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Aspartic vinyl sulfones: Inhibitors of a caspase-3-dependent pathway

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ABSTRACT

In this article we describe an expanded structure–activity relationship study for vinyl sulfones as caspase-3 inhibitors, a topic virtually unexplored in the existing literature. Most remarkably, and to our surprise, tripeptidyl vinyl sulfones were not active for caspase-3, opposite to other examples described in literature for peptidyl vinyl sulfones as potent cysteine protease inhibitors of clan CA. Moreover, the caspase-3 inhibitory activity of vinyl sulfones using an *in vitro* assay was then confirmed using a yeast cell-based assay. The results show that Fmoc-protected vinyl sulfones containing only the Asp moiety are inhibitors of a caspase-3-dependent pathway and the IC_{50} values obtained in the yeast assay are in the same order of magnitude of that obtained with the caspase-3 inhibitor tetrapeptidyl chloromethyl ketone, Ac-DEVD-CMK. This observation is consistent with appropriate cell permeability properties displayed by the vinyl sulfone inhibitors, as reflected by log *P* values ranging from 1.1 to 3.4. Overall, these results suggest that vinyl sulfones containing Asp at P_1 should be considered for further optimization as caspase inhibitors and modulators of caspase-3-dependent pathways.

1. Introduction

Caspases are a family of cysteine endoproteases involved in cytokine maturation and apoptosis. They represent one of the most specific protease families yet described, since they have an almost absolute requirement for an aspartic acid residue in the P_1 position of their substrate [1–3]. To date, only a few caspase inhibitors have entered preclinical studies with animal models of human diseases and their major disadvantage is the lack of selectivity. As a consequence, the search for effective caspase inhibitors as possible therapeutic agents against different diseases caused by excessive apoptosis, such as neurodegenerative disorders, is an important area of research [4–6].

One type of irreversible cysteine proteases inhibitors that has received special attention in the last few years are those based on Michael acceptor scaffolds. This class of inhibitors includes vinyl sulfones, which have been developed as highly potent inhibitors of many clan CA cysteine proteases [7]. Recently, we described the first structure–activity relationship study of peptidyl vinyl sulfones as caspase-3 inhibitors, enzymes that belong to clan CD [8]. Vinyl sulfones containing Asp at P_1 **1a**–**d** and **2a**–**h** (Fig. 1) were shown to be only moderate inhibitors of caspase-3, with IC_{50} values in the μM range. Dipeptidyl vinyl sulfones displayed improved activity over their counterparts containing Asp as the single amino acid residue, with this effect being particularly noticeably for the Fmoc-protected compounds. The most active compound, Fmoc-VD-VSMe **2g**, presented an IC_{50} value of 29 μM and was selective for caspase-3 over caspase-7 [8].

In this study, in order to expand the structure–activity relationships (SARs) and to improve the inhibitory potency of vinyl sulfones, we decided to extend the recognition structure by incorporating a third amino acid to derivatives **2**. The *in vitro* caspase-3 inhibition for other Fmoc dipeptidyl vinyl sulfones was also studied. Finally, the caspase-3 inhibitory effect of the vinyl sulfones active *in vitro* was also analysed using a yeast cell-based assay.

In order to search for modulators of caspase-3, an independent analysis of this caspase is required. However, the identification of at least 14 caspase family members, coupled with the complex patterns of caspase gene expression, the tightly synchronized cascade of activation and the extensive cross-talk amongst numerous signalling pathways in mammalian cells have hampered the pharmacological analysis of individual caspases in a cell environment [9]. To address this issue, yeast expressing human caspase-3 can be used to analyse the caspase-3 inhibitory effect of small molecules in

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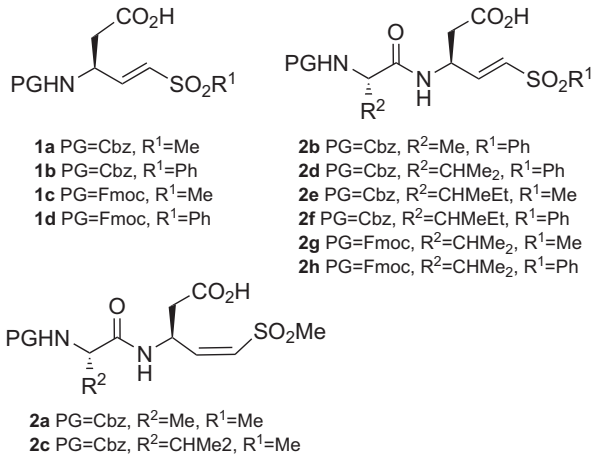


Fig. 1. Vinyl sulfone caspase-3 inhibitors.

a simpler eukaryotic cell system. In fact, many researchers have used yeast expressing human caspase-3 to uncover the mechanisms of endogenous regulation of this caspase [10–17].

