ELSEVIER

Biochemical Pharmacology 65 (2003) 755–763

Biochemical Pharmacology

www.elsevier.com/locate/biochempharm

Apoptotic cell death induction by F 11782 a novel dual catalytic inhibitor of topoisomerases I and II

Chantal Etiévant*, Anna Kruczynski, Jean-Marc Barret, Dominique Perrin, Bridget T. Hill

Division de Cancérologie Expérimentale I, Centre de Recherche Pierre Fabre, 17 Avenue Jean Moulin, F-81106 Castres Cedex 06, France

Received 22 April 2002; accepted 10 November 2002

Abstract

F 11782 (2″,3″-bis-pentafluorophenoxyacetyl-4″,6″ethylidene-β-D-glucoside of 4′-phosphate-4′-dimethylepipodophyllotoxin-2*N*-methyl glucamine salt), is a novel dual catalytic inhibitor of topoisomerases I and II characterised by marked *in vivo* antitumour activity, which also proved cytotoxic and exhibited DNA damaging properties *in vitro*. Mechanisms associated with this cell killing by F 11782 have been examined in P388 leukaemia cells. Treatment with F 11782 resulted in a dose-dependent DNA fragmentation coupled with the characteristic morphological features of apoptosis. Apoptosis-inducing concentrations of F 11782 induced caspases-3/7 activation accompanied by proteolytic cleavage of poly(ADP-ribose)-polymerase, which could be inhibited by the caspase inhibitor acetyl-Asp-Glu-Val-Asp-aldehyde. In addition, F 11782-induced apoptosis in P388 cells was associated with an increased expression of the pro-apototic Bax protein, without significant changes in the level of the anti-apoptotic Bcl-2 protein, and with modification at the mitochondrial membrane function. These results indicate that F 11782 leads to apoptosis through a caspase-3/7 dependent mechanism and suggest that the so-called ‘‘mitochondrial pathway’’ is implicated in F 11782-induced apoptosis in P388 cells.

Keywords: F 11782; Topoisomerases; Dual inhibitors; Apoptosis; P388 leukaemia; *In vitro*

1. Introduction

DNA topoisomerases I and II, implicated in topological changes in DNA essential for DNA replication and transcription, are the cellular targets of several clinically-useful anticancer agents [1,2]. The majority of these compounds, commonly referred to as ‘‘topoisomerase poisons’’, such as etoposide and camptothecin, exert their cytotoxicity by stabilising a ternary DNA–drug–enzyme complex (cleavable complex), which leads to DNA breaks considered to initiate apoptotic cell death [3]. Indeed, cells that die after treatment with topoisomerase-directed agents often display morphological and biochemical features of apoptosis [4]. A second emerging group of topoisomerase-interacting agents that inhibit the catalytic activity of topoisomerase II without stabilising cleavable complexes, include aclarubicin, bisdioxopiperazine derivatives and merbarone [5]. Although this second class of topoisomerase II-targeting agents is known to result in G_2-M arrest and subsequent apoptosis, the precise mechanisms triggering cell killing remain unclear.

Apoptosis, or programmed cell death, is a genetically regulated process occurring naturally in response to a variety of signals and resulting in numerous cellular changes such as membrane blebbing, cell shrinkage, nuclear condensation, DNA fragmentation and formation of apoptotic bodies [6]. The initiation and execution stages of apoptosis involve activation of a cascade of aspartate-specific cysteine proteases, namely the caspases [7]. Amongst these, caspase-3, is activated in many cell types during apoptosis. Once activated, caspase-3 cleaves specific substrates including the nuclear protein poly(ADP-ribose)-polymerase (PARP), involved in DNA repair and genome maintenance. Proteolytic cleavage of PARP was first recognised as a hallmark feature of apoptosis after exposure of human tumour cells to chemotherapeutic agents [8]. Furthermore, anti- or pro-apoptotic proteins belonging to the Bcl-2 protein family have been associated with the regulation of apoptosis induced by a variety of stimuli including chemotherapeutic drugs such as the topoisomerase inhibitors [9–13].

* Corresponding author. Tel.: +33-563-71-42-11; fax: +33-563-71-42-99.

E-mail address: chantal.etievant@pierre-fabre.com (C. Etiévant).

Abbreviations: PARP, poly(ADP-ribose)-polymerase; PBS, phosphate buffer saline; Ac-DEVD-CHO, acetyl-Asp-Glu-Val-Asp-aldehyde; Ac-DEVD-*p*-NA, acetyl-Asp-Glu-Val-Asp-*p*-nitraniline; MTT, (3-(4,5-dimethyl-thiazol-2-yl)-2,5-diphenyltetrazolium bromide).

0006-2952/02/$ – see front matter
PII: S0006-2952(02)01564-2

F 11782, is a novel pentafluorinated epipodophylloid characterised by marked *in vivo* antitumour activity [14], and a unique mode of interaction with topoisomerases I and II [15], preventing interaction of both enzymes with DNA, yet without either intercalating DNA or stabilising cleavable complexes *in vitro* [15,16]. Thus F 11782 appears to act as a catalytic inhibitor of both nuclear enzymes and is unusual in this respect. At the cellular level, F 11782 proved cytotoxic against a panel of murine leukaemia and human tumour cell lines, resulting in an accumulation of cells in the G_2-M phases of the cell cycle [17]. Furthermore, F 11782 induced DNA-strand breakage in tumour cells, but in a manner quite different from etoposide [18,19], and was shown to inhibit DNA repair synthesis, with a preferential effect on the incision step [20], thus emphasising the uniqueness of its overall mechanism of action.

The present study was undertaken to investigate whether the cytotoxicity of F 11782 was associated with an ability to induce apoptotic cell death, using various morphological and biochemical assays, in murine P388 leukaemia cells known to enter apoptosis after exposure to other cytotoxic agents.

2. Materials and methods

2.1. Cells and compounds

P388 murine leukaemia cells (National Cancer Institute, Tumour Repository), maintained *in vivo* in the peritoneal cavities of DBA/2 mice, were collected and adapted to *in vitro* cell culture in RPMI 1640 medium supplemented with 10% heat-activated horse serum, 4 mM L-glutamine, 1.25 μg/mL fungizone, 100 μg/mL penicillin-streptomycin, and 20 μM β-mercaptoethanol. F 11782 (2″,3″-bis-pentafluorophenoxyacetyl-4″,6″ethylidene-β-D-glucoside of 4′-phosphate-4′-dimethylepipodophyllotoxin-2*N*-methyl glucamine salt), recently named Tafluoposide, was provided by Pierre Fabre Médicament and solubilised in dimethyl sulfoxide (SDS).

