Pergamon

European Journal of Cancer, Vol. 34, No. 11, pp. 1756–1763, 1998

Printed in Great Britain
0959-8049/98/$—see front matter

PII: S0959-8049(98)00141-5

Original Paper

Selective Induction of Apoptosis in Multidrug Resistant HL60R Cells by the Thiazolobenzoimidazole Derivative 1-(2,6-difluorophenyl)-1*H*,3*H*-thiazolo [3,4-a] benzimidazole (TBZ)

S. Grimaudo,[1] M. Tolomeo,[2] A. Chimirri,[3] M. Zappala,[3] R.A. Gancitano[2] and N. D'Alessandro[4]

[1]Istituto Farmacochimico Facolta di Farmacia, Universita' di Palermo, Palermo; [2]Cattedra di Ematologia e Servizio A.I.D.S., Facolta' di Medicina, Universita' di Palermo, Palermo; [3]Dipartimento Farmaco-Chimico, Universita' di Messina, Messina; and [4]Istituto di Farmacologia, Facolta' di Medicina, Universita' di Palermo, Policlinico, via del Vespro 129, 90127 Palermo, Italy

We investigated the antitumour effects of 1-(2,6-difluorophenyl)-1*H*,3*H*-thiazolo [3,4-a]benzimidazole (TBZ) a new anti-HIV-1 agent, on human promyelocytic HL60 leukaemia, both a parental and a multidrug resistant form (HL60R). HL60R overexpresses P-glycoprotein and, like HL60, lacks p53 protein expression. HL60 and HL60R show similar levels of Bcl-2 protein. In contrast to the conventional chemotherapeutic agents daunorubicin, etoposide and mitoxantrone, TBZ caused equal or even greater cytotoxicity in HL60R than in HL60, and this result was associated with a more marked induction of apoptosis in the drug resistant cells. The antitumour activity of TBZ occurred in the range of concentrations higher than those required to exert antiviral activity. TBZ seems to act in the presence of P-glycoprotein and Bcl-2 and in the absence of p53 and is able to circumvent the mechanisms of drug resistance and anti-apoptosis present in HL60R cells. © **1998 Elsevier Science Ltd. All rights reserved.**

Key words: thiazolobenzimidazole, apoptosis, multidrug resistance, HL60 leukaemia
Eur J Cancer, Vol. 34, No. 11, pp. 1756–1763, 1998

INTRODUCTION

DRUG RESISTANCE, especially in its multiple forms (multidrug resistance, MDR), remains a major and difficult problem to resolve in cancer therapy. This phenomenon has often been ascribed to strictly pharmacological factors, such as the overexpression of the multidrug transporter P-glycoprotein [1–3], and, more recently, also to other variables closely associated with the biology of malignant transformation. The latter include factors which regulate the process of apoptosis in the cells [4–9]. Several chemotherapeutic agents can induce apoptosis in target cells [10–14], and the wild-type oncosuppressor p53 protein plays a critical role in triggering apoptosis following DNA damage or other forms of cell stress caused by such agents. However, loss of the p53 function can often result in an increased resistance to the apoptotic effects of the anticancer agents [15–18]. Various homologues of the Bcl-2 protein are also involved in apoptosis and they either promote or supress the process. Bcl-2 blocks apoptosis, and its overexpression in malignancies is associated with a poorer prognosis and failure to achieve response to chemotherapy [19–20]. Clearly, these findings should further the search of new strategies and agents to overcome the MDR process.

1-(2,6-difluorophenyl)-1*H*,3*H*-thiazolo[3,4-a]benzimidazole (TBZ) (Figure 1) is a new anti-HIV-1 agent and non-nucleoside reverse transcriptase inhibitor [21–27]. It has also shown some antitumour activity on human T-lymphoblastic CEM leukaemia cells [21, 25]. In this paper, we have investigated the antitumour activity of TBZ and report on its effect on the human promyelocytic HL60 leukaemia, both the parental (HL60) and MDR forms (HL60R). HL60R overexpresses P-glycoprotein and, like HL60, does not express p53 protein [28]. We also show that HL60 and HL60R have similar levels of Bcl-2 protein.

MATERIALS AND METHODS

Chemicals

Daunorubicin, etoposide and mitoxantrone were obtained from Sigma Chemical Company (St. Louis, Missouri, U.S.A.).

Correspondence to N. D'Alessandro.
Received 4 Nov. 1997; revised 16 Feb. 1998; accepted 18 Feb. 1998.

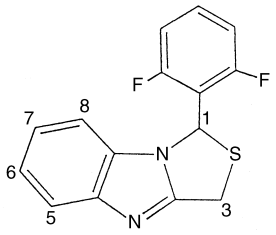

Figure 1. Chemical structure of 1-(2,6-difluorophenyl)-1*H*,3*H*-thiazolo[3,4-a]benzimidazole (TBZ).

Synthesis of TBZ

The synthesis of TBZ was carried out by a one-pot condensation–cyclisation reaction between 1,2-diaminobenzene, 2,6-difluorobenzaldehyde and 2-mercaptoethanoic acid in boiling benzene, according to procedures previously reported [21]. After normal work-up, the solid residue was recrystallised from ethanol to produce TBZ.

Drug preparation

TBZ was dissolved in dimethylsulphoxide (DMSO) in a stock solution at a concentration of 20 mM, stored at $-20°C$ and protected from light. In each experiment DMSO never exceeded 5‰ and this percentage did not interfere with cell growth.

