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Antitumor 1-nitroacridine derivative C-1748, induces apoptosis, necrosis or senescence in human colon carcinoma HCT8 and HT29 cells.

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ABSTRACT

C-1748 is a DNA binding agent with potent antitumor activity, especially towards prostate and colon carcinoma xenografts in mice. Here, we elucidated the nature of cellular response of human colon carcinoma HCT8 and HT29 cells to C-1748 treatment, at biological relevant concentrations (EC_{90} and their multiplicity). Cell cycle analysis showed gradual increase of HCT8 cells with sub-G1 DNA content (25% after 72 h) considered as apoptotic. Hypodiploid cell population increased up to 60% upon treatment with $4 \times EC_{90}$ concentration of the drug. Compared with HCT8 cells, the fraction of sub-G1 HT29 cells did not exceed 14%, even following 4-fold dose escalation. Morphological changes and biochemical markers such as: phosphatidylserine externalization, apoptotic DNA breaks, mitochondrial dysfunction, caspase activation, confirmed the presence of considerable amount of apoptotic HCT8 cells but only a low amount of apoptotic HT29 cells. Next, we demonstrated that HCT8 cells surviving after exposure to C-1748 were in state of senescence, based on altered cell morphology and expression of a pH 6-dependent β -galactosidase. On the contrary, no β -galactosidase staining was observed in HT29 cells after C-1748 treatment. Moreover, prolonged drug incubation (up to 168 h) resulted in massive detachment of cells from culture plates, what together with Annexin V/PI results, indicated that necrosis was the main response of HT29 cells to C-1748 treatment. We also determined the ability of C-1748 to induce reactive oxygen species (ROS) in colon cancer cells and demonstrated, that generation of ROS was not essential for C-1748-induced apoptosis and cytotoxic activity of this drug.

Key words:

C-1748; apoptosis; necrosis; senescence; reactive oxygen species (ROS);

1. INTRODUCTION

The 1-nitroacridines are DNA-binding and potent antitumor agents synthesized at Gdańsk University of Technology [1]. Among them, the most active compound Ledakrin (Nitracrine) was in 1970's registered as an antitumor drug in Poland [2]. Ledakrin was recommended especially for treatment of ovary carcinoma, but also effectively used against colon, breast and lung cancers [3, 4]. However, due to severe side effects and high general toxicity, Ledakrin was desisted from further clinical application. Modification of chemical properties of 1-nitroacridines, especially decreasing the ability of nitro group in position 1 to undergo reduction, by introducing to position 4, para to the nitro group, an electron donating-methyl group, resulted in development of 4-methyl-1-nitroacridines with high antitumor activity towards broader spectrum of tumors and lower toxicity than parental 1-nitroacridines [5, 6]. The most active 9-(2'-hydroxyethylamino)-4-methyl-1-nitroacridine (C-1748, Fig. 1), was found to have significant cytotoxic activity *in vitro* towards cancer cell lines and high antitumor activity against several prostate (LnCaP, JCA, PC3, TSU) and colon carcinoma (HCT8) xenografts in nude mice [6]. Pre-clinical toxicology studies in rodent and dogs showed C-1748 to have very low systemic toxicity along with a lowered mutagenic potential [7, 8, 9]. C-1748, as well as Ledakrin and other 1-nitroacridines, after metabolic activation bind covalently to DNA [10] and induced DNA crosslinking in tumor cells, which was crucial for biological activity of these compounds [11, 12, 13].

Recently, it has been shown that C-1748 significantly induced DNA double strand breaks in prostate cancer LnCaP cells and, to smaller extent, in leukemia HL60 cells, less sensitive to the action of this compound [6]. Moreover, in LnCaP cells, C-1748 treatment markedly downregulated androgen receptor but upregulated

1 estrogen receptor what was a further validation of the cytotoxicity of C-1748 in this
2 cells [6].
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4 The molecular mechanisms by which chemotherapeutic agents kill tumor cells
5 are numerous and depend on a variety of parameters including the drug and tumor
6 types. It is widely accepted that tumor cells exposed to cytotoxic drugs die mainly
7 from apoptosis. Moreover, in many types of tumor cells accelerated or premature
8 senescence, a form of growth arrest after exposure to ionizing radiation or drugs that
9 induce DNA damage, has been observed [14, 15]. It is still not clear what determines
10 if a cell undergoes senescence or apoptosis. Although most cells are capable of
11 both, these processes seem to be exclusive [16]. Moreover, in many types of cells
12 the influence of p53 function can promote senescence arrest and apoptotic cell death
13 [17]. However, there is also evidence for accelerated senescence that is independent
14 of p53 [18].
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31 The current studies were designed to elucidate the nature of the response of
32 human colon cancer HCT8 and HT29 cells to C-1748 in terms of growth arrest and/
33 or cell death. Additionally, we determined the ability of C-1748 to induce reactive
34 oxygen species (ROS) production, and investigated the correlation between ROS
35 generation and cell death and cytotoxic activity of the drug. In a view of recent finding
36 that a form of senescence arrest may occur in response to antitumor drugs, we also
37 examined the ability of C-1748 to induce senescence.
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2. MATERIALS AND METHODS

2.1. Chemicals and antibodies

9-(2'-hydroxyethylamino)-4-methyl-1-nitroacridine C-1748 was synthesized as monochloride by Dr Barbara Wysocka-Skrzela in the Department of Pharmaceutical Technology and Biochemistry at Gdańsk University of Technology, Poland. Stock solution of the drug in 50% ethanol was freshly prepared just before use. Proteinase K and N-acetyl-L-cysteine (NAC) were purchased from Merck (Darmstadt, Germany). RNase A, DAPI, PI, SA β -gal, melatonin, mouse monoclonal anti-p53 antibody, mouse monoclonal anti- β -actin antibody were obtained from Sigma-Aldrich (St. Louis, MO, USA). JC-1 was purchased from Molecular Probes (Eugene, OR, USA). The following chemicals were obtained from Roche Diagnostics (Mannheim, Germany): Annexin V-Fluos Staining Kit, terminal transferase, cobalt chloride, biotin-dUTP and TdT buffer. FITC-conjugated Monoclonal Active Caspase-3 Antibody Apoptosis Kit was purchased from BD Pharmingen (San Diego, CA, USA).

