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Cytotoxic activity of 3-nitropyrzolo[5,1-*c*][1,2,4]benzotriazine derivatives: a new series of anti-proliferative agents

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We report the synthesis and biological evaluation of a new series of 3-nitropyrzolo[5,1-*c*][1,2,4]benzotriazine derivatives (compounds 1–4) bearing appropriate substitutions in positions 7 and/or 8. The objective of this investigation was to study the effects of these substitutions on the cytotoxic activity of four new compounds against established human cancer cell lines (i.e. HT29 and HCT-8, colon carcinoma, MCF7, breast carcinoma, and A549, lung carcinoma cells). The inhibitory effects of compounds 1–4 on cell growth were assessed by the sulforhodamine B assay. Also, the effects of these compounds on cell cycle distribution of human colon carcinoma cells (HCT-8) were analyzed by flow cytometry. 3-Nitropyrzolo[5,1-*c*][1,2,4]benzotriazine derivatives displayed IC₅₀ values in the micromolar range on the growth of the four cell lines tested. Cell cycle perturbations induced on HCT-8 cells by study compounds at the IC₅₀ values consisted prevalently of a slight accumulation of cells in G₀/G₁ phase and a slight decrease in G₂/M phase. However, compound 3 induced a marked accumulation of cells into S phase with concomitant decrease in G₀/G₁ and G₂/M phases. Cytotoxicity data, compared to those obtained with 3-cyano-8-chloropyrzolo[5,1-*c*][1,2,4]benzotriazine

5-oxide (compound 5, NSC 683334) and other compounds previously synthesized in our laboratory, demonstrated a similar or even improved cytotoxic potency. Cell cycle perturbations caused by these compounds support the hypothesis that they may act by a direct or an indirect inhibition of DNA synthesis. *Anti-Cancer Drugs* 16:645–651 © 2005 Lippincott Williams & Wilkins.

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Keywords: apoptosis, cell cycle, cell growth inhibition, established human carcinoma cell lines, pyrzolo[5,1-*c*][1,2,4]benzotriazine system

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Introduction

A large number of compounds from many different chemical classes are reported in the literature as interfering with DNA-processing enzymes [1,2] or being DNA-intercalating agents [1,3,4].

Some derivatives of the pyrzolo[5,1-*c*][1,2,4]benzotriazine system have been extensively studied in our laboratory as benzodiazepine receptor ligands [5]. In a previous paper [6] we reported some variously substituted derivatives of this system which exhibited anti-tumor properties *in vitro* against leukemia cells more than against solid tumors; the nitro moiety appeared essential for the cytotoxic activity. Synthesis and biological evaluation of a new series of 3-nitropyrzolo[5,1-*c*][1,2,4]benzotriazine derivatives 1–4 bearing other substitutions with electron-drawers or electron-donors in the 7 and/or 8 position are reported here.

The aim of the present research was to investigate if these substitutions might improve the cytotoxic activity on solid tumors. Mitomycin C, doxorubicin and compound 5 (3-cyano-8-chloropyrzolo[5,1-*c*][1,2,4]benzo-

triazine 5-oxide) [6,7] were selected as reference compounds. The latter compound, which we previously synthesized [7], has been selected for anti-HIV and anti-cancer tests at the National Cancer Institute (NCI) [8]. This compound showed remarkable anti-proliferative activity against various human tumor cell lines (IC₅₀s ranging from 10⁻⁵ to 10⁻⁶ M) (unpublished data, see mean graph from the NCI [8]).

The *in vitro* inhibitory effects of the study compounds on the growth of human cancer cell lines (i.e. HT29 and HCT-8, colon carcinomas, MCF7, breast carcinoma, and A549, lung carcinoma cells) were assessed by the sulforhodamine (SRB) B assay [9]. Also, the effects of these compounds on cell cycle distribution of human colon carcinoma cells (HCT-8) were analyzed by flow cytometry [10].

