Chemistry & Biology, Vol. 12, 567–577, May, 2005, ©2005 Elsevier Ltd All rights reserved. DOI 10.1016/j.chembiol.2005.03.006

Selective Chemical Functional Probes of Granzymes A and B Reveal Granzyme B Is a Major Effector of Natural Killer Cell-Mediated Lysis of Target Cells

Sami Mahrus[1] and Charles S. Craik[1,2,*]
[1]Chemistry and Chemical Biology Graduate Program
[2]Department of Pharmaceutical Chemistry
University of California, San Francisco
San Francisco, California 94143

Summary

The mechanism of target cell lysis in cytotoxic lymphocyte-mediated death is not well understood, and the role of granzymes in this process is unclear. Chemical functional probes were thus prepared for the major granzymes A and B to deconvolute their role in natural killer cell-mediated lysis of target cells. These biotinylated and substrate specificity-based diphenyl phosphonates allowed facile evaluation of selectivity through activity-based profiling in cell lysates and intact cells. Both inhibitors were found to be extremely selective in vitro and in cells. Use of these inhibitors in cell-based assays revealed granzyme A to be a minor effector and granzyme B to be a major effector of target cell lysis by natural killer cells. These studies indicate that the proapoptotic granzyme B functions also as a pronecrotic effector of target cell death.

Introduction

Natural killer (NK) cells and cytotoxic T lymphocytes (CTL) function in immunity as the primary line of defense against viruses and other intracellular pathogens. These cytotoxic lymphocytes recognize host cells compromised by infection and kill them to halt pathogen proliferation. Death of infected target cells is predominantly mediated by members of the granzyme family of serine proteases in conjunction with the pore-forming protein perforin [1]. Through a mechanism referred to as granule exocytosis, vesicles containing the granzymes and perforin are secreted from the cytotoxic lymphocyte, and granule contents are taken up by the target cell into endocytic vesicles. Perforin then acts as an endosomolytic agent, allowing escape of the granzymes into the cytoplasm to carry out a series of proteolytic events that lead to several morphologic changes in the target cell. These include phosphatidylserine externalization, DNA damage, mitochondrial damage, and cell lysis, all of which culminate in death and elimination of the infected cell [2]. Although an essential component of immunity under normal conditions, aberrant cytotoxic lymphocyte activity can result in disease. In some cases, the granzymes may thus represent potential therapeutic targets [3–7].

Granzymes A and B are the most prominent members of the granzyme family, as numerous experiments have implicated them in the induction of target cell death. Notably, mice lacking either or both of these granzymes exhibit increased susceptibility to viral infections [8]. Additionally, far more is known about the molecular mechanism of action of these two granzymes than is known about the remaining "orphan" human granzymes, H, K, and M [9]. The substrate specificity of granzyme B is similar to that of apical caspases, and its function as an activator of apoptosis reflects this similarity. Granzyme B may also induce death through caspase-independent pathways. Major hallmarks of granzyme B-induced cellular damage are oligonucleosomal DNA fragmentation and mitochondrial damage [10]. An important pathway to granzyme A-induced damage involves cleavage and inactivation of SET (also known as PHAPII, TAF-Iβ, I_2^{PP2A}), which functions as an inhibitor of the DNase activity of the tumor metastasis suppressor NM23-H1. The resulting hallmark of granzyme A-induced damage is single-stranded DNA nicks mediated by NM23-H1 [11].

Most information concerning the function of granzymes A and B has been derived from genetic studies, or from reconstituted systems in which target cells are killed by exogenous addition of purified granzymes and perforin. Although genetic deletions in mice offer a high level of physiological relevance, compensation by related genes can sometimes obscure effects [12]. Compensation may be a particularly valid consideration in the case of the granzymes because several family members are highly homologous. Use of a reconstituted system is a practical approach to isolate effects of a specific granzyme, but it offers limited physiological relevance and can thus be susceptible to artifacts. A clear example of the potential discord between results obtained by using the two approaches comes from analysis of cell lysis, the standard morphological parameter for measurement of cytotoxic lymphocyte function. Cytotoxic lymphocytes from mice lacking either granzyme A, granzyme B, or both granzymes exhibit a relatively normal ability to lyse target cells [13]. In contrast, treatment of target cells with sublytic levels of perforin and either granzyme A or granzyme B leads to efficient cell lysis [14, 15].

As a means to study protein function, the use of selective chemical inhibitors can offer a high level of physiological relevance and a high level of temporal control that circumvents compensation by related genes [16]. Such an approach thus has the potential to complement the present understanding of the function of granzymes A and B. Toward the goal of establishing chemical functional probes for study of these two major granzymes, selective inhibitors targeted against granzymes A and B were developed for use in assays of cytotoxic lymphocyte function. Both inhibitors were designed to have the general architecture of tagged affinity labels. This design allowed for rapid evaluation of selectivity in the context of NK and target cell proteomes by SDS-PAGE analysis, followed by detection of a biotin label. Each inhibitor was found to be extremely selective for its target enzyme in the context of complex biochemical mixtures. The granzyme A- and B-targeted inhibitors were thus used to probe the con-

*Correspondence: craik@cgl.ucsf.edu

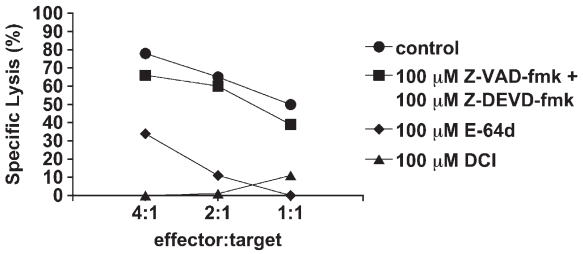

Figure 1. The Effect of Class-Specific Protease Inhibitors on NK Cell-Mediated Lysis of Target Cells

Lysis of K562 targets by NK-92 effectors is not inhibited by the caspase inhibitors Z-VAD-FMK and Z-DEVD-FMK, but it is inhibited by the cysteine protease inhibitor E-64d or the serine protease inhibitor DCI.

tributions of these two major granzymes to target cell lysis mediated by NK cells. In contrast to results obtained by using cytotoxic lymphocytes from granzyme-deficient mice, or reconstituted systems of purified granzymes and perforin, these studies revealed granzyme A to be a minor effector and granzyme B to be a major effector of target cell lysis by NK cells.

Results

Serine and Cysteine Protease Inhibitors, but Not Caspase Inhibitors, Block NK Cell-Mediated Lysis of Target Cells

To confirm prior findings related to which protease classes are required for cytotoxic lymphocyte-mediated cell lysis, cytotoxicity assays were carried out in the presence of the caspase inhibitors Z-VAD-FMK and Z-DEVD-FMK, the serine protease inhibitor DCI (3,4-dichloroisocoumarin), and the cysteine protease inhibitor E-64d (Figure 1). The NK cell line NK-92 and the NK-sensitive target cell line K562 were used as a model effector and target cell pair, respectively. A combination of Z-VAD-FMK and Z-DEVD-FMK only had a minor effect on cell lysis, in agreement with prior studies [17]. In contrast, DCI was found to be a potent inhibitor of lysis, again in agreement with prior studies [18]. The cysteine protease inhibitor E-64d was also found to be a potent inhibitor of lysis, possibly as a consequence of cathepsin C inhibition [19]. These results verify findings by other researchers and indicate that lysis of targets by NK cells is not dependent on caspases [17], but is dependent on serine proteases [18, 20]. Additionally, these results indicate that lysis is also dependent on cysteine proteases. While DCI is selective for serine proteases, some of its observed effect may be due to crossreactivity with cysteine proteases such as calpain [21].

The Five Human Granzymes Exhibit Divergent Extended Substrate Specificities

More selective inhibitors, particularly members of the granzyme family, would thus be useful for probing the role of serine proteases in target cell lysis. Since the activity of granzymes is highly dependent on extended and specific interactions with peptide ligands [22, 23], tailored peptide-based inhibitors may provide a means to selectively target individual granzymes in lysis assays. As a starting point for determining the feasibility of such an approach, the extended substrate specificities of all human granzymes were compared by using combinatorial libraries of protease substrates. The optimal P4-P3-P2-P1 (nomenclature for amino acid positions in substrates is Pn, Pn-1, ..., P2, P1 with amide bond hydrolysis occurring following P1) substrate specificities of the five human granzymes were determined to be IGNR for granzyme A, IEPD for granzyme B, PTSY for granzyme H, YRFK for granzyme K, and KVPL for granzyme M (Figure 2). These profiles indicate that the P4–P1 substrate specificities of the five human granzymes are quite distinct from one another. Such divergent specificities should allow preparation of granzyme-selective inhibitors.

Results obtained for granzymes A, B, and M are generally consistent with those obtained in previous studies with other positional scanning combinatorial libraries [23–25]. Although the optimal substrate specificity of granzyme A has previously been determined to be VANR, the single substrate Ac-IGNR-ACC was found to be approximately twice as sensitive as Ac-VANR-ACC (data not shown). Previous studies have demonstrated that granzyme H exhibits a preference for aromatic residues at P1 [26] and that granzyme K exhibits a preference for lysine and arginine at P1 [27]. The library profiles indicate that granzyme H, in fact, exhibits a strong preference for tyrosine over phenylalanine and tryptophan at this position, while granzyme K exhibits a strong preference for lysine over arginine. The P4–P2 substrate specificities of these two granzymes have not been previously characterized. The narrow specificity of the five granzymes at P1 is particularly remarkable. Since serine proteases derive much of the energy required for catalysis from binding P1, this property should aid in achieving selective chemical inhibition of a particular family member [28]. In instances in which some crossreactivity may be expected at P1, such as with granzymes A and K, distinct specificities at P4–P2 should further contribute to selectivity.

