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Induction of Apoptosis and Necrosis by Resistance Modifiers Benzazoles and Benzoxazines on Tumour Cell Line Mouse Lymphoma L5718 Mdr+cells

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Abstract. *Eighteen new fused heterocyclic compounds of benzazoles and benzoxazines were investigated for induction and inhibition of apoptosis on tumor cells (L5718, mouse lymphoma cell line containing the human mdr-1 gene). For evaluation of apoptosis, the cells were stained with FITC-labelled Annexin-V and propidium iodide and the results were analysed by flow cytometry. Nine of these substances were also checked for reversal of multidrug resistance. The reversal of multidrug resistance was determined by measuring the rhodamine-123 accumulation in the cancer cells. Rhodamine-123 shows a green fluorescence and its intracellular concentration correlates well with the inhibition of efflux pump activity. Three of the tested compounds, 5-(p-nitrobenzamido)-2-benzylbenzoxazole (BD-3), 6-methyl-2-(o-chlorophenyl) benzoxazole (A-9) and 5-(p-nitrophenoxyacetamido)-2-phenylbenzoxazole (D-30), showed an increased apoptotic effect on mouse lymphoma cells. Moreover, compounds BD-3, A-9 and 5-(2-thienylcarboxyamido)-2-phenylbenzoxazole (D-24) also amplified the apoptosis effect of 12H-benzo(a)phenothiazines (M-627). However, D-24, alone was not effective. Additionally, 2-(p-nitrobenzyl)benzoxazole (B-11), was also found to increase the apoptotic effect of M-627. On the other hand, 5-(p-nitrophenylacetamido)-2-phenylbenzoxazole (D-7) showed an anti-apoptotic effect. No positive correlation was found between the increased drug accumulation effect and the programmed cell death induced by the compounds studied.*

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In recent years, substituted benzoxazoles and related fused heterocyclic compounds such as benzimidazoles, benzothiazoles and benzoxazines have shown antibacterial and antifungal (1-5), antiviral (6), topoisomerase inhibiting (7, 8) and antitumour activities (9-13). A benzoxazole derivative, coded as L-697,661, was identified as a specific non-nucleoside reverse transcriptase inhibitor for the human immunodeficiency virus HIV-1 type and its use in combined therapy with zidovudine achieved a marked decrease of viraemia in some primary HIV-infected patients (14). Moreover, a new series of benzothiazoles showed potent inhibitory activity against human breast cancer cell lines *in vitro* and *in vivo*. Among them, the lysyl-amide of 2-(4-amino-3-methylphenyl)-5-fluorobenzothiazole has been selected for phase I clinical evaluation (15).

In this study, the induction of apoptosis or necrosis by 18 new resistance modifier fused heterocyclic compounds was investigated on multidrug resistant (mdr) tumour cell lines. Reversal of the multidrug resistance activities of 2, 5-disubstituted benzoxazoles, 2-phenoxymethyl-benzimidazole, 2-phenoxymethyl-benzothiazole and 6-chloro and/or 7-methyl-2H-3, 4-dihydro-1, 4-benzoxazine-3-on-2-acetic acid derivatives were investigated on human *mdr-1* gene-transfected mouse lymphoma cells (L5718). Rhodamine-123 was applied as an indicator substrate for the inhibition of efflux pump activity and the drug accumulation was followed by flow cytometry.

For the evaluation of apoptosis and necrosis, the cells were incubated with FITC-labelled Annexin-V and propidium iodide and the effects of the tested compounds were measured by flow cytometry and the results calculated by square analysis.