Cell culture

Human HL60 leukaemia cells as parental or MDR HL60R variants were grown in RPMI 1640 (GIBCO Grand Island, New York, U.S.A.) supplemented with 10% fetal calf serum (GIBCO), penicillin (100 U/ml) and streptomycin (100 µg/ml) in a 5% CO_2 atmosphere at 37°C.

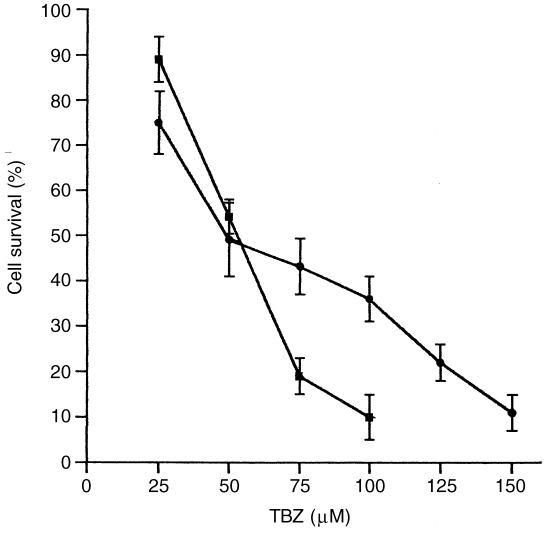

Figure 2. Cytotoxic activity of 1-(2,6-difluorophenyl)-1*H*,3*H*-thiazolo[3,4-a]benzimidazole (TBZ) in HL60 (●) and HL60R (■) cells. For further details see Materials and Methods.

Cytotoxicity assays

To evaluate the number of live and dead cells, the cells were stained with trypan blue and counted on a haemocytometer. The cells which showed trypan blue uptake were interpreted as non-viable. To determine the growth inhibitory activity of the drugs tested, 2×10^5 cells were plated into 25 mm wells (Costar, Cambridge, U.K.) in 1 ml of complete medium and treated with different concentrations of each drug. After 72 h incubation, the number of viable cells was determined and expressed as a percentage of control proliferation. IC_{50} and IC_{90} were the concentrations of drug able to inhibit the cell growth of 50% and 90%, respectively.

Flow cytometry analysis of apoptosis

The cells were washed twice in ice-cold phosphate buffered saline (PBS) and resuspended at 1×10^6/ml in a hypotonic fluorochrome solution containing propidium iodide (Sigma) 50 µg/ml in 0.1% sodium citrate plus 0.03% (v/v) Nonidet P-40 (Sigma). After 30 min of incubation in this solution, the samples were filtered through nylon cloth, 40 µm mesh, and their fluorescence was analysed, as single-parameter frequency histograms using a FACSort (Becton Dickinson, Mountain View, California, U.S.A.). Apoptosis was determined by evaluating the percentage of hypoploid nuclei accumulated in the sub-G_0–G_1 peak after labelling with propidium iodide.

Morphological evaluation of apoptosis and necrosis

The drugs' effects on apoptosis and necrosis were determined morphologically by fluorescent microscopy after labelling with acridine orange and ethidium bromide as described by Duke and Cohen [29]. Cells (2×10^5) were centrifuged (300 g) and the pellet was resuspended in 25 µl of the dye mixture. Ten microlitres of the mixture was placed on a microscope slide and covered with a 22 mm^2 coverslip and examined in oil immersion with a 100× objective using a fluorescent microscope. Live cells were determined by the uptake of acridine orange (green fluorescence) and exclusion of ethidium bromide (red fluorescence) stain. Live and dead apoptotic cells were identified by perinuclear condensation of chromatin stained by acridine orange or ethidium bromide, respectively, and by the formation of apoptotic bodies. Necrotic cells were identified by uniform labelling of the cells with ethidium bromide.

Evaluation of p53 and Bcl-2 protein expression

Cells (1×10^6) were permeabilised (in the assays of p53 and Bcl-2) or not (in the assays of P-glycoprotein) with

Table 1. *IC_{50} and IC_{90} of daunorubicin, etoposide and mitoxantrone in HL60 and HL60R cell lines*

	HL60		HL60R	
	IC_{50}	IC_{90}	IC_{50}	IC_{90}
Daunorubicin	0.005 (0.0017)*	0.01 (0.005)	1.0 (0.27)	7.5 (1.1)
Etoposide	0.123 (0.07)	0.35 (0.09)	5.0 (1.25)	41.2 (6.2)
Mitoxantrone	0.0025 (0.0008)	0.005 (0.001)	0.5 (0.008)	1.0 (0.28)

