Aminoethyl benzenesulfonyl fluoride and its hexapeptide (Ac-VFRSLK) conjugate are both in vitro inhibitors of subtilisin kexin isozyme-1

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Abstract Using a number of intramolecularly quenched fluorogenic (IQF) substrates encompassing the subtilisin kexin isozyme-1 (SKI-1)-mediated cleavage sites of various viral glycoproteins, it is revealed that 4-[2-Aminoethyl Benzene] SulfonylEluoride (AEBSF) can inhibit the proteolytic activity of SKI-1 mostly in a competitive manner. The measured IC₅₀ values range from 200 to 800 nM depending on the nature of the substrate used. This is the first in vitro demonstration of a nonpeptide inhibitor of SKI-1. In an effort to enhance the selectivity and potency of SKI-1 inhibition, a hexapeptidyl derivative containing SKI-1 consensus sequence, Ac-Val-Phe-Arg-Ser-Leu-Lys-AEBSF, was prepared. The peptide sequence was derived from the primary auto-activation site of prodomain of SKI-1 itself terminating at Leu-Lys¹³⁸ and contains the crucial P4-basic and P2 alkyl side chain containing hydrophobic amino acids. Like AEBSF, the hexapeptidyl-AEBSF analog blocked SKI-1 cleavages of all IQF-substrates tested but with enhanced efficiency.

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1. Introduction

Generation of bioactive proteins and polypeptides via cleavage of their inactive precursors after mono or paired basic residues is a wide spread process [1]. Less common is the recent finding that functionally active molecules can also be generated by selected proteolysis after either hydrophobic or small amino acid (aa) [2]. Subtilisin kexin isozyme-1 (SKI-1) [3] also known as site 1 protease (S1P) [4] defines the first mammalian subtilase with such substrate specificity. It is a widely expressed enzyme with various functional roles [3-5]. SKI-1 is a Ca²⁺dependent protease that cleaves protein precursors at the Cterminus of a non-basic amino acid within the sequence motif Arg/Lys-X- ϕ -Leu/Ser/Thr \downarrow , where X is any amino acid except Cys and ϕ is the alkyl side chain containing hydrophobic amino acid. SKI-1 is a subtype of basic amino acid cleaving enzymes called proprotein convertases (PCs) [1,2]. SKI-1 belongs to pyrolysin branch of subtilases compared to PCs that are within the kexin branch. So far, five physiological substrates of SKI-1 have been identified. These are proBDNF [3], SREBPs [4,6], the transcription factor ATF-6 [7,8], SREBP cleavage-activating protein involved in cartilage development in zebra fish [9] and the prodomain of SKI-1 itself [5]. Based on functional roles of some of these SKI-1 substrates, various cellular and biochemical studies, SKI-1 has been implicated in cholesterol metabolism, fatty acid synthesis, ER (endoplasmic reticulum) induced stress regulation and cartilage growth and development. Recent studies further indicated that this cellular protease also participates in the proteolytic activation of viral coat glycoproteins of highly infectious Lassa [10], lymphocytic choriomeningitis [11], and crimean congo hemorrhagic fever (CCHF) [12] viruses, suggesting its role in these pathogeneses. Owing to these findings, we became interested to develop small molecule inhibitors of SKI-1 that may find potential applications as therapeutic agents. Studies have so far revealed that the prodomain of SKI-1 and its derived peptides [5] and several bio-engineered forms of α 1-antitrypsin containing the SKI-1 motif ³⁷⁹RILL, RRLL or RRLV replacing the wild type sequence ³⁷⁹AIPM in the reactive site loop of this serpin [13] are able to inhibit SKI-1 mediated processing of SREBPs in cell based assay. Okada et al. [14] recently reported that the non-peptide general serine protease inhibitor 4-(2-Aminoethyl) Benzene Sulphonyl Fluoride (AEBSF) [15] prevented ER stress-induced SKI-1-mediated cleavage of activating transcription factor 6 (ATF6 α and β). It also inhibited the production of active form of SREBP-2 that was induced in response to sterol depletion. Both results indicated that AE-BSF is an ex vivo SKI-1 inhibitor. In order to confirm this finding, and as part of our ongoing efforts to develop potent and selective small molecule inhibitors of SKI-1, we have now carried out in vitro studies using recombinant SKI-1 and a number of synthetic fluorogenic SKI-1 substrates. These sub-

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Abbreviations: PC, proprotein convertase; SKI-1, subtilisin kexin isozyme-1, S1P, site 1 protease; vv, vaccinia virus; aa, amino acid(s); h, human; m, mouse; r, rat; MALDI-TOF, matrix-assisted laser desorption ionization-time of flight; Fmoc, *N*-(9-fluorenyl)-methoxycarbonyl; IQF, intramolecularly quenched fluorogenic; RP-HPLC, reverse phase high-performance liquid chromatography; Tyx, 3-nitro tyrosine; Abz, 2-aminobenzoic acid; TFA, trifluoroacetic acid; Dabcyl, 4-4'-dimethyl amino phenazo benzoic acid; Edans, 5-[(2'-amino ethyl)-amino] naph-thalene 1-sulfonic acid; PyBop, benzotriazol-1-yl-oxytripyrolidino-phosphonium hexafluorophosphate; HOBT, 1-hydroxybenzotriazole hydrate; Tris, Tris hydroxymethyl amino methane; Mes, 1-morpholino ethane sulfonic acid; cmv, cytomegalovirus