Materials and Methods

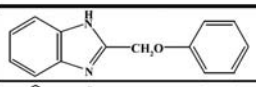
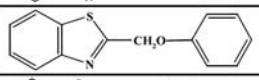
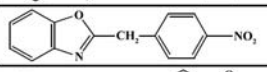
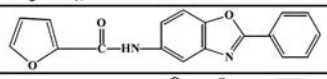
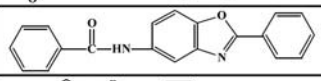
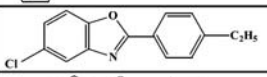
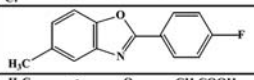
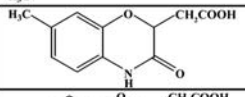
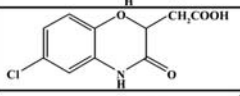
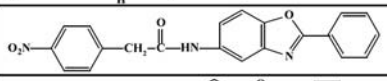
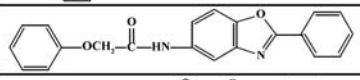
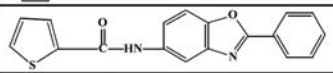
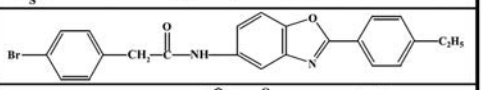
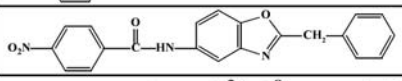
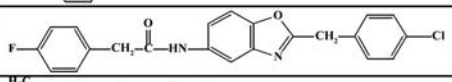
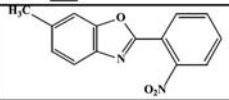
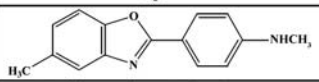
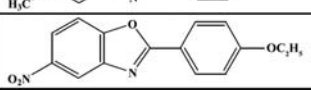
The benzazole and benzoxazine derivatives given in Table I, which were synthesized at the Pharmaceutical Chemistry Department of the Faculty of Pharmacy, Ankara University, Turkey, were investigated for their apoptosis induction or inhibition of apoptosis effects.

Apoptosis induction was measured according to the protocol of Alexis Biochemicals with minor modification (16). The cells of the mdr L5718 mouse cell line were transferred into small centrifuge tubes, centrifuged and resuspended in 1.0 mL binding buffer. After centrifugation, 750 μl supernatant fluid was removed and 3 mL Annexin-V-FITC was added to the samples. Following centrifugation, the cells were resuspended in 100 μl binding buffer and 1.0 μl from a 1.0 mg/mL propidium iodide stock solution was added to the samples. The fluorescent activity (FL-1 and FL-2) of the cells was measured and analysed on a Becton-Dickinson FACS-scan.

Apoptosis inhibition was measured after the addition of apoptosis inducers. The cells were incubated with M-627 (12H-benzo(a)phenothiazine) at 37˚C for 45 min in 50.0 μg/mL final concentration. After the incubation, the cells were washed 4 times with phosphate-buffered saline (PBS) and the tested compounds in DMSO solution were added (final concentration: 10.0 μg/mL) for 24 hours at 37˚C in a CO_2 incubator. After the incubation, the procedure was continued as above.

Reversal of multidrug resistance was measured by fluorescence uptake assay (17). The L5718Y mouse T cell lymphoma cells, that contain the human *mdr-1* gene, and the L5718Y parental cell line were grown in McCoys 5AA medium with 10% heat-inactivated horse serum, glutamine and antibiotics. The cells were adjusted to a density of $2x10^6$/mL and resuspended in serum-free McCoys 5AA medium before being distributed in 0.5 mL aliquots in Eppendorf centrifuge tubes. One μL of the 1.0 mg/mL stock solutions were added to the test compounds and the samples were incubated for 10 min at room temperature. Then 10 μL of the indicator rhodamine-123 (5.2 μM final concentration) was added to the samples and the cells were incubated for a further 20 min at 37˚C, washed twice and resuspended in 0.5 mL PBS for analysis. The fluorescence of the cell population was measured by flow cytometry using the Beckton Dickinson FACScan.

