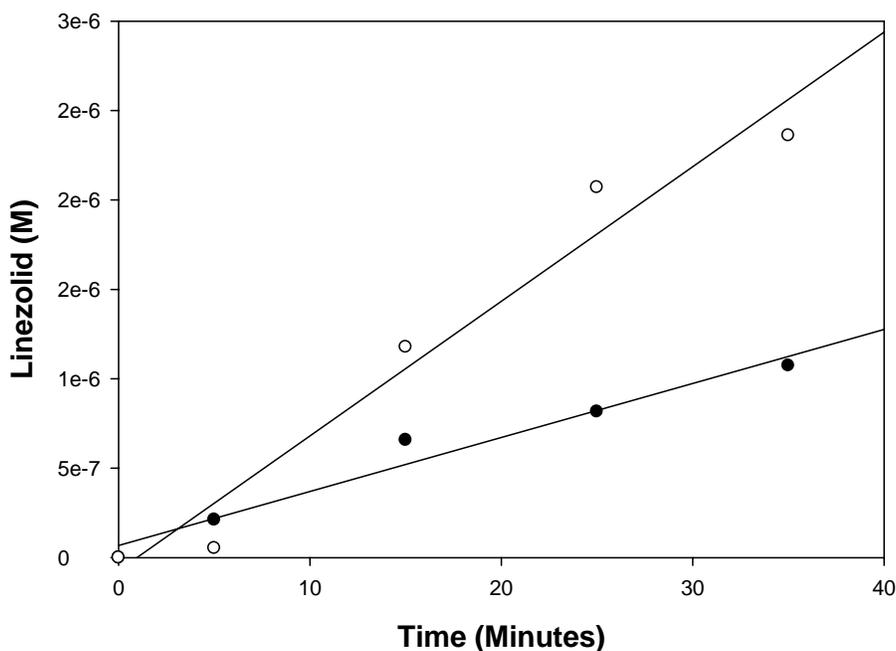


Figure 5.8: Plot of initial linezolid concentration (first 35 minutes) in the receiver compartment for AP to BL (●) and BL to AP (○) transport study of **5.1**.

An estimation of the P_{app} for the first 35 minutes show a two fold increase in the permeability of linezolid released from **5.1** for both the AP to BL and the BL to AP study (figure 5.9). The increase in the P_{app} values indicated that the sulfenamide prodrug contributes to an improvement in the permeability of linezolid. However, it should be noted that the apparent permeability values obtained for the first 35 minutes data underestimates the true permeability effect the sulfenamide prodrug has on the permeability of linezolid. This is due to the fact that a

significant amount of the prodrug, if not all the prodrug converts to linezolid within the 35 minute timeframe.

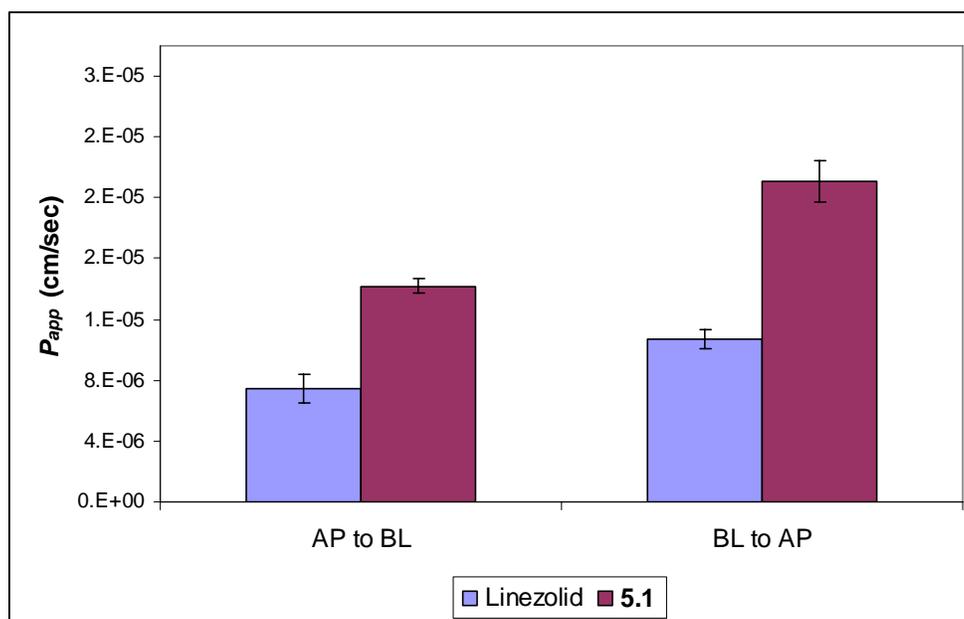


Figure 5.9: Comparison of the initial permeability (after 35 minutes) of linezolid from control study and linezolid released from **5.1** in AP to BL and BL to AP study of **5.1**.

In the study of **5.2**, no prodrug was detected in the donor compartment of the AP to BL study after 120 minutes (estimated $t_{1/2} = 24$ minutes) as shown in figure 5.10. On the other hand, after 180 minutes, approximately 2.5 % of **5.2** was still remaining in the donor compartment in the BL to AP direction study (estimated $t_{1/2} = 33$ minutes, figure 5.11), indicating that the degradation of **5.2** was occurring a lot faster on the AP side compared to the BL side. The faster reaction rates observed on the apical side leads to the conclusion that there is a higher concentration of free thiol species present on the apical side as opposed to the basolateral side.

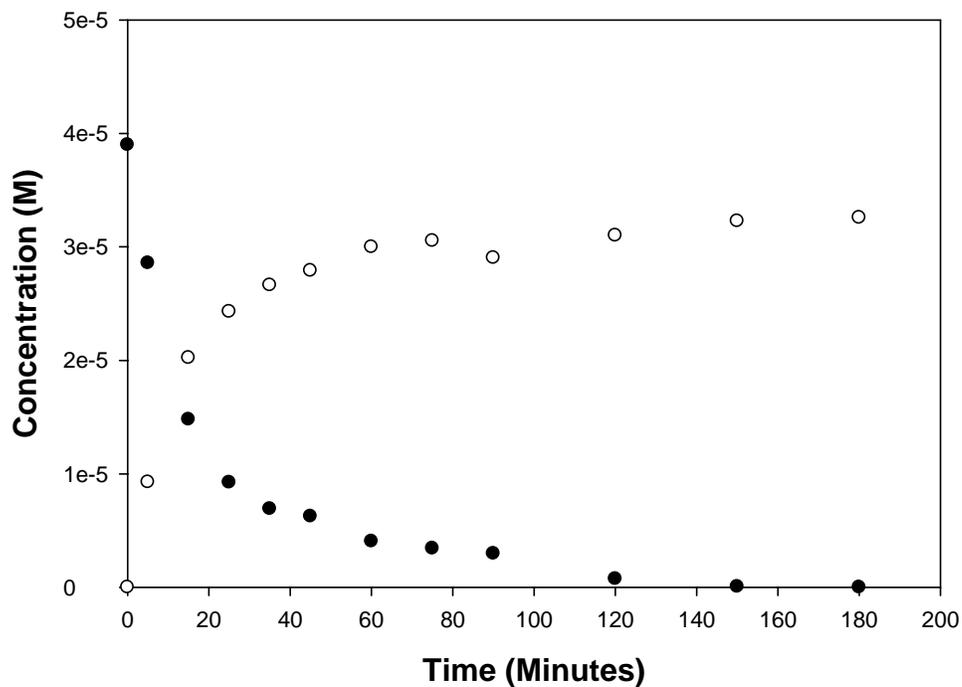


Figure 5.10: Plot of receiver donor compartment concentrations for the AP to BL study of **5.2** (●) and linezolid (○) with respect to time in the Caco-2 cell monolayer. Studies were carried out in HBSS at pH 7.4 and at 37°C.

The concentration versus time curved of the permeability studies of **5.2** did not yield in linear fits for all the data generated in 180 minutes as shown in figure 5.12a. As a result the terminal slope (a linear fit for the post 60 minute data, figure 5.12b) was used to estimate the P_{app} values of $6.57 (\pm 0.22) \times 10^{-6}$ cm/sec for the AP to BL study and $7.46 (\pm 0.09) \times 10^{-6}$ cm/sec for the BL to AP study.

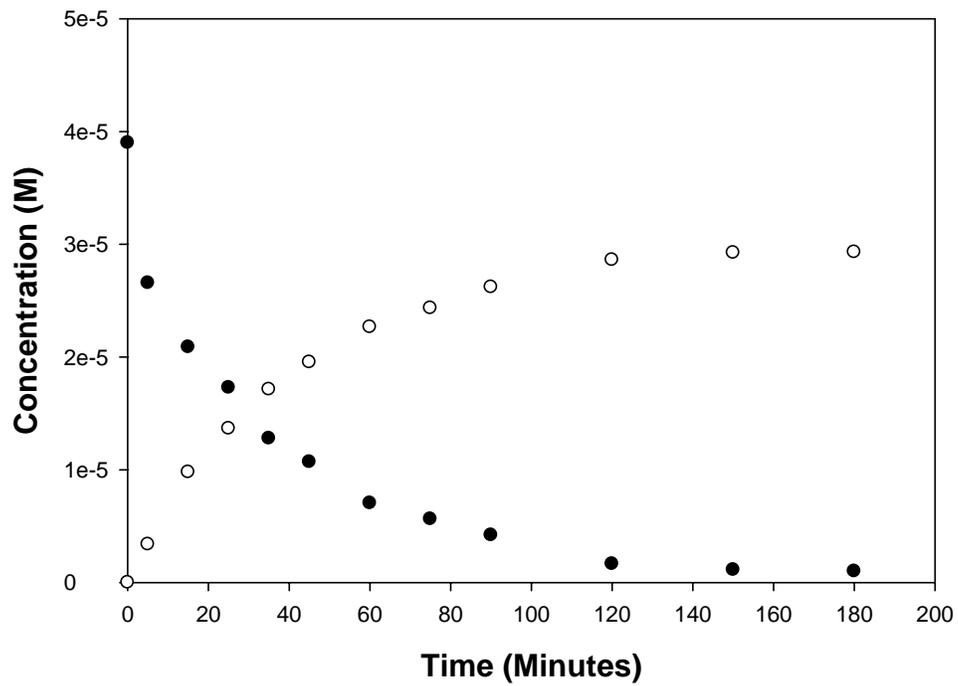


Figure 5.11: Plot of donor compartment concentrations for the BL to AP study of **5.2** (●) and linezolid (○) with respect to time in the Caco-2 cell monolayer. Studies were carried out in HBSS at pH 7.4 and at 37°C

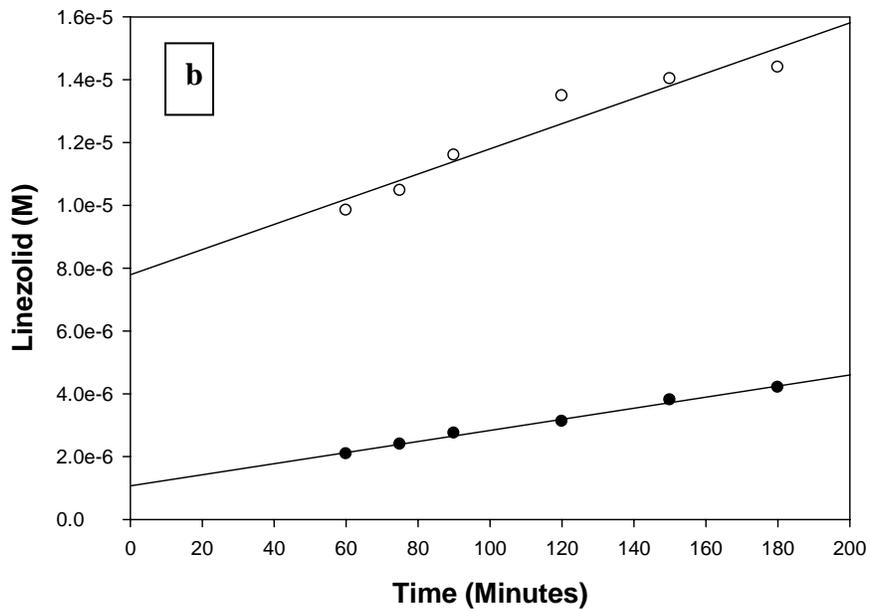
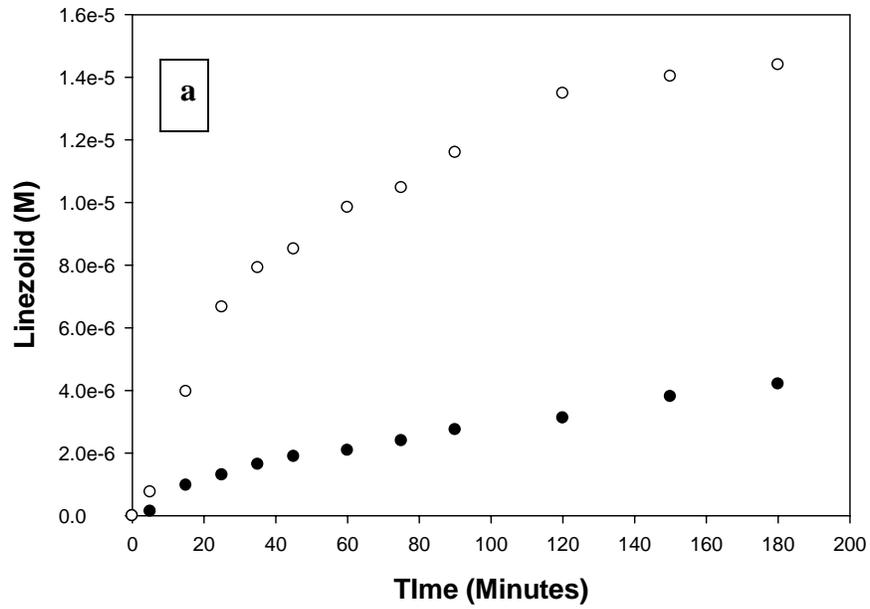


Figure 5.12 a & b: Plot of post 60 minute data for the receiver compartment concentrations of linezolid with respect to time in the AP to BL direction (●) and the BL to AP (○) direction for the transport study of **5.2** at pH 7.4 and at 37°C

A comparison of the estimated P_{app} values with the values obtained from the linezolid control study indicate no effect on the permeability of linezolid released from **5.2** compared to the linezolid control values. (See figure 5.13). In other words the permeability values estimated is consistent with the permeability of only linezolid from the donor compartment to the receiver compartment. No effect on the permeability of the linezolid is observed to occur from the sulfenamide prodrug due to the fact that all the prodrug converts to linezolid.