strates are derived from SKI-1 cleavage sites of various surface viral glycoproteins such as Lassa [10,16] and CCHF [12]. AEBSF, available in the form of its hydrochloride salt known as Pefabloc[®], SC is described as a serine protease inhibitor and an ideal substitute of phenylmethylsulfonyl fluoride (PMSF) and diisopropyl fluorophosphate (DFP) [17-20] due to its much lower toxicity, better stability at physiological pH and capacity to inhibit a broad range of serine proteases. This includes trypsin, chymotrypsin, plasmin, plasma kallikrein, thrombin, glandular kallikrein, tissue plasminogen activator, and subtilisin. Among other less known proteases, mention may be made of Proteinase K and serine threonine phosphatase [19,21]. It is mostly used to block unwanted detrimental effects of proteases during preparative protein purification. [19]. Depending on concentration and pH conditions, AEBSF can inhibit proteases in a reversible or irreversible manner [17-20]. In this study, we describe the SKI-1 inhibitory property of both AEBSF and its hexapeptidyl derivative that contains a SKI-1 recognition sequence which is likely to enhance the potency and selectivity of enzyme inhibition.

2. Materials and methods

2.1. Materials

4-(2-Aminoethyl) benzene sulfonyl fluoride was purchased as its hydrochloride salt from Fisher Scientific (Nepean, ON, Canada), while the intramolecularly quenched peptides were either bought from Bachem Chemical (King of Prussia, PA, USA) or synthesized in the laboratory. All N-(9-fluorenyl)-methoxycarbonyl (Fmoc) protected amino acids (L-configuration), the coupling reagents, HATU (O-hexafluorophospho-[7-azabenzotriazol-1-yl]-N,N,N',N'-tetramethyluronium), DIEA (diisopropyl ethyl amine), Fmoc-PAL-PEG-PS resin and the solvents were purchased from Applied Biosystems (Foster city, CA, USA), Calbiochem Novabiochem AG (San Diego, CA, USA), Chem-Impex International (Wood Dale, IL, USA) and Aldrich Chemical (Milwaukee, WI, USA). The 2-chlorotritylchloride resin was obtained from Calbiochem-Novabiochem AG. Amino acid analyses were performed following 24 h hydrolysis in 6 M HCl at 110 °C in a sealed tube using a Dionex BioLC system (model ICS-2500) equipped with an AminoPacTM PA10 (Dionex, Oakville, ON, Canada) anion-exchange analytical column and an electrochemical detector (www.dionex.com).

2.2. Peptide synthesis

Various intramolecularly quenched fluorogenic (IQF) peptide substrates used in this study are listed in Table 1. The C-terminal (CT) end of each peptide is in the amide form unless specified otherwise. Except 4-4'-dimethyl amino phenazo benzoic acid-cytomegalovirus-5-[(2'amino ethyl)-amino] naphthalene 1-sulfonic acid (Dabcyl-cmv-Edans) peptide, which was purchased from Bachem, all were synthesized on an automated solid phase peptide synthesizer (Pioneer model, PE-PerSeptive Biosystems, Framingham, MA, USA), following HATU/ DIEA-mediated Fmoc chemistry and Fmoc protected unloaded PAL-PEG-PS (polyamino linker polyethylene glycol polystyrene) resin [16]. For incorporation of hydrophobic amino acids, Val, Leu, Tyr, 3-nitro tyrosine (Tyx), Abz (2-amino benzoic acid) and hydroxy amino acids like Thr and Ser into the elongating peptide chain, an extended cycle (45 min) with or without a double coupling step was performed instead of normal standard (20 min) or fast (10 min) cycle. The following side chain protecting groups were used; pbf (2,2,4,6,7-pentame-thyldihydrobenzofuran-5-sulfonyl) for Arg; tBut (tertiary butyl) for Ser and tBoc (tertiary butyloxy carbonyl) for Lys.

2.3. Peptide purification

Crude peptides were purified by reverse phase high-performance liquid chromatography (RP-HPLC) on a Rainin Dynamax instrument equipped with SD-1 pump (Rainin Instrument, Emeryville, CA, USA), using a 10 μ m, 300 Å C₁₈ column {Jupiter, Phenomenex, column size: 0.46 × 25 cm (analytical) or 1 × 25 cm (semipreparative)}. The buffer system comprised of an aqueous 0.1% (v/v) TFA solution and an organic phase of CH₃CN containing 0.1% (v/v) TFA. Peptides were eluted with a 1% min⁻¹ linear gradient (5–60%) of 0.1% aqueous TFA/ CH₃CN at a flow rate adjusted to 1 mL/min (analytical run) or 2 mL/ min (semipreparative run), following a 5 min isocratic at 5% of 0.1% TFA/CH₃CN. The elution of the peptides was monitored on line by measurement of UV absorbency with wavelength set at 214 nm and/or fluorescence measurement at excitation and emission wavelengths fixed at 320 and 420 nm for Abz and nitro tyrosine pair or 355 and 495 nm for Dabcyl and Edans pair, respectively.

