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Inhibition of DNA topoisomerase I activity and induction of apoptosis by thiazacridine derivatives

Francisco W.A. Barros [a], Daniel P. Bezerra [b,*], Paulo M.P. Ferreira [c], Bruno C. Cavalcanti [a], Teresinha G. Silva [d], Marina G.R. Pitta [d], Maria do C.A. de Lima [d], Suely L. Galdino [d], Ivan da R. Pitta [d], Letícia V. Costa-Lotufo [a], Manoel O. Moraes [a], Rommel R. Burbano [e], Temenouga N. Guecheva [f], João A.P. Henriques [f], Cláudia Pessoa [a,*]

[a] Department of Physiology and Pharmacology, School of Medicine, Federal University of Ceará, Fortaleza, Ceará, Brazil
[b] Department of Physiology, Federal University of Sergipe, São Cristóvão, Sergipe, Brazil
[c] Department of Biological Sciences, Federal University of Piauí, Picos, Piauí, Brazil
[d] Department of Antibiotics, Federal, University of Pernambuco, Recife, Pernambuco, Brazil
[e] Institute of Biological Sciences, Federal University of Pará, Belém, Pará, Brazil
[f] Biotechnology Center, Federal University of Rio Grande do Sul, Porto Alegre, Rio Grande do Sul, Brazil

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ABSTRACT

Thiazacridine derivatives (ATZD) are a novel class of cytotoxic agents that combine an acridine and thiazolidine nucleus. In this study, the cytotoxic action of four ATZD were tested in human colon carcinoma HCT-8 cells: (5*Z*)-5-acridin-9-ylmethylene-3-(4-methylbenzyl)-thiazolidine-2,4-dione — **AC-4**; (5*ZE*)-5-acridin-9-ylmethylene-3-(4-bromo-benzyl)-thiazolidine-2,4-dione — **AC-7**; (5*Z*)-5-(acridin-9-ylmethylene)-3-(4-chloro-benzyl)-1,3-thiazolidine-2,4-dione — **AC-10**; and (5*ZE*)-5-(acridin-9-ylmethylene)-3-(4-fluoro-benzyl)-1,3-thiazolidine-2,4-dione — **AC-23**. All of the ATZD tested reduced the proliferation of HCT-8 cells in a concentration- and time-dependent manner. There were significant increases in internucleosomal DNA fragmentation without affecting membrane integrity. For morphological analyses, hematoxylin–eosin and acridine orange/ethidium bromide were used to stain HCT-8 cells treated with ATZD, which presented the typical hallmarks of apoptosis. ATZD also induced mitochondrial depolarisation and phosphatidylserine exposure and increased the activation of caspases 3/7 in HCT-8 cells, suggesting that this apoptotic cell death was caspase-dependent. In an assay using *Saccharomyces cerevisiae* mutants with defects in DNA topoisomerases 1 and 3, the ATZD showed enhanced activity, suggesting an interaction between ATZD and DNA topoisomerase enzyme activity. In addition, ATZD inhibited DNA topoisomerase I action in a cell-free system. Interestingly, these ATZD did not cause genotoxicity or inhibit the telomerase activity in human lymphocyte cultures at the experimental levels tested. In conclusion, the ATZD inhibited the DNA topoisomerase I activity and induced tumour cell death through apoptotic pathways.

Introduction

Topoisomerases are enzymes that regulate the overwinding or underwinding of DNA. They relax DNA supercoiling and perform catalytic functions during replication and transcription. There are two types of topoisomerases: type I enzymes that cleave one strand of DNA; and type II enzymes that cleave both strands. Both types of topoisomerases are essential for mammalian cell survival. Therefore, DNA topoisomerases are important targets for the development of cytotoxic agents (Miao et al., 2007; Moukharskaya and Verschraegen, 2012; Pommier et al., 2010; Vos et al., 2011). Topoisomerases I and II are important anticancer targets, and topoisomerase inhibitors such as camptothecin derivatives (e.g., topotecan and irinotecan), which are used clinically to inhibit the enzymatic activity of topoisomerase I (type I enzyme), and podophyllotoxin derivatives (e.g., etoposide and teniposide), which inhibit the enzymatic activity of topoisomerase II (type II enzyme) (Hartmann and Lipp, 2006) are used to block cancer growth.

Amsacrine (*m*-AMSA), an acridine derivative, was the first synthetic topoisomerase inhibitor approved for clinical treatment. Although *m*-AMSA is an intercalator and topoisomerase II inhibitor, its metabolism has been associated with the production of free radicals, which may cause serious harm to normal tissues (Belmont et al., 2007; Blasiak et al., 2003; Ketron et al., 2012; Sebestik et al., 2007).

A number of clinical and experimental studies have demonstrated that acridine and thiazolidine derivatives are promising cytotoxic

* Corresponding authors. Fax: +55 85 3366 8333.
E-mail addresses: danielpbezerra@gmail.com (D.P. Bezerra), cpessoa@ufc.br (C. Pessoa).

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agents. Recently, we described the synthesis of a novel class of cytotoxic agents, thiazacridine derivatives (ATZD), that couple the acridine and thiazolidine nucleus: (5*Z*)-5-acridin-9-ylmethylene-3-(4-methylbenzyl)-thiazolidine-2,4-dione (**AC-4**); (5*ZE*)-5-acridin-9-ylmethylene-3-(4-bromo-benzyl)-thiazolidine-2,4-dione (**AC-7**); (5*Z*)-5-(acridin-9-ylmethylene)-3-(4-chloro-benzyl)-1,3-thiazolidine-2,4-dione (**AC-10**); and (5*ZE*)-5-(acridin-9-ylmethylene)-3-(4-fluoro-benzyl)-1,3-thiazolidine-2,4-dione (**AC-23**). The chemical structures of these ATZD are illustrated in Fig. 1; their ability to interact with DNA was demonstrated using an electrochemical technique. These ATZD have demonstrated a solid tumour-selective cytotoxicity (Barros et al., 2012). Here, we study the mechanism of ATZD's selective cytotoxicity (**AC-4**, **AC-7**, **AC-10** and **AC-23**) in human colon carcinoma HCT-8 cells.

Material and methods

The synthesis of thiazacridine derivatives. The chemical data and synthetic procedures for (5*Z*)-5-acridin-9-ylmethylene-3-(4-methylbenzyl)-thiazolidine-2,4-dione (**AC-4**), (5*ZE*)-5-acridin-9-ylmethylene-3-(4-bromo-benzyl)-thiazolidine-2,4-dione (**AC-7**), (5*Z*)-5-(acridin-9-ylmethylene)-3-(4-chloro-benzyl)-1,3-thiazolidine-2,4-dione (**AC-10**) and (5*ZE*)-5-(acridin-9-ylmethylene)-3-(4-fluoro-benzyl)-1,3-thiazolidine-2,4-dione (**AC-23**) are reported elsewhere (Barros et al., 2012; Mourão et al., 2005; Silva et al., 2001). Thiazolidine-2,4-dione was *N*-(3)-alkylated in the presence of potassium hydroxide, which enabled the thiazolidine potassium salt to react with the substituted benzylhalide in a hot alcohol medium. The thiazacridine derivatives were synthesised by the nucleophilic addition of substituted 3-benzyl-thiazolidine-diones on 3-acridin-9-yl-2-cyano-acrylic acid ethyl ester. The mechanisms of cytotoxic action for the thiazacridine derivatives were studied as single *Z* isomers for **AC-4** and **AC-10**. The **AC-7** and **AC-23** compounds were studied as isomeric mixtures, but the *Z* isomer was the major stereoisomer.

