NOVEL INHIBITORS/INACTIVATORS OF CYSTEINE PROTEASES

An Undergraduate Research Scholars Thesis

by

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ABSTRACT

Novel Inhibitors/Inactivators of Cysteine Proteases. (May 2015)

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When operating aberrantly, enzymes contribute to the mechanisms of many different diseases, and as such, have proven to be promising targets for the discovery of new drugs. Proteases catalyze the hydrolysis of peptide bonds and proteins, the result of which is the inactivation or de-activation of physiological processes that are often contributory to disease. The cysteine proteases are also involved in pathogenesis including cancer, autosomal disorders, pulmonary disorders, and their action assists in the infectivity of the pathogenic protozoa that cause Chagas's disease and malaria. Despite their importance as therapeutic targets, currently no inhibitors of cysteine proteases have proven viable as treatments for these diseases. While numerous inhibitors of cysteine proteases exist, most of which inactivate these enzymes by covalent reaction, none have to date proven sufficiently specific to serve as drugs. We are beginning to develop novel types of covalent inactivators, which utilize detailed aspects of the mechanism and structure of cysteine proteases to render them highly selective toward their targets. The cysteine proteases human cathepsin C and cathepsin L were used as models to develop new inhibitors because they are available, well characterized kinetically, and the structure of human cathepsin C has been fully determined. This model served as a means to apply the same chemistry or similar to other enzymes such as falcipain-2, a drug target for malaria. Falcipain-2

was prepared as a recombinant protein. Three compounds, Cbz-Phe-Phe-CHO, Cbz-Phe-Phe-vinyl-phenylsulfone (Cbz-Phe-Phe-VSPh), and Cbz-Phe-Phe-2-vinylpyrimidine (Cbz-Phe-Phe-2VP), were shown to be time-dependent inhibitors/inactivators of falcipain-2 with apparent values of K_i = 0.25 nM, 32 nM, and 70 nM, respectively. Cbz-Phe-Phe-VSPh was a clinical candidate for treatment of malaria, but failed because it lacked sufficient specificity for falcipain-2. It does serve, however, as a "benchmark" inactivator for comparison with our novel inactivator Cbz-Phe-Phe-2VP. This inactivator has a pyrimidine ring that allows for "tuning" of its electrophilicity to create different levels of specificity. An increase in specificity for falcipain-2 could result in a decrease in side effects and more targeted clinical applications for treatment of malaria.

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NOMENCLATURE

Cbz – carbobenzoxy, a protecting group

AMC – 7-amino-4-methyl-coumarin

VSPh – vinyl-phenylsulfone

2VP – 2-vinyl-pyrimidine

CHAPTER I

INTRODUCTION

Many different diseases arise from the aberrant activities of enzymes that otherwise govern normal metabolic processes. Because of this important role, enzymes are excellent targets for drugs. Cathepsin C, cruzain, and falcipain, all part of the papain clan of the cysteine protease enzyme family, are involved in prevalent diseases and comprise validated targets for drug development. Cathepsin C is a target for cystic fibrosis treatment among other respiratory disorders, cruzain is critical for the life cycle of *Trypanosoma cruzi* – the causative agent of Chagas's disease, and falcipain-2 is critical for the life cycle of *Plasmodium falciparum* – the causative agent of malaria. Various covalent inhibitors of these enzymes have been developed including epoxides, peptidic aldehydes, α-halo-ketones, nitriles, and vinyl-sulfones. However, these electrophilic groups in general have proven to be too reactive or non-specific to achieve the necessary selectivity required for progression to therapeutic use. Cathepsin C is an ideal model with which to begin exploring new covalent agents because it is so well characterized, and its substrates are simple dipeptides[1,2]. This enzyme is also very useful in that it has a wide substrate specificity, so many different dipeptides can be used to create covalent inhibitors[2]. Additionally, the cysteine protease human cathepsin L is currently available, and has similar substrate specificity to falcipain-2, such that peptide-based inactivators of this enzyme may also be active against falcipain-2, and cathepsin L serves as a selectivity "marker" of inactivation. We began with both the H_2N-Gly-Phe and the Cbz-Phe-Phe peptide templates, the former of which comprises one of the most active dipeptide substrates of cathepsin C as H_2N-Gly-Phe-AMC[2]. The aldehyde analogues of the H_2N-Gly-Phe and Cbz-Phe-Phe dipeptides, H_2N-Gly-Phe-CHO