Materials and methods

Chemical compounds 1–4

New compounds 1–4 were synthesized according to our previously reported procedure [7]. The starting materials were represented by 1-(2-nitrophenyl)-5-aminopyrzolo-4-carboxylate with the appropriate substituent on the

phenyl ring in the 4 and 5 position. These compounds cyclize to pyrazolo[5,1-*c*][1,2,4]benzotriazine 5-oxide under alkaline conditions.

After decarboxylation and nitration of the 3 position, under standard conditions, we obtained the desired compounds 1–4.

The structures of the new compounds and new intermediates were confirmed by ¹H nuclear magnetic resonance and IR spectroscopy. The other experimental data are reported in Table 1. Compounds were solubilized in dimethylsulfoxide (DMSO) at 100 times the desired maximum test concentration (maximum final DMSO concentration of 0.1%; this concentration was not toxic) and stored frozen. Compounds were then diluted with complete media to obtain the 10 times desired final maximum test concentration. Additional serial dilutions were made to provide a total of five drug concentrations plus control. Aliquots of 20 μl of these different drug dilutions were added to the appropriate microtiter wells already containing 180 μl of medium, resulting in the required final drug concentrations. All chemicals were purchased from Sigma (St Louis, MO) unless otherwise specified.

Cell cultures

The cell lines used in this study were two human colon carcinoma cell lines (HT29 and HCT8), one breast carcinoma (MCF7) and one lung carcinoma (A549) cell line, obtained from the ATCC (Rockville, MD). The HT29, HCT8 and MCF7 cell lines were maintained in RPMI 1640 (Euroclone, Wetherby, UK) supplemented with 10% fetal calf serum (FCS) (Euroclone) and antibiotics (penicillin 100 U/ml and streptomycin 100 μg/ml). The A549 cell line was maintained in F12 (Euroclone) medium supplemented with 10% FCS and antibiotics. All

cell lines were incubated at 37°C, 5% CO₂/95% air and subcultured twice weekly. The cells had similar doubling times, varying between 20 and 25 h.

Cell growth inhibition studies

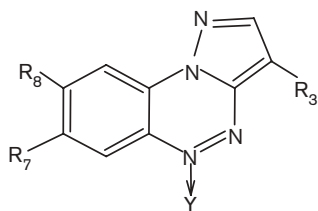
Exponentially growing cells were inoculated into 96-well microtiter plates at plating densities of 3000 cells/well. After cell inoculation, the microtiter plates were incubated at 37°C, 5% CO₂/95% air and 100% relative humidity for 24 h prior to the addition of experimental drugs. After 24 h, medium was removed and replaced with appropriate medium containing varying doses of experimental agents for 4, 24 and 72 h. After 4- and 24-h treatments, samples were washed and incubated for an additional 72 h in drug-free medium at 37°C, 5% CO₂/95% air and 100% relative humidity. According to the procedure described by Skehan *et al.* [9], the assay was terminated by the addition of cold TCA. Cells were fixed *in situ* by 10% TCA and stained by SRB solution at 0.4% (w/v) in 1% acetic acid. After staining, unbound dye was removed by washing 5 times with 1% acetic acid and the plates were air dried. Bound stain was subsequently solubilized with 10 mM Tris base and the absorbance was read on an automated plate reader at a wavelength of 540 nm. The IC₅₀ drug concentrations resulting in a 50% reduction in the net protein increase (as measured by SRB staining) in control cells during the drug incubation were determined after a 72-h exposure and at 72 h following 4- and 24-h exposures to compounds and subsequent washout.

