Identification and characterization of 3-substituted pyrazolyl esters as alternate substrates for cathepsin B: The confounding effects of DTT and cysteine in biological assays

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Abstract—Substituted pyrazole esters were identified as hits in a high throughput screen (HTS) of the NIH Molecular Libraries Small Molecule Repository (MLSMR) to identify inhibitors of the enzyme cathepsin B. Members of this class, along with functional group analogs, were synthesized in an effort to define the structural requirements for activity. Analog characterization was hampered by the need to include a reducing agent such as dithiothreitol (DTT) or cysteine in the assay, highlighting the caution required in interpreting biological data gathered in the presence of such nucleophiles. Despite the confounding effects of DTT and cysteine, our studies demonstrate that the pyrazole 1 acts as alternate substrate for cathepsin B, rather than as an inhibitor.

Keywords: Cathepsin B; DTT; Cysteine; Alternate substrate; HTS; MLMR; Pyrazole esters.

Cathepsins play an important role in cellular protein degradation through the lysosomal pathway,1 and as such, represent a significant class of drug targets, having been implicated in a variety of degenerative and invasive processes including: asthma, Alzheimer’s disease,2 cancer,3,4 diabetes, inflammatory diseases,5 liver disorders, multiple sclerosis, muscular dystrophy, and pancreatitis.6 The large number of disease states associated with the biological effects of the cathepsin protease family, and specifically cathepsin B, demands an understanding of their biological function. The availability of small molecule probes of the cathepsins thus holds the promise both for characterization of this ubiquitous enzyme class and for the discovery of new cysteine protease inhibitors of considerable biomedical value.3,7,8

Recently, the Penn Center for Molecular Discovery (PCMD)9 completed a high throughput screen (HTS) to identify small molecule probes (i.e., inhibitors) for the papain-like cysteine protease family, including cathepsins B, L, and S. While a number of both potent and selective inhibitors have been described previously,2-8 this project presented an opportunity to annotate the NIH Molecular Libraries Small Molecule Repository (MLSMR) through deposition of data in PubChem.10 As such, this work represents one of the first efforts to create a comprehensive, publicly available profile of small-molecule inhibitors of the cysteine protease class. Screening 63,332 members of the MLSMR against human liver cathepsin B resulted in a number of hits.11 Further confirmatory assays included IC50 determination and elimination of false positives resulting from non-specific redox chemistry.12 Based on these results, a family of substituted pyrazole esters was identified that displayed promising activity as inhibitors of cathepsin B (Table 1).13

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Table 1. Pyrazole HTS hits in the cathepsin B assay

<table>
<thead>
<tr>
<th>PubChem SID</th>
<th>X</th>
<th>R</th>
<th>IC50 (µM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>4249135 (1)</td>
<td>S</td>
<td>H</td>
<td>0.25 ± 0.03</td>
</tr>
<tr>
<td>4247730 (2)</td>
<td>S</td>
<td>F</td>
<td>0.44 ± 0.11</td>
</tr>
<tr>
<td>4245669 (3)</td>
<td>S</td>
<td>OMe</td>
<td>0.69 ± 0.10</td>
</tr>
<tr>
<td>844213 (4)</td>
<td>S</td>
<td>Me</td>
<td>1.99 ± 0.17</td>
</tr>
<tr>
<td>849441 (5)</td>
<td>O</td>
<td>Me</td>
<td>1.75 ± 0.06</td>
</tr>
<tr>
<td>845259 (6)</td>
<td>O</td>
<td>F</td>
<td>1.26 ± 0.04</td>
</tr>
</tbody>
</table>

*IC50 values are reported as means ± standard deviation (number of determinations = 3).

The pyrazole series (1–6) displayed suitable physicochemical properties (MW 347–379 and XlogP = 2.7–3.3) with structurally related analogs revealing a range of activities in our HTS analysis. Based on these attributes, members of this series were viewed as potential small molecule lead compounds for the inhibition of cathepsin B, and as such were selected for further study to understand and improve their biological profile.

Structurally related pyrazoles that were inactive in the HTS assay were compared to compounds 1–6 in a preliminary effort to understand the requirements for activity. Based on this analysis, we pinpointed the ester group as a functional moiety important for activity; we reasoned that the ester could act as an electrophilic site that would react with the active site cysteine. Alternatively, the compound could behave as an alternate substrate of the enzyme resulting in transesterification or hydrolysis of the ester functionality. To differentiate these two scenarios, a series of analogs were constructed and subjected to biochemical and analytical studies to define the mechanism of action. Herein we report the results of this effort.