2.2. Cell cycle analysis

Flow cytometric analyses of cellular DNA content by propidium iodide staining was performed as reported previously [17]. Briefly, P388 cells were incubated with test compound for 24 hr prior to centrifugation and washing. Cells were then prepared for DNA content measurements using the Coulter DNA-Prep reagent kit (Beckman Coulter) and 15,000 cells were analysed using a Coulter Epics XL flow cytometer. Percentages of cells in the various cell cycle phases were calculated using the M Cycle software. This software permits first a determination of the percentage of apoptotic cells in the sub-G_1 region within the total cell population (apoptotic peak), and secondly a calculation separately of the distribution of the non-apoptotic cells ($\geq G_1$) in the cell cycle phases.

2.3. DNA fragmentation measurements and cellular morphological assessments

DNA fragmentation was quantified as previously described [21]. P388 cells (2×10^5 cells/mL) prelabelled with 0.02 μCi/mL [^{14}C]thymidine, were treated with increasing concentrations of F 11782 for 24 hr, then pelleted and lysed in ice-cold phosphate buffered saline (PBS) buffer containing 0.5% Triton-X100 and 20 mM ethylenediaminetetraacetate (EDTA), pH 8 (30 min, 4°). The cell lysates were then centrifuged (12,000 *g*, 30 min, 4°) to separate low (supernatant) and high (pellet) molecular weight DNA. Radioactivity was measured in each fraction and the amount of fragmented [^{14}C]DNA (low molecular weight) was expressed as a percentage of the total [^{14}C]DNA. Percentages of DNA fragmentation in treated (F) and control (F_0) cells were calculated and final results were expressed as drug-specific DNA fragmentation (%) in treated cells using the following formula: $(F - F_0/100 - F_0)100$.

For morphological assessments, cells were prepared for electron microscopy as follows. Control or P388 cells treated with F 11782 for 24 hr were collected by centrifugation (400 *g*, 5 min at 4°). Cells were then fixed a first time with 50 nM potassium phosphate buffer, pH 7.4 containing 1.25% glutaraldehyde for at least 30 min at room temperature (RT), centrifuged (4000 *g*) and then fixed a second time for 2 hr at RT with 100 mM cacodylate buffer pH 7.2 containing 2.5% glutaraldehyde, before being washed twice with the previous buffer before incubation with 2% osmium tetraoxide (4 hr at 4°). Cells were then dehydrated (alcoholic gradient), impregnated with epoxy-1,2 propane and embedded in Epon resin (60° for 48 hr). Then 100–250 nm sections were prepared, mounted on copper grids, stained with uranyl acetate and lead citrate, before examination by transmission electron microscopy using an Philips EM-301 microscope.

2.4. Determination of caspase activation

Control or P388 cells (2×10^5 cells/mL) treated with F 11782 for 24 hr were washed once in ice-cold PBS and then lysed for 10 min on ice in a buffer containing 50 mM Hepes, pH 7.4, 0.1% Chaps, 1 mM DTT and 0.1 mM EDTA. After centrifugation (10,000 *g* for 5 min at 4°), caspase activity was determined in the supernatant (cytosol) in a 100 μL reaction volume using the caspases-3/7 specific coloured substrate, acetyl-Asp-Glu-Val-Asp-*p*-nitraniline (Ac-DEVD-*p*-NA from Biomol). Protein extracts (20 μg) were incubated with 200 μM substrate peptide in 50 mM Hepes, pH 7.4, 100 mM NaCl, 0.1% Chaps, 10 mM DTT, 1 mM EDTA and 10% glycerol. When used, the caspases-3/7 inhibitor acetyl-Asp-Glu-Val-Asp-aldehyde (Ac-DEVD-CHO from Biomol) was

added for 10 min prior to addition of the substrate peptide. Production of cleaved *p*-nitroaniline from the tetrapeptide substrate Ac-DEVD-*p*-NA was followed at a wavelength of 405 nm using a microplate reader (Dynatech MR 7000), allowing quantification of the total DEVD-specific protease activity. Assays were performed in triplicates and results are presented as average extinction ± SEM.

2.5. Combined cytotoxic effects with a caspase inhibitor

P388 cells, plated in 96-well microtiter plates, were either treated or not treated with 300 μM Ac-DEVD-CHO for 3 hr prior to the addition of increasing concentrations of F 11782 for a further 24 hr. The drug-induced growth-inhibiting effects were then determined using a colorimetric metabolic-dye-based 3-(4,5-dimethyl-thiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay as described earlier [16]. The effect of a defined association results from the fraction of cells affected by F 11782 alone, and the fraction of cells affected by the Ac-DEVD-CHO (inherent cytotoxic effect). Therefore, it is possible to calculate a theoretical additivity (TA) dose–response curve for F 11782 combined with the caspase inhibitor, using the formula:

$$\mathrm{TA}\,(\%) = F - \left(\mathrm{DEVD} \times \frac{F}{100}\right)$$

where *F*, effect of F 11782 alone; DEVD, Ac-DEVD-CHO-induced cell proliferation inhibition (%).

Based on this approach used earlier [16] and on each of the combinations tested here, the theoretical additivity dose–response curves have been calculated and then compared with the dose–response curves obtained experimentally. Data derived from two independent experiments are presented.

2.6. Western blots analyses

At different times after F 11782 addition *in vitro*, whole cell extracts (cell pellet boiled in sodium dodecyl sulphate (SDS) sample buffer) were prepared. Samples corresponding to 1.5×10^5 cells (40 μg protein) were subjected to SDS gel electrophoresis and the resolved proteins were transferred to nitrocellulose membranes (Amersham) and stained with the various primary antibodies after blocking (5% non fat milk + 10% foetal bovine serum). Horseradish peroxidase (HRP)-conjugated rabbit or goat antibodies (Interchim) were used as secondary antibodies (1/2000) prior to an enhanced chemoluminescence (ECL) detection procedure (Pierce). The following antibodies, anti-Bax (Upstate Biotechnology), anti-Bcl-2 (Santa Cruz Biotechnology) and anti-actin (Santa Cruz Biotechnology) were used at dilutions of 1/200, 1/100 and 1/100, respectively. Actin was taken as loading reference and used to standardise the quantification of each band after densitometric scanning (Fluor-S Multimager, Biorad). For PARP cleavage analyses an anti-PARP antibody recognising both 116-kDa native and 89-kDa degraded forms was used (Serotec) and both forms were quantified by densitometric scanning after exposure to increasing concentrations of F 11782. When used the caspases-3/7 inhibitor Ac-DEVD-CHO (300 μM) was added twice, 3 hr prior to and 3 hr after the addition of F 11782 for a further 24-hr period.