2.4. Matrix-assisted laser desorption time-of-flight (MALDI-TOF) mass spectrometry

All MALDI-TOF mass spectra were recorded using $1-2 \mu L$ sample and an identical volume of a saturated solution of CHCA (α -cyano-4hydroxycinnamic acid) or DHB, (1,2 dihydroxy benzoic acid) using Voyager DE (PE Biosystem, Framingham, MA, USA) instrument [16].

2.5. Synthesis of peptidyl-AEBSF

The peptide selected for conjugation to AEBSF for development of a SKI-1 inhibitor is the hexamer, Val-Phe-Arg-Ser-Leu-Lys. This sequence easily recognized by SKI-1 is derived from the region of primary activation site in the prodomain of SKI-1 itself [3-5]. Initially, the protected hexapeptide, H2N-Val-Phe-Arg(Pbf)-Ser(tBt)-Leu-Lys(-Boc)-COOH (1 mM scale), was prepared by solid phase Fmoc chemistry using the unloaded 2-chlorotritylchloride resin [22]. Acetylation was performed directly on the resin by the addition of 5% acetic anhydride and 2.1 equivalent DIEA (diisopropyl ethyl amine) in DCM (Dichloromethane) and shaking at 25 °C for 30 min. Excess reagent was removed by filtration and the peptide was carefully cleaved from the acid sensitive resin by treatment with acetic acid/trifluoro ethanol (TFE)/DCM (1:1:8) for 10 min and filtered into a receiving flask containing hexane. The procedure was repeated three times to ensure a complete release of the protected peptide from the resin. The combined filtrates in hexane were dried by rotary evaporator with further addition of hexane to azeotropically remove the acetic acid. The identity of the peptide was confirmed by MALDI-TOF mass spectrometry [observed m/z = 1212 (M + Na)⁺, calculated m/z = 1211] and amino acid analysis [16]. The protected peptide (30 mg, 0.025 mmol) was next activated with benzotriazol-1-yl-oxytripyrrolidinophosphonium hexafluorophosphate (PyBop; 50 mg, 0.1 mmol), 1-hydroxybenzotriazole hydrate (HOBT; 15 mg, 0.1 mmol) and DIEA (175 µL) in DMF (2 mL) at room temperature for 1 h. AEBSF.HCl (25 mg, 0.1 mmol) in DMF (2 mL) was added dropwise and stirred for 30 min. The reaction was monitored by thin layer chromatography. When judged complete (6 h), the solution was acidified with TFA in an ice bath. The product was recovered by preparative thin layer chromatography (TLC) and RP-HPLC and fully characterized by MALDI-TOF mass

Table 1

Inhibition parameters of AEBSF and its hexa-peptidyl derivative against SKI-1 activity as measured with various fluorogenic substrates

| Inhibitor | Substrate | Sequence | ^a IC ₅₀ (nM) | $K_{\rm i}$ (nM) |
|----------------------------------|--|--|------------------------------------|-------------------|
| AEBSF | Q-GPC ^{251–263} Dab-cmv-Edans ^{638–648} Q-CCHFV-A ^{512–523} | Abz-DIYISRRLL ²⁵⁹ ↓GTFT-Tyx-A Dabcyl-RGVVNA ⁶⁴³ ↓SSRLA-Edans Abz-SSGSRRLL ⁵¹⁹ ↓SEES-Tyx-A | 758 277 850 | 231 120 250 |
| Ac-Val-Phe-Arg-Ser-Leu-Lys-AEBSF | Q-GPC ^{251–263} | Abz-DIYISRRLL ²⁵⁹ ↓GTFT-Tyx-A | 150 | 57.7 |

^a All IC₅₀ values were measured with 50 μ M final concentration of the substrate. The values were derived from the average of at least two independent experiments. Both Q-GPC and Q-CCHFV-A peptides were prepared as described by us [12,16], while Dab-cmv-Edans peptide was obtained from Bachem Chemical [23].

spectrometry {observed m/z = 958 (M + H–H₂O), 976 (M + H)⁺, 998 (M + Na)⁺, calculated m/z = 975 (M + H)⁺} and by amino acid analysis as described earlier and in [16].

2.6. Source of recombinant SKI-1

The secreted soluble form of hSKI-1 used in this study was obtained by using a BTMD (Before Trans-Membrane Domain) hSKI-1 construct ending at **PGRYNQE**⁹⁹⁷ as previously described [3,5,16]. Following a 6 h infection of vaccinia virus (vv) infected BTMD SKI-1 construct in HK-293 cells, the conditioned medium containing active SKI-1 was concentrated (~15-fold) using centricon-30 (Amicon, USA), dialyzed and stored with 40% glycerol at -20 °C before use. The sample thus obtained was reasonably pure enzymatic wise. However, in order to compensate the effect of any contaminant and non-specific activity, each experiment was compared with the control, done in parallel with the medium obtained similarly from the expression of the empty vector.