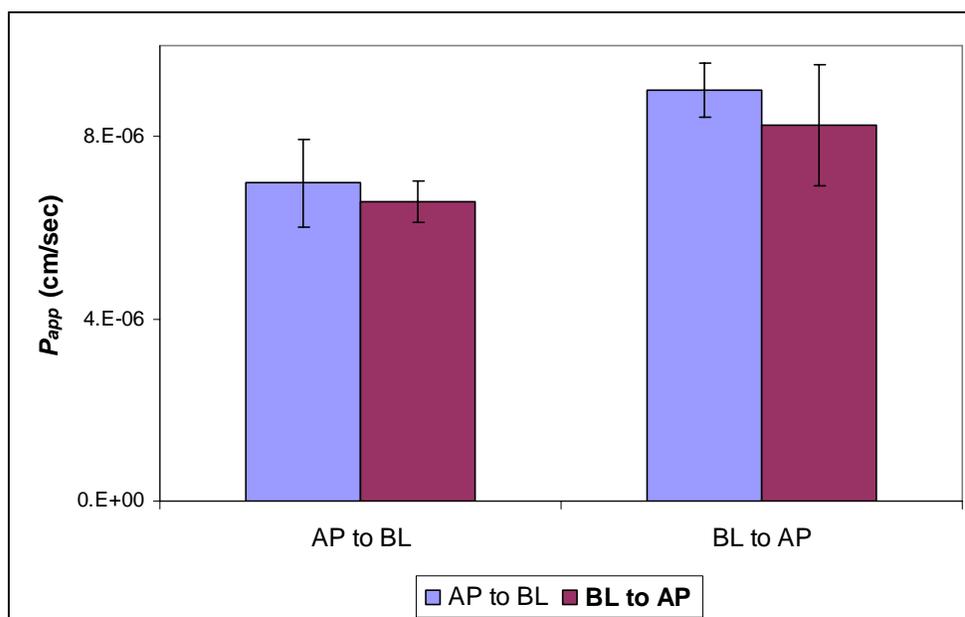


Figure 5.13: Comparison of the apparent permeability of linezolid from control study and linezolid released from the study of **5.2** in AP to BL and BL to AP study of **5.2**

A comparison of the initial P_{app} values estimated from linear fits of the permeability data from the first 35 minutes (figure 5.14), shows a three fold increase in the permeability of linezolid

released from **5.2** for the AP to BL study and a six fold increase in the permeability for the BL to AP study (see figure 5.15).

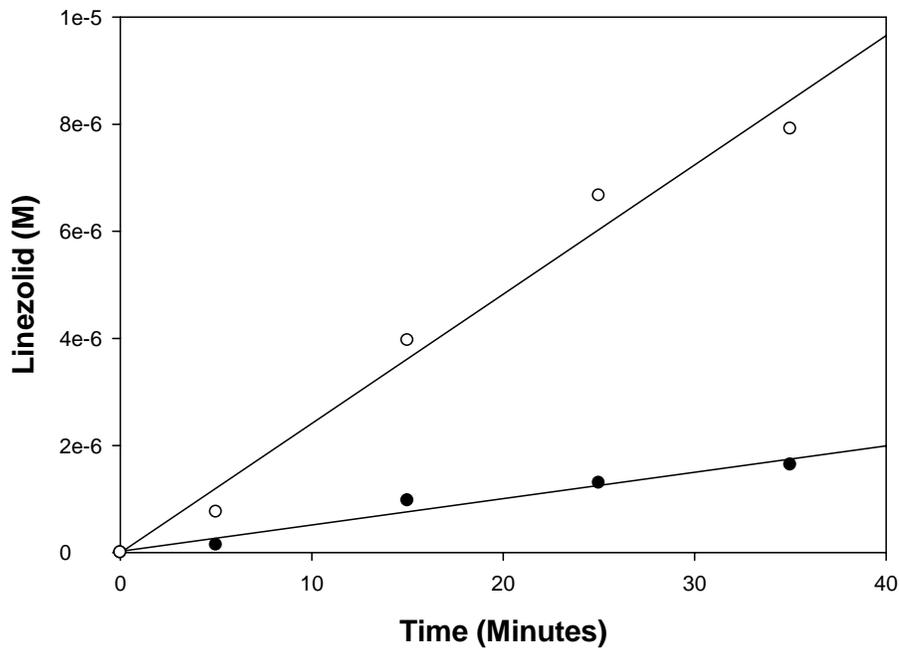


Figure 5.14: Plot of initial linezolid concentration (first 35 minutes) in the receiver compartment for AP to BL (●) and BL to AP (○) transport study of **5.2**.

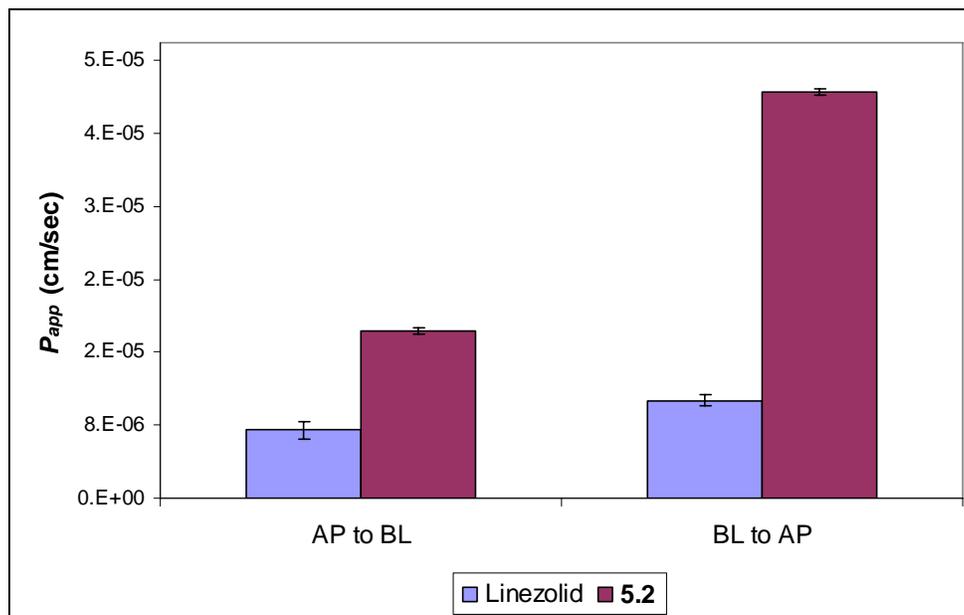


Figure 5.15: Comparison of the initial permeability (after 35 minutes) of linezolid from control study and linezolid released from **5.1** in AP to BL and BL to AP study of **5.1**.

As mentioned earlier, the initial permeability values underestimate the sulfenamide prodrugs' effect on the permeability of linezolid, but suggest that the sulfenamide prodrugs are contributing to improving the permeability of linezolid. Even though the prodrugs are not observed in the receiver compartment, it is expected that some of the prodrugs permeate the cell monolayer and convert to the parent drug within the cells, which then permeates into the receiver compartment as shown in figures 5.16 and 5.17. The overall effect of the prodrug on the permeability of the parent molecule is therefore limited by the rate of conversion of the sulfenamide prodrugs on the surface of the cells and within the cells to release the parent linezolid. The rate of conversion to the parent linezolid is determined by the concentration of the free protein thiols present on the surface of the cells and also within the cell.

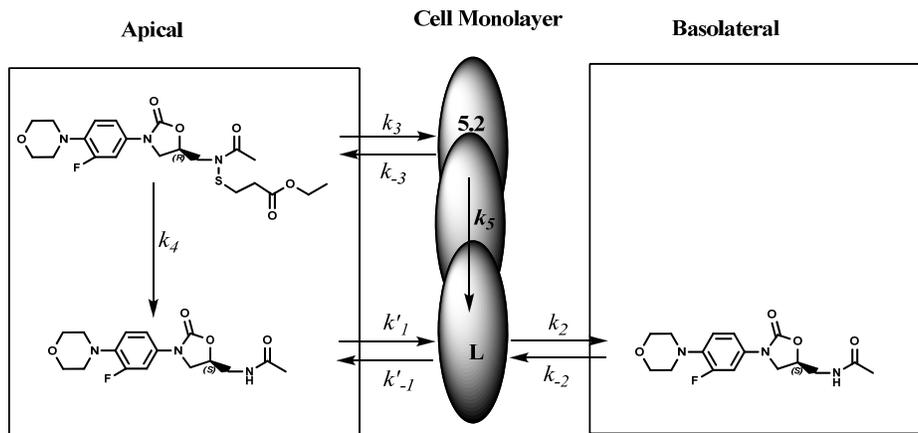


Figure 5.16: Illustration of the effect of sulfenamide prodrugs on the AP to BL permeability of Linezolid.

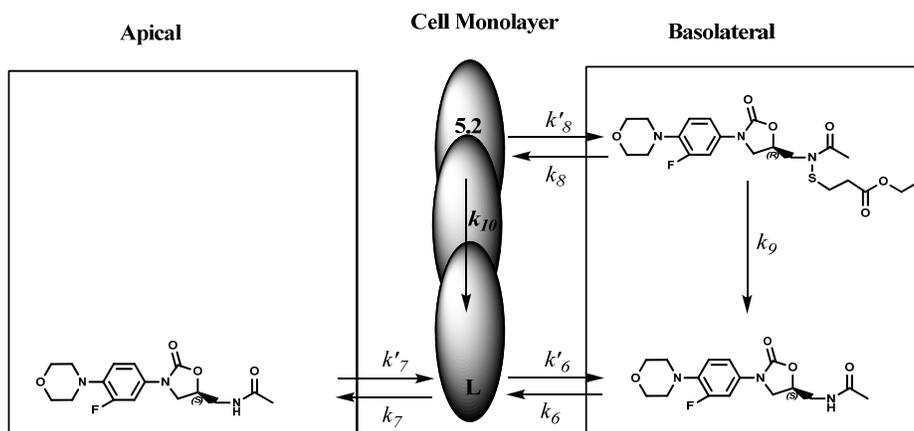


Figure 5.17: Illustration of the effect of sulfenamide prodrugs on the AP to BL permeability of Linezolid.

1.4. Conclusions

The permeability characteristic of sulfenamide prodrugs of the oxazolidinone antimicrobial agent - linezolid has been evaluated. Due to intracellular free thiol species and free thiol species found on the surface of the cells, the sulfenamide prodrugs degraded and were not detected in the receiver compartments. However, analysis of the data shows that the sulfenamide prodrugs have the potential to improve the permeability of the parent molecule. The permeability of the sulfenamide prodrugs is however limited by the concentration of the free thiols species on the cells and in the cells. In other words, the permeability of the sulfenamide prodrugs is limited by the rate of degradation of the sulfenamide in the presence of the free thiol species. The slower the degradation rate, the better the effect on the permeability of the parent drug. However, the instability of the sulfenamide prodrug in the presence of thiol species will limit the ability of the sulfenamides to be used as higher permeating prodrugs.

A recent study by Ren and Lien concluded that compounds with permeability equal to or greater than 5.0×10^{-6} cm/sec, have human GI absorption from 50 to 100%.¹³ All the estimated permeability values from the prodrugs studied fall above this value, suggesting that the prodrug will have a positive impact on the permeability and absorption characteristics of the parent molecules; however, it should be noted that absorption is not only dependent on good permeability. Other factors that play a role in the permeability of drugs include solubility, dissolution rates in the GIT, stability, molecular weight, and lipophilicity to mention a few. In the case of the sulfenamide prodrugs, the factor that plays a key role is the concentration of free thiol species in the GIT. A higher free thiol concentration means a rapid cleavage of the N-S bond to release the parent molecule, leading to no significant change in permeability and hence no effect on absorption. In the case of compounds with low permeability and low solubility, this could

result in the precipitation of the parent compound upon the fast release of the parent molecule from the prodrugs due to the cleavage of the N-S bond in the presence of the endogenous free thiol species.

On the positive side, these studies suggest that water soluble sulfenamide prodrugs of drugs with poor solubility but good permeability may prove useful. For example a water-soluble sulfenamide prodrug, on dissolution, could be cleaved by the free thiols in the GIT to quantitatively release the permeable parent drug, thus overcoming the slow dissolution rate of the parent drug.

5.4. References

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Chapter 6

Overall Summary, Conclusions and Future Work

6.1. Conclusions

The overall objective of this dissertation was to study sulfenamide derivatives of amide type -NH acids and evaluate their use as potential prodrugs. Specifically this dissertation focused on developing and studying sulfenamide prodrug models of antibacterial oxazolidinones. Our aim was to study the stability characteristics of sulfenamide prodrugs of amide type -NH acids under aqueous conditions and to understand the likely mechanism of breakdown of the sulfenamide prodrugs in the presence of free thiol species, which will serve as a predictor for the *in vivo* stability characteristics of the prodrugs. Finally this dissertation aimed at understanding the contribution of sulfenamide prodrugs to improving the permeability characteristics of amide type -NH acids.

Prodrug syntheses have been used in the enhancement of the physicochemical properties of drug candidates or molecules, especially for -NH acid molecules. However, there has been a void in the development of prodrugs for amide type -NH acids.¹⁻⁴ The -NH proton of the amide group of amide type -NH acids is a key hydrogen bond donor, which when removed, results in changes or disruption in physical properties such as the crystal lattice packing, melting point and solubility behavior.^{1,2} By replacing the -NH proton with an appropriate promoiety, it is possible to selectively modify the physicochemical properties of the parent molecule, such as solubility and lipophilicity. The application of sulfenamides as potential prodrugs provides an alternative prodrug approach to improving the drug properties of amide type -NH acids.

To successfully apply sulfenamides as an effective prodrug approach, the following criteria have to be met⁵⁻¹⁰

1. Successful synthesis of the prodrug molecules using a variety of promoieties.
2. The prodrug must exhibit adequate chemical and physical stability.

3. The prodrugs must possess enhanced properties relative to the parent drug molecule.
4. The prodrug must display the desired conversion back to the parent drug molecule in vivo.
5. The prodrug and promoiety chosen must exhibit non-toxic characteristics in vivo.

6.1.1. Synthesis and aqueous stability of sulfenamide prodrugs of oxazolidinones.

The synthesis of sulfenamide prodrugs of amide-type -NH acids using thiophthalimides as intermediates showed a significant improvement in the yields when compared to earlier attempts. A number of reasons can be attributed to this improvement. The thiophthalimides can be readily synthesized, isolated and purified prior to use. The purified thiophthalimide intermediates results in much cleaner reactions involving less steps to effectively isolate and purify the sulfenamide prodrugs. The drawback to using thiophthalimides is their instability in polar solvents as they readily breakdown in polar solvents to release phthalimide and a disulfide from the sulfenyl moiety.¹¹⁻¹³

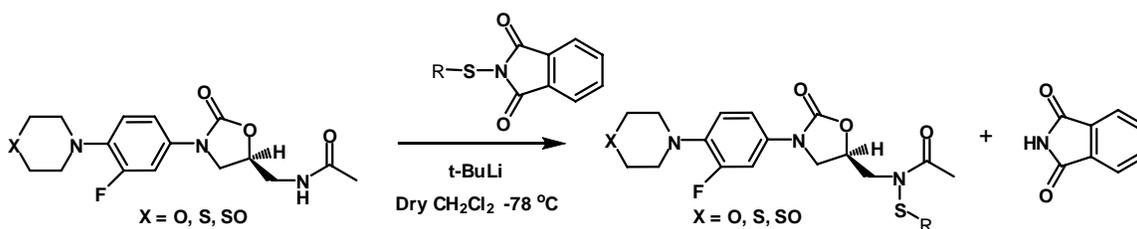


Figure 6.1: Synthesis of sulfenamide prodrugs using thiophthalimide intermediates.