* Standard deviation. Concentrations in µg/ml

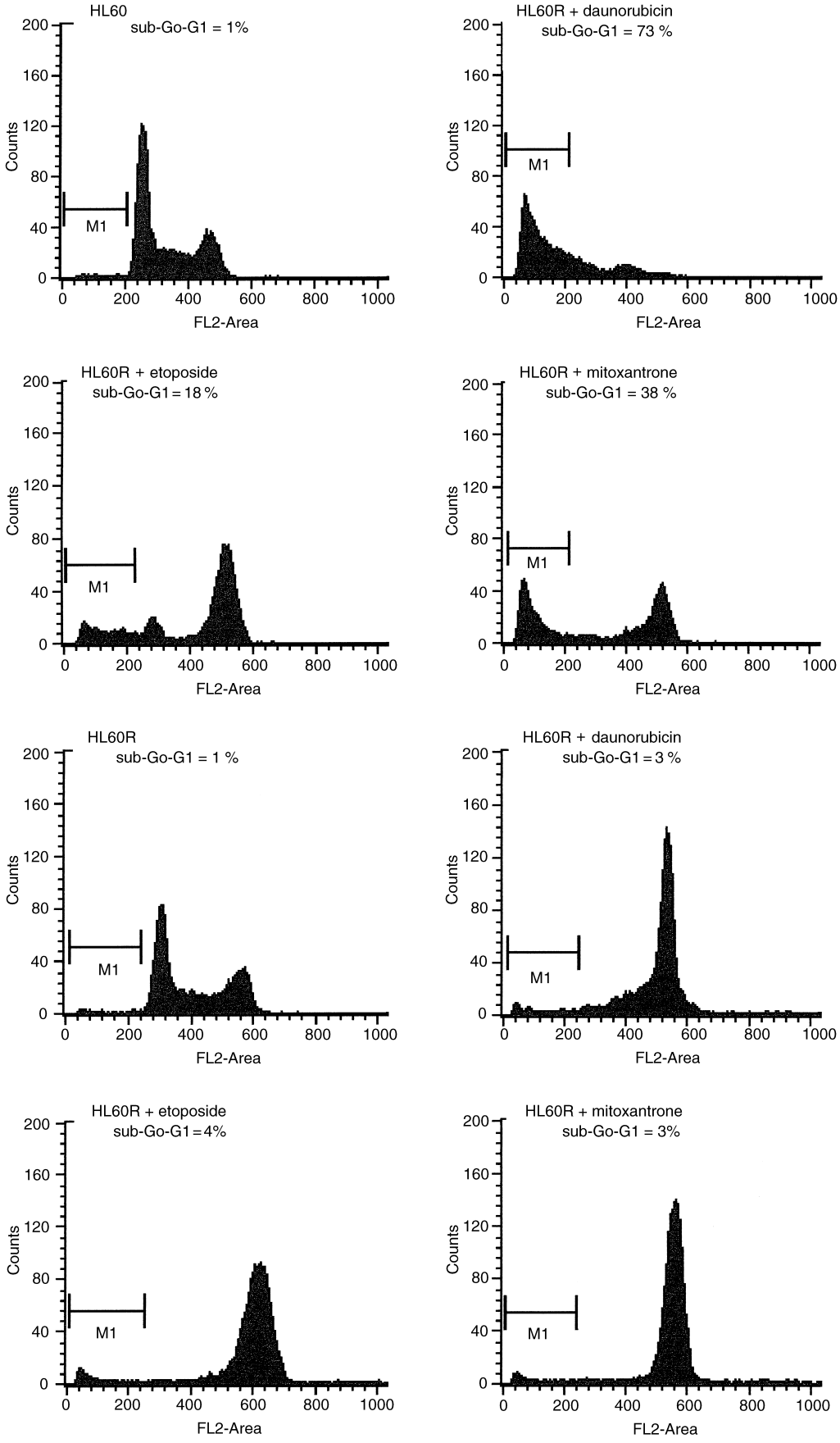

Figure 3. Flow cytometry assay of cell cycle distribution of HL60 and HL60R cells after 48 h exposure to the IC_{90}s of daunorubicin, etoposide or mitoxantrone in the cell lines.