2.7. Inhibition study

All enzymatic assays against recombinant SKI-1 were performed using either initial rate and/or stopped-time assays at 37 °C in a final volume of 100 µL in 96-wells flat bottom black plates (Microfluor, Dynatec Laboratories Inc., Rochester, NY, USA). The assays were performed in a buffer consisting of 25 mM Tris + 25 mM 1-morpholino ethane sulfonic acid (Mes) + 2.5 mM CaCl₂, pH 7.4. All SKI-1 assays were performed unless mentioned otherwise using a final concentration of 50 µM of each IQF substrate indicated below. (i) Lassa virus glycoprotein derived peptide Q-GPC²⁵¹⁻²⁶³: Abz-²⁵¹Asp-Ile-Tyr-Ile-Ser-Arg-Arg-Leu-Leu Gly-Thr-Phe-²⁶³Thr-**Tyx**-Ala [16], (ii) cmv protease derived peptide Dabcyl-cmv-Edans⁶³⁸⁻⁶⁴⁸: **Dabcyl**-⁶³⁸Arg-Gly-Val-Val-Asn-Ala Ser-Ser-Arg-Leu-⁶⁴⁸Ala-Edans [23], and (iii) the site A of glycoprotein of <u>Crimean-Congo Hemorrhagic Fever</u> <u>V</u>irus derived peptide Q-CCHFV-A⁵¹²⁻⁵²³: Abz-⁵¹²Ser-Ser-Gly-Ser-Arg-Arg-Leu-Leu Ser-Glu-Glu-523Ser-Tyx-Ala [12]. While both GPC and CCHFV fluorogenic peptides were developed previously by us, the cmv-peptide was purchased from Bachem Chemical. For measurement of both K_i (inhibition constant) and IC₅₀ (concentration necessary to achieve 50% inhibition of enzymatic activity) values, the inhibitor concentrations were varied over a range wide enough to yield residual activities of 25-75% of the control value. The kinetic parameters were evaluated using the program Grafit (v4.0, Erthacus Software, Distributor, Sigma Chemical, St. Louis, MO, USA). Standard curves and quench corrections were performed for accurate evaluation as described previously [16,24]. Enzyme sample, typically 10 µL of hSKI-1, was used for each assay. To these were added increasing concentrations of inhibitors ranging from 20 to 2500 µM in a total volume of 100 µl of buffer solution as described above, in a 96well plate at 37 °C. Following a 15 min pre-incubation, substrate solution (5 µL) was added and the sample was incubated again. The rate of substrate hydrolysis was obtained from changes in fluorescence readings and the values transformed into amounts of μ M/h of peptide cleaved by using the standard curve and quenching corrections as described in [24]. Non-linear regression of plots of hydrolysis rate vs. the inhibitor concentration was used for three sets of each substrate concentration. Data were collected in duplicates and each value is the mean of two independent experiments. For determination of site of cleavage, each digest was analyzed by RP-HPLC using a C18 analytical column followed by MALDI-TOF-MS of each isolated peak.

3. Results

3.1. Effect of AEBSF on SKI-1 mediated cleavage of Q-GPC

We have shown previously that one of the most potent substrates for in vitro assay of SKI-1 activity is an IQF peptide Q-GPC [Abz-²⁵¹Asp-Ile-Tyr-Ile-Ser-Arg-Arg-Leu-Leu↓Gly-Thr-Phe-²⁶³Thr-**Tyx**-Ala], derived from the activation site of Lassa viral glycoprotein [16]. This peptide was selectively and efficiently cleaved at **RRLL**²⁵⁹↓**GTF** site by SKI-1 resulting in the release of fluorescence measured at $\lambda_{ex} = 320$ nm and $\lambda_{em} = 420$ nm. To examine the effect of AEBSF on SKI-1

activity, Q-GPC was chosen as the initial substrate. Our results indicate that AEBSF bocks this cleavage in a dose-dependent manner. As shown in Fig. 1A using 50 µM Q-GPC as the substrate, the measured IC₅₀ value based on two independent experiments and correction of peptide content is \sim 758 nM. Dixon plots using three different concentrations (50, 25 and 12.5 µM) of Q-GPC and a 15-min pre-incubation period for the enzyme to interact with AEBSF before addition of the substrate showed that the inhibition constant K_i is 231 nM (Fig. 2, Table 1). It is further noted that the obtained three regression lines nearly pass through a common point of intersection, suggesting that the inhibition is mostly competitive and reversible in nature [16,24,25]. Based on the equation $K_{\rm i} = {\rm IC}_{50}/(1 + S/K_{\rm m})$ where S is the substrate concentration, $K_{\rm m}$ is the Michaelis–Menten constant, applicable to reversible and competitive inhibition [26], the estimated K_i for SKI-1 inhibition of Q-GPC cleavage by AEBSF is ~61 nM. This is more than 3-fold lower than the measured value of 231 nM. This may suggest that this inhibition may not be true competitive and possibly it is more like mixed type of inhibition. Thus, it is likely that AEBSF interacts with both catalytic and non-catalytic regions of SKI-1 in which case the above equation is not fully applicable. The inhibition character remains mostly unaltered over the range of pre-incubation time (2-15 min) used in this study. This suggests that the formation of the complex between AEBSF and SKI-1 enzyme is relatively rapid.

