



**HAL**  
open science

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

Ewa Augustin, Anna Moś-Rompa, Dorota Nowak-Ziatyk, Jerzy Konopa

► **To cite this version:**

Ewa Augustin, Anna Moś-Rompa, Dorota Nowak-Ziatyk, Jerzy Konopa. Antitumor 1-nitroacridine derivative C-1748, induces apoptosis, necrosis or senescence in human colon carcinoma HCT8 and HT29 cells. *Biochemical Pharmacology*, 2010, 79 (9), pp.1231. 10.1016/j.bcp.2009.12.012 . hal-00573915

**HAL Id: hal-00573915**

**<https://hal.science/hal-00573915>**

Submitted on 5 Mar 2011

**HAL** is a multi-disciplinary open access archive for the deposit and dissemination of scientific research documents, whether they are published or not. The documents may come from teaching and research institutions in France or abroad, or from public or private research centers.

L'archive ouverte pluridisciplinaire **HAL**, est destinée au dépôt et à la diffusion de documents scientifiques de niveau recherche, publiés ou non, émanant des établissements d'enseignement et de recherche français ou étrangers, des laboratoires publics ou privés.

## Accepted Manuscript

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

Authors: Ewa Augustin, Anna Moś-Rompa, Dorota Nowak-Ziatyk, Jerzy Konopa



PII: S0006-2952(09)01070-3  
DOI: doi:10.1016/j.bcp.2009.12.012  
Reference: BCP 10409

To appear in: *BCP*

Received date: 21-10-2009  
Revised date: 7-12-2009  
Accepted date: 10-12-2009

Please cite this article as: Augustin E, Moś-Rompa A, Antitumor 1-nitroacridine derivative C-1748, induces apoptosis, necrosis or senescence in human colon carcinoma HCT8 and HT29 cells, *Biochemical Pharmacology* (2008), doi:10.1016/j.bcp.2009.12.012

This is a PDF file of an unedited manuscript that has been accepted for publication. As a service to our customers we are providing this early version of the manuscript. The manuscript will undergo copyediting, typesetting, and review of the resulting proof before it is published in its final form. Please note that during the production process errors may be discovered which could affect the content, and all legal disclaimers that apply to the journal pertain.

1 Antitumor 1-nitroacridine derivative C-1748, induces apoptosis, necrosis or  
2 senescence in human colon carcinoma HCT8 and HT29 cells.  
3  
4  
5  
6

7 Ewa Augustin<sup>1\*</sup>, Anna Moś-Rompa<sup>1</sup>, Dorota Nowak-Ziatyk<sup>1</sup>, Jerzy Konopa<sup>1</sup>  
8  
9

10 <sup>1</sup>Department of Pharmaceutical Technology and Biochemistry, Gdańsk University of  
11 Technology, Chemical Faculty, Narutowicza Str. 11/12, 80-233 Gdańsk, Poland  
12  
13  
14  
15

16 e-mail address: [augustin@chem.pg.gda.pl](mailto:augustin@chem.pg.gda.pl) (E. Augustin)  
17

18 [annmos13@wp.pl](mailto:annmos13@wp.pl) (A. Moś-Rompa)  
19

20 [dorota-nowak@o2.pl](mailto:dorota-nowak@o2.pl) (D. Nowak-Ziatyk)  
21

22 [konopa@chem.pg.gda.pl](mailto:konopa@chem.pg.gda.pl) (J. Konopa)  
23  
24  
25  
26  
27  
28  
29  
30  
31

32 \*Corresponding author: Gdańsk University of Technology, Chemical Faculty,  
33 Department of Pharmaceutical Technology and Biochemistry, Narutowicza Str.  
34 11/12, 80-233 Gdańsk, Poland.  
35  
36  
37

38 Tel. 4858 347 14 68, fax: 4858 347 15 16  
39

40 E-mail address: [augustin@chem.pg.gda.pl](mailto:augustin@chem.pg.gda.pl) (E. Augustin)  
41  
42  
43  
44  
45  
46  
47  
48

49 Category 1: Antibiotics and Chemotherapeutics  
50  
51  
52  
53  
54  
55  
56  
57  
58  
59  
60  
61  
62  
63  
64  
65

**ABSTRACT**

1 C-1748 is a DNA binding agent with potent antitumor activity, especially towards  
2 prostate and colon carcinoma xenografts in mice. Here, we elucidated the nature of  
3 cellular response of human colon carcinoma HCT8 and HT29 cells to C-1748  
4 treatment, at biological relevant concentrations ( $EC_{90}$  and their multiplicity). Cell cycle  
5 analysis showed gradual increase of HCT8 cells with sub-G1 DNA content (25% after  
6 72 h) considered as apoptotic. Hypodiploid cell population increased up to 60% upon  
7 treatment with  $4 \times EC_{90}$  concentration of the drug. Compared with HCT8 cells, the  
8 fraction of sub-G1 HT29 cells did not exceed 14%, even following 4-fold dose  
9 escalation. Morphological changes and biochemical markers such as:  
10 phosphatidylserine externalization, apoptotic DNA breaks, mitochondrial dysfunction,  
11 caspase activation, confirmed the presence of considerable amount of apoptotic  
12 HCT8 cells but only a low amount of apoptotic HT29 cells. Next, we demonstrated  
13 that HCT8 cells surviving after exposure to C-1748 were in state of senescence,  
14 based on altered cell morphology and expression of a pH 6-dependent  $\beta$ -  
15 galactosidase. On the contrary, no  $\beta$ -galactosidase staining was observed in HT29  
16 cells after C-1748 treatment. Moreover, prolonged drug incubation (up to 168 h)  
17 resulted in massive detachment of cells from culture plates, what together with  
18 Annexin V/PI results, indicated that necrosis was the main response of HT29 cells to  
19 C-1748 treatment. We also determined the ability of C-1748 to induce reactive  
20 oxygen species (ROS) in colon cancer cells and demonstrated, that generation of  
21 ROS was not essential for C-1748-induced apoptosis and cytotoxic activity of this  
22 drug.  
23  
24  
25  
26  
27  
28  
29  
30  
31  
32  
33  
34  
35  
36  
37  
38  
39  
40  
41  
42  
43  
44  
45  
46  
47  
48  
49  
50  
51  
52  
53  
54  
55  
56  
57  
58  
59  
60  
61  
62  
63  
64  
65

**Key words:**

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

Accepted Manuscript

1  
2  
3  
4  
5  
6  
7  
8  
9  
10  
11  
12  
13  
14  
15  
16  
17  
18  
19  
20  
21  
22  
23  
24  
25  
26  
27  
28  
29  
30  
31  
32  
33  
34  
35  
36  
37  
38  
39  
40  
41  
42  
43  
44  
45  
46  
47  
48  
49  
50  
51  
52  
53  
54  
55  
56  
57  
58  
59  
60  
61  
62  
63  
64  
65

## 1. INTRODUCTION

1  
2 The 1-nitroacridines are DNA-binding and potent antitumor agents synthesized  
3  
4 at Gdańsk University of Technology [1]. Among them, the most active compound  
5  
6 Ledakrin (Nitracrine) was in 1970's registered as an antitumor drug in Poland [2].  
7  
8 Ledakrin was recommended especially for treatment of ovary carcinoma, but also  
9  
10 effectively used against colon, breast and lung cancers [3, 4]. However, due to  
11  
12 severe side effects and high general toxicity, Ledakrin was desisted from further  
13  
14 clinical application. Modification of chemical properties of 1-nitroacridines, especially  
15  
16 decreasing the ability of nitro group in position 1 to undergo reduction, by introducing  
17  
18 to position 4, para to the nitro group, an electron donating-methyl group, resulted in  
19  
20 development of 4-methyl-1-nitroacridines with high antitumor activity towards broader  
21  
22 spectrum of tumors and lower toxicity than parental 1-nitroacridines [5, 6]. The most  
23  
24 active 9-(2'-hydroxyethylamino)-4-methyl-1-nitroacridine (C-1748, Fig. 1), was found  
25  
26 to have significant cytotoxic activity *in vitro* towards cancer cell lines and high  
27  
28 antitumor activity against several prostate (LnCaP, JCA, PC3, TSU) and colon  
29  
30 carcinoma (HCT8) xenografts in nude mice [6]. Pre-clinical toxicology studies in  
31  
32 rodent and dogs showed C-1748 to have very low systemic toxicity along with a  
33  
34 lowered mutagenic potential [7, 8, 9]. C-1748, as well as Ledakrin and other 1-  
35  
36 nitroacridines, after metabolic activation bind covalently to DNA [10] and induced  
37  
38 DNA crosslinking in tumor cells, which was crucial for biological activity of these  
39  
40 compounds [11, 12, 13].  
41  
42  
43  
44  
45  
46  
47  
48  
49