2.7. Assessment of the mitochondrial inner transmembrane potential ($\Delta\Psi_m$)

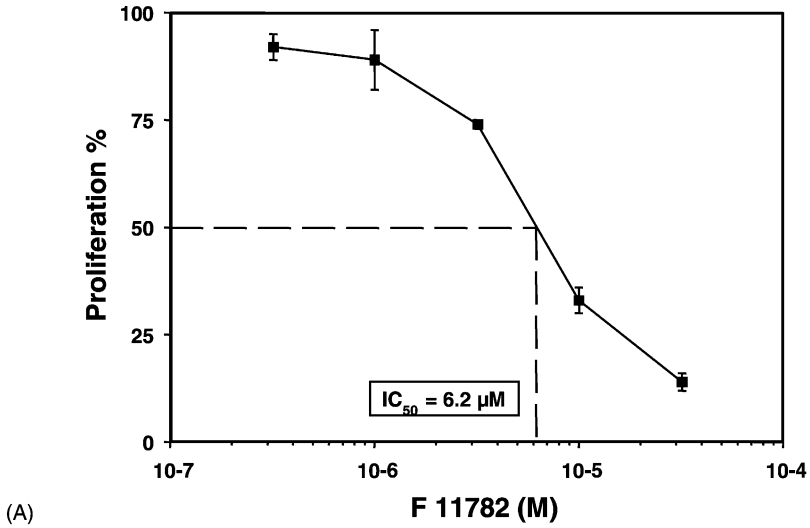
Flow cytometric analyses of $\Delta\Psi_m$ measurements were performed as reported earlier [22], with slight modifications. Briefly, exponentially-growing P388 cells were treated with increasing concentrations of F 11782 for 24 hr. After treatment, cells were stained with the $DiOC_6$ (Molecular Probes) fluorescent probe (5 nM final in PBS) for 15 min prior to fluorescence (FL1) measurements using Coulter Epics XL flow cytometer. The percentage of cells having a reduced $DiOC_6$ incorporation in treated or non-treated cells were determined, and the mean ± SEM values obtained from three independent experiments were calculated.

3. Results

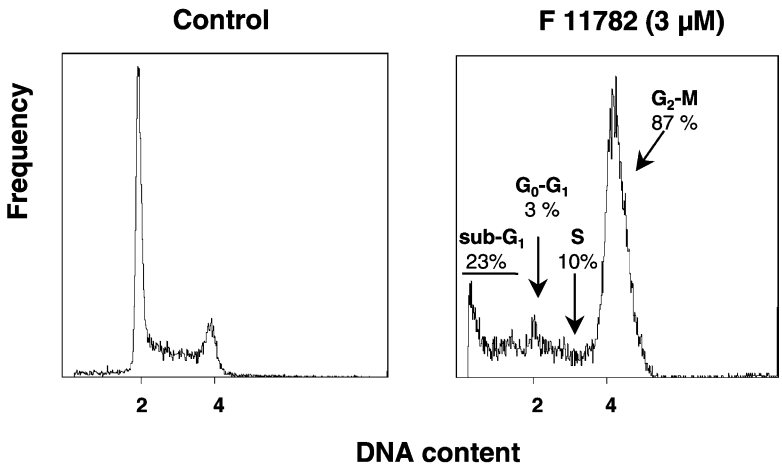
3.1. Effects of F 11782 on cell cycle distribution

The dose-dependent inhibitory effect of F 11782 against P388 leukaemia cells proliferation *in vitro* was first determined, after a 24-hr incubation period (Fig. 1A), using the MTT-based colorimetric assay described earlier [16]. A further investigation of the effects of F 11782 on the progression of P388 cells through the cell cycle, after this 24 hr incubation period, permitted the identification of cells with a hypodiploid DNA content (sub-G_1 cells illustrated in Fig. 1B), as well as a strong G_2-M blockage. No mitotic block was apparently associated with the G_2-M blockage, as determined microscopically after KCl-Giemsa cell labelling (data not shown).

The percentage of sub-G_1 cells (Fig. 1B) increased from 5 to 23% with increasing concentrations of F 11782 (0.5, 1 and 3 μM), with 1 and 3 μM F 11782 inducing 10 and 25% growth inhibition, respectively (Fig. 1A). The presence of cells with low DNA stainability, i.e. lower than that of G_1 cells, in cultured cells treated with cytotoxic agents, has been considered a marker of cell death by apoptosis [23], and has been linked to DNA fragmentation associated with apoptosis. Cell cycle analyses, together with cell proliferation measurements, thus suggest that, following F 11782 treatment, P388 cells accomplish apoptosis and that there is a good correspondence between the extent of apoptosis and growth inhibition. On the other hand, while 0.5 μM F 11782 was able to induce a significant accumulation of P388 cells in G_2-M, the percentage of sub-G_1 cells relative to untreated cells was unchanged and cell proliferation was unaffected.



(A)



(B)

(C)

Concentration of F 11782 (µM)	sub-G_1 population	Cell cycle distribution (%)		
		$G_0 - G_1$	S	G_2 - M
0	5	49 ± 2	39 ± 1	12 ± 1
0.5	5	8 ± 2	31 ± 1	61 ± 1
1	13	8 ± 1	7 ± 3	85 ± 3
3	23	3 ± 1	10 ± 2	87 ± 2

Fig. 1. Effects on cell proliferation and cell cycle distribution of logarithmically-growing P388 leukaemia cells following a 24-hr exposure to F 11782. (A) Concentration-dependent growth inhibiting effects of F 11782 after a 24-hr exposure, as measured by the metabolic-dye-based MTT assay described earlier [16]; (B) histogram of DNA content of untreated (control) cells and F 11782-treated cells; (C) percentage of sub-G_1 cells and cell cycle distribution of P388 cells treated with various concentrations of F 11782 for 24 hr. Means of duplicate experiments ± SEM.

These observations suggest that cells treated with this low concentration of F 11782 escape cell death.

