Design and characterization of a novel human Granzyme B inhibitor

Marcelo Marcet-Palacios, Catherine Ewen, Elliot Pittman, Brenda Duggan, Katia Carmine-Simmen, Richard P. Fahlman, and R. Chris Bleackley*

Department of Biochemistry, University of Alberta, Alberta, Edmonton, Canada

*To whom correspondence should be addressed. E-mail: chris.bleackley@ualberta.ca

Edited by Robin Leatherbarrow

Received 22 April 2014; Revised 15 October 2014; Accepted 31 October 2014

Abstract

The intracellular roles of Granzyme B (GrB) in immune-mediated cell killing have been extensively studied. Recent data also implicate GrB in extracellular pathways of inflammation, cytokine activation and autoimmunity. Targeting (GrB) provides a new pharmaceutical agent for various inflammatory disorders. Serpina3n is a mouse extracellular inhibitor of GrB. There is no apparent equivalent in humans. In this study, we used a novel applied genetics approach to engineer a new extracellular GrB serpin. A chimeric protein was generated in which the reactive center loop (RCL) of human extracellular antichymotrypsin (ACT) was replaced with that of serpina3n. This serpin contained 27 amino acid residues from the serpina3n RCL and the remaining 395 residues from human ACT. The insertion converted human ACT into a GrB-inhibitory serpin. Several critical residues were identified by scanning mutagenesis on the chimera and serpina3n. Targeted mutagenesis was conducted on wild-type human ACT by specifically substituting those critical residues, creating a novel inhibitor that contains 99.3% human ACT sequence with only three point mutations. Wild-type human ACT had a $k_{\text{ass}}$ for GrB of $2.26 \times 10^4$ M$^{-1}$ s$^{-1}$, whereas the novel inhibitor binds GrB with a $k_{\text{ass}}$ of $7.65 \times 10^5$ M$^{-1}$ s$^{-1}$. This new drug candidate can be developed in animal models and further tested in clinical trials to help us understand the role of GrB in numerous disorders.

Key words: drug, serpin, Granzyme B, apoptosis, autoimmune diseases

Introduction

Granzyme B (GrB) plays a key role in the cell-mediated death pathway of immunity. Apoptosis is vital for maintaining multicellular organisms through controlled removal of damaged or infected cells as well as unwanted cells during development (Afonina et al., 2010). Upon recognition of the target cell, cytotoxic T-lymphocytes (CTLs) and natural killer (NK) cells release granules containing perforin and GrB into the immunologic synapse. Perforin facilitates the entry of GrB into target cells. Once inside, the process of apoptosis begins with GrB activating a family of proteases known as caspases, by proteolytic cleavage (Callen and Martin, 2008). When activated, caspases cleave various intracellular substrates in the target cell to cause cell death (Fischer et al., 2003; Taylor et al., 2008). GrB also induces apoptosis through the mitochondrial/apoptosome pathway (Barry et al., 2000; Heibein et al., 2000). GrB proteolytically cleaves BH-only (Bid) protein, which leads to Bax/Bak oligomerization at the mitochondrial membrane, pore formation and the release of cytochrome c. Aside from these two traditional pathways of GrB in apoptosis, emerging evidence shows that GrB targets downstream or separate pathways to remove unwanted cells. GrB induces DNA fragmentation by cleavage of an inhibitor of DNase, DFF45/ICAD, resulting in a release of DNase (Thomas et al., 2000; Sharif-Askari et al., 2001). GrB can disrupt components of the cytoskeleton by targeting the c-terminus of $\alpha$-tubulin, which is critical for regulating microtubule polymerization (Sackett et al., 1985; Adrain et al., 2006; Goping et al., 2006). Thus, GrB may suppress the mitotic potential of tumour cells and limit viral replication as many viruses utilize the host microtubule network (Adrain et al., 2006). Recently, we discovered that GrB directly targets the translational...
machinery by proteolytic cleavage of the eukaryotic initiation factor eIF4G3 (Marcet-Palacios et al., 2011). This pathway prevents virus replication before the onset of apoptosis, supporting that GrB has non-apoptotic roles.