50  
51 Recently, it has been shown that C-1748 significantly induced DNA double  
52  
53 strand breaks in prostate cancer LnCaP cells and, to smaller extend, in leukemia  
54  
55 HL60 cells, less sensitive to the action of this compound [6]. Moreover, in LnCaP  
56  
57 cells, C-1748 treatment markedly downregulated androgen receptor but upregulated  
58  
59  
60  
61  
62  
63  
64  
65

1  
2  
3  
4  
5  
6  
7  
8  
9  
10  
11  
12  
13  
14  
15  
16  
17  
18  
19  
20  
21  
22  
23  
24  
25  
26  
27  
28  
29  
30  
31  
32  
33  
34  
35  
36  
37  
38  
39  
40  
41  
42  
43  
44  
45  
46  
47  
48  
49  
50  
51  
52  
53  
54  
55  
56  
57  
58  
59  
60  
61  
62  
63  
64  
65

estrogen receptor what was a further validation of the cytotoxicity of C-1748 in this cells [6].

The molecular mechanisms by which chemotherapeutic agents kill tumor cells are numerous and depend on a variety of parameters including the drug and tumor types. It is widely accepted that tumor cells exposed to cytotoxic drugs die mainly from apoptosis. Moreover, in many types of tumor cells accelerated or premature senescence, a form of growth arrest after exposure to ionizing radiation or drugs that induce DNA damage, has been observed [14, 15]. It is still not clear what determines if a cell undergoes senescence or apoptosis. Although most cells are capable of both, these processes seem to be exclusive [16]. Moreover, in many types of cells the influence of p53 function can promote senescence arrest and apoptotic cell death [17]. However, there is also evidence for accelerated senescence that is independent of p53 [18].

The current studies were designed to elucidate the nature of the response of human colon cancer HCT8 and HT29 cells to C-1748 in terms of growth arrest and/or cell death. Additionally, we determined the ability of C-1748 to induce reactive oxygen species (ROS) production, and investigated the correlation between ROS generation and cell death and cytotoxic activity of the drug. In a view of recent finding that a form of senescence arrest may occur in response to antitumor drugs, we also examined the ability of C-1748 to induce senescence.

## 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  $\beta$ -gal, melatonin, mouse monoclonal anti-p53 antibody, mouse monoclonal anti- $\beta$ -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 (Manheim, 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  $\mu$ g/ml streptomycin, 100 U/ml penicillin), in a 5% CO<sub>2</sub> atmosphere at 37°C. Under these growth conditions the cell-



1 doubling time was 24 h for both cell lines. All experiments were performed with cells  
2 in the exponential phase of growth.  
3

4 Cell growth inhibition was assessed by cell counting using Coulter Counter,  
5 model ZB1 according to the method describe by Bhuyan et al. [19]. Briefly, cells  
6 (1x10<sup>5</sup>) were seeded in 24-well microculture plates and treated with various C-1748  
7 concentrations for 72 h. A dose response curve was plotted and used to calculate  
8 drug concentration that yielded 90% of inhibition of cells growth (EC<sub>90</sub>).  
9  
10  
11  
12  
13  
14  
15  
16  
17  
18

### 19 *2.3. Cell cycle distribution by flow cytometry*

20 Asynchronous HCT8 and HT29 cells were exposed to C-1748 at  
21 concentrations corresponding to EC<sub>90</sub> values and their multiplicity for 3 - 72 h. After  
22 drug treatment, adherent cells were trypsinized, combined with nonadherent cells,  
23 collected by centrifugation for 5 min at 4°C, at 1,000 rpm, washed twice with ice-cold  
24 phosphate-buffered saline (PBS) and then fixed in ice-cold 80% ethanol overnight at  
25 -20°C. Next, cells were centrifuged for 5 min at 4°C, at 1,000 rpm, washed twice with  
26 PBS and resuspended in 1ml of DNA staining solution (20 µg/ml of propidium iodide  
27 PI and 100 µg/ml of RNase A in PBS), for 30 min in the dark. Flow cytometric  
28 analyses were performed using a FACScan cytometer (Becton Dickinson, San Jose,  
29 CA, USA). At least 1x10<sup>4</sup> cells were collected and the data analysis was performed  
30 using the WinMDI software (J. Trotter, Scripps Research Institute, San Diego, CA,  
31 USA). All experiments were repeated at least three times.  
32  
33  
34  
35  
36  
37  
38  
39  
40  
41  
42  
43  
44  
45  
46  
47  
48  
49  
50  
51  
52

### 53 *2.4. Cell morphology assessment*

54 Nuclear morphology was examined under fluorescence microscope  
55 (OLYMPUS BX60) after staining with 4',6-diaminidino-2-phenylindole (DAPI).  
56  
57  
58  
59  
60  
61  
62  
63  
64  
65

1 Briefly, after drug incubation, cells ( $5 \times 10^5$ ) were spun onto microscopic slides, fixed in  
2 methanol : acetic acid solution (3:1) for 15 min and stained with DAPI ( $1 \mu\text{g/ml}$ ) for 5  
3 min. Cells were regarded as apoptotic based on the presence of condensed,  
4 fragmented chromatin.  
5  
6  
7  
8  
9

## 10 11 12 2.5. TUNEL assay

13 Apoptotic DNA degradation following by C-1748 treatment was studied by the  
14 TUNEL assay. The commercially accessible Apoptosis Detection kit (Roche  
15 Diagnostics, Mannheim, Germany) was used and prepared according to the supplier's  
16 recommended protocol. Briefly, following treatment, cells ( $1 \times 10^6$ ) were centrifuged,  
17 washed twice in PBS and gently resuspended in 1% formaldehyde in PBS and kept  
18 on ice for 15 min. Fixed cells were washed with PBS and resuspended in 70%  
19 ethanol. Dehydrated cells were incubated overnight at  $-20^\circ\text{C}$ . After rehydration in  
20 PBS for 5 min cells were resuspended in 50  $\mu\text{l}$  of reaction buffer (5U terminal  
21 transferase, 2.5 mM cobalt chloride, 0.5 mM biotin-dUTP and TdT buffer containing  
22 0.2 M potassium cocadylate, 25 mM TRIS-HCl, 0.25 mg/ml BSA, pH 6.6) and  
23 incubated at  $37^\circ\text{C}$  for 30 min. Then, cells were washed with rinsing buffer (PBS  
24 containing 0.1% Triton X-100, 0.5% BSA), resuspended in 100  $\mu\text{l}$  of staining buffer  
25 containing 2.5  $\mu\text{g/ml}$  FITC-avidin, 4xSSC buffer, 0.1% Triton X-100 and 5% (w/v)  
26 non-fat dry milk, and incubated for 30 min at room temperature in the dark. The  
27 pellets were then washed twice with rinsing buffer, resuspended in PBS containing 5  
28  $\mu\text{g/ml}$  PI and 100  $\mu\text{g/ml}$  RNase A, and incubated at room temperature for 30 min. The  
29 red (PI) and green (FITC) fluorescence were measured with FACScan flow  
30 cytometer.  
31  
32  
33  
34  
35  
36  
37  
38  
39  
40  
41  
42  
43  
44  
45  
46  
47  
48  
49  
50  
51  
52  
53  
54  
55  
56  
57  
58  
59  
60  
61  
62  
63  
64  
65

## 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 ( $1 \times 10^6$ ) were trypsinized, rinsed with HEPES/NaOH buffer (10 mM HEPES pH 7.4, 140 mM NaCl, 5 mM  $\text{CaCl}_2$ ), pelleted and resuspended in 50  $\mu\text{l}$  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  $\mu\text{l}$  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 ( $5 \times 10^5$ ) 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 ( $1 \times 10^6$ ) stained with FITC-conjugated anti-active caspase-3 antibody, were analyzed by flow cytometry.

