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1,4-Benzothiazine Analogues and Apoptosis: Structure–Activity Relationship

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Abstract—We have previously shown 1,4-benzothiazine (1,4-B) derivatives induce thymocyte apoptosis in vitro and thymus cell loss in vivo. Apoptosis is mediated through a complex of biochemical events including phosphatidylcholine specific-phospholipase C (PC-PLC) activation, acidic sphingomyelinase (aSMase) activation and ceramide generation, caspase-8 and caspase-3 activation. As preliminary analysis of the structure–activity relationship (SAR) suggested some structural features were responsible for apoptosis, we synthesised several derivatives and tested for apoptosis activity at equimolar concentrations. In particular, we synthesised analogues that differed in the nature of skeleton (1,4-benzothiazine, 1,4-benzoxazine and 1,2,3,4-tetrahydroquinoline) and in the nature of side chain (imidazole, benzimidazole or piperazine as azole substituent; presence, absence or transformation of alcoholic group). Results of apoptosis induction indicate that transforming the 1,4-benzothiazine skeleton into 1,2,3,4-tetrahydroquinoline does not result in significant change. Transformation into 1,4-benzoxazine decreased activity. Replacing imidazole at the side chain with different piperazines also decreased activity while replacing it with benzimidazole does not change apoptotic activity. Finally, removal of the alcoholic group by dehydration to olefin, or by transforming it into ether, increased activity. Moreover, in an attempt to analyse further the SAR characteristics that are responsible for 1,4-B-activated apoptosis we tested the effect on caspase-8,-9 and-3 activation. 1,4-B analogues activate caspases and the structural requirements correlate with those responsible for apoptosis induction.

Introduction

1,4-Benzothiazine (1,4-B) derivatives are known to exert many effects in vivo and in vitro. In particular, 1,4-B-induced neurotoxic effects have been hypothesised to play a role in neurodegenerative diseases, such as Parkinson's Disease and Alzheimer Disease[1–6] and the in vivo anti-tumour efficacy of 1,4-B has been attributed to its cytotoxic activity that is directed against neoplastic cells.[7–9] Based on these observations, we previously performed experiments to analyse the mechanisms underlying 1,4-B-mediated cytotoxicity and observed that 1,4-B derivatives induce thymocytes apoptosis through a complex signalling pathway requiring the activation of different biochemical events that includes rapid activation of phosphatidylcholine specific-phospholipase C (PC-PLC) and of acidic sphingomyelinase (aSMase), loss of mitochondrial membrane potential ($\Delta\Psi$m), cytochrome *c* release and caspase activation.[10]

Preliminary structure–activity relationship (SAR) analysis, based on the assessment of apoptotic activity of some 1,4-B derivatives, focused attention on the sulphur oxidation state because an increase in the oxidation state resulted in a significant increase in apoptosis. Insertion of the side chain at different positions on the aromatic ring also influenced apoptosis and suggested that substitution at position 6 yielded in higher activity. Neither replacing imidazole with a triazole nor introducing a second CH_2-triazole group or a CH_2-imidazole group changed the apoptotic activity. Transformation of the alcoholic group into an ether group clearly increased activity. Side chain length also seemed to be involved as apoptotic activity decreased as the side chain shortened.

The present study examines the SAR closely and focuses on some structural modifications. We transformed the 1,4-benzothiazine skeleton into other fused rings such as 1,4-benzoxazine and 1,2,3,4-tetrahydroquinoline; we changed the azolic substituent by preparing benzimid-

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azolic and piperazinic analogues; we evaluated the effect of hydroxyl elimination at the side chain and assessed apoptosis induction using a number of 1,4-B analogues tested at equimolar concentration. For that purpose, we used mouse thymocytes, a cell population well sensitive to apoptosis induction that has been widely used to study several different apoptotic agents.[10–17]

Our results indicate that different analogues have different apoptosis-activating capabilities and the in vitro activity correlates with the capability to cause thymus cell loss in vivo.

In an attempt to analyse further the SAR features that are responsible for 1,4-B-activated apoptosis, we tested the effect on caspase-8,-9, and-3 activation and observed that 1,4-B analogues activate caspases and the structural requirements correlate with those responsible for the capability to induce apoptosis.

Chemistry

Scheme 1 outlines the synthesis of compounds listed in Table 1. Ketones **1**, **2** and **3** were prepared starting from 6-(2-bromoacetyl)-4-methyl-3,4-dihydro-2*H*-1,4-benzothiazin-3-one,[18] 6-(2-bromoacetyl)-4-methyl-3,4-dihydro-2*H*-1,4-benzoxazin-3-one[19] and 6-(2-bromoacetyl)-1-methyl-1,2,3,4-tetrahydroquinolin-2-one,[20] respectively, by reaction with the appropriate heterocycle.

Reduction with sodium borohydride supplied carbinols **4**, **5** and **6** that were converted respectively into **7**, **8** and **9** by alkylation with 4-chlorobenzyl chloride. Dehydration of carbinols provided the corresponding olefins **10**, **11** and **12**.

Results

1,4-Benzothiazine analogues induce thymocyte apoptosis: structure–activity relationships

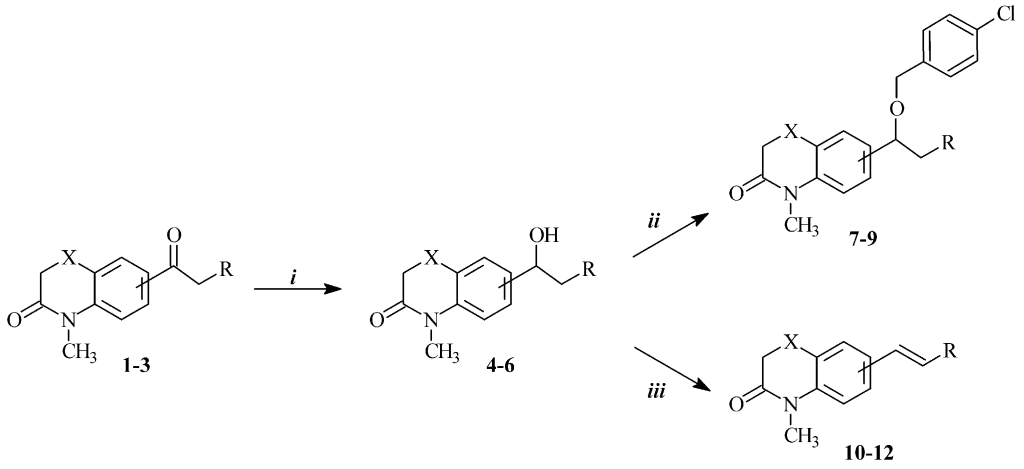
We have previously reported 1,4-B induce thymocyte apoptosis. Preliminary SAR analysis suggested the benzothiazinic skeleton and the side chain nature, position and length modulate apoptotic activity.[10]

To extend the SAR analysis further we synthesised new 1,4-B analogues and tested them, at equimolar concentrations ranging from 10^{-5} to 10^{-8} M, in comparison with **4a** and **7a** that, in previous studies, resulted to be the most effective compounds.[10] The derivatives we synthesised differed in skeleton (1,4-benzothiazine, 1,4-benzoxazine and 1,2,3,4-tetrahydroquinoline), azolic substituent in the side chain (1*H*-imidazole, 1*H*-benzimidazole, 1-(2-methoxyphenyl)piperazine, 2-methylpiperazine and 1-methylpiperazine) and side chain (presence, absence or transformation of alcoholic group).