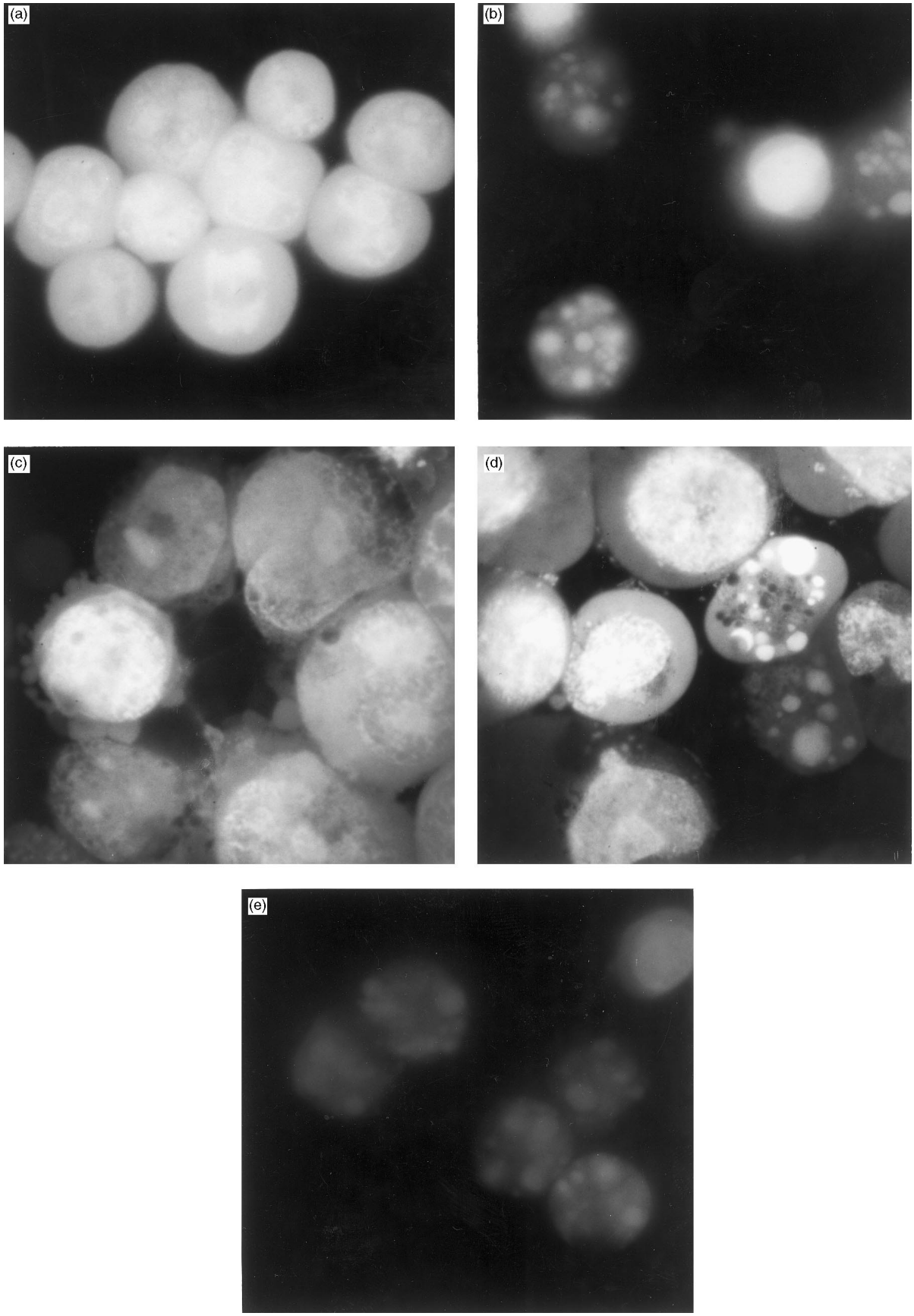

Figure 4. Morphology of cells after 48 h exposure to daunorubicin, etoposide, mitoxantrone or 1-(2,6-difluorophenyl)-1*H*,3*H*-thiazolo[3,4-a]benzimidazole (TBZ). (a) Untreated HL60 cells (HL60R showed a similar morphology); (b) HL60 cells exposed for 48 h to daunorubicin (IC_{90}); (c) HL60R cells exposed for 48 h to daunorubicin (IC_{90}); (d) HL60 cells exposed for 48 h to 75 μM TBZ; (e) HL60R cells exposed for 48 h to 75 μM TBZ.

Ortho-Permeafix (Ortho Diagnostic Systems, Raritan, New York, U.S.A.) and then washed twice with PBS. Monoclonal antibodies for P-glycoprotein (C1 FITC-conjugated; YLEM, Rome, Italy), p53 (DO7 FITC-conjugated; Dako A/S, Denmark, and PAB 240 PE-conjugated; Pharmingen, San Diego, California, U.S.A. or human Bcl-2 (clone 124 FITC-conjugated; Dako), as well as their appropriate FITC- or PE-conjugated negative controls, were added to the samples and the cells were incubated for 40 min. After two washes with PBS, the fluorescence was detected by flow cytometry.

Statistical analysis

The data reported in the figures represent the mean of three or more independent experiments and the bars show the standard deviation (SD).

RESULTS

Cytotoxicity assay

TBZ showed cytotoxic activity on both the parental and the MDR HL60 leukaemia cells (Figure 2). The activity was similar in the two cell lines up to a concentration of 50 μM. At higher concentrations, TBZ was more cytotoxic in the MDR cells. The IC_{50} of TBZ was approximately 50 μM in both cell lines. However, the IC_{90} was approximately 150 μM in the parental HL60 cells and 100 μM in the MDR cells. The activity of TBZ was then compared with that of three standard anticancer agents, daunorubicin, etoposide and mitoxantrone (Table 1). These drugs were active at concentrations lower than TBZ in the parental cells. Nevertheless, the IC_{50}s of daunorubicin, etoposide and mitoxantrone were 200, 400 and 20-fold higher, respectively, in the MDR than in the parental cells (Table 1).

Cytofluorimetric and morphological analysis of apoptosis

Figure 3 shows the DNA frequency distribution histograms of HL60 and HL60R cells following their treatment for 48 h with IC_{50}s of daunorubicin, etoposide or mitoxantrone. For the HL60 cell line, all three drugs and especially daunorubicin caused an evident increase in a sub-diploid peak, typical of apoptotic cells. The same phenomenon did not occur in the HL60R cells. The morphological examination gave very similar results. Many of the HL60 cells treated with the drugs had apoptotic features (Figure 4b) and their relative percentages were superimposable on those calculated on the basis of the cytofluorimetric analysis. In contrast, the majority of HL60R cells treated with daunorubicin, etoposide or mitoxantrone were alive, as indicated by their uniform staining with acridine orange. Approximately 8–10% of them showed necrotic features and were uniformly stained with ethidium bromide (Figure 4c). Morphological examination showed that TBZ induced moderate apoptosis in the parental HL60 cells, even after a 72 h exposure to concentrations of up to 100 μM (Figure 5a). In contrast, for MDR cells, approximately 40 and 80% were apoptotic after a 48 h exposure to 75 and 100 μM TBZ, respectively. 72 and 100% of the HL60R cells were apoptotic after 72 h of exposure to 75 and 100 μM TBZ, respectively (Figure 5b). Figure 4 (d,e) shows the morphology of HL60 and HL60R cells treated for 48 h with 75 μM TBZ. Figure 6 shows the DNA frequency distribution histograms of the HL60 or HL60R cells treated for 48 h with 75 μM TBZ and indicates the relative increases in the sub-diploid peak. An overall comparison of the apoptotic activity of TBZ, daunorubicin, etoposide and mitoxantrone in the parental and MDR HL60 cells is presented in Figure 7.