### 2.9. Determination of cellular senescence

1  
2 pH 6.0-dependent  $\beta$ -galactosidase expression was used as a marker for senescence  
3  
4 along with senescent related morphology. Following C-1748-treatment, cells ( $1 \times 10^5$ )  
5  
6 were washed twice with PBS and fixed in 2% formaldehyde and 0.2% glutaraldehyde  
7  
8 in PBS for 5 min. The cells were then washed again with PBS and stained with X-gal  
9  
10 ( 5-bromo-4-chloro-3-indolyl- $\beta$ -D-galactosidase) staining solution (1 mg/ml X-gal, 40  
11  
12 mM citric acid/sodium phosphate, pH 6.0, 5 mM potassium ferrocyanide, 5 mM  
13  
14 potassium ferricyanide, 150 mM NaCl, 2 mM  $MgCl_2$ ). Following overnight incubation  
15  
16 at 37°C, the cells were washed twice with PBS, and photographed under a light  
17  
18 microscope.  
19  
20  
21  
22

### 2.10. Western blot analysis

23  
24  
25  
26  
27  
28  
29  
30  
31  
32  
33  
34  
35  
36  
37  
38  
39  
40  
41  
42  
43  
44  
45  
46  
47  
48  
49  
50  
51  
52  
53  
54  
55  
56  
57  
58  
59  
60  
61  
62  
63  
64  
65  
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