and Cbz-Phe-Phe-CHO, respectively, are available by four synthetic steps[3], and these aldehydes proved useful as covalent inactivators of cathepsin C and cathepsin L/falcipain-2, given that they likely form a hemithioacetal with the active-site cysteines. The cysteine protease family can be potently inhibited/inactivated by vinyl-sulfone compounds because the thiol in the active site of the enzymes can perform a Michael reaction to the activated vinyl moiety, thereby becoming "covalently arrested" and unable to continue catalysis[4]. Vinyl-sulfone inhibitors have proven to be effective against cathepsin C, cathepsin L, as well as falcipain-2 and cruzain, and these compounds have been prepared from peptide aldehydes using Wittig and Horner-Wadsworth-Emmons chemistry[5,6]. These vinyl inhibitors have been effective, but their reactivity with the cysteine residue could be better controlled if the sulfone group was replaced with other groups that could still elicit a Michael reaction, but could also be easily altered to produce modulatable electrophilicity. If the reactivity of these inhibitors could be modulated in this manner, they would have a greater specificity against clinically important enzymes such as cathepsin C, cruzain, and falcipain-2. This increased specificity would mean that the drugs could be more effective at targeting these enzymes, which would result in a decrease in side effects and more targeted clinical applications.

CHAPTER II

METHODS

Synthesis of Inactivators

The inactivators used against cathepsin C, cathepsin L, and falcipain-2 were synthesized by a chemist in the lab. The aldehyde, Cbz-Phe-Phe-CHO, was synthesized in four synthetic steps, as shown in Figure 1. This aldehyde was used as a covalent inactivator of falcipain-2 by forming a covalent hemithioacetal with the active-site cysteine. The peptide aldehyde served two convenient roles: as a potent protease inactivator and as a chemical intermediate toward the final vinyl heterocycle product. The vinyl-pyrimidine inactivator was synthesized using the aldehyde by a Wittig reaction in which the aldehyde was reacted with a triphenylphosphonium ylide, as shown in Figure 1. The suspected mechanism of inactivation of this vinyl-pyrimidine inactivator (**6**) shown in Figure 2, in which proton transfer from His-162 in the enzyme's active site activates the pyrimidine ring and vinyl group for attack by the thiolate of Cys-25, forming a covalent bond between the enzyme and inactivator.

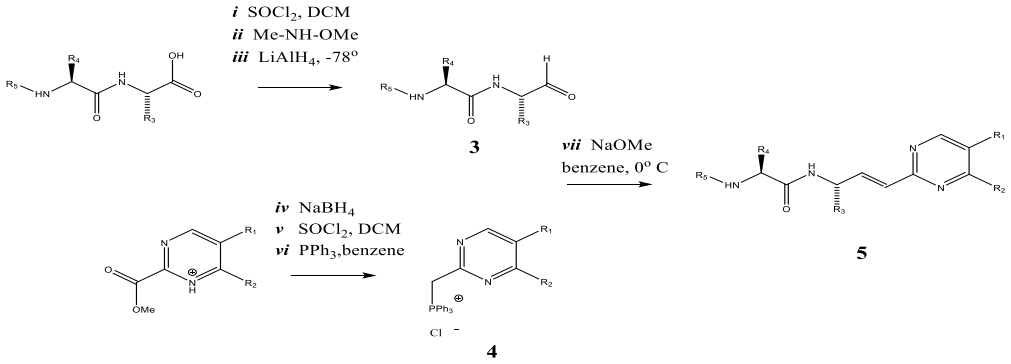

Figure 1. Synthesis of di-peptide aldehyde and di-peptide vinyl-pyrimidine inactivators. Di-peptide aldehydes **3** were made in three steps from the protected di-peptide carboxylic acids via formation of Weinreb amides, followed by reduction. Triphenylphosphonium ylides **4** were made in three steps from methyl esters of 2-carboxy-pyrimidines, and generic di-peptide 2-vinyl-pyrimidines **5** were formed by a single step Wittig condensation of **3** and **4**.

