Exploration of a Series of 5-Arylidene-2-thioxoimidazolidin-4-ones as Inhibitors of the Cytolytic Protein Perforin

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ABSTRACT: A series of novel 5-arylidene-2-thioxoimidazolidin-4-ones were investigated as inhibitors of the lymphocyte-expressed pore-forming protein perforin. Structure–activity relationships were explored through variation of an isodindolinone or 3,4-dihydroisoquinolinone subunit on a fixed 2-thioxoimidazolidin-4-one/thiophene core. The ability of the resulting compounds to inhibit the lytic activity of both isolated perforin protein and perforin delivered in situ by natural killer cells was determined. A number of compounds showed excellent activity at concentrations that were nontoxic to the killer cells, and several were a significant improvement on previous classes of inhibitors, being substantially more potent and soluble. Representative examples showed rapid and reversible binding to immobilized mouse perforin at low concentrations (≤2.5 μM) by surface plasmon resonance and prevented formation of perforin pores in target cells despite effective target cell engagement, as determined by calcium influx studies. Mouse PK studies of two analogues showed T_{1/2} values of 1.1–1.2 h (dose of 5 mg/kg iv) and MTDs of 60–80 mg/kg (ip).

INTRODUCTION

The key effector cells of the immune system, cytotoxic T lymphocytes (CTL), and natural killer (NK) cells eliminate virus-infected and transformed cells principally through the granule exocytosis pathway. CTLs and NK cells contain secretory vesicles (granules) that are used to store various cytotoxic proteins, including a group of proapoptotic serine proteases (granzymes) and perforin, a pore-forming glycoprotein. The crystal structure of monomeric murine perforin has been determined to 2.75 Å resolution and reveals a bent and twisted -sheet MACPF domain flanked by two clusters of fl-helices, an epidermal growth factor (EGF) domain, and a C-terminal C2 domain that mediates initial, calcium-dependent membrane-binding. The mechanism of pore formation has also been elucidated; upon exposure of perforin to the neutral, calcium-rich extracellular environment of the immune synapse, deprotonation of key aspartate residues and subsequent coordination of calcium takes place. This process triggers conformational changes that result in the assembly of highly ordered oligomers of 19–24 subunits that form a pore with an internal diameter of 130–200 Å. Site-directed mutagenesis has established that direct ionic interactions between the opposing faces of adjacent perforin monomers (Arg213 and Glu343 in particular) serve to assist pore self-assembly and stabilization of the resulting pore.

Perforin is encoded on a single-copy gene in both mice and humans, and while many of the granule components possess at least some degree of redundancy, perforin is absolutely essential for protective immunosurveillance. Perforin knockout mice show a reduced ability to reject many tumor xenografts, and when backcrossed with nonobese diabetic mice, the incidence of spontaneous diabetes is reduced from 77% in a perforin +/+ strain to 16% in perforin-defficient mice, with onset of disease markedly delayed. This is because perforin is critical to deliver an autoimmune “lethal hit” against the insulin-producing pancreatic β cells. Perforin-deficient mice also demonstrate increased susceptibility and failure to clear many viruses and other
in intracellular pathogens, and highly aggressive disseminated B cell lymphomas occur in the majority of animals over the age of 12 months. In humans, complete loss of perforin function results in familial hemophagocytic lymphohistiocytosis (FHL) syndrome, a severe immunoregulatory disorder usually diagnosed in infancy and characterized by severe anemia and hepatosplenomegaly, fever, and thrombocytopenia.13,14

There is abundant evidence to implicate inappropriate perforin activity in a number of human pathologies, including cerebral malaria, insulin-dependent diabetes, juvenile idiopathic arthritis, and postviral myocarditis,15−17 as well as therapy-induced conditions such as allograft rejection and graft-versus-host disease.18−20 Immunosuppressive agents used to treat these diseases are generally associated with a wide range of side effects, many of which arise from the unintended impact of the drug treatment on nontherapeutic targets.21,22 As perforin is expressed only by “killer lymphocytes”, a selective inhibitor should result in fewer off-target effects, meaning a small molecule inhibitor of perforin is of great interest as a new class of highly specific immunosuppressive agents.17

In our initial work in this area, we explored two series of compounds (1 and 2, Figure 1) that showed appreciable perforin-inhibitory activity but possessed limitations such as poor aqueous solubility or reduced activity in serum, precluding further development.23,24 We have substantially overcome these issues in our current series of 5-arylidene-2-thioxoimidazolidin-4-one-based inhibitors (3, 4)25,26 and now seek to maximize inhibitory activity and optimize “druglike” properties. This class of rhodanine-related heterocycle has recently become the subject of considerable debate in the medicinal chemistry community because of several publications classifying such substructures as either “privileged scaffolds” with potentially valuable biomolecular binding properties27−29 or “frequent/promiscuous hitters” and pan-assay interference compounds (PAINS) which should not be pursued further.30−32 While it is clear that these chemotypes should be carefully monitored in development, recent literature contains many examples of selective inhibitors of various target proteins containing such substructures.26,27,33,34 In addition, many of these moieties also occur in drugs that are clinically useful, providing templates with valuable structural features favoring protein binding which may be exploited in an advantageous way. Nevertheless, our current lead compound was subjected to and passed the PAINS filters30 (Professor Jonathan Baell, personal correspondence)35 possibly because of the presence of an extra nitrogen in the core five-membered ring.

Herein we will show that we have identified an exciting new class of inhibitors that unambiguously target perforin, offering up a novel mode of action and the potential to be further developed as immunosuppressive agents for the effective treatment of transplant rejection and selected autoimmune diseases.

■ RESULTS AND DISCUSSION

Lead compound 3 was selected from a small number of hits that showed reproducible perforin-inhibitory activity in a high throughput screen of approximately 100 000 compounds sourced from commercial libraries.36 Compound 3 can be readily disconnected into three subunits, a 2-thioxoimidazolidin-4-one (A), a furan (B), and a benzofuranone (C), lending itself to a study design where one unit can be independently varied in the presence of two other fixed subunits. We have reported previously our study of the structure−activity relationships (SAR) resulting from variation of the A- and B-subunits,26 and here we focus on the effect of introducing a wide range of C-subunits. Since thiophene 4 was one of the more potent compounds in our initial work, the current study explores the effect of replacing the benzofuranone C-subunit on a fixed 2-thioxoimidazolidin-4-one/thiophene scaffold. The requirement for substitution on the C-subunit aryl ring is explored, followed by a more detailed investigation focusing on compounds containing a bicyclic moiety.

**Synthesis of Target Compounds.** The compounds of Tables 1, 2, and 3 were prepared by heating a range of substituted aldehydes with 2-thioxoimidazolidin-4-one in acetic acid in the presence of β-alanine (Schemes 1−5). As a consequence, the majority of the chemistry that is described here involves the preparation of key aldehyde intermediates. For the compounds of Table 1, a small number of protected aldehydes (6−8) were prepared by Suzuki reaction of 2-(5-bromothiophen-2-yl)-1,3-dioxolane (5) with substituted phenylboronic acids, followed by deprotection (Scheme 1). The remaining aldehydes were commercially available with the exception of amides 34−42, which were prepared from carboxylic acid 28 using standard amide coupling conditions. All the required aldehydes 9−42 were then converted to final compounds 43−75.

Table 2 contains compounds where the benzofuranone of 3 has been replaced with a variety of substituted isoindoliones (118−135, 165, 166) and 3,4-dihydroxysoquinolin-1(2H)-ones (167−170). The isoindolione targets were bromination of methyl ester 76, followed by ring closure onto a variety of amines to give intermediate iodides 77−81 (Scheme 2). These iodides were then employed in a palladium-catalyzed reaction using an AgNO₃/KF activator system, as originally described by Mori et al.37 Coupling of the iodides with either 2-(thiophen-2-yl)-1,3-dioxolane or 2-(dimethoxymethyl)-thiophene at the vacant 5-position of the thiophene gave the desired protected aldehydes 82−86. Compound 82 was further modified through NaH/RX alkylations of the isoindolione NH to afford 87−92. The NMe derivative 87 was alkylated a second time using LDA and methyl iodide to give methylation on the isoindolione CH₂ (93) in 65% yield. Deprotections of 82−93 were carried out under acidic conditions to give the required aldehydes 94−106 which were then reacted with 2-thioxoimidazolidin-4-one to give final compounds 118−130 and 135. Likewise 136 (Table 3) was obtained by reaction of aldehyde 82 with imidazoline-2,4-dione. A small set of amino-substituted compounds (131−134) were also prepared from the C3 alcohol 101. The aldehyde substituent of 101 was initially protected as the dimethyl acetal 107. Then the alcohol converted to the...
mesylate 108 using mesyl chloride and TEA in THF. The mesylate was exchanged for iodide by heating with a large excess of NaI in acetone. Then the iodide of 109 was displaced with various amines to give intermediates 110–113. Deprotection as described above gave the key aldehydes 114–117 which were reacted with 2-thioimidazolidin-4-one to give final compounds 131–134.