66  
67  
68  
69  
70  
71  
72  
73  
74  
75  
76  
77  
78  
79  
80  
81  
82  
83  
84  
85  
86  
87  
88  
89  
90  
91  
92  
93  
94  
95  
96  
97  
98  
99  
100  
101  
102  
103  
104  
105  
106  
107  
108  
109  
110  
111  
112  
113  
114  
115  
116  
117  
118  
119  
120  
121  
122  
123  
124  
125  
126  
127  
128  
129  
130  
131  
132  
133  
134  
135  
136  
137  
138  
139  
140  
141  
142  
143  
144  
145  
146  
147  
148  
149  
150  
151  
152  
153  
154  
155  
156  
157  
158  
159  
160  
161  
162  
163  
164  
165  
166  
167  
168  
169  
170  
171  
172  
173  
174  
175  
176  
177  
178  
179  
180  
181  
182  
183  
184  
185  
186  
187  
188  
189  
190  
191  
192  
193  
194  
195  
196  
197  
198  
199  
200  
201  
202  
203  
204  
205  
206  
207  
208  
209  
210  
211  
212  
213  
214  
215  
216  
217  
218  
219  
220  
221  
222  
223  
224  
225  
226  
227  
228  
229  
230  
231  
232  
233  
234  
235  
236  
237  
238  
239  
240  
241  
242  
243  
244  
245  
246  
247  
248  
249  
250  
251  
252  
253  
254  
255  
256  
257  
258  
259  
260  
261  
262  
263  
264  
265  
266  
267  
268  
269  
270  
271  
272  
273  
274  
275  
276  
277  
278  
279  
280  
281  
282  
283  
284  
285  
286  
287  
288  
289  
290  
291  
292  
293  
294  
295  
296  
297  
298  
299  
300  
301  
302  
303  
304  
305  
306  
307  
308  
309  
310  
311  
312  
313  
314  
315  
316  
317  
318  
319  
320  
321  
322  
323  
324  
325  
326  
327  
328  
329  
330  
331  
332  
333  
334  
335  
336  
337  
338  
339  
340  
341  
342  
343  
344  
345  
346  
347  
348  
349  
350  
351  
352  
353  
354  
355  
356  
357  
358  
359  
360  
361  
362  
363  
364  
365  
366  
367  
368  
369  
370  
371  
372  
373  
374  
375  
376  
377  
378  
379  
380  
381  
382  
383  
384  
385  
386  
387  
388  
389  
390  
391  
392  
393  
394  
395  
396  
397  
398  
399  
400  
401  
402  
403  
404  
405  
406  
407  
408  
409  
410  
411  
412  
413  
414  
415  
416  
417  
418  
419  
420  
421  
422  
423  
424  
425  
426  
427  
428  
429  
430  
431  
432  
433  
434  
435  
436  
437  
438  
439  
440  
441  
442  
443  
444  
445  
446  
447  
448  
449  
450  
451  
452  
453  
454  
455  
456  
457  
458  
459  
460  
461  
462  
463  
464  
465  
466  
467  
468  
469  
470  
471  
472  
473  
474  
475  
476  
477  
478  
479  
480  
481  
482  
483  
484  
485  
486  
487  
488  
489  
490  
491  
492  
493  
494  
495  
496  
497  
498  
499  
500  
501  
502  
503  
504  
505  
506  
507  
508  
509  
510  
511  
512  
513  
514  
515  
516  
517  
518  
519  
520  
521  
522  
523  
524  
525  
526  
527  
528  
529  
530  
531  
532  
533  
534  
535  
536  
537  
538  
539  
540  
541  
542  
543  
544  
545  
546  
547  
548  
549  
550  
551  
552  
553  
554  
555  
556  
557  
558  
559  
560  
561  
562  
563  
564  
565  
566  
567  
568  
569  
570  
571  
572  
573  
574  
575  
576  
577  
578  
579  
580  
581  
582  
583  
584  
585  
586  
587  
588  
589  
590  
591  
592  
593  
594  
595  
596  
597  
598  
599  
600  
601  
602  
603  
604  
605  
606  
607  
608  
609  
610  
611  
612  
613  
614  
615  
616  
617  
618  
619  
620  
621  
622  
623  
624  
625  
626  
627  
628  
629  
630  
631  
632  
633  
634  
635  
636  
637  
638  
639  
640  
641  
642  
643  
644  
645  
646  
647  
648  
649  
650  
651  
652  
653  
654  
655  
656  
657  
658  
659  
660  
661  
662  
663  
664  
665  
666  
667  
668  
669  
670  
671  
672  
673  
674  
675  
676  
677  
678  
679  
680  
681  
682  
683  
684  
685  
686  
687  
688  
689  
690  
691  
692  
693  
694  
695  
696  
697  
698  
699  
700  
701  
702  
703  
704  
705  
706  
707  
708  
709  
710  
711  
712  
713  
714  
715  
716  
717  
718  
719  
720  
721  
722  
723  
724  
725  
726  
727  
728  
729  
730  
731  
732  
733  
734  
735  
736  
737  
738  
739  
740  
741  
742  
743  
744  
745  
746  
747  
748  
749  
750  
751  
752  
753  
754  
755  
756  
757  
758  
759  
760  
761  
762  
763  
764  
765  
766  
767  
768  
769  
770  
771  
772  
773  
774  
775  
776  
777  
778  
779  
780  
781  
782  
783  
784  
785  
786  
787  
788  
789  
790  
791  
792  
793  
794  
795  
796  
797  
798  
799  
800  
801  
802  
803  
804  
805  
806  
807  
808  
809  
810  
811  
812  
813  
814  
815  
816  
817  
818  
819  
820  
821  
822  
823  
824  
825  
826  
827  
828  
829  
830  
831  
832  
833  
834  
835  
836  
837  
838  
839  
840  
841  
842  
843  
844  
845  
846  
847  
848  
849  
850  
851  
852  
853  
854  
855  
856  
857  
858  
859  
860  
861  
862  
863  
864  
865  
866  
867  
868  
869  
870  
871  
872  
873  
874  
875  
876  
877  
878  
879  
880  
881  
882  
883  
884  
885  
886  
887  
888  
889  
890  
891  
892  
893  
894  
895  
896  
897  
898  
899  
900  
901  
902  
903  
904  
905  
906  
907  
908  
909  
910  
911  
912  
913  
914  
915  
916  
917  
918  
919  
920  
921  
922  
923  
924  
925  
926  
927  
928  
929  
930  
931  
932  
933  
934  
935  
936  
937  
938  
939  
940  
941  
942  
943  
944  
945  
946  
947  
948  
949  
950  
951  
952  
953  
954  
955  
956  
957  
958  
959  
960  
961  
962  
963  
964  
965  
966  
967  
968  
969  
970  
971  
972  
973  
974  
975  
976  
977  
978  
979  
980  
981  
982  
983  
984  
985  
986  
987  
988  
989  
990  
991  
992  
993  
994  
995  
996  
997  
998  
999  
1000  
1001  
1002  
1003  
1004  
1005  
1006  
1007  
1008  
1009  
1010  
1011  
1012  
1013  
1014  
1015  
1016  
1017  
1018  
1019  
1020  
1021  
1022  
1023  
1024  
1025  
1026  
1027  
1028  
1029  
1030  
1031  
1032  
1033  
1034  
1035  
1036  
1037  
1038  
1039  
1040  
1041  
1042  
1043  
1044  
1045  
1046  
1047  
1048  
1049  
1050  
1051  
1052  
1053  
1054  
1055  
1056  
1057  
1058  
1059  
1060  
1061  
1062  
1063  
1064  
1065  
1066  
1067  
1068  
1069  
1070  
1071  
1072  
1073  
1074  
1075  
1076  
1077  
1078  
1079  
1080  
1081  
1082  
1083  
1084  
1085  
1086  
1087  
1088  
1089  
1090  
1091  
1092  
1093  
1094  
1095  
1096  
1097  
1098  
1099  
1100  
1101  
1102  
1103  
1104  
1105  
1106  
1107  
1108  
1109  
1110  
1111  
1112  
1113  
1114  
1115  
1116  
1117  
1118  
1119  
1120  
1121  
1122  
1123  
1124  
1125  
1126  
1127  
1128  
1129  
1130  
1131  
1132  
1133  
1134  
1135  
1136  
1137  
1138  
1139  
1140  
1141  
1142  
1143  
1144  
1145  
1146  
1147  
1148  
1149  
1150  
1151  
1152  
1153  
1154  
1155  
1156  
1157  
1158  
1159  
1160  
1161  
1162  
1163  
1164  
1165  
1166  
1167  
1168  
1169  
1170  
1171  
1172  
1173  
1174  
1175  
1176  
1177  
1178  
1179  
1180  
1181  
1182  
1183  
1184  
1185  
1186  
1187  
1188  
1189  
1190  
1191  
1192  
1193  
1194  
1195  
1196  
1197  
1198  
1199  
1200  
1201  
1202  
1203  
1204  
1205  
1206  
1207  
1208  
1209  
1210  
1211  
1212  
1213  
1214  
1215  
1216  
1217  
1218  
1219  
1220  
1221  
1222  
1223  
1224  
1225  
1226  
1227  
1228  
1229  
1230  
1231  
1232  
1233  
1234  
1235  
1236  
1237  
1238  
1239  
1240  
1241  
1242  
1243  
1244  
1245  
1246  
1247  
1248  
1249  
1250  
1251  
1252  
1253  
1254  
1255  
1256  
1257  
1258  
1259  
1260  
1261  
1262  
1263  
1264  
1265  
1266  
1267  
1268  
1269  
1270  
1271  
1272  
1273  
1274  
1275  
1276  
1277  
1278  
1279  
1280  
1281  
1282  
1283  
1284  
1285  
1286  
1287  
1288  
1289  
1290  
1291  
1292  
1293  
1294  
1295  
1296  
1297  
1298  
1299  
1300  
1301  
1302  
1303  
1304  
1305  
1306  
1307  
1308  
1309  
1310  
1311  
1312  
1313  
1314  
1315  
1316  
1317  
1318  
1319  
1320  
1321  
1322  
1323  
1324  
1325  
1326  
1327  
1328  
1329  
1330  
1331  
1332  
1333  
1334  
1335  
1336  
1337  
1338  
1339  
1340  
1341  
1342  
1343  
1344  
1345  
1346  
1347  
1348  
1349  
1350  
1351  
1352  
1353  
1354  
1355  
1356  
1357  
1358  
1359  
1360  
1361  
1362  
1363  
1364  
1365  
1366  
1367  
1368  
1369  
1370  
1371  
1372  
1373  
1374  
1375  
1376  
1377  
1378  
1379  
1380  
1381  
1382  
1383  
1384  
1385  
1386  
1387  
1388  
1389  
1390  
1391  
1392  
1393  
1394  
1395  
1396  
1397  
1398  
1399  
1400  
1401  
1402  
1403  
1404  
1405  
1406  
1407  
1408  
1409  
1410  
1411  
1412  
1413  
1414  
1415  
1416  
1417  
1418  
1419  
1420  
1421  
1422  
1423  
1424  
1425  
1426  
1427  
1428  
1429  
1430  
1431  
1432  
1433  
1434  
1435  
1436  
1437  
1438  
1439  
1440  
1441  
1442  
1443  
1444  
1445  
1446  
1447  
1448  
1449  
1450  
1451  
1452  
1453  
1454  
1455  
1456  
1457  
1458  
1459  
1460  
1461  
1462  
1463  
1464  
1465  
1466  
1467  
1468  
1469  
1470  
1471  
1472  
1473  
1474  
1475  
1476  
1477  
1478  
1479  
1480  
1481  
1482  
1483  
1484  
1485  
1486  
1487  
1488  
1489  
1490  
1491  
1492  
1493  
1494  
1495  
1496  
1497  
1498  
1499  
1500  
1501  
1502  
1503  
1504  
1505  
1506  
1507  
1508  
1509  
1510  
1511  
1512  
1513  
1514  
1515  
1516  
1517  
1518  
1519  
1520  
1521  
1522  
1523  
1524  
1525  
1526  
1527  
1528  
1529  
1530  
1531  
1532  
1533  
1534  
1535  
1536  
1537  
1538  
1539  
1540  
1541  
1542  
1543  
1544  
1545  
1546  
1547  
1548  
1549  
1550  
1551  
1552  
1553  
1554  
1555  
1556  
1557  
1558  
1559  
1560  
1561  
1562  
1563  
1564  
1565  
1566  
1567  
1568  
1569  
1570  
1571  
1572  
1573  
1574  
1575  
1576  
1577  
1578  
1579  
1580  
1581  
1582  
1583  
1584  
1585  
1586  
1587  
1588  
1589  
1590  
1591  
1592  
1593  
1594  
1595  
1596  
1597  
1598  
1599  
1600  
1601  
1602  
1603  
1604  
1605  
1606  
1607  
1608  
1609  
1610  
1611  
1612  
1613  
1614  
1615  
1616  
1617  
1618  
1619  
1620  
1621  
1622  
1623  
1624  
1625  
1626  
1627  
1628  
1629  
1630  
1631  
1632  
1633  
1634  
1635  
1636  
1637  
1638  
1639  
1640  
1641  
1642  
1643  
1644  
1645  
1646  
1647  
1648  
1649  
1650  
1651  
1652  
1653  
1654  
1655  
1656  
1657  
1658  
1659  
1660  
1661  
1662  
1663  
1664  
1665  
1666  
1667  
1668  
1669  
1670  
1671  
1672  
1673  
1674  
1675  
1676  
1677  
1678  
1679  
1680  
1681  
1682  
1683  
1684  
1685  
1686  
1687  
1688  
1689  
1690  
1691  
1692  
1693  
1694  
1695  
1696  
1697  
1698  
1699  
1700  
1701  
1702  
1703  
1704  
1705  
1706  
1707  
1708  
1709  
1710  
1711  
1712  
1713  
1714  
1715  
1716  
1717  
1718  
1719  
1720  
1721  
1722  
1723  
1724  
1725  
1726  
1727  
1728  
1729  
1730  
1731  
1732  
1733  
1734  
1735  
1736  
1737  
1738  
1739  
1740  
1741  
1742  
1743  
1744  
1745  
1746  
1747  
1748  
1749  
1750  
1751  
1752  
1753  
1754  
1755  
1756  
1757  
1758  
1759  
1760  
1761  
1762  
1763  
1764  
1765  
1766  
1767  
1768  
1769  
1770  
1771  
1772  
1773  
1774  
1775  
1776  
1777  
1778  
1779  
1780  
1781  
1782  
1783  
1784  
1785  
1786  
1787  
1788  
1789  
1790  
1791  
1792  
1793  
1794  
1795  
1796  
1797  
1798  
1799  
1800  
1801  
1802  
1803  
1804  
1805  
1806  
1807  
1808  
1809  
1810  
1811  
1812  
1813  
1814  
1815  
1816  
1817  
1818  
1819  
1820  
1821  
1822  
1823  
1824  
1825  
1826  
1827  
1828  
1829  
1830  
1831  
1832  
1833  
1834  
1835  
1836  
1837  
1838  
1839  
1840  
1841  
1842  
1843  
1844  
1845  
1846  
1847  
1848  
1849  
1850  
1851  
1852  
1853  
1854  
1855  
1856  
1857  
1858  
1859  
1860  
1861  
1862  
1863  
1864  
1865  
1866  
1867  
1868  
1869  
1870  
1871  
1872  
1873  
1874  
1875  
1876  
1877  
1878  
1879  
1880  
1881  
1882  
1883  
1884  
1885  
1886  
1887  
1888  
1889  
1890  
1891  
1892  
1893  
1894  
1895  
1896  
1897  
1898  
1899  
1900  
1901  
1902  
1903  
1904  
1905  
1906  
1907  
1908  
1909  
1910  
1911  
1912  
1913  
1914  
1915  
1916  
1917  
1918  
1919  
1920  
1921  
1922  
1923  
1924  
1925  
1926  
1927  
1928  
1929  
1930  
1931  
1932  
1933  
1934  
1935  
1936  
1937  
1938  
1939  
1940  
1941  
1942  
1943  
1944  
1945  
1946  
1947  
1948  
1949  
1950  
1951  
1952  
1953  
1954  
1955  
1956  
1957  
1958  
1959  
1960  
1961  
1962  
1963  
1964  
1965  
1966  
1967  
1968  
1969  
1970  
1971  
1972  
1973  
1974  
1975  
1976  
1977  
1978  
1979  
1980  
1981  
1982  
1983  
1984  
1985  
1986  
1987  
1988  
1989  
1990  
1991  
1992  
1993  
1994  
1995  
1996  
1997  
1998  
1999  
2000  
2001  
2002  
2003  
2004  
2005  
2006  
2007  
2008  
2009  
2010  
2011  
2012  
2013  
2014  
2015  
2016  
2017  
2018  
2019  
2020  
2021  
2022  
2023  
2024  
2025  
2026  
2027  
2028  
2029  
2030  
2031  
2032  
2033  
2034  
2035  
2036  
2037  
2038  
2039  
2040  
2041  
2042  
2043  
2044  
2045  
2046  
2047  
2048  
2049  
2050  
2051  
2052  
2053  
2054  
2055  
2056  
2057  
2058  
2059  
2060  
2061  
2062  
2063  
2064  
2065  
2066  
2067  
2068  
2069  
2070  
2071  
2072  
2073  
2074  
2075  
2076  
2077  
2078  
2079  
2080  
2081  
2082  
2083  
2084  
2085  
2086  
2087  
2088  
2089  
2090  
2091  
2092  
2093  
2094  
2095  
2096  
2097  
2098  
2099  
2100  
2101  
2102  
2103  
2104  
2105  
2106  
2107  
2108  
2109  
2110  
2111  
2112  
2113  
2114  
2115  
2116  
2117  
2118  
2119  
2120  
2121  
2122  
2123  
2124  
2125  
2126  
2127  
2128  
2129  
2130  
2131  
2132  
2133  
2134  
2135  
2136  
2137  
2138  
2139  
2140  
2

