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Azaphenylalanine-based serine protease inhibitors induce caspase-mediated apoptosis

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ABSTRACT

Molecules regulating cell death constitute prominent therapeutic targets. The pro-apoptotic role of serine protease inhibitors prompted us to search for novel modulators of this process. We have tested some recently synthesized antithrombotic compounds for their potential to induce apoptotic cell death. Cell based analyses revealed that inhibitors built on the azaphenylalanine scaffold are, for B-cell lymphoma cells, severely cytotoxic, while other compounds tested were moderate or non-cytotoxic. These inhibitors induced the time and concentration dependent biochemical and morphological characteristics of apoptosis, such as DEVDase activation, loss of mitochondrial membrane potential, nuclear degradation and genomic DNA fragmentation. Most of the inhibitors proved to be selective for thrombin, with inhibition constants (K_i) in the nanomolar range. However, they could also inhibit at least one additional serine protease (trypsin, chymotrypsin and/or coagulation factor X) with K_i values in the nanomolar or low micromolar range. These serine protease inhibitors constitute novel apoptosis inducing compounds in B-cell lymphoma cells.

1. Introduction

Resistance to cytotoxic therapies has generated demands for the development of innovative anticancer strategies. The process of apoptotic cell death constitutes a novel target for cancer chemotherapy, since it underlies the homeostasis of multicellular organisms. Deregulation of apoptosis leads either to cell accumulation or cell loss, resulting in numerous illnesses including stroke, heart attack, neurodegenerative syndromes, osteoporosis, autoimmunity, inflammation and cancer (Fesik, 2005; Fischer and Schulze-Osthoff, 2005; Reed and Tomaselli, 2000). Consequently, the molecules involved in cell death regulation have emerged as interesting therapeutic targets.

Apoptotic cells undergo characteristic, highly conserved morphological changes, including cell shrinkage, plasma membrane blebbing, chromatin condensation, DNA and nuclear fragmentation, and formation of apoptotic bodies (Elmore, 2007; Wyllie et al., 1980). Caspases, the cysteine-dependent, aspartic acid specific proteases, act in a proteolytic cascade and are the main executers of apoptosis (Alnemri et al., 1996; Kumar, 2007; Riedl and Shi, 2004). However, in a number of cases cells undergo cell death without activation of caspases (Bröker et al., 2005; Chipuk and Green, 2005; Johnson, 2000; Kroemer and Martin, 2005). These caspase-independent cell death pathways are important safeguard mechanisms that protect the organism against unwanted and potentially harmful cells when caspase-mediated routes fail, but can also be triggered by cytotoxic agents or other death stimuli (Bröker et al., 2005). Several other proteases, such as the calcium-associated cysteine protease calpain, the lysosomal proteases cathepsins, and serine proteases participate in apoptosis (Johnson, 2000; Lankiewicz et al., 2000; Mlinaric-Rascan and Turk, 2003; Stenson-Cox et al., 2003).

The role of serine proteases is well established in numerous crucial physiological processes including digestion, immune response, blood coagulation, fibrinolysis and reproduction (Hedstrom, 2002), making them interesting targets for therapeutic intervention. A number of serine protease inhibitors are currently in clinical development, some already launched on the market. The target serine proteases for these inhibitors include thrombin, coagulation factor Xa, elastase, and urokinase (Abbenante and Fairlie, 2005). Recently, several serine proteases, such as granzymes A and B (Zapata et al., 1998), a trypsin-like mitochondrial protease HtrA2/Omi (Verhagen et al., 2002), a chymotrypsin-like apoptotic protein AP24 (Wright et al., 1994) and thrombin (Zain et al., 2000), have been shown to participate actively in the process of apoptosis in mammalian cells. Studies of serine proteases and their endogenous inhibitors, serpins, have shown that they function as both pro- and anti- apoptotic molecules (Moffit et al., 2007; Thiemmara et al., 2002). Thrombin, a trypsin-like serine protease, has been shown to exert a concentration-dependent dual effect on apoptosis or mitogenesis in tumor cell lines (Zain et al., 2000)