In addition, extracellular GrB has been implicated in a variety of disease states. Synovial GrB levels are elevated in subjects with chronic inflammatory conditions such as rheumatoid arthritis, with systemic GrB levels several hundred-fold higher in affected patients (Tak et al., 1994, 1999). GrB may mediate cartilage degradation in rheumatoid arthritis (Ronday et al., 2001; Goldbach-Mansky et al., 2005). Levels of circulating GrB are also higher in the bronchoalveolar lavage of atopic asthma patients compared with controls (Bratke et al., 2004). Aside from the postulate that GrB leaks into the immunologic synapse during intense activation of CTLs and NK cells (Afonina et al., 2010), other non-cytotoxic cells express GrB and could be contributing to the high circulating levels of GrB, e.g. dendritic cells, mast cells, basophils, B-lymphocytes, macrophages, keratinocytes, platelets and human articular chondrocytes (Rissoan et al., 2002; Horiuchi et al., 2003; Hernandez-Pigeon et al., 2006; Tschopp et al., 2006; Kim et al., 2007; Strik et al., 2007; Freihstät et al., 2009; Hagn et al., 2009).

GrB may have a role in atherosclerosis as GrB expression in atherosclerotic lesions correlates with disease severity (Choy et al., 2004), and higher levels of GrB are measured in patients with unstable plaques (Boivin et al., 2009). T-lymphocytes are the most abundant immune cell type expressing GrB in an atherosclerotic lesion, along with CD4+/CD8+ macrophages and foam cells (Choy et al., 2003; Baba et al., 2006; Kim et al., 2007; Daugherty et al., 2008, 2009). Clinically, increased GrB levels in plasma correspond to an increased incidence of cerebrovascular events and carotid plaque instability (Skjelland et al., 2007), possibly due to GrB’s activity on the extracellular matrix (ECM) (Choy et al., 2004; Buzza et al., 2005; Boivin et al., 2009). Numerous ECM substrates of GrB are reported in the literature (Hiebert and Granville, 2012), including vitronectin, fibronectin, laminin, decorin and the smooth muscle cell matrix (Choy et al., 2004; Buzza et al., 2005; Ang et al., 2011). Cleavage of ECM components by GrB may contribute to disease, not only through mechanical damage, but other mechanisms such as anokisis, a form of cell death that results from loss of cell-matrix interactions (Choy et al., 2004; Buzza et al., 2005; Boivin et al., 2009). Thus, GrB is a multifunctional protease with an important role in a variety of inflammatory diseases, making GrB a critical target for drug development (Boivin et al., 2009).

Normally, protease activity is tightly regulated to avoid unintended damage to host cells. Serpins are proteins that inhibit proteases by forming complexes with the proteases. Human protease inhibitor-9 (PI9) or SERPINB9 is an inhibitor of GrB. High levels of PI9 can be found in dendritic cells, lymphoid endothelial cells, non-lymphoid endothelial cells, and in cells of immune-privileged tissues such as the eye, testes, ovaries and placenta (Bladegroen et al., 2001). PI9 was originally identified in T-lymphocytes and thought to be protective against apoptosis induced by GrB (Hirst et al., 2003; Bots et al., 2006). It is an intracellular cytosolic protein, and there is no evidence that it has activity outside the cell. GrB retains 70% of its activity in human plasma, suggesting a lack of effective inhibitors in plasma (Kurschus et al., 2004). In support, Kurschus et al. (2004) have shown that GrB activity is not inhibited by human plasma in vitro.

Sipione et al. (2006) discovered a novel murine serpin secreted by sertoli cells known as serpina3n, which can bind and inhibit the activity of GrB. Serpina3n inhibits rupture in a mouse model of aortic aneurysm (Ang et al., 2011). As an extracellular protein, serpina3n is more plasma stable, but as a murine protein, would elicit an immune response if introduced in humans. Alpha-1 antichymotrypsin (ACT) is a human protein but unable to effectively bind GrB. Thus, our objective was to create a chimeric protein with the GrB inhibitory property and plasma stability of serpina3n and non-immunogenicity of human ACT.

Materials and methods

Materials

Cell lines
Jurkat cells were obtained from American Type Culture Collection. Cells were passaged when the cell density reached 10⁶ cells/ml. RPMI was supplemented with 10% fetal bovine serum, 0.06 mg/ml penicillin and 0.01 mg/ml streptomycin sulfate.