2.2. Cell culture and growth inhibition assay

HCT8 human ileocecal adenocarcinoma cell line was kindly provided by R.K. Tiwari from Department of Microbiology and Immunology, New York Medical College, Valhalla, USA and HT29 human adenocarcinoma colon cell line was obtained from D. Banerjee from Department of Medicine and Pharmacology, Cancer Institute of New Jersey, New Jersey, USA. HCT8 and HT29 cells were grown in RPMI 1640 medium (GIBCO BRL Life Technologies, Paisley, United Kingdom) and McCoy's 5A medium (Sigma-Aldrich, St Louis, MO, USA) respectively, supplemented with 10% heat-inactivated fetal bovine serum and antibiotics (100 μ g/ml streptomycin, 100 U/ml penicillin), in a 5% CO₂ atmosphere at 37°C. Under these growth conditions the cell-

doubling time was 24 h for both cell lines. All experiments were performed with cells in the exponential phase of growth.

Cell growth inhibition was assessed by cell counting using Coulter Counter, model ZB1 according to the method describe by Bhuyan et al. [19]. Briefly, cells (1×10^5) were seeded in 24-well microculture plates and treated with various C-1748 concentrations for 72 h. A dose response curve was plotted and used to calculate drug concentration that yielded 90% of inhibition of cells growth (EC_{90}).

2.3. Cell cycle distribution by flow cytometry

Asynchronous HCT8 and HT29 cells were exposed to C-1748 at concentrations corresponding to EC_{90} values and their multiplicity for 3 - 72 h. After drug treatment, adherent cells were trypsinized, combined with nonadherent cells, collected by centrifugation for 5 min at 4°C, at 1,000 rpm, washed twice with ice-cold phosphate-buffered saline (PBS) and then fixed in ice-cold 80% ethanol overnight at -20°C. Next, cells were centrifuged for 5 min at 4°C, at 1,000 rpm, washed twice with PBS and resuspended in 1ml of DNA staining solution (20 µg/ml of propidium iodide PI and 100 µg/ml of RNase A in PBS), for 30 min in the dark. Flow cytometric analyses were performed using a FACScan cytometer (Becton Dickinson, San Jose, CA, USA). At least 1×10^4 cells were collected and the data analysis was performed using the WinMDI software (J. Trotter, Scripps Research Institute, San Diego, CA, USA). All experiments were repeated at least three times.

2.4. Cell morphology assessment

Nuclear morphology was examined under fluorescence microscope (OLYMPUS BX60) after staining with 4',6-diaminidino-2-phenylindole (DAPI).

Briefly, after drug incubation, cells (5×10^5) were spun onto microscopic slides, fixed in methanol : acetic acid solution (3:1) for 15 min and stained with DAPI ($1 \mu\text{g/ml}$) for 5 min. Cells were regarded as apoptotic based on the presence of condensed, fragmented chromatin.

2.5. TUNEL assay

Apoptotic DNA degradation following by C-1748 treatment was studied by the TUNEL assay. The commercially accessible Apoptosis Detection kit (Roche Diagnostics, Mannheim, Germany) was used and prepared according to the supplier's recommended protocol. Briefly, following treatment, cells (1×10^6) were centrifuged, washed twice in PBS and gently resuspended in 1% formaldehyde in PBS and kept on ice for 15 min. Fixed cells were washed with PBS and resuspended in 70% ethanol. Dehydrated cells were incubated overnight at -20°C . After rehydration in PBS for 5 min cells were resuspended in 50 μl of reaction buffer (5U terminal transferase, 2.5 mM cobalt chloride, 0.5 mM biotin-dUTP and TdT buffer containing 0.2 M potassium cocadylate, 25 mM TRIS-HCl, 0.25 mg/ml BSA, pH 6.6) and incubated at 37°C for 30 min. Then, cells were washed with rinsing buffer (PBS containing 0.1% Triton X-100, 0.5% BSA), resuspended in 100 μl of staining buffer containing 2.5 $\mu\text{g/ml}$ FITC-avidin, 4xSSC buffer, 0.1% Triton X-100 and 5% (w/v) non-fat dry milk, and incubated for 30 min at room temperature in the dark. The pellets were then washed twice with rinsing buffer, resuspended in PBS containing 5 $\mu\text{g/ml}$ PI and 100 $\mu\text{g/ml}$ RNase A, and incubated at room temperature for 30 min. The red (PI) and green (FITC) fluorescence were measured with FACScan flow cytometer.

2.6. *Phosphatidylserine externalization*

Annexin V binding assay was performed using Annexin-V-Fluos Staining kit (Roche Diagnostics, Mannheim, Germany) according to the manufacturer's specification. Briefly, after drug incubation, cells (1×10^6) were trypsinized, rinsed with HEPES/NaOH buffer (10 mM Hepes pH 7.4, 140 mM NaCl, 5 mM CaCl_2), pelleted and resuspended in 50 μl of Annexin V-FITC diluted in binding buffer containing PI. Cells were incubated for 15 min at room temperature in the dark and diluted with 400 μl of HEPES/NaOH buffer and analyzed by flow cytometry within 1 h. Bivariate flow cytometry and data analysis were performed as described for TUNEL assay.

2.7. *Measurement of mitochondrial transmembrane potential ($\Delta\Psi_m$)*

Changes in mitochondrial membrane potential ($\Delta\Psi_m$) were analyzed by flow cytometry using JC-1 dye (5,5',6,6'-tetrachloro-1,1',3,3'-tetraethylbenzimidazolyl-carbocyanine iodide, Molecular Probes, Eugene, OR, USA). After treatment, cells (5×10^5) were trypsinized and washed twice with PBS and incubated with medium containing JC-1 (10 $\mu\text{g/ml}$) for 15 min at room temperature in the dark. Finally, cells were washed and resuspended in 1 ml of PBS for flow cytometry analysis. The loss of $\Delta\Psi_m$ was monitored based on a decrease in JC-1 red fluorescence concurrent with an increase in green fluorescence.

2.8. *Caspase-3 activity measurements*

Caspase-3 activation was determined using the Active Caspase-3 Apoptosis kit (BD Pharmingen, San Diego, CA, USA) according to the manufacturer's instruction. Cells (1×10^6) stained with FITC-conjugated anti-active caspase-3 antibody, were analyzed by flow cytometry.