To confirm both the activity and identity of the original hits, we resynthesized compounds 1–6.14,15 This exercise permitted both the development of an effective method to construct the pyrazole scaffold and the preparation of analogs. The synthesis of 1,3-disubstituted-5-amino-4-pyrazolones (1–6) began with commercially available cyanacetohydrazide (7) and the appropriate aryl-substituted sulfonyl chlorides, according to the procedure of Elgemeie et al.16,17 Reaction of 7 with the aryl-substituted sulfonyl chlorides in ethanol furnished the sulfonamides 8–11 which conveniently precipitated from solution (Scheme 1).

As expected, the major by-products, aryl ethyl sulfonates, remained soluble and were easily removed from 8–11 via filtration. Thus, on a 50 mmol scale, gram quantities of the required sulfonamide hydrazides could be readily prepared in high purity. Efficient cyclizations of sulfonamide hydrazides (8–11) to pyrazolones (12–15) occurred upon treatment with an aqueous solution of NaOH, followed by acidification (10% HCl) to precipitate the pyrazolones.18 The pyrazolones were used in the subsequent acylation step without purification. For example, treatment of 12–15 with 2-thiophenecarbonyl chloride or 2-furancarbonyl chloride in chloroform resulted in chemoselective19 acylation at the C3-oxygen versus the C5-amino group to furnish pyrazoles 1–6 in moderate to good yields after silica gel chromatography.

The availability of newly synthesized pyrazoles 1–6 allowed confirmation of the structures and observed biological activity of the initial hits. Pleasingly, both the structures and re-assay IC50 values of synthetic pyrazoles 1–6 were in agreement with the results obtained from the original screening experiments (Table 1).

As expected, the most electrophilic center for pyrazole ester 1 was the carbonyl carbon of the ester, with an electrostatic potential value of 128.0 kJ/mol. The carb-
bons of the epoxide ring in E64 had values of 104.3 kJ/mol and 104.7 kJ/mol for their electrostatic potential surface energies. The greater positive value of 128.0 kJ/mol suggests that the carbonyl carbon of I is more prone to nucleophilic attack than the epoxide carbons in the irreversible cathepsin B inhibitor E64. This difference in electrophilicity was confirmed experimentally (vide infra). Presumably, the stability of the pyrazolone by-product of I provides the driving force for attack of this atom center.

We reasoned that, modulating the electronic parameters of the electrophilic ester functionality in this series of compounds would quickly validate or eliminate the importance of this structural feature. To test this hypothesis, pyrazole 14 was employed as a common intermediate to construct ether 16, sulfonate 17, and isonicotinoyl ester 18 (Fig. 2).

Ether 16 was formed via an alkylation of 14 with 2-bromomethyl-thiophene in moderate yield. Sulfonate 17 and isonicotinoyl ester 18 were constructed under chemoselective acylation conditions similar to those employed to prepare pyrazoles 1–6. In support of the requirement of the ester functionality for biological activity, ether 16 and sulfonate 17 were found to be inactive. Surprisingly, however, related ester analog pyridine 18 was devoid of activity, despite its steric and electronic similarity to the thiophene moiety found in I. This unexpected result led us to question the stability of the pyrazole hits (e.g., hydrolysis or transesterification) under the assay conditions. To probe these issues, we undertook further biological characterization of I, as well as a detailed analysis of assay by-products. Kinetic analysis of the inhibition of cathepsin B by I revealed a competitive inhibition pattern, in which I increased the K_m of the substrate but had no effect on the V_max. The K_i of I was determined to be 0.9 μM (±) 0.25 μM (Fig. 3). As a control, a biochemical time course experiment was designed. Pyrazole I was combined with the assay buffer (phosphate, pH 6.8) over a series of time points (0, 2, 17, and 24 h). Samples at each time point were then incubated with cathepsin B (0.1 nM) in the buffer (100 mM) containing DTT (2 mM) under the standard assay conditions, and IC_{50} data were measured. Results from these experiments indicated a significant loss of inhibitory activity which correlated with the time of pre-incubation (Table 2).

Table 2. Time dependent pre-incubation of I with and without DTT: an analysis of I in the cathepsin B assay

<table>
<thead>
<tr>
<th>Pre-incubation time (h)</th>
<th>Assay buffer with DTT</th>
<th>Assay buffer without DTT</th>
<th>Non-buffered without DTT</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>1.4</td>
<td>0.8</td>
<td>0.8</td>
</tr>
<tr>
<td>2</td>
<td>9.0</td>
<td>1.4</td>
<td>1.7</td>
</tr>
<tr>
<td>17</td>
<td>45.4</td>
<td>0.7</td>
<td>1.8</td>
</tr>
<tr>
<td>24</td>
<td>&gt;50</td>
<td>3.2</td>
<td>5.0</td>
</tr>
</tbody>
</table>

^a IC_{50} values are calculated from a dose response curve of 16 2-fold dilutions.