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  
3 incubated with hydrogen peroxide (0.01%) were used as a positive control. All  
4 experiments were repeated at least three times.  
5  
6  
7  
8  
9

## 10 11 12 2.12. Statistical analysis

13  
14 Results are expressed as means  $\pm$  S.D. A paired *t*-test was used to compare  
15 two groups, and one-way ANOVA and Dunnett's *t*-test was used for multiple  
16 comparisons. The criterion for statistical significance was set at  $p < 0.05$ .  
17  
18  
19  
20  
21  
22  
23  
24  
25  
26  
27  
28  
29  
30  
31  
32  
33  
34  
35  
36  
37  
38  
39  
40  
41  
42  
43  
44  
45  
46  
47  
48  
49  
50  
51  
52  
53

## 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

1  
2  
3  
4  
5  
6  
7  
8  
9  
10  
11  
12  
13  
14  
15  
16  
17  
18  
19  
20  
21  
22  
23  
24  
25  
26  
27  
28  
29  
30  
31  
32  
33  
34  
35  
36  
37  
38  
39  
40  
41  
42  
43  
44  
45  
46  
47  
48  
49  
50  
51  
52  
53  
54  
55  
56  
57  
58  
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  $\mu\text{M}$ , 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% ( $\text{EC}_{90}$ ) demonstrated that HCT8 cells were more sensitive to C-1748 treatment than HT29 cells ( $\text{EC}_{90}$  0.04  $\mu\text{M}$  for HCT8 cells and 0.21  $\mu\text{M}$  for HT29 cells, respectively).  $\text{EC}_{90}$  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 ( $\text{EC}_{90}$  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 ( $4\times\text{EC}_{90}$ ), 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  $\text{EC}_{90}$  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).

1 Starting from 3 h of C-1748 exposure the population of polyploid cells increased and  
2 reached the highest level after 24 h (20%) and then began to decrease. Between 48  
3 and 72 h, the reduction in G2/M arrested and polyploid cells was accompanied by a  
4 and 72 h, the reduction in G2/M arrested and polyploid cells was accompanied by a  
5 concomitant increase in sub-G1 population (about 14% after 72 h, Tab. 1). In  
6  
7 contrary to HCT8 cells, increased concentration of C-1748 ( $4 \times EC_{90}$ ) did not result in  
8  
9 considerable increase of the number of HT29 cells undergoing apoptosis in terms of  
10  
11 sub-G1 DNA content (Fig. 2 C, Tab. 2).  
12  
13  
14  
15  
16  
17  
18

### 19 *3.2. Apoptotic cell morphology*

21 Flow cytometric analysis of cells in sub-G1 region indicated that colon cancer  
22 cells treated with C-1748 underwent apoptosis. To confirm these data, morphological  
23 changes of cells treated with C-1748 were assessed using fluorescence microscopy  
24 following DAPI staining. As shown in Fig. 3 A and B, untreated cells exhibited  
25 unchanged morphology, characteristic for colon cancer cells. After 12 h of C-1748  
26 treatment at  $EC_{90}$  concentration, only a few apoptotic HCT8 cells were visible.  
27 Prolonged drug incubation (48 and 72 h), as well as two-fold increased drug  
28 concentration, resulted in appearance of numerous cells exhibiting morphological  
29 changes typical for apoptosis, with cell shrinkage, chromatin condensation and  
30 fragmentation into discrete bodies. By contrast, only a few apoptotic HT29 cells were  
31 observed following C-1748 treatment at  $EC_{90}$  concentration and its multiplicity. At 72  
32 h of C-1748 exposure some of HT29 cells were shrunken and formed the structure of  
33 apoptotic bodies.  
34  
35  
36  
37  
38  
39  
40  
41  
42  
43  
44  
45  
46  
47  
48  
49  
50  
51

### 52 *3.3. DNA degradation*

53 TUNEL assay was used to investigate whether C-1748 induces apoptotic DNA  
54 fragmentation in human colon carcinoma cells. This method, based on the labeling  
55  
56  
57  
58  
59  
60  
61  
62  
63  
64  
65

1 the apoptotic DNA fragments by exogenous terminal deoxytransferase (TdT) and  
2 biotin-dUTP/FITC-avidin together with propidium iodide staining, allows to determine  
3 whether the DNA fragmentation observed in the treated cells occurred in particular  
4 phase of the cell cycle. As indicated in the Fig. 4 (right panel), the population of HCT8  
5 cells with apoptotic DNA breaks remained small after 3 h and 12 h of C-1748  
6 exposure at EC<sub>90</sub> concentration and increased to approximately 35% after 72 h. On  
7 the contrary, the population of HT29 cells with apoptotic DNA breaks was markedly  
8 smaller and reached the level of 12% after 72 h of C-1748 treatment. Biparametric  
9 flow cytometry analysis also revealed that cells with apoptotic DNA breaks derived  
10 from all phases of the cell cycle.  
11  
12  
13  
14  
15  
16  
17  
18  
19  
20  
21  
22  
23  
24  
25