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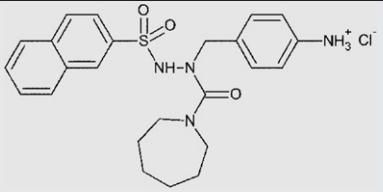
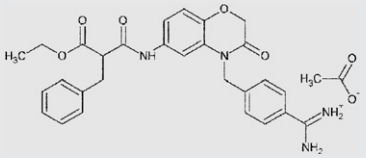
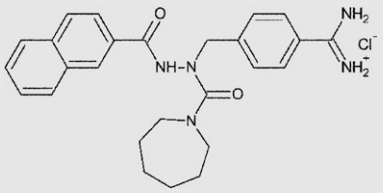
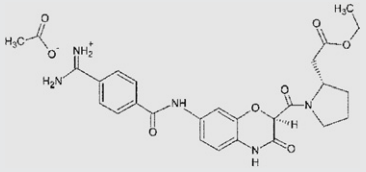
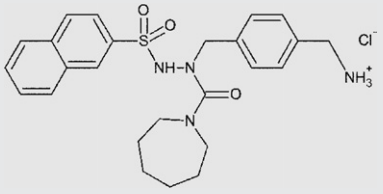
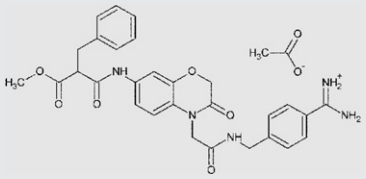
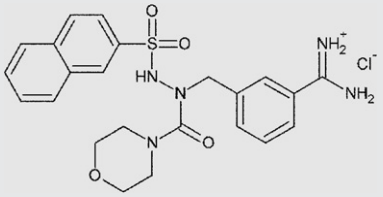
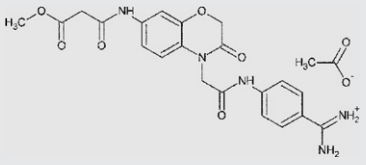
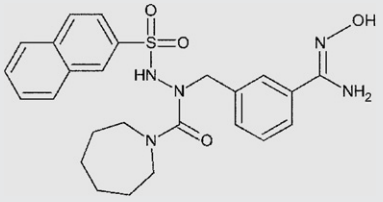
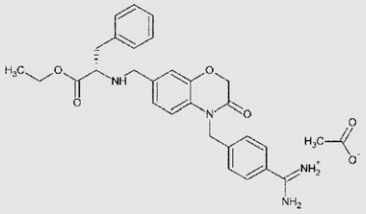
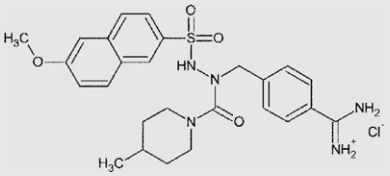
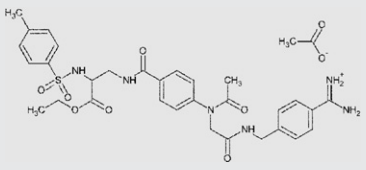
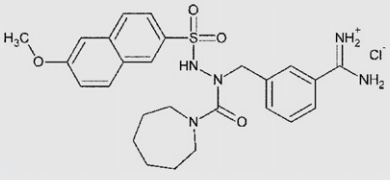
and a potential anti-apoptotic role has been reported for the serine protease myeloblastin (Bories et al., 1989). The inhibition of either chymotrypsin- or trypsin-like proteases with *N*-tosyl-L-phenylalanine chloromethyl ketone (TPCK) or *N*-tosyl-L-lysine chloromethyl ketone (TLCK), respectively, induces apoptosis in various cell lines (Hara et al., 1996; King et al., 2004; Murn et al., 2004; Zhu et al., 1997). This role of serine proteases in apoptosis suggests the use of serine protease inhibitors as alternative therapeutic agents for the modulation of apoptotic events (Moffit et al., 2007). An example of the successful development of a protease inhibitor modulating apoptosis is bortezomib, which has been approved for cancer treatment. It achieves its effects by selectively inhibiting the chymotryptic-like activity of the proteasome, a multicatalytic proteolytic enzyme (Abbenante and Fairlie, 2005; Adams and Kauffman, 2004).

In a search for novel modulating agents of apoptotic cell death we have investigated the pro-apoptotic activity of some recently

Table 1
Serine protease inhibitors for pro-apoptotic screening

	Compound number and structure	Ref[a]		Compound number and structure	Ref
1		Obreza et al. (2004b)	8		unp[b]
2		Obreza et al. (2004a)	9		Stefanic et al. (2004)
3		Obreza et al. (2004b)	10		unp
4		Smolnikar et al. (2007)	11		unp
5		Zega et al. (2004)	12		Stefanic Anderluh et al. (2005)
6		Zega et al. (2004)	13		unp
7		Zega et al. (2004)			

[a] Ref: references with description of synthesis.
[b] unp: unpublished.

synthesized serine protease inhibitors. The phenomenon of cell-death, with its accompanying biochemical and morphological characteristics, was evaluated on B-cell lymphoma models. We identified non-selective serine protease inhibitors based on the azaphenylalanine scaffold as potential inducers of caspase-dependent apoptotic cell death. These molecules could serve as a lead in developing novel modulators of cell death.

2. Materials and methods

2.1. Reagents

The novel serine protease inhibitors (compounds **1–13**) are listed in Table 1. The majority were synthesized as peptidomimetic antithrombotic compounds, including thrombin inhibitors, fibrinogen receptor antagonists, or compounds with dual thrombin inhibitory and fibrinogen receptor antagonistic activities.

TPCK and TLCK were obtained from Sigma-Aldrich (St. Louis, MO, USA). Bortezomib was from Janssen-Cilag International NV (Beerse, Belgium). Chymotrypsin, human leukocyte elastase (HLE), *N*-succinyl-Ala-Ala-Val *p*-nitroanilide (SAAVNA) and *N*-methoxysuccinyl-Ala-Ala-Pro-Val chloromethyl ketone (MSACK) were from Sigma-Aldrich. The chymotrypsin substrate Suc-Ala-Ala-Pro-Phe-AMC was from Bachem (Bachem AG, Bubendorf, Switzerland).

2.2. Synthesis of the compounds

The synthesis of the majority of compounds is described in the references listed in Table 1 (Obreza et al., 2004a,b; Smolnikar et al., 2007; Stefanic Anderluh et al., 2005; Stefanic et al., 2004; Zega et al., 2004). The synthesis of compounds **10, 11** and **13** is described in Supplementary data.

2.3. Cell culture

WEHI 231 and Ramos cell lines were cultured in RPMI 1640 medium (Sigma-Aldrich) supplemented with 10% fetal calf serum (Gibco, Grand Island, NY, USA), 2 nM L-glutamine, 100 U/ml penicillin, 100 µg/ml streptomycin and 50 µM 2-mercaptoethanol (all from Sigma-Aldrich) in a humidified chamber at 37 °C and 5% CO_2.