Strains and media for the yeast assays. The *Saccharomyces cerevisiae* strains in this study were acquired from Euroscarf (European *Saccharomyces cerevisiae* Archive for Functional Analysis). The following *S. cerevisiae* genotypes were used in this study: BY-4741 (*MATa*; *his3*Δ *1*; *leu2*Δ *0*; *met15*Δ *0*; *ura3*Δ *0*); *Top1*Δ (YOL006c), same as BY4741 with *YOL006c*::*kanMX4*; *Top3*Δ (YLR234w), same as BY4741 with *YLR234w*::*kanMX4*. The media, solutions and buffers were prepared as previously described (Burke et al., 2000). Complete medium (YPD), containing 1% yeast extract, 2% peptone and 2% glucose was used for routine growth. The stationary-phase cultures were obtained by inoculating an isolated colony into liquid YPD medium and incubating the culture at 28 °C for 72 h with shaking (for aeration). Cultures in the exponential phase were obtained by inoculating 5×10^6 cells/ml of the stationary-phase YPD culture into fresh YPD medium at 28 °C for 2 h. The cell concentrations were determined in a Neubauer chamber using a light microscope (LO, Laboroptik GmbH, Bad Homburg, Hessen, Germany).

Cell lines and cell culture. The cytotoxicity of ATZD was evaluated using human colon carcinoma HCT-8 cells donated by the Children's Mercy Hospital, Kansas City, MO, USA. The cells were maintained in RPMI-1640 medium supplemented with 10% foetal bovine serum, 2 mM glutamine, 100 μg/ml streptomycin and 100 U/ml penicillin. The cells were kept in tissue-culture flasks at 37 °C in a humidified atmosphere with 5% CO_2 and were harvested with a 0.15% trypsin–0.08% EDTA, phosphate-buffered saline solution (PBS).

The following experiments were performed to determine ATZD's cytotoxic mechanisms in HCT-8 cells. For all cell-based assays, the HCT-8 cells were seeded (0.7×10^5 cells/ml) and incubated overnight to allow the cells to adhere to the plate surface. Then, the cells were treated for 12- and/or 24-h at concentrations of 2.5, 5 and/or 10 μg/ml, corresponding to: 6.1, 12.2 and 24.4 μM for **AC-4**; 5.3, 10.6 and 21.2 μM for **AC-7**; 5.8, 11.6 and 23.2 μM for **AC-10**; 6.0, 12.1 and 24.1 μM for **AC-23**, respectively. The trypan blue exclusion test was performed before each experiment described below to assess cell viability. The negative control was treated with the vehicle (0.1% DMSO) used for diluting the tested substances. Amsacrine (*m*-AMSA, 0.3 μg/ml [0.8 μM], Sigma Chemical Co. St Louis, MO, USA) or doxorubicin (0.3 μg/ml [0.6 μM], Sigma Chemical Co. St Louis, MO, USA) was used as the positive control. The concentrations of ATZD used here were based on their IC_{50} value in this cell line (3.1 μg/ml for **AC-4**, 5.3 μg/ml for **AC-7**, 3.6 μg/ml for **AC-10** and 2.3 μg/ml for **AC-23**) as previously described (Barros et al., 2012).

Trypan blue dye exclusion test. Cell proliferation was determined using the Trypan blue dye exclusion test. After each incubation period, the cell proliferation was assessed. Cells that excluded trypan blue were counted using a Neubauer chamber.

BrdU incorporation assay. Twenty microliters of 5-bromo-20-deoxyuridine (BrdU, 10 mM) was added to each well and incubated for 3 h at 37 °C before 24-h of drug exposure. To assess the amount of BrdU incorporated into DNA, cells were harvested, transferred to cytospin slides (Shandon Southern Products Ltd., Sewickley Pennsylvania, USA) and allowed to dry for 2 h at room temperature. Cells that had incorporated BrdU were labelled by direct peroxidase immunocytochemistry using the chromogen diaminobenzidine. The slides were counterstained with hematoxylin, mounted and put under a

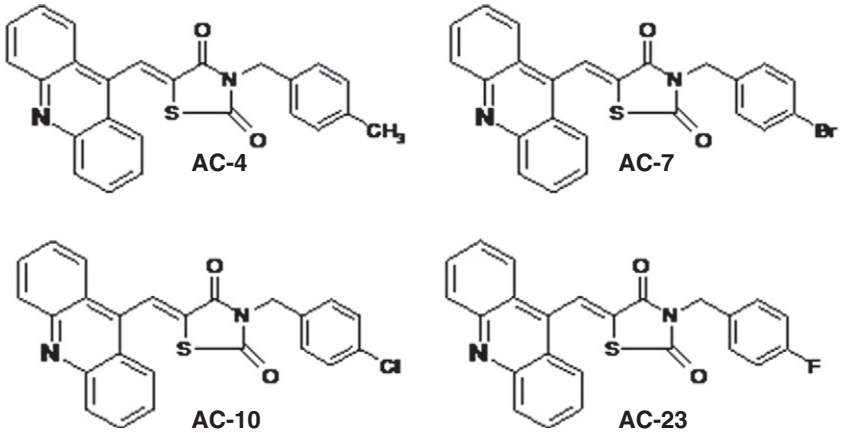

Fig. 1. The chemical structures of thiazacridine derivatives.

cover slip. A light microscopy (Olympus, Tokyo, Japan) was used to determine BrdU-positivity. Two hundred cells per sample were counted to determine the percent of BrdU-positive cells.

Morphological analyses using hematoxylin–eosin staining. Untreated or ATZD-treated HCT-8 cells were examined for morphological changes under a light microscopy (Metrimpex Hungary/PZO-Labimex Model Studar lab). To evaluate any alterations in morphology, cells from the cultures were harvested, transferred to a cytospin slide, fixed with methanol for 30 s, and stained with hematoxylin–eosin.

Morphological analyses using a fluorescence microscope. Cells were pelleted and resuspended in 25 μl of PBS. Then, 1 μl of aqueous acridine orange/ethidium bromide solution (AO/EB, 100 μg/ml) was added and the cells were observed under a fluorescence microscope (Olympus, Tokyo, Japan). Three hundred cells were counted per sample and classified as viable, apoptotic or necrotic (McGahon et al., 1995).

Cell membrane integrity. The integrity of the cell membrane was evaluated using the exclusion of propidium iodide (2 μg/ml, Sigma Chemical Co. St Louis, MO, USA). Cell fluorescence was determined by flow cytometry in a Guava EasyCyte Mini System cytometer using CytoSoft 4.1 software (Guava Technologies, Hayward, California, USA). Five thousand events were evaluated per experiment and the cellular debris was omitted from the analysis.

Cell cycle distribution. The cells were harvested in a lysis solution (citrate 0.1%, triton X-100 0.1% and propidium iodide 50 μg/ml) (Nicoletti et al., 1991), and the cell fluorescence was determined by flow cytometry, as described above.

Measurement of the mitochondrial transmembrane potential. The mitochondrial transmembrane potential was determined by the retention of rhodamine 123 dye (Gorman et al., 1997; Sureda et al., 1997). The cells were washed with PBS, incubated with rhodamine 123 (5 μg/ml, Sigma Chemical Co. St Louis, MO, USA) at 37 °C for 15 min in the dark and washed twice. The cells were then incubated again in PBS at 37 °C for 30 min in the dark and their fluorescence was measured by flow cytometry, as described above.

Annexin assay. Phosphatidylserine externalisation was analysed by flow cytometry (Vermes et al., 1995). A Guava® Nexin Assay Kit (Guava Technologies, Hayward, CA) determined which cells were apoptotic (early apoptotic + late apoptotic). The cells were washed twice with cold PBS and then re-suspended in 135 μl of PBS with 5 μl of 7-amino-actinomycin D (7-AAD) and 10 μl of Annexin V–PE. The cells were gently vortexed and incubated for 20 min at room temperature (20–25 °C) in the dark. Afterwards, the cells were analysed by flow cytometry, as described above.

Caspase 3/7 activation. Caspase 3/7 activity was analysed by flow cytometry using the Guava® EasyCyte Caspase 3/7 Kit (Guava Technologies, Hayward, CA). The cells were incubated with Fluorescent Labelled Inhibitor of Caspases (FLICATM) and maintained for 1 h at 37 °C in a CO_2 incubator. After incubation, 80 μl of wash buffer was added and the cells were centrifuged at 2000 rpm for 5 min. The resulting pellet was resuspended in 200 μl of wash buffer and centrifuged. The cells were then re-suspended in the working solution (propidium iodide and wash buffer) and analysed immediately using flow cytometry, as described above.