Indeed, the high degree of conservation of many pathways and cellular processes among yeast and human cells led several researchers to use yeast as a valuable cell model for functional studies of human proteins and as a drug screening tool [18–24]. Additionally, though yeast has a metacaspase (Yca1p), it was shown that the activity of human caspase-3 in yeast is independent of Yca1p activity [17]. Finally, it was shown that expression of an active form of caspase-3 in yeast caused a marked growth inhibition [10–17], which was reverted by endogenous inhibitors of caspase-3, such as mammalian IAPs (inhibitors of apoptosis) homologues (MIH) MIHA, MIHB and MIHC, and baculoviral caspase inhibitors p35 and p49 [12–16]. These studies established a correlation between yeast cell growth and caspase-3 activity.

2. Results and discussion

2.1. Chemistry

Dipeptidyl aldehydes **3a** and **3b** were synthesized as reported before [8] from the corresponding dipeptide acids using Weinreb chemistry and used without further purification. After Horner–Wadsworth–Emmons reaction between the dipeptidyl aldehydes **3a** and **3b** with the corresponding phosphonate, vinyl sulfones **4a**–**d** were obtained with yields of 36–53%. Deprotection of vinyl sulfones **4a**–**d** with TFA, afforded vinyl sulfones **2i**–**m** with yields of 88–93% (Scheme 1).

To improve the potency of the inhibitors, we decided to extend the molecular recognition by incorporating a third amino acid, valine, since it has been shown experimentally to be favourable at the P_3 position [25]. We had tried before, without success, to obtain the dipeptidyl vinyl sulfones **2** by Fmoc deprotection of the nitrogen atom of the vinyl sulfones **5a** and **5b**, using several different basic conditions (piperidine, triethylamine, DBU) [8]. However, recently this Fmoc deprotection was accomplished in the presence of Et_2NH [26]. Using the same methodology, vinyl sulfones **6a** and **6b** were obtained in a one pot reaction, by *N*-deprotection of vinyl sulfones **5a** and **5b** using Et_2NH in acetonitrile, followed by coupling with the dipeptide FmocValValOSu in DMF with yields of 54–57%. Deprotection of vinyl sulfones **6a** and **6b** with TFA, afforded vinyl sulfones **7a** and **7b** with yields of 69–73% (Scheme 2).

All of the proposed structures were established by NMR (^1H, ^{13}C, COSY and HMQC), IR, and MS. The stereochemistry around the double bond was established using the corresponding ^1H NMR coupling constant, where a double doublet ($J = 16.0$ Hz and 4 Hz) and a doublet ($J = 16.0$ Hz) are observed for the β and α proton relative to the sulfone group, respectively, confirming the presence of the *E* isomer on vinyl sulfones **2i**–**m** and **7a**, **7b**.

2.2. Biological activity

The vinyl sulfones synthesized were examined for their ability to inhibit the activity of recombinant human effector caspase-3. The IC_{50} values were determined using a fluorometric assay and the tetrapeptidyl chloromethyl ketone Ac-DEVD-CMK was used as positive control. Fmoc dipeptidyl vinyl sulfones with Ala at position P_2 **2i** and **2j** were inactive against caspase-3 for the tested concentrations (<200 μM), as we had observed previously for the Cbz-Ala-Asp vinyl sulfones **2a** and **2b** counterparts.

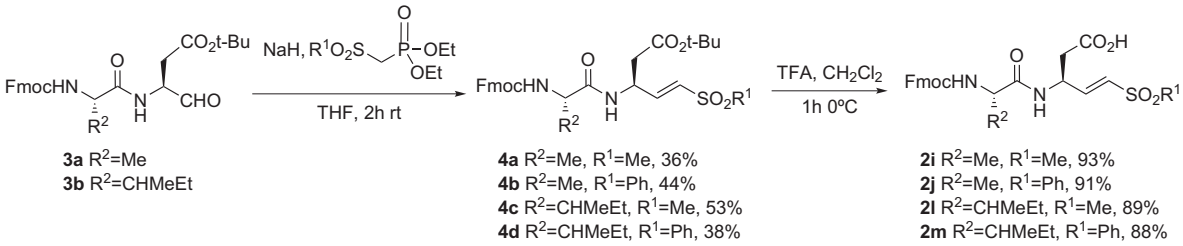
Surprisingly, the presence of Ile on vinyl sulfones **2l** and **2m** did not improve the activity related to the Fmoc-Val-Asp vinyl sulfones **2g** and **2h**, as was observed before for the Cbz dipeptidyl series. Most remarkably, and to our surprise, tripeptidyl vinyl sulfones were not active for caspase-3, opposite to other examples described in literature for peptidyl vinyl sulfones as potent cysteine protease inhibitors of clan CA [7]. In fact, the presence of a third amino acid on vinyl sulfones **2g** and **2h** also resulted in lost of activity against caspase-3, as observed for the Fmoc-tripeptidyl vinyl sulfones **7a** and **7b**.