2.4. Viability assay

WEHI 231 and Ramos cell lines were seeded in 96 well plates (10^5 cells/ml) and treated with the test compounds of interest, or with the corresponding concentration of vehicle (DMSO) as control. The viability of cells was assessed by the MTS test with a CellTiter 96® Aqueous One Solution Cell Proliferation Assay according to the manufacturer's instruction (Promega, Madison, WI, USA). After 24 h, the supplied tetrazolium compound was added to the medium (1:10), incubated for 2 h and the absorbance of the formazan product measured at 492 nm on an automated microplate reader Tecan Safire^2 (Tecan, Mannedorf/Zürich, Switzerland). The signal generated (color intensity) is directly proportional to the number of viable (metabolically active) cells in the wells. All measurements were performed in triplicate and cell viability was presented as the percentage of viability of vehicle-treated control cells. At least three independent determinations were performed for each experiment.

2.5. Determination of caspase activity

DEVDase activity was assayed as described (Mlinaric-Rascan and Turk, 2003). Total protein content in cell extracts was determined spectrophotometrically with the BCA Protein Assay Kit (Pierce, Rockford, IL, USA), following the manufacturer's instructions. Cell extracts were incubated for 30 min at 37 °C with 100 µM Ac-DEVD.AFC peptide substrate (Bachem). Release of 7-amino-4-trifluoromethyl coumarin (AFC) from the Ac-DEVD.AFC substrate was monitored for 40 min in a fluorescence microplate reader Tecan GENios SpectraFluor Plus (Tecan) at 495 nm excitation and 535 nm emission wavelengths. Steady-state hydrolysis rates were obtained from the linear part of the curves. Results were expressed as increase in fluorescence as a function of time ($\Delta F/\Delta t$).

2.6. Analysis of inter-nucleosomal DNA fragmentation

DNA was isolated from cells as described (Mlinaric-Rascan and Turk, 2003) and its concentration determined spectrophotometrically (NanoDrop ND-100 UV–Vis Spectrophotometer, Thermo Fisher Scientific, Waltham, MA, USA). Equal amounts of DNA per sample were electrophoresed through 1.8% agarose gels containing ethidium bromide in Tris borate/EDTA buffer. The DNA bands were visualized with a 254 nm UV transluminator (UVI Tec Ltd, Cambridge, UK) and compared to a 1 kbp DNA ladder standard (Promega).

2.7. Fluorescence microscopy

WEHI 231 cells were dually stained as described (Murn et al., 2004). After the staining of mitochondria with MitoTracker dye (MitoTracker Red CMXros, Molecular Probes, Eugene, OR, USA) the cells were fixed with 4% paraformaldehyde, washed in PBS and permeabilized with 0.5% Triton-X-100. Cells were washed again in PBS and mounted on glass slides with a drop of ProLong Gold antifade reagent with DAPI (Invitrogen Corporation, Carlsbad, CA, USA) for nuclear staining as described in the manufacturer's protocol. Cells were visualized under an Olympus IX 81 fluorescence microscope (Olympus, Tokyo, Japan) using the 100-fold magnification. Pictures were taken in z-stacks at fixed exposure time for each dye and processed using Cell^R Software (Olympus). To compare cells treated with 100 µM solutions of test compounds for 24 h with control cells (treated with the appropriate amount of the vehicle DMSO), all the pictures were processed at the same brightness adjustment for each dye.

2.8. Chymotrypsin inhibition assay

2.8.1. Determination of Km for chymotrypsin/Suc-Ala-Ala-Pro-Phe-AMC

A 1 nM solution of chymotrypsin was mixed with various concentrations of substrate Suc-Ala-Ala-Pro-Phe-AMC (1 µM, 10 µM, 20 µM, 30 µM, 50 µM, 80 µM, 100 µM, 150 µM, 200 µM, 300 µM, 400 µM and 500 µM), each in a final volume of 200 µl. All the solutions were prepared in 144 mM Tris–HCl, pH 7.8 (Jaulent and Leatherbarrow, 2004). Substrate hydrolysis was monitored at 30 °C on an automated microplate reader Tecan Safire^2 (Tecan, Mannedorf/Zürich, Switzerland) by measuring the fluorescence produced by AMC release (excitation at 360 nm, emission at 460 nm). Each experiment was conducted in triplicate. The initial rates of the reactions ($\Delta F/\Delta t$) were measured immediately after the addition of the substrate (within 200 s) and plotted versus substrate concentration with nonlinear regression to fit a Michaelis-Menten plot. The curve fit and kinetic parameters (K_m and v_{max}) were calculated using the GraphPad PRISM version 4.0 software (GraphPad Software, Inc., CA, USA). The recorded K_m was the mean of six independent experiments (66 ± 9 µM) and was used to calculate K_i values for the tested compounds.

2.8.2. Determination of inhibition constants for putative chymotrypsin inhibitors

The initial velocities of the hydrolysis reactions were measured under the same conditions as described for K_m determination. To 50 µl of each inhibitor solution (prepared in 144 mM Tris–HCl, pH 7.8) was added 50 µl of chymotrypsin solution (final concentration 1 nM) and incubated at 30 °C for 15 min. The reaction was started by the addition of

Table 2
Inhibition constants for synthetic serine protease inhibitors against thrombin, trypsin, coagulation factor Xa (FXa) and chymotrypsin

Inhibitor	K_i (μM)			
	Thrombin	Trypsin	FXa	Chymotr.[a]
1	3.3	27.1	46.6	14.6
2	3.8	2.4	2.9	6.3
3	0.92	13.0	92.2	60.0
4	0.017	0.31	2.8	>300
5	0.59	32.0	>75	53.5
6	0.045	18.0	>75	3.6
7	0.009	0.053	3.0	20.9
8	>300	>100	85.9	31.7
9	>300	0.97	78.6	>1000
10	20.5	0.48	NT[b]	2.6
11	74.3	7.7	51.5	>100
12	16.3	5.5	8.8	28.0
13	23.5	3.08	127.7	6.5

[a] Chymotrypsin.
[b] Not tested.