Drop test assay to determine the sensitivity of mutant S. cerevisiae strains with defective topoisomerases. The drop test assay determined the relative sensitivity of different *S. cerevisiae* strains to ATZD treatment. The following *S. cerevisiae* strains were used: BY-4741, *Top1*Δ and *Top3*Δ. Cells were treated with ATZD at concentrations of 50 and 100 μg/ml and more, 4 dilutions 1:10 were performed. A suspension of 2×10^5 cells/ml of *S. cerevisiae* in the exponential phase was used. An aliquot of 3 μl of each dilution was added to plates containing YEPD medium (YEL + agar). After 3–4 days of growth at 28 °C, the plates were photographed. *m*-AMSA served as the positive control.

DNA relaxation assay. The inhibitory effects of ATZD on human DNA topoisomerase I were measured using a Topo I Drug Screening Kit (TopoGEN, Inc.). Supercoiled (Form I) plasmid DNA (250 ng) was incubated with human Topo I (4 units) at 37 °C for 30 min in relaxation buffer (10 mM Tris buffer pH 7.9, 1 mM EDTA, 0.15 M NaCl, 0.1% BSA, 0.1 mM spermidine and 5% glycerol) in the presence or absence of ATZD (50 and 100 μg/ml, final 20 μl). The concentrations used were based on the positive control indicated in this Kit. CPT (100 μM) served as the positive control. The reaction was terminated by the addition of 10% SDS (2 μl) and proteinase K (50 μg/ml) and incubated at 37 °C for 30 min. The DNA samples were added to the loading dyes (2 μl) and subjected to electrophoresis on a 1% agarose gel for 90 min at room temperature and visualised with ethidium bromide.

Assessment of the genotoxic effect in human lymphocytes. A primary culture was obtained using a standard protocol and a Ficoll gradient. In addition, phytohemagglutinin (PHA) served as a mitogen to trigger cell division in T-lymphocytes. Peripheral blood was collected from four (two women and two men) healthy donors, 19–30 years of age with no history of smoking/drinking or chronic drug use. Venous blood (10 ml) was collected from each donor into heparinised vials. Lymphocytes were isolated with a Ficoll density gradient (Histopaque-1077; Sigma Diagnostics, Inc., St. Louis). The culture medium consisted of RPMI 1640 supplemented with 20% foetal bovine serum, phytohemagglutinin (final concentration: 2%), 2 mM glutamine, 100 U/ml penicillin and 100 μg/ml streptomycin at 37 °C with 5% CO_2 (Berthold, 1981; Brown and Lawce, 1997; Hutchins and Steel, 1983). For all of the experiments, cell viability was performed using the Trypan Blue assay. Ninety percent of the cells had to be viable before starting the experiments.

Alkaline comet assay. The alkaline (pH > 13) version of the comet assay (Single Cell Gel Electrophoresis) was performed, as described by Singh et al. (1988) with minor modifications (Hartmann and Speit, 1997). The slides were prepared in duplicate and 100 cells were screened per sample (50 cells from each duplicate slide) using a fluorescence microscope (Zeiss) equipped with a 515–560 nm excitation filter, a 590 nm barrier filter, and a 40× objective. The cells were visually scored and sorted into five classes according to tail length: (1) class 0: undamaged, without a tail; (2) class 1: with a tail shorter than the diameter of the head (nucleus); (3) class 2: with a tail length 1–2× the diameter of the head; (4) class 3: with a tail longer than 2× the diameter of the head; and (5) class 4: comets with no heads. A value of damage index (DI) was assigned to each comet according to its class, using the formula: $DI = (0\times n_0) + (1\times n_1) + (2\times n_2) + (3\times n_3) + (4\times n_4)$, where n = number of cells in each class analysed. The damage index ranged from 0 (completely undamaged: 100 cells × 0) to 400 (with maximum damage: 100 cells × 4). DI was based on migration length and on the amount of DNA in the tail and was considered a sensitive measure of DNA (Speit and Hartmann, 1999).

Chromosome aberration assay. We used naturally synchronised human peripheral blood lymphocytes with more than 95% of the cells in the G_0 phase (Bender et al., 1988; Wojcik et al., 1996). Short-term lymphocytes cultures, at a concentration of 0.3×10^6 cells/ml, were initiated according to a standard protocol (Preston et al., 1987). ATZD were studied at different phases of the cell cycle based on the protocol described by Cavalcanti et al. (2008) with minor modifications. Doxorubicin (0.3 μg/ml) served as a positive control. In the experimental procedures, when ATZD was added after 24-h, cells in both the G_1 and S

stages were exposed, whilst it can be assumed that when ATZD was added after 69 h, only cells in the G_2 stage were exposed. When ATZD was added at the same time as the PHA stimulation (in culture start, 0 h), the cells were exposed in the G_1 stage. To obtain a sufficient number of analysable metaphases, colchicine was added at a final concentration of 0.0016%, 2 h prior to harvesting. The cells were harvested by centrifugation, treated with 0.075 M KCl at 37 °C for 20 min, centrifuged and fixed in 1:3 (v/v) acetic acid:methanol. Finally, the slides were prepared, air-dried and stained with a 3% Giemsa solution (pH 6.8) for 8 min (Moorhead et al., 1960).

The slides were analysed with a light microscope; the structural and numerical CAs were examined during metaphase in the ATZD-treated cultures and the respective controls. The frequency of CAs (in 100 metaphases per culture) and the mitotic index (MI, number of metaphases per 2.000 lymphocytes per culture) were determined.

Telomerase inhibition assay. The ability of ATZD to inhibit telomerase action was measured by determining telomere length using fluorescence in situ hybridisation with probes to telomeric sequences (TELO-FISH), as described by Lansdorp (1995) and Lansdorp et al. (1996). Short-term lymphocyte cultures were initiated according to a standard protocol (Preston et al., 1987) and were fixed (methanol: acetic acid, 3:1) on slides. The slides were hybridised with the pan telomeric Star FISH probe. The measurement of telomere length determined in each nucleus, was acquired using the image capturing software Applied Special Imaging analysis system. The images were processed using the TFL-TELO software following the protocol (Poon et al., 1999).

Statistical analysis. The data are presented as the means ± standard error of the mean of *n* experiments. The differences among experimental groups were compared using a one-way analysis of variance (ANOVA) followed by a Newman–Keuls test ($p<0.05$). All analyses were carried out using the GRAPHPAD programme (Intuitive Software for Science, San Diego, California, USA).

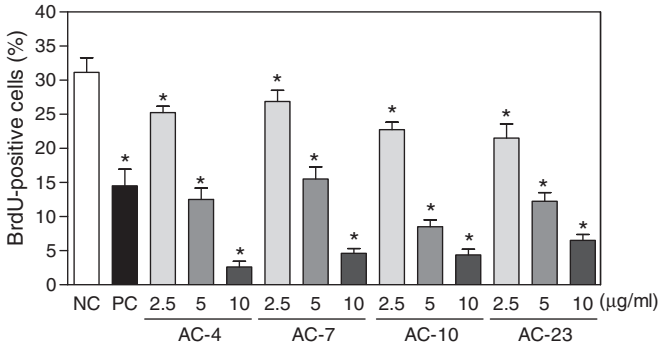

Fig. 3. The effect of thiazacridine derivatives on the proliferation of human colon carcinoma HCT-8 cells. To determine the extent of cell proliferation, inhibition BrdU incorporation was determined after a 24-h incubation. The data presented are the mean values ± S.E.M. from three independent experiments performed in duplicate. The negative control was treated with the same vehicle (NC, 0.1% DMSO) that diluted the tested substance. Amsacrine (PC, *m*-AMSA, 0.3 μg/ml) served as the positive control. *, $p<0.05$ compared to the negative control using an ANOVA followed by a Student Newman–Keuls tests.