### 26 *3.4. Annexin V/PI dual staining*

27  
28 To further confirm that C-1748 induces apoptosis, HCT8 and HT29 cells  
29 treated with the drug at EC<sub>90</sub> concentrations were stained with Annexin V-FITC and  
30 propidium iodide, and analyzed by flow cytometry. This assay is based on the  
31 translocation of phosphatidylserine (PS) from the inner leaflet of the plasma  
32 membrane to the cell surface in the early stage of apoptosis. Positive staining with  
33 fluorescently labelled Annexin V correlates with loss of plasma membrane polarity but  
34 precedes the complete loss of membrane integrity that accompanies later stages of  
35 cell death resulting from either apoptosis and necrosis. In contrast, PI can only enter  
36 cells after loss of their membrane integrity. Thus, dual staining with Annexin V and PI  
37 allows clear to discriminate between unaffected cells (Annexin V-/PI-), early apoptotic  
38 cells (Annexin V+/PI-), late apoptotic cells (Annexin V+/PI+) and primary necrotic  
39 cells (Annexin V-/PI+). As indicated in the Fig. 5 A, HCT8 cells remained largely  
40 unaffected for the first 6 h of C-1748 treatment. Prolonged drug incubation resulted in  
41  
42  
43  
44  
45  
46  
47  
48  
49  
50  
51  
52  
53  
54  
55  
56  
57  
58  
59  
60  
61  
62  
63  
64  
65



1  
2  
3  
4  
5  
6  
7  
8  
9  
10  
11  
12  
13  
14  
15  
16  
17  
18  
19  
20  
21  
22  
23  
24  
25  
26  
27  
28  
29  
30  
31  
32  
33  
34  
35  
36  
37  
38  
39  
40  
41  
42  
43  
44  
45  
46  
47  
48  
49  
50  
51  
52  
53  
54  
55  
56  
57  
58  
59  
60  
61  
62  
63  
64  
65

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 preceding 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<sub>90</sub> 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

1 72 h of exposure to C-1748. In the case of HT29 cells, the population with low  $\Delta\Psi_m$   
2 started to increase after 12 h of C-1748 treatment and reached 47% after prolonged  
3 incubation with the drug (72 h).  
4  
5  
6  
7  
8

### 9 3.6. Caspase-3 activity

10 It is well established that in most cases, apoptosis requires the participation of  
11 endogenous cellular enzymes – caspases. Among the identified caspases,  
12 activation of caspase-3 is a crucial in numerous types of cells leading to the  
13 execution of apoptosis [22, 23]. Therefore, we examined, whether caspase-3 was  
14 involved in apoptosis induced by C-1748 in colon cancer cells. After 3, 6 and 12 h of  
15 treatment with C-1748 at EC<sub>90</sub> concentration, we observed only a small amount of  
16 cells with active caspase-3 in both colon cancer cell lines (Fig. 6 B). However, in the  
17 case of HCT8 cells, 24 h of C-1748 treatment resulted in significant increase in  
18 number of cells with active caspase-3 (21%), and after 48 and 72 h of incubation, the  
19 percentage of cells with active caspase-3 reached about 40% and 60%, respectively.  
20 Compared to HCT8 cells, the percentage of HT29 cells expressing active caspase-3  
21 after C-1748 treatment, was markedly lower (17% after 72 h).  
22  
23  
24  
25  
26  
27  
28  
29  
30  
31  
32  
33  
34  
35  
36  
37  
38  
39  
40  
41  
42  
43

### 44 3.7. Induction of cellular senescence

45 DNA damage by a variety of compounds has been shown to induce cellular  
46 senescence in various cancer cells. Senescent cells are generally characterized by a  
47 reduction in proliferating capacity, adoption of a flattened and enlarged cell shape,  
48 and the appearance of senescence-associated  $\beta$ -galactosidase (SA- $\beta$ -gal) activity  
49 [24]. Because C-1748 induced apoptosis only in part of HCT8 and HT29 cells, we  
50 investigated whether these cells underwent senescence following C-1748 treatment.  
51  
52  
53  
54  
55  
56  
57  
58  
59  
60  
61  
62  
63  
64  
65

1  
2  
3  
4  
5  
6  
7  
8  
9  
10  
11  
12  
13  
14  
15  
16  
17  
18  
19  
20  
21  
22  
23  
24  
25  
26  
27  
28  
29  
30  
31  
32  
33  
34  
35  
36  
37  
38  
39  
40  
41  
42  
43  
44  
45  
46  
47  
48  
49  
50  
51  
52  
53  
54  
55  
56  
57  
58  
59  
60  
61  
62  
63  
64  
65

In HCT8 cells increased  $\beta$ -galactosidase staining relative to control was observed 72 h after treatment with C-1748. The number of SA- $\beta$ -gal-positive cells continued to increase and after 168 h of C-1748 exposure, the whole population of HCT8 cells presented  $\beta$ -galactosidase staining. Together with increased  $\beta$ -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  $\beta$ -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- $\beta$ -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

1  
2  
3  
4  
5  
6  
7  
8  
9  
10  
11  
12  
13  
14  
15  
16  
17  
18  
19  
20  
21  
22  
23  
24  
25  
26  
27  
28  
29  
30  
31  
32  
33  
34  
35  
36  
37  
38  
39  
40  
41  
42  
43  
44  
45  
46  
47  
48  
49  
50  
51  
52  
53  
54  
55  
56  
57  
58  
59  
60  
61  
62  
63  
64  
65

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 EC<sub>90</sub> 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% H<sub>2</sub>O<sub>2</sub> 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

1 crosslinks in cellular DNA [13]. Covalent binding and ability to form interstrand DNA  
2 crosslinks plays a crucial role for the biological activity of this drug [11, 12, 13]. It is  
3  
4 worth to point out that on the molecular level, C-1748 produced rather the same  
5 mechanism of action (interstrand DNA crosslinking) [11, 12, 13]. The question arises  
6  
7 whether such common mechanism of action will trigger the same cellular response in  
8  
9 different cancer cell lines.  
10  
11  
12  
13

14 The inhibition of tumor cells proliferation seems to be the first effect of action  
15 of anticancer drugs, including C-1748. The other cellular effects, such apoptosis,  
16 necrosis or senescence occur with some delay in relation to inhibition of cell  
17 proliferation. Therefore, it was very important to performed all experiments at  
18  
19 biologically relevant concentration of the drug. From the therapeutics point of view  
20 the most advantageous would be the concentration of the drug corresponding to its  
21  
22  $EC_{100}$  value. Because this concentration is impossible to estimate, in our studies we  
23 applied concentration of the drug corresponding to its  $EC_{90}$  values in all our  
24  
25 experiments (concentration which caused 90% of inhibition of cells proliferation).  
26  
27  
28  
29  
30  
31  
32  
33  
34  
35

36 Two colon cancer cell lines used in this work were selected due to their high  
37 sensitivity to C-1748. In addition, C-1748 exhibited potent antitumor activity against  
38  
39 HCT8 xenografts in mice [6].  
40  
41  
42

43 Cell cycle analysis showed time and dose-dependent appearance of sub-G1  
44 population of HCT8 cells, considered as apoptotic. In contrary to HCT8 cells, the  
45  
46 fraction of sub-G1 population of HT29 cells following C-1748 treatment was  
47  
48 considerable lower and did not exceed 14%, even following 4-fold increase of drug  
49  
50 concentration (Fig. 2 C), which suggested that apoptotic response of HT29 cells to C-  
51  
52 1748 treatment was somewhat weaker.  
53  
54  
55  
56  
57  
58  
59  
60  
61  
62  
63  
64  
65

1  
2 Apoptosis is characterized by several biochemical criteria, including different  
3 kinetics of phosphatidylserine (PS) exposure on the leaflet of the plasma membrane,  
4 changes in mitochondrial membrane permeability, caspase activation,  
5 internucleosomal DNA cleavage, release of intermembrane space mitochondrial  
6 proteins [26] as well as by a series of morphological changes such as formation of  
7 apoptotic bodies, nuclear and cytoplasmic condensation, chromatin fragmentation,  
8 shrinkage of cells and bleb formation [27]. However, necrosis is characterized mostly  
9 in negative terms by absence of caspase activation, cytochrome c release, DNA  
10 oligonucleosomal fragmentation, presence of plasma membrane permeabilization  
11 [28]. Therefore, identification of characteristic morphological and biochemical  
12 markers of apoptosis makes it possible to distinguish apoptosis from other forms of  
13 cell death.  
14  
15  
16  
17  
18  
19  
20  
21  
22  
23  
24  
25  
26  
27