Adenovirus-mediated internalization of GrB
GrB was internalized into Jurkat cells to induce apoptosis using a non-replicating strain of adenovirus (AD) (Froelich et al., 1996). AD was used at 200 plaque-forming unit (PFU) per cell. Experiments where Jurkat cells were treated with GrB and contained AD was referred to as ‘GrB/AD treatment’.

In vitro transcription translation
TNT quick-coupled transcription/translation kit (Promega, WI, USA) was used for in vitro transcription translation (IVTT). Individual reactions were performed as previously described (Marcet-Palacios et al., 2011) and analyzed by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) and autoradiography.

GrB binding
This technique was conducted with freshly made IVTT serpins or with serpins purified through fast protein liquid chromatography (FPLC). Equimolar concentrations of serpins and GrB were incubated in PBS for 30 min at 37°C. Radioactively labeled IVTT products were visualized after SDS–PAGE and autoradiography. FPLC-purified serpins were analyzed through SDS–PAGE and visualized by silver staining.

SDS–PAGE
Ten percent polyacrylamide gels were used. Electrophoresis was conducted in a mini protein 3 cell (Bio-Rad) with a Power Pac 1000 (Bio-Rad) (Marcet-Palacios et al., 2011).

RCL chimera
ACT chimera was obtained from GenScript (GenScript Corporation, NJ, USA). The reactive center loop (RCL) of murine protein serpina3n (residues E369–P395) was used to replace the RCL of human ACT (residues E371–P398) (Fig. 1C).

Site-directed mutagenesis
Both scanning and target mutagenesis were carried out using the Quikchange site-directed mutagenesis kit and oligonucleotide primers described in Table I. Following the PCR by Pfu Ultra High Fidelity DNA polymerase, the product was treated with DpnI, and then transformed into competent cells Escherichia coli DH5α. Using colony PCR, three to four colonies were analyzed from each reaction. Positive products were purified using a QIAquick™ PCR purification kit and subsequently sent for sequencing. Large-scale plasmid preps were made and used in IVTT reactions.
Purified GrB was pre-incubated with serpin inhibitors for 30 min, then added to 5 × 10⁴ Jurkat cells in 25 µl RPMI 1640 supplemented with 0.1% bovine serum albumin (w/v) in the presence of 200 PFU/cell Adenovirus 5. Cell death, as monitored by TUNEL-based DNA fragmentation, was assayed at 4 h following fixation with 4% paraformaldehyde, permeabilization with 0.1% saponin/PBS/2% FCS and stained with FITC-conjugated TUNEL label (terminal deoxynucleotidyl transferase-mediated dUTP nick-end labeling) (Roche Diagnostics Canada). Samples were then analyzed on a LSR II flow cytometer (BD Biosciences; San Jose, CA, USA) and the FCS Express 3 software (De Novo Software; Los Angeles, CA).

To prepare the target mutants of ACT for FPLC purification, 6× His-tag was added to the N-terminus of the mature form of ACT starting with the sequence NSPLDE. The resulting PCR fragments were digested with BamHI and inserted into the BamHI site of the expression vector pET-3c, followed by sequencing of the inserted fragments. The plasmid pET-3c with each mutant was inoculated in E. coli BL21 (DE3). From the overnight culture, a 1 : 20 dilution was grown at 37°C until OD600 was 0.5. Protein expression was induced with 0.2 mM isopropyl-β-D-1-thiogalactopyranoside, and the following cultivation was continued for 4 h at 37°C in the 4 l media. Cells were pelleted at 7000 g for 20 min. The pellet was resuspended in...
native lysis buffer (50 mM NaH2PO4, 300 mM NaCl, 10 mM imidazole, 5% glycerol, pH 8). Lysozyme (0.2 mg/ml) and protease inhibitor phenylmethylsulfonyl fluoride (1 mM) were added to the suspended cells. Sonication was conducted with a standard probe 80% power for six pulses for 15 s each. Immobilized metal ion affinity chromatography purification was conducted. Protein was eluted with Buffer B (50 mM NaH2PO4, 300 mM NaCl, 500 mM imidazole, 5% glycerol, pH 8) with 0–100% over 30 ml at 2.5 ml/min. During elution 2.5 ml fractions were collected. All samples were evaluated by SDS–PAGE to determine which fractions contained the protein of interest. Fractions containing the desired protein were pooled together and loaded into a PD-10 column pre-equilibrated with Q0 (20 mM TRIS pH 8, 0.1 mM EDTA, 0.5 M NaCl and 5% glycerol). Desalting was conducted by eluting with 4 ml of Q0. Further purification was conducted by passing the sample through a Hi-TrapQ HP column. Elution was conducted by passing Buffer B with 0–100% over 30 ml at 2.5 ml/min. During elution, 2.5 ml of the sample was collected. Sample desalting was conducted by passing the sample through a YM-10 column and eluted with Q0. Protein concentrations were determined and aliquots were flash frozen.