All the compounds were tested for apoptosis using two different assays: the PI assay, to evaluate DNA fragmentation,[15] and the Annexin-V assay, an early marker of apoptosis. Figure 1 illustrates the results of representative experiments, respectively PI and Annexin-V assays, showing the same activity pattern for the different analogues tested at the equimolar concentration of 10^{-5} M. In particular, we observed that:

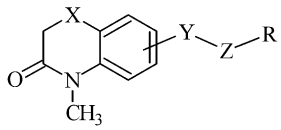
1. replacing the 1,4-benzothiazine skeleton with 1,2,3,4-tetrahydroquinoline results in slight changes (compare **9a** to **7a** and **6a** to **4a**), while replacement with 1,4-benzoxazine significantly decreases activity (compare **8a** with **7a**);
2. replacing the imidazole in the side chain with different piperazines significantly decreases activity (compare **7c** and **7e** to **7a**), while replacing imidazole with benzimidazole causes only slight changes in apoptotic activity (compare **4b** to **4a** and **7b** to **7a**);
3. removing the side chain alcoholic group by dehydration to olefin, significantly increases activity (compare **5a** to **11a**, **6a** to **12a** and **4a** to **10a**).

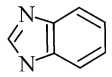
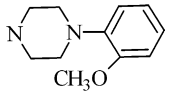
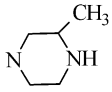
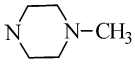
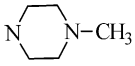
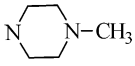
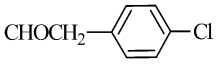
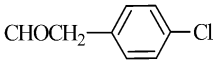
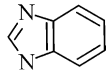
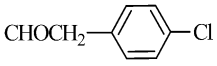
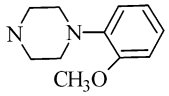
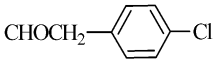
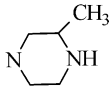
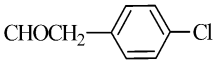
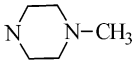
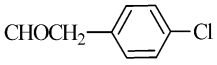
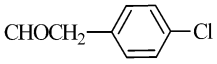
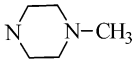
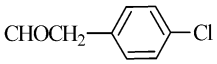
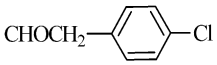
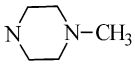
To analyse the apoptotic activity of these analogues we performed experiments using different drug concentra-



Scheme 1. (i) $NaBH_4$, CH_3OH; (ii) NaH, 4-chlorobenzyl chloride, DMF; (iii) AcOH, H_2SO_4.

Table 1.



Compd	X	Chain position	Y	Z	R
4a	S	6	CHOH	CH_2	
4b	S	6	CHOH	CH_2	
4c	S	6	CHOH	CH_2	
4d	S	6	CHOH	CH_2	
4e	S	6	CHOH	CH_2	
5a	O	6	CHOH	CH_2	
5e	O	6	CHOH	CH_2	
6a	CH_2	6[a]	CHOH	CH_2	
6e	CH_2	6[a]	CHOH	CH_2	
7a	S	6		CH_2	
7b	S	6		CH_2	
7c	S	6		CH_2	
7d	S	6		CH_2	
7e	S	6		CH_2	
8a	O	6		CH_2	
8e	O	6		CH_2	
9a	CH_2	6[a]		CH_2	
9e	CH_2	6[a]		CH_2	

(*continued on next page*)

Table 1 (*continued*)

Compd	X	Chain position	Y	Z	R
10a	S	6	CH	CH	
11a	O	6	CH	CH	
12a	CH_2	6[a]	CH	CH	

[a]Referred to 1,2,3,4-tetrahydroquinoline numeration.