3.2. *DNA fragmentation and morphological changes induced by F 11782*

Measurements of DNA fragmentation in [^{14}C]thymidine-prelabelled P388 cells, following a 24-hr incubation with a range of F 11782 concentrations (0.5–10 µM) revealed, as shown in Fig. 2A, that F 11782 induced DNA fragmentation in a dose-dependent manner, with mean values for DNA fragmentation ranging from 4 to 58%. The percentage of DNA fragmentation measured in cells treated with 1 µM F 11782 (30%) appeared high when compared to the percentage of sub-G_1 cells (13%) detected by flow cytometry (Fig. 1C), at this same concentration. Such a discrepancy

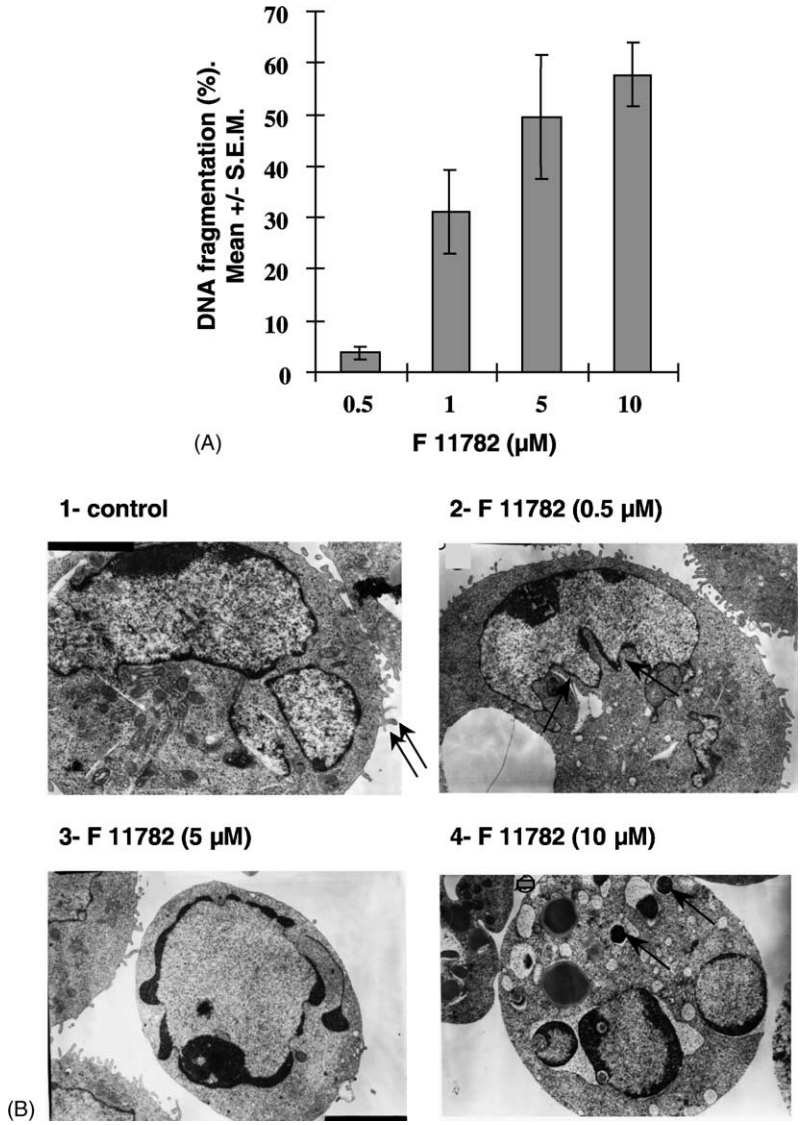


Fig. 2. Induction of apoptosis by F 11782. (A) F 11782-induced DNA fragmentation in P388 cells. [^{14}C]Thymidine prelabelled cells were exposed to 0.5–10 µM F 11782 for 24 hr. F 11782-induced fragmented DNA was measured using the intact chromatin precipitation assay, as detailed in Section 2. Results are expressed as means of triplicates ± SEM. (B) Electron microscopic examination of control cells (1; 18,200×) or cells exposed to 0.5–10 µM F 11782 (2–4; 11,400×) for 24 hr. (B1) Double arrows indicate specialised membrane structures in control cells; (B2) arrows indicate convolution of the nuclear membrane; (B3) condensed and marginated chromatin; (B4) arrows indicate a fragmented nuclei.

might be assigned to the specificity and sensitivity of the two different techniques used. Indeed, DNA fragmentation measured using the centrifugation method essentially identifies not only fragmentation linked to apoptosis (and necrosis) as commented on earlier [21], but also can reveal the DNA strand breakage activity of F 11782 [18,19]. Clearly, however, these DNA fragmentation data are consistent with a concentration-dependent induction of apoptosis by F 11782 in these P388 cells.

The ultrastructure of P388 cells was examined by transmission electron microscopy after exposure to F 11782 for 24 hr (Fig. 2B). Even at the lowest concentration of 0.5 µM, F 11782 induced certain morphological modifications, such as convolution of the nuclear membrane (Fig. 2B2). In the cell population treated with either 5 or 10 µM F 11782 most of the P388 cells had condensed, irregular, cresentic or beadlike marginated chromatin (Fig. 2B3), a fragmented nucleus (single arrow, Fig. 2B4), and condensed cytoplasm, yet with overall conservation of the cytoplasmic organelle structures themselves. Most F 11782-treated cells had also lost the specialised membrane structures observed in untreated cells (double arrows, Fig. 2B1), although the integrity of the plasma membrane was apparently maintained. Thus a large proportion of F 11782-treated cells displayed the typical morphological changes associated with apoptosis.

3.3. Effects of F 11782 on the activities of caspase proteases

Caspase activity was measured after a 24-hr exposure to F 11782 in cellular extracts of P388 cells using a

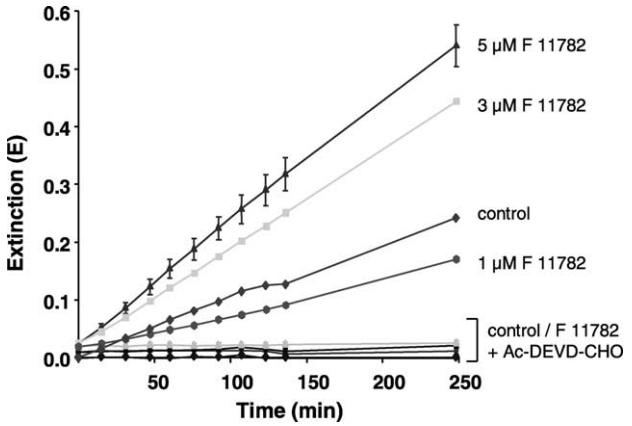


Fig. 3. Effects of F 11782 on caspases-3/7 activity. Enzymatic Ac-DEVD-*p*-NA cleavage activity in P388 cells extracts following a 24-hr incubation with 0 (control), 1, 3 or 5 µM F 11782. Twenty micrograms of protein from cell extracts prepared from control (untreated) or treated cells were assayed for caspases-3/7 dependent activity, over 250 min monitoring the rate of change of extinction at 405 nM, using the synthetic substrate Ac-DEVD-*p*-NA. Caspases-3/7 activation was also evaluated in the presence of 200 µM of Ac-DEVD-CHO, the caspases-3/7 inhibitor. Assays were performed in triplicates and results are expressed as the average of extinction values ± SEM.

colorimetric substrate (Ac-DEVD-*p*-NA) specific for caspases-3/7. As illustrated in Fig. 3A, F 11782 over the range of 3–5 µM, stimulated caspases-3/7 activities in a dose-dependent manner. No stimulation of caspases-3/7 activities was noted at 1 µM F 11782, a concentration inducing a clear G_2-M blockage, but with only a limited increase in the sub-G_1 cell population (Fig. 1C) and a marginal growth-inhibiting effect (Fig. 1A), thus suggesting only a small pro-apoptotic effect of F 11782 at 1 µM. Furthermore, Ac-DEVD-CHO was able to inhibit the F 11782-induced DEVD specific protease activation, confirming that caspases-3/7 were specifically activated.