GrB enzymatic assay
GrB activity and serpin-mediated inhibition were measured by a colorimetric assay as described previously (Ewen et al., 2003). Hydrolysis of the GrB substrate Ac-IEPD-pNA (Kaiya Biomedical, Seattle, WA) was monitored at A405. Human GrB was incubated for 30 min at 37°C to allow for GrB activity and serpin-mediated inhibition were measured by a colorimetric assay as previously described (Wang et al., 2006). Equimolar amounts (0.2 μM) of GrB and serpin molecules were combined and incubated at 37°C for 0–120 min to determine residual activity. The reaction buffer with Ac-IEPD-pNA was added and incubated at 37°C for 1 h.

Results
The RCL of serpina3n confers ACT the ability to bind to GrB
Serpina3n is an effective inhibitor of human GrB (Sipione et al., 2006). Serpins form an SDS-stable complex with their target enzymes. Radioactively labeled serpina3n bound purified human GrB or recombinant human GrB (rGrB) as demonstrated in an IVTT binding experiment (Fig. 1A). In this experiment, serpina3n ran at a MW of 47 000 but when bound to GrB, this shifted to MW 78 000. Human ACT did not bind GrB in this system, despite a high sequence homology between the two genes (Fig. 1B). To study the role of the RCL in conferring serpina3n its GrB-binding specificity, we replaced human ACT RCL with that of serpina3n (Fig. 1C). To build this chimera, the human ACT residues E371–P398 were extracted and replaced with the serpina3n residues E369–P395. This human ACT chimera (ACTWT) formed the SDS-stable complex with human GrB as demonstrated in Fig. 1B. Thus, the serpina3n RCL residues E369–P395 conferred human ACT the ability to bind GrB.

Critical serpina3n RCL residues are necessary to induce optimal binding to GrB
Serpina3n RCL spans 27 amino acids from E369–P396 (Fig. 1C). The P1 residue within this region is Met190. Point mutation of this residue to alanine, in either native serpina3n (serpina3nmmM191A) or in the human ACT chimera (ACTWT/RCL/MM191A), resulted in a loss of GrB binding (Fig. 1D). Interestingly, replacement of P1 Met residue by either aspartic acid or glutamic acid generated mutants that retain GrB-binding properties (Fig. 1D) in both the ACT chimera (ACTWT/RCL/MM191E/M191D) and serpina3n (serpina3nmmM191E/M191D).

To determine which residues within the RCL were critical in conferring serpina3n its GrB-binding specificity, we conducted scanning mutagenesis of residues P4–P4’ in both native serpina3n and ACTWT/RCL. Disappearance or reduction in the intensity of the 78 000 MW band indicated reduced binding. Replacement of residues in Positions P1 and P3 with alanine had the most significant impact in reducing native serpina3n binding to GrB (Fig. 2A), while scanning mutagenesis in ACTWT/RCL identified residues P3 and P4’ as important, in addition to the P1 site (Fig. 2B).