Table I. *Synthesized compounds tested for apoptosis- or necrosis-inducing effects on mouse lymphoma L5718 mdr+cells.*

Code	Structure of the compound
G-31	
E-18	
B-11	
D-6	
D-23	
A-24	
A-33	
BS-04	
BS-05	
D-7	
D-30	
D-24	
D-26	
BD-3	
BD-19	
A-9	
A-44	
A-49	

Results and Discussion

The apoptosis-inducing activity and resistance modifier effects of the tested benzazoles and bezoxazines, given in Table I, were systematically studied and the results are summarized in Tables II-VI.

Out of the 18 different fused heterocyclic compounds tested, some of the benzoxazole derivatives exhibited a variety of apoptosis- or necrosis-inducing effects on the mouse lymphoma L5718 mdr+cells. Three of the tested compounds, 5-(*p*-nitrobenzamido)-2-benzylbenzoxazole (BD-3), 6-methyl-2-(*o*-chlorophenyl)benzoxazole (A-9) and 5-phenoxyacetamido-2-phenylbenzoxazole (D-30), showed an increased apoptosis effect on the mouse lymphoma cells. Moreover, some of the compounds acted synergistically with M-627 in the induction of apoptosis. Compounds BD-3, A-9 and 5-(2-thienylcarboxyamido)-2-phenylbenzoxazole (D-24) also amplified the apoptosis effect of M-627, a well known apoptosis inducer synthesized by Mothohashi and co-workers in Tokyo (19). However, D-24, alone was not effective. Additionally, 2-(*p*-nitrobenzyl)benzoxazole (B-11),

Table II. *Apoptosis and necrosis by different benzazole and benzoxazine derivatives on mouse lymphoma L5718 mdr+ cells.*

Substances	Concentration µg/ml	Early apoptosis (%)	Apoptosis inhibition (%)	Necrosis (%)
Cell control without staining		0.02	0.04	0.10
Cell control Annexin-V+PI–		5.20	0.12	0.01
Cell control Annexin-V+PI+		0.02	0.05	6.78
Cell control Annexin-V–PI– 1.0% DMSO		3.21	7.18	1.88
M-627 control+	50.00	6.48	15.06	9.32
G-31	10.00	1.80	4.34	1.50
E-18	10.00	3.33	2.65	1.22
B-11	10.00	2.29	4.31	0.80
D-6	10.00	6.85	7.21	1.58
D-23	10.00	5.43	6.43	2.74
A-24	10.00	5.06	4.16	0.93
A-33	10.00	6.43	7.13	1.13
BS-04	10.00	4.58	6.20	1.79
BS-05	10.00	2.21	5.13	1.64

Table III. *Induction of apoptosis and necrosis by different benzazole and benzoxazine derivatives on mouse lymphoma L5718 mdr+ cells.*

Substances	Concentration µg/ml	Early apoptosis (%)	Apoptosis inhibition (%)	Necrosis (%)
Cell control without staining		0.01	0.02	0.03
Cell control Annexin-V+PI–		0.00	0.00	0.14
Cell control Annexin-V+PI+		0.20	11.08	0.63
Cell control in 1% DMSO				
M627 Annexin-V+PI+	50.00	13.18	73.37	0.31
D-7	10.00	0.17	16.01	1.23
D-30	10.00	0.23	23.06	2.91
D-24	10.00	0.30	18.86	0.98
D-26	10.00	0.16	17.08	1.13
BD-3	10.00	0.33	26.87	0.71
BD-19	10.00	0.13	15.50	1.05
A-9	10.00	0.12	24.84	0.89
A-44	10.00	0.09	21.86	0.57
A-49	10.00	0.23	19.20	1.15

Table IV. *Inhibition of apoptosis and necrosis by different benzazole and benzoxazine derivatives (E/A-1) on mouse lymphoma L5718 mdr+ cells.*