2.9. Determination of cellular senescence

pH 6.0-dependent β -galactosidase expression was used as a marker for senescence along with senescent related morphology. Following C-1748-treatment, cells (1×10^5) were washed twice with PBS and fixed in 2% formaldehyde and 0.2% glutaraldehyde in PBS for 5 min. The cells were then washed again with PBS and stained with X-gal (5-bromo-4-chloro-3-indolyl- β -D-galactosidase) staining solution (1 mg/ml X-gal, 40 mM citric acid/sodium phosphate, pH 6.0, 5 mM potassium ferrocyanide, 5 mM potassium ferricyanide, 150 mM NaCl, 2 mM MgCl_2). Following overnight incubation at 37°C, the cells were washed twice with PBS, and photographed under a light microscope.

2.10. Western blot analysis

Western blot analysis of whole cell lysates was performed as described previously [20]. Membrane was probed with primary mouse monoclonal anti-p53 antibody (Sigma-Aldrich, St. Louis, MO, USA) diluted 1:1000 and anti-actin (Sigma-Aldrich, St. Louis, MO, USA) diluted in 1:1000 and then incubated with horseradish peroxidase-conjugated secondary antibody (anti-mouse 1:2000 for p53, anti-mouse for actin 1:10 000) and visualized by enhanced chemiluminescence kit (Pierce Biotechnology, Rockford, IL, USA).

2.11. Measurement of ROS generation

Intracellular ROS generation was monitored by flow cytometry, using fluorescent product of DCFH-DA oxidation, 2', 7'- dichlorofluorescein, DCF (SIGMA, St Louis, MO, USA). HCT8 and HT29 cells were treated with C-1748 at concentration corresponding to EC_{90} value for the indicated times. Cells (1×10^6) were then harvested by trypsinization, washed twice with PBS, and loaded with 20 μM DCFH-

1 DA. Cells were incubated at 37°C for 30 min, washed twice in PBS, and green (DCF)
2 fluorescence was examined by flow cytometry on a FACScan cytometer. Samples
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4 incubated with hydrogen peroxide (0.01%) were used as a positive control. All
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6 experiments were repeated at least three times.
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10 11 12 *2.12. Statistical analysis*

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14 Results are expressed as means $\pm$ S.D. A paired *t*-test was used to compare
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16 two groups, and one-way ANOVA and Dunnett's *t*-test was used for multiple
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18 comparisons. The criterion for statistical significance was set at $p < 0.05$.
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54 **3. RESULTS**

55 56 57 58 *3.1. Effect of C-1748 on HCT8 and HT29 cell growth and cell cycle distribution* 59 60 61 62 63 64 65

The growth of the human colon cancer HCT8 and HT29 cells in the presence of various concentrations of C-1748, ranging from 0.001 to 10 μ M, was determined by cell counting using Coulter Counter. After a continuous 72 h exposure, C-1748 significantly inhibited growth of colon cancer cells in a concentration-dependent manner (data not shown). The concentration of the drug required to inhibit cell growth by 90% (EC_{90}) demonstrated that HCT8 cells were more sensitive to C-1748 treatment than HT29 cells (EC_{90} 0.04 μ M for HCT8 cells and 0.21 μ M for HT29 cells, respectively). EC_{90} concentration was used in all the subsequent experiments regarding the cellular response of colon cancer cells to C-1748.

The effect of the 1-nitroacridine derivative C-1748 on cell cycle progression of colon cancer cells, was determined by flow cytometry, following treatment with the drug (EC_{90} concentration) for various incubation times (3-72 h). Flow cytometry analyses showed that starting from 3 h of C-1748 treatment, HCT8 cells underwent a transient arrest in G1 and G2/M phases of the cell cycle, however the number of cells in these phases was gradually decreased during prolonged drug incubation (Fig. 2 A). These cell cycle changes were accompanied by a time-dependent appearance of typical for late stage of apoptosis, sub-G1 peak (25% after 72 h of treatment), as well as by the appearance of polyploid cell population (about 30% after 72 h of drug treatment, Tab. 1). The incubation of HCT8 cells with higher drug concentration ($4 \times EC_{90}$), significantly increased the percentage of sub-G1 cells (up to 60% after 72 h, Fig. 2 C, Tab. 2). Treatment of HT29 cells with C-1748 at EC_{90} concentration resulted in less evident perturbations of the cell cycle. Starting from 3 h of drug treatment, a slight increase in G1 phase was observed. Accumulation of cells at G2/M compartment was detectable after 24 h of drug treatment and following longer incubation, the number of cells in this phase significantly decreased (Fig. 2 B).

Starting from 3 h of C-1748 exposure the population of polyploid cells increased and reached the highest level after 24 h (20%) and then began to decrease. Between 48 and 72 h, the reduction in G2/M arrested and polyploid cells was accompanied by a concomitant increase in sub-G1 population (about 14% after 72 h, Tab. 1). In contrary to HCT8 cells, increased concentration of C-1748 ($4 \times EC_{90}$) did not result in considerable increase of the number of HT29 cells undergoing apoptosis in terms of sub-G1 DNA content (Fig. 2 C, Tab. 2).

3.2. Apoptotic cell morphology

Flow cytometric analysis of cells in sub-G1 region indicated that colon cancer cells treated with C-1748 underwent apoptosis. To confirm these data, morphological changes of cells treated with C-1748 were assessed using fluorescence microscopy following DAPI staining. As shown in Fig. 3 A and B, untreated cells exhibited unchanged morphology, characteristic for colon cancer cells. After 12 h of C-1748 treatment at EC_{90} concentration, only a few apoptotic HCT8 cells were visible. Prolonged drug incubation (48 and 72 h), as well as two-fold increased drug concentration, resulted in appearance of numerous cells exhibiting morphological changes typical for apoptosis, with cell shrinkage, chromatin condensation and fragmentation into discrete bodies. By contrast, only a few apoptotic HT29 cells were observed following C-1748 treatment at EC_{90} concentration and its multiplicity. At 72 h of C-1748 exposure some of HT29 cells were shrunken and formed the structure of apoptotic bodies.