The vinyl sulfones that were active *in vitro* were then studied using a yeast cell-based assay (Table 1). In this assay, inhibitors of caspase-3 are those compounds that stimulate the growth of yeast expressing human caspase-3 without interfering with the growth of control yeast (transformed with the vector without the cDNA encoding human caspase-3; pGALL).

To implement this assay, we firstly confirmed that, as previously reported by other authors [10–17], yeast expression of human reverse caspase-3, an active form of human caspase-3 (Fig. 2) caused a marked growth inhibition, when compared to control yeast (pGALL) (Fig. 3). Additionally, the reduction of this caspase-3-induced yeast growth inhibition by the commercial caspase-3 inhibitor, Ac-DEVD-CMK (Fig. 3) corroborated the correlation, between stimulation of growth of yeast expressing caspase-3 and caspase-3 inhibition, and validated this assay to search for small molecule inhibitors of caspase-3.

Subsequently, the effect of vinyl sulfones **1c**, **1d**, **2c** and **2e**–**h** on caspase-3 activity was also analysed using the yeast caspase-3 assay. For that, concentration–response curves for 5–100 μM vinyl sulfones and 5–50 μM Ac-DEVD-CMK (positive control) were obtained, considering the maximal growth stimulation achieved with 25 μM Ac-DEVD-CMK as 100% caspase-3 inhibition (Fig. 4A), and the IC_{50} value (lowest concentration of compound that caused 50% caspase-3 inhibition) was determined for each compound. The results obtained showed that all vinyl sulfones active *in vitro* behaved as inhibitors of a caspase-3 pathway in the cell-based assay (Table 1). In fact, vinyl sulfones **1c**, **1d**, **2c** and **2e**–**h**, did not significantly interfere with the growth of control yeast. This indicates that these vinyl sulfones had no effect on endogenous yeast proteins, such as yeast caspase-like. However, they inhibited human caspase-3-induced yeast growth inhibition. This reveals that they inhibited caspase-3 directly or indirectly by inhibition of a caspase-3-dependent pathway.

Based on Table 1, it was observed that the IC_{50} values for vinyl sulfones **1c**, **1d**, **2c** and **2e**–**h** determined in the yeast assay ranged from 16 to 35 μM and were within the same order of magnitude of that determined for Ac-DEVD-CMK (13 μM – Table 1). A remarkable



Scheme 1. Synthesis of Fmoc dipeptidyl vinyl sulfones **2i**–**m**.

increase in the activity of some vinyl sulfones, identified as weak inhibitors of caspase-3 in the *in vitro* assay, was observed in the yeast cell-based assay. In particular, vinyl sulfone **1d**, exhibited a proliferative effect (reduction of caspase-3-induced growth inhibition) in the same order of magnitude of the commercial caspase-3 inhibitor, Ac-DEVD-CMK (Fig. 4A,B and Table 1). These results indicate that, in spite of their moderate activity as inhibitors of caspase-3 [8], vinyl sulfones **1c**, **1d**, **2c**, **2e**–**f** and **2h** are inhibitors of a caspase-3-dependent pathway. In fact, since these compounds have a lower potency *in vitro* than in the yeast cell-based assay, we hypothesize that although they inhibit caspase-3 their main effect can be ascribed to the inhibition of a caspase-3-dependent pathway by modulation of other proteins of its pathway that indirectly inhibit caspase-3. In fact, the lower selectivity displayed by Fmoc-Asp vinyl sulfones **1c** and **1d** towards caspase-3 can be ascribed to the less extended recognition motif when compared to that of their dipeptide counterparts.

Overall, the results obtained from the yeast cell-based assay strongly suggest that the vinyl sulfones reported in the present study are inhibitors of a caspase-3-dependent pathway. Importantly, the calc log *P* values [27] for vinyl sulfones range from 1.1 to 3.4 (Table 2), i.e. significantly higher than that of Ac-DEVD-CMK (calc log $P = -0.6$), suggesting that they present appropriate cell permeability properties.

3. Conclusion

The comprehensive SAR study of vinyl sulfones as caspase-3 inhibitors, incorporating one, two and three amino acid residues, reveals that introduction of a third amino acid in dipeptidyl vinyl sulfones (i.e. converting **2g** and **2h** into **7a** and **7b**, respectively), did not improve the inhibitory activity determined using the biochemical assay. For all the series of vinyl sulfones synthesized, the compounds active against caspase-3 were then tested in a yeast cell system expressing this enzyme. This yeast assay revealed that Fmoc vinyl sulfones containing Asp at P_1 **1c** and **1d** were inhibitors of a caspase-3-dependent pathway in a cell system, with IC_{50} values similar to that obtained with the positive control (Ac-DEVD-CMK). In spite of their lack of selectivity to caspase-3, they can be considered potent modulators of a caspase-3-dependent pathway and therefore interesting compounds to be explored *in vivo*. Additionally, they can be used as lead compounds for the development of more potent and selective caspase-3 inhibitors. Further work must be carried out in order to identify the molecules of the caspase-3 pathway also modulated by these compounds.