Results

Thiazacridine derivatives inhibit the proliferation of human colon carcinoma in HCT-8 cells

Human colon carcinoma HCT-8 cells were treated with 2.5, 5 and 10 μg/ml of ATZD for 12- and/or 24-h and analysed in three different assays (trypan blue dye exclusion, propidium iodide exclusion and

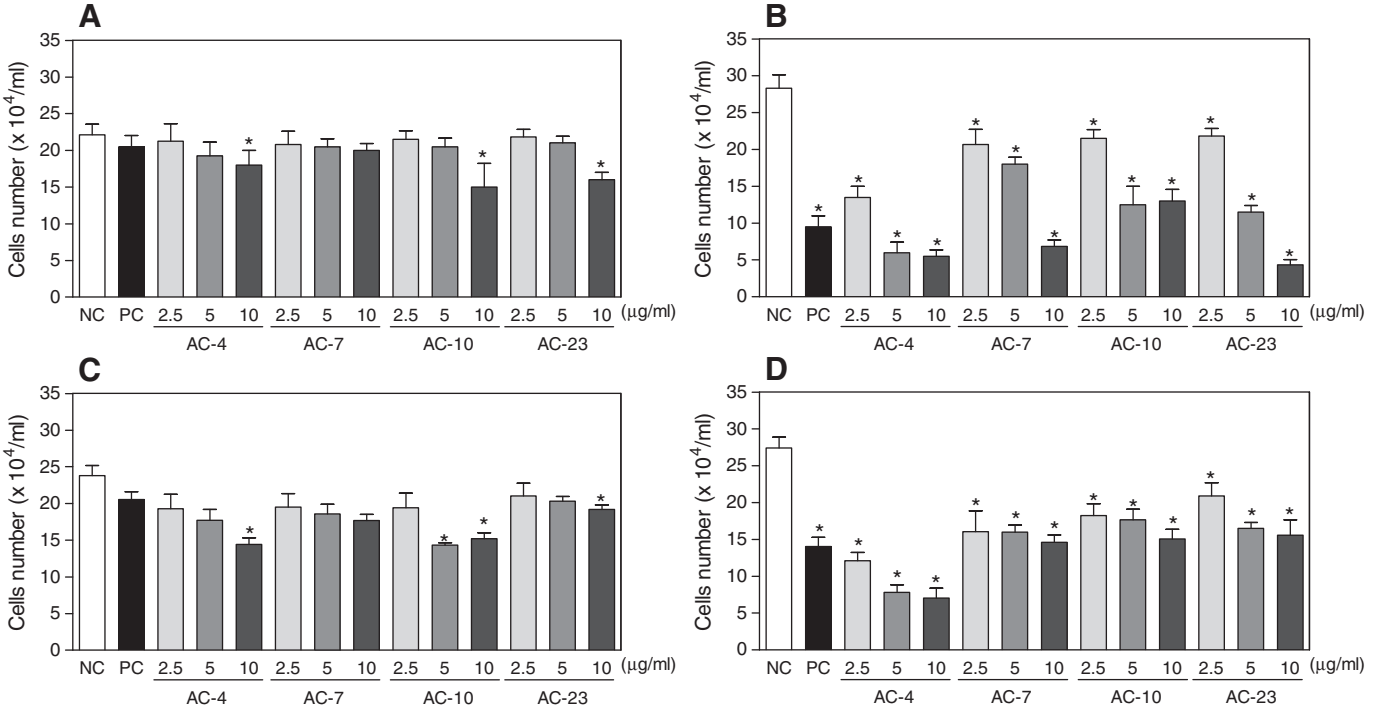

Fig. 2. The effect of thiazacridine derivatives on the proliferation of human colon carcinoma HCT-8 cells. A and B — the inhibition of cell proliferation was determined using the trypan blue dye exclusion method after 12- and 24-h incubations, respectively. C and D — the inhibition of cell proliferation was also determined using flow cytometry and propidium iodide after 12- and 24-h incubations, respectively. The data are presented as the mean values ± S.E.M. from three independent experiments performed in duplicate. The negative control was treated with the vehicle (NC, 0.1% DMSO) that diluted the test substance. Amsacrine (PC, *m*-AMSA, 0.3 μg/ml) was the positive control. For the flow cytometry analyses, 5000 events were analysed in each experiment. *, $p<0.05$ compared to the negative control using an ANOVA followed by a Student Newman–Keuls test.

Table 1
The effect of thiazacridine derivatives on the cell cycle distribution on human colon HCT-8 cells.

Drug	Concentration (μg/ml)	Cell cycle distribution (%)			
		Sub-G_1	G_1/G_0	S	G_2/M
After 12-h incubation					
NC	–	1.8 ± 0.1	62.8 ± 0.8	12.5 ± 0.3	15.7 ± 0.4
PC	0.3	1.5 ± 0.4	32.5 ± 1.7*	20.3 ± 2.4*	40.5 ± 2.1*
AC-4	2.5	3.8 ± 0.5*	58.2 ± 1.7	13.8 ± 0.9	19.7 ± 0.8*
	5	4.4 ± 0.3*	57.0 ± 1.1	15.2 ± 0.5	17.4 ± 1.3
	10	9.9 ± 1.1*	55.2 ± 2.7	14.5 ± 0.6	12.9 ± 0.9
AC-7	2.5	2.4 ± 0.4	58.3 ± 1.2	13.5 ± 0.6	19.2 ± 0.5*
	5	5.0 ± 0.6*	57.8 ± 1.3	15.3 ± 1.0	16.2 ± 0.4
	10	8.4 ± 0.9*	58.1 ± 1.6	15.3 ± 1.4	12.2 ± 0.6
AC-10	2.5	3.7 ± 0.5*	57.6 ± 1.6	14.1 ± 0.5	19.9 ± 0.5*
	5	5.3 ± 0.6*	58.2 ± 1.2	14.4 ± 0.3	17.6 ± 0.7
	10	6.9 ± 0.5*	58.4 ± 1.3	13.5 ± 0.6	16.7 ± 0.9
AC-23	2.5	3.3 ± 0.8	58.4 ± 1.0	14.7 ± 1.0	16.6 ± 0.3
	5	4.6 ± 1.0*	57.6 ± 0.7	16.6 ± 1.1	13.9 ± 0.5
	10	8.1 ± 0.8*	60.5 ± 0.3	13.0 ± 0.8	11.9 ± 0.5*
After 24-h incubation					
NC	–	4.7 ± 0.4	59.6 ± 0.8	14.4 ± 0.8	16.0 ± 0,3
PC	0.3	8.1 ± 1.1*	27.8 ± 1.6*	24.0 ± 2.3*	26.3 ± 1.0*
AC-4	2.5	13.9 ± 1.5*	55.2 ± 0.8	15.1 ± 1.1	17.5 ± 0.9
	5	24.7 ± 2.2*	51.8 ± 0.9*	15.1 ± 0.9	3.9 ± 0.8*
	10	43.3 ± 1.5*	44.0 ± 1.3*	10.0 ± 0.5*	3.7 ± 0.7*
AC-7	2.5	10.9 ± 0.8*	55.7 ± 1.5	14.9 ± 0.4	11.6 ± 0.4*
	5	13.7 ± 1.4*	54.9 ± 3.3*	13.6 ± 0.7	9.8 ± 0.7*
	10	26.6 ± 3.6*	54.6 ± 3.4*	8.9 ± 0.5*	5.2 ± 0.7*
AC-10	2.5	26.8 ± 0.7*	52.6 ± 2.3*	14.7 ± 0.8	10.8 ± 1.0*
	5	27.1 ± 1.0*	49.1 ± 3.1*	14.3 ± 0.6	9.7 ± 1.1*
	10	37.5 ± 1.4*	48.8 ± 1.8*	10.2 ± 0.5*	4.7 ± 0.5*
AC-23	2.5	12.1 ± 2.0*	56.3 ± 1.9	12.5 ± 0.5	10.8 ± 1.4*
	5	24.4 ± 2.1*	55.8 ± 3.3	11.1 ± 0.7	9.8 ± 1.2*
	10	28.9 ± 2.0*	52.2 ± 3.5*	6.4 ± 0.6*	4.5 ± 0.4*

The data are presented as the mean values ± S.E.M. from three independent experiments performed in duplicate. The negative control was treated with the same vehicle (NC, 0.1% DMSO) that diluted the tested substance. Amsacrine (PC, *m*-AMSA) served as the positive control. Five thousand events were analysed for the flow cytometry analysis in each experiment.