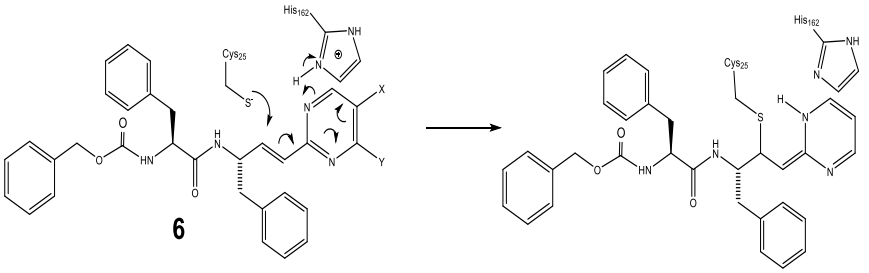

Figure 2. Proposed mechanism of inactivation by Cbz-Phe-Phe-2-vinylpyrimidine (**6**). The vinyl-pyrimidine likely inactivates enzymes in this proton-assisted mechanism.

Optimization of Peptide Substrates

The catalytic efficiencies of peptide substrates of cathepsin C, cathepsin L, and falcipain-2 were studied. Ideal substrates were selected based on size, solubility, and kinetic parameters. A panel of synthetic or commercially available peptide substrates of the form X-P_1-P_2-C(O)-NH-AMC were used. These peptide substrates contained a 7-amino-4-methylcoumarin (AMC) group at the scissile amide bond, which becomes fluorescent upon enzyme-catalyzed hydrolysis. P_1 and P_2 are naturally occurring (Phe, Arg, Leu, Val) amino acid side chains, and X is a carbobenzoxy or a similar substituent. We characterized the kinetic parameter K_m of a few AMC containing peptide substrates for which the lowest values of K_m comprised the substrates that are best recognized and hydrolyzed by cathepsin C, cathepsin L, and falcipain-2.

Characterization of Inactivators

The synthesized di-peptide-vinyl-pyrimidine, Cbz-Phe-Phe-2VP, was used to inactivate cathepsin L as a model for falcipain-2. The peptide-aldehyde inactivator, Cbz-Phe-Phe-CHO, a di-peptide-vinyl-phenylsulfone inactivator, Cbz-Phe-Phe-VSPh, and Cbz-Phe-Phe-2VP were used to inactivate falcipain-2. A time-dependent, tight-binding inhibition evaluation was performed in which enzyme and inhibitor were allowed to incubate for 30-60 minutes, followed by addition of substrate to initiate the reaction. The hydrolysis of the AMC group on the

substrate used, Cbz-Phe-Arg-AMC, produced fluorescence in the 360-460 nm range and a corresponding time course of the enzymatic reaction. The fluorescent intensity was measured and corresponded to thenzyme activity. As the enzyme and inhibitor associate, they form the complex EI, as shown in Figure 3. The EI complex is established quickly, in a matter of

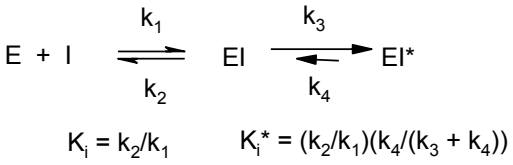

Figure 3. Time-dependent tight-binding inhibition

milliseconds. The EI* complex formed from the EI complex is the tight-binding complex, and is established over the course of a few minutes. The kinetic parameter, K_i^*, was determined for each of the inactivators according to the equation shown in Figure 3. This parameter is indicative of how potent the inhibitor is as a measure of the concentration of inhibitor required to produce half maximum inhibition. Thus, a smaller K_i^* value corresponds to a more potent inhibitor.