28  
29 In our study the induction of apoptosis by C-1748 in human colon cancer cells  
30 was confirmed by several biochemical markers, such as: DNA degradation (TUNEL  
31 assay), loss of mitochondrial membrane potential  $\Delta\Psi_m$ , caspase-3 activity and  
32 phosphatidylserine externalization, as well as by morphological examination of the  
33 cells. Cellular morphology of both tested human colon cancer cells progressively  
34 changed with increasing duration of C-1748 exposure. Prolonged drug incubation  
35 resulted in the appearance of disintegrated cells, as evidenced by apoptotic bodies,  
36 and cells with condensed nuclear chromatin (Fig. 3). These morphological  
37 observations together with the appearance of sub-G1 populations confirmed the  
38 presence of considerable amount of apoptotic HCT8 cells and only small amount of  
39 HT29 cells, following treatment with C-1748.  
40  
41  
42  
43  
44  
45  
46  
47  
48  
49  
50  
51  
52  
53  
54  
55

56 To corroborate cell morphology-based observations, the apoptotic process  
57 induced by C-1748 was additionally monitored by TUNEL assay. Exposure of colon  
58  
59  
60  
61  
62  
63  
64  
65

1 cancer HCT8 and HT29 cells to C-1748 at EC<sub>90</sub> concentration resulted in the  
2 appearance of TUNEL-positive cells. The fraction of cells with fragmented DNA was  
3  
4 35% in HCT8 cells and was smaller (12%) in HT29 cells (Fig. 4). These data  
5  
6 correlated with those obtained from cell cycle analysis, where 25% of the HCT8 and  
7  
8 14% of the HT29 all population represented cells with sub-G1 DNA content.  
9  
10 Moreover, cells with apoptotic DNA fragmentation were derived from each phase of  
11  
12 the cell cycle which was consistent with the lack of cell cycle arrest in the specific  
13  
14 phase.  
15  
16  
17  
18

19 Most apoptotic pathways converge on the mitochondria, inducing the  
20  
21 disruption of the mitochondrial transmembrane potential  $\Delta\Psi_m$ . The loss of  
22  
23 mitochondrial membrane potential  $\Delta\Psi_m$  is early and already irreversible stage of  
24  
25 apoptosis [21]. Moreover, mitochondrial dysfunction during apoptosis is very often  
26  
27 associated with the activation of caspases [29]. Exposure to C-1748 resulted in time-  
28  
29 dependent decrease of mitochondrial membrane potential  $\Delta\Psi_m$  in HCT8 and HT29  
30  
31 cells (Fig. 6 A). However, the significant increase in the number of cells with active  
32  
33 caspase-3 was observed in HCT8 cells. After 72 h of incubation with the drug, 60%  
34  
35 of HCT8 cells had active caspase-3, but in the case of HT29 cells caspase-3 was  
36  
37 activated only in 10% of cell population (Fig. 6 B). The activation of caspase-3  
38  
39 concomitantly with the dissipation of  $\Delta\Psi_m$  confirmed that apoptosis was the main  
40  
41 response of HCT8 cells to C-1748 treatment. In contrast, the disruption of  
42  
43 mitochondrial membrane potential  $\Delta\Psi_m$ , which also can result from membrane  
44  
45 permeabilization, together with the presence of only small population of HT29 cells  
46  
47 with active caspase-3 indicated that necrosis was responsible for the death of colon  
48  
49 cancer HT29 cells after C-1748 treatment. The increased permeabilization of cellular  
50  
51 membranes, including mitochondrial membrane which provides to decrease of  
52  
53  
54  
55  
56  
57  
58  
59  
60  
61  
62  
63  
64  
65



1  
2  
3  
4  
5  
6  
7  
8  
9  
10  
11  
12  
13  
14  
15  
16  
17  
18  
19  
20  
21  
22  
23  
24  
25  
26  
27  
28  
29  
30  
31  
32  
33  
34  
35  
36  
37  
38  
39  
40  
41  
42  
43  
44  
45  
46  
47  
48  
49  
50  
51  
52  
53  
54  
55  
56  
57  
58  
59  
60  
61  
62  
63  
64  
65

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  $\beta$ -galactosidase staining relative to control (Fig. 7 A). In contrast to HCT8 cells, no  $\beta$ -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  
2  
3  
4  
5  
6  
7  
8  
9  
10  
11  
12  
13  
14  
15  
16  
17  
18  
19  
20  
21  
22  
23  
24  
25  
26  
27  
28  
29  
30  
31  
32  
33  
34  
35  
36  
37  
38  
39  
40  
41  
42  
43  
44  
45  
46  
47  
48  
49  
50  
51  
52  
53  
54  
55  
56  
57  
58  
59  
60  
61  
62  
63  
64  
65

cells with wild p53 underwent mainly apoptosis and senescence. On the contrary, HT29 cells with mutated, non functional p53 underwent mainly necrosis (apoptotic response was very weak), and did not exhibit features of senescence. Western blot experiments revealed that the total p53 level gradually decreased in HCT8 cells upon C-1748 treatment and after 168 h there was no p53 protein signal from Western blot analysis (Fig.7 C). On the other hand, the number of SA- $\beta$ -gal-positive HCT8 cells continued to increase and after 168 h of C-1748 exposure the whole population presented  $\beta$ -galactosidase staining (Fig.7 A). These results suggested that senescence process induced by C-1748 was independent on p53 protein. Thus, further studies are needed to indicate other factors responsible for the diverse cellular response of colon cancer cells to C-1748 treatment.

C-1748 and other 1-nitroacridines require metabolic activation before they exhibit any cytotoxic and antitumor activity [13, 31]. It was shown that metabolic transformations of these compounds occurred in several steps including the reduction of nitro group. One electron reduction of nitro group is usually the first step of 1-nitroacridine's metabolism [31]. Nitroradical anion formed in this step is able to transform the molecular oxygen into ROS. Therefore, we determined the ability of C-1748 to induce ROS and we established the relevance of this effect for the induction of apoptosis and cytotoxic activity of this drug. In our study 2-fold increase in generation of DCF-sensitive ROS in HT29 cells was observed after 1 h of C-1748 treatment, whereas in HCT8 cells maximum level of ROS generation was reached after 4 h of incubation with the drug (Fig. 8 A). Moreover, two antioxidants: N-acety-L-cysteine (NAC) and melatonin, that neutralize reactive oxygen species, had no effect on cell death induced by C-1748, as well as on cell viability. Taken together, these results strongly suggest that oxidative stress may not be a casual factor in the

1 pathway of C-1748-mediated apoptosis and has no influence on cytotoxic activity of  
2 this drug.  
3

4 In summary, our results indicated that C-1748 at biological relevant  
5 concentration corresponding to  $EC_{90}$  values, induced different cellular response in  
6 human colon carcinoma cells. HCT8 cells underwent transient G1 and G2/M phase  
7 arrest followed by apoptosis. Moreover, prolonged drug treatment led to the induction  
8 of senescence of HCT8 cells. In contrast, the apoptotic response of HT29 cells to C-  
9 1748 exposure was significantly weaker and was preceded by rather stable G1  
10 phase arrest. However, HT29 cells did not undergo senescence and died mainly by  
11 necrosis. Additionally, we demonstrated that C-1748 at  $EC_{90}$  concentration induced  
12 reactive oxygen species (ROS), but generation of ROS was not essential for C-  
13 1748-induced apoptosis and cytotoxic activity of this drug.  
14  
15  
16  
17  
18  
19  
20  
21  
22  
23  
24  
25  
26  
27

28 Importantly, we demonstrated that one chemotherapeutic agent, C-1748,  
29 induces different cellular effects (apoptosis, necrosis, senescence) in two colon  
30 cancer cell lines. Moreover, such diverse cellular response was observed even within  
31 one colon cancer cell population. Similar observations have been made for other  
32 antitumor agents, such as adriamycin [32]. Therefore, the divergent cellular response  
33 triggered by the same chemotherapeutic agent should be especially considered in  
34 cancer therapy of heterogenous population of tumor cells in patients, e.g. in  
35 designing of multidrug therapy.  
36  
37  
38  
39  
40  
41  
42  
43  
44  
45  
46  
47  
48  
49

## 50 **5. ACKNOWLEDGMENTS**

51 This work was support by State Committee for Scientific Research (KBN), Poland,  
52 grant no. 3PO5A11824. The authors thank Dr. Anna Skwarska from our Department  
53 for critical reading of the article and for valuable comments. We also thank Prof.  
54  
55  
56  
57  
58  
59  
60  
61  
62  
63  
64  
65

Jacek Witkowski, Department of Pathophysiology, Medical University of Gdańsk, for  
help in flow cytometry studies.