Table I. Directed mutagenesis primer sequences used in mutant designed

<table>
<thead>
<tr>
<th>Mutant name</th>
<th>Primer sequence (positive strand)</th>
</tr>
</thead>
<tbody>
<tr>
<td>ACTmmRCL(M183A)</td>
<td>ACTmP1,P1,P3,P4,P4’</td>
</tr>
<tr>
<td>ACTmmRCL(M183D)</td>
<td>ACTmP1,P1,P3,P4,P4’</td>
</tr>
<tr>
<td>ACTmmRCL(M183E)</td>
<td>ACTmP1,P1,P3,P4,P4’</td>
</tr>
<tr>
<td>ACTmmM380A</td>
<td>ACTmP1,P1,P3,P4,P4’</td>
</tr>
<tr>
<td>ACTmmV381A</td>
<td>ACTmP1,P1,P3,P4,P4’</td>
</tr>
<tr>
<td>ACTmmM383A</td>
<td>ACTmP1,P1,P3,P4,P4’</td>
</tr>
<tr>
<td>ACTmmS384A</td>
<td>ACTmP1,P1,P3,P4,P4’</td>
</tr>
<tr>
<td>ACTmmK386A</td>
<td>ACTmP1,P1,P3,P4,P4’</td>
</tr>
<tr>
<td>ACTmmL387A</td>
<td>ACTmP1,P1,P3,P4,P4’</td>
</tr>
<tr>
<td>ACTmmP3</td>
<td>ACTmP1,P1,P3,P4,P4’</td>
</tr>
<tr>
<td>ACTmmP1,P3,P4</td>
<td>ACTmP1,P1,P3,P4,P4’</td>
</tr>
<tr>
<td>ACTmmP3,P1,P4,P4’</td>
<td>ACTmP1,P1,P3,P4,P4’</td>
</tr>
</tbody>
</table>

Three mutations in human ACT transform this protein into a GrB inhibitor
Following the identification of these four critical residues, we proceeded with the sequential point mutation into wild-type ACT (ACTWT) (Fig. 2C). Replacement of the P1 residue with methionine (ACTWT/P1) turned ACTWT into a GrB-binding protein (Fig. 2D). We then point-mutated this construct and replaced a leucine residue at the P3’ position with a lysine (ACTWT/P1,P3’). Although P3’ appeared...
Fig. 2. Scanning mutagenesis analysis of serpina3n (ser3n) and ACT identified three critical RCL residues. IVTT in the presence of radioactively labeled methionine (MetS35) was conducted for all tested serpins. Each radioactively labeled serpin was then incubated with purified GrB in a binding assay. Samples were then separated by SDS–PAGE, and developed through autoradiography. A shift in the serpin molecular weight was interpreted as formation of serpin–GrB complex.

(A) Serpina3n (ser3n) scanning mutagenesis where P4–P4’ residues were sequentially mutagenized to alanine. Mutagenesis of Ser3nmA383A was not conducted since there is an alanine already present at that P2’ site. We added the symbol asterisks to indicate this in the figure (Ser3nmA383A*). (B) ACTmRCL scanning mutagenesis where P4–P4’ residues were sequentially mutagenized to alanine. Scanning mutagenesis was not conducted on the ACT chimera (ACTmRCL) containing the ser3n RCL. We added the symbol asterisk to indicate in the figure that ACTmA385A was not mutagenized as an alanine is already present in the P2’ site. (C) Wild-type ACT (ACTWT) was mutagenized to contain a methionine in the P1 residue (ACTmP1). ACTmP1 was then mutated to contain a lysine in the P3’ residue (ACTmP1,P3’). ACTmP1,P3’ was mutated to contain a valine in P3 and a leucine in P4’ (ACTmP3,P1,P3’,P4’). The lysine residue in P3’ of ACTmP3,P1,P3’,P4’ was point mutated back to leucine, the original residue in P3’ of ACT (ACTmP3,P1,P4’). (D) ACT mutants described in (C) were assessed for their ability to form a complex with GrB.
to be a critical residue in serpina3n scanning mutagenesis, replacement of this leucine resulted in loss of GrB binding (Fig. 2D). A mutant containing all four point mutations (ACT<sup>mp3,p1,p3′,p4</sup>) bound GrB less efficiently than ACT<sup>p1</sup> mutant (Fig. 2D). However, removal of the P3′ lysine and replacement of this residue back to a leucine generated a mutant (ACT<sup>mp3,p1,p4</sup>) that could bind GrB as effectively as serpina3n.