The aqueous stability of the sulfenamide prodrug models with respect to pH from pH 1.1 to pH 7.5 resulted in a U shaped pH rate profile as shown in figure 6.2 with the maximum stability occurring between pH 4.0 and 4.5. The prodrugs degraded by hydrolysis to cleave mostly the N-S bond to release the parent molecule and a sulfenic acid, which further reacts, due to high aqueous instability, to generate sulfonamides as minor products. Estimated half lives and shelf lives of the sulfenamide prodrug models showed insufficient stability under the aqueous conditions studied. This low aqueous stability profile observed for the sulfenamides may limit the use of sulfenamide prodrugs in ready-to-use liquids as their aqueous stabilities fall well below the two year limit set by FDA guidelines. In the future it would be necessary to evaluate the solid state stability for possible formulations in the solid state such as in tablets or as a powder that is suspended in water when ready to administer. Also it would be useful to evaluate the stability of the sulfenamides in lipids for possible development in lipid formulations or in control release lipid capsules.

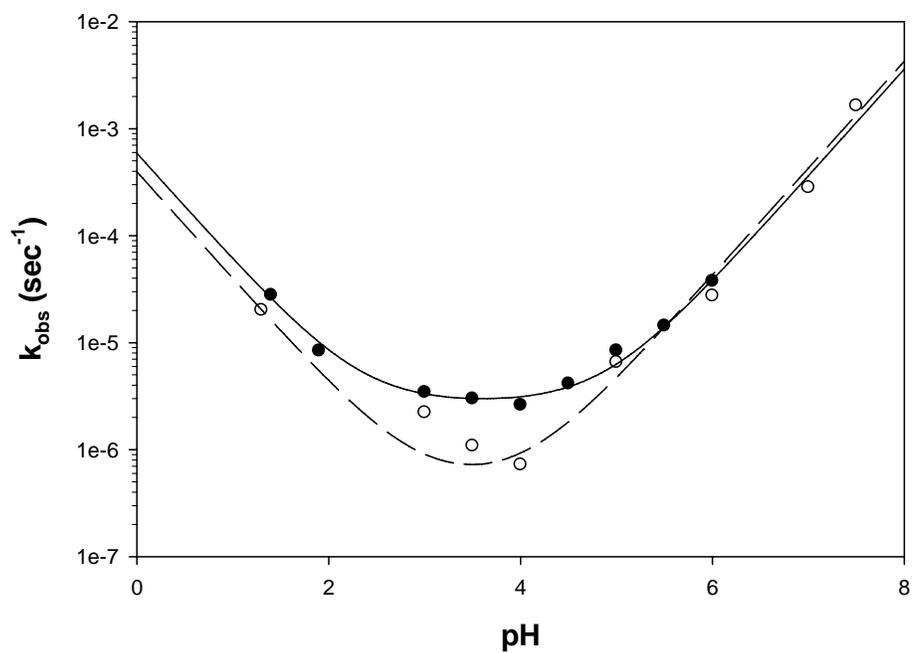


Figure 6.2: Plot of the observed rate constant (k_{obs}) for the loss of sulfenamide prodrug models of antimicrobial oxazolidinones.

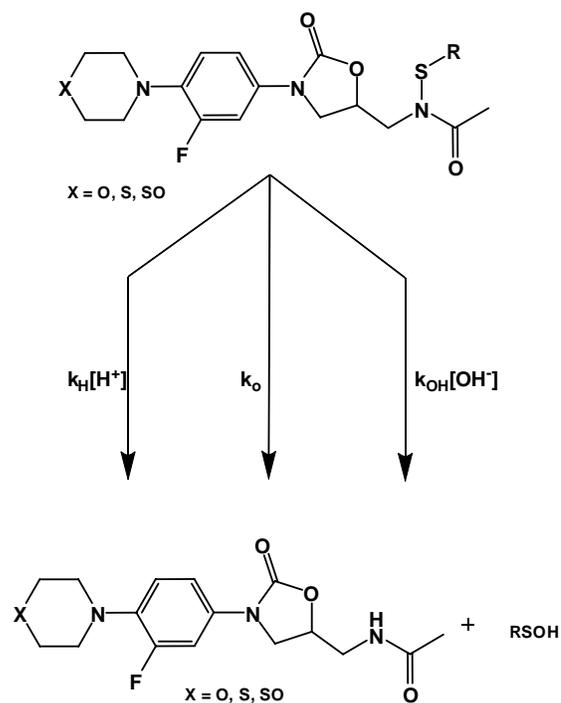


Figure 6.3: Macroscopic rate constants for the degradation of sulfenamide prodrug models of antimicrobial oxazolidinones under aqueous conditions

6.1.2. Understanding the mechanistic breakdown of the sulfenamide prodrugs *in vitro* and *in vivo*

Results from the study of sulfenamide prodrug models in the presence of thiol compounds of varying thiol *pKa* have shown that the thiolate ions are the species in solution that attack the sulfur of the sulfenamide bond leading to the breakdown of the sulfenamide prodrugs to release the parent molecule and a mixed disulfide. The second order rate constants of the reactions were found to be dependent on the dissociation constants of the thiol groups of the reacting species; however, the observed rate constants of the entire reactions were dependent on

both the dissociation constants of the thiol species and the pH of the reaction solution. Hence, it is expected that thiol species *in vivo* with *pKa* values closer to the physiological pH, will cause the cleavage of the sulfenamide bond once the sulfenamides are administered. The most likely *in vivo* small molecule thiol expected to cause the cleavage of the N-S bond is glutathione, with an average *in vivo* concentration of 0.1M in some tissues and cells. At such *in vivo* concentrations, glutathione is expected to cause the spontaneous cleavage of the sulfenamide prodrugs. Further *in vivo* studies have to be conducted to evaluate the conversion of the prodrugs to the parent molecules and the effects it has on the site of administration.

The N-S bond in the sulfenamide prodrugs has been found to be susceptible to nucleophilic attack by thiol species present on proteins. The reactivity is determined by both the *pKa* of the thiol and the accessibility of the prodrugs to the thiol groups on the protein. LCMS analysis of the reaction of the sulfenamide prodrugs with small molecule thiols indicated that a mixed disulfide is formed between the sulfenyl moiety and the thiol species, suggesting a similar disulfide formation between the sulfenyl moiety and protein thiols. The formation of mixed disulfides with protein thiols can lead to conformational changes of the protein that could lead to changes in the activity of the proteins. Also if the thiol species are found in the active site of the proteins, such as in the case of PRL1, this could lead to the inactivation of the entire protein, thus preventing the effective functioning of the protein. Another concern is immunological response from the formation of mixed disulfides as a result of the nucleophilic attack of the sulfenamide prodrugs by the protein thiols. Immunological responses including fever, skin eruptions, hepatotoxicity, and blood dyscrasias have been reported to occur as a result of conjugation of sulfur containing species with functional proteins.¹⁴⁻¹⁸

6.1.3. Contribution of sulfenamide prodrugs to the permeability of antimicrobial oxazolidinone; Linezolid.

The presence of free thiol containing proteins on the surface of Caco-2 cells and possibly within the cells resulted in the rapid degradation of the sulfenamide prodrugs prior to permeating to the receiver compartments of the permeability studies. The rapid degradation prevents the accurate estimation of the apparent permeability of the prodrugs. An evaluation of the linezolid in the receiver compartments under sink conditions suggests an improvement in the permeability of the parent linezolid. The data obtained also suggest that the improvement in the permeability is inversely related to the rate of conversion of the prodrug to the parent molecule. This rate of conversion is dependent on the concentration of the thiol species present on the surface of the cells and inside the cells. Absorption of the prodrug when administered orally will be dependent on the concentration of thiol containing species in the gut.¹⁹

6.2. Future Work

The synthesis of sulfenamide derivatives using thiophthalimides as intermediates proved to be successful, however more work need to be done to synthesize readily soluble sulfenamide derivatives of amide-type -NH acids. Once synthesized, they will allow for the study of the effects of the prodrugs on the solubility and stability of the parent molecules. Also, the low aqueous stability characteristics observed in chapter 2 suggest future studies of the solid state stability characteristics for possible solid state formulations of the sulfenamide prodrugs.

A study carried out by Hemenway *et al*, to evaluate the IV administration of sulfenamide prodrugs of carbamazepine indicated rapid conversion of the prodrug to the parent molecule with very similar pharmacokinetic profiles.^{20,21} However, it will be crucial to evaluate the oral

administration of sulfenamide prodrugs to evaluate their absorption across the intestinal cell wall. The *in vitro* studies of the stability of the sulfenamide prodrugs in the presence of small molecule thiols and thiol containing proteins in addition to the permeability studies in Caco-2 cell monolayers suggests the prodrugs will convert in the GIT; however, a reduced rate of conversion will result in an improvement in the permeability of the parent molecules by the prodrugs. The improvement in the permeability of the parent molecules by the prodrugs needs to be supported by *in vivo* data to determine the success of the sulfenamide prodrugs in improving the absorption of the parent molecule.

Finally, toxicity studies need to be out using a variety of sulfenamide prodrug models with different promoieties to evaluate the possible toxicity effects as a result of conjugation of the sulfenyl moiety with thiol containing proteins and glutathione.

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Appendix A

Preparation, characterization and in vivo conversion of new water-soluble sulfenamide prodrugs of carbamazepine

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Bioorganic & Medicinal Chemistry Letters 17 (2007) 6629–6632

Preparation, characterization and in vivo conversion of new water-soluble sulfenamide prodrugs of carbamazepine

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Abstract—Improved synthetic methods are reported for the preparation of sulfenamide derivatives of carbamazepine (CBZ) for evaluation as prodrugs. These sulfenamide prodrugs were designed to rapidly release CBZ in vivo by cleavage of the sulfenamide bond by chemical reaction with glutathione and other sulfhydryl compounds. Physicochemical characterization and in vivo conversion of a new prodrug of CBZ was evaluated to further establish the proof of concept of the sulfenamide prodrug approach. © 2007 Elsevier Ltd. All rights reserved.

Carbamazepine (CBZ, **1**) is effective in the treatment of partial and generalized tonic-clonic epileptic seizures,¹ and is less sedating and causes less cognitive impairment than other anticonvulsants such as phenytoin, phenobarbital, and primidone.² Unfortunately, CBZ's relatively poor aqueous solubility (120 µg mL⁻¹) hampers parenteral administration,³ and despite the need, there is no commercially available injectable formulation. The poor solubility of CBZ can be addressed to some extent by use of cosolvents and/or surface active agents,⁴ but the effectiveness of these approaches is limited and these additives are undesirable for intravenous (iv) formulations due to the risk of severe allergic reactions and formulation related toxicities.⁵ A water-soluble prodrug of CBZ that could be administered intravenously in a safe and effective formulation would be of considerable medical benefit.

There is a significant void in the literature for prodrug strategies available for weakly acidic NH-acid drugs such as amides, carbamates, and ureas.⁶ A new prodrug approach involving the preparation and evaluation of sulfenamide prodrugs of a variety of NH-acids has recently been reported by Guarino et al.^{6,7} The general

route for the synthesis of these derivatives involved the reaction of the appropriate disulfide with sulfonyl chloride to generate a sulfenyl chloride intermediate, which was then further reacted in situ with the NH-acid to form the sulfenamide derivative. One of the sulfenamide prodrugs synthesized and evaluated was **3**, an *O*-ethyl cysteinyl prodrug of CBZ. While the sulfenyl chloride synthetic route was considered adequate for most of the amine, amide and imide drug, and model compounds studied, the yields for this approach with the urea drug example CBZ were very poor (overall yield 1–2%). It was apparent that improved synthetic methods would be required to further the study of sulfenamide derivatives of CBZ for evaluation as prodrugs.

In this communication, we report improved synthetic methods for the preparation of sulfenamide derivatives of CBZ and physicochemical characterization, and in vivo evaluation of a new water-soluble sulfenamide prodrug. *N*-cysteamine-CBZ (**4**) was designed as a water-soluble prodrug of CBZ to regenerate CBZ in vivo by cleavage of the sulfenamide bond by chemical reaction with glutathione and other endogenous sulfhydryl compounds. A successful in vivo result, supporting the proof of concept of the sulfenamide prodrug strategy, could further the application of this approach to improve the physicochemical properties and/or delivery characteristics of other NH-acidic drugs, including ureas.

Thiophthalimides are well-known sulfur transfer agents,⁸ and were utilized as such here to attach various promoieties to the secondary nitrogen of CBZ. The

Keywords: Prodrugs; Carbamazepine; Sulfenamides; Stability; Glutathione; Solubility; Kinetics.

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reaction of *tert*-butyllithium with CBZ followed by *N*-(phenylthio)-phthalimide produce the sulfenamide *N*-(phenylthio)-CBZ (**2**) in good yield (76%, Scheme 1).⁹ Although not further studied here, compound **2** provides a sulfenyl urea model with a UV-active promoity to further investigate the stability and degradation reactions for this class of compounds.

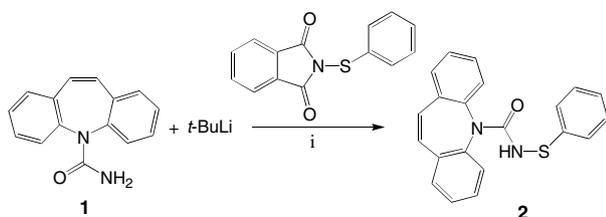
The improvement in synthetic yield of **3** over the sulfenyl chloride method is shown by the reaction of *tert*-butyllithium with CBZ followed by *N*-(Boc-Cys-OEt)-phthalimide, which yielded *N*-(Cys-OEt)-CBZ (**3**) with improved overall yield (55%, Scheme 2) compared to the overall yield using the synthetic method reported by Guarino et al.⁷

N-(Cys-OEt)-CBZ (**3**) is a water-soluble CBZ derivative that likely acts as a prodrug and the stability of **3** was recently characterized in detail.⁷ Cysteamine was selected as the promoity for this in vivo study to avoid any potential complication caused by hydrolysis of the ethyl ester functionality of **3**. The aqueous chemical stability of **4** was also found to be superior to that of **3**. The essential cysteamine derivative of phthalimide, *N*-(Boc-cysteamine)-phthalimide, was prepared by reaction of phthalimide with Boc-cysteamine in the presence of bromine and pyridine (96%, Scheme 3).