3.3. DNA degradation

TUNEL assay was used to investigate whether C-1748 induces apoptotic DNA fragmentation in human colon carcinoma cells. This method, based on the labeling

the apoptotic DNA fragments by exogenous terminal deoxytransferase (TdT) and biotin-dUTP/FITC-avidin together with propidium iodide staining, allows to determine whether the DNA fragmentation observed in the treated cells occurred in particular phase of the cell cycle. As indicated in the Fig. 4 (right panel), the population of HCT8 cells with apoptotic DNA breaks remained small after 3 h and 12 h of C-1748 exposure at EC_{90} concentration and increased to approximately 35% after 72 h. On the contrary, the population of HT29 cells with apoptotic DNA breaks was markedly smaller and reached the level of 12% after 72 h of C-1748 treatment. Biparametric flow cytometry analysis also revealed that cells with apoptotic DNA breaks derived from all phases of the cell cycle.

3.4. Annexin V/PI dual staining

To further confirm that C-1748 induces apoptosis, HCT8 and HT29 cells treated with the drug at EC_{90} concentrations were stained with Annexin V-FITC and propidium iodide, and analyzed by flow cytometry. This assay is based on the translocation of phosphatidylserine (PS) from the inner leaflet of the plasma membrane to the cell surface in the early stage of apoptosis. Positive staining with fluorescently labelled Annexin V correlates with loss of plasma membrane polarity but precedes the complete loss of membrane integrity that accompanies later stages of cell death resulting from either apoptosis and necrosis. In contrast, PI can only enter cells after loss of their membrane integrity. Thus, dual staining with Annexin V and PI allows clear to discriminate between unaffected cells (Annexin V-/PI-), early apoptotic cells (Annexin V+/PI-), late apoptotic cells (Annexin V+/PI+) and primary necrotic cells (Annexin V-/PI+). As indicated in the Fig. 5 A, HCT8 cells remained largely unaffected for the first 6 h of C-1748 treatment. Prolonged drug incubation resulted in

progressive appearance of early and late apoptotic cells, and after 72 h of drug exposure, these populations together reached ~ 48%. Moreover, upon drug treatment progression from early (Annexin V+/PI-) to late stages (Annexin V-/PI+) of apoptosis was visible. The fraction of Annexin V-/PI+ (primary necrosis) cells reached 8.8% after 72 h of incubation with C-1748. Treatment of HT29 cells with C-1748 resulted in significantly lower apoptotic cell population compared to HCT8 cells (Fig. 5 B). Populations of early and late apoptotic HT29 cells reached only about 10% after 72 h of C-1748 exposure. Interestingly, starting from 48 h, the population of necrotic cells (Annexin V-/PI+) significantly increased and at 72 h reached ~ 30%. Prolonged incubation with the drug (up to 144 h) resulted in intensive Annexin V-/PI+ (38%) as well as Annexin V+/PI+ (32%) staining without preceeding Annexin V+/PI- staining, and was accompanied by massive detachment of cells from culture plates (data not shown).

3.5. Changes in mitochondrial transmembrane potential

Mitochondrial transmembrane potential $\Delta\Psi_m$, an electrical potential required for several mitochondrial functions including ATP generation, is dramatically reduced early during apoptosis. The disruption of the $\Delta\Psi_m$ generally defines not only an early, but already irreversible stage of apoptosis [21]. The loss of $\Delta\Psi_m$ is routinely used as an indicator of mitochondria damage during apoptosis. Therefore, next we examined the effect of C-1748 on $\Delta\Psi_m$ using mitochondria specific membrane potential probe JC-1. As compared with untreated cells, a time-dependent increase in the proportion of HCT8 and HT29 cells with a reduced $\Delta\Psi_m$ was noted following C-1748 treatment at EC_{90} concentration (Fig. 6 A). Starting from 6 h, the population of HCT8 cells with low $\Delta\Psi_m$ steadily increased, and reached approximately 53% after

72 h of exposure to C-1748. In the case of HT29 cells, the population with low $\Delta\Psi_m$ started to increase after 12 h of C-1748 treatment and reached 47% after prolonged incubation with the drug (72 h).

3.6. Caspase-3 activity

It is well established that in most cases, apoptosis requires the participation of endogenous cellular enzymes – caspases. Among the identified caspases, activation of caspase-3 is a crucial in numerous types of cells leading to the execution of apoptosis [22, 23]. Therefore, we examined, whether caspase-3 was involved in apoptosis induced by C-1748 in colon cancer cells. After 3, 6 and 12 h of treatment with C-1748 at EC_{90} concentration, we observed only a small amount of cells with active caspase-3 in both colon cancer cell lines (Fig. 6 B). However, in the case of HCT8 cells, 24 h of C-1748 treatment resulted in significant increase in number of cells with active caspase-3 (21%), and after 48 and 72 h of incubation, the percentage of cells with active caspase-3 reached about 40% and 60%, respectively. Compared to HCT8 cells, the percentage of HT29 cells expressing active caspase-3 after C-1748 treatment, was markedly lower (17% after 72 h).

3.7. Induction of cellular senescence

DNA damage by a variety of compounds has been shown to induce cellular senescence in various cancer cells. Senescent cells are generally characterized by a reduction in proliferating capacity, adoption of a flattened and enlarged cell shape, and the appearance of senescence-associated β -galactosidase (SA- β -gal) activity [24]. Because C-1748 induced apoptosis only in part of HCT8 and HT29 cells, we investigated whether these cells underwent senescence following C-1748 treatment.

In HCT8 cells increased β -galactosidase staining relative to control was observed 72 h after treatment with C-1748. The number of SA- β -gal-positive cells continued to increase and after 168 h of C-1748 exposure, the whole population of HCT8 cells presented β -galactosidase staining. Together with increased β -galactosidase staining, all HCT8 cells had an enlarged and flattened morphology typical for senescent cells (Fig.7 A). In contrast, we did not observe any β -galactosidase staining, as well as senescent morphology, in HT29 cells after C-1748 treatment (Fig. 7 B).

Several recent studies have shown that drug-induced senescence depends on p53 status [17]. However, there are also evidences, that senescence has been observed in cells both with and without functional p53 [18]. Therefore we conducted experiments to examine the influence of p53 protein on the senescence process induced by C-1748 in HCT8 cells. Western blot analysis revealed that the total p53 level gradually decreased following C-1748 treatment (Fig.7 C). Simultaneously, the number of SA- β -gal-positive cells increased after prolonged drug incubation up to 168 h (Fig. 7A). The obtained results suggested that senescence process induced by C-1748 in HCT8 cells was independent on p53 status.