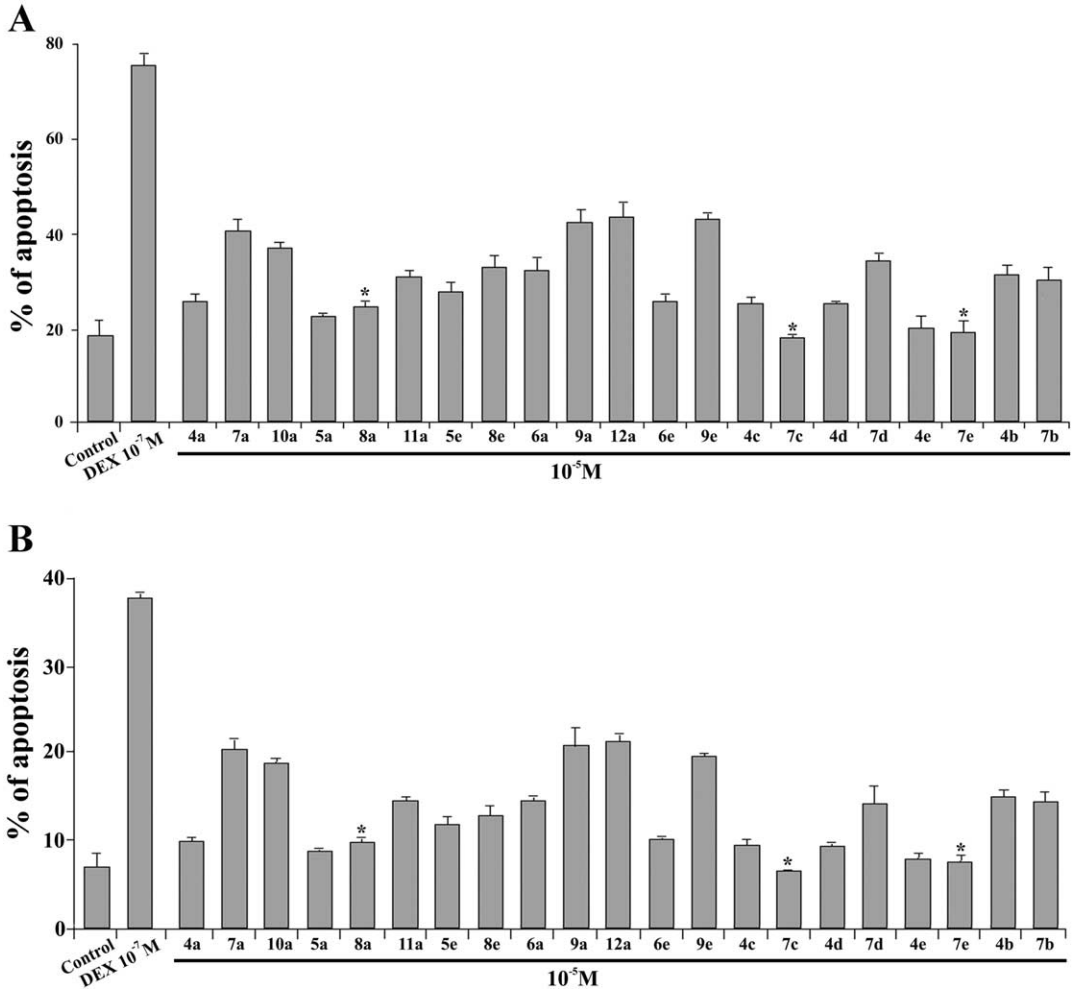


Figure 1. 1,4-Benzothiazine analogues induce apoptosis in mouse thymocytes. Mouse thymocytes were treated with the indicated derivatives (10^{-5} M) and the apoptosis was evaluated by PI (A) and Anexin V staining (B). Fluorescence intensity was measured by flow cytometry and data were analysed by Lysis II program. Mean values ± SE of three different experiments in duplicate are reported. *$p < 0.001$ as compared to **10a**.

tions (Fig. 2). The results of representative experiments, obtained by PI assay, confirm the findings reported in Figure 1. In particular, Figure 2A indicates that replacing of the sulphur atom of 1,4-benzothiazine with an oxygen atom (compare **8a** to **7a**), but not with a methylene group (compare **9a** to **7a**), significantly decreases the activity at all the active drug concentrations (10^{-5}, 10^{-6} M). Substituting different piperazines for imidazole at the side chain, significantly decreases apoptotic activity (Figure 2B, compare **7d**, **7e** and **7c** to **7a**) which did not occur when benzimidazole was used (Fig. 2C, compare **7b** to **7a** and **4b** to **4a**). Eliminating the alcoholic group (**10a** and **12a**) or transforming it into ether (**7a** and **9a**), in both the benzothiazinic (Fig. 2D, compare **10a** and **7a** to **4a**) and the tetrahydroquinolinic (Fig. 2E, compare **12a** and **9a** to **6a**) derivatives, increases apoptotic activity. These results confirm data reported in Figure 1.

In vivo activity has similar SAR requirements

We have previously reported that active 1,4-B induce thymus cell loss to a similar extent as treatment with dexamethasone (DEX), a drug that is well known to

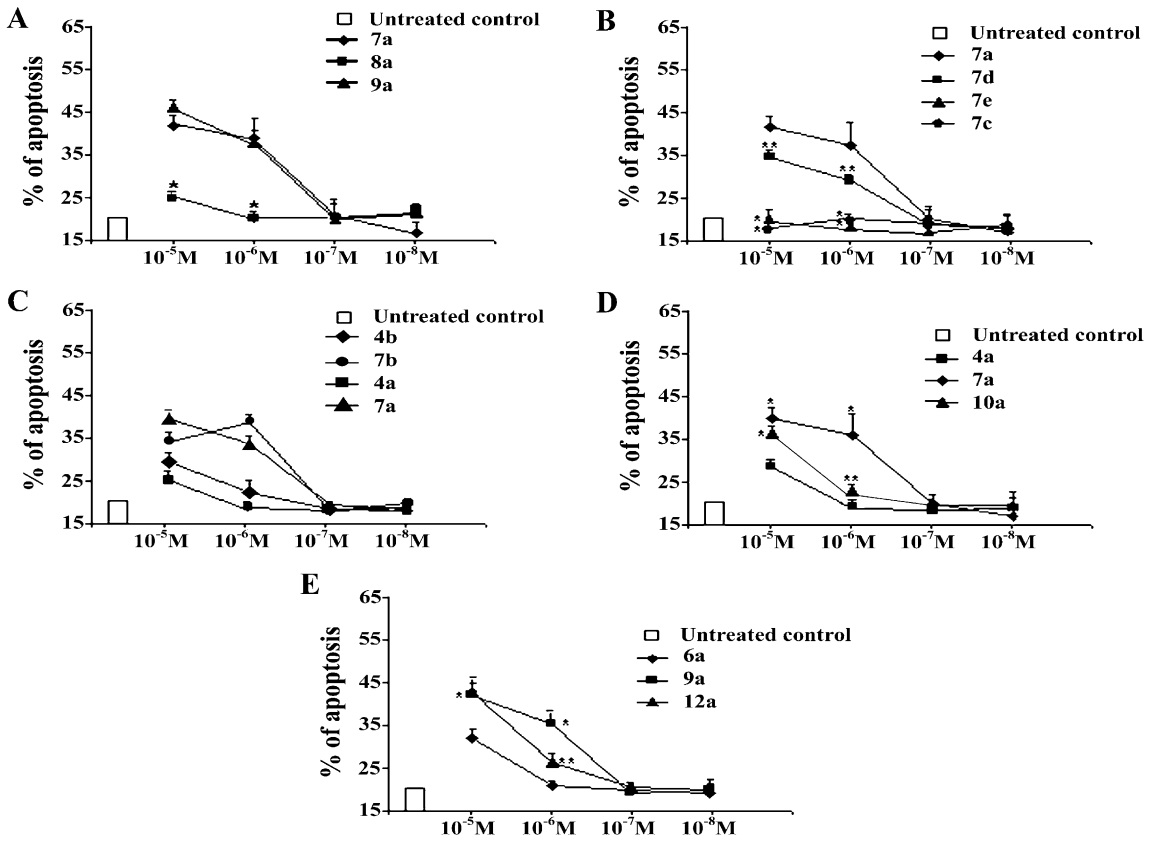


Figure 2. Dose–response curve of different 1,4-benzothiazine analogues in relationship to chemical modification. Mouse thymocytes were treated with the indicated analogues at concentrations ranging from 10^{-5} to 10^{-8} M. After 18 h, the cells were stained by PI and apoptosis evaluated by flow cytometry (see Fig. legend 1). *$p < 0.001$; **$p < 0.05$.

cause thymus cell death.[15] Here we evaluated the effect of in vivo treatment with newly synthesised derivatives on thymocyte number. Results of a representative experiment (media of 8 mice/group) are shown in Figure 3. Treatment with some, but not all, derivatives significantly reduced the number of thymocyte. Piperazine substituted derivatives in particular do not induce thymus cell loss (compare **7c** and **7e** with untreated control). Removing the side chain alcoholic group by dehydration to olefin (**10a**, **12a**) or by transforming it into an ether group (**9a**) increases apoptotic activity (compare **9a**, **10a** and **12a** with untreated control) in a dose-dependent manner. Similar results were obtained with doses ranging from 1 to 10 mg/kg ip and with other apoptotic analogues (data not shown).