* $p<0.05$ compared to negative control by ANOVA followed by a Student Newman–Keuls test.

BrdU incorporation). ATZD reduced the proliferation of HCT-8 cells in a concentration- and time-dependent manner.

After a 12-h incubation, cell proliferation was reduced at higher concentration tested, which was confirmed by trypan blue dye exclusion and propidium iodide exclusion ($p<0.05$, Figs. 2A, C). After a 24-h incubation, ATZD reduced cell number ($p<0.05$) at all concentrations tested using trypan blue dye exclusion (Fig. 2B), propidium iodide exclusion (Fig. 2D) and BrdU incorporation (Fig. 3). *m*-AMSA, the positive control, also reduced HCT-8 cell proliferation.

Thiazacridine derivatives preferentially caused human colon carcinoma HCT-8 cells to transition from the G_2/M phase to DNA fragmentation

The effects that these ATZD had on cell cycle progression were evaluated using flow cytometry after 12- and 24-h. All DNA that was sub-diploid in size (sub-G_1) was considered to be caused by internucleosomal DNA fragmentation. Table 1 indicates the cell cycle distribution obtained. After a 12-h incubation, the ATZD treated with **AC-4**, **AC-7** and **AC-10** (2.5 μg/ml) caused a small increase in the number of cells in the G_2/M phase compared with the negative control (15.7%, $p<0.05$). For the ATZD-treated cells, the percentage of cells in the G_2/M phase were 19.7%, 19.2% and 19.9%, for **AC-4**, **AC-7** and **AC-10**, respectively. After a 24-h incubation, the cells in the G_0/G_1 and S phases remained mostly unchanged; however, there were fewer cells in the G_2/M phase. Additionally, all ATZD caused significant internucleosomal DNA fragmentation at all of the concentrations tested ($p<0.05$), which implies that ATZD preferentially caused cells from the G_2/M phase to transition into sub-G_1. Cells treated with *m*-AMSA served as the positive control, and had an increased number of cells in the G_2/M interval and a significant amount of internucleosomal DNA fragmentation.

Thiazacridine derivatives induce apoptosis in human colon carcinoma HCT-8 cells

After 12- and 24-h incubations, the effects of ATZD were evaluated based on cell morphology using hematoxylin–eosin and acridine

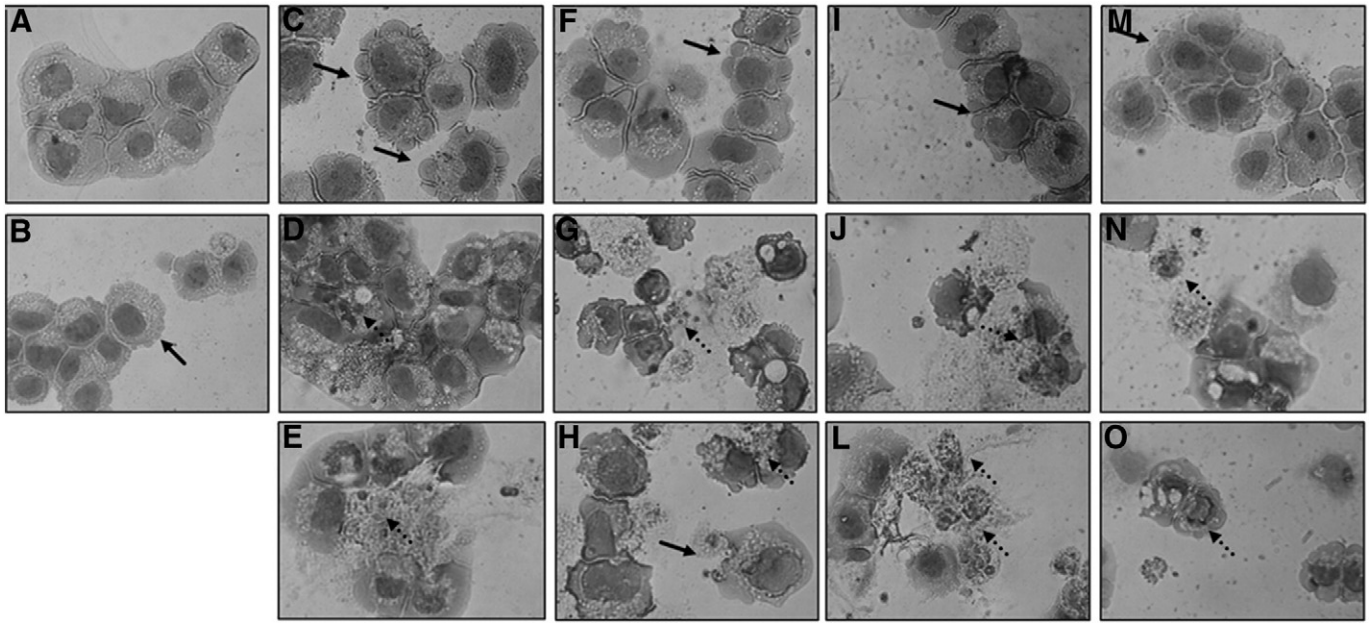

Fig. 4. The effect of thiazacridine derivatives on the cell morphology of human colon carcinoma HCT-8 cells. The cells were stained with hematoxylin-eosin and analysed by optical microscopy after a 24-h incubation with **AC-4**, **AC-7**, **AC10** and **AC23** at concentrations of 2.5 (C, F, I, M), 5 (D, G, J, N) and 10 μg/ml (E, H, L, O), respectively. The negative control (A) was treated with the same vehicle (0.1% DMSO) that diluted the tested substance. Amsacrine (*m*-AMSA, 0.3 μg/ml) served as the positive control (B). The continuous arrows show the apoptotic bodies and the non-continuous arrows indicate nuclear fragmentation.

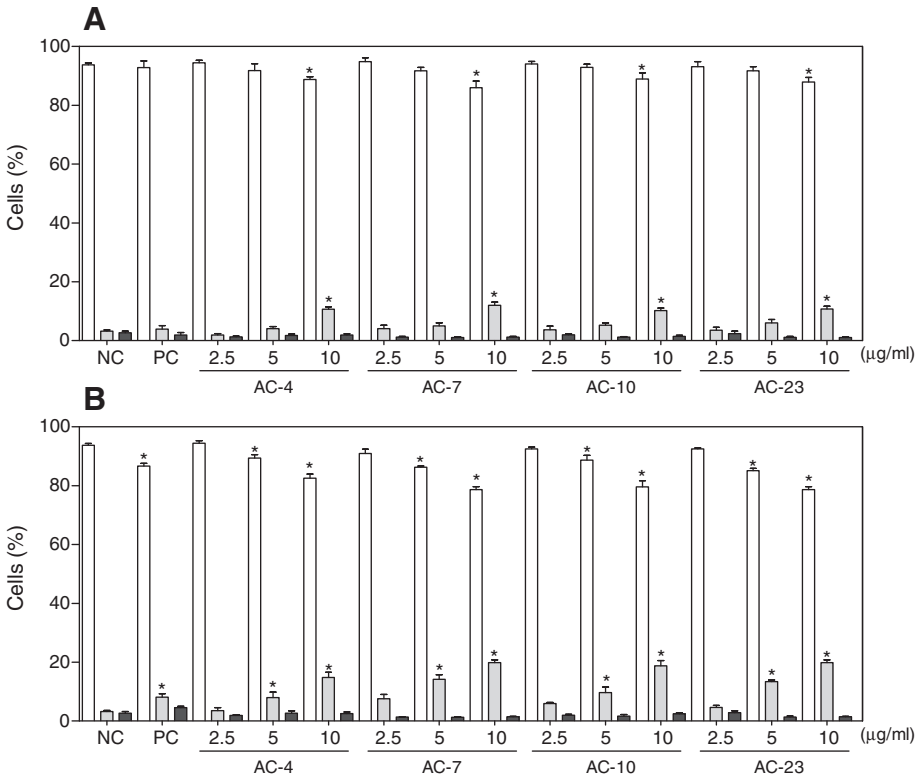

Fig. 5. The effect of thiazacridine derivatives on the viability of human colon carcinoma HCT-8 cells. A and B — cell viability (viable cells – white bar; apoptotic cells – grey bar; and necrotic cells – black bar) was determined by fluorescence microscopy using acridine orange/ethidium bromide after 12- and 24-h incubations, respectively. The data are presented as the mean values ± S.E.M. from three independent experiments performed in duplicate. The negative control was treated with the same vehicle (NC, 0.1% DMSO) that diluted the tested substance. Amsacrine (PC, *m*-AMSA, 0.3 μg/ml) served as the positive control. *, $p<0.05$ compared to negative control by ANOVA followed by a Student Newman–Keuls test.