3.2. Effect of AEBSF on SKI-1 mediated cleavage of cmv peptide

Recent study from our laboratory (Basak et al., in preparation) indicated that SKI-1 cleaves quite efficiently the IQF peptide, Dab-cmv-Edans^{638–648} [Dabcyl-RGVVNA⁶⁴³] SSRLA-Edans], derived from the maturational site of human (h) cmv protease at the correct physiological site [23]. When this peptide (50 μ M) was used as a substrate for SKI-1, again



Fig. 1. Inactivation of proteolytic activity of recombinant human SKI-1 towards cleavages of Q-GPC^{251–263} [Abz-DIYISRR LL²⁵⁹]GTFT-Tyx-A] (A) and Dab-cmv-Edans^{638–648} Dabcyl-RGVVNA⁶⁴³]SSRLA-Edans (B) by varying concentrations of AEBSF.HCl. A 15 min pre-incubation between the enzyme and AEBSF is followed by addition of the substrate (50 μ M final concentration). The residual enzymatic activity after a period of 150 min was estimated by measuring the fluorescence intensity at excitation and emission wave lengths fixed at 320 and 420 nm for Q-GPC^{251–263} and 355 and 495 nm for Dab-cmv-Edans^{638–648}, respectively. The enzyme used in this study was obtained from BTMD-SKI-1 construct and is partially purified by centricon filtration and dialysis as described in Section 2. Control experiments were performed with media obtained similarly from empty vector. Each data point was the representative of at least two independent experiments carried simultaneously.



Fig. 2. Dixon plots showing the inhibition of SKI activity by varying concentrations of AEBSF.HCl as measured against three different concentrations (12.5, 25 and 50 μ M) of the substrate, Q-GPC^{251–263} [Abz-DIYISRRLL²⁵⁹]GTFT-Tyx-A]. The inhibition constant K_i was measured from the intersections of any two regression lines and the values were averaged as shown (for details see Section 2).

AEBSF is able to block the cleavage quite effectively. In fact, more than 95% of SKI-1 inhibition was achieved with 1 μ M AEBSF and the measured IC₅₀ value was 277 nM (Fig. 1B) which is nearly 2.7-fold lower than 758 nM value observed with Q-GPC. The measured K_i value with this substrate based on Dixon plot (not shown) is ~120 nM, nearly 2.3-fold lower than that for Q-GPC. Mass spectral analysis of 3 h crude digest of Dab-cmv-Edans^{638–648} indicated that SKI-1 cleaved Dabcyl-cmv-Edans primarily at the correct NA⁶⁴³↓SS physiological site. Thus, MS spectrum showed peaks at (i) 1627

 $(M + H)^+$, 1649 $(M + Na)^+$ for the undigested peptide (calculated mass = 1626), (ii) 866 $(M + H)^+$, 888 $(M + Na)^+$ for the N-terminal (NT) fragment **Dabcyl-R-G-V-V-N-A-OH** (calculated mass = 865) and (iii) 779 (not observed possibly due to further degradation) for the CT fragment, **S-S-R-L-A-Edans** (Fig. 3). Upon prolonged incubation (18 h), a minor cleavage at **Dabcyl-R-G-V-V-N-A-S-S** \downarrow **R-L-A-Edans** was also observed leading to the formation of **Dabcyl-R-G-V-V-N-A-S-S-OH** [*m*/*z* = 1041, (M + H)⁺, calculated = 1040] (not shown).

A comparison of MALDI-TOF MS data of 3 h crude digests of Dabcyl-cmv-Edans (10 µg) with an identical amount of recombinant SKI-1 (10 µL) in the absence and presence of varying amounts (12.5–250 nM) of AEBSF is shown in Fig. 3. The figure shows that as the amount of added AEBSF is increased, there is less formation of cleavage fragment (peak at m/z = 866 for the NT piece) and more of undigested substrate (peak at m/z = 1627), suggesting that AEBSF blocks SKI-1 mediated cleavage of cmv peptide.

3.3. Dose-dependent effect of AEBSF on SKI-1 mediated cleavage of Q-GPC peptide using mass spectra

Similar to that observed with cmv peptide, the cleavage of Q-GPC peptide by recombinant SKI-1 can also be efficiently hindered in vitro by AEBSF in a concentration-dependent manner. Thus, as shown by MALDI-TOF MS data in Fig. 4, with the increase in amount of added AEBSF, the intensity of the peak for the NT cleavage product (m/z = 1268) following 6 h digestion with SKI-1 was gradually diminished while the peak intensity of the undigested Q-GPC peptide (m/z = 1950) is enhanced. In these experiments, the CT cleaved product at m/z = 702 was not detectable possibly due to further degradation, lack of charge and high hydrophobicity.