Substrate Specificity-Based Phosphonate Inhibitors as Affinity Labels of Granzymes A and B

Granzymes A and B have a clear role in NK cell-mediated cytotoxicity, but their role in induction of lysis is unclear. To explore the contribution of the major granzymes A and B to lysis of targets by NK cells, substrate specificity-based inhibitors of these two proteases were developed. These were designed to have the general architecture of affinity labels containing a diphenyl phosphonate electrophile, specificity elements to impart selectivity, and a biotin tag (Figures 3A and 3B). 4-amidinophenylglycine was used at the P1 position of the granzyme A inhibitor in place of arginine based on precedent [28]. Although it has been reported that granzyme B is not inhibited by an acylated diphenyl phosphonate ester analog of aspartic acid [29], peptide phosphonates based on the extended substrate specificity of granzyme B have not previously been described. Time-dependent inhibition and covalent labeling of recombinant granzymes A and B indi-

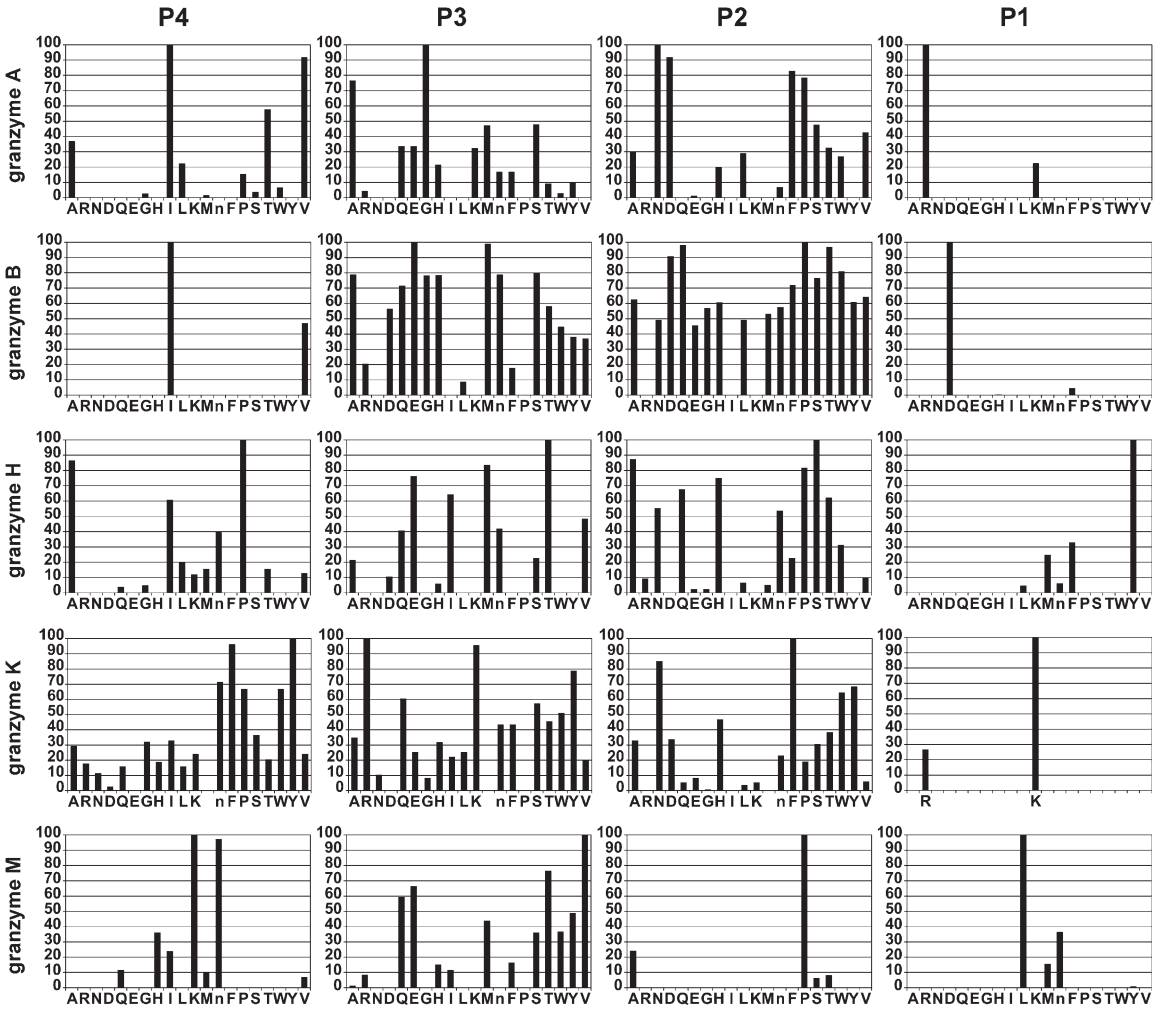

Figure 2. Substrate Specificity of Human Granzymes

Recombinant granzymes A, B, H, and M were profiled at positions P4–P1 by using a completely diversified PS-SCL of ACC substrates. Granzyme K was profiled at P4–P2 by using a PS-SCL of ACC substrates that is fixed as lysine at P1, and was profiled at P1 by using the P1-Arg and P1-Lys wells of the completely diversified PS-SCL. The y axis represents relative activity, and the x axis represents the fixed P4, P3, P2, or P1 amino acid (with norleucine represented by n). Activities for each enzyme are relative to the highest activity at each of the four substrate positions.

cate that bio-x-IGN(AmPhg)^P-$(OPh)_2$ and bio-x-IEPD^P-$(OPh)_2$ function as irreversible inhibitors of their target enzymes (Figures 3A and 3B). Activity assays of recombinant enzymes also indicate that both compounds are selective for their target enzymes in the context of the granzyme family (Table 1).

The Granzyme A- and B-Targeted Inhibitors Are Selective in the Context of an NK Cell Proteome

To evaluate the selectivity of bio-x-IGN(AmPhg)^P-$(OPh)_2$ and bio-x-IEPD^P-$(OPh)_2$ in the context of relevant complex biochemical mixtures, NK cell lysates were treated with each inhibitor and analyzed by SDS-PAGE, followed by avidin blotting. Treatment of NK-92 cell lysates with each inhibitor led to selective labeling of a single protein in a concentration-dependent manner (Figures 4A and 4B). Labeling was activity based since, in both cases, no labeling was detected when lysates were preinactivated by denaturation with 2% SDS. Immunoprecipitation of human granzyme A or B from lysates treated with inhibitors followed by avidin blotting of immunoprecipitates demonstrates that the protein labeled by bio-x-IGN(AmPhg)^P-$(OPh)_2$ is granzyme A and the protein labeled by bio-x-IEPD^P-$(OPh)_2$ is granzyme B (Figures 4C and 4D). Immunodepletion of lysates removed the vast majority of biotinylated protein, indicating that the inhibitors do not crossreact with proteins of similar molecular weight to granzymes A and B. Altogether, these labeling experiments indicate that bio-x-IGN(AmPhg)^P-$(OPh)_2$ and bio-x-IEPD^P-$(OPh)_2$ are remarkably selective for their target enzymes in the context of an NK cell proteome.

Treatment of intact NK-92 cells with bio-x-IGN(AmPhg)^P-$(OPh)_2$ or bio-x-IEPD^P-$(OPh)_2$ followed by extensive washing of the cells also led to selective labeling of granzymes A and B (Figures 4E and 4F). At

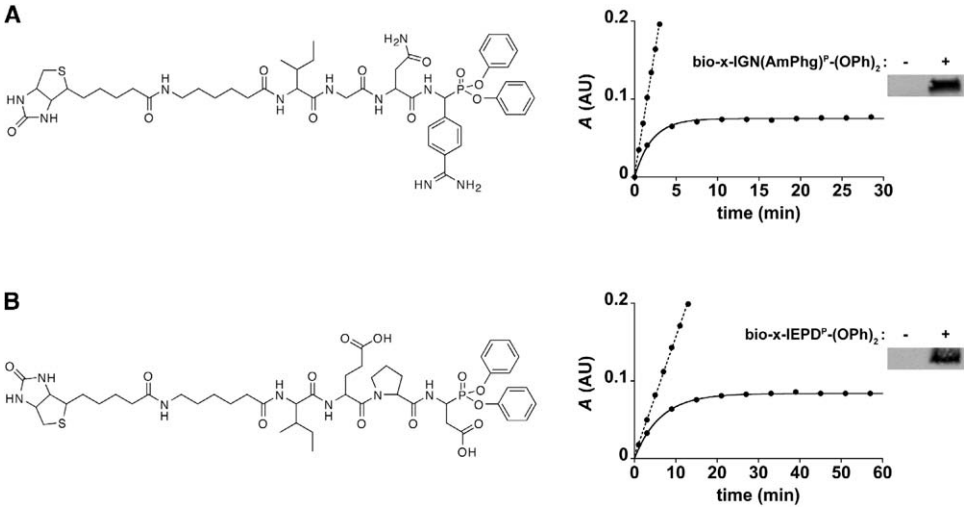

Figure 3. The Phosphonates Bio-x-IGN(AmPhg)^P-(OPh)$_2$ and Bio-x-IEPD^P-(OPh)$_2$ Are Irreversible Inhibitors of Their Target Enzymes

(A) Bio-x-IGN(AmPhg)^P-(OPh)$_2$ inhibits the activity of recombinant granzyme A (1 μM) with Suc-AAPR-*p*NA (1 mM) in a time-dependent manner (dashed line, control; solid line, 10 μM inhibitor). Incubation of recombinant granzyme A (1 ng) with bio-x-IGN(AmPhg)^P-(OPh)$_2$ (1 μM) for 1 hr, followed by 6 hr of dialysis, SDS-PAGE analysis, and avidin blotting, indicates covalent labeling of the enzyme.