4. Experimental protocols

4.1. Chemistry

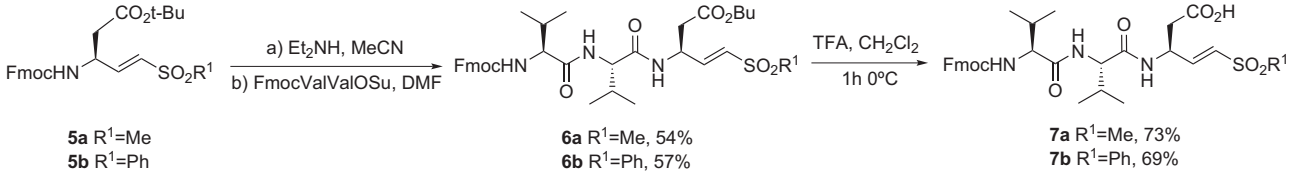
All reagents and solvents were obtained from commercial suppliers and were used without further purification. Melting points were determined using a Kofler camera Bock monoscope M and are uncorrected. The infrared spectra were collected on a Nicolet Impact 400 FTIR infrared spectrophotometer. High resolution mass spectra (HMRS) were performed in Unidade de Espectrometria de Masas, Santiago de Compostela. Elemental analyses were carried out on a C. Erba Model 1106 (Elemental Analyzer for C, H and N) and the results are within ±0.4% of the theoretical values. Merck Silica Gel 60 F254 plates were used as analytical TLC; flash column chromatography was performed on Merck Silica Gel (200–400 mesh). ^{1}H and ^{13}C NMR spectra were recorded on a Bruker 400 Ultra-Shield (400 MHz). ^{1}H and ^{13}C chemical shifts are expressed in δ (ppm) referenced to the solvent used and the proton coupling constants (J) in hertz.

4.1.1. General procedure for the preparation of vinyl sulfones *4a–d*

To a suspension of NaH 60% (1.8 mmol, 1.1 equiv) in THF (5 ml), at 0 °C, was added 1 equiv of the appropriate phosphonate. The resulting solution was stirred at temperature room for 30 min. A solution of 1 equiv of the appropriate aldehyde **3** in THF (16 ml) was added and stirred at room temperature for 2 h. The solvent was removed under reduced pressure, and the residue was dissolved in CH_2Cl_2. The organic solution was washed with brine, dried, and concentrated. The resulting residue was flash chromatographed.

4.1.2. General procedure for the preparation of vinyl sulfones *6a–d*

A solution of the appropriate vinyl sulfone **5** [8] (1 mmol, 1 equiv) in a 1:1 mixture of MeCN/Et_2NH (5 ml) was stirred at room temperature for 2 h. After this time, the solvent was removed under vacuum and the resulting residue was dissolved in DMF (3 ml). To this solution was added the protected dipeptide (FmocValValOSu,



Scheme 2. Synthesis of Fmoc-tripeptidyl vinyl sulfones **7a** and **7b**.

Table 1
Inhibition of human caspase-3 using a yeast cell system by vinyl sulfones [PG–N–X–CH=CHSO$_2$R].

Compound	PG	X	R	IC_{50} (μM)[a]	Growth stimulation (%)[b]
				Cell system	(Control yeast)
1c	Fmoc	Asp	Me	23.7 ± 4.5	1.7 ± 3.2
1d	Fmoc	Asp	Ph	16.2 ± 2.0	2.8 ± 2.3
2c	Cbz	Val-Asp	Me	35.3 ± 3.3*	−1.0 ± 0.4
2e	Cbz	Ile-Asp	Me	30.5 ± 3.5*	−3.3 ± 2.1
2f	Cbz	Ile-Asp	Ph	28.3 ± 8.9	1.9 ± 2.2
2g	Fmoc	Val-Asp	Me	34.0 ± 4.3*	−7.0 ± 0.1
2h	Fmoc	Val-Asp	Ph	29.9 ± 3.4*	1.3 ± 3.9
Ac-DEVD-CMK	–	–	–	13.4 ± 2.8	1.7 ± 3.2

The percentage of growth stimulation was calculated considering 100% growth the number of CFU obtained with transformed yeast incubated with DMSO only.

[a] IC_{50} values, were obtained from concentration–response curves of 5–100 μM vinyl sulfones and 5–50 μM Ac-DEVD-CMK, which were obtained considering the maximal growth stimulation obtained with 25 μM Ac-DEVD-CMK as 100% caspase-3 inhibition. Data are means ± s.e.m. of IC_{50} values obtained from 4–5 independent experiments with 6 replicates each. IC_{50} value significantly higher than that obtained with Ac-DEVD-CMK, $^*P < 0.05$.

[b] Each compound was tested in control yeast at the concentration that caused the maximal effect on yeast expressing caspase-3. Positive values indicate stimulation of yeast growth. Data are means ± s.e.m. of 4 independent experiments with 6 replicates each.