100 μl of substrate. Inhibition constants were computed using Eq. (1), where I is the concentration of the inhibitor, v_0 is the rate of the uninhibited reaction, v_i is the rate of the inhibited reaction, S is the substrate concentration and K_m is the Michaelis–Menten constant:

$$K_i = \frac{I}{\left(\frac{v_0}{v_i} - 1\right)\left(1 + \frac{S}{K_m}\right)} \quad (1)$$

Each inhibitor was assayed at two concentrations (ranging from 3 to 100 μM depending on solubility and accessibility) at two substrate concentrations (50 μM and 100 μM), and all the reactions were performed in triplicate. The K_i values were determined as mean values of all measurements.

2.9. Human leukocyte elastase inhibition assay

2.9.1. Determination of Km for human leukocyte elastase (HLE)/SAAVNA

A 42 nM solution of HLE was mixed with increasing concentrations of the substrate SAAVNA (75 μM, 250 μM, 500 μM and 1500 μM) in a final volume of 200 μl. All the solutions were prepared in HBSA buffer, pH 7.5, 0.05% Triton X-100. The release of *p*-nitroanilide and consequent increase of absorbance at 410 nm was measured at 30 °C on an automated microplate reader Tecan Safire^2. Each experiment was conducted in duplicate. The initial rates of the reactions ($\Delta A/\Delta t$) were calculated from the initial, linear parts of slopes within 10 min from the start of the reaction. K_m was calculated as described for chymotrypsin. The mean value of K_m from four independent experiments was 0.76±0.07 mM, which was used to calculate K_i values of inhibitors.

2.9.2. Determination of inhibition constants for HLE

50 μl of each inhibitor solution (prepared in HBSA buffer, pH 7.5, 0.05% TritonX-100) was incubated with 50 μl of chymotrypsin (final concentration 1 nM) at 25 °C. After 15 min, 100 μl of substrate was added to start the reaction. Each inhibitor was assayed at two different concentrations (12.5 μM and 25 μM) at a substrate concentration 1500 μM. All the reactions were performed in duplicate. K_i was determined as for chymotrypsin. To monitor the validity of the test, we used MSACK, an irreversible inhibitor of HLE.

2.10. Statistical analysis

One sample Student's *t*-test was used to test whether the average percentage of viable cells after treatment differs significantly from the 100% viability of control cells. A P value of <0.05 (two-sided) was considered significant.

3. Results

3.1. Novel serine protease inhibitors possess cross-inhibitory capacity

Novel compounds were designed and synthesized mostly as thrombin inhibitors or compounds with dual thrombin inhibitory and fibrinogen receptor antagonistic properties. These compounds also displayed high to moderate selectivity for thrombin over other serine proteases such as factor Xa or trypsin.

Compounds **1–7** (Table 1) are azaphenylalanine derivatives, synthesized mostly as putative non-covalent thrombin inhibitors. Compounds **8–13**, built on a 1,4-benzoxazinone scaffold, were conceived as potential peptidomimetic antithrombotic compounds with both thrombin inhibitory and fibrinogen receptor antagonistic activity. The ability of the compounds to inhibit the enzymatic action of thrombin, trypsin and factor Xa was determined previously with amidolytic enzyme assays using chromogenic substrates as described in the references listed in Table 1 (Obreza et al., 2004a,b; Smolnikar et al., 2007; Stefanic Anderluh et al., 2005; Stefanic et al., 2004; Zega et al., 2004).

The ability of compounds **1–13** to inhibit chymotrypsin was assayed using Suc-Ala-Ala-Pro-Phe-AMC as substrate. The validity of the method was confirmed by comparison of the measured K_m of chymotrypsin for this substrate (66±9 μM) with the reported value of 70±12 μM (Delaria et al., 1997). The inhibitory constants (K_i) of the compounds for thrombin, FXa, trypsin and chymotrypsin are presented in Table 2. Compounds **1–13**, covered a wide range of potencies