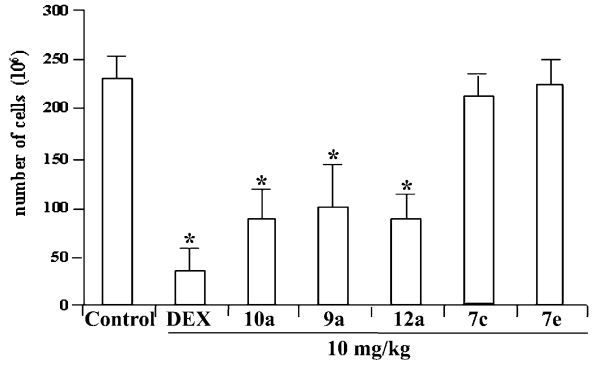


Figure 3. Effect of 1,4-benzothiazine analogues on in vivo thymocyte apoptosis. DEX or the indicated derivatives were injected ip in mice. Thymocytes were collected 24 h later and the cell death was evaluated by cell count (tripan blu exclusion). Mean values ± SE of three different experiments in duplicate are reported. *$p < 0.001$ as compared to untreated controls.

These results confirm that treatment with new derivatives reduces the number of thymocyte in vivo. This activity depends on structural features that are similar to those responsible for apoptotic activity in vitro.

Caspase-3, -8 and -9 activation: role of compound structure

Several ICE-family cysteine proteases (caspases) have been hypothesised to play a prominent role in T cell and thymocyte apoptosis.[21,22] In particular, two major sequential pathways of caspase activation may exist: the caspase-8 and caspase-3 (caspase 8/3) pathway which is activated by death receptors and the cytochrome *c*-dependent caspase-9 activation and the consequent activation of caspase-3 (caspase 9/3) pathway which is activated by non-receptor signals.[23] We have previously reported that 1,4-B-induced apoptosis correlates with caspase-8, -9 and -3 activation.[10]

To evaluate the role of SAR in 1,4-B-induced apoptosis we tested caspase activation by western blotting using specific antibodies against caspase-3, -8 and -9. Figure 4 shows the results of a representative experiment. Caspase activation, revealed as pro-caspase cleavage to lower

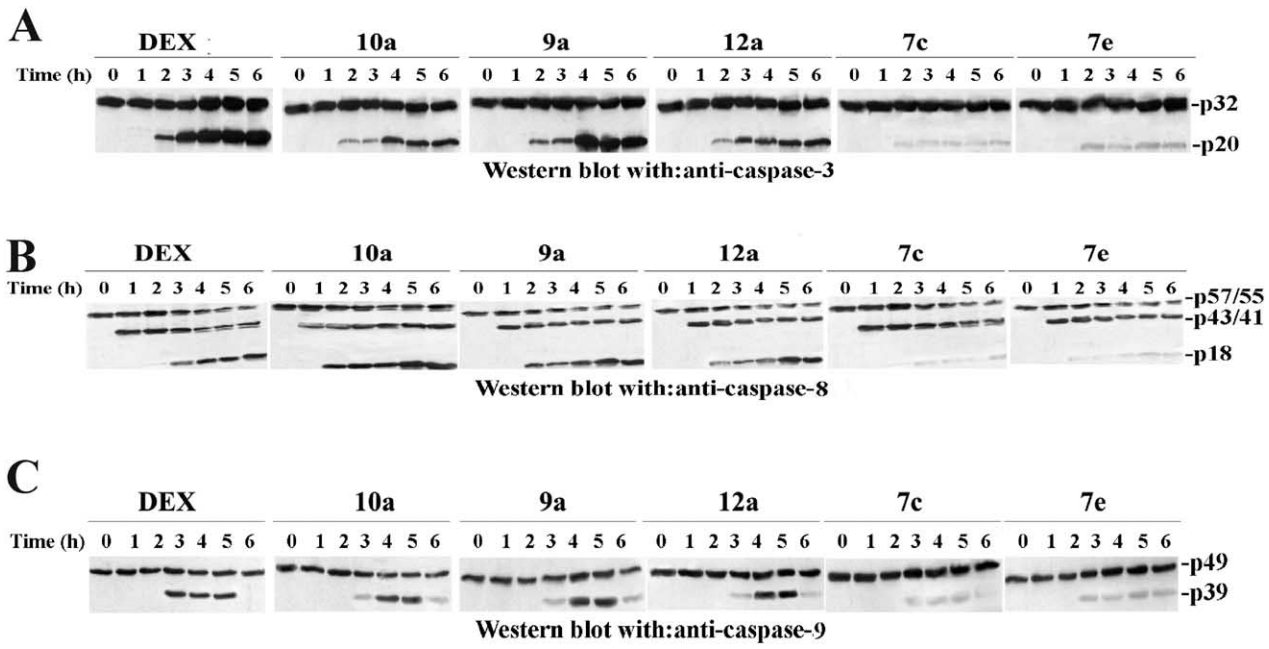


Figure 4. 1,4-Benzothiazine analogues activate caspase-3,-8 and-9. Murine thymocytes were untreated or treated with DEX (10^{-7} M), or 1,4-benzothiazine analogues (10^{-5} M). Pro-caspase cleavage was evaluated at different time-points ranging from 1 to 6 h. Caspase activation (pro-caspase cleavage) was assayed by Western blot with specific antibodies.

molecular weight peptides, was tested at different times (1–6 h after treatment). Caspase activation follows the same SAR required for in vitro apoptosis induction and in vivo thymus cell number reduction (Figs 1–3). In fact, apoptotic analogues (**9a**, **10a**, **12a**) strongly activate caspases, while piperazine derivatives (**7c** and **7e**), which have low or not apoptotic activity in vitro and thymus cytotoxicity in vivo, induced weak activation of all tested caspases.

As another control, we evaluated the effect of DEX on caspase activation. DEX has been reported to activate caspase-8 and consequently caspase-3 and -9.[24] Results in the Fig. 4 confirm these findings showing that DEX activates all those caspases.

These results indicate that treatment with 1,4-B analogues induces caspase activation, which is characteristic of apoptosis, and that this effect depends on structure requirements for apoptosis.

Discussion

The present paper investigated the SAR of 1,4-B derivatives to induce thymocyte apoptosis. To this aim we synthesised and tested the capability of different analogues to induce caspase activation and apoptosis in vitro, and to decrease thymus cell number in vivo.

Transformation of 1,4-benzothiazine nucleus into 1,2,3,4-tetrahydroquinoline does not result in significant change while transformation into 1,4-benzoxazine decreases activity. Replacing imidazole in the side chain with different piperazines decreases activity while substitution with benzimidazole does not modify apoptotic activity. Removal of the alcoholic group by dehydration to olefin or by transformation into ether increases activity.

1,4-B derivatives have been shown to exert several pharmacological effects in various in vivo and in vitro experimental systems. In particular, 1,4-B derivatives exert cytotoxic activity on neurons and neoplastic cells, suggesting a role in neurodegenerative diseases and a potential value in the treatment of tumours.

All these different effects may be of relevance in the development of 1,4-B pharmacology for therapeutic applications. However, the cytotoxic activity, as observed in different experimental systems, requires further characterisation.