**Human ACT<sup>mp3,p1,p4′</sup> mutant prevents GrB-mediated killing of Jurkat cells**

To determine the ability of human ACT<sup>mp3,p1,p4′</sup> mutant to protect Jurkat cells against GrB, we treated Jurkat cells with GrB/AD and measured the percentage of TUNEL-positive Jurkat cells (Fig. 3) in the presence of each mutant. For this experiment, mutants were HA tagged, then generated through IVTT and purified through affinity chromatography. We found that although ACT<sup>WT</sup>, ACT<sup>mp1</sup> and ACT<sup>mp3,p1,p3′,p4′</sup> were unable to prevent TUNEL staining, both serpina3n and ACT<sup>mp3,p1,p4′</sup> significantly reduce Jurkat cell killing. Although ACT<sup>mp1</sup> showed strong binding to GrB (Fig. 2D), this candidate failed to protect Jurkat cells against GrB/AD (Fig. 3).

**Human ACT<sup>mp3,p1,p4′</sup> mutant is an effective GrB inhibitor**

In order to obtain sufficient quantities for analysis, the constructs were subcloned in pET3c, the leader sequence was removed, and a His-tag was added as described in the Materials and methods section. To examine the kinetic properties of this novel GrB inhibitor, serpina3n, ACT<sup>WT</sup>, ACT<sup>mp1</sup>, ACT<sup>mp3,p1,p3′,p4′</sup> and ACT<sup>mp3,p1,p4′</sup> were purified by FPLC (Fig. 4A). Hydrolysis of the GrB substrate Ac-IEPD-pNA demonstrated that in the range of GrB concentrations tested, ACT<sup>mp3,p1,p4′</sup> was a more effective inhibitor of GrB than ACT<sup>mp1</sup>. At 0.5 μM ACT<sup>mp1</sup>, there was a significant decrease in Ac-IEPD-pNA hydrolysis. This effect disappeared at lower concentrations of ACT<sup>mp1</sup>. We concluded from these data that ACT<sup>mp1</sup> would be an inferior drug candidate to ACT<sup>mp3,p1,p4′</sup>.

The initial interaction between a serpin and a protease results in the formation of a reversible Michaelis complex. Following this interaction, a covalent bond is formed between the protease and the serpin resulting in an irreversible complex. The association constant ($k_{ass}$) is a kinetic factor that defines the rate at which the Michaelis complex is converted into the irreversible covalent complex. For a $k_{ass}$ between a serpin and a protease to be relevant, it must be in the order of $10^{-7}$–$10^{-5}$ M$^{-1}$ s$^{-1}$. We measured the $k_{ass}$ of serpina3n, ACT<sup>WT</sup> and ACT mutants (Table II). The $k_{ass}$ of ACT<sup>mp3,p1,p4′</sup> for human GrB was 7.65 × 10$^{-5}$ M$^{-1}$ s$^{-1}$. Thus, the three point mutations conferred ACT<sup>mp3,p1,p4′</sup> a $k_{ass}$ 34-fold higher than ACT<sup>WT</sup> and not significantly different than $k_{ass}$ of serpina3n (Fig. 4C). Interestingly, ACT<sup>mp3,p1,p4′</sup> resembled serpina3n in that it can also bind elastase (Fig. 5). ACT<sup>WT</sup> did not bind GrB, GrA or elastase (data not shown).

**Discussion**

GrB levels are elevated in patients with various autoimmune disorders and blocking GrB activity improves symptoms in animal models. Interestingly, a naturally occurring extracellular protease inhibitor (serpin) of GrB has not been identified and GrB activity appears to be unregulated in inflammatory states. Targeting GrB provides a novel pharmaceutical method to control various inflammatory disorders.
In this study, we designed a novel GrB inhibitor using the backbone of ACT, the secretory human ortholog of serpina3n (Horvath et al., 2005). Serpins interact with their proteases through a domain called the RCL. The amino acid residues in the RCL confer serpins their specificity for the particular protease they inhibit. We took wild type ACT and replaced its RCL domain with that of the GrB inhibitor serpina3n. This mutation resulted in a chimera that bound and inhibited GrB. Scanning mutagenesis analysis on this chimera and in the native serpins revealed the S3 pocket is defined by polar residues capable of forming hydrogen bonds, which explains the preference for Glu at P3. In serpina3n or PI9 are yet to be resolved and are necessary to understand the relevance of the dramatic change of amino acid sequences from a GrB substrate to a GrB inhibitor.