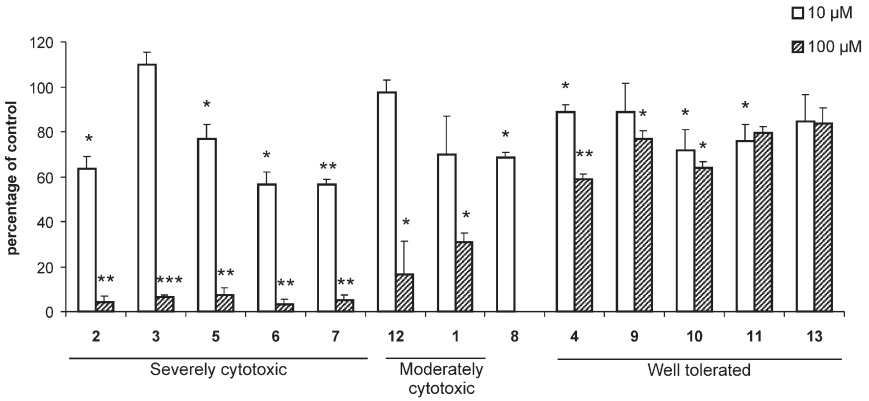


Fig. 1. A subgroup of screened inhibitors is severely cytotoxic. WEHI 231 cells (10^5 cells/ml/100 μl/well) were incubated with 10 and 100 μM inhibitors (1–13) for 24 h. Control cells were treated with the appropriate amount of vehicle (DMSO) in the medium, and grown under the same conditions. The number of viable cells remaining after 24 h of treatment with 100 μM of inhibitors, measured with the MTS test, is expressed as a percentage of that in control cells. The bars represent the average of results in three wells of a representative experiment. Error bars depict standard deviations. Significantly different values from control (vehicle-treated cells) are denoted: *$P<0.05$, **$P\leq0.001$, ***$P\leq0.0001$. Inhibitor 8 was not sufficiently soluble at 100 μM to carry out the cytotoxicity test.

for thrombin inhibition, from low nanomolar (compound **7**) to low micromolar (compounds **1, 2**) to almost inactive (compounds **8, 9**). Azaphenylalanine scaffold based compounds were selective for thrombin, except for compound **2** which was designed as a general serine protease inhibitor. Compound 2 proved to be a nonselective serine protease inhibitor, with K_i for thrombin, trypsin, factor Xa and chymotrypsin ranging from 2.4 to 6.3 μM.

Compounds **8–13**, designed as both thrombin inhibitors and fibrinogen receptor antagonists, displayed the lowest thrombin inhibitory capacities of the tested substances and were more inhibitory for other serine proteases than for thrombin; such as compound **8** for chymotrypsin (K_i=31.7 μM) and compounds **9–13** for trypsin. Compound **12** was the least selective inhibitor in this group, its K_i ranging from 5.5 to 28.0 μM for all the serine proteases tested.

The inhibition of human leukocyte elastase (HLE) by compounds **2, 3, 5, 6, 7**, TPCK and TLCK was examined, using SAAVNA as a substrate. The K_m value (0.76±0.07 mM) was closely similar to the reported value of 0.77±0.04 mM (Stein, 1983). The compounds did not inhibit HLE, except for compound **5** which caused a small decrease in initial reaction rate (data not shown), giving a mean value of K_i of 190 μM. The irreversible inhibitor MSACK inhibited the enzyme completely at concentrations of 12.5 and 25 μM.

3.2. Serine protease inhibitors built on the azaphenylalanine scaffold are severely cytotoxic to WEHI 231 and Ramos cells

In a pre-screening cytotoxicity test performed on WEHI 231 cells with the MTS cell proliferation assay, a subgroup of the azaphenylalanine derivatives (compounds **2, 3, 5, 6** and **7**) displayed severe cytotoxicity at 100 μM concentration (Fig. 1). The residual viability of WEHI 231 cells after 24 h of treatment with 100 μM of those compounds was less than 10% of that of control cells treated with vehicle only. Compounds **1** and **12** were moderately cytotoxic at 100 μM**,** with residual viability after 24 h of treatment of 31% for compound **1** and 16% for compound **12**. Compound **8** also triggered a moderate degree of cell death at a 10 μM concentration; however it could not be tested at 100 μM due to low solubility. Compounds

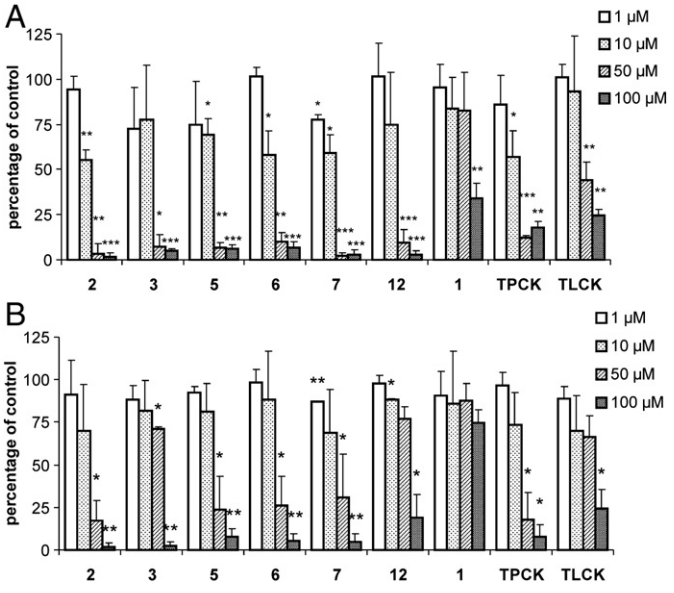


Fig. 2. The cytotoxicity of serine protease inhibitors against (A) WEHI 231 and (B) Ramos cells. Results of MTS cell proliferation assay after 24 h of treatment. The number of viable cells remaining after 24 h of treatment with increasing concentrations of inhibitors is expressed as a percentage of that in control cells. Bars represent the average of three independent experiments. Error bars depict standard deviations. Significantly different values from control (vehicle-treated cells) are denoted: *P<0.05, **P≤0.001, ***P≤0.0001.