We have recently shown that 1,4-B analogues, such as for example, **7a**, induce thymus cell loss in vivo, thymocyte apoptosis and activate a number of apoptotic signals.[10] The apoptotic activity correlates with PC-PLC and aSMase and caspase activation. Moreover, preliminary results suggested that the benzothiazinic skeleton and the side chain nature, position and length could modulate the apoptotic activity.

We synthesised and tested new compounds at equimolar concentrations to analyse the SAR into apoptotic activity. For that purpose, we used mouse thymocyte because this cell population is highly sensitive to apoptosis.

The apoptotic effect correlates with specific structural characteristics of 1,4-B. Of interest, in vivo treatment with 1,4-B analogues induces significant thymocyte loss which has been associated with other apoptosis inducers such as, for example, corticosteroids.[15]

Moreover, since caspase activation plays a crucial role in apoptosis, we also evaluated caspase-3, -8 and -9 activation. Our results indicate caspase activation correlates well with apoptosis induction.

In conclusion, depending on their chemical structure, some of the newly synthesised 1,4-B analogues induce cell death. Apoptosis correlates with caspase activation and in vivo thymus cell loss.

The findings in this report could account for some of the pharmacological effects associated with 1,4-B treatment, including its well-described neurotoxicity and anti-tumour activity.

Future studies will design and synthesise more active molecules and analyse the role of 1,4-B-induced apoptosis in other experimental systems including in vivo neurotoxicity and anti-tumour activity.

Experimental

Melting points determined in capillary tubes (Electrothermal, Model 9100, melting point apparatus) were uncorrected. Element analysis was performed on a Carlo Erba element analyser 1106, and the data for C, H, and N are within ±0.4% of the theoretical values. ^{1}H NMR spectra were recorded at 200 MHz (Bruker DPX spectrometer) with Me_4Si as internal standard. Chemical shifts are given in ppm (δ) and the spectral data are consistent with the assigned structures. Reagents and solvents were purchased from common commercial suppliers and used as received. Column chromatography separations were carried out on Merck silica gel 40 (mesh 70–230). Yields of purified product were not optimised. All starting materials were commercially available unless otherwise indicated.

General procedure for preparing heterocyclic derivatives 1a–e; 2a,e; 3a,e

This preparation is illustrated by the synthesis of **6-[2-(1*H*-1-imidazolyl)acetyl]-1-methyl-1,2,3,4-tetrahydroquinolin-2-one (3a).**

1*H*-Imidazole (0.73 g, 10.70 mmol) was added to a solution of 6-(2-bromoacetyl)-1-methyl-1,2,3,4-tetrahydroquinolin-2-one[20] (0.85 g, 3.60 mmol) in $CHCl_3$ (10 mL) and stirred at room temperature for 24 h, then it was refluxed for 3 h. The mixture was evaporated to dryness; the residue was treated with EtOH and filtered. **3a** (0.70 g, 73%) was obtained as a white solid, mp 188–189 °C, ^{1}H NMR ($CDCl_3$) δ: 2.60–2.70 (2H, m, C*H*$_2$CH$_2$CO), 2.90–3.10 (2H, m, CH$_2$C*H*$_2$CO), 3.40 (3H, s, NCH_3), 5.40 (2H, s, CH_2N), 7.00, 7.15 and 7.60 (each 1H, s, imidazolic H), 7.06 (1H, d, J = 8.3 Hz, H-8), 7.80 (1H, d, J = 1.5 Hz, H-5), 7.90 (1H, dd, J = 8.3 and 1.5 Hz, H-7).

By reaction of 6-(2-bromoacetyl)-1-methyl-1,2,3,4-tetrahydroquinolin-2-one with 1-methylpiperazine, **1-methyl-6-[2-(4-methylpiperazinyl)acetyl]-1,2,3,4-tetrahydroquinolin-2-one** (**3e**) was obtained as an oil, 74% yield, ^{1}H NMR ($CDCl_3$) δ 2.40 (3H, s, piperazinic NCH_3); 2.50–2.80 (10H, m, piperazinic H and C*H*$_2$CH$_2$CO); 2.80–3.10 (2H, m, CH$_2$C*H*$_2$CO); 3.40 (3H, s, NCH_3); 3.80 (2H, s, $COCH_2N$); 7.15 (1H, d, J = 8.4 Hz, H-8); 7.80 (1H, d, J = 1.5 Hz, H-5); 8.00 (1H, dd, J = 8.4 and 1.5 Hz, H-7).

Starting from 6-(2-bromoacetyl)-4-methyl-3,4-dihydro-2*H*-1,4-benzothiazin-3-one,[18] by reaction with 1*H*-imidazole, 1*H*-benzimidazole, 1-(2-methoxyphenyl)piperazine, 2-methylpiperazine and 1-methylpiperazine respectively the following products were prepared: **6-[2-(1*H*-1-imidazolyl)acetyl]-4-methyl-3,4-dihydro-2*H*-1,4-benzothiazin-3-one (1a)**;[18] **6-[2-(1*H*-1-benzo[*d*]imidazolyl)acetyl]-4-methyl-3,4-dihydro-2*H*-1,4-benzothiazin-3-one (1b)**, mp 199-201 °C, 87% yield, ^{1}H NMR (DMSO-d_6) δ 3.50 (3H, s, NCH_3); 3.65 (2H, s, SCH_2); 6.10 (2H, s, CH_2N); 7.20–7.85 (7H, m, aromatic H); 8.25 (1H, s, benzimidazolic H); **6-[2-[4-(2-methoxyphenyl)piperazinyl]acetyl]-4-methyl-3,4-dihydro-2*H*-1,4-benzothiazin-3-one (1c)**, mp 149–152 °C, 73% yield, ^{1}H NMR ($CDCl_3$) δ 2.90–3.00 and 3.05–3.10 (each 4H, m, piperazinic H); 3.35–3.60 (5H, m, NCH_3 and $COCH_2N$); 3.80–4.00 (5H, m, OCH_3 and SCH_2); 6.80–7.10 (4H, m, aromatic H); 7.50 (1H, d, J = 8.0 Hz, H-8); 7.75 (1H, dd, J = 8.0 and 1.0 Hz, H-7); 7.85 (1H, d, J = 1.0 Hz, H-5); **4-methyl-6-[2-(3-methylpiperazinyl)acetyl]-3,4-dihydro-2*H*-1,4-benzothiazin-3-one (1d)**, oil, 62% yield, ^{1}H NMR ($CDCl_3$) δ 1.10 (3H, d, J = 6.3 Hz, CHC*H*$_3$); 1.85–2.00 (1H, m, piperazinic H); 2.15–2.30 (1H, m, piperazinic H); 2.70 (1H, bs, NH); 2.80–3.15 (5H, m, piperazinic H); 3.40 (2H, s, SCH_2); 3.50 (3H, s, NCH_3); 3.75 (2H, s, $COCH_2N$); 7.45 (1H, d, J = 7.6 Hz, H-8); 7.70 (1H, dd, J = 7.6 and 2.1 Hz, H-7); 7.80 (1H, d, J = 2.1 Hz, H-5); **4-methyl-6-[2-(4-methylpiperazinyl)acetyl]-3,4-dihydro-2*H*-1,4-benzothiazin-3-one (1e)**, mp 218–220 °C, 60% yield, ^{1}H NMR ($CDCl_3$) δ 2.25 (3H, s, piperazinic NCH_3); 2.45–2.65 (8H, m, piperazinic H); 3.30 (2H, s, SCH_2); 3.35 (3H, s, NCH_3); 3.75 (2H, s, $COCH_2N$); 7.35 (1H, d, J = 8.0 Hz, H-8); 7.60 (1H, dd, J = 8.0 and 2.0 Hz, H-7); 7.75 (1H, d, J = 2.0 Hz, H-5).