Scheme 3 describes the preparation of “reverse” and five- and six-membered lactams 165–170 (Table 2). The five-membered lactam boronates 147 and 148 were prepared from the corresponding bromides 137 and 138 by Pd-catalyzed reaction with bis(pinacolato) diboron. The four six-membered lactam boronates 149–152 were prepared in the same manner from the bromides 143–146 which were in turn synthesized from the carbamates 139–142 according to literature procedures.38,39 The boronates were then each employed in a Suzuki reaction with bromide 5 to give compounds 153–158 which were deprotected and reacted with 2-thioimidazolidin-4-one to afford final compounds 165–170. For comparison with the isoindolinones, a single indole-based compound, 3-acetylindole 178, was also prepared (Scheme 4). 5-Bromoindole 171 was protected and acetylated according to literature procedures,40,41 followed by displacement of the benzenesulfonyl protecting group to give the corresponding N-methylindole 174. Subsequent Suzuki coupling, deprotection, and reaction with 2-thioimidazolidin-4-one gave target compound 178.

For the compounds of Table 3, protected bromothiophene- (179), bromobenzene- (180, 181), bromopyridine- (182), bromoindole- (183, 184), or bromoquinolone- (185) carboxylic acids were reacted with either commercially available 5-(4,4,5,5-tetramethyl-1,3,2-dioxaborolan-2-yl)isoindolin-1-one or boronate 149 as described above, deprotected to aldehydes 195–203, and converted to final products 204–212 (Scheme 5).

In Vitro Activities of the 5-Arylidene-2-thioimidazolidin-4-ones. In the first instance, the isobenzofuran-1(3H)-one C-subunit was replaced with a large range of substituted-benzene derivatives (Table 1). Substitution on this ring is clearly required, with phenyl (43) possessing no detectable activity. Each position was then surveyed with a range of substituents, and while methyl and methoxy proved to be inactive (see Supporting Information), a variety of electron-withdrawing substituents (halogen, CF₃, cyano, hydroxyl, carboxyl) did produce activity. The results for a series of chloro (44–46), fluoro (47, 48), and CF₃ (50, 51) compounds show a clear preference for substitution at the 4-position. Carboxy-based examples (59–61) were among the most potent, although in this instance the 3-carboxamide (60, IC₅₀ = 0.79 μM) was slightly better than its 4-isomer (61, IC₅₀ = 1.56 μM). Methylation of the carboxamide NH of 61 resulted in a reduction of activity (67, 68), while amide-linked weakly basic side chains (69–71) were generally poor. The corresponding amide-linked hydroxylated side chains (72–75), however, exhibited significant activity (IC₅₀ = 2.44–4.35 μM) but were still not as potent as the primary amide 61. Other compounds with good activity were the 4-Br (55, 1.69 μM), 4-SMe (56, 1.15 μM), and 4-acyl (57, 1.67 μM). The “acyclic” analogue of 4, compound 62, showed a 4-fold reduction in activity over its cyclic counterpart (IC₅₀ = 3.03 μM compared to 0.78 μM).

Although the above study (Table 1) produced some potent compounds, none were an improvement on lead compound 4. An alternative approach where the entire isobenzofuran-1(3H)-one unit was replaced with an isoindolin-1-one was therefore implemented (Table 2), resulting in a series of compounds considered less susceptible to hydrolysis (amide versus ester) and where the nitrogen atom provides an additional position at which a side chain can be appended. 

Isoindolin-1-one 118 showed a reduction of activity over lactone 4 (from IC₅₀ = 0.78 μM to IC₅₀ = 2.55 μM). Activity was restored through substitution on the nitrogen atom, with both methyl-substituted (119, IC₅₀ = 0.51 μM) and ethyl-substituted (120, IC₅₀ = 0.60 μM) compounds more potent than the lead compound 4. A more bulky substituent such as isopropyl (123, IC₅₀ = 4.42 μM) was less acceptable, but the acetate 128 and carbamate 129 both showed excellent activity (IC₅₀ = 0.93 and 0.55 μM, respectively). In this series it also appears possible to introduce various side chains off the isoindolinone and maintain activity, with the two alcohols 125 and 126 among the most

### Table 1. Inhibitory Activities of C-Subunit Variants

| compd | R               | IC₅₀ (μM) |
|-------|-----------------|----------|
| 4     | see Figure 1    | 0.78     |
| 43    | H               | >20      |
| 44    | 2-Cl            | >20      |
| 45    | 3-Cl            | 7.19     |
| 46    | 4-Cl            | 5.22     |
| 47    | 3-F             | 10.77    |
| 48    | 4-F             | 6.69     |
| 49    | 3,4-di-F        | 4.59     |
| 50    | 3-CF₃           | 5.66     |
| 51    | 4-CF₃           | 4.66     |
| 52    | 3-CN            | 9.68     |
| 53    | 4-OH            | 7.72     |
| 54    | 3-CF₃, 4-Cl     | 3.09     |
| 55    | 4-Br            | 1.69     |
| 56    | 4-SMe           | 1.15     |
| 57    | 4-Ac            | 1.67     |
| 58    | 4-CH₂OAc        | 3.04     |
| 59    | 4-COOMe         | 1.62     |
| 60    | 3-CNH₂          | 0.79     |
| 61    | 4-CNH₂          | 1.56     |
| 62    | 3-Me, 4-COOMe   | 3.03     |
| 63    | 4-SO₂Me         | 13.80    |
| 64    | 4-NHSO₂Me       | 7.70     |
| 65    | 3-pyridyl       | 12.89    |
| 66    | 4-pyridyl       | 10.06    |
| 67    | 4-CNHMe         | 4.53     |
| 68    | 4-CNO₂Me        | 6.93     |
| 69    | 4-COMorpholine  | >20      |
| 70    | 4-CNH(CH₂)₂morpholine | >20  |
| 71    | 4-CNH(CH₂)₃morpholine | 8.13  |
| 72    | 4-CNH(CH₂)OH    | 3.64     |
| 73    | 4-CNH(CH₂)₂OH   | 4.35     |
| 74    | 4-CNHCH₂CH(CH₃)OH | 6.50  |
| 75    | 4-CNHCH(CH₃)OH| 2.44     |

“Testing was carried out over a range of concentrations, with the IC₅₀ being equal to the concentration at which 50% inhibition of the lysis of Jurkat cells by purified recombinant perforin was observed, as measured by ¹¹C release. Values are the average of at least three independent IC₅₀ determinations.”

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potent compounds of the class (IC$_{50}$ = 0.78 and 0.53 μM). Diol 127 and O-acetate 124 also demonstrated good activity, but when a basic amino substituent was introduced in an attempt to improve solubility, activity was virtually abolished with only morpholine (130, not reproducible), piperidine 132, and pyrrolidine 134 (IC$_{50}$ = 11.78 and 8.93 μM) showing any evidence of activity. Introduction of a methyl substituent on the lactam ring (135) resulted in poorer activity than the corresponding unsubstituted analogue (135 IC$_{50}$ = 3.21 μM vs 119 IC$_{50}$ = 0.51 μM).

Since the 3-carboxamide 60 was 2-fold more potent than 4-carboxamide 61 in the substituted benzene series (Table 1), the effect of reversing the orientation of the lactam ring was investigated (165). The same result was observed; the IC$_{50}$ for the compound with the lactam amide in the 3-position 165 (relative to the thiophene) was around 2-fold better than the corresponding lactam amide in the 4-position (118) (IC$_{50}$ = 1.38 μM compared to 2.55 μM). Expansion from a five-membered lactam ring (118) to a six-membered lactam ring (167) also resulted in a significant increase in activity (from IC$_{50}$ = 2.55 to IC$_{50}$ = 1.14 μM) and improved a further 2-fold (to 0.64 μM) through introduction of the “reverse” 3-lactam amide (168). N-Methylation of 165, 167, and 168 to give 166, 169, and 170, respectively, did not result in the same increase in activity seen when 118 was alkylated to give the analogous N-methyl compound 119.