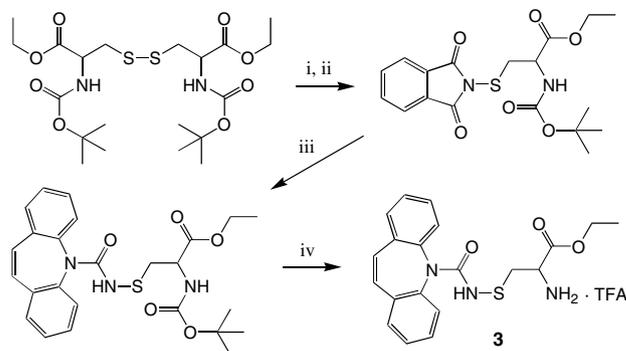
N-(Boc-cysteamine)-phthalimide was then reacted with lithium CBZ to yield *N*-(Boc-cysteamine)-CBZ, followed by reaction with TFA to yield **4** as a white powder in good overall yield (66%, Scheme 4).

The white powder **4** dissolved rapidly in pure water with apparent solubility in excess of 100 mg mL⁻¹ (final pH value of 2.6). The chemical stability of **4** was determined in aqueous solution at various pH values. Figure 1 shows the pH-rate profile for the aqueous degradation of **4** at 70 °C.

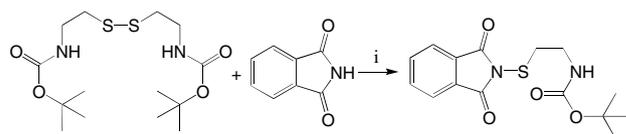
Eq. 1 expresses the observed rate constant (k_{obs}) for the loss of **4** in terms of rate and equilibrium constants as a function of hydrogen ion concentration, or pH where $[\text{H}^+] = 10^{-\text{pH}}$ and $K_w = 1.51 \times 10^{-13}$ at 70 °C.¹⁰ The rates of hydrolytic reactions of the ionized and neutral forms of **4** include acid catalyzed hydrolysis (k_{H}), water hydrolysis (k_{o}), and base catalyzed hydrolysis (k_{OH}) of the protonated form and base catalyzed hydrolysis of the neutral form (k'_{OH}).



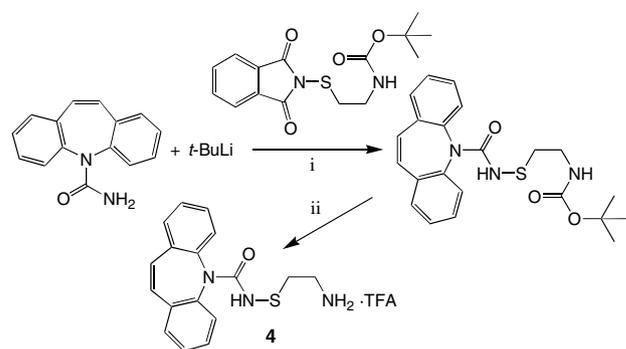
Scheme 1. Synthesis of *N*-(phenylthio)-CBZ (**2**). Reagents and condition: (i) -78 °C, THF, Ar.



Scheme 2. Improved synthesis of **3**. Reagents: (i) SO₂Cl₂, THF; (ii) potassium phthalimide, THF; (iii) deprotonated CBZ (with *t*-BuLi), THF; (iv) TFA, CH₂Cl₂.



Scheme 3. Synthesis *N*-(Boc-cysteamine)-phthalimide. Reagents: (i) Br₂, pyridine, CH₃CN.



Scheme 4. Preparation of **4**. Reagents and condition: (i) -78 °C, THF, Ar; (ii) TFA, CH₂Cl₂.

$$k_{\text{obs}} = \frac{k_{\text{H}}[\text{H}^+]^2 + k_{\text{o}}[\text{H}^+] + k_{\text{OH}}K_w + \frac{k'_{\text{OH}}K_aK_w}{[\text{H}^+]}}{[\text{H}^+] + K_a} \quad (1)$$

The results for k_{obs} from the accelerated stability studies of **4** conducted at pH 4 were $9.39 \times 10^{-6} \text{ s}^{-1}$ for 70 °C, $1.67 \times 10^{-6} \text{ s}^{-1}$ for 60 °C, and $3.96 \times 10^{-7} \text{ s}^{-1}$ for 50 °C. The Eyring behavior was assumed to be linear and the values obtained were: $\Delta H^\ddagger = 143 \text{ kJ mol}^{-1}$ (34.2 kcal mol⁻¹) and $\Delta S^\ddagger = 74 \text{ J mol}^{-1} \text{ K}^{-1}$ ($R^2 = 0.9949$). The ΔH^\ddagger value appears to be relatively high compared to the activation energies reported for the hydrolysis of many other drug compounds.¹¹ The predicted hydrolysis rate (k_{obs}) for the aqueous degradation of **4** extrapolated to 25 °C at pH 4.0 is $3.8 \times 10^{-9} \text{ s}^{-1}$, which corresponds to a half-life ($t_{1/2}$) of 5.7 years and a time for 10% degradation (t_{90}) of 320 days. Extrapolation to 4 °C predicts a k_{obs} value of $4.4 \times 10^{-11} \text{ s}^{-1}$ and a t_{90} value of 75 years indicating that a refrigerated ready-to-use injectable formulation of **4** with a shelf-life of >2 years may be possible. While the time for 10% degradation (t_{90}) appears to be more than adequate

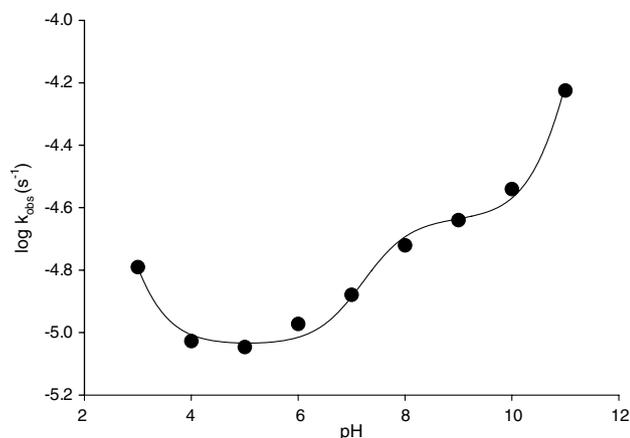


Figure 1. pH-rate profile for the aqueous degradation of **4** at 70 °C. The solid line represents the fit of the data to Eq. 1. The values for the rate constants obtained from the fit of the pH-rate profile were: $k_H = 7.06 \times 10^{-3} \text{ s}^{-1} \text{ M}^{-1}$, $k_O = 9.04 \times 10^{-6} \text{ s}^{-1}$, $k_{OH} = 5.92 \text{ s}^{-1} \text{ M}^{-1}$, and $k'_{OH} = 1.01 \times 10^{-10} \text{ s}^{-1} \text{ M}^{-1}$, and the value obtained for the pK_a of **4** was 7.4.

for this indication, it is likely that the precipitation of insoluble degradation products would be the limiting factor.

A 59 μM solution of **4** in 25 mM isotonic pH 7.4 phosphate buffer was found to rapidly and completely convert to CBZ in the presence of a 10-fold molar excess of L-cysteine at 25 °C. The conversion was nearly instantaneous as the immediate HPLC analysis of this sample showed no detectable **4**. Similarly, it was found that **4** rapidly and completely converted to CBZ in rat whole blood at ambient temperature.

A limited animal study was performed to establish the bioavailability of CBZ following iv administration of **4** in rats. A crossover design was chosen because of the large degree of interanimal variation seen in pharmacokinetics studies with CBZ in rats.¹² Rats were given an iv bolus dose of CBZ control (dissolved in 10% HP- β -CD) followed 48 h later by an equimolar iv dose of **4** in sterile saline or vice versa in a crossover design.¹³ *N*-methylmaleimide was added to drawn blood samples to trap any available sulfhydryl compounds to limit the ex vivo conversion of **4** to CBZ to less than 1.8% during blood sample processing, refrigerated storage, and analysis. The iv bolus dose administration of **4** resulted in the immediate appearance of the peak plasma level of CBZ and no intact **4** was observed in any blood samples. The plasma concentration versus time profiles of CBZ from iv doses of **4** and CBZ control for rat 1 are shown in Figure 2. The area under the curve values for CBZ were 964 $\mu\text{g min mL}^{-1}$ for CBZ from **4** and 959 $\mu\text{g min mL}^{-1}$ for CBZ from control.

A second rat showed a similar plasma CBZ profile from administration of **4**, but only one data point was able to be collected for the CBZ control dose due to failure of the carotid artery from blood clot blockage. Values for the dose amounts of CBZ (D_{CBZ}) and **4** (D_4), AUC values for CBZ from **4** and control, and CBZ C_{max} values

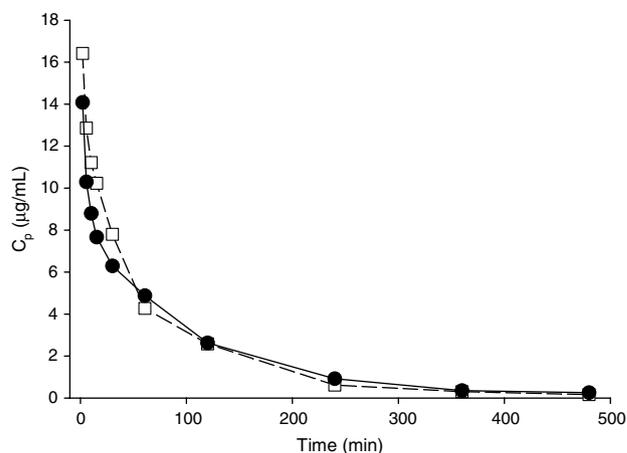


Figure 2. Plasma concentration of CBZ versus time profile following crossover iv administration of **4** (●) and CBZ control (□) in rat 1.

are listed in Table 1. Compound **4** was not detected in any blood or urine samples.

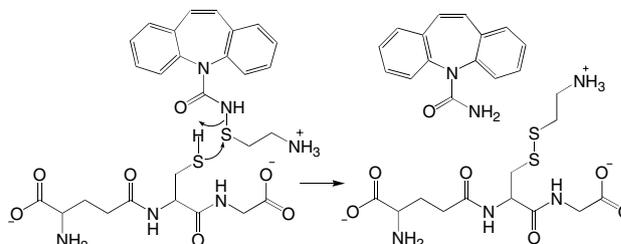
All rats dosed with CBZ and/or **4** did not seem to exhibit any pain or discomfort with the injections. Prior to dosing, rats appeared active, and within minutes following dosing the rats calmed down and went to sleep and were no longer active for several hours. The observed behavior was identical following doses of CBZ and **4**.

In summary, these results show that **4** acts as a prodrug of CBZ and the conversion of **4** to CBZ is rapid and quantitative in vitro and in vivo. This supports the hypothesis that sulfenamide prodrugs release parent NH-acidic drugs by reaction with glutathione and other endogenous sulfhydryl containing compounds (Scheme 5).

Table 1. Values for dose amounts of CBZ (D_{CBZ}) and **4** (D_4), AUC values for CBZ from **4** and control, and peak plasma CBZ concentration (C_{max})

	Rat 1	Rat 2
Rat mass (g)	187	227
D_{CBZ} (mg)	2.54	3.08
D_4 (mg)	4.54	5.50
CBZ AUC _{control} ^a ($\mu\text{g min}^{-1} \text{ mL}^{-1}$)	959	ND
CBZ AUC ₄ ^a ($\mu\text{g min}^{-1} \text{ mL}^{-1}$)	964	545
C_{max} (control) ($\mu\text{g mL}^{-1}$)	16.4	17.4
C_{max} (from 4) ($\mu\text{g mL}^{-1}$)	14.1	16.3

^a Values determined by trapezoidal method.



Scheme 5. Proposed conversion mechanism for reaction of **4** with glutathione to release to CBZ in vivo.

Glutathione activity is known to be high in red blood cells and tissues such as the liver and kidney of rats and humans.¹⁴ The rapid and complete conversion of **4** to CBZ in rat whole blood is consistent with the proposed mechanism of conversion via reaction with glutathione.

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- N*-(phenylthio)CBZ (**2**). ¹H NMR (400 MHz, CDCl₃) δ 5.76 (s, 1H), 6.97 (s, 2H), 7.16–7.27 (m, 2H), 7.28–7.34 (m, 3H), 7.34–7.44 (m, 4H), 7.45–7.51 (m, 2H), 7.52–7.57 (m, 2H).
N-(OEt-Cys)-CBZ (**3**). ¹H NMR (400 MHz, DMSO-*d*₆) δ 1.15 (t, 3H), 2.22–2.36 (m, 1H), 3.03–3.14 (m, 1H), 4.00–4.08 (m, 1H), 4.16 (q, 2H), 6.97 (s, 2H), 7.09 (s, 1H), 7.31–7.38 (m, 2H), 7.39–7.45 (br m, 6H), 8.41 (br s, 3H).
N-cysteamine-CBZ (**4**). ¹H NMR (400 MHz, DMSO-*d*₆) δ 2.74 (t, 2H), 2.89 (q, 2H), 7.02 (s, 2H), 7.06 (s, 1H), 7.33–7.42 (m, 2H), 7.45–7.50 (m, 6H), 7.81 (br s, 3H). The addition of two drops of D₂O to the NMR sample resulted in the disappearance of the peaks at 7.06 and 7.81. HRMS (TOF ES⁺) *m/z* calcd C₁₇H₁₈N₃OS (M+H)⁺ 312.1171, found 312.1155.
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- Animal studies*. Adult male Sprague–Dawley rats (200–227 g) were purchased from Harlan (Indianapolis, IN) with jugular and carotid artery pre-cannulated. Rats were attached to a swivel device allowing the rat free mobility and access to water during dosing and blood sampling experiments, but fasted for 12 h prior to dosing. A 13.58 mg kg⁻¹ CBZ control dose solubilized with 10% HP-β-CD in sterile saline followed by an equivalent dose of **4** (24.30 mg kg⁻¹) in sterile saline (or vice versa) were administered intravenously in a crossover design following a 48-h washout period. Each dose was prepared in excess to provide the specified dose of **1** or **4** in a volume of 1.00 mL. Dosing syringes and needles were completely filled and the 1.00- mL dose was administered as indicated by the change in position of the syringe plug. Doses were given iv through the jugular vein cannula as a bolus dose over 1 min. Blood samples were collected manually. Blood (200 μL) was drawn from the carotid artery cannula at 2, 15, 30, 60, 120, 240, 360, and 480 min post-dose. Excess blood was returned and 200 μL of normal saline was infused into the jugular vein cannula following each blood sample to replace lost blood volume. A volume of heparinized saline (100 UI mL⁻¹) equivalent to the cannula volume was infused into the cannula to create a heparin lock after replacing the lost blood volume. Drawn blood samples were immediately added to a solution of 0.10 M *N*-methylmaleimide and 1.0 M acetic acid in methanol (1–10 parts blood) and vortexed. Processed samples were stored at 4 °C until analyzed.
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Appendix B

Prodrug strategies to overcome poor water solubility.