Cell cycle analysis

HCT-8 cells were exposed to agents at concentrations of the test compounds equal to IC₅₀ of a 72-h continuous exposure. At different times (24, 48 and 72 h), floating cells were collected, adherent cells were trypsinized and pooled with the floating ones, and then fixed in 70%

Table 1 Structures and chemical data of the new pyrazolo[5,1-*c*][1,2,4]benzotriazines

Compounds	R ₃	R ₇	R ₈	Y	Formula (MW)	Melting point ^a (°C)	Recrystallization solvent	Yield (%)
1	NO ₂	NO ₂	CH ₃	O	C ₁₀ H ₆ N ₆ O ₅ (290.19)	234	– ^a	–
2	NO ₂	CH ₃		O	C ₁₀ H ₇ N ₅ O ₃ (245.20)	260	EtOH	80
3	NO ₂	CF ₃		O	C ₁₀ H ₄ F ₃ N ₅ O ₃ (299.18)	236	EtOH	57
4	NO ₂		CH ₃		C ₁₀ H ₇ N ₅ O ₂ (229.20)	264	isopropanol	70
5 (reference compound [17])	CN		Cl	O				



^aPurified by column chromatography (cyclohexane:ethylacetate 2:1).

Table 2 IC₅₀ values (μM)^a of study compounds as determined by the SRB assay

Treatment	Cell type			
	HCT-8	HT29	MCF7	A549
4-h exposure ^b				
1	3.58	>10	5.29	>10
2	6.36	>10	6.09	>10
3	9.42	>10	6.5	>10
4	>10	>10	7.4	>10
5	5.84	>10	>10	>10
MMC	0.16	0.47	0.36	0.47
DOX	0.177	0.375	0.175	0.34
24-h exposure ^b				
1	2.64	4.7	2.57	2.32
2	2.35	2.39	1.76	1.95
3	4.53	6.96	6.45	5.88
4	3.91	5.2	4.16	6.09
5	3.69	>10	>10	>10
MMC	0.077	0.06	0.066	0.11
DOX	0.129	0.089	0.088	0.12
72-h continuous exposure				
1	2.08 ± 0.78	2.67 ± 0.36	3.64 ± 1.1	1.64 ± 0.2
2	1.60 ± 0.11	2.39	2.26 ± 0.83	1.64 ± 0.19
3	3.16 ± 1.00	3.43 ± 0.55	2.08 ± 0.29	2.32 ± 0.72
4	3.31 ± 0.95	3.71 ± 0.26	4.64 ± 0.88	4.64 ± 0.36
5	2.75	>10	2.08	>10
MMC	0.075 ± 0.01	0.088	0.092 ± 0.016	0.095 ± 0.015
DOX	0.07 ± 0.018	0.067 ± 0.01	0.064	0.053 ± 0.024

^aMean ± SE of at least three determinations or mean of two independent experiments performed with quadruplicate cultures at each drug concentration tested.

^bIC₅₀ values after a 72-h drug washout.

ice-cold ethanol and stored at 4°C. Cells were then rehydrated in phosphate-buffered saline and stained with propidium iodide (PI, 50 μg/ml) solution containing RNase A (5 U/ml) for 30 min [10]. PI-stained cells were analyzed for DNA content with a FACStar cell sorter (Becton Dickinson, Mountain View, CA) equipped with an argon ion laser (Model Innova 90; Coherent, Palo Alto, CA) operating at 500 mW output at 488 nm. The red fluorescence emitted by PI was collected by a 620-nm long-pass filter, recorded as a measure of the amount of DNA-bound PI and displayed on a linear scale. Data from 20 000 cells were analyzed with forward and scattered light, and red fluorescence was recorded with Consort 30 software. Non-cellular material was excluded from the analysis by gating side angle and forward angle light scattering characteristics of each sample so that we could assume that cells with a DNA content less than 2*n* that appeared during the time course were apoptotic cells. The percentage of cells in the various cycle phases was determined using WinMDI2.8 Windows Multiple Document Interface Flow Cytometry Application (Cylchred Windows 95, version 1.02).