Compounds 136 and 204–212 (Table 3) combine those B-subunits which were previously identified as having excellent activity in the isobenzofuranone-based series, with selected isoudolinones and 3,4-dihydroisoquinolin-1(2H)-ones from the current series. Compound 136 contains a imidazolidine-2,4-dione A-subunit and is the only example to show an improvement, being 3-fold better than the analogous isobenzofuranone reported in our initial paper (IC$_{50}$ = 0.40 μM compared to 1.19 μM). All other compounds (204–212) contain a 2-thioximidazolidin-4-one A-subunit and are 1.5- to 4-fold poorer than the corresponding isobenzofuranones (see Figure 1 in Supporting Information).

Those compounds that showed good inhibition of the purified protein were then also evaluated for their ability to inhibit perforin produced by an intact NK cell line (KHYG-1; see Experimental Section for details); this assay is a better model for in vivo studies, as the perforin is delivered by a functional NK cell. Elimination of any nonspecific toxicity against the effector cell, viability was also measured 24 h after the NK cells were exposed to the compounds (4 h, 37 °C), then washed and replated in culture. Elimination of any nonspecific

| compd | structure | X | R | inhibition of Jurkat cell lysis, IC$_{50}$ (μM)$^a$ |
|-------|-----------|---|---|----------------------------------|
| 118$^b$ | I | CH$_2$ | H | 2.55 |
| 119 | I | CH$_2$ | Me | 0.51 |
| 120 | I | CH$_2$ | Et | 0.60 |
| 121 | I | CH$_2$ | n-Pr | 1.52 |
| 122 | I | CH$_2$ | n-Bu | 1.18 |
| 123 | I | CH$_2$ | i-Pr | 4.42 |
| 124 | I | CH$_2$ | CH$_3$CH$_2$OAc | 1.17 |
| 125 | I | CH$_2$ | CH$_3$CH$_2$OH | 0.78 |
| 126 | I | CH$_2$ | CH$_3$CH$_2$CH$_2$OH | 0.53 |
| 127 | I | CH$_2$ | CH$_3$CH(OH)CH$_2$OH | 1.26 |
| 128 | I | CH$_2$ | Ac | 0.93 |
| 129 | I | CH$_2$ | C(O)OEt | 0.55 |
| 130 | I | CH$_2$ | CH$_3$CH$_2$morph | variable |
| 131 | I | CH$_2$ | CH$_3$CH$_2$CH$_2$NM$_2$ | >20 |
| 132 | I | CH$_2$ | CH$_3$CH$_2$CH$_2$pip | 11.78 |
| 133 | I | CH$_2$ | CH$_3$CH$_2$CH$_2$NMepiperazine | >20 |
| 134 | I | CH$_2$ | CH$_3$CH$_2$CH$_2$pyrrolidine | 8.93 |
| 135 | I | CH(CH$_3$)$_2$ | Me | 3.21 |
| 165$^c$ | II | CH$_2$ | H | 1.38 |
| 166 | II | CH$_2$ | Me | 1.89 |
| 167 | II | (CH$_2$)$_2$ | H | 1.14 |
| 168 | II | (CH$_2$)$_2$ | H | 0.64 |
| 169 | II | (CH$_2$)$_2$ | Me | 2.33 |
| 170 | II | (CH$_2$)$_2$ | Me | 4.76 |
| 178$^d$ | | | | 5.30 |

$^a$Testing was carried out over a range of concentrations, with the IC$_{50}$ being equal to the concentration at which 50% inhibition of the lysis of Jurkat cells by purified recombinant perforin was observed, as measured by $^{51}$Cr release. Values are the average of at least three independent IC$_{50}$ determinations.

$^b$Preparation of compounds 118–135 described in Scheme 2.

$^c$Preparation of compounds 165–170 described in Scheme 3.

$^d$Compound 178 contains a 3-acetylinodole C-subunit, and its synthesis is described in Scheme 4.
Table 3. Inhibitory Activities of Optimized A-, B-, and C-Subunits

| compd | X⁺ | n | inhibition of Jurkat cell lysis, IC₅₀ (μM) |
|-------|----|---|---------------------------------|
| 136   | 2,5-thiophene⁺ | 1 | 0.40                            |
| 204   | 2,4-thiophene   | 1 | 3.00                            |
| 205   | 1,4-benzenne    | 1 | 5.55                            |
| 206   | 1,3-benzenne    | 1 | 3.44                            |
| 207   | 3,6-pyridine    | 1 | 0.63                            |
| 208   | 2,6-(1H-indole) | 1 | 1.58                            |
| 209   | 2,5-(1H-indole) | 2 | 2.50                            |
| 210   | 2,5-(1H-indole) | 2 | 1.66                            |
| 211   | 2,6-pyrimidine  | 2 | 1.45                            |
| 212   | 2,6-pyrimidine  | 2 | 2.55                            |

“Structure of the B-subunit. bTesting was carried out over a range of concentrations, with the IC₅₀ being equal to the concentration at which 50% inhibition of the lysis of Jurkat cells by purified recombinant perforin was observed, as measured by ⁵¹Cr release. Values are the average of at least three independent IC₅₀ determinations.” The 2-thioxoimidazolidin-4-one A-subunit is replaced with a imidazolidine-2,4-dione.

toxicity is important because CTLs and NK cells play a key role in the overall immunological response, meaning that any potential perforin-targeted treatment must allow rapid recovery of these cytotoxic effector cells. For the purposes of this study, compounds were classified as toxic if NK cell viability fell below 70% at 24 h.

The data contained in Table 4 were used to identify compounds suitable for advancement into in vivo pharmacokinetic studies, as a prelude to in vivo efficacy testing. In the absence of serum, the majority of compounds possessed similar or improved inhibitory activity compared to previous lead 4, notably 118, 135, and 167, which all showed excellent activity in this more demanding system (56%, 90%, 83% compared to 53%). As reported with previous series, a number of compounds were adversely affected in the presence of serum, precluding their further consideration (125, 126, 129, 168, 207). The solubility of all compounds was substantially improved by their conversion to sodium salts, resulting in an enhanced drug profile, although some examples (118, 119, 129, 136, 168) still proved too insoluble in this form for administration in vivo. Compounds 119 and 167 also showed some evidence of toxicity toward the NK cells (28% and 38% survival at 20 μM, respectively). The compound with the best overall profile for an in vivo candidate was 135, combining superior activity in the presence and absence of serum (90% and 67%, respectively) with good viability (74%) and aqueous solubility (1408 μg/mL). In addition, when 167 was tested at a lower concentration of 5 μM, the resultant lack of NK toxicity suggested a possible therapeutic window, meaning this compound was also selected as an in vivo candidate.

In Vivo Pharmacokinetics of Selected Compounds. Compounds 135 and 167 were evaluated for plasma pharmacokinetics in male CD-1 mice. Blood samples were collected at multiple time-points after dosing at 5 mg/kg in a solution of 20% hydroxypropyl-β-cyclodextrin by intravenous injection. Both compounds were determined to have acceptable drug exposures (AUC = 1151 and 955 h·ng/mL, respectively), despite high clearance and volume of distribution values (Table 5). On this basis, 135 and 167 were progressed to maximum tolerated dose (MTD) studies by single or multiple doses (intraperitoneally) to establish an appropriate level for efficacy studies. Both compounds were found to be well tolerated after a single dose of 80 and 60 mg/kg, respectively, while when administered twice daily over 3 days, 60 and 40 mg/kg were found to be suitable for multiple dosing.

Binding Affinity of Inhibitors to Perforin Protein. While several series of potent inhibitors have now been identified, little is known about the mechanism by which the cytolytic activity of perforin action is blocked. In order to find out more about the nature and degree of inhibitor binding, recombinant purified mouse perforin was immobilized on a surface plasmon resonance (SPR) S-series CMS sensor chip using standard amide coupling methodology. Then solutions of in vivo candidates 135 and 167 in buffer were passed over the covalently bound protein, and relative binding affinities were determined using a Biacore T200 SPR machine.