Valentino J. Stella , Kwame W. Nti-Addae
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Prodrug strategies to overcome poor water solubility[☆]

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Abstract

Drug design in recent years has attempted to explore new chemical spaces resulting in more complex, larger molecular weight molecules, often with limited water solubility. To deliver molecules with these properties, pharmaceutical scientists have explored many different techniques. An older but time-tested strategy is the design of bioreversible, more water-soluble derivatives of the problematic molecule, or prodrugs. This review explores the use of prodrugs to effect improved oral and parenteral delivery of poorly water-soluble problematic drugs, using both marketed as well as investigational prodrugs as examples. Prodrug interventions should be considered early in the drug discovery paradigm rather than as a technique of last resort. Their importance is supported by the increasing percentage of approved new drug entities that are, in fact, prodrugs. © 2007 Elsevier B.V. All rights reserved.

Keywords: Prodrug; Solubility; Novel strategy; Phosphates; Ionizable and non-ionizable prodrugs; Bioreversible; Parenteral; Oral delivery

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1. Introduction — why prodrugs?

The use of a prodrug strategy as a chemical/biochemical approach to overcome various barriers which can hinder drug

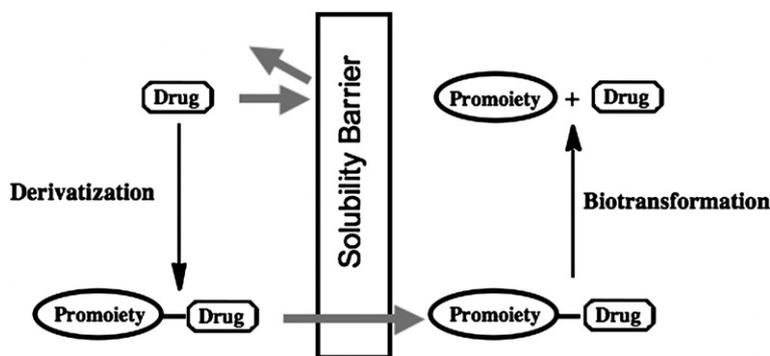
delivery, including solubility, is the subject of two new books and some recent reviews [1–4]. The focus of this paper is the use of prodrugs to overcome poor water solubility, not only of already marketed drugs with solubility limitations, but more important, how the prodrug strategy should become an integral part of the drug design paradigm.

Why prodrugs now, when the concept has been around for many years? One main reason is the recent slump in the number of drugs approved by worldwide regulatory agencies. This, in

[☆] This review is part of the *Advanced Drug Delivery Reviews* theme issue on “Drug solubility: How to measure it, how to improve it”.

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Scheme 1. An illustration of the prodrug concept.

turn, can be attributed to changes in drug candidate identification methodologies implemented years earlier, the conservative nature of the pharmaceutical industry, and ever tougher regulatory and safety standards. Because of new high throughput receptor based screens/assays (HTS) initiated in the late 1980s and the use of combinatorial chemistry approaches to drug design, many drug candidates during this era had pharmacokinetic/pharmacodynamic and physical/chemical properties that limited their chance of being developed into pharmaceutical products. In a recent series of edited books [5,6] these issues were discussed in great detail. During the mid-to late-1990s, the introduction of HTS for pharmaceutical properties, such as solubility, stability and metabolic stability and cellular permeability led to better identified molecules that were more likely to be “developable”. Data mining exercises such as those published by Lipinski and co-workers [7–9] supported the need to design drugs with delivery in mind.

While one can be critical of our drug design colleagues for some of the current failures, those currently responsible for new drug design strategies have embraced the need for studies that identify early, drug candidates with greater chance for success because of good physicochemical and ADME properties. On the other hand, it can be argued that because we ignored some of this for a period, we did begin to explore chemical space that we may not have gone to if we only looked for molecules with desirable delivery properties.

Solubility has been identified as a critical parameter and one amenable to manipulation via a prodrug strategy [1–4,10–13]. The previous chapters in this ADDR issue have identified structural elements that contribute to poor water solubility mainly where it affects the oral delivery of drug molecules, and some of the formulation approaches that can be used to overcome solubility limitations. These formulation strategies can often perform very well for low-dose potent molecules and for molecules whose solubility is just below that needed for delivery. Does that mean that we should not explore molecules with more severe solubility limitations as drug candidates? At some limit, the answer is yes. That limit may be when the free or unbound drug concentration in plasma is below that which could be achieved due to solubility limitations i.e., the highest concentration of free, unbound drug in plasma must be its solubility, and more realistically, some fraction of that value. However, provided one is not too close to this limitation, one

can manipulate drug solubility for delivery purposes through the use of a prodrug strategy.

The benefits of the prodrug strategy are most often illustrated by Scheme 1 below. For the purposes of this paper, the barrier to drug delivery is solubility.

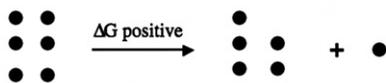
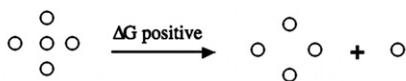
2. Structure/solubility relationships and solubility manipulation through prodrugs

In the course of consulting to the pharmaceutical industry for over 30 years (V.S.), the proposal of molecules with limited water solubility as drug candidates has been observed repeatedly. Some of these molecules performed poorly, as expected, while others performed better than expected based on their solubility characteristics. Over the years, some general trends have become clear:

First, and obvious, the larger the dose the bigger is the delivery challenge. This applies even to drugs displaying moderately low, as opposed to extremely low solubility [14]. Second, drugs with high melting points and crystallinity that display poor water and lipid solubility (lipid solubility here meaning solubility in relatively non-polar organic solvents, not in hot DMSO) have a higher probability of being the “bad actors”. These are the so-called “brick dust” molecules. Third, drugs with low melting points that display poor water solubility but high lipid solubility often perform better, orally, than expected. The reason for this is now obvious, the content of the gastrointestinal tract is conducive to dissolving such molecules because they have properties not unlike many fatty food components that are dispersed and dissolved by the presence of bile acids and lecithin mixed micelles in the GIT [15–17]. These drug molecules are often referred to as “grease ball” molecules.

Fourth, solubility is often assumed to be the etiology of poor oral performance when in fact, in many cases, poor water solubility masks poor permeability characteristics e.g. because the drug is an efflux candidate, or lacks oral bioavailability due to presystemic metabolism.

A way to illustrate points two and three above is to refer to the Scheme 2, a variation of one suggested earlier by Hildebrand and Scott [18].

Step 1. Removal of a Molecule from its Crystal Lattice**Step 2. Creating a Void in the Solvent****Step 3. Release of Solvation Energy**

Scheme 2. An illustration of the three steps needed for drug solubility [18,74].

As illustrated in [Scheme 2](#), high crystal packing energy requires a lot of free energy to free a molecule from its crystal (*step 1*) and that has to be compensated for by the release of solvation energy in *step 3*. *Step 2* illustrates that the bigger the molecule, the larger the cavity required in the solvent and thus, for a solvent like water, the more hydrogen bonds need to be broken. For molecules that do not have high crystal packing energy, such as low melting solids and oils, water solubility limitations are usually due to poor interactions with the solvent, water.

3. Oral drug delivery of poorly water-soluble compounds

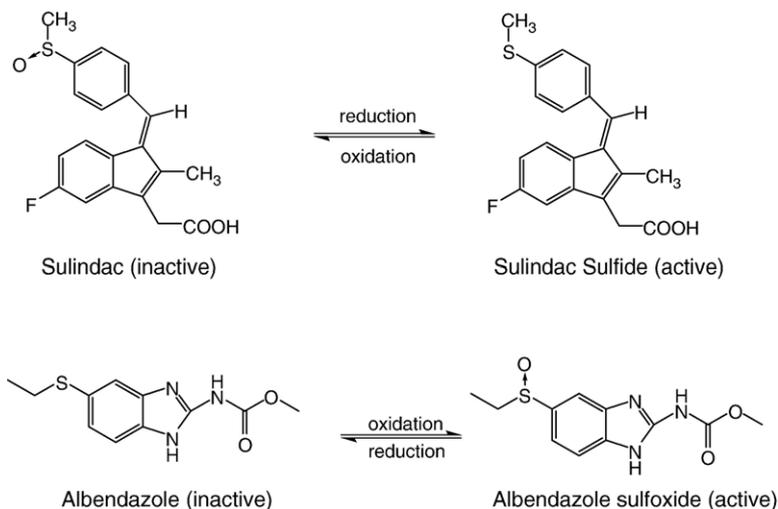
How does this discussion relate to prodrug design to effect better delivery of poorly water-soluble molecules? First, we need to make the assumption, often not validated in the early phases of a drug discovery effort, that solubility is the major culprit affecting poor performance. Next, we believe that it is helpful to identify the etiology of the poor solubility. Is the molecule one that displays high crystallinity, a “brick dust” molecule, or is it a low melting or “grease ball” molecule? Obviously, a continuum exists, with most molecules not fitting either extreme. If the molecule has the characteristics of a

“grease ball” molecule and usual formulation approaches do not work, solubility enhancement through the use of a polar promoiety may prove useful, as shown in the following examples. If one has a “brick dust” molecule a polar promoiety may work, as might strategies that disrupt the intermolecular interactions that led to the high crystallinity (see [Section 3.2](#)).

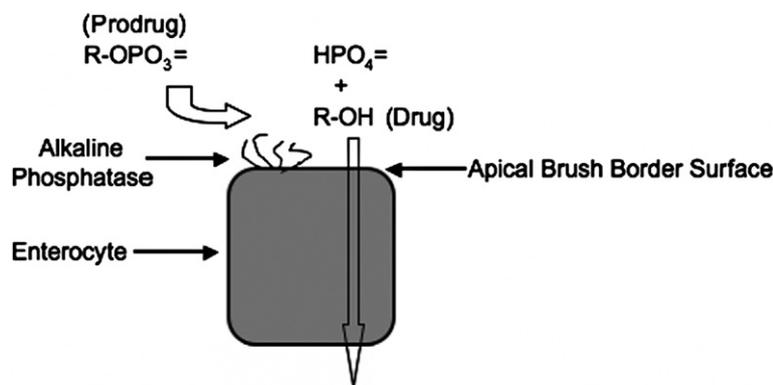
3.1. Enhanced water solubility for enhanced oral drug delivery via the addition of polar functionalities

Conventional wisdom dictates that placing a polar functional group in the structure of a molecule with limited aqueous solubility should enhance solubility. In the case of a prodrug, that functionality would have to be removed/modified, either chemically or enzymatically, to regenerate the parent drug. There are surprisingly few examples of commercially approved prodrugs that were designed to enhance water solubility for improved oral delivery; parenteral delivery examples will be considered in [Section 4](#).

Placing a non-ionizable functionality in the structure may meet the solubility enhancing goal if improved solvation compensates for any increase in intermolecular interactions occur in the crystal. An excellent example of a non-ionizable group used to effect better oral delivery is the drug sulindac as a more water-soluble delivery form of its sulfide metabolite, which is an effective NSAID [19]. The sulfoxide group is more polar, and therefore has better interaction with the solvent than the reduced sulfide. This example is illustrated in [Scheme 3](#). Sulindac is also said to be less locally irritating because it is a poorer inhibitor of prostaglandin synthetase activity [19]. The reversible metabolic reduction/oxidation seen with sulindac is an interesting prodrug example whose characteristics are not shared by many other examples. Note, however, that the drug albandazole ([Scheme 3](#)) is also reversibly metabolized to its sulfoxide. Here the albandazole sulfoxide is not only the more active component but is much more water-soluble [20]. That is, the innovators in this case chose the wrong molecule to develop; the active drug had better properties than its precursor. In their



Scheme 3. Oxidation/reduction equilibrium between sulfoxides and sulfides [19,98,99].



Scheme 4. Scheme showing the conversion of a phosphate ester prodrug ($R-OPO_3^-$) to its parent drug ($R-OH$) by the brush-border enzyme, alkaline phosphatase, and the subsequent passive absorption of $R-OH$ [21].

defense, this is an older drug and at the time it was developed it was not known that the sulfoxide was the more active component.

Many prodrugs designed to increase water solubility involve the addition of an ionizable moiety to the parent molecule. Because charged molecules have greater difficulty crossing biological membranes, one must balance increased water solubility with the potential for decreased permeability. For example, one might argue that a phosphate ester of a drug with an alcohol functionality in its structure would produce a poorly membrane permeable prodrug. However, phosphate esters have been shown to be very effective at improving the delivery of poorly water-soluble parent drug molecules after oral delivery. The reason for the success of phosphate esters is illustrated in Scheme 4.

For $R-OH$, a highly permeable drug, bioavailability after oral dosing is limited by slow dissolution. The prodrug, $R-OPO_3^-$ is much more soluble, rapidly dissolves in the content of the GIT but is cleaved to $R-OH$ by the presence of the enzyme, alkaline phosphatase, seen in abundance on the brush border surface of the cells lining the small intestine, the enterocytes. $R-OH$, being permeable, readily crosses the enterocyte membranes and enters the systemic circulation. The process can be viewed as a semi-coupled metabolism/transport event. Others have noted limitations to this approach: first, the phosphate prodrug must be a good substrate for alkaline phosphatase; second, $R-OH$ must be permeable once cleaved; and third, too rapid a cleavage of a very insoluble $R-OH$ can result in precipitation of $R-OH$, and thus poor re-dissolution [21]. Table 1 lists some examples of phosphate ester prodrugs of sparingly water-soluble drugs that have been used to effect better oral delivery.