Morphological analysis

HCT-8 cells were harvested following a 48-h exposure to 72-h IC₅₀ values of test compounds; attached (control) cells and detached cells were collected separately, counted, centrifuged and suspended in 50 μg/ml PI. After staining, morphology was determined by examining the cytospin slides using fluorescence microscopy interfaced with a computerized image analysis system. The

percentage of dead floating cells was evaluated by the Trypan blue dye exclusion test.

Results

Cell growth-inhibitory effects

Cell growth inhibition by study compounds 1–4 was evaluated on a panel of established human tumor cell lines: colon carcinoma cells (HCT-8, HT29), breast carcinoma cells (MCF7) and lung carcinoma cells (A549) as a function of exposure time and concentrations. For comparison purposes, the cytotoxicity of doxorubicin (DOX), mitomycin C (MMC) and 8-chloro-pyrazolo[5,1-*c*][1,2,4]benzotriazine-3-carbonitrile 5-oxide (compound 5) was evaluated under the same conditions. The results from these experiments are summarized in terms of IC₅₀ values in Table 2 and Fig. 1.

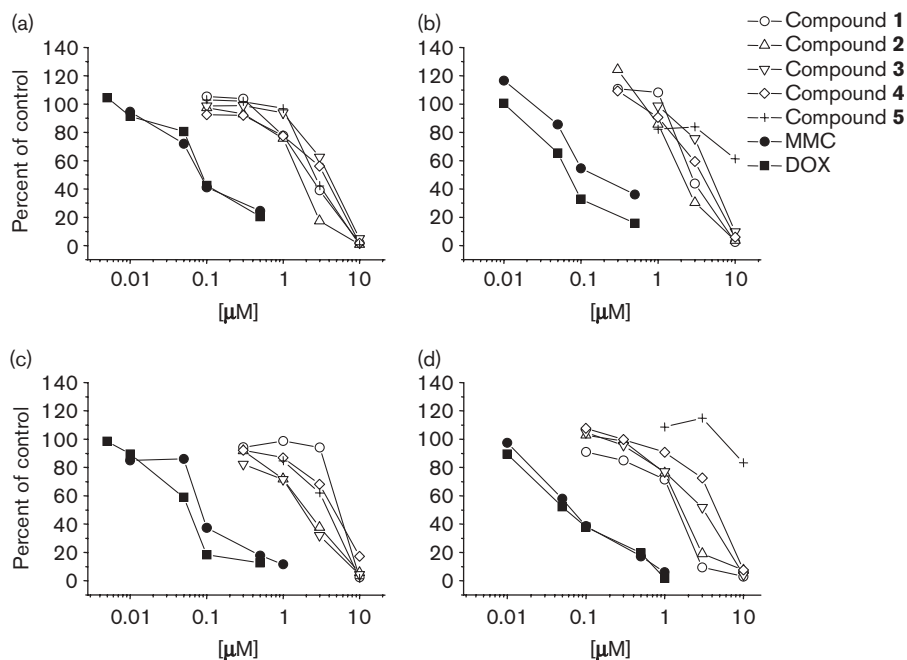
Cytotoxicity after a 4-h exposure

After a short-term exposure, in HCT-8 and MCF7 cells IC₅₀ values ranged from 3.6 to 9.4 μM for all compounds, with the exception of compound 4 in HCT-8 cells and compound 5 in MCF7 cells (IC₅₀ > 10 μM); in HT29 and A549 cells all compounds exhibited IC₅₀ > 10 μM. All cell lines were highly sensitive to DOX and MMC, with IC₅₀ values ranging from 0.2 and 0.5 μM.