Fig. 3. MALDI-TOF mass spectral analyses of 3 h crude digests of Dab-cmv-Edans^{638–648} (10 μ g) by identical amount of recombinant hSKI-1 (10 μ L) in the presence of various amounts (12.5, 62.5, 125 and 250 nM) of AEBSF.HCl. The peaks at *m*/*z* 1627 and 866 were attributed to the undigested substrate (Dab-cmv-Edans^{638–648} (Dabcyl-RGVVNA⁶⁴³ SSRLA-Edans, calculated mass = 1626) and the NT fragment (Dabcyl-RGVVNA-OH, calculated mass = 864). The corresponding CT fragment could not be detected under the condition used.



Fig. 4. MALDI-TOF mass spectral analyses of 6 h crude digests of Q-GPC^{251–263} (10 μ g) by identical amount of recombinant hSKI-1 (10 μ L) in the presence of various amounts (12.5, 62.5, 125 and 250 nM) of AEBSF.HCl. The peaks at *m*/*z* 1950 and 1268 were attributed to the undigested substrate, (Q-GPC^{251–263} = Abz-DIYISRRLL²⁵⁹ GTFT-Tyx-A, calculated mass = 1949) and and the NT fragment (Abz-DIYISRRLL-OH, calculated mass = 1267). The corresponding CT fragment could not be detected under the condition used.

3.4. Effect of AEBSF on SKI-1 mediated cleavage of Q-CCHFV-A peptide

Recently, we reported that the envelope surface glycoprotein of CCHFV is cleaved at the NT A-site (RRLL⁵¹⁹ JSE) by SKI-1, while the other site (C-terminal B-site, RKPL¹⁰⁴⁰ FL) is cleaved by a non-PC like enzyme [12]. Based on this observation, we developed an IQF peptide (Q-CCHFV⁵¹²⁻⁵²³) derived from the A-site as a substrate for SKI-1 (Table 1). As shown in this table, A EBSF also prevents the cleavage of Q-CCHFV⁵¹²⁻⁵²³ by recombinant SKI-1. A comparison of measured IC₅₀ value (~850 nM) with those obtained with Q-GPC²⁵¹⁻²⁶³ and Dab-cmv-Edans⁶³⁸⁻⁶⁴⁸ showed that its inhibition by AEBSF is comparable to that for Q-GPC²⁵¹⁻²⁶³ substrate. We also observed by mass spectra that AEBSF upon incubation does not react chemically with any of the substrates used in this study except Q-CCHFV⁵¹²⁻⁵²³, which upon prolonged incubation forms a stable chemical adduct with AEBSF as shown in Fig. 5. Thus, following 18 h incubation it exhibited strong peaks at m/z = 1706 and 1891 in the mass spectrum for the undigested substrate and the substrate-AEBSF adduct, respectively. The increase of 185 mass unit for the adduct indicates that AEBSF (molecular weight = 203) and the peptide (molecular weight = 1706) are covalently linked to each other by elimination of a molecule of water. The calculated molecular weight [1706 (mass for peptide) + 203 (mass for AEBSF) – H_2O (a molecule of water)=1891] is in consistent with the observed value. It is interesting to note that such adduct formation was not detectable at lower incubation time (less than 4 h) nor with the two other substrates even after long incubation. It is presumed that AEBSF may have coupled with either of the two glutamic acid residues present only in Q-CCHFV-A substrate.

3.5. Synthesis of peptidyl-AEBSF

The chemical synthesis of hexapeptide conjugate of AEBSF, namely Ac-Val-Phe-Arg-Ser-Leu-Lys-AEBSF, is described schematically in Fig. 6. The protected hexapeptide acid Ac-Val-Phe-Arg(Pbf)-Ser(tBut)-Leu-Lys(Boc)-OH, obtained by solid phase Fmoc chemistry using the unloaded 2-chlorotritylchloride resin (calculated mass = 1190, observed = 1191), was conjugated to AEBSF using PyBop/HOBT/DIEA. It was then fully deprotected with reagent K [16] yielding a complex mixture which was partially purified by preparative TLC. The final purification was accomplished by RP-HPLC (Fig. 7A). All HPLC peaks were collected and examined by mass spectra. The peak eluting at retention time 49.01 min yielded the desired compound Ac-Val-Phe-Arg-Ser-Leu-Lys-AEBSF (molecular weight calculated = 975), observed $m/z = 976 (M + H)^+$, 998 $(M + Na)^+$, and 958 $(M + H - H_2O)^+$ (Fig. 7B). The inset within B shows the expanded portion of the mass spectrum near the molecular weight region.

3.6. Effect of Ac-VFRSLK-AEBSF on the activity of SKI-1

When Ac-VFRSLK-AEBSF was pre-incubated for 15 min with recombinant SKI-1 and the mixture tested for residual enzyme activity against Q-GPC^{251–263}, there was a significant drop in fluorescence release depending on the concentration of Ac-VFRSLK-AEBSF used compared to control AEBSF alone. In fact, addition of 1 μ M Ac-VFRSLK-AEBSF completely abrogated the cleavage of Q-GPC^{251–263} by recombinant SKI-1. The measured IC₅₀ value of 150 nM is nearly 5-fold lower than AEBSF itself. This suggests that coupling of AEBSF via its amino terminal to a peptide sequence compatible with SKI-1 recognition significantly enhances SKI-1 inhibition by the resultant molecule. However, when K_i was determined by using Dixon plots based on end



Fig. 5. MALDI-TOF mass spectra of Q-CCHFV-A⁵¹²⁻⁵²³ (Abz-SSGSRRLL⁵¹⁹ SEES-Tyx-A) following incubation at 37 °C with equimolar quantity of AEBSF.HCl in aqueous solution for various lengths of time as indicated. Small aliquots were withdrawn at specific times for recording the mass spectra.