For illustrative purposes, two examples are worthy of further discussion. The first is fosamprenavir, a phosphate prodrug of the HIV protease inhibitor, amprenavir. Amprenavir was originally formulated in a 150 mg capsule containing TPGS, PEG 400 and propylene glycol, requiring patients to take 8 capsules to achieve a dose of 1600 mg twice a day, clearly at a competitive disadvantage to other lower dose and more conveniently administered antiAIDS drugs. Amprenavir has a secondary alcohol group in its structure that was synthetically

phosphorylated to produce fosamprenavir. Although phosphate esters had previously been used to effect improved oral delivery and had been extensively discussed in the literature [21–23], the commercial success of fosamprenavir has made an impact. Fosamprenavir is in the form of a calcium salt, chosen because of its superior pharmaceutical properties compared to the disodium salt, and is approximately 10 times more soluble than amprenavir. Because of this superior solubility, even more so at low pH values where the calcium salt dissociates, fosamprenavir can be formulated as a 700 mg tablet (equivalent to 600 mg of amprenavir) thus reducing the dosing to 2 tablets twice a day. The oral availability of amprenavir from fosamprenavir is essentially equivalent to amprenavir from the original capsules. Many advantages ensue, the first being the more convenient dosing to the patients, the second being the competitive advantage of the product in the market and the third being the longer patent clock provided by the fosamprenavir patent [24–27].

The second example is fosphenytoin, a prodrug of phenytoin. Although fosphenytoin is not marketed for oral use, it results in very good oral delivery of phenytoin, an erratically absorbed, poorly water-soluble drug [21,28,29]. Phenytoin is a hydantoin drug with only the two N–H groups at the 1- and 3-positions in the hydantoin ring structure readily available for derivatization. Phenytoin does not have a ready “handle” on to which a phosphate group can be attached. Varia et al. [30–33], recognized that phenytoin reacts with formaldehyde under basic pH conditions to form 3-hydroxymethylphenytoin. In the absence of excess formaldehyde, 3-hydroxymethylphenytoin, at physiological pH and temperature, readily releases the formaldehyde with a half-life in the order of a few seconds. Thus the hydroxyl group on 3-hydroxymethylphenytoin provides a synthetic “handle” that can be phosphorylated to produce fosphenytoin [29,30]. This example demonstrates the use of a “spacer” or “linker” group in prodrug chemistry, whereby a specific function group can be attached not to the molecule itself but via the spacer group. Phenytoin oral availability from fosphenytoin is excellent [34]. The parenteral use of fosphenytoin will be mentioned in Section 4. Some of the examples presented in this paper will provide further illustrations of the use of a spacer group.

Table 1
Examples (with references) of phosphate prodrugs showing enhanced oral delivery

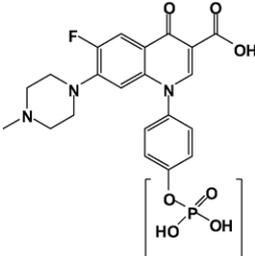
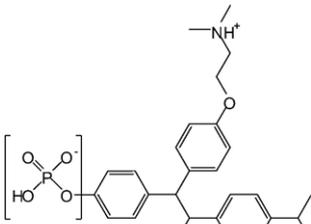
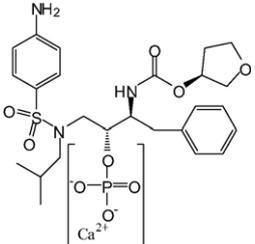
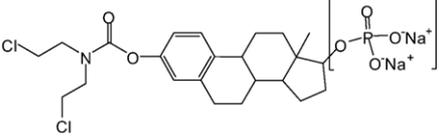
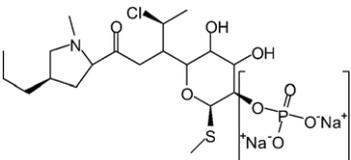
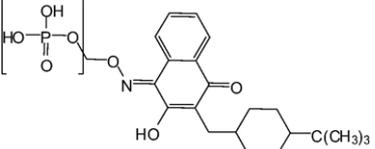
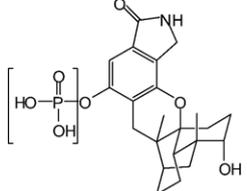
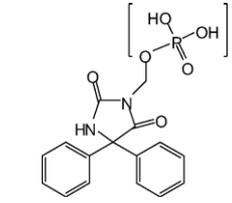
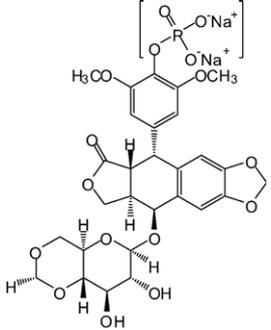
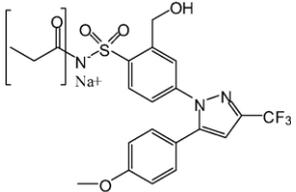
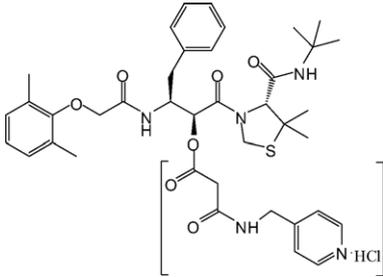
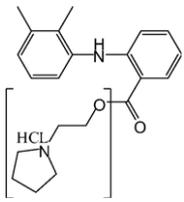
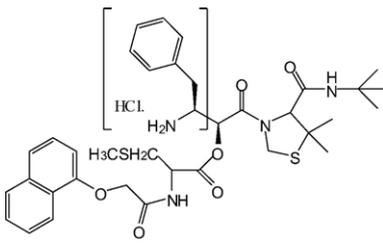
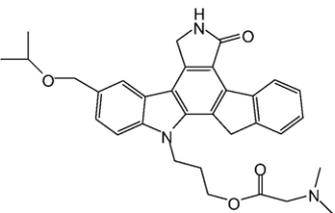
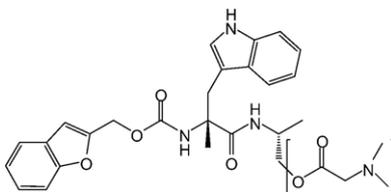
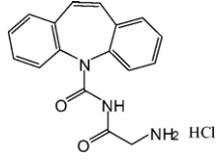
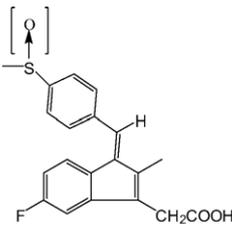
Prodrug	Ref	Prodrug	Ref
 <p>PA2808 (parent: PA2789).</p>	[75]	 <p>Miproxifene phosphate (parent: miproxifene)</p>	[21]
 <p>Fosamprenavir (parent: amprenavir)</p>	[26,76–79]	 <p>Estramustine phosphate (parent: estramustine)</p>	[80,81]
 <p>Clindamycin phosphate (parent: clindamycin)</p>	[82,83]	 <p>(Parent: bupuravaquone)</p>	[84]
 <p>Stachyflin phosphate (parent: stachyflin)</p>	[85]	 <p>Cam 5223 (parent: CAM 4451)</p>	[29,86]
 <p>Etoposide disodium phosphate (parent: etoposide)</p>	[87–89]		[90]

Table 2
Examples (with references) of non-phosphate prodrugs on poorly water-soluble parent drugs intended for enhanced oral delivery

Prodrug	Ref	Prodrug	Ref
 <p>(Parent: celecoxib)</p>	[91,92]	 <p>(Parent: KNI-727)</p>	[93–95]
 <p>(Parent: mefenamic acid)</p>	[96]	 <p>(Parent: KNI-272)</p>	[94,95]
 <p>CEP 7055 (parent: CEP 5214)</p>	[97,98]	 <p>CAM 4562 (parent: CAM 4451)</p>	[90]
 <p><i>N</i>-gly-carbamazepine (parent: carbamazepine)</p>	[57]	 <p>Sulindac sulfoxide(Clinoril®)</p>	[19,99,100]

Phosphate groups are not the only way to increase water solubility. Table 2 contains examples of water-soluble prodrugs designed to effect better oral availability. Most prevalent are prodrugs that employ an amine group capable of being protonated to form a more water-soluble salt. Unlike the phosphate group, amine group containing prodrugs are often capable of being absorbed intact from the GIT because the neutral free-base form of the drug is present in sufficient quantities to allow permeation across biomembranes.

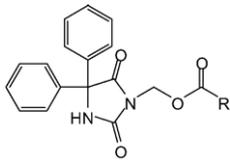
The table illustrates the diversity of pro-groups that have been utilized, including some instances where the prodrug requires significant rearrangement to regenerate the parent drug.

3.2. Decreased crystal packing and enhanced oral availability, the role of the GI tract contents

A strategy not often considered to effect better oral delivery of poorly soluble drugs is one that attempts to convert a “brick dust” molecule to a “grease ball” molecule. The rationale for this approach is as follows. The Noyes–Whitney model, mathematically defined by Eq. (1) describes the dissolution rate (DR) of a drug under sink conditions,

$$DR = \frac{D^*A^*C_s}{h} \quad (1)$$

Table 3
Physical properties of phenytoin and some 3-acyloxymethyl phenytoin prodrugs [37]

	Mp (°C)	pH 6.4 buffer solubility ^a ($M \times 10^5$)	Cyclohexane solubility ($M \times 10^3$)	SIBLM ^b solubility ($M \times 10^4$)	DR pH 6.8 buffer $\times 10^{-11}$ mol/cm ² /s	DR SIBLM $\times 10^{-11}$ mol/cm ² /s
	Phenytoin	296	8.0	0.16	5.5	10.1
$R = -\text{CH}_3$	158–9	3.4	0.19	2.7	5.1	13.5
$R = -\text{C}_2\text{H}_5$	172–4	1.24	0.24	1.3	1.4	5.9
$R = -\text{C}_3\text{H}_7$	134–5	0.85	1.08	1.4	1.1	6.0
$R = -\text{C}_4\text{H}_9$	89–92	4.3	14.7	4.3	1.9	28.4
$R = -\text{C}_5\text{H}_{11}$	107–8	0.13	4.5	1.9	0.2	9.8
$R = -\text{C}_6\text{H}_{13}$	87–8	0.05	9.9	2.1	0.07	10.0
$R = -\text{C}_7\text{H}_{15}$	67.5–8	0.03	150	5.4	0.04	55.9
$R = -\text{C}_8\text{H}_{17}$	78.5–80	ND ^c	48	3.4	ND ^c	33.8
$R = -\text{C}_9\text{H}_{19}$	56–7	ND ^c	701	6.1	ND ^c	30.7

^a Isotonic pH 6.4 phosphate buffer.

^b SIBLM is described by Stella et al. [37].

^c ND, the properties could not be determined because the concentrations were below the limit of quantification.

where D is the diffusion coefficient, A is the surface area available to the dissolution media, h is the unstirred film thickness and C_s is the equilibrium solubility in the dissolution media. Although this equation has some limitations, it illustrates that DR should be proportional to solubility, C_s , but solubility in what? Although the aqueous solubility should help define the DR in water, the GIT fluids in animal species commonly used in preclinical work do not consist of just water, and also not of simple buffer solutions typically used for solubility determinations, e.g. pH 6.4 isotonic aqueous, phosphate buffer. Because of the presence of mixed micelles of bile salts and lecithin as well as food digestion products, the GIT presents an environment conducive to dissolving the poorly soluble, lipophilic compounds which can be solubilized by this complex milieu [14–17,35–38].

A systematic study of this concept was presented in a series of papers that used phenytoin as a model compound [35–38]. The N–H at the 3-position of phenytoin is known to hydrogen bond with the carbonyl of a second phenytoin molecule. Thus, removing or blocking the N–H group at the 3-position dramatically changes the properties of the molecule. The melting points of a series of 3-acyloxymethyl prodrugs of phenytoin is shown in Table 3 along with their solubility in water, cyclohexane and in a bile salt/lecithin mixture used to simulate the GIT contents (referred to here by the acronym SIBLM, for simulated bile salt, lecithin mixture). Also included in Table 3 are the dissolution rates for a few of the derivatives in pH 6.4, phosphate buffer and SIBLM. Note the melting behavior of the pentanoyl- ($\text{C}_4\text{H}_8\text{CO}-$) and octanoyl- ($\text{C}_7\text{H}_{15}\text{CO}-$) derivatives, which have melting points lower than their higher and lower homologs, and the relationship between melting point and the solubility of the various prodrugs in the hydrocarbon solvent, cyclohexane. Similar behavior was seen in various triglyceride and fatty acid esters suitable for soft gelatin capsule formulations [38].

Clearly if one were to only consider water solubility and DR in water, phenytoin itself would be the superior candidate. When one considers the DR in the SIBLM however, the choice becomes less clear, with the DR of the octanoate prodrug superior to that of phenytoin in this medium [36,37]. The oral, absolute bioavailability of phenytoin from phenytoin, the pentanoate and the octanoate was assessed in fed and fasted dogs. The results are given in Table 4. The bioavailability of phenytoin is enhanced in the fed state, consistent with an increase in DR in the presence of the enhanced levels of bile salts and lecithin triggered by food [36,37]. The bioavailability of phenytoin from the two prodrugs is superior in both the fed and fasted state despite their lower aqueous solubility. In the fed state, phenytoin bioavailability from the pentanoate and the octanoate is close to complete, even though the octanoate had limited aqueous solubility and no measurable DR in water.