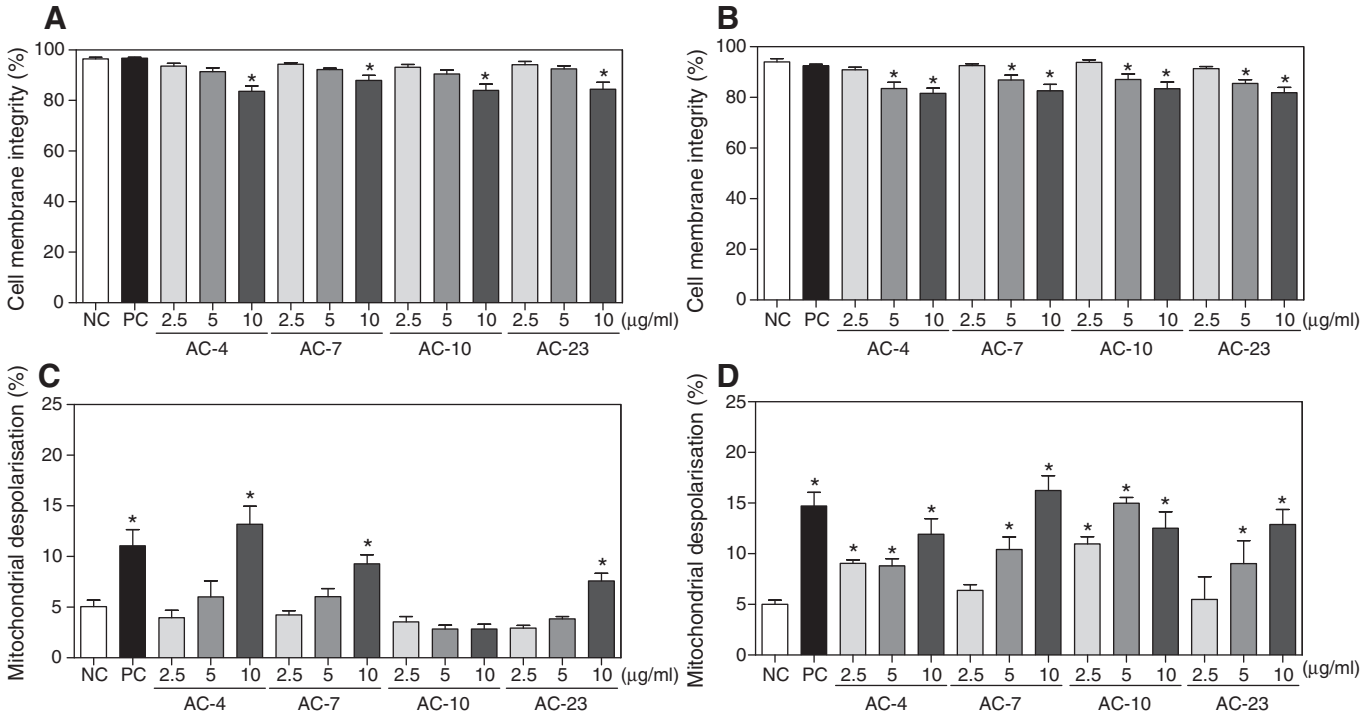

Fig. 6. The effect of thiazacridine derivatives on the viability of human colon carcinoma HCT-8 cells. A and B — the integrity of the cell membranes was determined by flow cytometry using propidium iodide after 12- and 24-h incubations, respectively. C and D — the mitochondrial membrane potential was determined by flow cytometry using rhodamine 123 after 12- and 24-h incubations, respectively. The data are presented as the mean values ± S.E.M. from three independent experiments performed in duplicate. The negative control was treated with the same vehicle (NC, 0.1% DMSO) that diluted the tested substance. Amsacrine (PC, *m*-AMSA, 0.3 μg/ml) served as the positive control. Five thousand events were analysed for the flow cytometry analysis in each experiment. *, $p<0.05$ compared to the negative control using an ANOVA followed by a Student Newman–Keuls test.

orange/ethidium bromide staining. The integrity of the cell membrane and the mitochondrial membrane potential were also determined by flow cytometry. Additionally, after a 24-h incubation, phosphatidylserine externalisation and caspase 3/7 activation were measured by flow cytometry.

After a 12-h incubation, HCT-8 cells either treated or untreated with ATZD, were tested at all concentrations and presented slight morphological changes (data not shown). On the other hand, after a 24-h incubation, morphological examination of HCT-8 cells showed severe drug-mediated changes. The hematoxylin–eosin stained HCT-8 cells treated with ATZD presented a morphology consistent with apoptosis, including a reduction in cell volume, chromatin condensation and nuclei fragmentation (Fig. 4). The acridine orange/ethidium bromide stained and treated cells also displayed a morphology consistent with apoptosis, in a time- and concentration-dependent manner ($p<0.05$, Fig. 5). *m*-AMSA, served as the positive control, which also induced morphological changes consistent with apoptosis.

The integrity of the cell membrane is a parameter of cell viability that differs between apoptotic and necrotic cells. After 12- or 24-h of exposure, ATZD induced a slight disruption in the plasmatic membrane, which was only observed at the higher concentrations tested (Figs. 6A, B). As cited above, the internucleosomal DNA fragmentation was markedly increased in ATZD-treated cells ($p<0.05$, Table 1). Both of these modifications are characteristics of apoptotic cells. In addition, ATZD induced mitochondrial depolarisation in a time- and concentration-dependent manner ($p<0.05$, Figs. 6C, D). *m*-AMSA served as the positive control, which also induced mitochondrial depolarisation and DNA fragmentation without affecting the membrane's integrity.

In addition, phosphatidylserine externalisation (**AC-4** and **AC-10** at concentrations of 2.5 and 5 μg/ml) and caspase 3/7 activation (**AC-4**, **AC-10** and **AC-23** at concentrations of 5 and 10 μg/ml) were measured in ATZD-treated cells after a 24-h incubation. Phosphatidylserine exposure ($p<0.05$, Fig. 7A) and an increase in caspase 3/7 activation ($p<0.05$, Fig. 7B) were also observed, suggesting that a caspase-dependent apoptotic cell death had occurred. Doxorubicin served as the positive control and also induced phosphatidylserine exposure and increased caspase 3/7 activation.

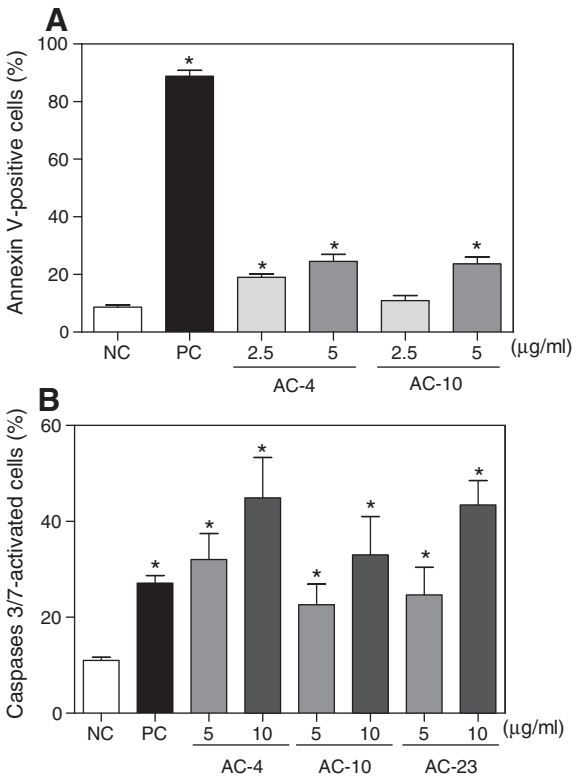

Fig. 7. The effect of thiazacridine derivatives on the viability of human colon carcinoma HCT-8 cells. A — the cell viability was determined by flow cytometry using Annexin V–PE. B — the activity of caspase 3/7 was determined by flow cytometry using propidium iodide and Flica. The data are presented as the mean values ± S.E.M. from three independent experiments performed in duplicate after a 24-h incubation. The negative control was treated with the same vehicle (NC, 0.1% DMSO) that diluted the tested substance. Doxorubicin (PC, 0.3 μg/ml) was the positive control. For the flow cytometry analysis, 5000 events were analysed in each experiment. *, $p<0.05$ compared to the negative control using an ANOVA followed by a Student Newman–Keuls test.