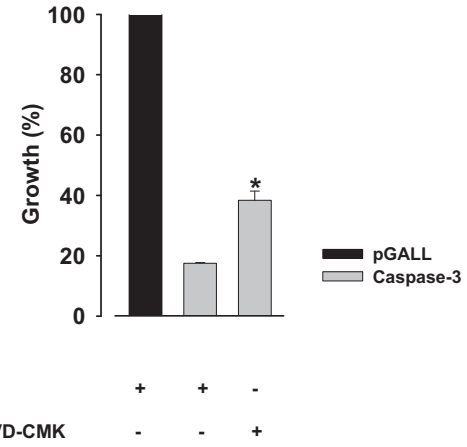


Fig. 3. Expression of human caspase-3 caused a marked yeast growth inhibition that was significantly reduced by the commercial caspase-3 inhibitor Ac-DEVD-CMK. Transformed yeast cells were incubated in galactose selective medium with 25 μM Ac-DEVD-CMK or DMSO only to 0.3 OD_{600}. Yeast growth was evaluated by CFU counts, considering the number of CFU obtained with control yeast (pGALL) incubated only with DMSO as 100% growth. Data are means ± s.e.m. of 4 independent experiments with 6 replicates each. Value of yeast expressing caspase-3 incubated with Ac-DEVD-CMK significantly higher than that obtained with DMSO only, $^*P < 0.05$.

1 equiv) and the mixture stirred for 12 h at room temperature. The reaction mixture was diluted with H_2O, extracted with AcOEt (3×) and the organic layers combined, dried under Na_2SO_4 and the solvent removed under vacuum. The resulting residue was flash chromatographed.

*4.1.3. General procedure for the preparation of vinyl sulfones **2i–m** and **7a**, **7b***

The appropriate vinyl sulfone was treated with TFA at 0 °C for 1 h. TFA was removed under vacuum and the final products were recrystallized from ethyl acetate/hexane as white solids.

*4.1.3.1. FmocAlaAspVSMe (**2i**).* Obtained in 93% yield. M.p. 97–98 °C; IR (NaCl): 3421, 3318, 3074, 2959, 1714, 1682, 1510 cm^{-1}; ^1H NMR (400 MHz, $CDCl_3$) δ 7.91 (1H, d, $J = 8.0$ Hz), 7.77 (2H, d, $J = 8.0$ Hz), 7.59 (2H, d, $J = 8.0$ Hz), 7.42 (2H, t, $J = 8.0$ Hz), 7.28 (2H, t, $J = 8.0$ Hz), 6.63 (1H, dd, $J = 16.0$, 4.0 Hz, C*H*=CHSO$_2$Me), 6.56 (1H, d, $J = 16.0$ Hz, CH=C*H*SO$_2$Me), 5.50 (1H, m), 5.21 (1H, sl), 4.43 (2H, m), 4.30 (1H, m), 4.16 (1H, m), 3.12 (3H, s), 2.51 (1H, m), 2.48 (1H, m), 1.24 (3H, d, $J = 8.0$ Hz); ^{13}C NMR (100 MHz, $CDCl_3$) δ 173.86, 171.90, 160.65, 158.68, 144.95, 141.31, 139.57, 128.78, 127.56, 124.34, 122.93, 66.68, 50.69, 50.33, 48.17, 39.89, 38.13, 18.44; HRMS-ESI-TOF: *m*/*z* calcd $C_{24}H_{26}N_2O_7SNa$ ($M^+ + Na$) 509.1358, found 509.1364.

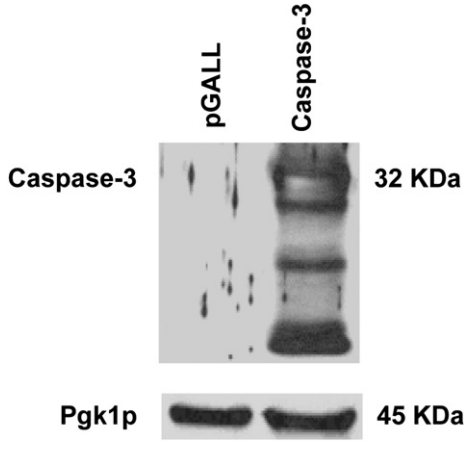


Fig. 2. Expression of human reverse caspase-3 in yeast was confirmed by Western blot analysis. Control yeast (pGALL) and yeast expressing caspase-3 were incubated in galactose selective medium to 0.3 OD_{600}. Full length procaspase (32 kDa) and the respective cleaved fragments (processed caspase-3) were detected using an anti-caspase-3 polyclonal antibody. Detection of Pgk1p was used as loading control. Immunoblots were developed by chemiluminescence.