For GrB, degrades the extracellular component of the ECM in cartilage tissue (Froelich et al., 1999), while Froelich et al. (2007), suggested that GrB degrades aggrecan, an integral component of the ECM in cartilage tissue (Froelich et al., 1999). Clearly this cannot be the explanation for the result seen with serpina3n as P4 is a hydrophobic Leu. Serpina3n (FVPM) and PI9 (VVAE) display P4–P1 residues that differ from the consensus IEPD. The 3D structure of human GrB in complex with IEPD was later resolved and used to elucidate the interactions of the enzymes’ S4–S1 sites with the tetrapeptide (Rottunda et al., 2001). The Asp specificity of GrB is due in large part to an interaction with Arg226 (Caputo et al., 1994). However, the enzyme can also cleave synthetic substrates at Glu and Met (Poe et al., 1991).

We found that targeted mutagenesis of the PI1 residue in either serpina3n or ACTmRCL to alanine resulted in complete loss of binding to GrB (Fig. 1D) confirming the importance of the PI1 residue. In addition, mutagenesis of the PI1 residue to glutamic acid or aspartic acid generated mutants that recovered their ability to bind to GrB (Fig. 1D). These results were not surprising given that reported serpin inhibitors of GrB have aspartic acid, as is the case for CrmA (Quan et al., 1995), and glutamic acid for PI9 (Bird et al., 1998). We noted that although the aspartic acid mutants ACTmRCLm318ID and serpina3n were able to bind GrB (Fig. 1D), degradation fragments were also observed, showing that these mutants were substrates for GrB. This agrees with literature showing that GrB has a strong preference for aspartic acid in the PI1 site as a result of the strong salt-bridge formed between the S1 residue Arg226 of GrB and the PI1 residue on GrB substrates (Estevez-Perpina et al., 2000).

The S3 pocket is defined by polar residues capable of forming hydrogen bonds, which explains the preference for Glu at P3. In both serpina3n and PI9, this amino acid is a hydrophobic Val. It is interesting that mutation of this Val in both of these serpins resulted in a reduction of GrB binding. Mutation of the P4’ amino acid in PI9 (Glu to Ala) resulted in a reduction of GrB binding. This was explained by the lack of a salt bridge between the Glu 344 and Lys 27 (Sun et al., 2001). Clearly this cannot be the explanation for the result seen with serpina3n as P4’ is a hydrophobic Leu. Serpina3n (FVPM) and PI9 (VVAE) display P4–P1 residues that differ from the consensus IEPD. This is in agreement with the lack of a salt bridge between the Glu 344 and Lys 27 (Sun et al., 2001). Clearly this cannot be the explanation for the result seen with serpina3n as P4’ is a hydrophobic Leu. Serpina3n (FVPM) and PI9 (VVAE) display P4–P1 residues that differ from the consensus IEPD. Crystal structures of GrB in complex with either serpina3n or PI9 are yet to be resolved and are necessary to understand the relevance of the dramatic change of amino acid sequences from a GrB substrate to a GrB inhibitor.

GrB is expressed extracellularly and the lack of an intrinsic extracellular inhibitor of GrB is puzzling. Extracellular GrB inhibitors like SERPINB1 (Poe et al., 1991) have been suggested but studies have shown little in vitro inhibitory activity (Tremblay et al., 2000). High levels of extracellular GrB in disorders like multiple sclerosis, rheumatoid arthritis, chronic obstructive pulmonary disease, cystic fibrosis and sepsis, has linked GrB to the pathogenesis of these conditions. Specific animal studies show that GrB induces selective neuronal death in a multiple sclerosis mouse model (Haule et al., 2011) and inhibition of GrB with serine proteinases correlates with NK cell-mediated cytology in rats (Trapani et al., 1996). GrB may contribute to the pathogenesis of rheumatoid arthritis, as GrB levels are upregulated in plasma and synovial fluid of affected patients (Tak et al., 1999), while Froelich et al. showed that GrB degrades aggrecan, an integral component of the ECM in cartilage tissue (Froelich et al., 1993). Using a model of abdominal aortic aneurysm, Chamberlain and Ranum (2012) determined that GrB knockout mice had a reduced incidence of aneurysm rupture, while the mouse extracellular GrB inhibitor serpina3n also reduce aneurysm rupture and degrades the