CHAPTER III

RESULTS

Optimization of Peptide Substrates

Kinetic parameters of peptide substrates were characterized for cathepsin C, cathepsin L, and falcipain-2 for those substrates that were most efficient and best recognized by their respective enzymes. The best substrate for cathepsin C was determined to be H_2N-Gly-Phe-AMC[2]. The best substrate for both cathepsin L and falcipain-2 was determined to be Cbz-Phe-Arg-AMC. The Michaelis-Menten model was used to calculate the K_m for each enzyme and substrate, as shown in Table I.

Table I. The Km values for cathepsin C, cathepsin L, and falcipain-2 and their respective substrates

Enzyme	Substrate	K_m
cathepsin C	H_2N-Gly-Phe-AMC	75 μM ±15
cathepsin L	Cbz-Phe-Phe-AMC	20 μM
falcipain-2	Cbz-Phe-Phe-AMC	13 μM ±2

Characterization of Inactivators

Characterization of Cbz-Phe-Phe-CHO

The inactivator Cbz-Phe-Phe-CHO was used to inactivate falcipain-2. A time-dependent, tight-binding inhibition evaluation was run in which 25 nM falcipain-2 was mixed with Cbz-Phe-Phe-CHO, and the reaction mixture was allowed to pre-incubate for 30 minutes before addition of

100 μM Cbz-Phe-Arg-AMC as the substrate to initiate the reaction. The tight-binding inhibition time course is shown in Figure 5. As the concentration of Cbz-Phe-Phe-CHO increased, the rate

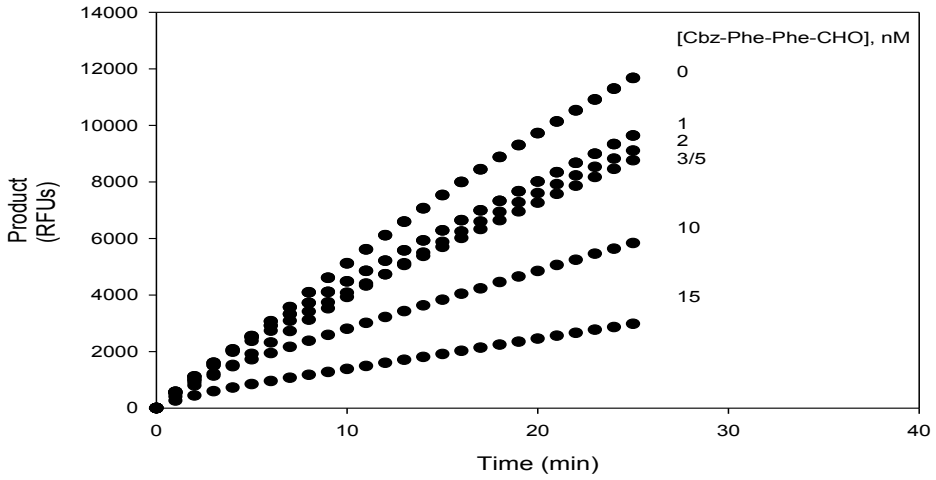

Figure 4. Tight-binding inhibition time course shows inhibition of falcipain-2 by Cbz-Phe-Phe-CHO. Falcipain-2 activity decreases as concentration of Cbz-Phe-Phe-CHO increases.

of falcipain-2 activity decreased, following a time course that was characteristic of tight-binding inhibition and shown to be extremely potent. The K_i^* was calculated from fitting of the data to the equation shown in Figure 5 using SigmaPlot to be 250 ± 10 pM.

$$P_t = v_s t + \left(\frac{v_i - v_s}{k_{obs}}\right)\left(1 - e^{k_{obs} t}\right)$$

Figure 5. Tight-binding inhibition equation in which P_t is equal to the AMC formed at time t, v_s and v_i are the steady-state and initial velocities of the cysteine protease, and k_{obs} is the apparent rate of conversion of EI to EI^* at a given concentration of inhibitor (I).