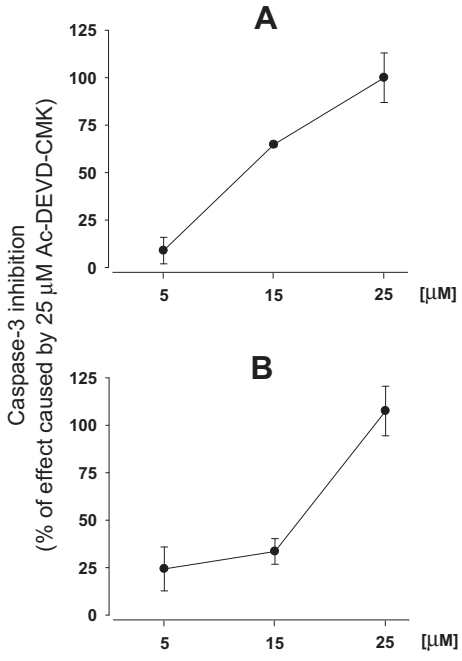


Fig. 4. Concentration–response curves for (A) Ac-DEVD-CMK and (B) vinyl sulfone **1d**. The percentage of growth stimulation caused by compounds was calculated considering the number of CFU obtained with yeast expressing caspase-3 incubated with DMSO only as 100% growth. Concentration–response curves were obtained considering the maximal growth stimulation obtained with 25 μM Ac-DEVD-CMK as 100% caspase-3 inhibition. Data are means ± s.e.m. of 4–5 independent experiments.

Table 2
Relevant parameters for preliminary permeation properties of vinyl sulfones **1c**, **1d**, **2c** and **2e**–**h** (data for Ac-DEVD-CMK are also included for comparison).

Compound	PG	X	R	log P^a
1c	Fmoc	Asp	Me	2.22
1d	Fmoc	Asp	Ph	3.34
2c	Cbz	Val-Asp	Me	1.16
2e	Cbz	Ile-Asp	Me	1.53
2f	Cbz	Ile-Asp	Ph	2.38
2g	Fmoc	Val-Asp	Me	2.33
2h	Fmoc	Val-Asp	Ph	3.43
Ac-DEVD-CMK	–	–	–	−0.63

[a] Estimated by the ALOGPS 2.1 algorithm [27].

*4.1.3.2. FmocAlaAspVSPh (**2j**).* Obtained in 91% yield. M.p. 99–100 °C; IR (NaCl): 3421, 3305, 3061, 2932, 1714, 1657, 1510 cm^{-1}; ^1H NMR (400 MHz, $CDCl_3$) δ 8.02 (1H, d, $J = 8.0$ Hz), 7.74 (2H, d, $J = 8.0$ Hz), 7.56–7.23 (11H, m), 6.72 (1H, dd, $J = 16.0$, 4.0 Hz, C*H*=CHSO$_2$Ph), 6.66 (1H, d, $J = 16.0$ Hz, CH=C*H*SO$_2$Ph), 5.29 (1H, m), 5.22 (1H, sl), 4.43 (2H, m), 4.30 (1H, m), 4.16 (1H, m), 3.12 (3H, s), 2.81 (1H, m), 2.75 (1H, m), 1.25 (3H, d, $J = 8$ Hz); ^{13}C NMR (100 MHz, $CDCl_3$) δ 173.72, 171.76, 160.51, 144.81, 140.75, 139.43, 136.77, 135.44, 133.04, 129.08, 128.63, 128.14, 127.41, 124.20, 122.79, 66.54, 50.54, 50.18, 48.03, 39.75, 18.29; HRMS-ESI-TOF: *m/z* calcd $C_{29}H_{28}N_2O_7SNa$ ($M^+ + Na$) 571.1515, found 571.1523.

*4.1.3.3. FmocIleAspVSMe (**2l**).* Obtained in 89% yield. M.p. 87–88 °C; IR (NaCl): 3305, 2971, 1727, 1657, 1523 cm^{-1}; ^1H NMR (400 MHz, $CDCl_3$) δ 8.01 (1H, d, $J = 8.0$ Hz), 7.59 (2H, d, $J = 8.0$ Hz), 7.45 (2H, d, $J = 8.0$ Hz), 7.41 (2H, t, $J = 8.0$ Hz), 7.32 (2H, t, $J = 8.0$ Hz), 6.73 (1H, dd, $J = 16.0$, 4.0 Hz, C*H*=CHSO$_2$Me), 6.66 (1H, d, $J = 16.0$ Hz, CH=C*H*SO$_2$Me), 5.50 (1H, m), 5.23 (1H, sl), 4.43 (2H, m), 4.30 (1H, m), 4.16 (1H, m), 3.02 (3H, s), 2.72 (1H, m), 2.60 (1H, m), 1.51 (1H, sl), 1.23 (1H, sl), 0.97 (6H, m); ^{13}C NMR (100 MHz, $CDCl_3$) δ 173.20, 171.85, 160.83, 158.64, 144.91, 141.27, 139.53, 128.73, 127.51, 124.30, 122.89, 66.63, 60.57, 50.28, 48.12, 39.85, 38.08, 35.66, 25.05, 16.37, 11.66; HRMS-ESI-TOF: *m/z* calcd $C_{27}H_{32}N_2O_7SNa$ ($M^+ + Na$) 551.1828, found 551.1835.