Table II. $k_{\text{ass}}$ for serpina3n, wild-type ACT (ACTWT) and ACT mutants ACTmP3,P1,P4’ and ACTmP3,P1,P3,P4’

<table>
<thead>
<tr>
<th>Serpin name</th>
<th>$k_{\text{ass}}$ (M$^{-1}$ s$^{-1}$)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Serpina3n</td>
<td>6.71 $\times$ 10$^3$</td>
</tr>
<tr>
<td>ACTWT</td>
<td>2.26 $\times$ 10$^4$</td>
</tr>
<tr>
<td>ACTmP1</td>
<td>3.18 $\times$ 10$^5$</td>
</tr>
<tr>
<td>ACTmP3,P1,P4’</td>
<td>7.65 $\times$ 10$^5$</td>
</tr>
<tr>
<td>ACTmP3,P1,P3,P4’</td>
<td>3.71 $\times$ 10$^4$</td>
</tr>
</tbody>
</table>

Fig. 5. Mutant ACTmP3,P1,P4’ binds GrB and elastase. Purified ACTmP3,P1,P4’ was incubated with recombinant GrB, GrA and elastase. Samples were separated by SDS-PAGE and visualized by coomassie blue staining.
ECM protein decorin (Ang et al., 2011). Extracellular GrB also cleaves and activates soluble cytokines like IL-18 (Omoto et al., 2010) and IL-1α (Afonina et al., 2011), contributing to the progression of inflammatory disorders.

Our objective for this study was to create a new protein-based GrB inhibitor that could be used to elucidate the biology of GrB and potentially be used as a drug candidate for various diseases. GrB inhibitors have been designed and used as powerful tools to study the biology of GrB. Available inhibitors can be grouped into six major classes: peptide aldehydes, peptide chloromethyl ketones, acylating agents, iso-coumarins and suicide GrB-based inhibitors (Kam et al., 2000).

Protein-based inhibitors like ACTm3,P1,P4 have several advantages to smaller synthetic inhibitors. Small synthetic inhibitors are more likely to bind to unintended sites resulting in non-specific responses and would likely have more side effects as a pharmaceutical agent. Small synthetic peptide-based drugs can be more specific but these drugs are less stable biologically and have inherent physico-chemical vulnerabilities (Banerjee and Onyukel, 2012; Schall et al., 2012).

In this manuscript, we described the design and characterized the enzymatic properties of a novel GrB inhibitor. ACTm3,P1,P4 is based on a protein (ACT) that is already found extracellularly with a minimal three-amino acid change that transforms this enzyme into a powerful GrB inhibitor. To our knowledge, this is the first report in which a set of specific mutations has been introduced into a human serpin to generate a GrB inhibitor. Purified human serpins like α-antitrypsin and antithrombin H have been clinically tested in the treatment of emphysema (Mast et al., 1990), antitrypsin deficiency (Mordwinkin and Louie, 2007) and antithrombin deficiency during the acute phase of thermal injury (Kowal-Vern et al., 2001). Thus, anti-protases are effective means of reducing the expression of circulating proteases in the treatment of several disorders.

Although the literature indicates that GrB could play a role in numerous conditions, there are no clinical studies using GrB inhibitors. Our novel chimera, ACTm3,P1,P4, has inherent properties that make it a suitable drug candidate in the treatment of autoimmune inflammatory disorders.

Acknowledgements

The authors acknowledge the support of the Canadian Institutes of Health and Research (CIHR). They thank Nancy Ehrman and Diane Marganski for their support. They thank the Biological Sciences Technology Department and the School of Sustainable Building and Environmental Management from the Northern Alberta Institute of Technology for their encouragement and support. The authors have no conflicting financial interests.

References

New inhibitor for the enzyme Granzyme B