The observations made with the phenytoin prodrugs is also consistent with the studies of Shaw et al. [39] who showed superior oral activity seen with a *N*-acyl prodrug of a urea

Table 4

Absolute phenytoin bioavailability of oral suspensions, in fasted and fed beagle dogs, from phenytoin and the pentanoate ($R = -\text{C}_4\text{H}_9$) and octanoate ($R = -\text{C}_7\text{H}_{15}$) esters of 3-hydroxymethyl phenytoin, lipid soluble, all low melting point prodrugs of phenytoin [35]

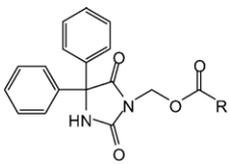
	Fasted absolute % bioavailability ($\pm\%$ SD)	Fed absolute % bioavailability ($\pm\%$ SD)
	Phenytoin	21.0 (6.9)
$R = -\text{C}_4\text{H}_9$	44.2 (16.2)	84.2 (16.5)
$R = -\text{C}_7\text{H}_{15}$	40.7 (19.8)	77.5 (22.1)

Table 5
 Examples (with references) of water-soluble prodrugs for parenteral delivery of poorly water-soluble parent molecules

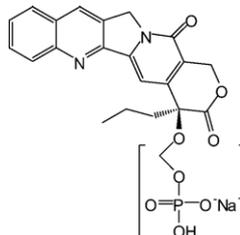
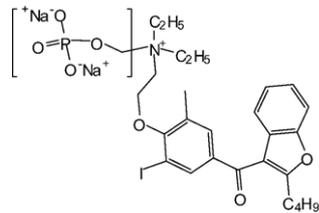
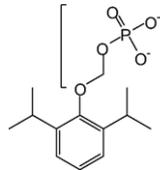
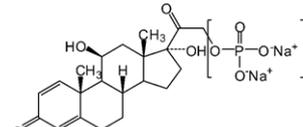
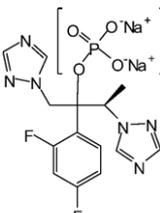
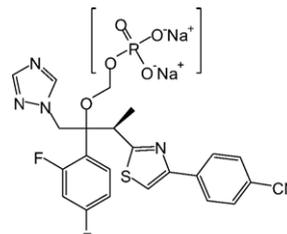
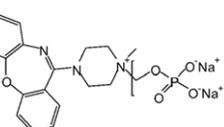
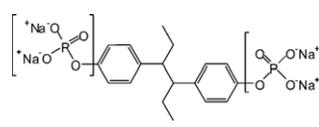
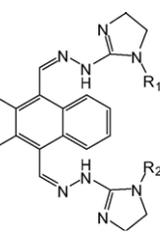
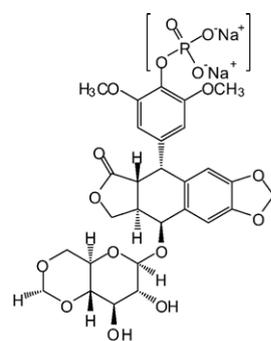
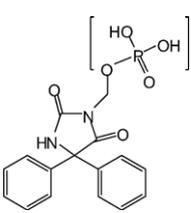
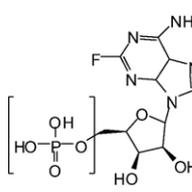
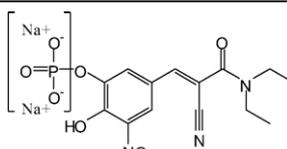
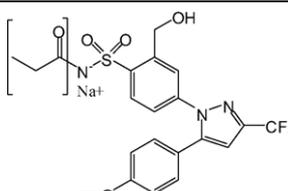
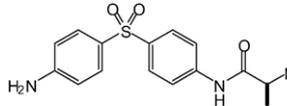
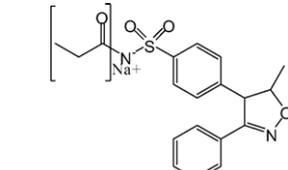
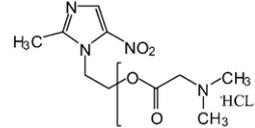
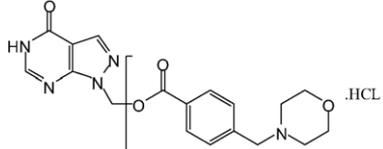
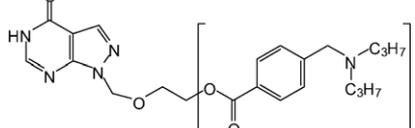
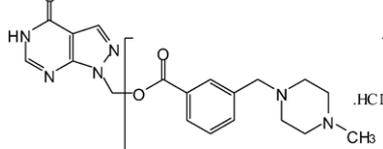
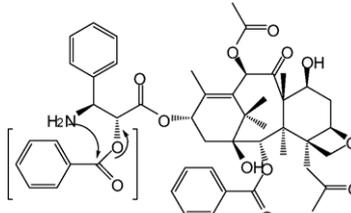
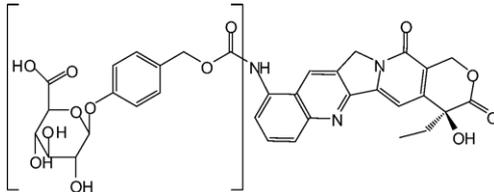
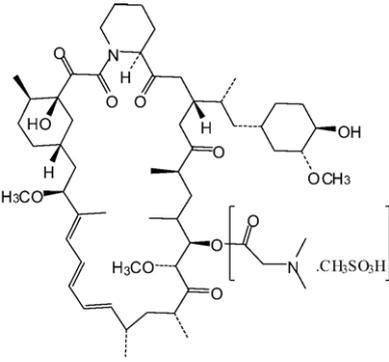
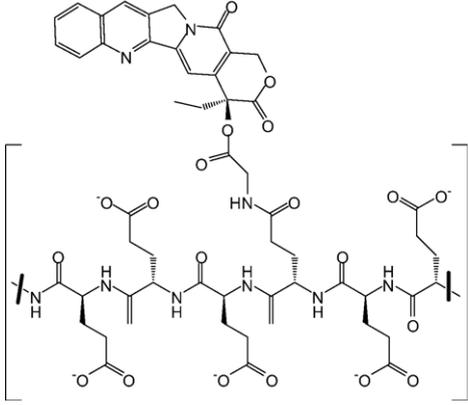
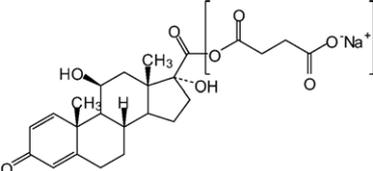
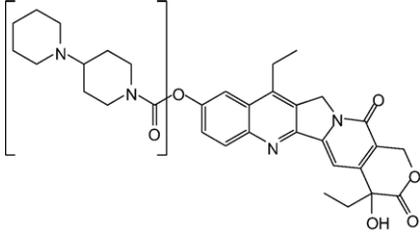
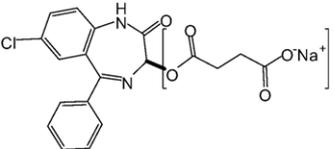
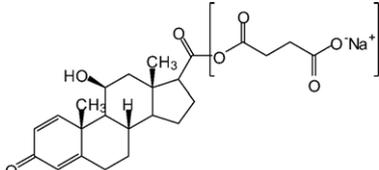
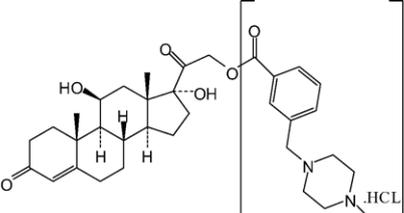
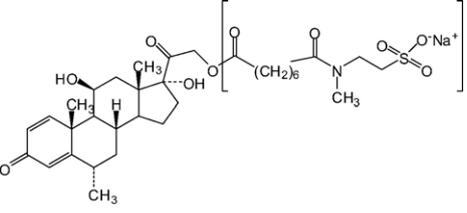
Prodrug	Ref	Prodrug	Ref
 <p>20-Phosphoryloxymethyl camptothecin (parent: camptothecin)</p>	[47,71]	 <p>Aminodarone disodium phosphate (parent: aminodarone)</p>	[101]
 <p>Aquavan (parent: propofol)</p>	[43,102,103]	 <p>Prednisolone sodium phosphate (parent: prednisolone)</p>	[51,104]
 <p>Fosfluconazole (parent: fluconazole (Difluca®))</p>	[105,106]	 <p>(Parent: ravuconazole)</p>	[107,108]
 <p>(Parent: loxapine)</p>	[109,110]	 <p>(Parent: diethylstilbestrol)</p>	[111,112]
 <p>(Parent: bisantrene)</p>	[113,114]	 <p>Etoposide disodium phosphate (parent: etoposide)</p>	[87–89]
 <p>Fosphenytoin (parent: phenytoin)</p>	[29,86]	 <p>Fludarabine phosphate (Fludara®) (parent: vidarabine)</p>	[115,116]

Table 5 (continued)

Prodrug	Ref	Prodrug	Ref
 <p>Comtan® (Entacapone phosphate) (parent: entacapone)</p>	[21,117]	 <p>(Parent: celecoxib)</p>	[91,92]
 <p>1. R = Glycine 2. R = -L- alanine 3. R = -D- alanine 4. R = -L- leucine 5. R = -D- leucine 6. R = -L- phenylalanine 7. R = -L- phenylalanine 8. R = -L- lysine 9. R = -D- lysine</p> <p>(Parent: dapsone)</p>	[118,119]	 <p>Parecoxib sodium (parent: valdecoxib)</p>	[3,120–123]
 <p>Metronidazole <i>N,N</i>-dimethylglycinate (parent: metronidazole)</p>	[124]	 <p>(Parent: allopurinol)</p>	[125]
 <p>Aminomethylbenzoate ester of acyclovir (parent: acyclovir)</p>	[126]	 <p>(Parent: allopurinol)</p>	[125]
 <p>Isotaxel (parent: paclitaxel)</p>	[127]	 <p>(Parent: 9-aminocamptothecin)</p>	[128,129]

(continued on next page)

Table 5 (continued)

Prodrug	Ref	Prodrug	Ref
 <p>(Parent: rapamycin)</p>	[130,131]	 <p>Poly-(L-glutamic acid)-gly-camptothecin (parent: 20 (S)-camptothecin)</p>	[129,132,133]
 <p>Prednisolone sodium succinate (parent: prednisolone)</p>	[51,104]	 <p>Irinotecan (parent: camptothecin)</p>	[133]
 <p>Oxazepam sodium succinate (parent: oxazepam)</p>	[134–136]	 <p>Hydroxydione sodium succinate (parent: hydroxydione)</p>	[137,138]
 <p>(Parent: hydrocortisone)</p>	[139]	 <p>Promedrol® (parent: methylprednisolone)</p>	[63,140]

molecule. The parent molecule had a melting point of >320 °C while an *N*-benzoyl derivative had a significantly lower melting point, lower water solubility but superior activity after oral dosing to dogs [39].

4. Parenteral drug delivery

Because this paper relates to aqueous solubility issues, parenteral formulations of prodrugs intended for prolonged

drug release, including pegylated drugs, are not discussed here. The reader is directed to more extensive reviews on this subject [40–42]. The examples presented here will focus on water-soluble prodrugs intended for rapid drug release after IV, SC or IM administration. Although sparingly water-soluble drugs can be formulated with suitable excipients for parenteral use, most formulation techniques present their own challenges with respect to safety and chemical and physical stability. Also, when large increases in solubility are needed, prodrugs often present the best chance of success. Some of the best and most successful marketed prodrugs are those intended for parenteral use. Table 5 provides a cross-section of marketed and investigational parenteral prodrugs.

4.1. Enhanced water solubility via the addition of polar functionalities

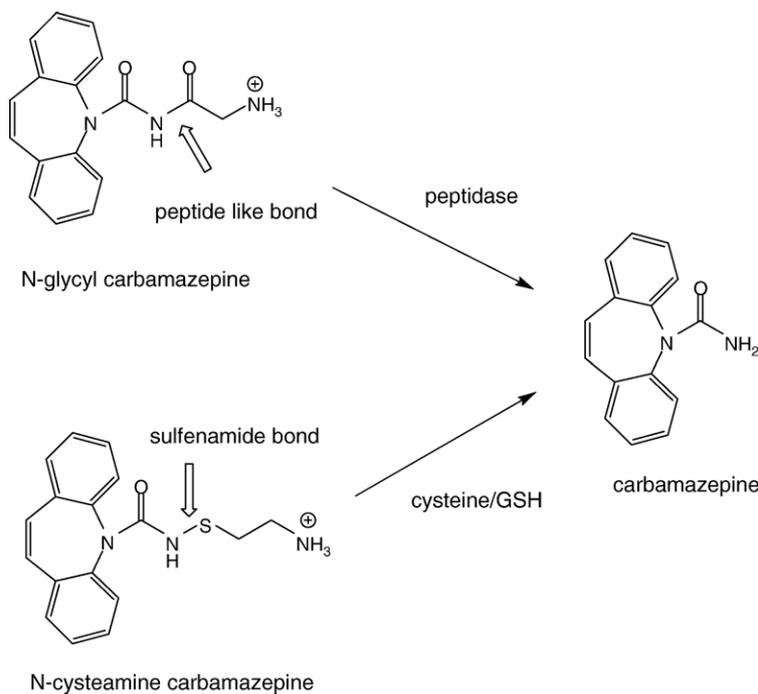
The majority of parenteral prodrugs listed in Table 5 involve prodrug modifications whereby a polar, most often an ionizable moiety, is utilized to increase water solubility. Many recent examples have focused on the use of the phosphate group either directly linked to the parent drug, where possible, or through a linker group such as formaldehyde [29,43–47]. Unlike some other moieties, many phosphate esters show good chemical stability while undergoing rapid and often quantitative cleavage *in vivo* via alkaline phosphatases [22,29–32,47–49]. Hemisuccinate esters on the other hand, are chemically metastable and weak substrates for esterase cleavage. This has resulted in incomplete conversion to the parent drug [50–55]. Three of the earliest parenteral prodrugs are chloramphenicol, prednisolone and methylprednisolone hemisuccinates [50–55].

An interesting phosphate prodrug undergoing clinical trials is aquavan, a water-soluble, pain free prodrug of the anesthetic

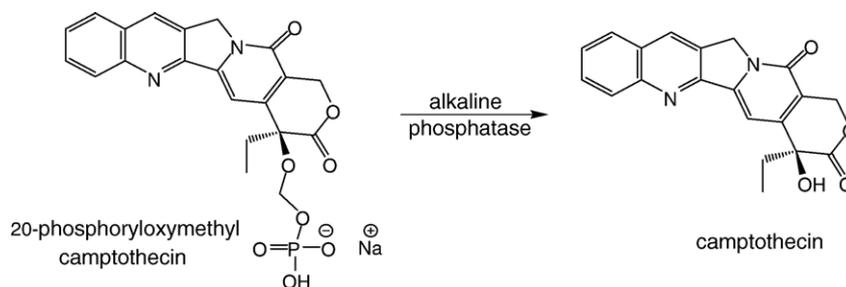
drug, propofol (see Table 5). Propofol is used in general anesthesia and in ICU use for maintaining patients in an induced coma. Because of its fast onset and offset rate, and minimal after-effects, it would be an ideal anesthetic if it did not cause significant brachial pain and acutely lower blood pressure. Furthermore it is formulated in an o/w emulsion, which can lead to hyperlipidemia when used long-term to maintain ICU patients in a coma. Stella et al. [56] synthesized and evaluated a water-soluble, phosphoryloxymethyl ether prodrug of propofol, aquavan, which was subsequently clinically evaluated [43–46]. It is currently in the final stages of Phase III clinical trials for light sedation with further trials planned. In clinical studies, aquavan shows no brachial pain as the arm veins only “see” the prodrug and not the pain-causing propofol. Additionally, the drop in blood pressure is less severe. And because the prodrug can be formulated in a purely aqueous vehicle, hyperlipidemia is not a problem.

Direct phosphorylation of propofol did produce a water-soluble form of propofol but studies in animals showed slower conversion to propofol, presumably because of the steric hindrance by the *ortho*-di-isopropyl groups, than the formaldehyde linked molecule. The directly phosphorylated molecule also showed an unfavorable EEG behavior in experimental animals [unpublished results].

Carbamazepine is an antiseizure drug for which there is no parenteral form. Recently, Hemenway [57] evaluated two water-soluble prodrugs of carbamazepine (see Scheme 5). The first is the *N*-glycyl derivative. It was argued that even though the *N*-glycyl derivative is an acyl urea, the glycine-carbamazepine bond resembles a peptide bond and so may be subject to cleavage by peptidases. *N*-glycylcarbamazepine was found to be a peptidase substrate and to be rapidly and quantitatively cleaved to carbamazepine in rats after i.v. administration in rats



Scheme 5. Two water-soluble prodrugs of carbamazepine [57].