3.4. Effects of F 11782 on PARP cleavage and cell growth inhibition

Caspase-3 is primarily responsible for the cleavage of PARP (116-kDa) during cell death, to produce an 89-kDa fragment, detectable by immunoblotting, using an antibody which recognises both native and degraded forms. A dose-dependent cleavage of PARP was observed (Fig. 4A), after a 24-hr exposure to 1–10 µM F 11782, with only 10% PARP cleavage product recorded at 1 µM, again suggesting the low pro-apototic effects of F 11782 at this concentration, yet with 26 and 48% being noted at the higher concentrations tested (Fig. 4A). Ac-DEVD-CHO (300 µM) inhibited ≥90% of the PARP cleavage induced by 5 µM F 11782 (Fig. 4B). These results provide evidence that caspase-3 mediates F 11782-induced PARP cleavage in P388 cells.

We then measured the cytotoxic effects induced by a 24-hr exposure to 1–10 µM F 11782 alone or in combination with the caspases-3/7 inhibitor Ac-DEVD-CHO (300 µM)

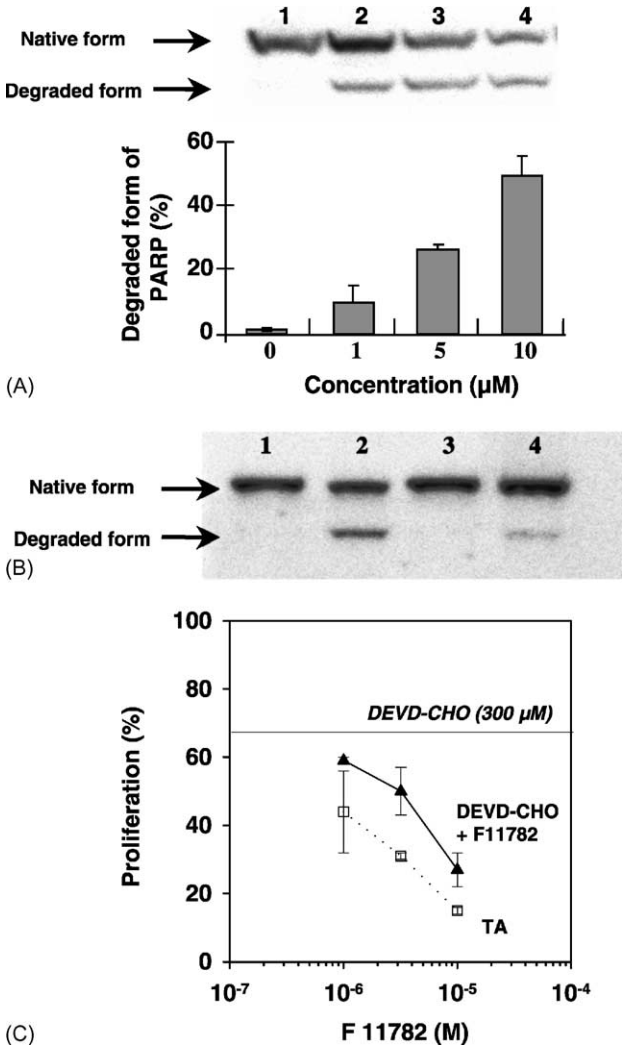


Fig. 4. Effects of the caspase inhibitor Ac-DEVD-CHO on F 11782-induced PARP cleavage and P388 cell proliferation. (A) Cleavage of PARP after a 24-hr incubation time of control P388 cells (lane 1) or cells treated with 1 µM (lane 2), 5 µM (lane 3) or 10 µM (lane 4) F 11782, as analysed by Western blot using an antibody against native and degraded forms of PARP. Results expressed as means (±SEM) from two to three independent experiments of the percentage of degraded form vs. total (degraded plus native) PARP forms. (B) Western blot of PARP cleavage in control cells (lane 1), or cells treated with 5 µM F 11782 alone (lane 2), 300 µM Ac-DEVD-CHO alone (lane 3), or both compounds (lane 4). When used, the caspase inhibitor was added 3 hr prior to and 3 hr after the addition of F 11782 for a further 24-hr. Results presented are representative blots of three separated experiments. (C) Effects of 300 µM Ac-DEVD-CHO on F 11782-induced cell proliferation inhibition. P388 cells were incubated for 3 hr with or without Ac-DEVD-CHO prior to the addition of increasing concentrations of F 11782 for a further 24-hr. Cell proliferation was then determined relative to control untreated cells for each condition. The theoretical addidity curve (□) was calculated (see Section 2) and compared with dose–response curve obtained experimentally with these associations (▲). The horizontal bar represent the inherent growth-inhibiting effect of the caspase inhibitor. Vertical bars indicate standard deviation from two independent experiments.

added 3 hr prior to F 11782. In order to take into account the inherent cytotoxic effect of the caspase inhibitor Ac-DEVD-CHO (resulting in approximately 35% of growth inhibition) in the combination, response curves obtained

experimentally were compared with a calculated theoretical additivity (TA) dose–response curve as described previously [16] and described in Section 2. A comparison of the experimental (□) and the calculated TA (▲) dose–response curves (Fig. 4C) revealed that the association was less than additive, suggesting antagonistic effects of combining Ac-DEVD-CHO and F 11782. Similar data were obtained evaluating combinations of the caspases-3/7 inhibitor and etoposide in experiments run in parallel (data not shown).