Substances	Concentration µg/ml	Early apoptosis (%)	Apoptosis inhibition (%)	Necrosis (%)
Cell control without staining		0.02	0.04	0.10
Cell control Annexin-V+PI–		2.20	3.15	0.02
Cell control Annexin-V–PI+		1.02	1.05	0.78
Cell control Annexin-V+PI– 1.0% DMSO		1.96	5.17	1.00
Cell control Annexin-V–PI– in 1% DMSO		4.21	5.18	1.68
M-627 control+	50.00	81.04	96.06	3.32
M-627/G-31	10.00	62.68	84.34	1.50
M-627/E-18	10.00	54.33	76.65	1.82
M-627/B-11	10.00	83.29	89.31	0.90
M-627/D-6	10.00	71.85	87.51	1.08
M-627/D-23	10.00	71.29	87.46	4.34
M-627/A-24	10.00	79.47	88.95	3.93
M-627/A-33	10.00	78.05	88.61	3.72
M-627/BS-04	10.00	80.18	88.15	1.72
M-627/BS-05	10.00	80.43	93.86	3.96

Table V. *Inhibition of apoptosis and necrosis by different benzazole and benzoxazine derivatives on mouse lymphoma L5718 mdr+ cells.*

Substances	Concentration μg/ml	Early apoptosis (%)	Apoptosis inhibition (%)	Necrosis (%)
Cell control without staining		0.01	0.02	0.03
Cell control Annexin-V–PI–		0.00	0.00	0.24
Cell control Annexin-V+PI+		2.01	4.52	0.85
Cell control in 1% DMSO				
M627 Annexin-V+PI+	50.00	11.74	34.19	0.56
M627/D-7	10.00	1.90	21.54	0.72
M627/D-30	10.00	1.88	37.10	0.52
M627/D-24	10.00	2.65	45.15	0.27
M627/D-26	10.00	2.32	39.73	0.65
M627/BD-3	10.00	2.22	47.16	0.41
M627/BD-19	10.00	2.15	38.41	1.05
M627/A-9	10.00	2.42	51.24	0.45
M627/A-44	10.00	2.78	38.69	0.44
M627/A-49	10.00	1.71	30.49	0.42

Table VI. *Correlation between apoptosis induction and reversal of multidrug resistance.*

Substances	Early apoptosis (%)	Total apoptosis (%)	Necrosis (%)	Apoptosis inhibition[1] (%)	Reversal of multidrug resistance (%)
G-31	1.80	4.34	1.50	+11.72	1.10
D-6	6.85	7.21	1.58	+8.55	8.62
D-23	5.43	6.43	2.74	+8.60	1.32
A-33	6.43	7.13	1.13	+7.11	2.76
BS-04	4.58	6.20	1.79	+7.91	1.08
BS-05	2.21	5.13	1.64	+2.20	1.07
D-24	0.30	18.86	0.98	-10.96	4.68
BD-19	0.13	15.50	1.05	-4.22	1.41
A-44	0.09	21.86	0.57	-4.50	0.99

[1]apoptosis inhibition= apoptosis by M-627 alone (%) / apoptosis by M-627 with substance (%).

which has a nitro group at the *para* position of the 2-benzyl moiety , also increased the apoptotic effect of M-627.

On the other hand, 5-(*p*-nitrophenylacetamido)-2-phenylbenzoxazole (D-7) inhibited the apoptosis-inducing effect of M-627, showing a similar molecular structure to BD-3, but, holding a *p*-nitrophenylacetamido group on position 5 at the fused ring system instead of *p*-nitrobenzamido, having a methylene bridge to increase the length of the substituent.

Apparently the induction of apoptosis does not depend on specific chemical structures, since various structurally-unrelated compounds were able to induce apoptosis. There was no significant difference in the effects of stereo isomers (18) when the apoptosis-inducing effect of various enantiomers and stereoisomeric pairs was studied. No correlation between the reversal of multidrug resistance and the induction or inhibition of apoptosis was found.

In conclusion, the results of our investigation showed that some new fused heterocyclic resistance modifier compounds, alone or in combination, influence (either increase, or reduce) the programmed cell death of multidrug-resistant cancer cells. The multiple beneficial effects should be further studied in tumor xenograft transplanted animal experiments.

Acknowledgements

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