Starting from 6-(2-bromoacetyl)-4-methyl-3,4-dihydro-2*H*-1,4-benzoxazin-3-one,[19] by reaction with 1*H*-imidazole, **6-[2-(1*H*-1-imidazolyl)acetyl]-4-methyl-3,4-dihydro-2*H*-1,4-benzoxazin-3-one (2a)**[19] was obtained and, by reaction with 1-methylpiperazine, **4-methyl-6-[2-(4-methylpiperazinyl)acetyl]-3,4-dihydro-2*H*-1,4-benzoxazin-3-one(2e)** was obtained, mp 228–230 °C, 50% yield, ^{1}H NMR (DMSO-d_6) δ 2.80 (3H, s, piperazinic NCH_3); 2.75–3.00 and 3.05–3.30 (each 4H, m, piperazinic H); 3.40 (3H, s, NCH_3); 4.00 (2H, s, $COCH_2N$); 4.75 (2H, s, OCH_2); 7.15 (1H, d, J = 7.9 Hz, H-8); 7.60–7.80 (2H, m, H-5 and H-7).

Preparation of carbinol derivatives 4a–e, 5a,e and 6a,e

The procedure is illustrated by the synthesis of **6-[1-hydroxy-2-(1*H*-1-imidazolyl)ethyl]-1-methyl-1,2,3,4-tetrahydroquinolin-2-one (6a)**.

To a solution of **3a** (0.30 g, 1.11 mmol) in MeOH (10 mL) $NaBH_4$ (0.06 g, 1.66 mmol) was added in small fractions over 1 h. The mixture was then evaporated to dryness and the residue chromatographed eluting with $CHCl_3$/MeOH 90:10 to give **6a** (0.28 g, 94%) as an amorphous solid, ^{1}H NMR ($CDCl_3$) δ 2.55–2.70 and 2.80–3.00 (each 2H, m, CH_2CH_2CO), 3.35 (3H, s, NCH_3), 4.05–4.20 (2H, m, CHOHC*H*$_2$), 4.85–5.00 (1H, m, C*H*OH), 5.45 (1H, bs, CHO*H*), 6.90–7.70 (6H, m, aromatic H).

Starting from **1a–e**, **2a**,**e** and **3e**, derivatives **4a–e**, **5a**,**e** and **6e**, respectively, were obtained by a similar procedure: **6-[1-hydroxy-2-(1*H*-1-imidazolyl)ethyl]-4-methyl-3,4-dihydro-2*H*-1,4-benzothiazin-3-one (4a)**;[18] **6-[1-hydroxy-2-(1*H*-1-benzo[*d*]imidazolyl) ethyl]-4-methyl-3,4- dihydro-2*H*-1,4-benzothiazin-3-one (4b)**, amorphous solid, 87% yield, ^{1}H NMR ($CDCl_3$) δ 3.25 (3H, s, NCH_3); 3.35 (2H, s, SCH_2); 4.30 (1H, dd, $J=14.4$ and 7.7 Hz, CHOHC*H*H); 4.38 (1H, dd, $J=14.4$ and 3.6 Hz, CHOHCH*H*); 5.20 (1H, dd, $J=7.7$ and 3.6 Hz, C*H*OH); 5.70 (1H, bs, CHO*H*); 7.10–7.50 (7H, m, aromatic H); 7.80 (1H, s, benzimidazolic H); **6-[1-hydroxy- 2-[4-(2-methoxyphenyl)piperazinyl]ethyl]-4-methyl-3,4-dihydro-2*H*-1,4-benzothiazin-3-one (4c)**, mp 194–196 °C, 76% yield, ^{1}H NMR ($CDCl_3$) δ 2.80–3.25 (10H, m, piperazinic H and CHOHCH_2); 3.40 (2H, s, SCH_2); 3.50 (3H, s, NCH_3); 3.85 (3H, s, OCH_3); 4.85 (1H, bs, CHO*H*); 6.85–7.40 (7H, m, aromatic H); **6-[1-hydroxy- 2-(3-methylpiperazinyl)ethyl]-4-methyl-3,4-dihydro-2*H*-1,4-benzothiazin-3-one (4d)**, oil, 66% yield, ^{1}H NMR ($CDCl_3$) δ 1.10 (3H, m, CHCH_3); 1.80–3.20 (11H, m, piperazinic H, NH and CHO*H*CH_2); 3.35 (2H, s, SCH_2); 3.45 (3H, s, NCH_3); 4.65–4.75 (1H, m, C*H*OH); 6.90 (1H, dd, $J=8.0$ and 0.5 Hz, H-7); 7.15 (1H, d, $J=0.5$ Hz, H-5); 7.30 (1H, d, $J=8.0$ Hz, H-8); **6-[1-hydroxy-2-(4-methylpiperazinyl)ethyl]-4-methyl-3,4-dihydro-2*H*-1,4-benzothiazin-3-one (4e)**, amorphous solid, 74% yield, ^{1}H NMR ($CDCl_3$) δ 2.25 (3H, s, piperazinic NCH_3); 2.45–2.70 (8H, m, piperazinic H); 2.75–2.85 (2H, m, CHOHCH_2); 3.45 (2H, s, SCH_2); 3.50 (3H, s, NCH_3); 4.00 (1H, bs, CHO*H*); 4.75 (1H, dd, $J=10.1$ and 3.6 Hz, C*H*OH); 7.00 (1H, dd, $J=7.9$ and 1.2 Hz, H-7); 7.20 (1H, d, $J=1.2$ Hz, H-5); 7.40 (1H, d, $J=7.9$ Hz, H-8); **6-[1-hydroxy-2-(1*H*-1-imidazolyl)ethyl]-4-methyl-3,4-dihydro-2*H*-1,4-benzoxazin-3-one (5a)**;[19] **6-[1-hydroxy-2-(4-methylpiperazinyl)ethyl]-4-methyl-3,4-dihydro-2*H*-1,4-benzoxazin-3-one (5e)**, oil, 94% yield, ^{1}H NMR ($CDCl_3$) δ 2.35 (3H, s, piperazinic NCH_3); 2.45–2.75 (8H, m, piperazinic H); 2.75–3.00 (2H, m, CHOHCH_2); 3.40 (3H, s, NCH_3); 3.65 (1H, bs, CHO*H*); 4.65 (2H, s, OCH_2); 4.75 (1H, dd, $J=9.9$ and 4.0 Hz, C*H*OH); 6.85–7.10 (3H, m, aromatic H); **6-[1-hydroxy-2-(4-methylpiperazinyl)ethyl] - 1 - methyl - 1,2,3,4 - tetrahydroquinolin-2-one (6e)**, mp 128–130 °C, 81% yield, ^{1}H NMR ($CDCl_3$) δ 2.35 (3H, s, piperazinic NCH_3); 2.50-2.70 (10H, m, piperazinic H and CH_2CH_2CO); 2.85–3.00 (4H, m, CH_2CH_2CO and CHOHCH_2); 3.35 (3H, s, NCH_3); 4.75 (1H, dd, $J=9.1$ and 4.8 Hz, C*H*OH); 7.00 (1H, d, $J=8.1$ Hz, H-8); 7.25–7.35 (2H, m, H-5 and H-7).