Cytotoxicity after a 24-h exposure

After an intermediate exposure, HCT-8 cells displayed a similar degree of sensitivity to all study compounds (IC₅₀ values ranging from 2.3 to 4.5 μM). MCF7 cell line exhibited IC₅₀ values between 1.8 and 6.4 μM, but

Fig. 1



Drug sensitivity profiles of HCT-8 (a), HT29 (b), MCF7 (c) and A549 (d) cell lines. Each point is the percentage of growth with respect to the control upon incubation with increasing amounts of the compounds and evaluated after a 72-h exposure. Each percentage is the mean \pm SE of three determinations or the mean of two independent experiments performed with quadruplicate cultures at each drug concentration tested.

was not sensitive to compound 5 ($\text{IC}_{50} > 10 \mu\text{M}$). Prolongation of exposure time induced a higher degree of sensitivity to study compounds also in HT29 and A549 cells (IC_{50} values ranging from 2.3 to 7.0 μM), with the exception of compound 5 ($\text{IC}_{50} > 10 \mu\text{M}$). IC_{50} values for DOX and MMC ranged from 0.07 and 0.13 μM in all cell lines.

Cytotoxicity after a 72-h exposure

After a long-term exposure, the inhibitory cell growth effects of study compounds were similar in all cell lines and ranged from 1.6 and 4.6 μM . HT29 and A549 cells remained not sensitive to compound 5 ($\text{IC}_{50} > 10 \mu\text{M}$). IC_{50} values for DOX and MMC ranged from 0.05 and 0.09 μM in all cell lines.

Cell cycle effects

Analysis of cell cycle perturbations induced by study compounds was performed on a representative cell line (HCT-8). Figure 2 shows the cell cycle effects after exposure to 72-h IC_{50} values of the various study compounds.

Continuous exposure of 24 h to compounds 1, 4 and DOX appeared not to cause any significant cell cycle perturbation. After the same exposure time, in the presence of compound 2, a substantial accumulation of cells in the G_2/M phase was observed with a considerable decrease in

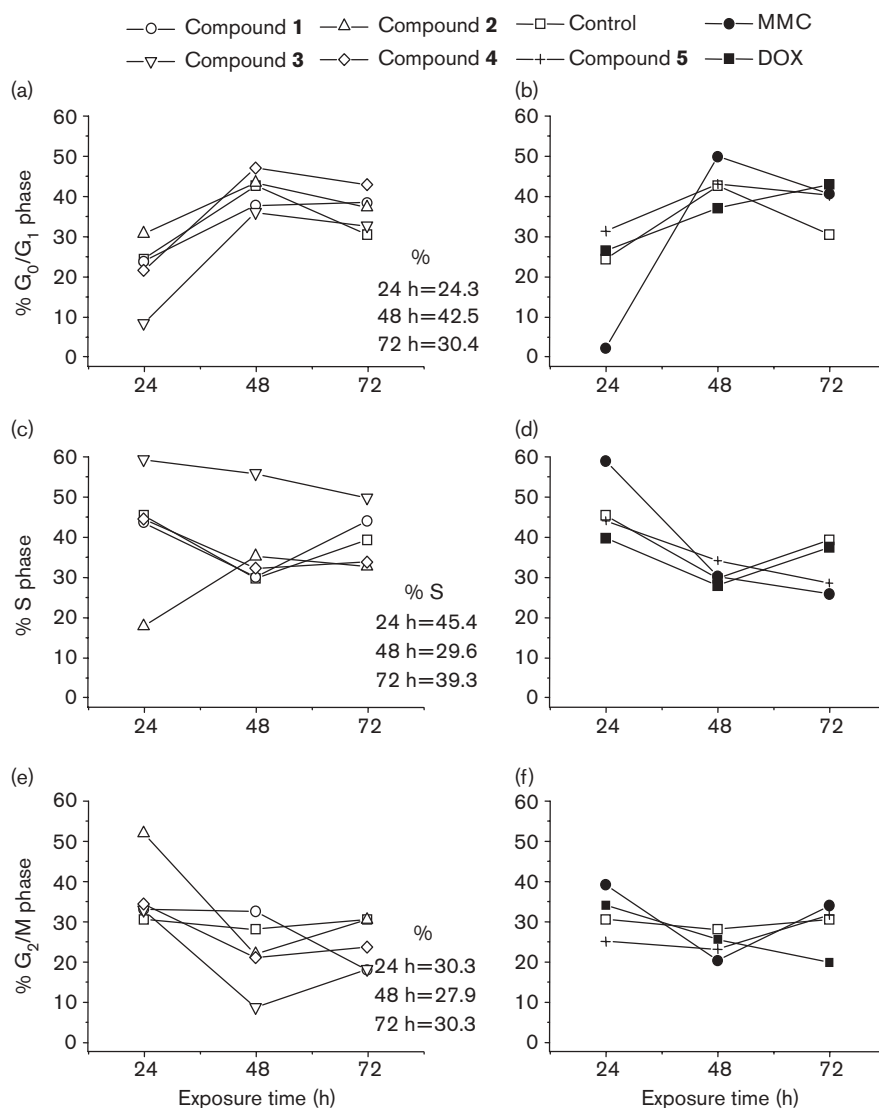
the S phase. Compound 3 and MMC caused a marked accumulation of cells in the S phase with a notable decrease of the G_0/G_1 fraction. Compound 5 did not appear to cause any significant change in cell cycle distribution.