(B) Bio-x-IEPD^P-(OPh)$_2$ inhibits the activity of recombinant granzyme B (100 nM) with Ac-IEPD-*p*NA (1 mM) in a time-dependent manner (dashed line, control; solid line, 10 μM inhibitor). Incubation of recombinant granzyme B (1 ng) with bio-x-IEPD^P-(OPh)$_2$ (1 μM) for 1 hr, followed by 6 hr of dialysis, SDS-PAGE analysis, and avidin blotting, indicates covalent labeling of the enzyme.

higher concentrations, the two inhibitors can access intracellular granzymes A and B, and they are thus partially cell permeable. Labeling in intact cells was also found to be time dependent, further indicating that this labeling is not a result of a reaction between granzymes and inhibitors upon disruption of plasma membrane integrity during preparation of lysates (data not shown). Lack of labeling saturation in intact cells may be due to a limit in cell permeability or to the fact that granzymes are maintained in a partially inactive state in the acidic granules of NK cells (pH ~5). The activity of serine proteases is optimal at close to neutral pH and decreases at lower pH [30]. Reactivity between phosphonate inhibitors and the catalytic serine residue of granzymes is thus expected to be decreased in the acidic environment of intact granules. Treatment of intact NK cells with nonbiotinylated inhibitors, followed by preparation of lysates, and evaluation of inhibition by labeling with biotinylated inhibitors also revealed only partial granzyme inactivation (data not shown).

Table 1. Inhibition of Human Granzymes by Bio-x-IGN(AmPhg)^P-(OPh)$_2$ and Bio-x-IEPD^P-(OPh)$_2$

	$k_{obs}/[I]$ (M^{-1} s^{-1})	
Granzyme	Bio-x-IGN(AmPhg)^P-(OPh)$_2$	Bio-x-IEPD^P-(OPh)$_2$
A	2000 ± 200	NI
B	NI[a]	460 ± 35
H	NI	NI
K	NI	NI
M	NI	NI

Inhibition constants represent the average, and errors represent the standard deviation from three separate experiments.

[a] NI indicates less than 10% inhibition after 4 hr of incubation with 100 μM inhibitor.

Use of Granzyme A- and B-Targeted Inhibitors as Functional Probes Indicates that Granzyme B Is a Major Mediator of NK Cell-Mediated Lysis of Target Cells

Although the amidine of bio-x-IGN(AmPhg)^P-(OPh)$_2$ and the two acids of bio-x-IEPD^P-(OPh)$_2$ are likely to be detrimental to cell permeability, labeling of granzymes A and B in intact NK cells demonstrates that the two inhibitors are partially cell permeable. To shed light on the roles of granzymes A and B in the lysis of target cells mediated by NK cells, cytotoxicity assays with NK-92 effectors and K562 targets were carried out in the presence of bio-x-IGN(AmPhg)^P-(OPh)$_2$ and bio-x-IEPD^P-(OPh)$_2$. The granzyme B inhibitor blocked lysis by more than 75%, while the granzyme A inhibitor had a far less pronounced effect (Figure 5A). The effect observed with bio-x-IEPD^P-(OPh)$_2$ in cytotoxicity assays was found to be dependent on preincubation of cells with the inhibitor. This may be indicative of an active form of inhibitor uptake such as pinocytosis, which has previously been observed for other peptide-based compounds [31]. Studies were also conducted to examine the effect of both inhibitors on the processing of the granzyme A protein substrate SET and the granzyme B protein substrate caspase-3, but technical difficulties related to low sensitivity in Western blots and adventitious proteolysis during sample preparation precluded clean interpretation of results.

To confirm that bio-x-IEPD^P-(OPh)$_2$ exerts its effect through the blockade of a protein in effector cells, the inhibitor was preincubated for 4 hr with effectors,

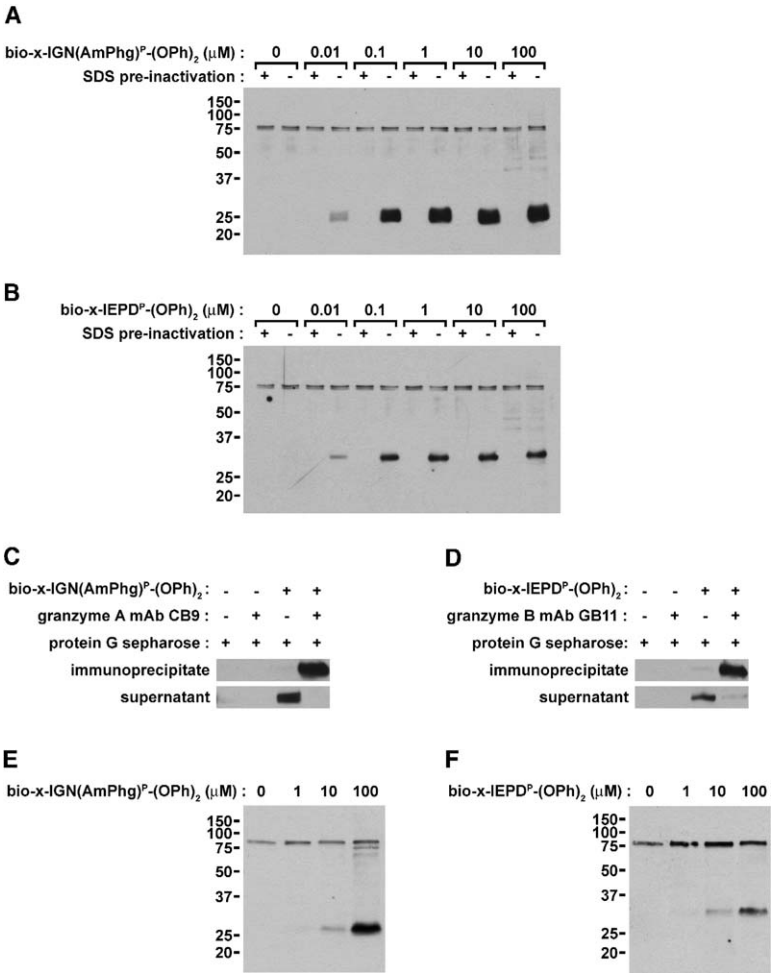

Figure 4. Selective Targeting of Granzyme A by Bio-x-IGN(AmPhg)^P-(OPh)$_2$ and Granzyme B by Bio-x-IEPD^P-(OPh)$_2$ in NK Cell Lysates and Intact NK Cells

(A) Concentration-dependent and activity-based labeling of a single protein in NK-92 cell lysates by bio-x-IGN(AmPhg)^P-(OPh)$_2$.

(B) Concentration-dependent and activity-based labeling of a single protein in NK-92 cell lysates by bio-x-IEPD^P-(OPh)$_2$.

(C) Immunoprecipitation of NK-92 cell lysates indicates that the protein labeled by bio-x-IGN(AmPhg)^P-(OPh)$_2$ is granzyme A.

(D) Immunoprecipitation of NK-92 cell lysates indicated that the protein labeled by bio-x-IEPD^P-(OPh)$_2$ is granzyme B.

(E) Concentration-dependent labeling of granzyme A in intact NK-92 cells by bio-x-IGN(AmPhg)^P-(OPh)$_2$.

(F) Concentration-dependent labeling of granzyme B in intact NK-92 cells by bio-x-IEPD^P-(OPh)$_2$.

targets, or both prior to the start of killing assays. Such longer incubations with targets were not compatible with time-resolved fluorimetric cytotoxicity assays because of prohibitively high background TDA release. Cell mixtures were instead analyzed by flow cytometry for propidium iodide incorporation by using APC-conjugated anti-CD56 to distinguish effectors from targets (Figure 5B). In the absence of bio-x-IEPD^P-(OPh)$_2$, 31.4% of targets were found to be propidium iodide positive. When only targets were preincubated with bio-x-IEPD^P-(OPh)$_2$, 29.6% of targets were found to be propidium iodide positive. When only effectors were preincubated with bio-x-IEPD^P-(OPh)$_2$, 21.6% of targets were found to be propidium iodide positive. Finally, when both targets and effectors were preincubated with bio-x-IEPD^P-(OPh)$_2$, 17.0% of targets were found to be propidium iodide positive. Inhibition of lysis by bio-x-IEPD^P-(OPh)$_2$ is thus more dependent on preincubation with effectors than with targets. These results indicate that bio-x-IEPD^P-(OPh)$_2$ acts on a protein found in effector cells and also validate the effect observed in the time-resolved fluorimetric cytotoxicity assay (Figure 5A). The magnitude of inhibition by bio-x-IEPD^P-(OPh)$_2$ in the cytometry assay is an underestimate because of a consistent background of ~5% propidium iodide-positive targets and because dead cells that have disintegrated to debris are not accounted for.