3.8. Generation of ROS by C-1748 and effects of antioxidants on cell viability and C-1748-induced cell death

A numerous of evidence has accumulated indicating involvement of oxidative stress in the mode of action of various bioactive substances, including anti-cancer agents [25]. Reactive oxygen species (ROS) are frequently generated by redox cycling via electron transfer groups, including nitro group in aromatic compounds like C-1748. Stimulation of formation of ROS can lead to oxidative stress, involved in the

induction of necrotic or apoptotic cell death. To determine whether this event occurs in C-1748-induced apoptosis, we examined the generation of ROS by flow cytometric analysis following staining with 2', 7'- dichlorofluorescein diacetate (DCFH-DA), which is converted to highly fluorescent 2', 7'- dichlorofluorescein (DCF) with the presence of intracellular ROS. HCT8 and HT29 cells were incubated with C-1748 at EC₉₀ concentration for 0, 1, 2, 3, 4, 5 and 24 h prior to collection, and analyzed by flow cytometry for DCF fluorescence. Cells with a high level of DCF fluorescence, correlated with ROS generation, were shifted right on the x axis. As shown in Fig. 8 A, an approximately 2-fold increase in generation of DCF-sensitive ROS in HT29 cells was evident as early as 1 h after C-1748 treatment and then showed a steady level up to 24 h. In HCT8 cells, a small increase in ROS generation was detected after 1 h of C-1748 exposure and reached its maximum levels within 4 h. For comparison, in positive controls, in which HCT8 or HT29 cells were treated with 0.01% H₂O₂ for 30 min prior to analysis, an approximately 2-fold increase in DCF fluorescence intensity was observed (data not shown). To further elucidate whether the ability to generate of ROS is essential for C-1748-induced apoptosis, we investigated the effects of antioxidants on C-1748-induced cell death as well as their influence on cell viability following C-1748 exposure. The colon cancer cells were pretreated with increasing concentrations of N-acetyl-L-cysteine (NAC) or melatonin for 1 h and then were incubated for further 72 h with the drug (HCT8→0.04 μM; HT29→0.21 μM). After treatment, cell viability and the phosphatidylserine (PS) externalization as a marker of apoptotic cells were determined. As shown in Fig. 8 B and 8 C, two studied antioxidants: NAC and melatonin had no effect on cell viability. Moreover, NAC did not protect HCT8 cells against C-1748-induced apoptosis because the level of Annexin V positive/PI negative cells did not change following

pretreatment with antioxidant (Fig. 8 D with reference to results presented on Fig. 5 A).

4. DISCUSSION

The aim of the current study was to investigate and characterize the cellular response of human colon cancer HCT8 and HT29 cells to C-1748 treatment.

It was previously shown that C-1748 binds to DNA non-covalently (by intercalation) and covalently, and after metabolic activation forms interstrand

crosslinks in cellular DNA [13]. Covalent binding and ability to form interstrand DNA crosslinks plays a crucial role for the biological activity of this drug [11, 12, 13]. It is worth to point out that on the molecular level, C-1748 produced rather the same mechanism of action (interstrand DNA crosslinking) [11, 12, 13]. The question arises whether such common mechanism of action will trigger the same cellular response in different cancer cell lines.

The inhibition of tumor cells proliferation seems to be the first effect of action of anticancer drugs, including C-1748. The other cellular effects, such apoptosis, necrosis or senescence occur with some delay in relation to inhibition of cell proliferation. Therefore, it was very important to performed all experiments at biologically relevant concentration of the drug. From the therapeutics point of view the most advantageous would be the concentration of the drug corresponding to its EC_{100} value. Because this concentration is impossible to estimate, in our studies we applied concentration of the drug corresponding to its EC_{90} values in all our experiments (concentration which caused 90% of inhibition of cells proliferation).

Two colon cancer cell lines used in this work were selected due to their high sensitivity to C-1748. In addition, C-1748 exhibited potent antitumor activity against HCT8 xenografts in mice [6].

Cell cycle analysis showed time and dose-dependent appearance of sub-G1 population of HCT8 cells, considered as apoptotic. In contrary to HCT8 cells, the fraction of sub-G1 population of HT29 cells following C-1748 treatment was considerable lower and did not exceed 14%, even following 4-fold increase of drug concentration (Fig. 2 C), which suggested that apoptotic response of HT29 cells to C-1748 treatment was somewhat weaker.

Apoptosis is characterized by several biochemical criteria, including different kinetics of phosphatidylserine (PS) exposure on the leaflet of the plasma membrane, changes in mitochondrial membrane permeability, caspase activation, internucleosomal DNA cleavage, release of intermembrane space mitochondrial proteins [26] as well as by a series of morphological changes such as formation of apoptotic bodies, nuclear and cytoplasmic condensation, chromatin fragmentation, shrinkage of cells and bleb formation [27]. However, necrosis is characterized mostly in negative terms by absence of caspase activation, cytochrome c release, DNA oligonucleosomal fragmentation, presence of plasma membrane permeabilization [28]. Therefore, identification of characteristic morphological and biochemical markers of apoptosis makes it possible to distinguish apoptosis from other forms of cell death.

In our study the induction of apoptosis by C-1748 in human colon cancer cells was confirmed by several biochemical markers, such as: DNA degradation (TUNEL assay), loss of mitochondrial membrane potential $\Delta\Psi_m$, caspase-3 activity and phosphatidylserine externalization, as well as by morphological examination of the cells. Cellular morphology of both tested human colon cancer cells progressively changed with increasing duration of C-1748 exposure. Prolonged drug incubation resulted in the appearance of disintegrated cells, as evidenced by apoptotic bodies, and cells with condensed nuclear chromatin (Fig. 3). These morphological observations together with the appearance of sub-G1 populations confirmed the presence of considerable amount of apoptotic HCT8 cells and only small amount of HT29 cells, following treatment with C-1748.