At later time points (48 and 72 h) compound 4 induced a modest accumulation of cells in G_0/G_1 accompanied by a slight decrease of G_2/M cells. A similar trend was observed as far as compound 1 and DOX were concerned. The early accumulation of cells in the G_2/M phase and $\text{S}/\text{G}_2/\text{M}$ interface noted with compound 2 and MMC at 24 h rapidly decreased at 48 h. At 72 h no substantial cell cycle perturbations were observed with compound 2, while a slight cell decrease in S phase and a slight cell accumulation in G_0/G_1 was observed with MMC. Cell accumulation in the S phase reported with compound 3 was instead persistent within time until 72 h. No substantial late cell cycle effects were observed with compound 5. With this compound only an increased slight block of cells in the G_0/G_1 phase accompanied by a modest decrease of cells in the S phase was in fact observed at 72 h.

Morphological effects

Detached HCT8 cells were collected 48 h following incubation with 72-h IC_{50} s of the study compounds. These cells showed an apoptotic morphology with

Fig. 2



Cell cycle distribution of HCT-8 cells treated with study compounds (a, c and e) and reference compounds (b, d and f) for 72 h with IC₅₀ values. Their cell cycle phase distribution was determined by flow cytometry, as described in Materials and methods. The percentage of control cells in the various phases of the cell cycle is also listed.