Levels of Granzymes A and B in NK Cell Lines Correlate with Cytotoxicity

Labeling with both inhibitors was observed in lysates of the NK cell lines NK-92 and NKL, but not in lysates of the control cell lines K562 and Daudi (Figure 6A). The activity-based profiles of granzymes A and B across cell lines are generally consistent with Western blots of the same lysates for granzymes A and B (Figure 6A). Interestingly, densitometric quantitation of these avidin and Western blots reveals that, on a per cell basis, very low levels of granzyme B are found in the cell line NKL (Figure 6B). Even with high sample loading (2×10^6 cells per lane in Figure 6 versus 5×10^4 cells per lane in Figure 4), granzyme B is not easily detected in NKL lysates with the commonly used anti-human granzyme B monoclonal antibody 2C5. Activity-based profiling with bio-x-IEPD^P-(OPh)$_2$ appears to be a more sensitive method for detection of granzyme B, allowing for a comparison between granzyme B levels in NK-92 and NKL cells. Labeling with inhibitors indicates that while 30% less granzyme A is found in NKL cells than in NK-92 cells, 85% less granzyme B is found in NKL cells than in NK-92 cells. In agreement with the effect ob-

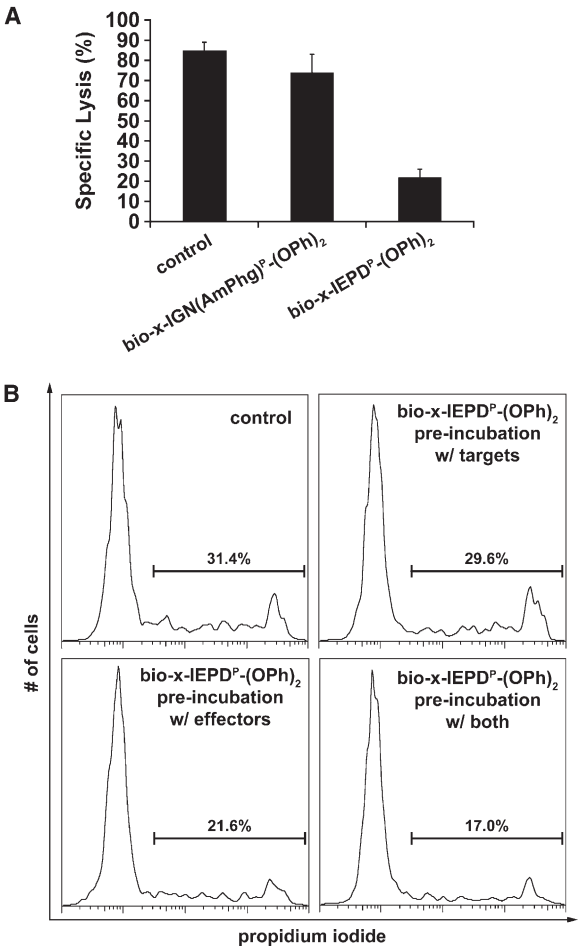

Figure 5. Granzyme B Functions as a Major Effector of NK Cell-Mediated Lysis of Target Cells

(A) Bio-x-IEPD^P-(OPh)$_2$, but not bio-x-IGN(AmPhg)^P-(OPh)$_2$, inhibits lysis of K562 targets by NK-92 effectors. Effectors were preincubated for 4 hr, and targets were preincubated for 1 hr alone, with 100 μM bio-x-IGN(AmPhg)^P-(OPh)$_2$, or with 100 μM bio-x-IEPD^P-(OPh)$_2$ prior to mixing at an effector to target cell ratio of four. Inhibitors were also present during the cytotoxicity assay at the same concentrations. Data represent the average, and error bars represent the standard deviation from four separate experiments.

(B) The effect of bio-x-IEPD^P-(OPh)$_2$ is dependent on preincubation with effectors, but it is not dependent on preincubation with targets. 100 μM bio-x-IEPD^P-(OPh)$_2$ was preincubated for 4 hr with effectors, targets, or both prior to mixing at an effector to target cell ratio of four. Following incubation, cell mixtures were analyzed by flow cytometry for propidium iodide incorporation by using APC-conjugated anti-CD56 to distinguish effectors from targets.

served with bio-x-IEPD^P-(OPh)$_2$ in the cell lysis assays, the decreased granzyme B levels in NKL cells correlate with the lower lytic potential exhibited by this cell line (Figure 6C).

Discussion

Genetic studies of granzymes A and B have greatly contributed to the understanding of these two proteases as effectors of cell-based immunity. The increased susceptibility to viral infection exhibited by mice lacking either one or both of these granzymes is especially

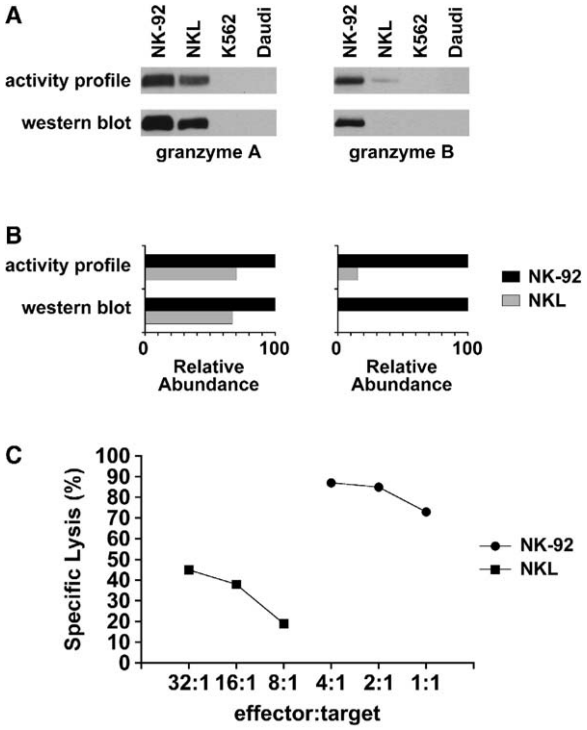

Figure 6. Levels of Granzymes A and B in Different Cell Lines Are Consistent with Granzyme B Serving a More Prominent Role in Target Cell Lysis than Granzyme A

(A) Activity-based profiling of granzyme A with 100 μM bio-x-IGN(AmPhg)^P-(OPh)$_2$, granzyme B with 100 μM bio-x-IEPD^P-(OPh)$_2$, and Western blotting of granzymes A and B across different cell lines.

(B) Densitometric quantitation of activity-based profiles and Western blots reveals markedly lower levels of granzyme B, but not granzyme A, in the cell line NKL relative to the cell line NK-92.

(C) Lower levels of granzyme B in the cell line NKL correlate with decreased cytotoxicity relative to the cell line NK-92.

compelling evidence for their important role in immunity [8]. Although knowledge gained from granzyme A and B knockout mice exemplifies how traditional genetics continues to be an immensely powerful tool for study of protein function, the utility and advantages of chemical genetics are gaining increasing recognition [16]. In particular, use of chemical inhibitors to block protein function offers a level of temporal control that is unparalleled, even in comparison to gene silencing by RNA interference. A common drawback of using small molecules as functional probes is crossreactivity with unintended targets, particularly when the intended target is highly homologous to a set of other proteins. An elegant and generalizable solution to this problem, which combines synthetic chemistry and genetics, has been applied to the study of protein kinases [32]. A similarly generalizable approach has yet to be developed for proteases.

The inhibitors described herein were developed with the goal of establishing chemical probes to further current understanding of granzymes A and B. As a means to account for potential inhibitor crossreactivity, these compounds were designed as tagged affinity labels.

Such design permitted rapid and facile evaluation of selectivity in the context of complex mixtures. The granzyme A inhibitor bio-x-IGN(AmPhg)^P-$(OPh)_2$ and the granzyme B inhibitor bio-x-IEPD^P-$(OPh)_2$ were both found to be remarkably selective based on labeling in cell lysates and intact cells. Selectivity among all five human granzymes was also verified by activity. Much like peptide acyloxymethylketone and fluoromethylketone inhibitors of caspases [33], the granzyme A and B inhibitors described in this work demonstrate how tailoring of specificity determinants and choice of an appropriate electrophile facilitate preparation of extremely selective peptide-based inhibitors of proteases. Whereas electrophiles such as chloromethylketones and aldehydes react with active site nucleophiles of a variety of enzymes, fluorophosphate inhibitors are selective for serine hydrolases [34], and peptidyl diphenyl phosphonate esters exhibit an additional level of selectivity for serine proteases [35]. The selectivity of bio-x-IGN(AmPhg)^P-$(OPh)_2$ and bio-x-IEPD^P-$(OPh)_2$ in a cellular context makes them ideal functional probes for studies of cytotoxic lymphocyte function.

Experiments with lymphocytes from knockout mice, and those with reconstituted systems of purified perforin and granzymes, are generally in agreement regarding the roles of granzymes A and B in DNA damage [13, 15, 36]. In contrast, results regarding the role of these proteases in lysis are conflicting [13–15]. Target cell lysis was originally attributed to the formation of large pores in the plasma membrane of target cells by perforin polymers. But this model was updated following the discovery that these pores are not large enough to allow passage of the macromolecular complex between granzymes and the proteoglycan serglycin [37, 38]. The current model of granule exocytosis involves uptake of granzyme-serglycin complexes and perforin by target cells into endosomes. Through an unknown mechanism, perforin then acts as an endosomolytic agent to allow escape of the granzymes into the cytoplasm [37, 38]. Although perforin is absolutely required for lysis of target cells by cytotoxic lymphocytes [39], it is unlikely to be the sole effector of lysis under physiological conditions. The first among several indications that serine proteases play a role in this process came from experiments in which general serine protease inhibitors such as DCI and DFP (diisopropyl phosphofluoridate) were observed to block the cytolytic activity of cytotoxic lymphocytes [18, 20].