Thiazacridine derivatives inhibits DNA topoisomerase I action

Because ATZD interact with DNA, they are potential topoisomerase inhibitors. The effect of ATZD on DNA topoisomerase activity was evaluated in a yeast-based assay and in a cell-free assay.

First, the effects of ATZD were evaluated using a drop test assay in a mutant strain of *S. cerevisiae* that was defective in topoisomerase type I (Fig. 8). The type IB topoisomerases (topoisomerase 1 in yeast) relax both positively and negatively supercoiled DNA, whereas type IA topoisomerases (topoisomerase 3 in yeast) preferentially relax negatively supercoiled DNA. At a concentration of 50 μg/ml, the ATZD were more resistant in yeast mutants that lacked topoisomerase 1 (*Top1*Δ) activity compared with the wild-type strain (BY-4741), indicating that these molecules may induce lesions in topoisomerase 1. In ATZD at higher concentration (100 μg/ml), the *Top1*Δ mutant was more sensitive than the wild-type strain, which indicates that an additional cytotoxicity mechanism (i.e., interaction with topoisomerase II) may be involved. Moreover, the strain without topoisomerase 3, but with topoisomerase 1, (*Top3*Δ), was more sensitive to the ATZD, with the exception of **AC-23**. *m*-AMSA served as the positive control, which showed similar effects.

In addition, the effect of ATZD on topoisomerase I activity was evaluated in a cell-free system. Purified human DNA topoisomerase I was incubated with ATZD (50 and 100 μg/ml) in the presence of supercoiled plasmid DNA; the products of this reaction were subjected to electrophoresis on agarose gels to separate the closed and open circular DNAs. Relaxation of the DNA strand was inhibited in both of the concentrations tested (Fig. 9). CPT served as the positive control because it also inhibits DNA topoisomerase I.

Thiazacridine derivatives do not cause genotoxicity or inhibit telomerase activity in human lymphocytes

The genotoxicity of ATZD (**AC-4**, **AC-7**, **AC-10** and **AC-23**) was evaluated in human lymphocyte cultures using an alkaline comet assay at concentrations of 2.5, 5 and 10 μg/ml. The genotoxicity of ATZD (**AC-4** and **AC-10**) was also evaluated in human lymphocyte cultures using a chromosome aberration assay at concentrations of 2.5, 5 and 10 μg/ml. The ability of ATZD (**AC-4** and **AC-10**) to inhibit telomerase action was performed using a pan telomeric probe at a concentration of 2.5 μg/ml. None of the ATZD showed genotoxic activity or anti-telomerase activity at any experimental concentrations tested (data not shown). Doxorubicin served as the positive control, and demonstrated potent genotoxic activity.

Discussion

The present work demonstrates the mechanism by which ATZD (**AC-4**, **AC-7**, **AC-10** and **AC-23**) are cytotoxic in human colon carcinoma HCT-8 cells. As cited above, these agents were recently synthesised as a novel class of solid tumour-selective cytotoxic agents. These ATZD exhibit a relatively high cytotoxicity in colon carcinoma (HCT-8, HCT-15, SW-620 and COLO-205), prostate carcinoma (PC-3 and DU-145), ovarian carcinoma (OVCAR-8), melanoma (UACC-62 and MDA-MB-435) and glioblastoma (SF-295) tumour cell lines. However, these compounds were not active in leukaemia (HL-60, K-562 and

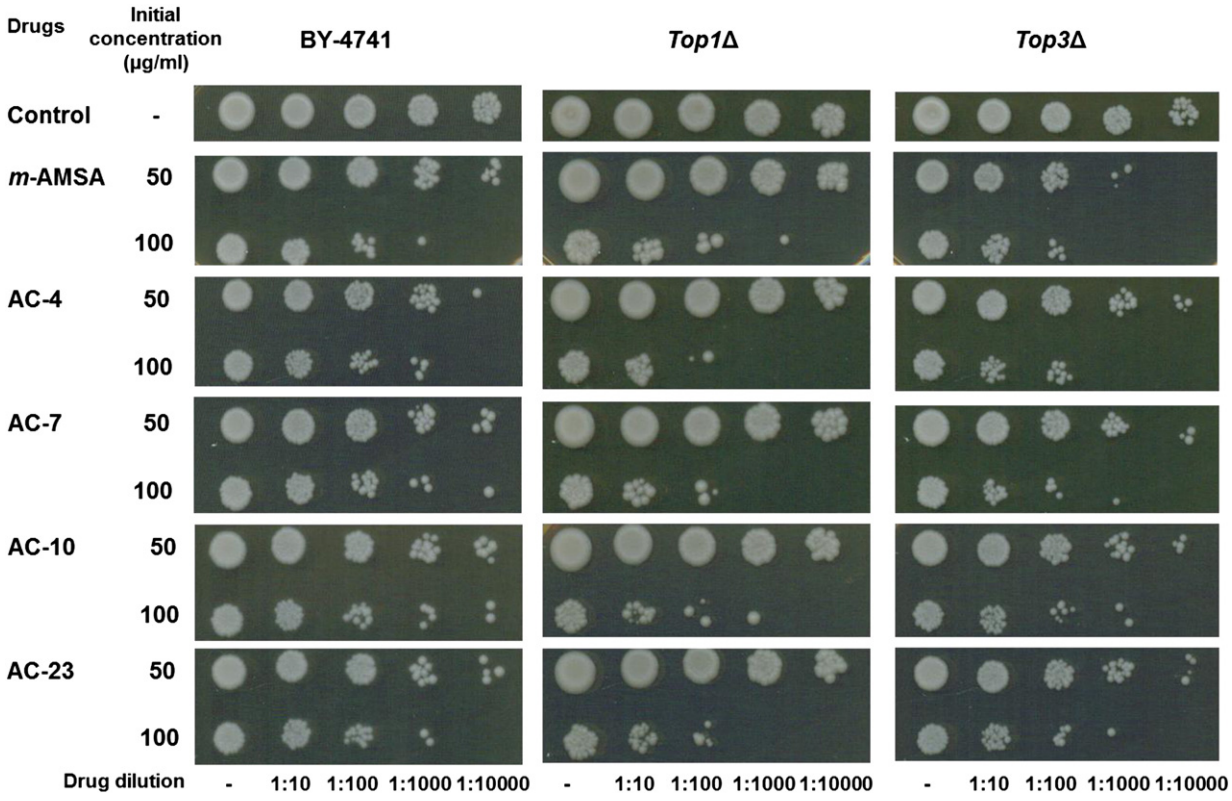

Fig. 8. The sensitivity of a wild-type strain of *Saccharomyces cerevisiae* and mutants with defective topoisomerases. The sensitivity of topoisomerases type I to thiazacridine derivatives was determined by a drop test assay. A suspension of *S. cerevisiae* cells in the exponential phase of growth was treated for 24-h in the absence or in the presence of thiazacridine derivatives at the indicated concentrations. The diluted cell cultures (10^7–10^3 from left to right) were spotted on YPD agar plates. Amsacrine (*m*-AMSA) served as the positive control. BY-4741: Wild-type strain; *top1*Δ: without topoisomerase 1; and *top3*Δ: without topoisomerase 3.