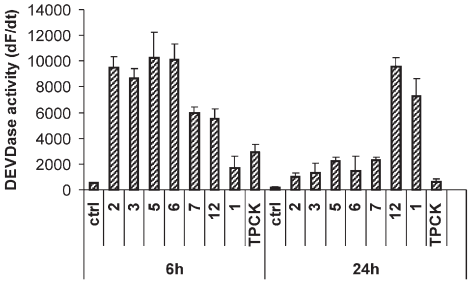


Fig. 3. The 'severely toxic' subgroup of inhibitors provokes rapid induction of caspase-3-like activity. WEHI 231 cells were incubated with 100 μM inhibitors for 6 and 24 h. Caspase activity was determined spectrofluorimetrically by measuring DEVDase activity in whole cell lysates using fluorogenic Ac-DEVD.AFC substrate. The results are presented as changes in fluorescence as a function of time. Bars represent a representative experiment conducted in triplicate. Error bars depict standard deviations. Abbreviations: ctrl, control cells treated with appropriate amount of vehicle (DMSO).

4, 9, 10, 11 and **13** were well tolerated by WEHI 231 at a 100 μM concentration, with residual viability after 24 h of treatment ranging from 60% to 84%.

Based on the cytotoxicity level and good solubility under experimental conditions, seven compounds (**1, 2, 3, 5, 6, 7** and **12**) were selected for further testing on the murine B cell line WEHI 231 and the human B cell line Ramos. Their cytotoxicity was compared with those of known serine protease inhibitors, TPCK and TLCK, and was classified into subgroups of (1) *severely cytotoxic* and (2) *moderate inducers of cytotoxicity*. The first subgroup includes inhibitors **2, 3, 5, 6** and **7**, all of which exhibited more pronounced cytotoxic effects than TPCK or TLCK (Fig. 2). A 24-hour treatment with 100 μM inhibitors **2, 3, 5, 6** and **7** led to over 90% decrease in cell proliferation rates in both WEHI 231 and Ramos cells. Inhibitor **12** was also severely cytotoxic for murine WEHI 231 cells, however it proved less effective on Ramos cells, where the residual viability after 24-hour treatment was 19%. The selective cytotoxicity of compound **12** for WEHI 231 cells is more pronounced at 50 μM concentration, where the residual viability in the murine B cell line is approximately 10%, compared to 77% in Ramos.

Inhibitor **1** had milder cytotoxic effects on the murine B cell line WEHI 231 than TPCK or TLCK (Fig. 2A) and was classified as *moderate inducer of cytotoxicity*. However, the same treatment provoked only a small decrease in cell viability in the human B cell line Ramos, where the residual viability was approximately 75% (Fig. 2B).

3.3. Cytotoxic serine protease inhibitors induce apoptotic cell death

To elucidate the mode of cell death provoked by the serine protease inhibitors **1, 2, 3, 5, 6, 7** and **12**, we examined whether the observed cytotoxic effects are caused by caspase-dependent apoptosis. Cell extracts were prepared from untreated controls and from WEHI 231 cells incubated in the presence of 100 μM of inhibitors for 6 and 24 h, the time points previously determined as optimal (Murn et al., 2004). Caspase-3-like activity, assayed with Ac-DEVD-AMC substrate, peaked at 6 h of incubation with compounds **2, 3, 5, 6** and **7** and subsequently decreased (Fig. 3). These results demonstrate a correlation between caspase activation and decreased cell viability, indicating immediate cell death (within 24 h) after the increases in DEVDase activity induced by the inhibitors. Less cytotoxic inhibitors **1** and **12** exhibited slower kinetics of DEVD-ase activity induction, peaking at 24 h.

Irregular shape and cell shrinkage, typical of apoptosis, were observed when treating cells with inhibitors **2, 3, 5, 6, 7** and **12**. The morphological characteristics of apoptotic cells are shown on representative transmission pictures (Fig. 4A) for compounds **2** and **12**.

Loss of mitochondrial membrane potential is another prominent characteristic of xenobiotic-induced apoptotic cell death. As with previous findings for TPCK- and TLCK-induced apoptosis, treatment of WEHI 231 cells with the inhibitors revealed that cell death was induced