Preparation of ether derivatives 7a–e, 8a,e and 9a,e

The procedure is illustrated by the synthesis of **6-[1-[(4-chlorobenzyl)oxy]-2-(1*H*-1-imidazolyl)ethyl]-1-methyl-1,2,3,4-tetrahydroquinolin-2-one** (**9a**).

To a solution of **6a** (0.15 g, 0.55 mmol) in dry DMF (5 mL), NaH (60% mineral oil dispersion, 0.07 g, 1.66 mmol) was added in small fractions to prevent any heating. 4-Chlorobenzyl chloride (0.36 g, 2.24 mmol) in DMF (2 mL) was then added dropwise. The mixture was stirred at room temperature for 30 min and the hydride excess was decomposed with small amounts of EtOAc. The mixture was suspended in water and extracted with EtOAc, the combined organic layers were evaporated to dryness to afford a crude residue that was purified by chromatography eluting with $CHCl_3$ to give **9a** (0.13 g, 60%) as an oil, ^{1}H NMR ($CDCl_3$) δ 2.60–2.75 and 2.80–3.00 (each 2H, m, CH_2CH_2CO), 3.35 (3H, s, NCH_3), 4.05–4.25 (3H, m, C*H*OCH_2 and CH_2N), 4.35–4.50 (2H, m, OCH_2Ph), 6.90–7.40 (10H, m, aromatic H).

In a similar way, starting from **4a–e**, **5a**,**e** and **6e**, derivatives **7a–e**, **8a**,**e** and **9e** were synthesised respectively: **6-[1-[(4-chlorobenzyl)oxy]-2-(1*H*-1-imidazolyl)ethyl]-4-methyl-3,4-dihydro-2*H*-1,4-benzothiazin-3-one (7a)**;[18] **6-[1-[(4-chlorobenzyl)oxy]-2-(1*H*-1-benzo[*d*]imidazolyl) ethyl]-4-methyl-3,4-dihydro-2*H*-1,4-benzothiazin-3-one (7b)**, oil, 20% yield, ^{1}H NMR ($CDCl_3$) δ 3.30 (3H, s, NCH_3); 3.45 (2H, s, SCH_2); 4.20 and 4.50 (each 1H, d, $J=11.9$ Hz, OCH_2Ph); 4.40 (2H, d, $J=6.0$ Hz, CH_2N); 4.70 (1H, t, $J=6.0$ Hz, C*H*CH_2N); 6.80–7.90 (11H, m, aromatic H); 8.05 (1H, s, benzimidazolic H); **6-[1-[(4-chlorobenzyl)oxy]-2-[4-(2-methoxyphenyl) piperazinyl]-ethyl]-4-methyl-3,4-dihydro-2*H*-1,4-benzothiazin-3-one (7c)**, amorphous solid, 10% yield, ^{1}H NMR ($CDCl_3$) δ 2.75–3.35 (10H, m, piperazinic H and CHCH_2); 3.40 (2H, s, SCH_2); 3.45 (3H, s, NCH_3); 3.85 (3H, s, OCH_3); 4.40–4.75 (3H, m, OCH_2 and C*H*CH_2); 6.80–7.40 (11H, m, aromatic H); **6-[1-[(4-chlorobenzyl)oxy]-2-(3-methylpiperazinyl)ethyl]-4-methyl-3,4-dihydro-2*H*-1,4-benzothiazin-3-one (7d)**, amorphous solid, 10% yield, ^{1}H NMR ($CDCl_3$) δ 1.20–1.30 (3H, m, CHCH_3); 2.05–3.10 (10H, m, piperazinic H, CHCH_2 and NH); 3.20 and 4.05 (each 1H, d, $J=13.4$ Hz, OCH_2Ph); 3.40 (2H, s, SCH_2); 3.50 (3H, s, NCH_3); 4.70–4.80 (1H, m, CHO); 7.00 (1H, d, $J=8.1$ Hz, H-8); 7.20 (1H, d, $J=1.2$ Hz, H-5); 7.25-7.45 (5H, m, aromatic H); **6-[1-[(4-chlorobenzyl)oxy]-2-(4-methylpiperazinyl)ethyl]-4-methyl-3,4-dihydro-2*H*-1,4-benzothiazin-3-one (7e)**, amorphous solid, 15% yield, ^{1}H NMR ($CDCl_3$) δ 2.40 (3H, s, piperazinic NCH_3); 2.50–2.90 (10H, m, piperazinic H and CHCH_2N); 3.50 (5H, s, NCH_3 and SCH_2); 4.35 and 4.50 (each 1H, d, $J=12.1$ Hz, OCH_2Ph); 4.55 (1H, t, $J=5.3$ Hz, C*H*CH_2N); 7.00–7.50 (7H, m, aromatic H); **6-[1-[(4-chlorobenzyl)oxy]-2-(1*H*-1-imidazolyl)ethyl]-4-methyl-3,4-dihydro-2*H*-1,4-benzoxazin-3-one (8a)**;[19] **6-[1-[(4-chlorobenzyl)oxy]-2-(4-methylpiperazinyl)ethyl]-4-methyl-3,4-dihydro-2*H*-1,4-benzoxazin-3-one (8e)**, amorphous solid, 28% yield, ^{1}H NMR ($CDCl_3$) δ 2.30 (3H, s, piperazinic NCH_3); 2.45–2.75 (9H, m, piperazinic H and CHCH*H*N); 2.85 (1H, dd, $J=13.5$ and 8.7 Hz, CHC*H*HN); 3.40 (3H, s, NCH_3); 4.30 (1H, d, $J=12.3$, OC*H*HPh); 4.45–4.55 (2H, m, OCH*H*Ph and CH_2C*H*O); 4.70 (2H, s, OCH_2CO); 6.90-7.45 (7H, m, aromatic H); **6-[1-[(4-chlorobenzyl)oxy]-2-(4-methylpiperazinyl)ethyl]-1-methyl-1,2,3,4-tetrahydroquinolin-2-one (9e)**, oil, 14% yield, ^{1}H NMR ($CDCl_3$) δ 2.25 (3H, s, piperazinic NCH_3); 2.50–3.00 (14H, m, piperazinic H, CHCH_2N and CH_2CH_2CO); 3.40 (3H, s, NCH_3); 4.30 (1H, d, $J=12.4$ Hz, OC*H*HPh); 4.40–4.60 (2H, m, OCH*H*Ph and CH_2C*H*O); 6.90–7.45 (7H, m, aromatic H).