*4.1.3.4. FmocIleAspVSPh (**2m**).* Obtained in 88% yield. M.p. 111–112 °C; IR (NaCl): 3429, 2974, 1705, 1523 cm^{-1}; ^1H NMR (400 MHz, $CDCl_3$) δ 8.00 (1H, d, $J = 8.0$ Hz), 7.75 (2H, d, $J = 8.0$ Hz), 7.61–7.23 (11H, m), 6.73 (1H, dd, $J = 16.0$, 4.0 Hz, C*H*=CHSO$_2$Ph), 6.63 (1H, d, $J = 16.0$ Hz, CH=C*H*SO$_2$Ph), 5.46 (1H, m), 5.24 (1H, sl), 4.49 (2H, m), 4.23 (1H, m), 4.15 (1H, m), 2.73 (1H, m), 2.58 (1H, m), 1.51 (1H, sl), 1.27 (1H, sl), 0.68 (6H, m); ^{13}C NMR (100 MHz, $CDCl_3$) δ 173.11, 171.76, 160.73, 144.81, 140.75, 139.43, 136.77, 135.44, 133.04, 129.08, 128.63, 128.14, 127.41, 124.20, 122.79, 66.54, 60.47, 50.18, 48.03, 39.75, 35.56, 24.95, 16.27, 11.56; HRMS-ESI-TOF: *m/z* calcd $C_{32}H_{34}N_2O_7SNa$ ($M^+ + Na$) 613.1984, found 613.1993.

*4.1.3.5. FmocValValAspVSMe (**7a**).* Obtained in 69% yield. M.p. 96–97 °C; IR (NaCl): 3283, 2949, 1741, 1700, 1655, 1522 cm^{-1}; ^1H NMR (400 MHz, $CDCl_3$) δ 9.23 (1H, sl), 8,12 (1H, sl), 7.76 (2H, d, $J = 8.0$ Hz), 7.41–7.33 (6H, m), 6.74 (1H, dd, $J = 16.0$, 4.0 Hz, C*H*=CHSO$_2$Me), 6.47 (1H, d, $J = 16.0$ Hz, CH=C*H*SO$_2$Me), 4.70 (2H, d, $J = 8.0$ Hz), 4.60 (1H, sl), 4.54 (1H, sl), 4.47 (2H, m), 3.28 (1H, sl), 2.93 (3H, s), 2.78 (1H, dd, $J = 8.0$, 4.0 Hz), 2.57 (1H, dd, $J = 8.0$, 4.0 Hz), 2.03 (1H, m), 1.85 (1H, m), 1.07 (6H, d, $J = 8.0$ Hz), 1.00 (6H, d, $J = 8.0$ Hz); ^{13}C NMR (100 MHz, $CDCl_3$) δ 172.39, 172.21, 171.81, 160.79, 158.60, 144.87, 144.87, 141.23, 139.49, 128.69, 127.47, 124.26, 122.85, 66.54, 59.11, 58.33, 50.19, 48.03, 39.76, 37.99, 30.48, 19.04; HRMS-ESI-TOF: *m/z* calcd $C_{31}H_{39}N_3O_8SNa$ ($M^+ + Na$) 636.2356, found 636.2363.

*4.1.3.6. FmocValValAspVSPh (**7b**).* Obtained in 73% yield. M.p. 86–87 °C; IR (NaCl): 3314, 2992, 1740, 1702, 1666, 1516 cm^{-1}; ^1H NMR (400 MHz, $CDCl_3$) δ 9.50 (1H, sl), 7.88 (2H, d, $J = 8.0$ Hz), 7.79 (2H, d, $J = 8.0$ Hz), 7.71 (1H, m), 7.58 (2H, m), 7.43 (4H, m), 7.33 (2H, m), 7.16 (1H, sl), 6.50 (1H, dd, $J = 16.0$, 4.0 Hz, C*H*=CHSO$_2$Ph), 6.25 (1H, d, $J = 16.0$ Hz, CH=C*H*SO$_2$Ph), 4.70 (2H, d, $J = 8.0$ Hz), 4.60 (1H, sl), 4.46 (1H, m), 4.39 (2H, sl), 4.15 (1H, sl), 2.83 (1H, dd, $J = 8.0$, 4.0 Hz), 2.62 (2H, m), 2.27 (1H, m), 1.11 (6H, d, $J = 8.0$ Hz), 0.91 (6H, d, $J = 8.0$ Hz); ^{13}C NMR (100 MHz, $CDCl_3$) δ 172.34, 172.16, 171.76, 160.73, 144.81, 140.75, 139.43, 136.77, 135.44, 133.03, 129.09, 128.64, 128.64, 127.42, 124.21, 122.79, 66.56, 59.13, 58.35, 50.22, 48.05, 39.78, 30.50, 19.06; HRMS-ESI-TOF: *m/z* calcd $C_{36}H_{41}N_3O_8SNa$ ($M^+ + Na$) 698.2512, found 698.2516.