Inhibitors 135 and 167 were found to bind reversibly to the immobilized perforin, and during injection a steady-state response was achieved within seconds (Figures 2 and 3 in Supporting Information). At the end of the injection the compounds dissociated rapidly with an almost immediate return to baseline resonance response. Any nonspecific binding to the sensor chip surface was accounted for through the response in the reference flow cell. Because of the relative insolubility of the compounds in the running buffer, low analyte concentrations were required (≤2.5 μM) and the relative resonance responses at steady state were typically below 10 response units (RU). However, although the absolute response values were low, it is clear that 135 and 167 can bind to perforin even at low concentrations. These data demonstrate for the first time that these inhibitors possess the ability to bind reversibly to perforin monomers. Prior to this experiment it was also not known whether immobilization of perforin monomers to the sensor chip surface via (random) amide couplings could render potential...
Scheme 2a

“Reagents and conditions: (i) (a) NBS, AIBN, benzene, reflux, 6 h, (b) R,NH2, THF, rt; (ii) 2-(thiophen-2-yl)-1,3-dioxolane or 2-(dimethoxymethyl)thiophene, PdCl2(PPh3)2, KF, AgNO3, DMSO, 100 °C, 2 h; (iii) (a) NaH, DMF, 0 °C to rt, (b) R,X, DMF, rt, 1 h; (iv) Ac2O, reflux, 2 h; (v) (a) LDA, THF, −78 °C, 0.25 h, (b) MeI, THF, −78 °C-rt; (vi) 1 M HCl, acetone, rt; (vii) p-TsOH, acetone/water, 50 °C, 5 h; (viii) (MeO)2CH, p-TsOH, 4 Å molecular sieves, MeOH, reflux, 15 h; (ix) MsCl, TEA, THF, rt; (x) NaI, acetone, 70 °C, 1 h; (xi) R1R2NH, DMA, rt; (xii) 2-thioxoimidazolidin-4-one, β-alanine, AcOH, reflux, 15 h; (xiii) hydrantoin, β-alanine, AcOH, reflux, 15 h (136).

CONCLUSIONS

The current study has resulted in further optimization of a novel new series of small-molecule inhibitors of the pore-forming protein perforin. By building on our previous studies, we have designed compounds that possess enhanced druglike properties compared to earlier structures. We also report new mechanistic evidence that reveals a specificity for the granule exocytosis pathway, of which perforin is an integral component.

Structure—activity relationships for variation of the C-subunit on a 2-thioxoimidazolidin-4-one/thiophene scaffold showed a need for substitution, especially at the 4-position, for simple substituted-benzene derivatives (Table 1). In this series the 3- and 4-carboxamides 60 and 61 proved to be the most potent, although this was limited to primary amides, as the introduction of N-substitution and extended hydroxyalkyl or aminoalkyl side chains (67–75) resulted in a loss of activity. The “acyclic” analogue of the lead compound (62) also showed an almost 4-fold reduction in activity, suggesting retention of a bicyclic C-subunit to be the best approach. The isobenzofuranone of 4 was therefore replaced with a variety of isomeric isoindolinones and 3,4-dihydroisoquinolin-1(2H)-ones. N-Substitution on the isoindoline nitrogen was beneficial, with methyl (119), ethyl (120), hydroxyethyl (125), and hydroxypropyl (126) substituents all demonstrating excellent activity (IC50 = 0.51, 0.60, 0.78, 0.53 μM, respectively). Comparable substitution on the 3,4-dihydroisoquinolin-1(2H)-one nitrogen did not improve activity (comparing 167/169 and 168/170 pairings), although these compounds (167, 168) still showed potent inhibitory activity in their own right. Lastly, a series of hybrid compounds were prepared (Table 3), combining B-subunits that were previously identified as conferring good activity with the above optimized binding sites for inhibitors inaccessible. Combined, these findings show that SPR biosensor analysis can be a useful technique to determine perforin protein—drug binding interactions.

Investigation of the Mechanism of Perforin Inhibition.

In order to investigate whether 167 inhibited perforin directly in the context of the physiological immune synapse, we also used real-time microscopy to analyze the functional interaction between primary human natural killer cells and their cognate model targets, HeLa cells. When the killer cell engages with its target cell, a cascade of downstream signaling events leads to the influx of extracellular Ca2+ (as detected by the calcium ionophore Fluo-4). As shown recently,42 Ca2+ in the target cell, a cascade of downstream signaling events leads to the formation of the perforin pore was blocked in the remaining 30% of synapses, despite effective target cell engagement (Figure 2). These data demonstrate that 167 directly inhibits perforin-induced lysis through reduction of cell membrane binding and/or prevention of transmembrane pore formation, thus preventing target cell death.
C-subunits. In this instance, the isoindolinones were all poorer than the corresponding isobenzofuranones (Figure 1 of Supporting Information).

A smaller subset of active compounds were then screened for their ability to inhibit perforin-dependent lysis induced by whole NK cells, resulting in the selection of 135 and 167 as suitable candidates for in vivo studies. Problems observed previously, such as poor aqueous solubility, inactivity in serum, and toxicity have been substantially overcome in the present series. The mechanism of perforin inhibition by these compounds has also been investigated further, and for the first time, compounds with in vitro inhibitory activity have been shown to

Scheme 3

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Reagents and conditions: (i) (a) NaH, DMF, 0 °C to rt, (b) MeI, DMF, rt, 1 h; (ii) bis(pinacolato)diboron, KOAc, Pd(dppf)Cl₂, DMSO, 90 °C, 5 h; (iii) P₂O₅, POCl₃, reflux, 2 h; (iv) 5, 2 M Na₂CO₃, toluene/EtOH, PdCl₂(dppf), reflux, 2 h; (v) 1 M HCl, acetone, rt; (vi) 2-thioxoimidazolidin-4-one, β-alanine, AcOH, reflux, 15 h.
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Scheme 4

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Reagents and conditions: (i) PhSO₂Cl, NaOH, Bu₄N⁺HSO₄⁻, CH₂Cl₂, rt; (ii) Ac₂O, AlCl₃, CH₂Cl₂, rt; (iii) MeOH, Bu₄N⁺Br⁻, CsCO₃, toluene, 65 °C, 1 h; (iv) 2 M KHCO₃/DMF (3:1), Pd(dppf)Cl₂, 70 °C, 1 h; (v) acetone/2 M HCl (3:1), rt; (vi) 2-thioxoimidazolidin-4-one, β-alanine, AcOH, reflux, 15 h.
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Table 4. Additional Testing on Selected Compounds

| compd | Jurkat IC50 (μM) | KHYG-1 inhibition (% at 20 μM) | KHYG-1 viability (μg/mL) | solubility (μg/mL) | solubility (μg/mL) |
|-------|-----------------|-------------------------------|------------------------|-------------------|-------------------|
|       | no serum 10% serum |                               |                        |                   |                   |
| 4     | 0.78 53 ± 2.6 38.9 ± 3.5 | 88 ± 3.0 23.0 [167]            | 0.80 0.59 [140]       |                   |                   |
| 118   | 2.55 56 ± 7.2 55 ± 6.5 | 95 ± 3.5 0.54 [167]            | 3.5 38 [167]          |                   |                   |
| 119   | 0.51 42 ± 5.5 37 ± 2.4 | 28 ± 1.5 0.13 [167]            | 3.5 38 [167]          |                   |                   |
| 125   | 0.78 43 ± 5.8 24 ± 3.4 | 81 ± 0.5 43.0 [167]            | 3.5 38 [167]          |                   |                   |
| 126   | 0.53 48 ± 7.9 17 ± 3.0 | 90 ± 3.0 1.78 [167]            | 3.5 38 [167]          |                   |                   |
| 129   | 0.55 46 ± 5.9 35 ± 9.5 | 93 ± 4.1 0.18 [167]            | 3.5 38 [167]          |                   |                   |
| 132   | 3.21 90 ± 4.0 67 ± 6.1 | 74 ± 8.3 1.32 [167]            | 3.5 38 [167]          |                   |                   |
| 136   | 0.40 49 ± 9.8 50 ± 7.1 | 93 ± 3.0 11.6 [167]            | 3.5 38 [167]          |                   |                   |
| 167   | 1.17 83 ± 4.6 81 ± 5.3 | 38 ± 7.8 0.53 [167]            | 3.5 38 [167]          |                   |                   |
| 167/* | 0.51 42 ± 5.5 37 ± 2.4 | 28 ± 1.5 0.13 [167]            | 3.5 38 [167]          |                   |                   |
| 168   | 0.64 51 ± 4.0 23 ± 7.1 | 87 ± 4.1 0.13 [167]            | 3.5 38 [167]          |                   |                   |
| 207   | 0.63 43 ± 5.4 13 ± 3.8 | 92 ± 2.6 0.59 [167]            | 3.5 38 [167]          |                   |                   |

**Inhibition by compounds (20 μM) of the perforin-induced lysis of KS62 target cells when co-incubated with KHYG-1 human NK cells (see Experimental Section). Percent inhibition calculated compared to untreated control. As for footnote a, but in the presence of 10% mouse serum. Viability of KHYG-1 NK cells after 24 h by Trypan blue exclusion assay (see Experimental Section). All results are the average of at least three separate determinations ± SEM. Compound 167 was also tested at 5 μM to test for activity under less toxic conditions. Insufficient material to prepare sodium salt.**

The use of purified perforin protein rather than perforin delivered by an intact killer cell.