Fig. 6. It shows the schematic diagram for the synthesis of hexapeptidyl-AEBSF derivative. The final product was obtained following complete deprotection and purification by preparative thin-layer chromatography and RP-HPLC in combination as described in Section 2. Synthetic scheme for pentapeptidyl-AEBSF.

time assay (Fig. 8A) and progress curve assay (not shown) using three separate concentrations of the substrate Q-GPC, the measured value was 57 nM (Fig. 8, Table 1). The value was confirmed by two independent experiments. This is ~3-fold higher than that calculated from the equation $K_i = (IC_{50}/(1 + S/K_m))$ valid for true competitive inhibition, where $S = 50 \mu$ M and $K_m = 4.9 \mu$ M [16]. This variation again suggests that

like AEBSF, its peptidyl analog Ac-VFRSLK-AEBSF may also interact with SKI-1 at multiple sites including the catalytic (competitive inhibition).

The dose-dependent inhibition of SKI-1 activity by Ac-VFRSLK-AEBSF as measured against Q-GPC substrate was also shown by progress curves in Fig. 8B. Clearly, there is a gradual loss in SKI-1 activity as revealed by the slope of each



Fig. 7. (A) RP-HPLC chromatogram of crude material obtained after coupling of Ac-V-F-R(Pbf)-S(tBt)-L-K(Boc)-OH with AEBSF, removal of all protecting groups except the acetyl (Ac) group and partial purification by preparative thin-layer chromatography. Various peaks obtained were collected and examined by MALDI-TOF mass spectrometry. The peak with retention time (R_t) = 49.01 min was characterized by mass spectrometry (B) as the correct product. (B) The MALDI-TOF mass spectrum of peak eluting at 49.01 min. It displayed peaks at m/z 976 and 998 corresponding to the molecular size of Ac-VFRSLK-AEBSF (calculated mass = 975) and its sodium adduct (calculated mass = 997). The peak at m/z = 558 was attributed to the loss of a water (18 mass unit) molecule from the parent molecular ion.

progress curve with the increase in the amount of added peptidyl-AEBSF.

4. Discussions

This study indicates that aromatic sulfonyl fluoride, AEBSF, is capable of blocking in vitro the proteolytic cleavage of three synthetic IQF substrates, Q-GPC²⁵¹⁻²⁶³, Dab-cmv-Edans⁶³⁸⁻⁶⁴⁸ and Q-CCHFV⁵¹²⁻⁵²³ by recombinant SKI-1 (Table 1). The measured IC₅₀ values in all cases are in medium to high nM range. Under identical condition, SKI-1 inhibits the cleavage of Dab-cmv-Edans⁶³⁸⁻⁶⁴⁸ 2.7 and 3-fold more effectively than Q-GPC²⁵¹⁻²⁶³ and Q-CCHFV⁵¹²⁻⁵²³, respectively. Although AEBSF was previously described as a general inhibitor of serine proteases of all sub-types such as trypsin, chymotrypsin, plasma kallikrein and elastase, no reports are available about its effect on mammalian subtilases except a recent publication where it was demonstrated that AEBSF blocks ex vivo the SKI-1 cleavage of stress responsive transcription factors ATF6 α and β [14]. Since ATF6 plays a major role in unfolded protein response, this study showed the potential of AEBSF in this event via its inhibitory action on SKI-1 activity. Our in vitro study supports the above ex vivo data and is the first direct demonstration of inhibition SKI-1 activity by this nonpeptide AEBSF molecule using small synthetic fluorogenic substrates. The study also demonstrates that the potency of inhibition may be substrate dependent, since the most potent inhibition was observed with Dab-cmv-Edans⁶³⁸⁻⁶⁴⁸ followed, respectively, by Q-GPC²⁵¹⁻²⁶³ and Q-CCHFV⁵¹²⁻⁵²³. This work further indicates that in the absence of P4 Arg, SKI-1 can accept P6-Arg as long as there is P2 and/or P1 alkyl side chain containing hydrophobic residue - an observation not reported until recently involving the N-terminal processing of mammalian prosomatostatin (PSST) by SKI-1. It was shown that SKI-1 cleaves PSST at the amino terminal segment ⁴Asp-Pro-Arg-Leu-Arg-Gln-Phe-Leu↓Gln-Lys-Ser-Leu¹⁵, where a single mutation by Ala of either Arg did not fully abrogate the cleavage while double mutations caused a complete loss of cleavage by SKI-1 [27]. Thus, the Arg⁸ to Ala variant [¹Ala-Pro-Ser-Asp-Pro-Arg-Leu-Ala⁸-Gln-Phe-Leu |Gln-Lys-Ser-Leu¹⁵] is still processed by SKI-1 though to a reduced level. This suggests that the presence of Arg at P4 is not an absolute requirement for recognition by SKI-1 and absence of such residue can be partly compensated by the presence of P6-Arg residue. This is in consistent with the present study. Furthermore, our data indicated that the cleavage of a P6-Arg containing substrate such as Dabcyl-cmv-Edans with hydrophobic Val residues at both P4 and P3 positions is more efficiently blocked by AEBSF compared to substrates that contain P4-Arg. It may be mentioned that unlike AEBSF, other sulfonyl fluoride analogs such as PMSF [phenyl-methane-sulfonyl fluoride] and aPMSF [4-amidino-phenyl-methane-sulfonyl fluoride] were described as poor inhibitors of SKI-1 [5]. This could be related to the presence of aromatic hydrophobic ring system directly attached to the sulfonyl fluoride moiety that may interact better with SKI-1 compared to the alkyl chain attached sulfonyl fluorides. One function of the aryl ring is to diminish the electrophilicity of attached fluorine atom and therefore prevent covalent modification of the enzyme. It is likely that such molecules may associate with the catalytic residues of the enzyme via non-covalent interactions. To our knowledge, this is only the second report where SKI-1 cleaves substrates which do not possess a P4-Arg residue but instead contains a P6-Arg residue and a P4 hydrophobic residue.