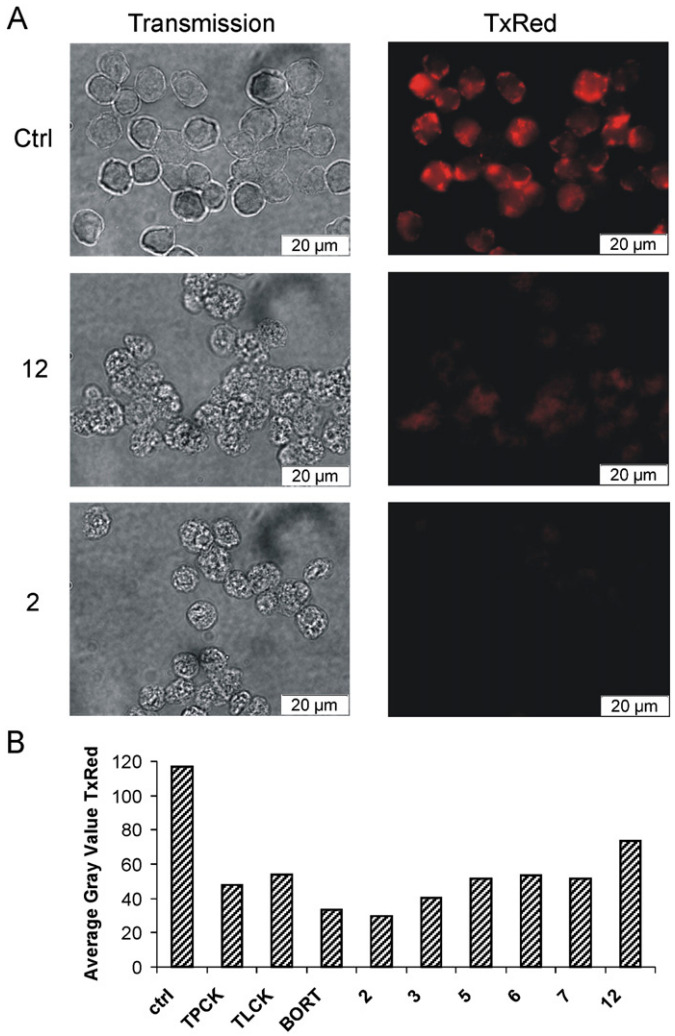


Fig. 4. Serine protease inhibitors cause the loss of mitochondrial membrane potential. WEHI 231 cells were incubated with 100 µM inhibitor for 24 h and dually stained with the nuclear stain DAPI and MitoTracker Red CMXRos (TxRed). Pictures were acquired in z-stacks using an Olympus IX 81 fluorescence microscope (Olympus, Tokyo, Japan) and processed using Cell^R Software (Olympus). (A) Images of representative bright-field phase contrast and immunofluorescence photomicrographs showing the decrease or absence of red staining of active mitochondria (TxRed) after treatment with compounds **12** and **2**, respectively, compared with untreated cells (ctrl). Z stacks were acquired at fixed exposure time (300 ms for TxRed) and processed with the same brightness adjustments (min 250, max 600 for TxRed). (B) Chart depicting the TexRed intensity profiles. The average gray values for TxRed were measured after the 24 h treatment of WEHI 231 cells with serine protease inhibitors. The measurement was performed after 3D blind deconvolution of the acquired z-stacks, using Cell^R Software (Olympus). The results are representative of three independent experiments. Abbreviations: ctrl, control cells treated with appropriate amount of vehicle (DMSO); bort, bortezomib.

via the mitochondrial pathway. Staining cells with mitochondria-sensitive MitoTracker Red CMXRos dye, whose sequestration into mitochondria is sensitive to transmembrane potential, revealed a significant decrease in fluorescence intensity in cells exposed to inhibitors, compared with untreated cells (Fig. 4). Inhibitor **2** provoked the most prominent decrease in fluorescence intensity (measured as average gray value), meanwhile the least pronounced decrease was observed in cells treated with inhibitor **12** (Fig. 4B). The latter is consistent with slower caspase activation observed with inhibitor **12** (Fig. 3).

3.4. Novel cytotoxic serine protease inhibitors induce inter-nucleosomal DNA cleavage

Treatment of WEHI231 cells with 100 µM concentrations of compounds **2, 3, 5, 6, 7** for 24 h triggered inter-nucleosomal cleavage, as shown in Fig. 5. Compound 12 displayed a similar laddering pattern (result not shown). DNA laddering patterns were compared to those shown to characterize TPCK and TLCK induced apoptotic changes in WEHI 231 cells (Murn et al., 2004) and to that provoked by 100 nM of bortezomib, an inhibitor of the chymotryptic activity of the proteasome. The inhibitors **2, 3, 5, 6, 7** and **12**, together with TLCK and bortezomib, induced DNA laddering typical of apoptosis. TPCK-induced apoptotic changes, in the absence of DNA laddering, were detected, as shown previously (Murn et al., 2004). DAPI staining of DNA confirmed nuclear fragmentation in cells treated with bortezomib, TLCK, inhibitors **3** and **12** in comparison to control cells treated with vehicle (DMSO) (Fig. 5B). Similar results were observed when treating cells with inhibitors **2, 5, 6** and **7** (results not shown). TPCK induced nuclear shrinkage in the absence of DNA fragmentation (Fig. 5B).