Together, the data we have amassed demonstrate that these series of compounds are selective inhibitors of perforin and not nonspecific promiscuous binders. We have previously shown that these compounds do not inhibit the mechanistically related pore-forming protein pneumolysin and that we observe a wide range of activities (0.36 to >20 μM) against both isolated perforin and perforin produced by whole NK cells. Furthermore, the membrane-piercing terminal components of the mammalian membrane attack complex of complement (MAC), which share significant structural similarity with perforin and alternative, receptor-mediated proapoptotic pathways used by cytotoxic lymphocytes, are not inhibited (data not shown). These observations are recapitulated in the current report by the clear SAR demonstrated by the C-subunit (IC50 of 0.51 to >20 μM), irrespective of the presence of a 5-arylidene-2-thioxoimidazolidin-4-one as the A-subunit. A range of activities was also observed against perforin released by intact NK cells, a far more rigorous model that requires the compound to block perforin released into the immunological synapse that transiently forms between the killer cell and its target. Our live cell imaging analyses clearly show for the first time that perforin operating within a functional immune synapse, an intricate microenvironment comprising many proteins taking part in various complex signaling pathways, can be selectively targeted to inhibit perforin-dependent apoptosis of the target cell.

In summary, we have taken significant steps toward elucidating the role of perforin in the granule exocytosis pathway, using small-molecule inhibitors to investigate binding affinity and probe the mechanism of membrane interaction and subsequent pore formation. We have found compounds 135 and 167 to be both potent and soluble inhibitors of perforin in vitro. These compounds also possess appropriate in vivo pharmacokinetic characteristics and toxicity profiles for their selection as in vivo efficacy candidates in our pursuit of a novel class of perforin.

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**Table 5. In Vivo Pharmacokinetics of Compounds 135 and 167**

| compd | T1/2 (h) | Cmax (ng/mL) | AUC0-∞ (b·ng/mL) | V (mL/kg) | Cl (mL h⁻¹ kg⁻¹) |
|-------|---------|-------------|----------------|----------|-----------------|
| 135   | 1.15    | 4488        | 1151           | 1478     | 4346            |
| 167   | 1.30    | 4513        | 955            | 2750     | 5237            |

**Pharmacokinetic parameters derived from the plasma concentration–time profiles for each compound following a 5 mg/kg iv dose. The results were processed using a bolus iv dose and two-compartmental model with Phoenix WinNonlin 6.2 (Pharsight Corporation, St. Louis, MO). The derived parameters are maximum plasma concentration (Cmax), the area under the curve (AUC), plasma half-life (T1/2), volume of distribution (V), and clearance (Cl).**
inhibitors suitable for the effective treatment of autoimmune-based diseases and transplant rejection.

■ EXPERIMENTAL SECTION

Medicinal Chemistry. Analyses were performed by the Microchemical Laboratory, University of Otago, Dunedin, New Zealand. Melting points were determined using an Electrothermal model 9200 and are as read. NMR spectra were measured on a Bruker Avance 400 spectrometer and referenced to Me₄Si. Mass spectra were recorded either on a Varian VG 7070 spectrometer at nominal 5000 resolution or a Finnigan MAT 900Q spectrometer. All final compound purities were determined to be >95% by HPLC.

Representative general procedures are given below. Full experimental information for all remaining final compounds and intermediates is given in Supporting Information.

General Procedure A: (4-(5-(1,3-Dioxolan-2-yl)thiophen-2-yl)phenyl)methanol (6) (Scheme 1, R = CH₂OH). 5-Bromo-2-thiophenecarboxaldehyde was protected as the cyclic acetal 5 according to a literature procedure. The acetal 5 (278 mg, 1.18 mmol) was then dissolved in toluene (12 mL), to which was added a suspension of 4-(hydroxymethyl)benzeneboronic acid (150 mg, 0.99 mmol) in EtOH (4 mL). A solution of 2 M Na₂CO₃ (3 mL) and Pd(dppf)Cl₂ (40 mg, 0.05 mmol) was added, and the entire mixture was heated at reflux under nitrogen for 2 h. When the mixture was cooled, all solvents were removed under reduced pressure and the resulting residue was partitioned between water (50 mL) and CH₂Cl₂ (50 mL). Two further CH₂Cl₂ (50 mL) extractions were performed. Then the combined organic fractions were dried (Na₂SO₄), filtered, and the solvent was removed under reduced pressure to afford a residue which was purified by flash column chromatography on silica gel (20% EtOAc/hexanes as eluant) to give 6 as a pale yellow solid (171 mg, 66%).

1H NMR [400 MHz, (CD₃)₂SO] δ 7.60 (d, J = 8.3 Hz, 2 H), 7.36 (d, J = 3.6 Hz, 1 H), 7.35 (d, J = 8.4 Hz, 2 H), 7.20 (d, J = 3.8 Hz, 1 H), 6.04 (s, 1 H), 5.19 (t, J = 5.6 Hz, 1 H), 4.51 (d, J = 5.5 Hz, 2 H), 4.10−4.07 (m, 2 H), 3.93−3.80 (m, 2 H), 3.79−3.76 (m, 2 H), 3.74−3.71 (m, 2 H), 3.69−3.66 (m, 2 H), 3.64−3.61 (m, 2 H), 3.59−3.56 (m, 2 H), 3.54−3.51 (m, 2 H), 3.49−3.46 (m, 2 H), 3.44−3.41 (m, 2 H), 3.40−3.37 (m, 2 H), 3.37−3.34 (m, 2 H), 3.33−3.30 (m, 2 H), 3.30−3.27 (m, 2 H), 3.26−3.23 (m, 2 H), 3.22−3.19 (m, 2 H), 3.19−3.16 (m, 2 H), 3.15−3.12 (m, 2 H), 3.12−3.09 (m, 2 H), 3.08−3.05 (m, 2 H), 3.05−3.02 (m, 2 H), 3.02−2.99 (m, 2 H), 2.99−2.96 (m, 2 H), 2.96−2.93 (m, 2 H), 2.93−2.90 (m, 2 H), 2.90−2.87 (m, 2 H), 2.87−2.84 (m, 2 H), 2.84−2.81 (m, 2 H), 2.81−2.78 (m, 2 H), 2.78−2.75 (m, 2 H), 2.75−2.72 (m, 2 H), 2.72−2.69 (m, 2 H), 2.69−2.66 (m, 2 H), 2.66−2.63 (m, 2 H), 2.63−2.60 (m, 2 H), 2.60−2.57 (m, 2 H), 2.57−2.54 (m, 2 H), 2.54−2.51 (m, 2 H), 2.51−2.48 (m, 2 H), 2.48−2.45 (m, 2 H), 2.45−2.42 (m, 2 H), 2.42−2.39 (m, 2 H), 2.39−2.36 (m, 2 H), 2.36−2.33 (m, 2 H), 2.33−2.30 (m, 2 H), 2.30−2.27 (m, 2 H), 2.27−2.24 (m, 2 H), 2.24−2.21 (m, 2 H), 2.21−2.18 (m, 2 H), 2.18−2.15 (m, 2 H), 2.15−2.12 (m, 2 H), 2.12−2.09 (m, 2 H), 2.09−2.06 (m, 2 H), 2.06−2.03 (m, 2 H), 2.03−2.00 (m, 2 H), 2.00−1.97 (m, 2 H), 1.97−1.94 (m, 2 H), 1.94−1.91 (m, 2 H), 1.91−1.88 (m, 2 H), 1.88−1.85 (m, 2 H), 1.85−1.82 (m, 2 H), 1.82−1.79 (m, 2 H), 1.79−1.76 (m, 2 H), 1.76−1.73 (m, 2 H), 1.73−1.70 (m, 2 H), 1.70−1.67 (m, 2 H), 1.67−1.64 (m, 2 H), 1.64−1.61 (m, 2 H), 1.61−1.58 (m, 2 H), 1.58−1.55 (m, 2 H), 1.55−1.52 (m, 2 H), 1.52−1.49 (m, 2 H), 1.49−1.46 (m, 2 H), 1.46−1.43 (m, 2 H), 1.43−1.40 (m, 2 H), 1.40−1.37 (m, 2 H), 1.37−1.34 (m, 2 H), 1.34−1.31 (m, 2 H), 1.31−1.28 (m, 2 H), 1.28−1.25 (m, 2 H), 1.25−1.22 (m, 2 H), 1.22−1.19 (m, 2 H), 1.19−1.16 (m, 2 H), 1.16−1.13 (m, 2 H), 1.13−1.10 (m, 2 H), 1.10−1.07 (m, 2 H), 1.07−1.04 (m, 2 H), 1.04−1.01 (m, 2 H), 1.01−0.98 (m, 2 H), 0.98−0.95 (m, 2 H), 0.95−0.92 (m, 2 H), 0.92−0.89 (m, 2 H), 0.89−0.86 (m, 2 H), 0.86−0.83 (m, 2 H), 0.83−0.80 (m, 2 H), 0.80−0.77 (m, 2 H), 0.77−0.74 (m, 2 H), 0.74−0.71 (m, 2 H), 0.71−0.68 (m, 2 H), 0.68−0.65 (m, 2 H), 0.65−0.62 (m, 2 H), 0.62−0.59 (m, 2 H), 0.59−0.56 (m, 2 H), 0.56−0.53 (m, 2 H), 0.53−0.50 (m, 2 H), 0.50−0.47 (m, 2 H), 0.47−0.44 (m, 2 H), 0.44−0.41 (m, 2 H), 0.41−0.38 (m, 2 H), 0.38−0.35 (m, 2 H), 0.35−0.32 (m, 2 H), 0.32−0.29 (m, 2 H), 0.29−0.26 (m, 2 H), 0.26−0.23 (m, 2 H), 0.23−0.20 (m, 2 H), 0.20−0.17 (m, 2 H), 0.17−0.14 (m, 2 H), 0.14−0.11 (m, 2 H), 0.11−0.08 (m, 2 H), 0.08−0.05 (m, 2 H), 0.05−0.02 (m, 2 H), 0.02−0.00 (m, 2 H).