The IC_{50} values of pyrazole I increased from 1.42 μM at a pre-incubation time of 0 h to >50 μM at an incubation time of 24 h. Two additional experiments were conducted to pinpoint the cause of the time dependent loss of activity: (1) pyrazole I was added to an assay buffer that did not contain DTT (Column 2, Table 2); and (2) pyrazole I was added to a non-buffered solution also without DTT (Column 3, Table 2). Both experiments revealed that the IC_{50} values changed slightly from the 0
to 24 h incubation time. Thus, the presence of DTT was deemed largely responsible for the significant loss in activity.

With these results in hand, we set out to define more precisely the role of DTT in the inactivation of 1. Analytical experiments were designed to mimic the biochemical pre-incubation experiment, wherein the aqueous solutions of 1 with and without DTT would be analyzed via LC–MS for hydrolysis products (e.g., pyrazolone 12). The LC–MS analysis corroborated the previous study and also provided an additional detail, the loss of 1 was the result of its conversion to pyrazolone 12 (Table 3).

Table 3. Time dependent pre-incubation of 1 with and without DTT: an analysis of 1 by LC–MS

<table>
<thead>
<tr>
<th>Incubation time (h)</th>
<th>Assay buffer with DTT</th>
<th>Assay buffer without DTT</th>
<th>Non-buffered with DTT</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>72</td>
<td>5</td>
<td>5</td>
</tr>
<tr>
<td>2</td>
<td>80</td>
<td>5</td>
<td>6</td>
</tr>
<tr>
<td>16</td>
<td>87</td>
<td>13</td>
<td>11</td>
</tr>
<tr>
<td>24</td>
<td>88</td>
<td>17</td>
<td>9</td>
</tr>
</tbody>
</table>

* LC–MS analysis conducted on a 6.5 min run time (100 μM sample concentration).

For example, when 1 was incubated in the DTT containing assay buffer, conversion to 12 occurred quickly with 28% of 1 remaining after 1 h, and only 12% after 24 h. Hydrolysis of 1 to 12 also occurred, albeit slowly, in both buffer without DTT and non-buffered water. However, on the time-scale of the HTS assay, the contribution of this degradation pathway to the activity was negligible. Additional experiments indicated that when E64 was exposed to DTT (under the same conditions as listed in Table 3) it was found to be stable with no E64 decomposition and no DTT-E64 adduct formed, as monitored by LC–MS.

Anticipating that 12 could arise via nucleophilic displacement of the thiophene ester with DTT, we examined the LC–MS data for the anticipated transesterification DTT-thiophene ester product 19 (Scheme 2).

The molecular ion corresponding to 19 (M+1 = 265) was observed in the LC–MS analysis of the assay buffer with DTT. As further verification, adduct 19 was synthesized independently using DTT and 2-thiophencarboxyl chloride, and used as a standard in the LC–MS analysis. Bioassay of 19 with cathepsin B revealed no activity. Taken together, these data reveal the basis for the diminished IC_{50} values following extended pre-incubation times.

The inherent redox chemistry associated with DTT is well known, both in connection with the identification of false positives in cysteine protease assays and with enzymes such as phosphatases. To avoid the redox issues of DTT, cysteine is often used in place of DTT due to its lower oxidation potential. Less well appreciated, however, is the propensity of DTT to act as a nucleophile, thereby confounding bioassay results. Unfortunately, in our case, replacement of DTT with cysteine does not alleviate the problem. Indeed, the same fast conversion of 1-12 was observed, via LC–MS, in the presence of cysteine. Thus, the series of pyrazole esters is susceptible to both the hydrolysis and transesterification pathways. We were, however, intrigued by the apparent lack of any activity of 18, despite the similar size and electronic attributes of the ester moiety. To explore this observation, we analyzed the rate of hydrolysis and transesterification of 18. Surprisingly, incubation of 18 in assay buffer, with and without DTT, and a non-buffered aqueous solution, revealed rapid conversion (ca. 1 h) to pyrazolone 14, regardless of the presence of DTT (Table 4).

Table 4. Time dependent pre-incubation of 18 with and without DTT: an analysis of 18 by LC–MS

<table>
<thead>
<tr>
<th>Incubation time (h)</th>
<th>Assay buffer with DTT</th>
<th>Assay buffer without DTT</th>
<th>Non-buffered without DTT</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>&gt;99</td>
<td>41</td>
<td>15</td>
</tr>
<tr>
<td>2</td>
<td>&gt;99</td>
<td>80</td>
<td>24</td>
</tr>
<tr>
<td>16</td>
<td>&gt;99</td>
<td>&gt;99</td>
<td>95</td>
</tr>
<tr>
<td>24</td>
<td>&gt;99</td>
<td>&gt;99</td>
<td>99</td>
</tr>
</tbody>
</table>

* LC–MS analysis conducted on a 6.5 min run time (100 μM sample concentration).