Determination of P-glycoprotein, p53 and Bcl-2 protein

Flow cytometry determinations showed that, in contrast to HL60 cells, HL60R were clearly positive for P-glycoprotein expression (Figure 8). There was a lack of expression of p53 protein in both HL60 and HL60R cells (data not shown). HL60 and HL60R cells showed a superimposable expression of Bcl-2 protein (Figure 8).

DISCUSSION

TBZ (NSC 625487) has been identified as the most active derivative of a new class of anti-HIV agents, the thiazolobenzimidazoles [21–27]. At a concentration of approximately 1 μM, TBZ has been shown to inhibit HIV-1-induced cell killing and viral replication in several human cell lines, as well as fresh human peripheral blood lymphocytes and macrophages [21, 22, 25–27]. It is an inhibitor of HIV-1 reverse transcriptase [22, 25–27].

In this paper, we investigated the antitumour effects that the compound exerts on the human promyelocytic HL60 leukaemia and on one MDR, P-glycoprotein expressing, variant (HL60R) of this cell line. The pro-apoptotic p53 protein is not present in HL60 cells, owing to major deletions of its gene [28] and, as expected, HL60R cells also lack p53 protein. In contrast, HL60 and HL60R cells showed similar levels of the anti-apoptotic factor Bcl-2 protein.

TBZ exhibited cytotoxic activity on the cell lines when used at concentrations higher than those reported as necessary to exert antiviral activity *in vitro* [21, 22, 25–27]. Interestingly, the activity of the compound on the drug sensitive and resistant cells was different from that shown by conventional anticancer agents such as daunorubicin, etoposide and mitoxantrone. First, TBZ produced equal or even greater cytotoxicity in the drug resistant cells. The IC_{50} of TBZ was in fact 50 μM in both the cell lines, and the IC_{90} was 150 μM in HL60 and 100 μM in HL60R. Moreover, TBZ induced remarkable apoptosis in the drug resistant cells. For example, 40 and 80% of the HL60R cells were apoptotic after 48 h of treatment with 75 and 100 μM TBZ, respectively. In contrast, HL60 cells showed no necrotic and scarce apoptotic

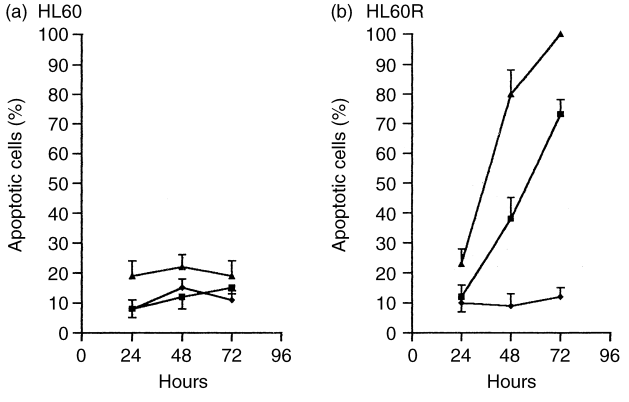

Figure 5. Percentage of apoptotic cells after 24, 48 or 72 h exposure to 1-(2,6-difluorophenyl)-1*H*,3*H*-thiazolo[3,4-a]benzimidazole (TBZ): 50 μM (◆); 75 μM (■); 100 μM (▲). Apoptotic cells were evaluated by morphological examination as described in Materials and Methods.

effects, even after 72 h of exposure to high concentrations of TBZ. The chemotherapeutic drugs caused evident apoptosis in the parental cells and were unable to induce it in the drug resistant ones, even when added at fully cytotoxic concentrations (IC_{90}s). With reference to the latter result, daunorubicin, etoposide, and mitoxantrone all induce cytotoxicity and apoptosis, at least in part, through interaction with topoisomerase II [12–14, 30, 31]. Thus, it is possible that, apart from P-glycoprotein expression, HL60R cells are also endowed with a qualitative and/or quantitative defects of the DNA enzyme, as observed in other tumour cell lines resistant to these agents ([32] and for a review, see [33]).