To corroborate cell morphology-based observations, the apoptotic process induced by C-1748 was additionally monitored by TUNEL assay. Exposure of colon

1 cancer HCT8 and HT29 cells to C-1748 at EC₉₀ concentration resulted in the
2 appearance of TUNEL-positive cells. The fraction of cells with fragmented DNA was
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4 35% in HCT8 cells and was smaller (12%) in HT29 cells (Fig. 4). These data
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6 correlated with those obtained from cell cycle analysis, where 25% of the HCT8 and
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8 14% of the HT29 all population represented cells with sub-G1 DNA content.
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10 Moreover, cells with apoptotic DNA fragmentation were derived from each phase of
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12 the cell cycle which was consistent with the lack of cell cycle arrest in the specific
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14 phase.
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19 Most apoptotic pathways converge on the mitochondria, inducing the
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21 disruption of the mitochondrial transmembrane potential $\Delta\Psi_m$. The loss of
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23 mitochondrial membrane potential $\Delta\Psi_m$ is early and already irreversible stage of
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25 apoptosis [21]. Moreover, mitochondrial dysfunction during apoptosis is very often
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27 associated with the activation of caspases [29]. Exposure to C-1748 resulted in time-
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29 dependent decrease of mitochondrial membrane potential $\Delta\Psi_m$ in HCT8 and HT29
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31 cells (Fig. 6 A). However, the significant increase in the number of cells with active
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33 caspase-3 was observed in HCT8 cells. After 72 h of incubation with the drug, 60%
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35 of HCT8 cells had active caspase-3, but in the case of HT29 cells caspase-3 was
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37 activated only in 10% of cell population (Fig. 6 B). The activation of caspase-3
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39 concomitantly with the dissipation of $\Delta\Psi_m$ confirmed that apoptosis was the main
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41 response of HCT8 cells to C-1748 treatment. In contrast, the disruption of
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43 mitochondrial membrane potential $\Delta\Psi_m$, which also can result from membrane
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45 permeabilization, together with the presence of only small population of HT29 cells
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47 with active caspase-3 indicated that necrosis was responsible for the death of colon
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49 cancer HT29 cells after C-1748 treatment. The increased permeabilization of cellular
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51 membranes, including mitochondrial membrane which provides to decrease of
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mitochondrial transmembrane potential $\Delta\Psi_m$ has been very often observed during necrosis [30].

The mode of cell death induced by C-1748 was further confirmed by Annexin V/PI dual staining. In HCT8 cells the percentage of Annexin V+/PI+ cells was increased by 48% at 72 h of C-1748 treatment, whereas in HT29 cells, the fraction of early and late apoptotic cells reached only 10% after 72 h of drug treatment (Fig. 5 A and B). Simultaneously, 72 h incubation resulted in dramatic increase of necrotic (Annexin V-/PI+) HT29 cells (30%). This effect was further augmented upon 144 h of drug exposure. Compared with HT29 cells, only small population of necrotic cells (~9% after 72 h) was observed in HCT8 cells treated with C-1748. Collectively, these findings confirmed that apoptosis plays a major role in death of HCT8 cells in response to C-1748, whereas HT29 cells die mainly by necrosis.

Next, we investigated whether C-1748-treated cells, which did not die by apoptosis or necrosis, might undergo accelerated senescence. We demonstrated that the HCT8 cells, surviving after exposure to C-1748, were in state of senescence, based on altered cell morphology and increased β -galactosidase staining relative to control (Fig. 7 A). In contrast to HCT8 cells, no β -galactosidase staining could be observed in HT29 cells after C-1748 treatment (Fig. 7 B). Moreover, the prolonged drug incubation (up to 168 h) resulted in massive detachment of cells from culture plates. These observations, together with Annexin V/PI results, clearly indicate that necrosis plays the main role in the response of HT29 cells to C-1748 exposure.

It is worth to point out, that on the cellular level C-1748, at biologically relevant concentrations corresponding to its EC_{90} values, induces different cellular response (apoptosis, necrosis or senescence) in human colon carcinoma cell lines. A possible explanation of such diverse cellular response may be the status of p53 protein. HCT8

1 cells with wild p53 underwent mainly apoptosis and senescence. On the contrary,
2 HT29 cells with mutated, non functional p53 underwent mainly necrosis (apoptotic
3 response was very weak), and did not exhibit features of senescence. Western blot
4 experiments revealed that the total p53 level gradually decreased in HCT8 cells upon
5 C-1748 treatment and after 168 h there was no p53 protein signal from Western blot
6 analysis (Fig.7 C). On the other hand, the number of SA- β -gal-positive HCT8 cells
7 continued to increase and after 168 h of C-1748 exposure the whole population
8 presented β -galactosidase staining (Fig.7 A). These results suggested that
9 senescence process induced by C-1748 was independent on p53 protein. Thus,
10 further studies are needed to indicate other factors responsible for the diverse cellular
11 response of colon cancer cells to C-1748 treatment.
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27 C-1748 and other 1-nitroacridines require metabolic activation before they
28 exhibit any cytotoxic and antitumor activity [13, 31]. It was shown that metabolic
29 transformations of these compounds occurred in several steps including the
30 reduction of nitro group. One electron reduction of nitro group is usually the first step
31 of 1-nitroacridine's metabolism [31]. Nitroradical anion formed in this step is able to
32 transform the molecular oxygen into ROS. Therefore, we determined the ability of C-
33 1748 to induce ROS and we established the relevance of this effect for the induction
34 of apoptosis and cytotoxic activity of this drug. In our study 2-fold increase in
35 generation of DCF-sensitive ROS in HT29 cells was observed after 1 h of C-1748
36 treatment, whereas in HCT8 cells maximum level of ROS generation was reached
37 after 4 h of incubation with the drug (Fig. 8 A). Moreover, two antioxidants: N-acety-L-
38 cysteine (NAC) and melatonin, that neutralize reactive oxygen species, had no effect
39 on cell death induced by C-1748, as well as on cell viability. Taken together, these
40 results strongly suggest that oxidative stress may not be a casual factor in the
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pathway of C-1748-mediated apoptosis and has no influence on cytotoxic activity of this drug.

In summary, our results indicated that C-1748 at biological relevant concentration corresponding to EC_{90} values, induced different cellular response in human colon carcinoma cells. HCT8 cells underwent transient G1 and G2/M phase arrest followed by apoptosis. Moreover, prolonged drug treatment led to the induction of senescence of HCT8 cells. In contrast, the apoptotic response of HT29 cells to C-1748 exposure was significantly weaker and was preceded by rather stable G1 phase arrest. However, HT29 cells did not undergo senescence and died mainly by necrosis. Additionally, we demonstrated that C-1748 at EC_{90} concentration induced reactive oxygen species (ROS), but generation of ROS was not essential for C-1748-induced apoptosis and cytotoxic activity of this drug.