Use of bio-x-IGN(AmPhg)^P-$(OPh)_2$ and bio-x-IEPD^P-$(OPh)_2$ in cytotoxicity assays has herein allowed dissection of the contribution of granzymes A and B to lysis of target cells by NK cells. The granzyme B inhibitor bio-x-IEPD^P-$(OPh)_2$ potently inhibited lysis of K562 target cells by NK-92 effector cells, while the granzyme A inhibitor bio-x-IGN(AmPhg)^P-$(OPh)_2$ had a less pronounced effect. Lack of a pronounced effect on lysis in the presence of bio-x-IGN(AmPhg)^P-$(OPh)_2$ may be a result of inefficient granzyme A inhibition in the cell-based assay, possibly due to poor cell permeability. Alternatively, lack of a pronounced effect may be an indication that granzyme A is only a minor effector of lysis. The latter scenario appears more likely given that both bio-x-IGN(AmPhg)^P-$(OPh)_2$ and bio-x-IEPD^P-$(OPh)_2$ are efficient inhibitors of their target enzymes in vitro, that labeling indicates that both access their target enzymes with approximately the same efficiency in intact NK cells, and that the charged and thus presumably sparingly cell-permeable bio-x-IEPD^P-$(OPh)_2$ is active in the same assay. More importantly, a greater than 75% blockade of lysis by bio-x-IEPD^P-$(OPh)_2$ in a standard assay indicates that, even if the remaining 25% lysis is completely attributable to granzyme A, granzyme B functions as a major effector of target cell lysis, and granzyme A likely functions as a relatively minor effector in the same process.

This finding stands in contrast to experiments with cytotoxic lymphocytes from knockout mice, which support a role for neither granzyme in lysis [13], and experiments with reconstituted systems, which support a role for both granzymes in lysis [14, 15]. A potential reconciliation for results from all three methods of study is that: 1) no marked effect in lysis is observed for either granzyme in genetic studies because of compensation by other granzymes; 2) a marked effect is observed for both granzymes in reconstituted systems because the concentrations and/or mode of delivery of granzymes and perforin are not physiological; 3) a marked effect is observed for granzyme B, but not for granzyme A, in pharmacological studies because temporal control circumvents compensation, and because no alterations are made to the concentrations and mode of delivery of granzymes and perforin. Studies are underway to determine whether granzyme B functions as a major mediator of lysis in systems other than the model of NK-92 effectors and K562 targets used in this work.

A role for granzyme B in lysis has also subsequently been verified by using a potent and selective reversible inhibitor of granzyme B obtained from Merck [40]. This compound exhibits an inhibitory effect similar to that of bio-x-IEPD^P-$(OPh)_2$ in our lysis assays (data not shown). A role for granzyme B in lysis is also consistent with levels of this protease in the cell lines NK-92 and NKL. Far higher effector to target cell ratios are necessary to achieve maximal lysis with the NKL line than with the NK-92 line [41, 42]. Although similar granzyme A levels were found in both cell lines, significantly lower granzyme B levels were found in the NKL line. While small-molecule inhibitors of granzyme A that are selective in vitro have been reported to be active in cell lysis assays [43, 44], their selectivity in a cellular context has not been verified. It is therefore possible that the observed effect is a result of crossreactivity with an unknown target or granzyme B. Many of these compounds are based on an isocoumarin scaffold, which is known to inhibit not only serine proteases but also some cysteine proteases and proteasome activities [21]. Possible crossreactivity of these compounds with cysteine proteases is particularly relevant since use of E-64d demonstrates that a blockade of cysteine protease activity also affects lysis.

Cytotoxic lymphocyte-mediated death undoubtedly has an apoptotic component, manifested in hallmarks such as caspase activation, oligonucleosomal DNA fragmentation, and mitochondrial depolarization. On the other hand, rapid lysis of target cells clearly indicates that cytotoxic lymphocyte-mediated death has a necrotic component as well. This necrosis is not likely to be a secondary effect of apoptosis since it occurs indepen-

dently of several apoptotic hallmarks [17] and occurs more rapidly than secondary necrosis [45]. Because granzyme B activates caspases and cleaves several known caspase substrates, it has generally been thought of as a proapoptotic effector of death. The work presented herein directly implicates granzyme B in death pathways culminating in lysis, and therefore indicates that granzyme B is a pronecrotic as well as proapoptotic effector of death. Although the molecular mechanism of lysis in cytotoxic lymphocyte-mediated death is not well understood, it is tempting to speculate that plasma membrane disruption is a downstream result of granzyme B-mediated processing of specific protein substrates. It has been demonstrated that cleavage of plasma membrane Ca^{2+} pumps by caspases in apoptotic neurons leads to secondary necrosis [46]. Whether granzyme B alters the osmotic balance of target cells through a similar mechanism remains to be determined. The granzyme B inhibitor bio-x-IEPD^{P}-$(OPh)_2$ should serve as a useful tool for dissection of pronecrotic pathways involving granzyme B and for identification of macromolecular substrates in these pathways.

The granule exocytosis pathway has been linked to autoimmune disorders such as allograft rejection [3, 4], rheumatoid arthritis [5], diabetes [6], and graft-versus-host disease [7]. Some of the granzymes may represent potential therapeutic targets for these disorders. In particular, an interesting molecular link exists between granzyme B and autoimmunity since several protein substrates of granzyme B are known autoantigens [47]. The finding that granzyme B is a key mediator of target cell lysis sheds new light on its potential role in the progression of autoimmune disease. Uptake of apoptotic cells by phagocytes, which occurs under conditions of normal tissue turnover, generally results in the induction of tolerance to self. In contrast, uptake of necrotic cells by phagocytes, which occurs following cytotoxic lymphocyte-mediated lysis of virally infected cells, results in T cell activation and induction of an inflammatory response [48]. As a major mediator of cell lysis by cytotoxic lymphocytes, granzyme B functions not only as an effector of death, but also as a key propagator of the immune response. In this sense, chemical blockade of granzyme B can be predicted to result in an anti-inflammatory effect that attenuates autoimmunity. It is noteworthy that the granule exocytosis pathway is linked to several of the autoimmune disorders for which the proinflammatory cytokine TNF-α is a therapeutic target [49]. The work presented herein indicates that granzyme B may be a viable alternate point of intervention for treatment of these disorders.

Significance

The substrate specificity-based diphenyl phosphonates prepared herein for granzymes A and B were found to be remarkably selective for their target enzymes, even in the context of complex biochemical mixtures. As affinity labels, these inhibitors function as sensitive activity-based probes of granzymes A and B. More significantly, use of these inhibitors in cell-based assays has yielded new, to our knowledge, insight into the function of granzymes A and B that could not be easily deduced from prior genetic or biochemical experiments.

Results regarding the roles of granzymes A and B in the lysis of target cells mediated by cytotoxic lymphocytes are conflicting. It is possible that this conflict is a result of compensation in the case of genetic experiments and low physiological relevance in the case of biochemical experiments. Development of bio-x-IGN(AmPhg)^{P}-$(OPh)_2$ and bio-x-IEPD^{P}-$(OPh)_2$ has allowed for a selective chemical blockade of granzymes A and B in cell-based assays as an alternative approach for probing the function of these two major granzymes. This pharmacological approach has demonstrated that granzyme B functions as a major effector of NK cell-mediated lysis of target cells, while granzyme A likely plays a secondary role in this process.

This is an interesting finding because it directly implicates granzyme B in pronecrotic pathways of death in addition to the proapoptotic pathways it is already known to trigger through the caspases. Importantly and in agreement with prior studies, NK cell-mediated target cell lysis was found to be caspase independent, indicating that bio-x-IEPD^{P}-$(OPh)_2$ does not exert its effect through caspase inhibition. Because phagocytic uptake of virally infected cells that have undergone necrotic death induces a proinflammatory immune response, it is likely that granzyme B functions as a key propagator as well as a mediator of the immune response following pathogen infection.

Experimental Procedures

Reagents

Unless otherwise specified, all solvents and chemicals were obtained from Sigma-Aldrich. Amino acids and resins used for peptide synthesis were obtained from Novabiochem. DCI was obtained from Roche. Z-VAD(OMe)-FMK, Z-D(OMe)E(OMe)VD(OMe)-FMK, and recombinant human granzyme K from *E. coli* were obtained from Calbiochem. E-64d, Suc-AAPR-*p*NA, Ac-IEPD-*p*NA, Suc-FLF-SBzl, and Z-K-SBzl were obtained from Bachem. Ac-KVPL-ACC was prepared as previously described [23]. The cell lines NK-92 and K562 were obtained from the American Type Culture Collection (ATCC). The cell lines NKL and Daudi were generous gifts from Dr. Lewis Lanier (University of California, San Francisco).

Heterologous Expression and Purification of Human Granzymes A, B, H, and M

Recombinant human granzymes A and M from *P. pastoris* were prepared and purified as previously described [23, 24]. The cDNA encoding mature human granzyme B was a generous gift from Dr. Sandra Waugh Ruggles (Catalyst Biosciences), and the cDNA encoding mature human granzyme H was amplified from I.M.A.G.E. clone 5936395. Recombinant human granzymes B and H were prepared and purified in a manner analogous to that reported for recombinant human granzymes A and M.