Characterization of Cbz-Phe-Phe-VSPh

The inactivator Cbz-Phe-Phe-VSPh was used to inactivate falcipain-2. A time-dependent, tight-binding inhibition evaluation was run in which 130 nM falcipain-2 was mixed with Cbz-Phe-Phe-VSPh, and the reaction mixture was allowed to pre-incubate for 60 minutes before addition of 50 μM Cbz-Phe-Arg-AMC as the substrate to initiate the reaction. The tight-binding inhibition time course is shown in Figure 6. As the concentration of Cbz-Phe-Phe-VSPh increased, the rate

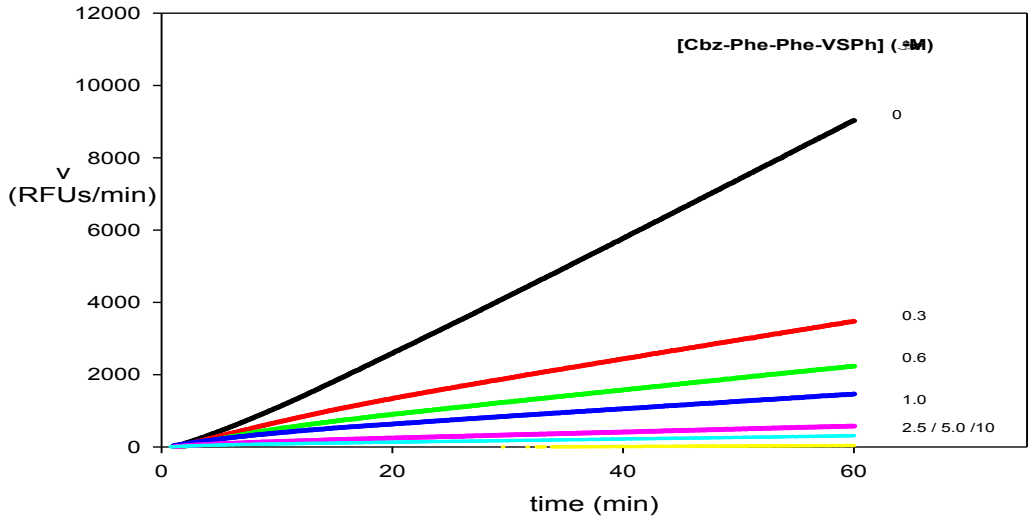

Figure 6. Tight-binding inhibition time course shows inhibition of falcipain-2 by Cbz-Phe-Phe-VSPh. Falcipain-2 activity decreases as concentration of Cbz-Phe-Phe-VSPh increases.

of falcipain-2 activity decreased. The K_i^* was calculated from fitting of the data to the equation shown in Figure 5 using SigmaPlot to be 32 ± 1 nM.

Characterization of Cbz-Phe-Phe-2VP

The novel inactivator Cbz-Phe-Phe-2VP was first used to inactivate cathepsin L as a model for falcipain-2. A time-dependent, tight-binding evaluation was run in which cathepsin L was mixed

with Cbz-Phe-Phe-2VP, and the reaction mixture was allowed to pre-incubate for 60 minutes before addition of 20 μM Cbz-Phe-Arg-AMC as the substrate to initiate the reaction. As the concentration of Cbz-Phe-Phe-2VP is increased, the rate of cathepsin L decreased. The vinyl-pyrimidine inhibitor was an apparent competitive inhibitor of cathepsin L. The K_i^* value was calculated from fitting of the data to the equation shown in Figure 5 using SigmaPlot to be 1.0 ± 0.1 μM.

The novel inactivator Cbz-Phe-Phe-2VP was used to inactivate falcipain-2. A time-dependent, tight-binding inhibition evaluation was run in which 130 nM falcipain-2 was mixed with Cbz-Phe-Phe-2VP, and the reaction mixture was allowed to pre-incubate for 60 minutes before addition of 50 μM Cbz-Phe-Arg-AMC as the substrate to initiate the reaction. The tight-binding inhibition time course is shown in Figure 7. As the concentration of Cbz-Phe-Phe-2VP increased, the rate of falcipain-2 activity decreased. The K_i^* was calculated using SigmaPlot and the equation in Figure 3 to be 70 ± 20 nM.