Scheme 6. A water-soluble prodrug of camptothecin [71].

[13,50,57]. On oral dosing, *N*-glycylcarbamazepine resulted in superior oral bioavailability of carbamazepine compared to the parent drug. A limitation seen with the glycine derivative as a parenteral prodrug was its marginal chemical stability, requiring the drug to be formulated as a freeze-dried product for reconstitution.

A second carbamazepine prodrug that was more chemically stable was the *N*-cysteamine derivative [13,50,57,58] (Scheme 5). Guarino et al. [59], earlier showed that sulfenamide derivatives of ureas and amides are quite chemically stable but readily revert to the parent urea or amide in the presence of cysteine, glutathione and other sulfhydryl molecules. After i.v. administration to rats, *N*-cysteamine carbamazepine resulted in rapid and quantitative conversion to carbamazepine. The use of sulfenamides as prodrugs of acidic N–H molecules was recently reviewed by Guarino and Stella [60,61].

4.2. Formulation challenges

Parenteral formulations present some unique challenges compared to oral formulations. The two most obvious are the need for sterility and that the formulation for i.v. use must be free of significant particulates. Sterility can be achieved through either sterile filtration or terminal heat sterilization. Because prodrugs, by design, are intended to be metastable by being cleaved by a chemical or enzyme mediated pathway to the parent drug, most prodrug solutions cannot be heat sterilized; they are too chemically unstable at room temperature to be formulated as ready-to-use solutions, even when *in vivo* performance requires enzymatic versus chemical cleavage. As a result, many parenteral prodrugs are formulated as freeze-dried products for reconstitution.

A second significant challenge, also related to chemical stability, is the precipitation limit to the shelf-life of many prodrug solutions. Since most products must maintain a content specification of $\pm 10\%$ of labeled amount, the shelf-life of many drugs is usually determined by the time to degrade 10% of the drug content. This is not always the case for many water-soluble prodrugs of poorly water-soluble parent drugs. The chemical degradation of a prodrug often results in formation of the sparingly soluble parent drug as the primary degradation product. When the parent drug is formed in excess of its solubility in the formulation, it can precipitate, taking the product out of specification due to the present of particulates/precipitates. Consider the following example. Fosphenytoin is formulated as

a 50 mg/mL, mole equivalent solution of sodium phenytoin. At pH 7.4 in the presence of fosphenytoin, phenytoin has a solubility of about 45 $\mu\text{g/mL}$ in the absence of a phenytoin solubility enhancer [62]. Therefore, the shelf-life of fosphenytoin at pH 7.4 is dictated by the time for fosphenytoin to degrade from 50 mg/mL to 49.955 mg/mL, or the time for only approximately 0.1% of the fosphenytoin to degrade to phenytoin. Even this calculation assumes that the fosphenytoin API contains 0% phenytoin as an impurity at time zero. For this reason fosphenytoin was initially formulated at pH > 8.5 because phenytoin is not the principal degradation product in this pH range and the products formed are water-soluble. However, because of the greater intrinsic instability at pH > 8.5 compared to pH 7.4, the formulation requires refrigeration, limiting its use to areas with ready access to refrigeration. Narisawa and Stella [62] solved this problem by formulating fosphenytoin at pH 7.4–8 at room temperature in the presence of 60 mM sulfobutylether- β -cyclodextrin, which is capable of selectively solubilizing the formed phenytoin in the presence of high fosphenytoin concentrations. Thus a sophisticated formulation approach combined with a prodrug was able to provide a unique solution to the solubility/stability issues.

Some water-soluble prodrugs can solubilize their sparingly water-soluble degradation products because the prodrugs tend to be amphiphilic and capable of forming micelles [63–65]. Thus, the shelf-life may be longer than the value that would be expected by considering just the solubility of the parent drug. Consider the investigational prodrug, 20-phosphoryloxymethyl camptothecin (Scheme 6) [47]. This prodrug is formulated at pH 4 for reasons related to the facile E-ring opening at higher pH values [47]. Table 6 shows the increase in solubility of camptothecin in the presence of increasing concentration of 20-phosphoryloxymethyl camptothecin at pH 4. In this case, the

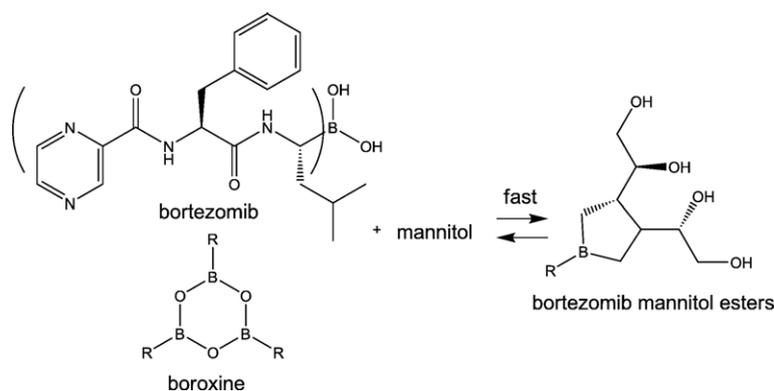
Table 6
Solubility of camptothecin at 25 °C in 20 mM aqueous citrate buffer, pH 4.0, in the presence of increasing concentrations of its 20-phosphoryloxymethyl ether prodrug (see Scheme 6 for structures) [71]

20-phosphoryloxymethyl camptothecin concentration, mg/mL (camptothecin equivalents)	Camptothecin solubility, mg/mL
0 (0)	2–3
1.3 (1)	45
3.3 (2.5)	95
6.6 (5)	175
13.1 (10)	290

prodrug does not appear to form micelles, but rather a complex with the camptothecin “sandwiched” between two molecules of the prodrug [unpublished results]. Although the shelf-life of 20-phosphoryloxymethyl camptothecin is still determined by the conversion to and possible precipitation of camptothecin, the shelf-life is much longer than that predicted by assuming no interaction between the camptothecin and its prodrug.

4.3. Bortezomib (Velcade®), a novel example

An example of a recent “surprising” prodrug is the boronic acid, proteasome inhibitor, bortezomib, marketed as Velcade, which is used to treat lymphomas. Although the chemical structure of bortezomib is usually drawn as illustrated in [Scheme 7](#), in the solid state as the pure API, it actually exists as the very insoluble boroxine, a cyclic boronic acid anhydride. When placed in water, the boroxine dissociates to form an equilibrium between itself and the monomeric bortezomib resulting in an apparent water solubility of about 0.5–1 mg/mL, still not sufficient for formulation purposes. As part of work performed in collaboration with the innovator and the National Cancer Institute, bortezomib was found to be oxidatively unstable [66], requiring the development of a freeze-dried formulation. A stable formulation was found by first by dissolving bortezomib in warm *t*-butyl alcohol, then adding water (to 50–60% water) and mannitol (1%, bulking agent), followed by freeze-drying. On reconstitution, bortezomib was found to rapidly dissolve and was significantly more soluble due to the *in situ* formation of boronic acid esters by reaction with diol groups of mannitol during the freeze-drying process [67]. The mannitol esters play two roles, first the esters themselves may be more soluble, and second, in forming the esters during the freeze-drying step, it prevents or competes with formation of the less soluble and slowly dissolving boroxine. The kinetics of formation and dissociation of the diol esters has (to our knowledge) not been published for bortezomib but studies in our laboratory with other boronic acids have suggested that the half-lives are in the order of seconds. The FDA allowed the innovator significant leeway in defining bortezomib as the active agent while the final formulation contains the diol ester prodrugs. On *in vivo* administration, it was successfully argued that the mannitol esters rapidly and quantitatively dissociate [68].



Scheme 7.

5. What have you done for me lately?

Many still question the use of a prodrug strategy as a problem solving technique. Some of these concerns are valid, namely, the additional time and cost when a prodrug approach is needed to solve a specific formulation or delivery problem. It can be argued that if the prodrug strategy became an integral part of the drug design paradigm, little additional time and expense is needed. The prodrug becomes the NCE — that happens to have an active metabolite.

According to Hedge and Schmidt [69], of the 24 NCEs approved in 2005, 19 were small molecules, while five were polypeptides or macromolecules (biotech products). Of the 19 small molecules, two were clearly identified as prodrugs (10.5%). There was one pegylated antiviral approved that was probably a prodrug (raising the percentage to 15.8%). There were two other small molecule antivirals that owed their activity to being phosphorylated and are therefore technically prodrugs but not always valued as such. If one includes all five molecules, 26.3% of all drugs approved in 2005 were prodrugs, with 15.8% clearly by design. Similar evaluations over longer time periods show that a significant number of drugs marketed over the last 15 years were prodrugs [70]. Ettmayer et al. [3] confirmed this trend, stating that 6.9% of all marketed drugs in Germany are prodrugs.

As stated earlier, there many successful examples of parenteral, water-soluble prodrugs of poorly water-soluble drugs. In fact, other than prodrugs to overcome permeability barriers, they are by far some of the best prodrug examples. Surprisingly, there are few marketed prodrugs designed to improve the oral delivery of sparingly water-soluble drugs. This is, however, an active area of interest and one that is likely to lead to many new products in the future.

6. Why the occasional failures?

The reticence of some to explore a prodrug solution to a problem can usually be traced to previous failures or by management wanting a fast solution that will not delay compound development. Previous failures can usually be traced to a poor choice of drug candidate to begin with, poor choice of prodrug strategy, misidentification of the etiology of the

delivery barrier, unrealistic expectations, generation of a new problem in solving the initial problem, or misleading results in animal models with respect to improved availability.

When a drug displays poor water solubility, it is almost always assumed that poor performance after oral dosing was related to the solubility limitation. This is often reinforced when a solution form of the drug in a co-solvent mixture or a SEDDS formulation outperforms a suspension of the drug. However, the problem may lie elsewhere. Other barriers, such as poor permeability or first pass metabolism may well be at play. Especially in the last five years, there has been growing awareness about the role of efflux pump transporters such as *P*-glycoprotein (Pgp) in curtailing transport of molecules across biomembranes, even for those molecules with apparently ideal *P* properties.

An additional difficulty is getting reliable pharmacokinetic parameters for a poorly soluble drug from *i.v.* studies. How do you administer a poorly soluble drug *i.v.* without the use of exotic vehicles? Therefore, it becomes difficult and sometimes impossible to determine, with accuracy, the absolute bioavailability of a drug after oral dosing and questionable whether one can identify the barrier(s) to delivery. For similar reasons, identifying whether a drug is a Pgp substrate (permeability studies that require the drug to be present in reasonable concentrations on the apical side of a cell mono-layer) or is rapidly cleared (requiring accurate and precise pharmacokinetic measurements) can also be a challenge.

Therefore, when one does not “know” the cause, one often “assumes,” and poor solubility is nearly always assumed to be the cause of the poor performance. However, poor permeability and presystemic metabolism can be hidden by poor water solubility. We studied the parenteral and oral availability of the anticancer drug camptothecin from its 20-phosphonoxyethyl prodrug [12,47,71]. After *i.v.* administration, the prodrug quantitatively released camptothecin [71]. It was argued that the poor oral availability of camptothecin was due to its low water solubility of about 3 µg/mL and ensuing slow dissolution rate. The oral bioavailability of camptothecin in rats from an oral suspension was found to be 2%, while its availability from a complex co-solvent vehicle was 4%, or double that from the suspension. The oral availability of camptothecin from its water-soluble phosphate prodrug was also only 4%. At the time, we did not realize that camptothecin was a very good Pgp substrate. Thus, the poor oral availability of camptothecin was not only due to poor water solubility, but also its poor permeability. Presystemic metabolism of camptothecin may also have been contributing to the poor oral availability in the rat model.

The second relates to the comment that in solving one problem a second can be created. As seen earlier, to increase water solubility one often attempts to incorporate a water-solubilizing moiety in the prodrug structure. A favorite of chemists is an amino group (1° – 3°). In forming the prodrug, the molecular weight is also increased. The combination of an amine group and increased molecular weight increases the potential for the prodrug to be a Pgp substrate [72,73]. So, while solubility and dissolution may be increased, the new molecule, the prodrug, may be less permeable and thus poorly absorbed.

The third relates to choice of animal models. One could spend a lot of time on this point and one would be not the much wiser for it. The recently published new book by Testa and Mayer [2] contains many excellent discussions of the bioreversible, enzymes based mechanisms for prodrugs and differences seen between animal species. Clearly a prodrug strategy that relies on only a single animal model for initial screening of prodrugs can lead to both false positives as well as negatives, but this is not unique to prodrugs, analog assessment suffers from the same limitations. With respect to oral availability assessments, there are several differences among species which may affect drug dissolution and absorption. For example, bile flow is continuous in the rat whereas in all other species, increased bile flow is often triggered by feeding. In the dog, the bile acid and lecithin content of the small intestinal fluids after meals is much higher than in many other animal species. With respect to enzyme activity, it should be noted that glucuronidation is poor in dogs. By contrast, esterase activity in rodents appears to be generally quite high. Understanding the limitations of the animal species chosen helps to recognize the potential dangers associated with interpreting the results in terms of human application and refines the choice of prodrug candidates in a second animal model. Ultimately however, one still needs to make an educated “guess”. If that “guess” is off, and the prodrug strategy does not meet with success in a clinical trial, there will be less willingness to invest in prodrug strategies going forward. However, it seems appropriate to mention at this juncture that this same reticence is rarely applied to analog development, *i.e.*, just because one does not synthesize a new blockbuster drug on the first try, one does not conclude that the analog approach is not a good way to discover a new drug!

7. Conclusion

Prodrugs continue to be an exciting area of research. The heightened interest of late comes from the fact that more and more drug candidates present significant delivery challenges, with poor water solubility being an increasingly frequent problem. The relatively high percentage of recently approved drugs that are, in fact, prodrugs supports claims for the heightened recent interest in prodrugs. We hope this paper will encourage creative prodrug research to help solve some of the unmet drug delivery challenges, especially those related to limited solubility.

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Glossary

- ADDR:* advanced drug delivery reviews
ADME: absorption, distribution, metabolism and elimination
AIDS: autoimmune deficiency syndrome
BCS: biopharmaceutics classification system
DMSO: dimethylsulfoxide
DR: dissolution rate
GIT: gastrointestinal tract
HIV: human immunodeficiency virus
HTS: high throughput screening
ICU: intensive care unit
i.m.: intramuscular
i.v.: intravenous
NCE: new chemical entity
PEG 400: polyethylene glycol 400
Pgp: P-glycoprotein
s.c.: subcutaneous
SEDDS: self-emulsifying drug delivery system
SIBLM: simulated intestinal bile salt and lecithin mixture
TPGS: d- α -tocopheryl polyethylene glycol 1000 succinate