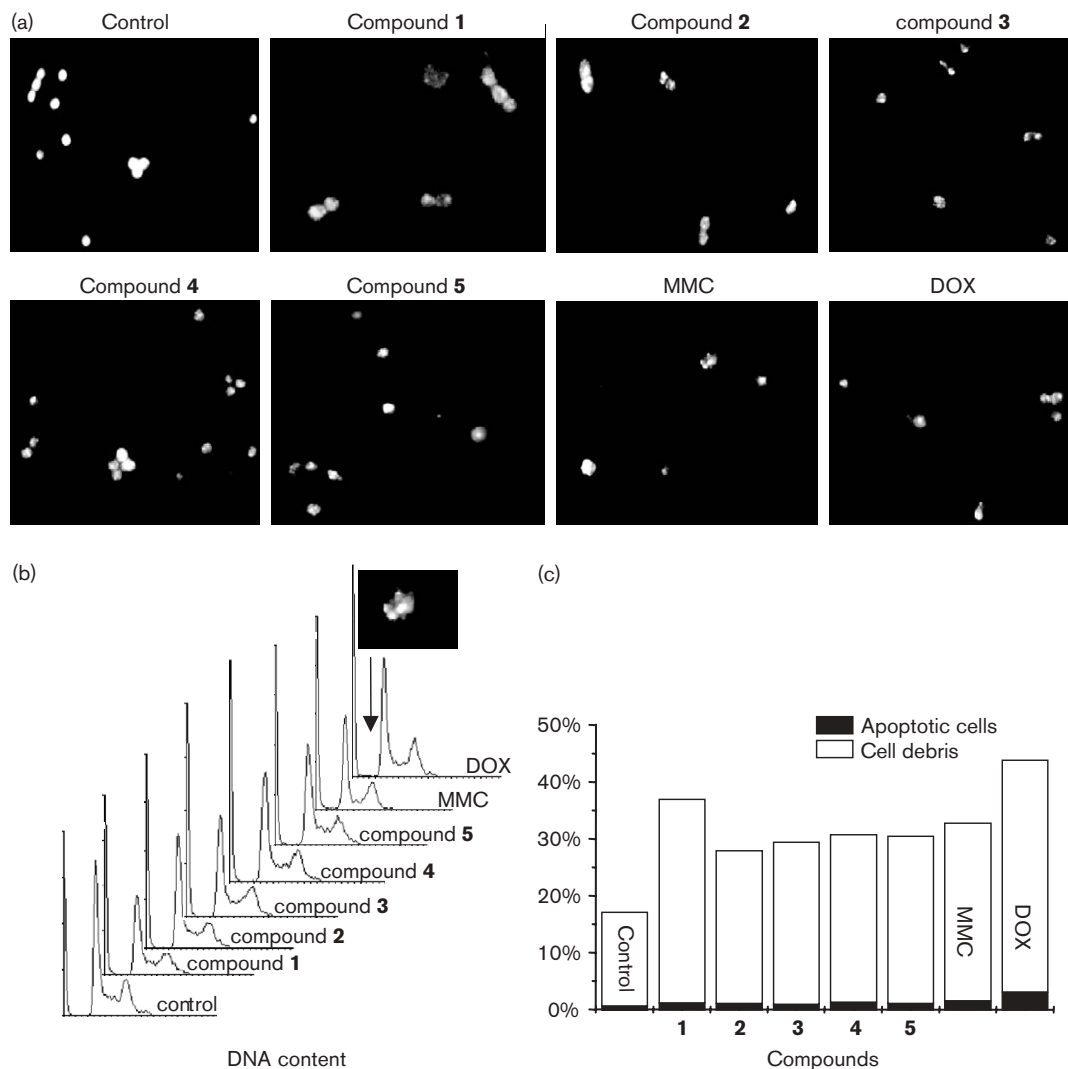
compacted and fragmented nuclei when compared with attached control cells where the chromatin was diffuse (Fig. 3a). Moreover, 58–80% of detached cells excluded Trypan blue 48 h after exposure to IC₅₀ agent values, meaning that their membranes remained intact. Figure 3(b and c) illustrates the percentage of HCT-8 cells with sub-G₀/G₁ DNA content calculated on the total cell population 48 h after drug exposure; at this time we had a higher number of floating cells. Spontaneous apoptosis was observed in the 0.5% of the control cells with 16% of cell debris. Only minimal, non-significant increases in the percentage of HCT-8 apoptotic cells were observed with study compounds and control drugs, ranging from 0.9 to

2.5%. On the contrary, abundant cellular debris, as demonstrated by the DNA histograms, was present, ranging from 27 to 41%.

Discussion

Previous investigations have already revealed favorable cytotoxic properties for some 3-nitropyrazolo[5,1-*c*][1,2,4]benzotriazine derivatives, especially against leukemia cells, and low cytotoxic activity against solid tumors [6]. This further stimulated our interest, and thus we continued our investigation with the synthesis and study of this new class of 3-nitropyrazolo[5,1-*c*]

Fig. 3



Fluorescent microscopy showing normal nuclear morphology of HCT-8 control untreated attached cells and apoptotic nuclei of detached cells from HCT-8 48 h following exposure to IC_{50} values of compounds (a); the DNA histograms (b) and the percentage of apoptotic cells and cell debris (c), evaluated as explained in the text, are also reported.