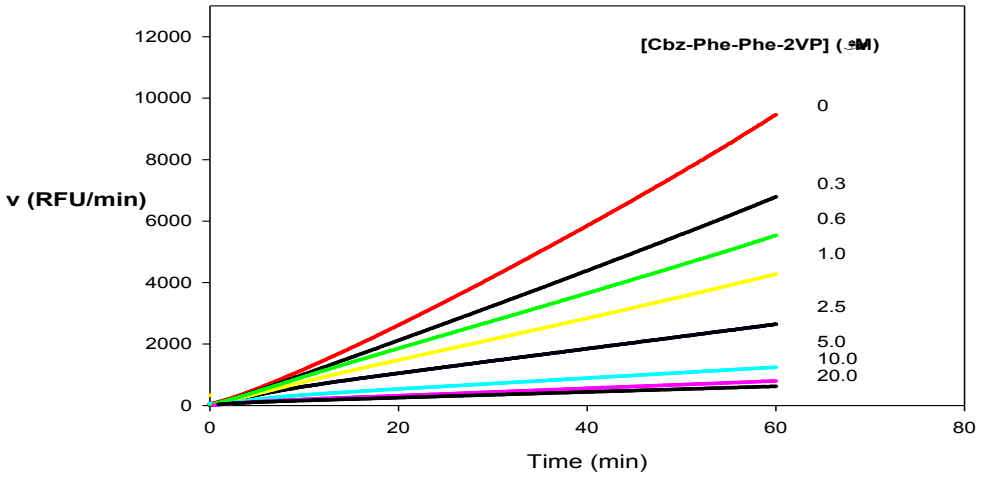

Figure 7. Tight-binding inhibition time course shows inhibition of falcipain-2 by Cbz-Phe-Phe-2VP. Falcipain-2 activity decreases as concentration of Cbz-Phe-Phe-2VP increases.

CHAPTER IV

CONCLUSIONS

The inactivators that we synthesized all effectively inhibited falcipain-2, some demonstrating tight-binding inhibition/inactivation. The K_i^* values that we determined in the time-dependent tight-binding inhibition reactions are shown in Table II. The most potent inactivator, Cbz-Phe-Phe-CHO, is likely to be too non-specific to be a potential drug candidate,

Table II. Inhibition Constant K_i^* values describing the inhibition of enzyme with each inactivator

Inactivator	Enzyme	Inhibition Constant K_i^* [5]
Cbz-Phe-Phe-CHO	falcipain-2	0.25 ± 0.01
Cbz-Phe-Phe-VSPh	falcipain-2	32 ± 1
Cbz-Phe-Phe-2VP	cathepsin L	2950
	falcipain-2	70 ± 20

although its inhibition constant indicates very tight-binding. The next most potent inactivator, Cbz-Phe-Phe-VSPh, achieved Phase I clinical trials to use as an inhibitor against falcipain-2 for the treatment of malaria. It failed, however, because it was not specific enough against falcipain. Our novel inactivator, Cbz-Phe-Phe-2VP, did not bind as tightly to falcipain-2 as Cbz-Phe-Phe-CHO and Cbz-Phe-Phe-2VP, but it still effectively inhibited it with apparent tight-binding inhibition. When compared to its inhibition of cathepsin L, the Cbz-Phe-Phe-2VP inhibition of falcipain-2 was much more potent. This suggests that our novel inactivator has some level of specificity for falcipain-2 as opposed to other enzymes even when compared to its interactions

with those that have similar substrate specificities, such as cathepsin L. The next steps toward creating a potential drug candidate for malaria by targeting falcipain-2 are to further characterize our compound and to compare its inhibition of falcipain-2 to that of other enzymes in the cysteine protease family. This will help us better understand its specificity for falcipain-2, which in turn could lead to a more effective drug candidate. Substitution of the pyrimidine ring on the novel inactivator, Cbz-Phe-Phe-2VP, with electron-donating and electron-withdrawing groups will allow for "tuning" of the electrophilicity of the vinyl "warhead" of the peptidomimetic inhibitor. This change in electrophilicity will change the ability of the compound to react with specific enzymes. With the flexibility that this gives us, a compound that is both potent and specific against the malarial enzyme falcipain-2 will likely be seen in the near future.

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