Importantly, we demonstrated that one chemotherapeutic agent, C-1748, induces different cellular effects (apoptosis, necrosis, senescence) in two colon cancer cell lines. Moreover, such diverse cellular response was observed even within one colon cancer cell population. Similar observations have been made for other antitumor agents, such as adriamycin [32]. Therefore, the divergent cellular response triggered by the same chemotherapeutic agent should be especially considered in cancer therapy of heterogenous population of tumor cells in patients, e.g. in designing of multidrug therapy.

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FIGURE LEGENDS

Figure 1.

Chemical structure of 9-(2'-hydroxyethylamino)-4-methyl-1-nitroacridine derivative,
C-1748.

1
2 *Figure 2.*

3
4 Changes in DNA content following treatment with C-1748 at EC₉₀ concentration for
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6 the time indicated. HCT8 (A) and HT29 (B) cells were fixed in ethanol, stained with PI
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8 and their DNA content was measured by FACScan. Representative histograms of
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10 three independent experiments are shown. (C) The percentage of sub-G1 fraction of
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12 HCT8 and HT29 cells treated with C-1748 at 1xEC₉₀ and 4xEC₉₀ concentrations. The
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14 percentage was calculated by deconvolution of the DNA content histograms.
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21 *Figure 3.*

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23 Changes in nuclear morphology of HCT8 (A) and HT29 (B) cells following treatment
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25 with C-1748 at 1xEC₉₀ and 2xEC₉₀ concentrations. Representative pictures of cells
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27 stained with DAPI and examined under a fluorescent microscope (magnification,
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29 x400). Cells with condensed, fragmented chromatin were considered as apoptotic.
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34 *Figure 4.*

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36 TUNEL assay for HCT8 and HT29 cells exposed to C-1748 at EC₉₀ concentration for
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38 the time indicated. *Left panel*, bivariate flow cytometry histograms of representative
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40 TUNEL assay data. The gated regions are TUNEL positive cells. *Right panel*,
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42 quantitation of the percentage of TUNEL positive cells (mean values from three
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44 experiments $\pm$ SD).
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53 *Figure 5.*

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55 Flow cytometric analysis of membrane alteration in HCT8 cells (A) and HT29 cells (B)
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57 treated with C-1748 at EC₉₀ concentration. *Left panel*, representative bivariate flow
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cytometry histograms of Annexin V signal versus PI signal. Bottom left quadrant represents live cells (Annexin V negative, PI negative); bottom right quadrant represents early apoptotic cells (Annexin V positive, PI negative); top right quadrant represents late apoptotic cells (Annexin V positive, PI positive); top left quadrant represents primary necrotic cells (Annexin V negative, PI positive). *Right panel*, percentages of unaffected, early and late apoptotic, as well as necrotic cells, based on flow cytometry analysis. Data are representative of three independent experiments.

Figure 6.

Mitochondrial events and caspase-3 activation during cell death induced by C-1748 in colon cancer cells. (A) *left panel*, dissipation of mitochondrial membrane potential $\Delta\Psi_m$ in HCT8 and HT29 cells exposed to C-1748 at EC_{90} concentration. The loss of $\Delta\Psi_m$ was analyzed by flow cytometry after staining cells with JC-1. Events shifted to a lower JC-1 red fluorescence and high green were gated as measures of cells with low $\Delta\Psi_m$. *Right panel*, quantitative representation of the flow cytometry data with fraction of HCT8 and HT29 cells with low $\Delta\Psi_m$. Data shown are mean values ($\pm$ SD) from two independent experiments. (B) *left panel*, representative bivariate flow cytometry histograms of caspase-3 activation in HCT8 and HT29 cells following treatment with C-1748 at EC_{90} concentration. Caspase-3 activation was determined by flow cytometry after staining cells with FITC-conjugated antibody that specifically recognizes the active form of caspase-3. *Right panel*, quantitation of the percentage of caspase-3 positive cells based on flow cytometry analysis, and the mean $\pm$ SD of two experiments is shown.

Figure 7.

Characterization of the time course of senescence-associated β -galactosidase (SA- β -Gal) expression in C-1748-treated HCT8 (A) and HT29 (B) cells. Control HCT8 and HT29 cells (C/168 h) were treated with vehicle for 168 h. Following C-1748 exposure at EC₉₀ concentration, cells were fixed and incubated with X-Gal at pH 6.0 which produced blue coloration in SA- β -Gal-positive cells. Cells were photographed under a light microscope (magnification, x200). (C) Expression of p53 protein in HCT8 cells following exposure to C-1748 at EC₉₀ concentration. After the indicated times, cells were lysed and total protein was extracted, separated by SDS-PAGE, electrotransferred to nitrocellulose membrane, and subjected to immunoblotting with anti-p53 antibody. Data correspond to a representative experiment one of two, which gave similar results.

Figure 8.

Generation of ROS induced by C-1748 and effect of antioxidants on C-1748-induced cell death. (A) *top panel*, ROS generation. HCT8 and HT29 cells were treated with vehicle (control, C), or with C-1748 at concentration corresponding to EC₉₀ value for indicated times, or with 0.01% H₂O₂ for 30 min (positive control). The harvested cells were then incubated with 20 μ M DCFH-DA for 30 min and analyzed by flow cytometry, *top*. *Bottom panel*, Fluorescence intensity of DCF was quantified, and the percentage of change in ROS production was determined relative to the control. Results are mean $\pm$ SD of three independent experiments. (B), effect of N-acetyl-L-cysteine or (C), melatonin on the cytotoxic effect of C-1748 in HCT8 and HT29 human colon cancer cell lines. The cells were pretreated with increasing concentrations of NAC or melatonin for 1 h, respectively, and then exposed to C-

1748 (HCT8→0.04 μ M; HT29→0.21 μ M) for 72 h. Cell viability was detected by MTT assay. C, Control-untreated cells; C + 5, cells treated with 5 mM NAC alone; C + 100, cells treated with 100 μ M melatonin alone. Data are mean values from three experiments ($\pm$ SD). (D), effect of NAC on phosphatidylserine externalization in HCT8 cells following C-1748 exposure. HCT8 cells were pretreated with 5 mM NAC for 1 h and then exposed to 0.04 μ M C-1748 for indicated times. After the treatment, cells were stained with Annexin V and propidium iodide (PI) and then analyzed by flow cytometry. C + 5, cells treated with 5 mM NAC alone. Bar graphs represent quantitation of unaffected, early and late apoptotic as well as necrotic cells based on the flow cytometry analysis. Data are representative of three independent experiments.