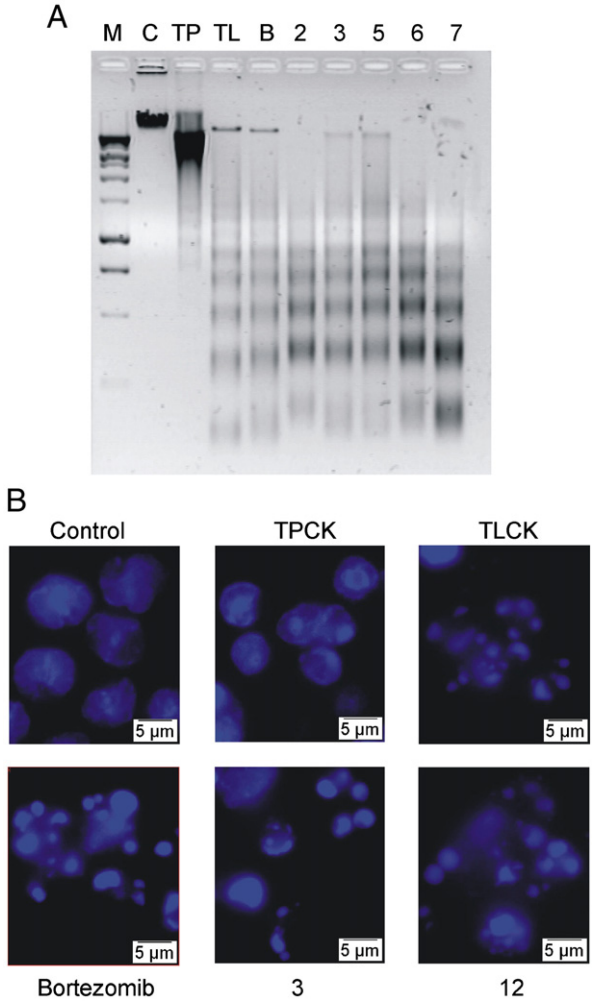


Fig. 5. DNA fragmentation triggered by serine protease inhibitors. (A) DNA-laddering. WEHI 231 cells were incubated with 100 µM of inhibitors or 100 nM bortezomib for 24 h and the extracted DNA subjected to agarose gel electrophoresis. For the comparison of laddering patterns, equal amounts of DNA per sample were loaded on the gel. Inhibitors **2**, **3**, **5**, **6**, **7**, TLCK, and bortezomib produced a DNA laddering pattern typical for apoptosis. In comparison to control cells, TPCK provoked some high molecular weight fragmentation in complete absence of low molecular weight DNA fragments. (B) DAPI staining of nuclei. After the treatment of WEHI 231 cells with 100 µM of inhibitors (100 nM bortezomib) for 24 h, the cells were fixed, permeabilized and mounted on glass slides with a drop of ProLong Gold antifade reagent with DAPI. Abbreviations: M, DNA laddering marker (1 kb DNA Ladder, Promega); C, control cells treated with appropriate amount of vehicle (DMSO); TP, TPCK; TL, TLCK; B, bortezomib.

4. Discussion

Recent evidence implicating serine proteases in apoptotic pathways led us to screen for serine protease inhibitors as apoptosis-inducing agents, since drugs interfering with molecular modes of apoptosis could overcome the resistance of cancer cells to chemotherapy. We have previously shown that the inhibition of anti-apoptotic serine proteases governs the onset of the caspase-dependent apoptotic cascade, by using inhibitors of chymotrypsin and trypsin-like proteases, TPCK and TLCK (Murn et al., 2004). DNA fragmentation is a hallmark of apoptotic cell dismissal, and is believed to occur as a two step process: cleavage of high molecular weight DNA by a caspase-activated DNase (CAD) being followed by inter-nucleosomal DNA cleavage. We and others have demonstrated the involvement of a serine protease in the terminal stage of apoptosis, where chymotrypsin-like protease activity is required for inter-nucleosomal DNA fragmentation in apoptotic cells (Eitel et al., 1999; Fearnhead et al., 1995; Hara et al., 1996; Murn et al., 2004; Zhu et al., 1997). DNA fragmentation was abrogated in TPCK-pre-treated WEHI 231 cells undergoing apoptosis triggered either by anti-IgM or TLCK (Murn et al., 2004).

In the present work we have further examined the role of serine proteases in malignant cell proliferation by the use of novel inhibitors of serine proteases, synthesized in our group. The inhibitors included azaphenylalanine derivatives (compounds **1–7**) and compounds, built on a 1,4-benzoxazinone scaffold (**8–13**). In general we observed major differences between the two groups of inhibitors, used for biochemical screening. The azaphenylalanine derivatives **2, 3, 5, 6** and **7** showed severely cytotoxic activity on both murine WEHI 231 and human Ramos B cell lymphoma, while the 1,4-benzoxazinone derivatives **8–11** and open ring analogue **13** did not show cytotoxic activity in the initial screening. The only exception was compound **12**, designed as a dual thrombin and fibrinogen receptor antagonist, which conveyed moderate cytotoxicity. However, compound **12** proved less effective on human Ramos cells in comparison to murine WEHI 231 cells. Inhibitor **12** also exhibited slower kinetics of DEVD-ase activity induction, peaking after 24 h of treatment, compared to a rapid DEVD-ase activity increase (within 6 h) provoked by inhibitors **2, 3, 5, 6** and **7**.

The structure-activity relationship (SAR) study of azaphenylalanine derivatives **1–7** showed that the cytotoxic effect was particularly sensitive to modifications at terminal basic residues. Greater basicity of benzamidines **2, 6** and **7** resulted in increased cytotoxic effect, which was also observed in compound **5** with amidoxime group, which is a well-known prodrug form of amidines. Interestingly, benzamidine **4** showed virtually no cytotoxic effect although the only unique structural feature is morpholine ring, which in itself should not contribute to such difference. The position of functional group on aromatic ring and methoxy group on naphthalene do not affect activity significantly. Aniline derivative **1** is significantly less basic than benzamidines and is only moderately cytotoxic, while benzylamine **3** is effective on murine WEHI 231 cells and significantly less on human Ramos cells (at a 50 μM concentration). The azaphenylalanine derivative **1** exhibited similar kinetics of DEVD-ase activation to compound **12** and expressed a selective cytotoxicity on murine WEHI 231 cells, while it was well tolerated by human Ramos B cells.

The severely cytotoxic compounds **2**, **3**, **5**, **6**, **7** and moderately cytotoxic compound **12** were assayed for biochemical and morphological characteristics of apoptotic cell death. In addition to DEVDase activation, the selected serine protease inhibitors provoked cell shrinkage, loss of mitochondrial membrane potential, nuclear degradation and genomic DNA fragmentation.

Except for inhibitor **2**, the compounds are selective inhibitors of thrombin, with K_i values in the nanomolar range. However, all the compounds could inhibit at least one other serine protease (trypsin, coagulation factor Xa and/or chymotrypsin) with K_i values in the nanomolar or low micromolar range.

To summarise, our findings demonstrate that the azaphenylalanine subgroup of novel serine protease inhibitors (compounds **2, 3, 5, 6, 7**) exert severe cytotoxicity on both murine and human B lymphoma. They induce apoptotic cell death characterized by rapid activation of caspases, followed by mitochondrial dysfunction and inter-nucleosomal DNA cleavage. These novel apoptosis inducing molecules will serve in our extended research as a lead for developing novel modulators of cell death.

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The authors declare no competing financial interests.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.ejphar.2008.11.008.

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