Figure 2. Effect of 167 in the context of the physiological immune synapse.
(m, 2 H). LRMS (APCI') calc. for C_{17}H_{15}N_{2}O_{3}S_{2} 359 (MH+), found 359. Anal. (C_{17}H_{14}N_{2}O_{3}S_{2}) C, H, N.

General Procedure B: 5-(4-(Hydroxymethyl)phenyl)-thiophene-2-carbaldehyde (24) (Scheme 1, R = CH_{2}OH). Compound 6 (171 mg, 0.65 mmol) was dissolved in acetonitrile (10 mL), to which was added 1 M HCl (2 mL). The mixture was stirred under reduced pressure to afford a pale yellow solid which was transferred into CH_{2}Cl_{2} (2 × 50 mL). The combined organic fractions were evaporated down to give 24 as a yellow solid (142 mg, 100%). \(^{1}H\) NMR [400 MHz, (CD_{3})_{2}SO] \(δ\) 9.90 (s, 1 H), 8.03 (d, \(J = 3.9\) Hz, 1 H), 7.76 (d, \(J = 8.3\) Hz, 2 H), 7.72 (d, \(J = 4.0\) Hz, 1 H), 7.42 (d, \(J = 8.4\) Hz, 2 H), 5.26 (t, \(J = 5.7\) Hz, 1 H), 4.54 (d, \(J = 5.6\) Hz, 2 H). LRMS (APCI') calc. for C_{13}H_{12}NO_{2}S 246 (MH+), found 246.

General Procedure C. (E,Z)-4-(5-(5-Oxo-2-thioxoimidazolidin-4-yldiene)methyl)thiophen-2-yl)benzyl Acetate (58) (Scheme 1, R = CH_{2}OAc). 5-((4-Hydroxymethyl)phenyl)thiophene-2-carbaldehyde (24) (140 mg, 0.64 mmol), 2-thioxoimidazolidone-4-one (89 mg, 0.77 mmol), and \(β\)-alanine (69 mg, 0.77 mmol) were suspended in AcOH (5 mL), and the mixture was heated at reflux for 15 h. When the mixture was cooled, an orange solid crystallized out of solution which was collected by filtration and purified by flash column chromatography on silica gel (20% THF/hexanes), giving 58 as an orange solid (18%), mp (THF/\(\text{Me}_{2}\text{CO}) 230–234 °C. \(^{1}H\) NMR [400 MHz, (CD_{3})_{2}SO] \(δ\) 9.95 (s, 1 H), 8.44 (d, \(J = 4.0\) Hz, 1 H), 7.72 (d, \(J = 4.0\) Hz, 1 H), 7.65 (d, \(J = 4.0\) Hz, 1 H), 7.44 (d, \(J = 8.4\) Hz, 2 H), 6.63 (s, 1 H), 5.10 (s, 2 H), 2.08 (s, 3 H). LRMS (APCI') calc. for C_{17}H_{15}N_{2}O_{3}S 359 (MH+), found 359. Anal. (C_{17}H_{14}N_{2}O_{3}S) C, H, N.

General Procedure D: 4-(5-Formylthiophen-2-yl)-N-methylbenzamide (34) (Scheme 1, R = CONR_{2}, where NR_{2} = NHMe). 4-(5-Formylthiophen-2-yl)benzoic acid (28) (85 mg, 0.37 mmol) was dissolved in THF (5 mL), to which was added pyridine (289 mg, 3.67 mmol), followed by pentafluorophenyl trifluoracetate (PF_{5}-TFA). This mixture was stirred at room temperature for 15 h. Then all solvent was removed under reduced pressure to afford a crude solid which was dissolved in EtOAc (25 mL) and washed with 1 M HCl (25 mL), water (20 mL). The combined organic fractions were evaporated down to give a crude solid. Purification by column chromatography on silica gel (5% EtOAc/CH_{2}Cl_{2} as eluant). The bromide (1.08 mg, 3.04 mmol) was dissolved in THF (10 mL), and 0.1 M Na_{2}SO_{4} was added. The mixture was stirred for 0.5 h at 0 °C. Then the resulting suspension was stirred for 0.5 h at 100 °C. The mixture was allowed to cool, then filtered, and the resulting filtrate was heated at reflux temperature for 6 h. The mixture was then concentrated under reduced pressure to give a solid yellow oil which was purified by flash column chromatography on silica gel (5% acetonitrile/CH_{2}Cl_{2} as eluant). 5-((4-Hydroxymethyl)phenyl)thiophene-2-carbaldehyde (24) (Scheme 1, R = CH_{2}OH). Compound 6 (171 mg, 0.65 mmol) was dissolved in acetonitrile (10 mL), to which was added 1 M HCl (2 mL). The mixture was stirred under reduced pressure to afford a pale yellow solid which was transferred into CH_{2}Cl_{2} (2 × 50 mL). The combined organic fractions were evaporated down to give 24 as a yellow solid (142 mg, 100%). \(^{1}H\) NMR [400 MHz, (CD_{3})_{2}SO] \(δ\) 9.90 (s, 1 H), 8.03 (d, \(J = 3.9\) Hz, 1 H), 7.76 (d, \(J = 8.3\) Hz, 2 H), 7.72 (d, \(J = 4.0\) Hz, 1 H), 7.42 (d, \(J = 8.4\) Hz, 2 H), 5.26 (t, \(J = 5.7\) Hz, 1 H), 4.54 (d, \(J = 5.6\) Hz, 2 H). LRMS (APCI') calc. for C_{13}H_{12}NO_{2}S 246 (MH+), found 246.

General Procedure D: 4-(5-Formylthiophen-2-yl)-N-methylbenzamide (34) (Scheme 1, R = CONR_{2}, where NR_{2} = NHMe). 4-(5-Formylthiophen-2-yl)benzoic acid (28) (85 mg, 0.37 mmol) was dissolved in THF (5 mL), to which was added pyridine (289 mg, 3.67 mmol), followed by pentafluorophenyl trifluoracetate (PF_{5}-TFA). This mixture was stirred at room temperature for 15 h. Then all solvent was removed under reduced pressure to afford a crude solid which was dissolved in EtOAc (25 mL) and washed with 1 M HCl (25 mL), water (20 mL). The combined organic fractions were evaporated down to give a crude solid. Purification by column chromatography on silica gel (5% EtOAc/CH_{2}Cl_{2} as eluant). The bromide (1.08 mg, 3.04 mmol) was dissolved in THF (10 mL), and 0.1 M Na_{2}SO_{4} was added. The mixture was stirred for 0.5 h at 0 °C. Then the resulting suspension was stirred for 0.5 h at 100 °C. The mixture was allowed to cool, then filtered, and the resulting filtrate was heated at reflux temperature for 6 h. The mixture was then concentrated under reduced pressure to give a solid yellow oil which was purified by flash column chromatography on silica gel (5% acetonitrile/CH_{2}Cl_{2} as eluant) to give 87 as a yellow solid (82%). \(^{1}H\) NMR [400 MHz, (CD_{3})_{2}SO] \(δ\) 7.87 (d, \(J = 0.7\) Hz, 1 H), 7.76 (d, \(J = 7.9, 1.6\) Hz, 1 H), 7.67 (d, \(J = 8.0\) Hz, 1 H), 7.53 (d, \(J = 3.8\) Hz, 1 H), 7.25 (d, \(J = 3.8\) Hz, 1 H), 6.07 (s, 1 H), 4.49 (s, 2 H), 3.66 (t, \(J = 4.0\) Hz, 1 H), 2.08 (s, 3 H). LRMS (APCI') calc. for C_{14}H_{15}O_{3}S 263 (MH+), found 263.
flash column chromatography on silica gel (10% acetone/CH2Cl2 as eluant) to give 78 as a crystalline white solid (562 mg, 59% over two steps). 1H NMR (400 MHz, CDCl3) δ 8.02 (d, J = 7.1 Hz, 1 H), 7.84 (d, J = 7.9, 1.4 Hz, 1 H), 7.45 (d, J = 7.9 Hz, 1 H), 4.82 (t, J = 5.4 Hz, 1 H), 4.52 (s, 2 H), 3.58–3.64 (m, 2 H), 3.32–3.57 (m, 2 H). LRMS (APCI+), calcld for C15H18N3O2S, found 304 (MH+), reported 304.