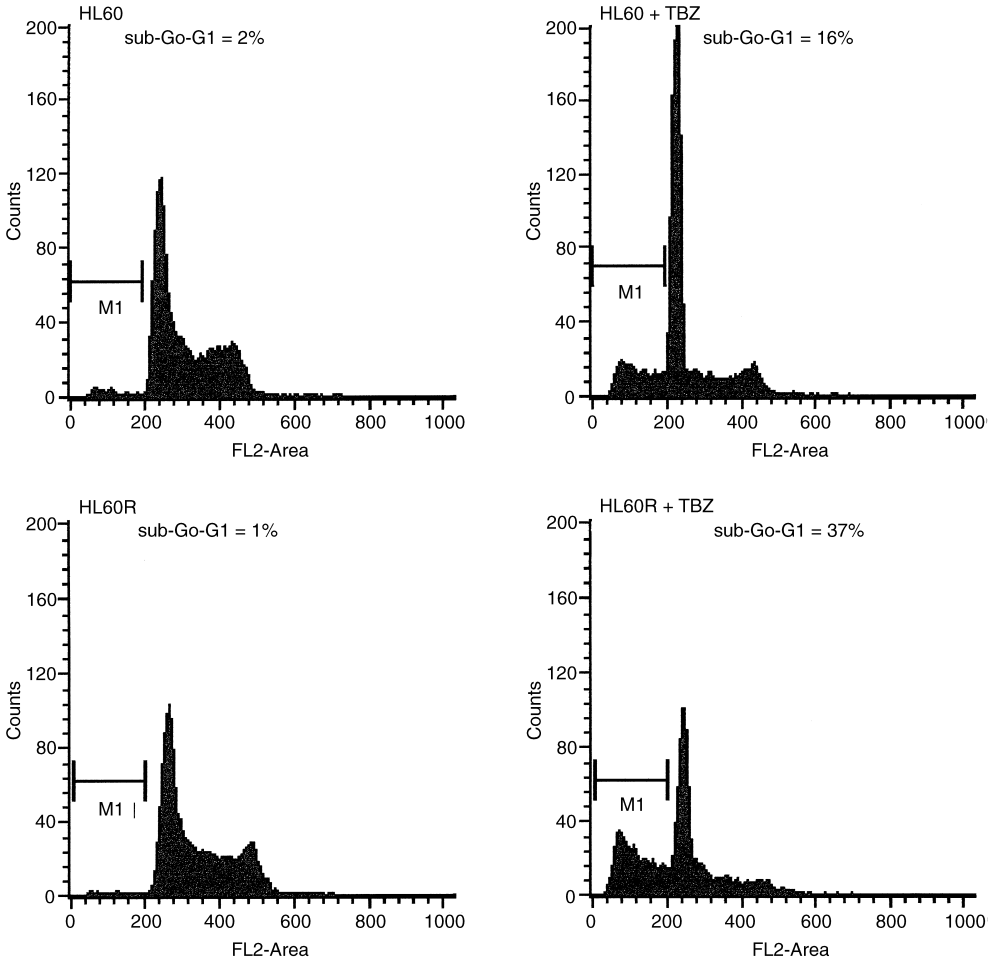

Figure 6. Flow cytometric assay of cell cycle distribution of HL60 and HL60R cells after 48 h exposure to 75 μM 1-(2,6-difluorophenyl)-1*H*,3*H*-thiazolo[3,4-a]benzimidazole (TBZ).

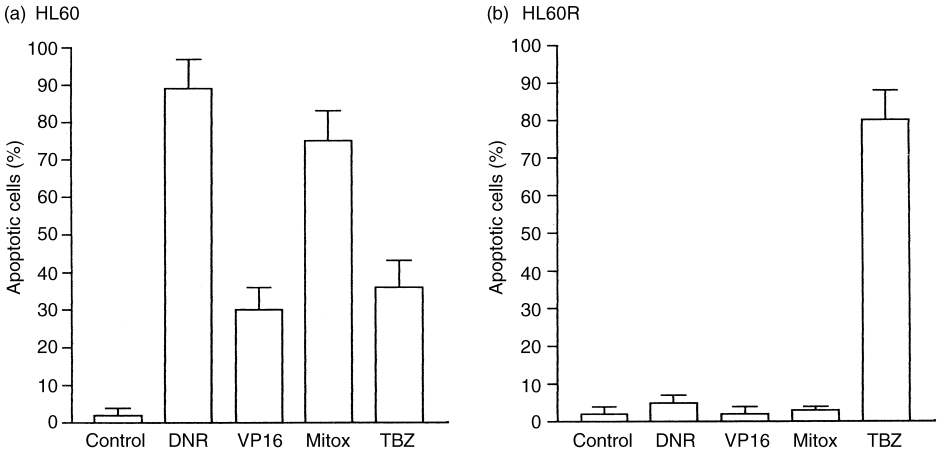

Figure 7. Percentage of apoptotic cells in (a) HL60 and (b) HL60R cells after 48 h exposure to daunorubicin (DNR), etoposide (VP16), mitoxantrone (Mitox) or 1-(2,6-difluorophenyl)-1*H*,3*H*-thiazolo[3,4-a]benzimidazole (TBZ) at IC_{90}s.