Positional Scanning Synthetic Combinatorial Libraries

Human granzymes A, B, H, and M were profiled by using a completely diversified PS-SCL (positional scanning synthetic combinatorial library) of tetrapeptide ACC (7-amino-4-carbamoylmethylcoumarin) substrates (Y. Choe and C.S.C., unpublished data). Human granzyme K was profiled by using the Ac-XXXR-ACC and Ac-XXXK-ACC wells of the completely diversified PS-SCL, and a P1-Lys PS-SCL of tetrapeptide ACC substrates [50]. Screening of libraries was carried out as previously described [50].

Synthesis of Bio-x-IGN(AmPhg)^P-(OPh)$_2$

Detailed synthetic procedures are included as Supplemental Data available online. In general, the diphenyl phosphonate ester analog of 4-amidinophenylglycine, H-(AmPhg)^P-(OPh)$_2$, was prepared essentially as previously described [51] and was purified by HPLC. The peptide biotinyl-Ahx-Ile-Gly-Asn(Trt)-OH was prepared on 2-chlorotrityl chloride resin by using standard fluorenylmethoxycarbonyl chemistry and was purified by HPLC. Phosphonate and peptide were coupled by using PyBOP and diisopropylethylamine in dimethylformamide, and the product biotinyl-Ahx-Ile-Gly-Asn(Trt)-(AmPhg)^P-(OPh)$_2$ was obtained following HPLC purification. The trityl protecting group was removed in 95% trifluoroacetic acid, 2.5% water, and 2.5% triisopropylsilane. The final product, biotinyl-Ahx-Ile-Gly-Asn-(AmPhg)^P-(OPh)$_2$, was obtained following HPLC purification and was characterized by mass spectrometry. Mass calcd for $C_{48}H_{65}N_{10}O_{10}PS$: 1004.4. MALDI-TOF *m/z* found: 1005.7 $(M+H)^+$.

Synthesis of Bio-x-IEPD^P-(OPh)$_2$

Detailed synthetic procedures are included as Supplemental Data. In general, the side chain-protected diphenyl phosphonate ester analog of aspartic acid, H-Asp(ONb)^P-(OPh)$_2$, was prepared by using *p*-nitrobenzyl formylacetate in the condensation reaction developed by Oleksyszyn et al. [52] and was purified by HPLC. The peptide biotinyl-Ahx-Ile-Glu(OBzl)-Pro-OH was prepared on 2-chlorotrityl chloride resin by using standard fluorenylmethoxycarbonyl chemistry and was purified by HPLC. Phosphonate and peptide were coupled by using PyBOP and diisopropylethylamine in dimethylformamide, and the product biotinyl-Ahx-Ile-Glu(OBzl)-Pro-Asp(ONb)^P-(OPh)$_2$ was obtained following HPLC purification. Benzyl and *p*-nitrobenzyl protecting groups were removed by catalytic hydrogenation in ethanol. The final product, biotinyl-Ahx-Ile-Glu-Pro-Asp^P-(OPh)$_2$, was obtained following HPLC purification and was characterized by mass spectrometry. Mass calcd for $C_{47}H_{66}N_7O_{13}PS$: 999.4. MALDI-TOF *m/z* found: 999.7 $(M+H)^+$.

Inhibitor Kinetics

The pseudo second-order rate constants $k_{obs}/[I]$ for inhibition of recombinant granzyme A (100 nM) by bio-x-IGN(AmPhg)^P-(OPh)$_2$ (1 μM) and recombinant granzyme B (1 μM) by bio-x-IEPD^P-(OPh)$_2$ (10 μM) were determined according to the method of Kitz and Wilson [53]. Enzymes and inhibitors were incubated at 25°C in the same assay buffer as that used for PS-SCL studies, and they were diluted 100-fold into assay buffer at fixed intervals. The remaining enzymatic activity was determined by using Z-K-SBzl (1 mM) for granzyme A and Ac-IEPD-*p*NA (1 mM) for granzyme B. Crossreactivity of both inhibitors among granzymes was determined by incubation of each granzyme (1 μM) with either inhibitor (100 μM) at 25°C in assay buffer for 4 hr. The remaining enzymatic activity was determined following 100-fold dilutions into assay buffer by using Z-K-SBzl (1 mM) for granzymes A and K, Ac-IEPD-*p*NA (1 mM) for granzyme B, Suc-FLF-SBzl (100 μM) for granzyme H, and Ac-KVPL-ACC (1 mM) for granzyme M. Final DMSO concentrations in assays never exceeded 2%. Hydrolysis of SBzl substrates was monitored spectrophotometrically at 405 in the presence of DTNB (5,5′-dithio-bis(2-nitrobenzoic acid)) (100 μM). Hydrolysis of Ac-IEPD-*p*NA was monitored spectrophotometrically at 405 nm. Hydrolysis of Ac-KVPL-ACC was monitored as described for library assays.

Affinity Labeling of Cell Lysates

Bio-x-IGN(AmPhg)^P-(OPh)$_2$ or bio-x-IEPD^P-(OPh)$_2$ from DMSO stocks was added to 25 μl 10^6 cell/ml or 10^7 cell/ml lysates of NK-92, NKL, K562, or Daudi cells prepared in PBST (phosphate-buffered saline with 0.1% Triton X-100). Final inhibitor concentrations were as indicated in the Results section. Final DMSO concentrations were 1%. Lysates were incubated with inhibitors for 1 hr at 25°C. 12% SDS (5 μl) was added to lysates either before incubation for preinactivated samples or following incubation to quench reactions. Following the addition of 6× gel loading buffer (6 μl) to the resulting lysates, samples were boiled for 2 min, and sample volumes corresponding to 5×10^4 cells/well for inhibitor titrations or to 2×10^6 cells/well for activity profiling across cell lines were analyzed by SDS-PAGE and avidin blotting as described below.

Affinity Labeling of Intact NK Cells

Bio-x-IGN(AmPhg)^P-(OPh)$_2$ or bio-x-IEPD^P-(OPh)$_2$ from DMSO stocks was added to 25 μl 10^7 intact NK-92 cells/ml in RPMI 1640 containing 10% fetal bovine serum. Final inhibitor concentrations were as indicated in the Results section. Final DMSO concentrations were 1%. Cells were incubated with inhibitors for 4 hr at 37°C, washed with PBS (5 × 500 μl), and lysed in PBST (250 μl). Following the addition of 6× gel loading buffer (10 μl) to 50 μl of the resulting lysates, samples were boiled for 2 min, and sample volumes corresponding to 1×10^5 cells/well were analyzed by SDS-PAGE and avidin blotting as described below.

Immunoprecipitation

Bio-x-IGN(AmPhg)^P-(OPh)$_2$ or bio-x-IEPD^P-(OPh)$_2$ from DMSO stocks was added to 25 μl 10^7 cell/ml NK-92 cell lysates prepared in PBST. Final inhibitor concentrations were 100 μM, and final DMSO concentrations were 1%. Cell lysates were incubated with inhibitors for 1 hr at 25°C and were diluted into PBS (215 μl) containing 2.5 μg anti-human granzyme A monoclonal antibody CB9 (BD Biosciences) or anti-human granzyme B monoclonal antibody GB11 (Serotec). Diluted lysates were incubated with antibodies for 1 hr at 25°C, and a 50% slurry of protein G sepharose (Amersham Pharmacia) in PBST (20 μl) was added. Lysates were incubated with sepharose beads for 1 hr at 25°C, and beads were separated from supernatant and washed with PBS (3 × 500 μl). 12% SDS (5 μl) and 6× gel loading buffer (6 μl) were added to 25 μl of the original supernatant. PBS (50 μl), 12% SDS (5 μl), and 6× gel loading buffer (6 μl) were added to the beads. Samples were boiled for 2 min, and sample volumes corresponding to 1×10^5 cells/well for supernatant or 60 μl for beads were then analyzed by SDS-PAGE and avidin blotting as described below.

Avidin Blot Analysis

Protein samples were separated by SDS-PAGE and transferred to a nitrocellulose membrane. The membrane was blocked in TBST (Tris-buffered saline with 0.1% Triton X-100) containing 5% nonfat dry milk, washed with TBST, incubated in a solution of VECTASTAIN ABC Standard (Vector Laboratories) biotin detection reagent in TBST according to manufacturer's instructions, and washed with TBST. Avidin bound protein bands were then detected by enhanced chemiluminescence (Amersham Biosciences).

Immunoblot Analysis

Protein samples were separated by SDS-PAGE and transferred to a nitrocellulose membrane (Schleicher & Schuell). The membrane was blocked in TBST containing 5% nonfat dry milk, incubated in a solution of anti-human granzyme A monoclonal antibody GA6 (Serotec) (1 μg/ml) or a solution of anti-human granzyme B monoclonal antibody 2C5 (BD Biosciences) (1 μg/ml) in TBST containing 5% nonfat dry milk, washed with TBST, incubated in a 1:10,000 dilution of horseradish peroxidase-conjugated secondary antibody (Bio-Rad) in TBST containing 5% nonfat dry milk, and washed again with TBST. Antibody bound protein bands were then detected by enhanced chemiluminescence (Amersham Biosciences).