Preparation of olefin derivatives 10a–e, 11a,e and 12a,e

The procedure is illustrated by the synthesis of **6-[2-(1*H*-1-imidazolyl)-1-ethenyl]-1-methyl-1,2,3,4-tetrahydroquinolin-2-one (12a)**.

Compound **9a** (0.10 g, 0.37 mmol) was added to a mixture of H_2SO_4 concd. (0.5 mL) and AcOH (1.5 mL) and stirred at 110 °C for 30 min, then poured into ice/water, neutralised with a saturated solution of $NaHCO_3$ and extracted with EtOAc. The residue was chromatographed eluting with $CHCl_3$/MeOH 97:3. **12a** was obtained as an oil (0.06 g, 60%), ^{1}H NMR ($CDCl_3$) δ 2.60–2.75 and 2.80–3.00 (each 2H, m, CH_2CH_2CO); 3.35 (3H, s, NCH_3); 6.20 (1H, d, $J = 14.5$ Hz, C*H*=CHN); 6.90 (1H, d, $J = 8.3$ Hz, H-8); 7.10–7.40 (5H, m, CH=C*H*N and aromatic H); 7.80 (1H, s, imidazolic H).

Starting from **4a** and **5a,** by a similar procedure, derivatives **10a** and **11a,** respectively, were obtained: **6-[2-(1*H*-1-imidazolyl)-1-ethenyl]-4-methyl-3,4-dihydro-2*H*-1,4-benzothiazin-3-one (10a)**, amorphous solid, 43% yield, ^{1}H NMR ($CDCl_3$) δ 3.50 (2H, s, SCH_2); 3.55 (3H, s, NCH_3); 6.80 and 7.45 (each 1H, d, $J = 14.5$ Hz, CH=CH); 7.05–8.00 (6H, m, aromatic H); **6-[2-(1*H*-1-imidazolyl)-1-ethenyl]-4-methyl-3,4-dihydro-2*H*-1,4-benzoxazin-3-one (11a)**, oil, 30% yield, ^{1}H NMR ($CDCl_3$) δ 3.30 (3H, s, NCH_3); 4.65 (2H, s, OCH_2); 6.65 and 7.25 (each 1H, d, $J = 14.2$ Hz, CH=CH); 6.90–7.20 (5H, m, aromatic H); 7.70 (1H, s, imidazolic H).

Cell system and treatments

Thymocytes, from 4- to 6-week-old C3H/HeN mice, were enriched by cell passage through nylon wool columns. For in vitro experiments, thymocytes were cultured for 15 h with or without different drug concentrations as indicated in the legends to figures. For in vivo experiments, thymus cells were collected 24 h after in vivo ip injection of the indicated drugs.

Apoptosis evaluation by propidium iodide solution

Apoptosis was measured by flow cytometry as described elsewhere.[15] After culturing, cells were centrifuged and the pellets were gently resuspended in 1.5 mL hypotonic propidium iodide solution (PI, 50 μg/mL in 0.1% sodium citrate plus 0.1% Triton X-100, Sigma). Tubes were kept at 4 °C in the dark overnight. The PI-fluorescence of individual nuclei was measured by flow cytometry with standard FACScan equipment (Becton Dickinson). The nuclei traversed the light beam of a 488 nm Argon laser. A 560 nm dichroid mirror (DM 570) and a 600 nm band pass filter (band width 35 nm) were used to collect the red fluorescence due to PI DNA staining, and data were recorded in logarithmic scale in a Hewlett Packard (HP 9000, model 310) computer. The percentage of apoptotic cell nuclei (sub-diploid DNA peak in the DNA fluorescence histogram) was calculated with specific FACScan research software (Lysis II).

Apoptosis evaluation using annexin-V-labeling

In the early stages of apoptosis, changes occur at the cell surface. One of these plasma membrane alterations is the translocation of phosphatidylserine (PS) from the inner part of the plasma membrane to the outer layer, to expose PS at the external surface of the cell. Annexin V is a Ca^{2+}-dependent phospholipid-binding protein with high affinity for PS. This protein can hence be used as a sensitive probe for PS exposure upon the outer leaflet of the cell membrane and is therefore suited to detect apoptotic cells in cell populations.

The analysis of phosphatidylserine on the outer leaflet of apoptotic cell-membranes is performed by using Annexin-V-Fluos and propidium iodide (PI) for the differentiation from necrotic cells or labelling with a cell surface marker for cell characterization. After treatments, cells were centrifuged at 1200 rpm for 10 min, resuspended in 500 μL of Annexin-V-Fluos in a Hepes buffer (10 mM Hepes/NaOH, pH 7.4, 140 mM NaCl, 5 mM $CaCl_2$) containing PI 50 μg/mL for 15 min at room temperature and analysed by flow cytometry.

Western blotting to evaluate activation of caspase-3, -8 and -9

Cells were washed once with ice-cold PBS and lysed by incubating for 30 min on ice in 100 μL of lyses buffer (20 mM Tris–HCl, 0.15 M NaCl, 5 mM EDTA, 100 mM PMSF, 2.5 mM leupeptin, 2.5 mM aprotinin). After centrifugation at 15,000 rpm for 15 min, extracted proteins were separated on a 12 or 15% SDS-polyacrilamide gel and electrophoretically transferred to a nitrocellulose transfer membrane (Schleicher & Schuell, Keene, NH, USA). Membrane was blocked with TBST (20 mM Tris–HCL pH 7.5, 150 mM NaCl, 0.1% Tween 20) containing 5% skimmed milk for 1 h at room temperature, and each antibody was applied overnight at 4 °C in the same blocking solution. Anti-caspase-3 antibody and anti-caspase-8 antibody were purchased from Santa Cruz and anti-caspase-9 antibody was purchased from New England Biolabs, Beverly, MA, USA. After incubation membranes were washed with TBST and incubated for 1 h with horseradish peroxidase-labelled goat anti-rabbit (for anti-caspase-8 and -9) or anti-mouse (for caspase-3) IgG (Pierce). The antigen–antibody complexes were revealed by enhanced chemiluminescence following the manufacturer's instructions (Pierce).

Statistical analysis

All the experiments here shown were repeated at least three times. For data analysis, Student's *t* test was used with the STATPAC Computerized Program, and *p* values < 0.05 were considered significant.

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