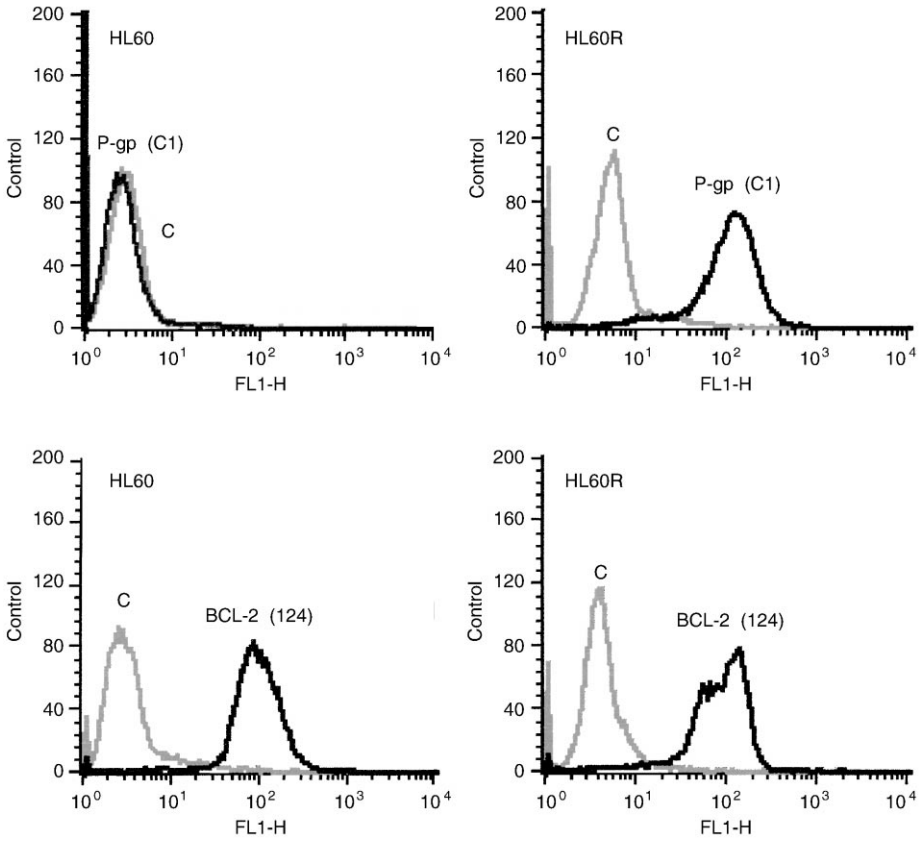

Figure 8. Evaluation of the expression of P-glycoprotein or Bcl-2 protein by flow cytometry. C indicates the fluorescence associated with the respective isotypic negative monoclonal antibodies.

In conclusion, TBZ was able to overcome the P-glycoprotein mechanism in the HL60R cells, thus suggesting that it is not a suitable substrate for the multidrug transporter. In addition, it caused more apoptosis in the drug resistant cells. This result could not be explained by a variation in the p53 or Bcl-2 protein levels of the cell line. It suggests that TBZ induced apoptosis through a pathway different from that related to the chemotherapeutic agents and needs to be better clarified at the molecular level. Nevertheless, Del Bufalo and colleagues [34] observed similar effects using lonidamine, a derivative of indazole-3-carboxylic acid. Lonidamine induced apoptosis in two drug resistant human cancers, the MCF-7 ADR human breast cancer cell line and the LB9 glioblastoma multiform cell line, but not in the corresponding parental tumours. These effects occurred independently of p53 and Bcl-2 expression and were attributed to the interference of lonidamine with the mitochondrial function and/or energetic metabolism of the cells; drug resistant cells can in fact exhibit an enhanced rate of glycolysis and an increased demand for ATP production [35].

Although information on *in vivo* pharmacology and toxicology of TBZ is still scanty, it has been shown that the plasma concentrations required to kill HL60 and HL60R cells (i.e. $>50\,\mu M$) are maintained for 1–2 h following the administration of the maximum tolerated intravenous dose of TBZ to mice [24]. TBZ is rapidly inactivated *in vivo* [24], and synthetic efforts are in progress to block its metabolism with retention of activity. Since the present findings have suggested a possible selective activity of TBZ on MDR tumour cells, we think that further studies aimed at better defining the possible specificity and mechanisms of its antitumour and pro-apoptotic action should be carried out.

1. Rothenberg M, Ling V. Multidrug resistance: molecular biology and clinical relevance. *J Natl Cancer Inst* 1989, **81**, 907–910.
2. Dietel M, Albrechts C. Second international symposium on cytostatic drug resistance. *Cancer Res* 1993, **53**, 2683–2688.
3. Gottesman MM. How cancer cells evade chemotherapy: sixteenth Richard and Hinda Rosenthal foundation award lecture. *Cancer Res* 1993, **53**, 747–754.
4. Martin SJ, Green DR. Apoptosis and cancer: the failure of controls on cell death and cell survival. *Crit Rev Oncol Hematol* 1995, **18**, 137–153.
5. Boobis AR, Fawthrop DJ, Davies DS. Mechanisms of cell death. *Trend Pharm Sci* 1989, **10**, 275–280.
6. Kerr MR, Wyllie AH, Currie AR. Apoptosis: a basic biological phenomenon with wide-ranging implications in tissue kinetics. *Br J Cancer* 1972, **26**, 239–257.
7. Williams GT. Programmed cell death: apoptosis and oncogenesis. *Cell* 1991, **65**, 1097–1098.
8. Williams GT, Smith CA. Molecular regulation of apoptosis: genetic controls on cell death. *Cell* 1993, **74**, 777–779.
9. Carson DA, Ribeiro JM. Apoptosis and disease. *Lancet* 1993, **341**, 1251–1254.
10. Lennon SV, Martin SJ, Cotter TG. Dose-dependent induction of apoptosis in human tumor cell lines by widely diverging stimuli. *Cell Prolif* 1991, **24**, 203–214.
11. Collins RJ, Harmon BV, Souvlis T, Pope JH, Kerr JFR. Effects of cycloheximide on B-chronic lymphocytic leukaemic and normal lymphocytes *in vitro*: induction of apoptosis. *Br J Cancer* 1991, **64**, 518–522.
12. Walker PR, Smith C, Youdale T, Leblanc J, Whitfield JF, Sikorska M. Topoisomerase II-reactive drugs induce apoptosis in thymocytes. *Cancer Res* 1991, **51**, 1078–1085.