Cell-Mediated Cytotoxicity Assay

The lysis assay employed was a time-resolved fluorimetric assay based on preloading of target cells with BATDA (Perkin Elmer), an acetoxymethyl ester of the Eu^{3+} fluorescence-enhancing ligand TDA, and quantitation of EuTDA chelate fluorescence following addition of cell media to a solution of Eu^{3+}. Results obtained by using this assay are similar to those obtained by using the more traditional radioactive ^{51}Cr release assay [54]. K562 cells were incubated at 10^6 cells/ml in RPMI 1640 containing 10% fetal bovine serum with BATDA (20–80 μM) for 2 hr at 37°C. These targets were then washed five times with 1 ml PBS. Assays were conducted in round-bottom 96-well plates with 5000 targets per well. The number of effectors corresponded to the effector to target cell ratios indicated in the Results section. A total volume of 200 μl RPMI 1640 containing 10% fetal bovine serum was used per well. Targets and effectors were mixed, and plates were centrifuged and incubated for 4 hr at 37°C. Maximum release was determined by the addition of 0.05% Triton X-100 to wells containing 5000 targets 30

min prior to the end of the assay. Spontaneous release was determined by using media from wells containing 5000 targets. Media from each well (10 μl) were added to 96-well plates containing 50 μM $EuCl_3 \cdot 6H_2O$ in 300 mM acetate (pH 4.0) (100 μl). Following agitation of plates for 5 min, time-resolved fluorescence was measured by using an excitation wavelength of 289 nm and an emission wavelength of 616 nm. Percent specific lysis was calculated as 100 × (experimental release – spontaneous release)/(maximum release – spontaneous release). Each experimental, spontaneous, or maximum release value used in this calculation was an average of values from triplicate wells.

Inhibitors in Cell-Mediated Cytotoxicity Assays

Efficacy of inhibitors in the cytotoxicity assay described above was evaluated by preincubating effectors with each inhibitor in RPMI 1640 containing 10% fetal bovine serum (100 μl) for 4 hr at 37°C, preincubating targets (after BATDA preloading) with each inhibitor in RPMI 1640 containing 10% fetal bovine serum (100 μl) for 1 hr at 37°C, and then mixing both to start the cytotoxicity assay as described above. DCI was preincubated with effectors for 1 hr instead of 4. Final inhibitor concentrations were 100 μM. Final DMSO concentrations were 1%. Inhibitors were not toxic at the concentrations used in assays, as evaluated by trypan blue exclusion. The inhibitors bio-x-IGN(AmPhg)^P-$(OPh)_2$ and bio-x-IEPD^P-$(OPh)_2$ were used at a maximal concentration of 100 μM since concentrations higher than 200 μM were found to be toxic.

Flow Cytometry

NK-92 cells and K562 cells at 2 × 10^6 cells/ml in RPMI 1640 containing 10% fetal bovine serum (50 μl in both cases) were incubated at 37°C for 4 hr alone or in the presence of bio-x-IEPD^P-$(OPh)_2$ (100 μM). Final DMSO concentrations were 1% in all cases. Following inhibitor preincubations, NK-92 cells (40 μl) were mixed with K562 cells (10 μl) and incubated at 37°C for another 2 hr. PBS (425 μl), APC-conjugated anti-CD56 antibody (BD Pharmingen) (20 μl), and 10 μg/ml propidium iodide (Molecular Probes) were then added to the cell mixture. Cells were incubated in the dark at 25°C for 30 min, and samples were analyzed on a BD FACSCalibur flow cytometry system.

Supplemental Data

Supplemental Data containing detailed synthetic procedures for the preparation of bio-x-IGN(AmPhg)^P-$(OPh)_2$ and bio-x-IEPD^P-$(OPh)_2$ are available at http://www.chembiol.com/cgi/content/full/12/5/567/DC1/.

Acknowledgments

We thank Prof. Lewis Lanier, Prof. Kevan Shokat, Prof. Arthur Weiss, and members of the Craik lab for helpful discussions throughout the course of this work. We also thank Dr. Nancy Thornberry (Merck) for the generous gift of the reversible small-molecule granzyme B inhibitor first reported by Willoughby et al. [40]. This work was supported in part by National Institutes of Health Grant CA 72006.

Received: January 24, 2005
Revised: March 5, 2005
Accepted: March 30, 2005
Published: May 20, 2005

References

1. Henkart, P.A. (1994). Lymphocyte-mediated cytotoxicity: two pathways and multiple effector molecules. Immunity *1*, 343–346.
2. Russell, J.H., and Ley, T.J. (2002). Lymphocyte-mediated cytotoxicity. Annu. Rev. Immunol. *20*, 323–370.
3. Choy, J.C., Kerjner, A., Wong, B.W., McManus, B.M., and Granville, D.J. (2004). Perforin mediates endothelial cell death and resultant transplant vascular disease in cardiac allografts. Am. J. Pathol. *165*, 127–133.
4. Li, B., Hartono, C., Ding, R., Sharma, V.K., Ramaswamy, R., Qian, B., Serur, D., Mouradian, J., Schwartz, J.E., and Suthanthiran, M. (2001). Noninvasive diagnosis of renal-allograft rejection by measurement of messenger RNA for perforin and granzyme B in urine. N. Engl. J. Med. *344*, 947–954.
5. Tak, P.P., Spaeny-Dekking, L., Kraan, M.C., Breedveld, F.C., Froelich, C.J., and Hack, C.E. (1999). The levels of soluble granzyme A and B are elevated in plasma and synovial fluid of patients with rheumatoid arthritis (RA). Clin. Exp. Immunol. *116*, 366–370.
6. Kagi, D., Odermatt, B., Seiler, P., Zinkernagel, R.M., Mak, T.W., and Hengartner, H. (1997). Reduced incidence and delayed onset of diabetes in perforin-deficient nonobese diabetic mice. J. Exp. Med. *186*, 989–997.
7. Graubert, T.A., Russell, J.H., and Ley, T.J. (1996). The role of granzyme B in murine models of acute graft-versus-host disease and graft rejection. Blood *87*, 1232–1237.
8. Mullbacher, A., Waring, P., Tha Hla, R., Tran, T., Chin, S., Stehle, T., Museteanu, C., and Simon, M.M. (1999). Granzymes are the essential downstream effector molecules for the control of primary virus infections by cytolytic leukocytes. Proc. Natl. Acad. Sci. USA *96*, 13950–13955.
9. Grossman, W.J., Revell, P.A., Lu, Z.H., Johnson, H., Bredemeyer, A.J., and Ley, T.J. (2003). The orphan granzymes of humans and mice. Curr. Opin. Immunol. *15*, 544–552.
10. Trapani, J.A., and Sutton, V.R. (2003). Granzyme B: pro-apoptotic, antiviral and antitumor functions. Curr. Opin. Immunol. *15*, 533–543.
11. Lieberman, J., and Fan, Z. (2003). Nuclear war: the granzyme A-bomb. Curr. Opin. Immunol. *15*, 553–559.
12. Carmeliet, P., Schoonjans, L., Kieckens, L., Ream, B., Degen, J., Bronson, R., De Vos, R., van den Oord, J.J., Collen, D., and Mulligan, R.C. (1994). Physiological consequences of loss of plasminogen activator gene function in mice. Nature *368*, 419–424.
13. Simon, M.M., Hausmann, M., Tran, T., Ebnet, K., Tschopp, J., ThaHla, R., and Mullbacher, A. (1997). In vitro- and ex vivo-derived cytolytic leukocytes from granzyme A x B double knockout mice are defective in granule-mediated apoptosis but not lysis of target cells. J. Exp. Med. *186*, 1781–1786.
14. Beresford, P.J., Xia, Z., Greenberg, A.H., and Lieberman, J. (1999). Granzyme A loading induces rapid cytolysis and a novel form of DNA damage independently of caspase activation. Immunity *10*, 585–594.
15. Trapani, J.A., Jans, D.A., Jans, P.J., Smyth, M.J., Browne, K.A., and Sutton, V.R. (1998). Efficient nuclear targeting of granzyme B and the nuclear consequences of apoptosis induced by granzyme B and perforin are caspase-dependent, but cell death is caspase-independent. J. Biol. Chem. *273*, 27934–27938.
16. Alaimo, P.J., Shogren-Knaak, M.A., and Shokat, K.M. (2001). Chemical genetic approaches for the elucidation of signaling pathways. Curr. Opin. Chem. Biol. *5*, 360–367.
17. Sarin, A., Haddad, E.K., and Henkart, P.A. (1998). Caspase dependence of target cell damage induced by cytotoxic lymphocytes. J. Immunol. *161*, 2810–2816.
18. Hudig, D., Gregg, N.J., Kam, C.M., and Powers, J.C. (1987). Lymphocyte granule-mediated cytolysis requires serine protease activity. Biochem. Biophys. Res. Commun. *149*, 882–888.
19. Pham, C.T., and Ley, T.J. (1999). Dipeptidyl peptidase I is required for the processing and activation of granzymes A and B in vivo. Proc. Natl. Acad. Sci. USA *96*, 8627–8632.
20. Hudig, D., Redelman, D., and Minning, L.L. (1984). The requirement for proteinase activity for human lymphocyte-mediated natural cytotoxicity (NK): evidence that the proteinase is serine dependent and has aromatic amino acid specificity of cleavage. J. Immunol. *133*, 2647–2654.
21. Powers, J.C., Asgian, J.L., Ekici, O.D., and James, K.E. (2002). Irreversible inhibitors of serine, cysteine, and threonine proteases. Chem. Rev. *102*, 4639–4750.
22. Harris, J.L., Peterson, E.P., Hudig, D., Thornberry, N.A., and Craik, C.S. (1998). Definition and redesign of the extended sub-