Our study also revealed that attachment of a peptide sequence compatible with SKI-1 recognition motif such as Val-Phe-Arg-Ser-Leu-Lys to the amino function of AEBSF led to an enhancement of SKI-1 inhibition compared to AEBSF alone. Our initial choice of hexapeptide sequence is based on the autocatalytic activation site of prodomain of SKI-1 itself [3-5]. Other sequence motifs (like RSLL \downarrow , RRLL \downarrow and RLRQFL) more notably present in the SKI-1 processing sites of SREBP-2 [3,4,7], glycoproteins of Lassa [10], CCHF [12] viruses and PSST (27) could be attached to AEBSF in order to develop more potent and selective SKI-1 inhibitors. Moreover, in peptidyl-AEBSF conjugate the aromatic group of AEBSF moiety may be substituted by a non-aromatic group or none at all in order to examine the role of aryl group if any on SKI-1 inhibition. Thus, in future study, a tripeptide such as RRL as well as the tetrapeptide RRLL will be conjugated separately to AEBSF along with other peptides to understand better the recognition motif of SKI-1. Among other variations, small non-aromatic chains mimicking Leu residue may be substituted to examine their effects on SKI-1 inactivation. Studies involving a variety of these and other peptidyl aryl and nonaryl sulfonyl fluorides may provide further insight into the mechanism of inhibition and also development of novel SKI-1 inhibitors. The formation of a covalent adduct between the Q-CCHFV-A peptide and AEBSF with the removal of a molecule of water upon prolonged incubation indicated that certain



Fig. 8. (A) Dixon plots showing the inhibition of SKI activity by varying concentrations of Ac-VFRSLK-AEBSF as measured against the substrate, Q-GPC^{251–263} [Abz-DIYISRRLL²⁵⁹ GTFT-Tyx-A]. The inhibition constant K_i was measured from the intersections of any two regression lines and the values were averaged as shown (see Section 2). (B) Progress curves showing the gradual inhibition of SKI-1 activity by various concentrations of Ac-VFRSLK-AEBSF. The activity of SKI-1 was monitored using Q-GPC (50 μ M) as the substrate.

substrates, particularly those with multiple acidic amino acids like Glu, may react with AEBSF and may therefore not be suitable for monitoring SKI-1 activity.

The mechanism of enzyme inhibition by AEBSF and related derivatives has been described in the literature. It is now known that AEBSF rapidly reacts with the catalytic serine residue, forming a stable acylated enzyme resulting in a loss of proteolytic activity. The inhibitors may vary in many respects, including specificity for particular proteases and the rate at which they inactivate these proteases. In many cases, non-covalent interactions have also been reported [17-20]. It is quite likely that SKI-1 is also inhibited in a non-covalent manner as indicated by Dixon plots and progress curves where in many cases a higher inhibition is noted at the initial period. Another important finding of this study is that SKI-1 cleaves in vitro the synthetic cmv peptide at the correct RGVVNA⁶⁴³ USS maturational site. This may suggest a possible role of this enzyme in the activation of cmv protease and hence in the pathogenesis of this infection. Consequently, SKI-1 specific inhibitors may be useful in the intervention of this and other infections such as Lassa (10), CCHFV (12) where its role has already been established.

5. Conclusion

Our study revealed for the first time that a simple aromatic sulfonyl fluoride (Ar–SO₂–F) that inhibits most serine proteases is also capable of inhibiting in vitro the activity of subtilase SKI-1. The study also demonstrated that peptidyl arylsufonyl fluoride may represent a novel class of SKI-1 inhibitors. The peptide sequence preceding the attached sulfonyl fluoride moiety is crucial for such inhibition and more studies involving a variety of peptide sequences and appropriate sulfonyl fluoride function are needed for final confirmation. Future study will reveal any potential antiviral and other biochemical activity of such small molecule based SKI-1 inhibitors.

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