[1,2,4]benzotriazine derivatives **1–4** bearing other substitutions with electron-drawers or electron-donors in positions 7 and/or 8. Our data demonstrate that the new series of 3-nitropyrazolo[5,1-*c*][1,2,4]benzotriazine derivatives exerts cytotoxic activity at micromolar concentrations against human colon, breast and lung carcinoma cell lines. In all tumor cell lines tested, the growth-inhibitory effects of the study compounds were dependent on both drug concentrations and exposure times.

The differences in sensitivity to study compounds observed in the various cell lines after a short-term exposure (4 h) became less pronounced following longer-term exposures (24 and 72 h). When HT29 and A549 cells

were exposed to study compounds for 4 h, 50% cell growth inhibition was not achieved even at the highest concentration tested (10 μ M). The same result was observed in HCT-8 and MCF7 cells with short-term exposure to compounds **4** and **5**. Increasing the exposure time from 4 to 24 h notably decreased the IC_{50} values observed for all cell lines, whereas an increase in the exposure time from 24 to 72 h only slightly decreased the IC_{50} values for the four cell lines.

We also studied the cell kinetic perturbations produced by *in vitro* exposure to study compounds of one representative tumor cell line (HCT-8). At 72-h IC_{50} values, this resulted in cell cycle perturbations

comparable to those of MMC and DOX, except for compound **3** which induced a marked accumulation of cells into the S phase with a concomitant decrease in G₀/G₁ and G₂/M phases.

Since apoptosis is a major mode of cell death induced by several DNA-damaging agents [11], we evaluated the induction of apoptosis of colon carcinoma cells (HCT-8) treated with IC₅₀ values of the study compounds. Apoptotic cells were detected as the population of cells with sub-G₀/G₁ DNA content. Further analysis by fluorescent microscopy of the PI-stained detached cell morphology helped us to confirm the presence of apoptotic cells in the cell culture which may be the same recorded with flow cytometry as the sub-G₀/G₁ DNA peak. Previous studies by other authors have shown that only detached cells exhibit apoptotic morphology, while attached treated cells have a morphology similar to that of attached untreated control cells [12–14].

Our results indicate the presence of a modest percentage (up to 2.5%) of cells with sub-G₀/G₁ DNA content in all samples after a 48-h exposure to agents. On the other hand, the presence of abundant cellular debris, provided by the DNA histograms, suggests cell killing. One possible explanation is that all compounds induce extensive fragmentation of DNA, generating very small DNA fragments or single nucleotides that cannot be detected with flow cytometry.

In conclusion, this study has demonstrated that 3-nitropyrazolo[5,1-*c*][1,2,4]benzotriazine derivatives possess cytotoxic activity in the micromolar range against human tumor cell lines under aerobic conditions. This activity is similar or even improved as compared to 3-cyano-8-chloropyrazolo[5,1-*c*][1,2,4]benzotriazine 5-oxide **5**, previously synthesized in our laboratory, but significantly lower than that of DOX and MMC. The effects on the cell cycle induced by the 3-nitropyrazolo[5,1-*c*][1,2,4]benzotriazine derivatives were, however, similar to those of DOX (e.g. compounds **1**, **3** and **4**) and MMC (e.g. compounds **2** and **5**). These results do not necessarily imply that the cytotoxic mechanism of the tested compounds is similar to that of DOX or MMC, but the structural features of these agents suggest that DNA may be one of the targets of their cytotoxic action. Although these data do not provide an explanation

for the anti-proliferative effects of 3-nitropyrazolo[5,1-*c*][1,2,4]benzotriazine derivatives, they confirm the critical role of the nitro group on cytotoxicity independently of substituents in positions 7 and 8. These results warrant further investigations to clarify pathways through which alterations of the cell cycle lead to cell growth inhibition.

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