3.5. Effects of F 11782 on the expression of Bcl-2 and Bax

Cellular apoptosis is highly regulated by pro-apoptotic and anti-apoptotic modulators, including members of the Bcl-2 family. Amongst them, Bcl-2 prevents the initiation of the apoptotic programme, at least in part by hetero-dimerizing with its pro-apototic relative Bax [24]. Experiments were conducted to examine whether the expression of Bcl-2 and Bax were modified in P388 cells, after 6-, 18- or 24-hr of treatment with F 11782 (1–10 μM). The immunoblot analysis presented in Fig. 5A indicates that treatment of cells with increasing concentrations of F 11782, over this time period, did not significantly influenced the expression of Bcl-2 protein. On the other hand (Fig. 5B), a concentration dependent increase in Bax expression was noted after a 24-hr treatment with 1, 5 and 10 μM F 11782 (1.3-, 1.5- and 2.0-fold the control value, respectively), resulting in an increase in the Bax/Bcl-2 ratio for the cells treated with 5 and 10 μM F 11782 (ratios of 1.3 and 1.9, respectively). This induction of Bax after 5 μM F 11782 though appears only minimal when considering that exposure of cells to 3 or 5 μM F 11782 for 24 hr induced apoptosis in 23–30% of cells, as shown respectively by cell cycle analyses (Fig. 1) and DNA fragmentation assays (Fig. 2). However, these last two techniques do not exclude measurement of necrotic cells together with apoptotic cells, and this may account, at least in part, for this apparent discrepancy. Moreover, an increase in the Bax/Bcl-2 ratio, associated with the increase in Bax expression (1.6-fold), was also noted after 18 hr for cells treated with the highest concentration of F 11782 (10 μM). Comparable data were obtained with etoposide tested at 5 μM, an equicytotoxic concentration to 10 μM F 11782, with a Bax/Bcl-2 ratio of 1.8 and 2 after 18 and 24 hr treatment, respectively (data not shown). These data implicating a modification of Bax expression, and a resulting increase in the Bax/Bcl-2 ratio are consistent with other evidence of caspase activation by F 11782 in these P388 cells.

3.6. Modification of mitochondrial inner membrane potential by F 11782

Castedo *et al.* [25] reported that apoptotic cells often manifest a decrease of the mitochondrial inner membrane

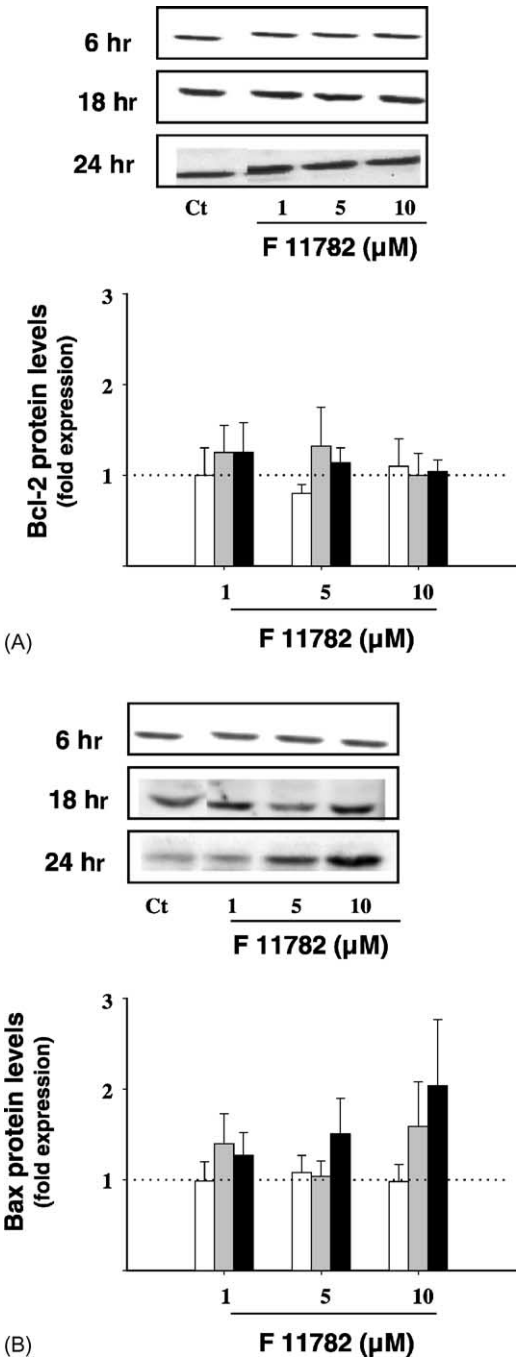


Fig. 5. Effects of F 11782 on levels of Bcl-2 and Bax proteins in P388 cells. Representative Western blots and quantitative analysis of Bcl-2 (A), or Bax protein levels (B), in cells exposed to F 11782 (1–10 μM) for 6 hr (white bars), 18 hr (grey bars) or 24 hr (black bars). Results, from three to five independent experiments, are expressed as average protein levels (fold expression) relative to control cells (dotted line) ± SEM.

potential ($\Delta\Psi_m$) that constitutes an irreversible event in the apoptotic process regulated by members of the Bcl-2/Bax family. Experiments were performed in order to examine whether $\Delta\Psi_m$ was modified in P388 cells, after a 24-hr treatment with F 11782 (1–10 μM), the time at which the highest Bax expression increase was noted. A concentration-dependent increase in the proportion (%) of cells with

a reduced $\Delta\Psi_m$ was noted following F 11782 treatment (42 ± 3, 74 ± 5 and $84 \pm 4\%$ after 1, 5 or 10 μM F 11782, respectively), relative to control (untreated) cells ($14 \pm 0.8\%$). These initial data are consistent with an alteration of mitochondrial membrane function associated with F 11782-induced apoptosis in these P388 cells.

4. Discussion

Previous studies have indicated that the novel fluorinated epipodophylloid, F 11782, which was shown uniquely to inhibit the catalytic activity of both topoisomerases I and II without stabilisation of cleavable complexes *in vitro* [15], and possessed markedly superior antitumour activity *in vivo* compared both to etoposide [14] and to an bisdioxopiperazine, a topoisomerase II catalytic inhibitor [17], also proved cytotoxic *in vitro* against a panel of human and murine tumour cell lines [16]. Whilst it has been proposed that the cell killing mechanism of the topoisomerase poisons correlates with their ability to induce cleavable complexes [26], the mechanism(s) responsible for the cytotoxicity of catalytic inhibitors of the topoisomerases, which do not stabilise cleavable complexes, remain to be elucidated. Studies of catalytic inhibitors, such as bisdioxopiperazines or merbarone, have suggested that the cytotoxicity of such compounds may involve a decatenation-sensing checkpoint which might be activated by abnormal mitoses resulting from topoisomerase II inhibition and polyploidisation [27–29]. However, a recent study [17] clearly showed that in response to F 11782 treatment, there was an accumulation of cells in the G_2-M phase of the cell cycle without any polyploidisation, suggesting that F 11782 and the bisdioxopiperazines function as quite distinctive catalytic inhibitors of topoisomerase II. Moreover, in recent studies, F 11782, unlike other catalytic inhibitors, was shown to induce DNA stand breaks [17–19], and was identified as a potent inhibitor of nucleotide excision repair (NER) [20]. These results emphasis the unique mode of action of this novel molecule. The goal of this study was to investigate the capacity of F 11782 to induce apoptosis and to identify the biochemical mechanisms of such apoptosis, using murine leukaemia P388 cells, a cell line sensitive to the cytotoxic effects of this compound and susceptible to apoptosis.