4.2. Caspase-3 in vitro assays

Caspase-3 fluorometric assay was used, which is based on the hydrolysis of the peptide substrate acetyl-Asp-Glu-Val-Asp-7-amido-4-methylcoumarin (Ac-DEVD-AMC) by caspase-3, resulting in the release of the fluorescent 7-amido-4-methylcoumarin (AMC) moiety. Briefly, 5 μl of stock solution in assay buffer (10 U/μl) of caspase-3 (caspase-3, human, recombinant, Calbiochem) was added to 5 μl of the tested inhibitors at various concentrations. The reaction was initiated by the addition of 190 μl of substrate to a final concentration of 200 μM in assay buffer (20 mM HEPES, 2 mM EDTA, 0.1% CHAPS, and 5 mM DTT, pH 7.4). Liberation of AMC was monitored continuously at 37 °C using a Tecan infinite M200 (Tecan, Switzerland) 96-well plate reader (white plates from Greiner bio-one, Germany) using an excitation wavelength of 360 nm and an emission wavelength of 460 nm. Inhibitors stock solutions were prepared in DMSO, and serial dilutions were made in DMSO. Controls were performed using enzyme alone, substrate alone, enzyme with DMSO and a positive control (Ac-DEVD-CMK, Calbiochem).

4.3. Plasmids, yeast strain, transformation and growth conditions

Constructed yeast expression plasmid pGALL-*LEU2* encoding human reverse caspase-3, an active form of human caspase-3, and the respective empty vector were kindly provided by Dr. C.J. Hawkins (Children's Cancer Centre, Royal Children's Hospital, Parkville, Australia). Plasmids have a galactose-inducible *GAL1/10 promoter*.

Saccharomyces cerevisiae CG379 (*α ade5 his7-2 leu2-112 trp1-289 ura3-52 [Kil-O];* Yeast Genetic Stock Center) strain was transformed by the lithium acetate method as described [24]. Transformed cells were routinely grown in a minimal selective medium with 2% (w/v) glucose, 0.67% (w/v) yeast nitrogen base without amino acids (Difco) and all the amino acids required for yeast growth (50 μg/ml) except leucine, at 30 °C, with mechanical shaking (200 r.p.m.) to approximately 1 optical density (OD_{600}; *Jenway 6310 Spectrophotometer*). To induce expression of human caspase-3, yeast cells were diluted to 0.05 OD_{600} in a minimal selective medium with 2% (w/v) galactose and raffinose, instead of glucose, and 3% (v/v) glycerol, and grown at 30 °C with mechanical shaking (200 r.p.m.) to 0.3 OD_{600}.

4.3.1. Western blot analysis

Preparation of yeast extracts and Western blot analysis were performed basically as described [24]. Expression of human caspase-3 in yeast was detected using the anti-caspase-3 rabbit polyclonal antibody (1:200; Santa Cruz Biotechnology), followed by the anti-rabbit horseradish peroxidase (HRP)-conjugated secondary antibody (1:5000; Santa Cruz Biotechnology). For loading control, membranes were stripped and reprobed with the anti-Pgk1p mouse monoclonal antibody (1:5000; Molecular probes) followed by the

anti-mouse HRP-conjugated secondary antibody (1:5000; Santa Cruz Biotechnology). Immunoblots were developed by enhanced chemiluminescence.

4.3.2. Yeast caspase-3 assay

To analyse the effect of compounds on the growth of yeast expressing human caspase-3 and control yeast (transformed with the vector without the cDNA encoding human caspase-3; pGALL), transformed cells were incubated in galactose selective medium with 5–100 μM vinyl sulfones, 5–50 μM Ac-DEVD-CMK (positive control; caspase-3 inhibitor III; Calbiochem) or 0.1% DMSO only to 0.3 OD_{600}. Cell growth was analysed by counting the number of colony-forming units (CFU) per ml obtained after 2 days incubation, at 30 °C, on Sabouraud Dextrose Agar plates. The percentage of growth stimulation of yeast expressing caspase-3 (proportional to the degree of caspase-3 inhibition) caused by compounds was calculated considering the number of CFU obtained with yeast expressing caspase-3 incubated only with DMSO as 100% growth. Concentration–response curves for 5–100 μM vinyl sulfones and 5–50 μM Ac-DEVD-CMK were obtained, considering the maximal growth stimulation achieved with 25 μM Ac-DEVD-CMK as 100% caspase-3 inhibition, and the lowest concentration that caused 50% caspase-3 inhibition (IC_{50}) calculated.

4.3.3. Statistical analysis

Data were analysed statistically using the *SigmaStat 3.5 software*. Differences between means were tested for significance using the unpaired Student's *t* test ($P < 0.05$).

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Appendix. Supplementary data

Supplementary data associated with this article can be found in the online version, at doi:10.1016/j.ejmech.2011.02.067.

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