The ease of hydrolysis and esterification of 18 is likely a reflection of the fact that the pyridine ring is partially protonated under the conditions. Thus, within the timescale of the cathepsin B assay (1 h incubation with the enzyme), a negligible amount 18 would be present.

Taken together, the results support the hypothesis that 1 acts as an alternate cathepsin B substrate rather than an
inhibitor. Presumably, the active site cysteine acts much like DTT to transesterify 1, and thereby forms a transient thiophenyl-enzyme intermediate. 7,22,23 To test this scenario, a stoichiometric reaction was devised to incubate pyrazole ester 1 (1 equiv) with and without cathepsin B (2 equiv) (Table 5).

Table 5. Standard assay conditions with and without cathepsin B: a stoichiometric reaction analyzed by LC–MS

<table>
<thead>
<tr>
<th>Assay conditions with cathepsin B</th>
<th>Assay conditions without cathepsin B</th>
</tr>
</thead>
<tbody>
<tr>
<td>0% remaining 1</td>
<td>50% remaining 1</td>
</tr>
</tbody>
</table>

*aLC–MS analysis conducted on a 6.5 minute run time (20 µM cathepsin B; 10 µM 1).

Under these stoichiometric reaction conditions, in the presence of cathepsin B, pyrazole 1 was fully converted to 12 after only 15 min. However, under identical conditions, in the absence of cathepsin B, 50% of pyrazole 1 remained. 8,24 We conclude that pyrazole esters such as 1 are competitive substrates for the enzyme cathepsin B.

In summary, we have demonstrated that pyrazole 1 acts as an alternate substrate for the cysteine protease, cathepsin B. Synthesis and evaluation of related analogs revealed the potential reactivity of the ester functionality with the nucleophilic enzyme active site cysteine to form a transient thiphenoyl-enzyme intermediate. Initially, the similar reactivity of DTT and cysteine in the bioassay confounded the HTS and subsequent assay results due to the nucleophilic properties of the thiol sulfur. Thus, it is important for the biological and chemical communities to consider the potential of DTT and cysteine to act as nucleophiles in assay systems where substrates contain electrophilic functionality.

Acknowledgments

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References and notes

13. Compounds were serially diluted in DMSO and transferred into the assay microplate using a 100-nL pipette to give 16 dilutions ranging from 50 µM to 1.5 nM. Triplicate plates were set up in this manner to give three independently calculated IC50 values for each compound. Cathepsin B (Calbiochem 219362) was activated by incubating with assay buffer for 15 min. Assay buffer consisted of 100 mM sodium-potassium phosphate, pH 6.8 (86 mM potassium phosphate, monobasic; 7 mM sodium phosphate, monobasic; 7 mM sodium phosphate, tribasic), 1 mM EDTA, and 2 mM DTT. Upon activation, cathepsin B was incubated with Z-Arg-Arg-AMC substrate (15 µM) and test compound in 10 µL of assay buffer for 1 h at room temperature. Fluorescence of AMC released was read on a Perkin-Elmer Envision microplate reader (excitation 355 nm, emission 460 nm).
14. Characterization data for 1. Mp = 167 °C; IR (thin film, CHCl3) 3479, 3328, 1733, 1621, 1189, 610 cm–1; 1H NMR (500 MHz, DMSO-d6) δ 8.11 (m, 1H), 7.98 (m, 1H), 7.94 (m, 2H), 7.79 (m, 1H), 7.69 (m, 2H), 7.29 (m, 1H), 6.57 (s, 3H), 5.36 (s, 1H); 13C NMR (125 MHz, DMSO-d6) δ 158.4, 158.0, 152.4, 136.5, 136.2, 135.9, 134.9, 130.4, 129.8, 128.9, 127.2, 80.5; high resolution mass spectrum (ES+) m/z 350.0265 [(M+H)+; calcd for C14H11N3O4S2H+: 350.0269].
15. A single crystal X-ray structure of pyrazole ester 2 was also obtained to verify its structure.
20. PC Spartan is available from Wavefunction, Inc., 18401 Von Karman Avenue, Suite 370 Irvine, CA 92612.

24. Reversibility of inhibition was demonstrated by incubating I at a concentration 10× IC50 with enzyme at 100-fold greater than the usual assay concentration for 15-min. Following the 15 min incubation the mixture was diluted 100-fold into assay buffer containing substrate, and the enzyme was assayed. This dilution gave the usual assay concentration of enzyme, and I at 0.1× IC50, a concentration at which most of the enzymatic activity should be regained if the compound is rapidly reversible.

25. Pyrazolones 12, 14, and 15 were found to be inactive in the cathepsin B assay.


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