Initially it was noted that F 11782, which induced cell cycle arrest in P388 cells in the G_2 phase, also induced the appearance of a sub-G_1 cell population, considered as a marker of cell death by apoptosis. Consistent with this observation it was shown that F 11782 induced a dose-dependent DNA fragmentation, which is an end-stage apoptotic event. Furthermore, F 11782-treated cells displayed the characteristic morphological features of apoptosis, i.e. loss of specialised membrane structures, convolution and blebbing of cytoplasmic and nuclear membranes, condensed and marginated chromatin in the nucleus and generation of apoptotic bodies. Apoptosis induction was also clearly identified in A549 human tumour cells treated with F 11782, as shown by measuring cytoplasmic histone-associated-DNA-fragments using an enzyme-immunoassay (data not shown).

Further studies showed that exposure of P388 cells to F 11782 for 24 hr caused a dose-dependent activation of caspases-3/7 proteases, which play a central role in the biological processing of apoptosis induced by topoisomerase-targeted drugs [4]. Caspase-3 cleaves important cellular targets including PARP. PARP cleavage is considered as a valuable indicator of an early stage of apoptosis, which, indirectly demonstrates caspases activation. In P388 leukaemia cells, the F 11782-induced caspase-3 activation was shown to be associated with PARP degradation. Both caspase activation and PARP cleavage were prevented by the addition of Ac-DEVD-CHO, a caspases-3/7 inhibitor which also demonstrated antagonistic cytotoxicity when combined with F 11782. Together these data suggest that the dual catalytic inhibitor of topoisomerases I and II, F 11782, triggers cell death through apoptotic mechanisms involving the activation of caspases-3/7 in these P388 leukaemic cells.

One of the best characterised regulators of apoptosis is the Bcl-2-family of proteins. Bcl-2 is an intracellular suppressor of apoptosis which functions by heterodimerizing with its pro-apoptotic relative Bax [2]. Bcl-2 prolongs cell survival, at least in part, by preventing Bax from releasing cytochrome *c* from mitochondria [30,31]. Either no change in protein levels or increases in Bax protein and/or underexpression of Bcl-2 protein have been reported in cells treated with topoisomerase inhibitors [9–12]. Here we showed that treatment with F 11782 induced a dose-dependent increase of Bax protein expression in P388 cells after a 24-hr treatment, associated with no change in Bcl-2 protein expression. As a consequence, the Bax/Bcl-2 ratio was increased, which may contribute to caspase activation and apoptosis induction through the so-called mitochondrial pathway [31,32]. Indeed, at least two major apoptotic pathways have been described in mammalian cells, the ''mitochondrial pathway'' implicating a pro-apoptotic member of the Bcl-2 family and the death receptor pathway implicating the cell surface receptor Fas/Apo-1/CD95 and death ligands such as FasL and TRAIL [13], both pathways converging at the level of caspase-3 activation. As mentioned by Mow *et al.* [32], the mitochondrial pathway is used extensively in response to cellular stress, including DNA damage. Thus, the recently described DNA damaging properties of F 11782 [18,19] may support a potential role for the mitochondrial pathway, suggested by the F 11782-induced increase in the Bax/Bcl-2 ratio, in the induction of apoptosis by F 11782. Moreover, initial studies identified a the dose-dependent disruption of the mitochondrial inner membrane potential ($\Delta\Psi_m$), after a 24-hr treatment with F 11782, so serving to add weight to the suggested putative role of the mitochondrial apoptotic pathway in F 11782-induced apoptosis.

In summary, these data indicate that F 11782, a catalytic inhibitor of topoisomerases I and II that does not stabilised

cleavable complexes, induces apoptosis in P388 leukaemia cells, as first characterised by flow cytometric studies. F 11782-induced apoptosis was associated with DNA fragmentation and morphological features of programmed cells death. These apoptotic mechanisms were mediated by caspases-3/7 activation and PARP cleavage, and associated with an increase in Bax expression, without any concomitant change in Bcl-2 levels, as well as with alterations in mitochondrial membrane function. Overall, these studies describing the pro-apoptotic properties of F 11782 complement and extend our previous *in vitro* and *in vivo* studies aimed at an improved understanding of the mechanism of action of this unique compound. The data presented here contribute further to the interesting preclinical profile of activity of this novel agent, F 11782, now scheduled for Phase I clinical trials.

Acknowledgments

The authors wish to thank Nathalie Cabrol, Nathalie Chansard, Eric Chazottes, Christine Jean, Sylvie Rigaud and Sandrine Salicio and for their technical assistance.