CEM), breast carcinoma (MDA-MB-231, HS-578-T and MX-1) or normal lymphoblast (PBMC) cells (Barros et al., 2012). Here, we demonstrate the effects of ATZD on cell proliferation, cell cycle progress and apoptotic-induction using HCT-8 cells as a model. Studies in a yeast-based assay and a cell-free assay examine how ATZD interfere in topoisomerase I activity.

The ATZD inhibit human colon carcinoma HCT-8 cell proliferation in a concentration- and time-dependent manner, and their cytotoxic activity was assessed using different assays. Previously, we demonstrated that ATZD exhibited relatively high cytotoxicity against colon carcinomas and that the highlight of these ATZD was their selectivity toward solid tumours because these ATZD were not active in leukaemias or normal lymphoblasts (Barros et al., 2012). The pyrazoloacridines, bisannulated acridines, aminoderivatives of azapyranoxanthenone and pyranoisoflavones have also been cited as solid tumour-selective cytotoxic agents (Gao et al., 2011; Kolokythas et al., 2006; Sebolt, et al., 1987; Thale et al., 2002). Therefore, this feature is noteworthy but the mechanisms accounting for this selectivity are poorly understood.

The population of cells in the G_2/M phase was shifted to the sub-G_1 population in ATZD-treated HCT-8 cells, whilst few changes occurred in the population of cells in the G_0/G_1 or S phases. This indicates that the ATZD preferentially guide cells from the G_2/M phase into apoptosis. Manipulating the regulatory events at this checkpoint is a promising approach that will improve the efficiency of cytotoxic drugs and overcome drug resistance (Links et al., 1998). In addition, HCT-8 cells treated with ATZD presented typical hallmarks of apoptosis. Selective apoptosis, the deletion of certain cells in tissues without concomitant inflammation, is advantageous in tissue homeostasis. The induction of apoptosis is one of the main mechanisms that inhibit cancer growth and proliferation and is used by several antitumor agents (Los et al., 2003; Schultz and Harrington, 2003). Moreover, ATZD treatment induces mitochondrial depolarisation, phosphatidylserine exposure and an increase in caspase 3/7 activation, which suggests that ATZD treatment leads to a caspase-dependent apoptotic cell death. Caspases play an essential role in apoptosis (Fan et al., 2005; Kitazumi and Tsukahara, 2011): these caspases are responsible for the cleavage of cellular proteins, such as cytoskeletal components, which leads to the morphological changes previously observed in the cells that undergo apoptosis (Kothakota et al., 1997).

The mechanism by which acridine and thiazolidine derivatives act has been continuously researched. Thiazolidine derivatives activate peroxisome proliferator-activated receptors (Barros et al., 2010). Meanwhile, acridine derivatives used in cancer chemotherapy have biological targets, such as DNA topoisomerases I and/or II, telomerase/telomeres and kinases (Castillo-González et al., 2009; Guo et al., 2009; Oppegard et al., 2009). Our understanding of ATZD's cytotoxic mechanisms have been limited to results from double stranded-DNA biosensors and single stranded-DNA solutions, which show a positive interaction with these ATZD that couple acridine and thiazolidine (Barros et al., 2012). Here, we demonstrate that ATZD inhibit DNA topoisomerase I activity.

The cytotoxicity of DNA topoisomerase I inhibitors is caused by blocking DNA topoisomerase I cleavage complexes or by inhibiting DNA topoisomerase I catalytic activity. Then, DNA topoisomerase I inhibitors work by stabilising the DNA topoisomerase I cleavage complexes, which cause DNA damage (Hsiang et al., 1989; Pommier et al., 1998; Stewart et al., 1998). Because malignant cells often contain greater amounts of DNA topoisomerase I than normal cells, tumour cells should be more sensitive to the toxic effects of these inhibitors. The malignant cells that often contain great amounts of DNA topoisomerase I include colon adenocarcinoma, several types of non-Hodgkin's lymphoma, leukaemias, melanoma and carcinomas of the

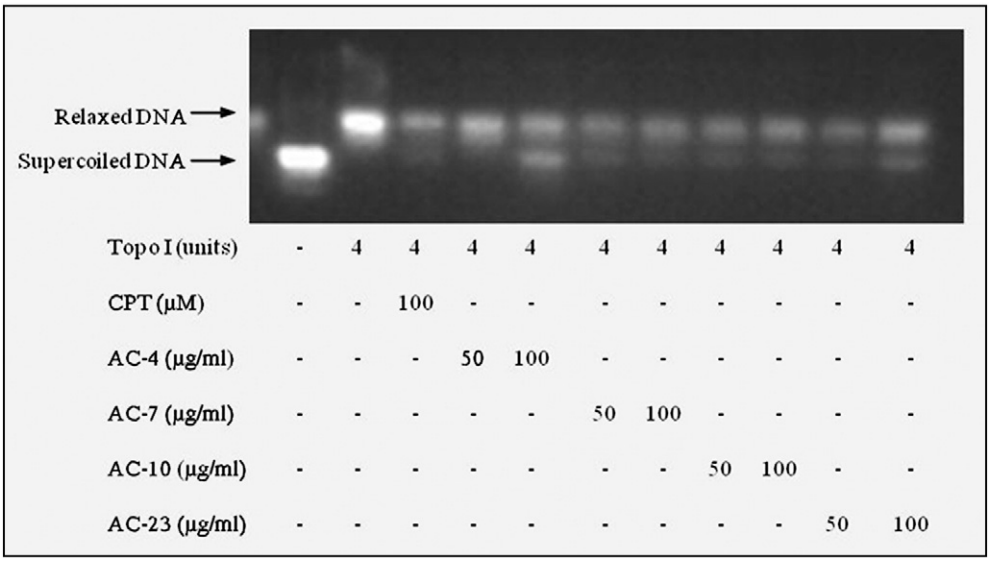

Fig. 9. The inhibition of topoisomerase I-mediated DNA supercoiling in the presence of thiazacridine derivatives. The supercoiled DNA (250 ng) was incubated with 4 units of topoisomerase I in the presence or absence of thiazacridine derivatives at the indicated concentrations. The negative control was treated with the same vehicle that diluted the tested substance. Camptothecin (CPT) served as the positive control. The DNA was analysed by electrophoresis using a 1% agarose gel. The gels were stained with ethidium bromide and photographed under UV light.

stomach, breast and lung (Potmesil, 1994). This partially explains the selective cytotoxic effects of ATZD. However, the exact mechanism of this selective antitumor activity remains to be determined.

Previous studies have reported that some acridine and thiazolidine derivatives are somatic- and germ-cell mutagenic agents capable of inducing both numerical and structural chromosome aberrations in vitro and in vivo (Attia, 2008; Attia, in press; Kao-Shan et al., 1984; Nishi et al., 1989). These compounds are highly cytotoxic/genotoxic to normal lymphocyte cells. Therefore, to improve our understanding of the ATZD's cytotoxic actions, we assessed their genotoxic effects in human peripheral lymphocytes. Previously, the cytotoxicity of these compounds was assessed against normal lymphocyte cells (Barros et al., 2012); however, the genotoxicity had not been investigated. The genotoxic effects of ATZD were determined using an alkaline comet assay and a chromosome aberration assay; the anti-telomerase activity was determined using a pan telomeric probe. In our studies, none of these ATZD agents showed genotoxicity and/or anti-telomerase activity in cultured human lymphocytes at the experimentally tested concentrations. Therefore, unlike the acridine and thiazolidine derivatives, ATZD did not cause cytotoxicity, genotoxicity and the inhibition of telomerase activity in human lymphocytes.

In this manuscript, we show that the ATZD are solid tumour-selective cytotoxic agents that inhibit DNA topoisomerase I activity and induce tumour cell death through caspase-dependent apoptosis pathways without causing genotoxicity in human lymphocytes. These data confirm that these ATZD are promising anticancer drugs.

Conflicts of interest

The authors declare no conflicts of interest.

Acknowledgments

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