General Procedure H: 5-(2-(Hydroxyethyl)-1-oxoisoindolin-5-yl)thiophene-2-carboxaldehyde (100) (Scheme 2, R1 = CH2CH2OH, R5 = H). 2-Thiophenecarboxaldehyde was protected as the dimethyl acetal according to a literature procedure, then reacted with 78 according to general procedure E to give 83. Deprotection directly to the aldehyde was then carried out as follows. Compound 83 (430 mg, 1.29 mmol) was dissolved in acetonitrile (32 mL), to which were added water (8 mL) and p-toluenesulfonic acid (100 mg). This mixture was stirred at 50 °C for 5 h. Upon cooling, the reaction mixture was diluted with water (100 mL) and extracted with CH2Cl2 (3 × 50 mL). Purification by flash column chromatography on silica gel (5% MeOH/CH2Cl2 as eluant) gave 100 as a yellow solid (378 mg, 71% over two steps). 1H NMR (400 MHz, CDCl3) δ 7.94 (s, 1 H), 8.08 (d, J = 4.0 Hz, 1 H), 8.04 (d, J = 0.8 Hz, 1 H), 7.92 (dd, J = 7.9, 1.6 Hz, 1 H), 7.85 (d, J = 4.0 Hz, 1 H), 7.75 (d, J = 8.0, 1.5 Hz, 1 H), 4.83 (t, J = 5.4 Hz, 1 H), 4.62 (s, 2 H), 3.56–3.67 (m, 4 H). LRMS (APCI+), calcld for C15H17NO3S, found 288 (MH+), reported 288.

(E)-2-(1-Oxo-5-(5-(5-oxo-2-thioxoimidazolidin-4-ylidene) methyl)thiophen-2-yl) isoxazolidin-2-yl)methyl Acetate (124) (Scheme 2, R1 = CH2CH2CH2OH, R4 = H, R5 = H). Hydrolysis of 122 (161 mg, 0.42 mmol) was carried out by removing the previous reaction solvent (AcOH, 4 mL) from the reaction mixture under reduced pressure and treating the crude O-acetate with K2CO3 (289 mg, 2.09 mmol) in a mixture of MeOH (10 mL) and water (2 mL) at room temperature for 1 h. The MeOH was removed under reduced pressure to give an orange-black oil which was cooled to 0 °C with an ice/water and acidicified with 1 M HCl. The resulting solid was collected by filtration, allowed to air-dry, then stirred in warm MeOH until a solid (72%), mp (MeOH) 261–264 °C. 1H NMR (400 MHz, CDCl3) δ 12.39 (s, 1 H), 11.98 (s, 1 H), 7.92–7.96 (m, 1 H, J = 7.8 Hz), 7.84 (d, J = 4.0 Hz, 1 H), 7.74 (d, J = 8.0, 1.5 Hz, 1 H), 7.77 (d, J = 4.0 Hz, 1 H), 7.73 (d, J = 7.9 Hz, 1 H), 6.65 (s, 1 H, J = 11.98 Hz), 4.59 (s, 2 H), 4.26 (t, J = 5.4 Hz, 2 H), 3.78 (t, J = 5.3, 2 H, 2 H), 2.00 (s, 3 H).

General Procedure I: (E)-2-(2-Hydroxyethyl)-5-(5-(5-oxo-2-thioxoimidazolidin-4-ylidene)methyl)thiophen-2-yl)-isoxazolidin-2-one (125) (Scheme 2, R1 = CH2CH2CH2OH, R4 = H, R5 = H). Reaction of 105 with 2-thiolydantoin according to general procedure C gave 124 as a red powder (57%), mp (AcOH) 261–264 °C. 1H NMR (400 MHz, CDCl3) δ 7.38–7.32 (m, 2 H), 7.68 (d, J = 7.6 Hz, 1 H), 7.55 (d, J = 3.7 Hz, 1 H), 7.12 (d, J = 3.7, 0.8 Hz, 1 H), 5.66 (d, J = 0.6 Hz, 1 H), 4.53 (s, 2 H), 4.25 (s, J = 6.2 Hz, 2 H), 3.63 (s, J = 6.9 Hz, 2 H), 3.32 (s, 6 H), 3.18 (s, 3 H), 2.04 (pentet, J = 6.6 Hz, 2 H). LRMS (APCI+), calcld for C15H22N2O3S, found 426.

5-(5-(Dimethoxymethyl)thiophen-2-yl)-1-oxoisoindolin-5-ylpropyl Methanesulfonate (108) (Scheme 2, R4 = OMs). Alcoholic (1.29 g, 3.72 mmol) was dissolved in dry THF (40 mL). Triethylamine (3.76 g, 37.2 mmol) and methanesulfonyl chloride (1.70 g, 14.9 mmol) were added, and the reaction mixture was stirred at room temperature for 3.5 h. The mixture was diluted with EtOAc (200 mL), which was washed with water (100 mL), saturated NaHCO3 (100 mL), and brine (100 mL). The organic layer was dried (Na2SO4), filtered and the solvent removed under reduced pressure to give a residue which was purified by flash column chromatography on silica gel (5% acetone/CH2Cl2 as eluant), giving 108 as a waxy yellow solid (1.58 g, 100%). 1H NMR (400 MHz, CDCl3) δ 7.86 (d, J = 0.7 Hz, 1 H), 7.06 (d, J = 7.9, 1.5 Hz, 1 H), 7.69 (d, J = 7.6 Hz, 1 H), 7.75 (d, J = 3.7 Hz, 1 H), 7.12 (d, J = 3.7, 0.8 Hz, 1 H), 5.56 (d, J = 0.6 Hz, 1 H), 4.53 (s, 2 H), 4.25 (t, J = 6.2 Hz, 2 H), 3.63 (s, J = 6.9 Hz, 2 H), 3.32 (s, 6 H), 3.18 (s, 3 H), 2.04 (pentet, J = 6.6 Hz, 2 H). LRMS (APCI+), calcld for C15H22NO3S, found 458 (MH+), reported 458. 1H NMR (400 MHz, CDCl3) δ 7.58 (d, J = 7.9 Hz, 1 H), 7.54 (d, J = 3.7 Hz, 1 H), 7.52 (d, J = 3.8, 0.8 Hz, 1 H), 5.66 (d, J = 0.7 Hz, 1 H), 4.51 (s, 2 H), 4.49 (t, J = 5.1 Hz, 1 H), 3.57 (t, J = 7.2 Hz, 2 H), 3.46 (q, J = 5.9 Hz, 2 H), 3.32 (s, 6 H), 1.76 (pentet, J = 6.7 Hz, 2 H). LRMS (APCI+), calcld for C15H22NO3S, found 458 (MH+), reported 458.
(131) (Scheme 2, R1 = (CH3)3NMe2, R2 = H). Reaction of 114 with 2-thioxoydantin according to general procedure C, followed by isolation according to general procedure J, gave 131 as a dark orange solid (51%), mp (MeOH) 212 °C (dec).1H NMR [400 MHz, (CD3)2SO] δ 11.47 (v brs, 2 H), 7.91 (br s, 1 H), 7.82 (dd, J = 8.1, 1.4 Hz, 1 H), 7.67–7.73 (m, 3 H), 6.52 (s, 1 H), 4.53 (s, 2 H), 3.07 (s, 3 H). LRMS (APCI+) calcld for C9H9BrNO, 247 (M+), found 247.