strate specificity of granzyme B. J. Biol. Chem. *273*, 27364–27373.
23. Mahrus, S., Kisiel, W., and Craik, C.S. (2004). Granzyme M is a regulatory protease that inactivates proteinase inhibitor *9*, an endogenous inhibitor of granzyme B. J. Biol. Chem. *279*, 54275–54282.
24. Bell, J.K., Goetz, D.H., Mahrus, S., Harris, J.L., Fletterick, R.J., and Craik, C.S. (2003). The oligomeric structure of human granzyme A is a determinant of its extended substrate specificity. Nat. Struct. Biol. *10*, 527–534.
25. Thornberry, N.A., Rano, T.A., Peterson, E.P., Rasper, D.M., Timkey, T., Garcia-Calvo, M., Houtzager, V.M., Nordstrom, P.A., Roy, S., Vaillancourt, J.P., et al. (1997). A combinatorial approach defines specificities of members of the caspase family and granzyme B. Functional relationships established for key mediators of apoptosis. J. Biol. Chem. *272*, 17907–17911.
26. Edwards, K.M., Kam, C.M., Powers, J.C., and Trapani, J.A. (1999). The human cytotoxic T cell granule serine protease granzyme H has chymotrypsin-like (chymase) activity and is taken up into cytoplasmic vesicles reminiscent of granzyme B-containing endosomes. J. Biol. Chem. *274*, 30468–30473.
27. Wilharm, E., Parry, M.A., Friebel, R., Tschesche, H., Matschiner, G., Sommerhoff, C.P., and Jenne, D.E. (1999). Generation of catalytically active granzyme K from *Escherichia coli* inclusion bodies and identification of efficient granzyme K inhibitors in human plasma. J. Biol. Chem. *274*, 27331–27337.
28. Jackson, D.S., Fraser, S.A., Ni, L.M., Kam, C.M., Winkler, U., Johnson, D.A., Froelich, C.J., Hudig, D., and Powers, J.C. (1998). Synthesis and evaluation of diphenyl phosphonate esters as inhibitors of the trypsin-like granzymes A and K and mast cell tryptase. J. Med. Chem. *41*, 2289–2301.
29. Hamilton, R., Walker, B., and Walker, B.J. (1998). Synthesis and proteinase inhibitory properties of diphenyl phosphonate analogues of aspartic and glutamic acids. Bioorg. Med. Chem. Lett. *8*, 1655–1660.
30. Craik, C.S., Roczniak, S., Largman, C., and Rutter, W.J. (1987). The catalytic role of the active site aspartic acid in serine proteases. Science *237*, 909–913.
31. Walensky, L.D., Kung, A.L., Escher, I., Malia, T.J., Barbuto, S., Wright, R.D., Wagner, G., Verdine, G.L., and Korsmeyer, S.J. (2004). Activation of apoptosis in vivo by a hydrocarbon-stapled BH3 helix. Science *305*, 1466–1470.
32. Bishop, A.C., Ubersax, J.A., Petsch, D.T., Matheos, D.P., Gray, N.S., Blethrow, J., Shimizu, E., Tsien, J.Z., Schultz, P.G., Rose, M.D., et al. (2000). A chemical switch for inhibitor-sensitive alleles of any protein kinase. Nature *407*, 395–401.
33. Thornberry, N.A., Peterson, E.P., Zhao, J.J., Howard, A.D., Griffin, P.R., and Chapman, K.T. (1994). Inactivation of interleukin-1 beta converting enzyme by peptide (acyloxy)methyl ketones. Biochemistry *33*, 3934–3940.
34. Kidd, D., Liu, Y., and Cravatt, B.F. (2001). Profiling serine hydrolase activities in complex proteomes. Biochemistry *40*, 4005–4015.
35. Oleksyszyn, J., and Powers, J.C. (1991). Irreversible inhibition of serine proteases by peptide derivatives of (alpha-aminoalkyl)phosphonate diphenyl esters. Biochemistry *30*, 485–493.
36. Fan, Z., Beresford, P.J., Oh, D.Y., Zhang, D., and Lieberman, J. (2003). Tumor suppressor NM23–H1 is a granzyme A-activated DNase during CTL-mediated apoptosis, and the nucleosome assembly protein SET is its inhibitor. Cell *112*, 659–672.
37. Browne, K.A., Blink, E., Sutton, V.R., Froelich, C.J., Jans, D.A., and Trapani, J.A. (1999). Cytosolic delivery of granzyme B by bacterial toxins: evidence that endosomal disruption, in addition to transmembrane pore formation, is an important function of perforin. Mol. Cell. Biol. *19*, 8604–8615.
38. Metkar, S.S., Wang, B., Aguilar-Santelises, M., Raja, S.M., Uhlin-Hansen, L., Podack, E., Trapani, J.A., and Froelich, C.J. (2002). Cytotoxic cell granule-mediated apoptosis: perforin delivers granzyme B-serglycin complexes into target cells without plasma membrane pore formation. Immunity *16*, 417–428.
39. Kagi, D., Ledermann, B., Burki, K., Seiler, P., Odermatt, B., Olsen, K.J., Podack, E.R., Zinkernagel, R.M., and Hengartner, H. (1994). Cytotoxicity mediated by T cells and natural killer cells is greatly impaired in perforin-deficient mice. Nature *369*, 31–37.
40. Willoughby, C.A., Bull, H.G., Garcia-Calvo, M., Jiang, J., Chapman, K.T., and Thornberry, N.A. (2002). Discovery of potent, selective human granzyme B inhibitors that inhibit CTL mediated apoptosis. Bioorg. Med. Chem. Lett. *12*, 2197–2200.
41. Robertson, M.J., Cochran, K.J., Cameron, C., Le, J.M., Tantravahi, R., and Ritz, J. (1996). Characterization of a cell line, NKL, derived from an aggressive human natural killer cell leukemia. Exp. Hematol. *24*, 406–415.
42. Gong, J.H., Maki, G., and Klingemann, H.G. (1994). Characterization of a human cell line (NK-92) with phenotypical and functional characteristics of activated natural killer cells. Leukemia *8*, 652–658.
43. Poe, M., Wu, J.K., Blake, J.T., Zweerink, H.J., and Sigal, N.H. (1991). The enzymatic activity of human cytotoxic T-lymphocyte granzyme A and cytolysis mediated by cytotoxic T-lymphocytes are potently inhibited by a synthetic antiprotease, FUT-175. Arch. Biochem. Biophys. *284*, 215–218.
44. Odake, S., Kam, C.M., Narasimhan, L., Poe, M., Blake, J.T., Krahenbuhl, O., Tschopp, J., and Powers, J.C. (1991). Human and murine cytotoxic T lymphocyte serine proteases: subsite mapping with peptide thioester substrates and inhibition of enzyme activity and cytolysis by isocoumarins. Biochemistry *30*, 2217–2227.
45. Martin, S.J., Reutelingsperger, C.P., McGahon, A.J., Rader, J.A., van Schie, R.C., LaFace, D.M., and Green, D.R. (1995). Early redistribution of plasma membrane phosphatidylserine is a general feature of apoptosis regardless of the initiating stimulus: inhibition by overexpression of Bcl-2 and Abl. J. Exp. Med. *182*, 1545–1556.
46. Schwab, B.L., Guerini, D., Didszun, C., Bano, D., Ferrando-May, E., Fava, E., Tam, J., Xu, D., Xanthoudakis, S., Nicholson, D.W., et al. (2002). Cleavage of plasma membrane calcium pumps by caspases: a link between apoptosis and necrosis. Cell Death Differ. *9*, 818–831.
47. Casciola-Rosen, L., Andrade, F., Ulanet, D., Wong, W.B., and Rosen, A. (1999). Cleavage by granzyme B is strongly predictive of autoantigen status: implications for initiation of autoimmunity. J. Exp. Med. *190*, 815–826.
48. Green, D.R., and Beere, H.M. (2000). Apoptosis. Gone but not forgotten. Nature *405*, 28–29.
49. Aggarwal, B.B. (2003). Signalling pathways of the TNF superfamily: a double-edged sword. Nat. Rev. Immunol. *3*, 745–756.
50. Harris, J.L., Backes, B.J., Leonetti, F., Mahrus, S., Ellman, J.A., and Craik, C.S. (2000). Rapid and general profiling of protease specificity by using combinatorial fluorogenic substrate libraries. Proc. Natl. Acad. Sci. USA *97*, 7754–7759.
51. Oleksyszyn, J., Boduszek, B., Kam, C.M., and Powers, J.C. (1994). Novel amidine-containing peptidyl phosphonates as irreversible inhibitors for blood coagulation and related serine proteases. J. Med. Chem. *37*, 226–231.
52. Oleksyszyn, J., Subotkowska, L., and Mastalerz, P. (1979). Diphenyl 1-Aminoalkanephosphonates. Synthesis, 985–986.
53. Kitz, R., and Wilson, I.B. (1962). Esters of methanesulfonic acid as irreversible inhibitors of acetylcholinesterase. J. Biol. Chem. *237*, 3245–3249.
54. Blomberg, K., Hautala, R., Lovgren, J., Mukkala, V.M., Lindqvist, C., and Akerman, K. (1996). Time-resolved fluorometric assay for natural killer activity using target cells labelled with a fluorescence enhancing ligand. J. Immunol. Methods *193*, 199–206.