References

[1] D.A. Burden DA, N. Oscheroff N. Mechanism of action of eukaryotic DNA topoisomerase II and drugs targeted to the enzyme. Biochim Biophys Acta 1998;1400:139–54.
[2] Y. Pommier Y, P. Pourquier P, Y. Fan Y, D. Strumberg D. Mechanism of action of eukaryotic DNA topoisomerase I and drugs targeted to the enzyme. Biochim Biophys Acta 1998;1400:83–106.
[3] B. Gatto B, G. Capranico G, M. Palumbo M. Drugs acting on DNA topoisomerases: recent advances and future perspectives. Curr Pharm Des 1999;5:195–215.
[4] S.H. Kaufmann SH. Cell death induced by topoisomerase-targeted drugs: more questions than answers. Biochim Biophys Acta 1998; 1400:195–211.
[5] T. Andoh T, R. Ishida R. Catalytic inhibitors of DNA topoisomerase II. Biochim Biophys Acta 1998;1400:155–71.
[6] A.H. Wyllie AH. Apoptosis: an overview. Br Med Bull 1997;53:451–65.
[7] G.M. Cohen GM. Caspases: the executioners of apoptosis. Biochem J 1997;326:1–16.
[8] F.C. Kaufmann FC, S. Desnoyer S, Y. Ottaviano Y, N.E. Davidson NE, G.G. Poirier GG. Specific proteolytic cleavage of PARP: an early marker of chemotherapy-induced apoptosis. Cancer Res 1993;53:3976–85.
[9] T.S. Huang TS, C.H. Shu CH, Y.L. Shih YL, H.C. Huang HC, Y.C. Su YC, Y. Chao Y, W.K. Yang WK, J. Whang-Peng J. Protein tyrosine phosphatase activities are involved in apoptotic cancer cell death induced by GL331, a new homolog of etoposide. Cancer Lett 1996;110:77–85.
[10] L. Garcia-Bermejo L, C. Perez C, N.E. Vilaboa NE, P. Aller P. cAMP increasing agents attenuate the generation of apoptosis by etoposide in promonocytic leukemia cells. J Cell Sci 1998;111:637–44.
[11] M. Sawada M, S. Nakashima S, Y. Banno Y, H. Yamakawa H, K. Hayashi K, K. Takenaka K, Y. Nishimura Y, N. Sakai N, Y. Nozawa Y. Ordering of ceramide formation, caspase activation, and Bax/Bcl-2 expression during etoposide-induced apoptosis in C6 glioma cells. Cell Death Differ 2000;7:761–72.
[12] J. Kluza J, A. Lansiaux A, N. Wattez N, C. Mahieu C, N. Osheroff N, C. Bailly C. Apoptotic response of HL-60 human leukemia cells to the antitumor drug TAS-103. Cancer Res 2000;60:4077–84.
[13] I. Petak I, J.A. Houghton JA. Shared pathways: death receptors and cytotoxic drugs in cancer therapy. Pathol Oncol Res 2001;7:95–106.
[14] A. Kruczynski A, C. Etiévant C, D. Perrin D, T. Imbert T, F. Colpaert F, B.T. Hill BT. Preclinical antitumour activity of F 11782, a novel dual catalytic inhibitor of topoisomerases. Br J Cancer 2000;83:1516–24.
[15] D. Perrin D, B. van Hille B, J.M. Barret JM, A. Kruczynski A, C. Etiévant C, T. Imbert T, B.T. Hill BT. F 11782, a novel non-intercalating dual inhibitor of topoisomerases I and II with an original mechanism of action. Biochem Pharmacol 2000;59:807–19.
[16] C. Etiévant C, A. Kruczynski A, J.-M. Barret J-M, D. Perrin D, B. van Hille B, Y. Guminski Y, B.T. Hill BT. F 11782, a dual inhibitor of topoisomerases I and II with an original mechanism of action in vitro, and markedly superior in vivo antitumour activity, relative to three other dual topoisomerase inhibitors, intoplicin, aclurubicin and TAS-103. Cancer Chemother Pharmacol 2000;46:101–13.
[17] B. van Hille B, C. Etiévant C, J.-M. Barret J-M, A. Kruczynski A, B.T. Hill BT. Characterization of the biological and biochemical activities of F 11782 and the bisdioxipiperazines, ICRF-187 and ICRF-193, two types of topoisomerase II catalytic inhibitors with distinctive mechanisms of action. Anticancer Drugs 2000;11:829–41.
[18] J.-M. Barret J-M, D. Montaudon D, C. Etiévant C, D. Perrin D, A. Kruczynski A, J. Robert J, B.T. Hill BT. Detection of DNA-strand breaks in cells treated with F 11782, a catalytic inhibitor of topoisomerases I and II. Anticancer Res 2000;20:4557–62.
[19] J.-M. Barret J-M, B.T. Hill BT, P. Olive P. Characterization of DNA-strand breakage induced in V79 cells by F 11782, a catalytic inhibitor of topoisomerases. Br J Cancer 2000;83:1740–6.
[20] J.M. Barret JM, M. Cadou M, B.T. Hill BT. Inhibition of nucleotide excision repair and sensitisation of cells to DNA cross-linking anticancer drugs by F 11782, a novel fluorinated epipodophylloid. Biochem Pharmacol 2002;63:251–8.
[21] R. Bertrand R, M. Sarang M, J. Jenkin J, D. Kerrigan D, Y. Pommier Y. Differential induction of secondary fragmentation by topoisomerase II inhibitors in human tumor cell lines with amplified c-*myc* expression. Cancer Res 1991;51:6280–5.
[22] A. Quillet-Mary A, J.-P. Faffrézou J-P, V. Mansat V, C. Bordier C, J. Naval J, G. Laurent G. Implication of mitochondrial hydrogen peroxide generation in ceramide-induced apoptosis. J Biol Chem 1997;272:21388–95.
[23] I. Nicoletti I, G. Migliorati G, M.C. Pagliacci MC, F. Grignani F, C. Riccardi C. A rapid and simple method for measuring thymocyte apoptosis by propidium iodide staining and flow cytometry. J Immunol Methods 1991;139:271–9.
[24] L.Z. Penn LZ. Apoptosis modulators as cancer therapeutics. Curr Opin Invest Drugs 2001;2:684–92.
[25] M. Castedo M, K. Ferri K, T. Roumier T, D. Metivier D, N. Zamzami N, G. Kroemer G. Quantitation of mitochondrial alterations associated with apoptosis. J Immunol Methods 2002;265:39–47.
[26] A.Y. Chen AY, L.F. Liu LF. DNA topoisomerases: essential enzymes and lethal targets. Annu Rev Pharmacol Toxicol 1994;34:191–218.
[27] R. Ishida R, T. Miki T, T. Narita T, R. Yui R, M. Sato M, K.R. Utsumi KR, K. Tanabe K, T. Andoh T. Inhibition of intracellular topoisomerase II by antitumor bis(2,6-dioxopiperazine) derivatives: mode of cell growth inhibition distinct from that of cleavable complex-forming type inhibitors. Cancer Res 1991;51:4909–16.
[28] M. Chen M, W.T. Beck WT. Differences in inhibition of chromosome separation and G2 arrest by DNA topoisomerase II inhibitors merbarone and VM-26. Cancer Res 1995;55:1509–16.
[29] P.B. Deming PB, C.A. Cistulli CA, H. Zhao H, P.R. Graves PR, H. Piwnica-Worms H, R.S. Paules RS, C.S. Downes CS, W.K. Kaufmann WK. The human decatenation checkpoint. Proc Natl Acad Sci USA 2001;98:12044–9.
[30] J.C. Reed JC. Bcl-2 family proteins. Oncogene 1998;17:3225–36.
[31] M.O. Hengartner MO. The biochemistry of apoptosis. Nature 2000;407: 770–2.
[32] B.M. Mow BM, A.L. Blajeski AL, J. Chandra J, S.H. Kaufmann SH. Apoptosis and the response to anticancer therapy. Curr Opin Oncol 2001;13:453–62.