(128) 2,3-Dimethyl-5-(5-((5-oxo-2-thioxoimidazolidin-4-ylidene)methyl)thiophen-2-yl)isoindolin-1-one (106). Reaction of 106 with 2-thioxoydantin according to general procedure C gave 135 as an orange solid (89%), mp (AcOH) >310 °C.1H NMR [400 MHz, (CD3)2SO] observe E- and Z-isomers separately δ 11.97–12.34 (m, 2 H), 7.91–7.96 (m, 2 H), 7.82 (d, J = 4.0 Hz, 0.85 Hz), 7.75–7.78 (m, 1 H), 7.69 (d, J = 4.0 Hz, 0.15 Hz), 7.66 (d, J = 8.6 Hz, 1 H), 6.84 (s, 0.15 Hz), 6.66 (s, 0.15 Hz), 4.40 (s, 2 H), 3.10 (s, 3 H). LRMS (APCI+) calcld for C9H8NO3S, 354 (M+–H), found 354.

Inhibition of Peroxin-Mediated Lysis of Sheep Red Blood Cells (SRBC). As reported previously,26 compound 3 was identified from screening a commercial library of ~100,000 compounds for the ability to reproducibly inhibit perforin-mediated lysis of SRBC at a compound concentration of 100 μM.

Inhibition of Peroxin-Mediated Lysis of Jurkat Cells. The ability of the compounds to inhibit the lysis of nucleated (Jurkat T lymphoma) cells in the presence of 0.1% BSA was measured by release of 51Cr label. Jurkat target cells were labeled by incubation in medium with 100 μCi 51Cr for 1 h. The cells were then washed three times to remove unincorporated isotope and resuspended at 1 × 107 cells per milliliter in RPMI buffer supplemented with 0.1% BSA. Each test compound was preincubated to concentrations of 20, 10, 5, 2.5, and 1.25 μM with recombinant perforin for 30 min with DMSO as a negative control. 51Cr labeled Jurkat cells were then added, and the cells were incubated at 37 °C for 4 h. The supernatant was collected and assessed for its radioactivity content on a γ counter (Wallac Wizard 1470 automatic γ counter). Each data point was performed in triplicate, and an IC50 was calculated from the range of concentrations described above. Compounds with IC50 < 1 μM were titrated to lower concentrations in the same manner as above, to determine a reproducible IC50.

KHYG-1 Cytotoxicity Assay. KHYG-1 cells were washed and resuspended in RPMI + 0.1% BSA at 4 × 105 cells/mL, and an amount of 50 μL of KHYG-1 cells was dispensed to each well of a 96-well V-bottom plate. RPMI (50 μL) + 0.1% BSA or 10% (final concentration) of serum was added to each well. Then test compounds were added to KHYG-1 cells at various concentrations up to 20 μM and incubated at room temperature for 20 min. 1 × 105 K562 target cells were labeled with 75 μCi 51Cr in 200 μL of RPMI for 90 min at 37 °C. Cells were washed as described above and resuspended at 1 × 105 cells/mL of RPMI + 0.1% BSA. An amount of 50 μL of 11Cr labeled K562 leukemia target cells was added to each well of the KHYG-1 plate (effector/target 2:1) and incubated at 37 °C for 4 h. 51Cr release was assayed using a Skatron harvesting press and radioactivity estimated on a Wallac Wizard 1470 automatic γ counter (Turku, Finland). The percentage of specific cytotoxicity was calculated using the following formula:

\[
\text{% specific lysis} = \frac{\text{experimental release} - \text{spontaneous release}}{\text{maximum release} - \text{spontaneous release}} \times 100
\]

and expressed as the mean of triplicate assays ± standard error of the mean.

Toxicity to KHYG-1 NK Cells. The toxicity assay was carried out in exactly the same manner as the killing assay above, but instead of adding the labeled K562 target cells, 100 μL of RPMI + 0.1% BSA was added. Cells were incubated for 4 h at 37 °C and then washed 3× in RPMI + 0.1% BSA. Cells were then resuspended in 200 μL of complete medium and incubated for 18–24 h at 37 °C. Trypan blue was added to each well. Viable (clear) cells and total (clear + blue) cells were counted, and the percentage of viable cells was calculated compared to DMSO treated control cells (percent viability).

Plasma Pharmacokinetics and MTD Determinations. Pharmacokinetic studies were carried out in healthy CD-1 male mice (n = 3 at each time point) using intravenous administration of 135 and 167 at a concentration of 5 mg/kg in 20% 2-hydroxypropyl-β-cyclodextrin (Sigma-Aldrich). Blood was collected by cardiac puncture at 5 and 30
min and 1, 2, 4, and 6 h postdose in ice-cold K3HEDTA tubes. Plasma samples were separated by centrifuging at 4000g for 8 min and stored at −80 °C until analysis. Frozen plasma was thawed on wet ice on the day of analysis. Then the sample (10 μL) was transferred to a clean microcentrifuge tube and mixed with three volumes of ice-cold acetonitrile/methanol (3:1 v/v) [containing 213 as internal standard (Table 6, Supporting Information)] to precipitate the plasma proteins. The tubes were centrifuged at 15000g for 10 min to obtain the clear supernatant which was mixed with 0.01% formic acid—water (1:1), and concentration of the parent drug was measured through injection of 10–20 μL into an LC–MS/MS (Agilent 6410 series triple quadrupole mass spectrometry detector system). A Zorbax SB-C18, 50 mm × 2.1 mm 5 μm column was used at a 0.5 mL/min flow rate, and elution was with a gradient of organic phase (A), acetonitrile in water (80:20 v/v) containing 0.01% formic acid and aqueous phase (B), water containing 0.01% formic acid. LC–MS/MS analysis was carried out using multiple reaction monitoring. Parent drug was quantified against a standard curve 0–250 ng/mL in control mouse plasma. Plasma samples from 5 min, 30 min, and 1 h were diluted with control mouse plasma (1:20 for 5 min, 1:10 for 30 min and 1 h samples) to fit within this range. The resultant concentration vs time data were fitted (using Phoenix WinNonlin 6.2; Pharsight Corporation, St. Louis, MO) to calculate pharmacokinetic parameters including half-life (T1/2), maximum concentration (Cmax), area under the curve (AUC0–t), volume of distribution (V), and clearance (Cl).

The MTD was determined by single dose or b.i.d. × 3 intraperitoneal administration through a classical dose escalation design in C57BL/6 mice and defined as a dose level that was tolerated by mice (n = 2–3 mice/group) with a body weight loss of <10% and no death or detectable sickness. Mice were observed for four days postdose. All animal experiments were approved by the Animal Ethics Committee of the University of Auckland.

Measurement of Inhibitor Binding by SPR. Binding experiments with surface immobilized wild-type perforin monomers on a S-series CMS sensor chip (GE Healthcare Life Sciences) were carried out using a Biacore T200 SPR machine (GE Healthcare Life Sciences). Perforin monomer solution (25 μg/mL in 10 mM sodium acetate, pH 5.5) was injected over the sensor chip surface under standard amide coupling conditions to obtain covalent binding of the protein (density = 4257 RU). The initial and running buffers were made from a 10X stock solution of HEPES buffered saline (HBS). 10X HBS (50 mL) was diluted to 500 mL with water and filtered through a 0.22 μm filter (initial buffer). This initial buffer (380 mL) was added to DMSO (20 mL) to prepare the 5% DMSO running buffer. Stock solutions (2.5 mM) of compound were prepared in 100% DMSO. These solutions were diluted in HEPES initial buffer to 5% DMSO (125 μM) and then subsequently diluted to the required concentrations using HEPES running buffer containing 5% DMSO to maintain a constant DMSO concentration. A solvent correction was run before and after the injections to account for differences in DMSO concentrations between buffer and analyte solutions.

Analysis of the Cytotoxic Immunological Synapse. Time-lapse microscopy and image analysis were performed as described previously.7 In brief, primary human natural killer cells were isolated from the peripheral blood of four unrelated donors using negative selection, as described in the above reference. Natural killer cells were labeled with 1 μM Fluo-4AM for 20 min at 37 °C/5% CO2, and then washed and preincubated for 10 min in RPMI culture medium containing 0.2% (v/v) DMSO or 20 μM 167/0.2% (v/v) DMSO at room temperature before combining with HeLa cell targets preseeded in chamber slides. The final mixture contained complete RPMI-1640 culture medium supplemented with 100 μM propidium iodide, 0.2% (v/v) DMSO or 20 μM 167/0.2% (v/v) DMSO. All experiments were performed at 37 °C/5% CO2.

ASSOCIATED CONTENT

■ Supporting Information

Tables of all compounds numbered individually, selected figures, experimental, SPR, NMR, HRMS, HPLC, biological data (as indicated in the text), and combustion analysis results of all final compounds. This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes

The authors declare no competing financial interest.

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■ ABBREVIATIONS USED

NK, natural killer; CTL, cytotoxic T lymphocyte; MACPF, membrane attack complex/perforin; EGF, epidermal growth factor; FLH, familial hemophagocytic lymphohistiocytosis; SPR, surface plasmon resonance

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