Table 1. Percentage of sub-G1 and cell cycle distribution of HCT8 and HT29 cells treated with 0.04 μ M (1xEC₉₀) or 0,21 μ M (1xEC₉₀) C-1748 respectively, for the time indicated. Data are presented as mean $\pm$ SD of triplicate experiments.

Cell line	Time of treatment [h]	Sub-G1	G1	S	G2/M	> 4n
HCT8	0	1.5 $\pm$ 0.10	35.6 $\pm$ 0.30	23.5 $\pm$ 0.50	39.4 $\pm$ 1.20	4.3 $\pm$ 0.50
	3	1.4 $\pm$ 0.25	41.4 $\pm$ 2.28	8.0 $\pm$ 2.10	40.0 $\pm$ 1.25	9.7 $\pm$ 0.25
	6	1.7 $\pm$ 0.35	27.4 $\pm$ 1.39	12.2 $\pm$ 0.80	47.3 $\pm$ 4.08	12.1 $\pm$ 1.32
	24	7.6 $\pm$ 0.61	20.3 $\pm$ 1.24	15.9 $\pm$ 1.34	33.2 $\pm$ 4.45	24.0 $\pm$ 0.13
	48	17.9 $\pm$ 1.21	18.5 $\pm$ 0.98	12.1 $\pm$ 3.40	27.5 $\pm$ 1.36	24.5 $\pm$ 0.74
	72	25.0 $\pm$ 1.79	14.0 $\pm$ 0.40	11.2 $\pm$ 3.40	19.7 $\pm$ 0.54	30.8 $\pm$ 3.78
HT29	0	2.7 $\pm$ 0.30	45.0 $\pm$ 0.80	13.9 $\pm$ 1.80	28.2 $\pm$ 0.20	9.5 $\pm$ 0.15
	3	1.6 $\pm$ 0.15	50.5 $\pm$ 4.09	15.3 $\pm$ 0.21	25.1 $\pm$ 1.56	8.1 $\pm$ 0.10
	6	1.8 $\pm$ 0.37	45.4 $\pm$ 0.66	14.6 $\pm$ 1.15	27.8 $\pm$ 2.60	10.5 $\pm$ 1.10
	24	4.1 $\pm$ 0.12	30.6 $\pm$ 1.46	11.8 $\pm$ 0.94	32.9 $\pm$ 2.84	20.8 $\pm$ 1.25
	48	9.6 $\pm$ 0.55	43.9 $\pm$ 2.91	13.2 $\pm$ 2.16	24.6 $\pm$ 2.51	9.5 $\pm$ 0.13
	72	14.0 $\pm$ 0.29	46.5 $\pm$ 5.64	17.0 $\pm$ 1.61	16.0 $\pm$ 0.35	6.9 $\pm$ 0.50

Table 2. Percentage of sub-G1 and cell cycle distribution of HCT8 and HT29 cells treated with 0.16 μ M (4xEC₉₀) or 0,84 μ M (4xEC₉₀) C-1748 respectively, for the time indicated. Data are presented as mean $\pm$ SD of triplicate experiments.

Cell line	Time of treatment [h]	Sub-G1	G1	S	G2/M	> 4n
HCT8	0	1.5 $\pm$ 0.10	35.6 $\pm$ 0.30	23.5 $\pm$ 0.50	39.4 $\pm$ 1.2	4.3 $\pm$ 0.50
	3	2.0 $\pm$ 0.04	31.3 $\pm$ 0.50	12.5 $\pm$ 2.86	31.7 $\pm$ 5.69	21.2 $\pm$ 2.8
	6	2.0 $\pm$ 0,45	23.7 $\pm$ 0.37	13.2 $\pm$ 2.93	35.8 $\pm$ 2.31	24.0 $\pm$ 0.90
	24	48.8 $\pm$ 0.10	15.2 $\pm$ 2.16	8.0 $\pm$ 1.34	15.0 $\pm$ 0.47	13.2 $\pm$ 2.4
	48	60.4 $\pm$ 4.55	15.8 $\pm$ 0.15	10.5 $\pm$ 0.30	8.9 $\pm$ 0.35	5.0 $\pm$ 0.85
	72	57.8 $\pm$ 0.03	15.3 $\pm$ 0.73	11.7 $\pm$ 0.58	9.3 $\pm$ 0.54	6.3 $\pm$ 0.47
HT29	0	2.7 $\pm$ 0.30	45.0 $\pm$ 0.80	13.9 $\pm$ 1.80	28.2 $\pm$ 0.20	9.5 $\pm$ 0.15
	3	2.4 $\pm$ 0.30	38.1 $\pm$ 2.09	31.0 $\pm$ 3.54	12.4 $\pm$ 0.23	17.1 $\pm$ 2.43
	6	2.3 $\pm$ 0.12	33.9 $\pm$ 1.97	28.9 $\pm$ 0.68	16.1 $\pm$ 1.80	19.7 $\pm$ 3.50
	24	9.9 $\pm$ 0.18	30.5 $\pm$ 0.86	25.4 $\pm$ 1.35	13.1 $\pm$ 2.30	14.7 $\pm$ 0.47
	48	14.5 $\pm$ 0.23	37.5 $\pm$ 2.54	23.0 $\pm$ 0.80	12.9 $\pm$ 0.34	13.2 $\pm$ 0.59
	72	18.9 $\pm$ 0.23	32.2 $\pm$ 1.56	19.0 $\pm$ 0.95	11.9 $\pm$ 0.12	17.9 $\pm$ 1.12

A)

1 x EC₉₀

0 h

2 x EC₉₀

12 h

24 h

48 h

72 h

B)

1 x EC₉₀

0 h

2 x EC₉₀

12 h

24 h

48 h

72 h

A)

B)

A)

B)

A)

C)

B)