13. Del Bino G, Darzynkiewicz Z. Camptothecin, teniposide, or 4′-(9-Acridinylamino)-3-methanesulfon-m-anisidide, but not mitoxantrone or doxorubicin induces degradation of nuclear DNA in the S phase of HL-60 cells. *Cancer Res* 1991, **51**, 1165–1169.
14. Kapuscinski J, Darzynkiewicz Z. Relationship between the pharmacological activity of antitumor drugs ametantrone and mitoxantrone (novantrone) and their ability to condense nucleic acids. *Proc Natl Acad Sci USA* 1986, **83**, 6302–6306.
15. Rotter V, Foord O, Navot N. In search of the functions of normal p53 protein. *Trend Cell Biol* 1993, **3**, 46–49.
16. Lowe SW, Schmitt EM, Smith SW, Osborne BA, Jacks T. p53 is required for radiation-induced apoptosis in mouse thymocytes. *Nature* 1993, **362**, 847–849.
17. Lowe SW, Ruley BE, Jacks T, Housman DE. p53-dependent apoptosis modulates the cytotoxicity of anticancer agents. *Cell* 1993, **74**, 951–967.
18. Kinzler KW, Bogelstein B. Clinical implications of basic research: cancer therapy meets p53. *New Eng J Med* 1994, **331**, 49–50.
19. Hockenbery D, Nunez G, Milliman C, Schreiber RD, Korsmeyer SJ. Bcl-2 is an inner mitochondrial membrane protein that blocks programmed cell death. *Nature* 1990, **348**, 334–338.
20. Miyashita T, Reed JC. Bcl-2 oncoprotein blocks chemotherapy-induced apoptosis in a human leukemia cell line. *Blood* 1993, **81**, 151–157.
21. Chimirri A, Grasso S, Monforte AM, Monforte P, Zappala' M. Anti-HIV agents II: synthesis and in vitro anti-HIV activity of novel 1H,3H-thiazolo[3,4-a]benzimidazoles. *Il Farmaco* 1991, **46**, 925–933.
22. Hizi A, Tal R, Shaharabany M, *et al.* Specific inhibition of the reverse transcriptase of human immunodeficiency virus type 1 and the chimeric enzymes of human immunodeficiency virus type 1 and type 2 by nonnucleoside inhibitors. *Antimicrob Agents Chemother* 1993, **37**, 1037–1042.
23. Tinwalla AY, Hoesterey BL, Xiang T, Anderson BD. Solubilization of thiazolobenzimidazole using a combination of pH adjustment and complexation with 2-hydroxypropyl-β-cyclodextrin. *Pharm Res* 1993, **10**, 1136–1143.
24. El Dareer SM, Tillery KF, Rose LM, *et al.* Metabolism and disposition of a thiazolobenzimidazole active against human immunodeficiency virus-1. *Drug Metab Disp* 1993, **21**, 231–235.
25. Buckheit RW, Hollingshead MG, Germany-Decker J, *et al.* Thiazolobenzimidazole: biological and biochemical anti-retroviral activity of a new nonnucleoside reverse transcriptase inhibitor. *Antiviral Res* 1993, **21**, 247–265.
26. Artico M. Non nucleoside anti-HIV reverse transcriptase inhibitors (NNRTIs); a chemical survey from lead compounds to select drugs for clinical trials. *Il Farmaco* 1996, **51**, 305–331.
27. Chimirri A, Grasso S. Molica C, *et al.* Structural features and anti-HIV activity of the isomers of 1-(2′,6′-difluorophenyl)-1H,3H-thiazolo[3,4-a]benzimidazole, a potent non-nucleoside HIV-1 reverse transcriptase inhibitor. *Antiviral Chem Chemother* 1997, **8**, 83–90.
28. Wolf D, Rotter V. Major deletions in the gene encoding the p53 expression in HL60 cells. *Proc Natl Acad Sci USA* 1985, **82**, 790–794.
29. Duke RC, Cohen JJ. Morphological and biochemical assays of apoptosis. In JE Coligan, AM Kruisbeak, eds. *Current Protocols in Immunology*. New York, John Wiley & Sons, 1992, 3.17.1–3.17.16.
30. Kaufmann SH. Induction of endonucleolytic DNA cleavage in human acute myelogenous leukemia cells by etoposide, camptothecin and other cytotoxic anticancer drugs: a cautionary note. *Cancer Res* 1989, **49**, 5870–5878.
31. Bertrand R, Kerrigan D, Sarang M, Pommier Y. Cell death induced by topoisomerase inhibitors. Role of calcium in mammalian cells. *Biochem Pharmacol* 1991, **42**, 77–85.
32. Harker GW, Slade DL, Parr RL, Feldhoff PW, Sullivan DM, Hoguin MH. Alterations in the topoisomerase IIalpha gene, messenger RNA, and subcellular protein distribution as well as reduced expression of the DNA topoisomerase II beta enzyme in a mitoxantrone-resistant HL-60 human leukemia cell line. *Cancer Res* 1995, **55**, 1707–1716.
33. Flandina C, Flugy A, Borsellino N, D'Alessandro N. Development and partial characterization of a human T-lymphoblastic leukemic (CCRF-CEM) cell line resistant to etoposide. Analysis of possible circumventing approaches. *J. Chemother* 1996, **8**, 465–471.
34. Del Bufalo D, Biroccio A, Soddu S, *et al.* Lonidamine induces apoptosis in drug-resistant cells independently of the p53 gene. *J Clin Invest* 1996, **98**, 1165–1173.
35. Lyon RC, Cohen JS, Faustino PJ, Megninand F, Myers CE. Glucose metabolism in drug-sensitive and drug-resistant human breast cancer cells monitored by magnetic resonance spectroscopy. *Cancer Res* 1988, **48**, 876–877.

Acknowledgement—This paper was partially supported from Associazione Italiana per la Ricerca sul Cancro.