Accepted Manuscript

## 6. REFERENCES

1. Ledochowski A. Ledakrin – anticancerous medicine 1-nitro-9(3-dimethylamino)-acridine x 2HCl x H<sub>2</sub>O. *Materia Medica Polona* 1976;8:237-42.
2. Gniazdowski M, Szmigiero L. Nitracrine and its congeners – an overview. *General Pharmacol* 1995;26:473-81.

- 1  
2  
3  
4  
5  
6  
7  
8  
9  
10  
11  
12  
13  
14  
15  
16  
17  
18  
19  
20  
21  
22  
23  
24  
25  
26  
27  
28  
29  
30  
31  
32  
33  
34  
35  
36  
37  
38  
39  
40  
41  
42  
43  
44  
45  
46  
47  
48  
49  
50  
51  
52  
53  
54  
55  
56  
57  
58  
59  
60  
61  
62  
63  
64  
65
3. Bratkowska – Seniów B, Dziedzic J, Fengler I, Steuden W, Szymaniec S, Wysocka M. Morphologic blood pathern in patients treated with Ledakrin. *Materia Medica Polona* 1976;8:295-301.
4. Kwaśniewska – Rokocińska C, Święcicki J, Drosik K. Preliminary observations on the Ledakrin effects in treatment of patients with lung carcinoma. *Materia Medica Polona* 1976;8:289-95.
5. Chen Y, Ashok BT, Venkata P, Garikapaty VPS, Liu X, Szostek A, et al. Pre-clinical studies of 1-nitroacridine derivatives: Effective anti-tumor agents for prostate cancer. *Proc Am Assoc Cancer Res* 2002;43:77.
6. Tadi K, Ashok BT, Chen Y, Banerjee D, Wysocka-Skrzela B, Konopa J, et al. Pre-clinical evaluation of 1-nitroacridine derived chemotherapeutic agent that has preferential cytotoxic activity towards prostate cancer. *Cancer Biol Ther* 2007;6:1632-7.
7. Ashok BT, Tadi K, Garikapaty VPS, Chen Y, Huang Q, Banerjee D, et al. Pre-clinical toxicological evaluation of a putative prostate cancer specific 1-nitroacridine derivative in rodent. *Anti-Cancer Drugs* 2007;18:87-94.
8. Ashok BT, Tadi K, Banerjee D, Konopa J, Iatropoulos M, Tiwari RK. Pre-clinical toxicology and pathology of 9-(2'-hydroxyethylamino)-4-methyl-1-nitroacridine (C-1748), a novel anti-cancer agent in male Beagle dogs. *Life Sci* 2006;79:1334-42.
9. Narayanan R, Tiwari P, Inos D, Ashok BT. Comparative analysis of mutagenic potency of 1-nitroacridine derivatives. *Life Sci* 2005;77:2312-23.
10. Konopa J, Wysocka-Skrzela B, Szostek A, Tiwari R. Potent antitumor 4-substituted-1-nitroacridines DNA crosslinking agents. *Proc EORTC-NCI-AACR, Boston*, 2003:66.

- 1  
2  
3  
4  
5  
6  
7  
8  
9  
10  
11  
12  
13  
14  
15  
16  
17  
18  
19  
20  
21  
22  
23  
24  
25  
26  
27  
28  
29  
30  
31  
32  
33  
34  
35  
36  
37  
38  
39  
40  
41  
42  
43  
44  
45  
46  
47  
48  
49  
50  
51  
52  
53  
54  
55  
56  
57  
58  
59  
60  
61  
62  
63  
64  
65
11. Konopa J, Pawlak J, Pawlak K. The mode of action of cytotoxic and antitumor 1-nitroacridines. III. In vivo interstrand crosslinking of DNA of mammalian or bacterial cells by 1-nitroacridines. *Chem Biol Interact* 1983;43:175-97.
  12. Pawlak K, Pawlak J, Konopa J. Cytotoxic and antitumor activity of 1-nitroacridines as an aftereffect of their interstrand DNA cross-linking. *Cancer Res* 1984;44:4289-96.
  13. Szostek A, Wysocka-Skrzela B, Tiwari RK, Konopa J. Interstrand crosslinking of DNA in tumor cells by a new group of antitumor 1-nitroacridines. *Int J Cancer* 2002;13:441.
  14. Schmitt CA. Cellular senescence and cancer treatment. *Biochim Biophys Acta* 2007;1775:5-20.
  15. Gewirtz DA, Holt SE, Elmore LW. Accelerated senescence: An emerging role in tumor cell response to chemotherapy and radiation. *Biochem Pharmacol* 2008;76:947-57.
  16. Campisi J, d'Adda di Fagagna F. Cellular senescence: when bad things happen to good cells. *Nat Rev Mol Cell Biol* 2007;8:729-40.
  17. Hattangadi DK, DeMasters GA, Walker DT, Jones KR, Di X, Newsham IF, et al. Influence of p53 and caspase 3 activity on cell death and senescence in response to methotrexate in the breast tumor cell. *Biochem Pharmacol* 2004;68:1699-1708.
  18. Roberson RS, Kussick SJ, Valeries E, Chen SY, Wu DY. Escape from therapy-induced accelerated cellular senescence in p53-null lung cancer cells in human lung cancers. *Cancer Res* 2005;65:2795-803.

- 1  
2  
3  
4  
5  
6  
7  
8  
9  
10  
11  
12  
13  
14  
15  
16  
17  
18  
19  
20  
21  
22  
23  
24  
25  
26  
27  
28  
29  
30  
31  
32  
33  
34  
35  
36  
37  
38  
39  
40  
41  
42  
43  
44  
45  
46  
47  
48  
49  
50  
51  
52  
53  
54  
55  
56  
57  
58  
59  
60  
61  
62  
63  
64  
65
19. Bhuyan K, Smith KS, Adams EG, Petzold GL, Mc Govren JP. Lethality, DNA alkylation and cell cycle effects of adozelesin (U-73975) on rodent and human cells. *Cancer Res* 1992;52:5687-92.
20. Augustin E, Moś-Rompa A, Skwarska A, Witkowski JM, Konopa J. Induction of G2/M phase arrest and apoptosis of human leukemia cells by potent antitumor triazoloacridinone C-1305. *Biochem Pharmacol* 2006;72:1668-79.
21. Ly JD, Grubb DR, Lawen A. The mitochondrial membrane potential ( $\Delta\psi(m)$ ) in apoptosis. *Apoptosis* 2003;8:116-28.
22. Janicke RU, Sprengart ML, Wati MR, Porter AG. Emerging role of caspase-3 in apoptosis. *Cell Death Differ* 1999;6:99-104.
23. Yang S, Zhou Q, Yang X. Caspase-3 status is a determinant of differential responses to genistein between MD-MB-231 and MCF-7 breast cancer cells. *Biochim Biophys Acta* 2007;1773:903-11.
24. Dimri GP, Lee X, Basile G, Acosta M, Scott G, Roskelley C, et al. A biomarker that identifies senescent human cells in culture and aging skin in vivo. *Proc Natl Acad Sci USA* 1995;92:9363-7.
25. Kovacic P, Osuna JA. Mechanisms of anti-cancer agents: emphasis on oxidative stress and electron transfer. *Curr Pharm Design* 2000;6:277-309.
26. Krysko DV, Berghe TV, D'Herde K, Vandenabeele P. Apoptosis and necrosis: detection, discrimination and phagocytosis. *Methods* 2008;44:205-21.
27. Rello S, Stockert JC, Moreno V, Gamez A, Pacheco M, et al. Morphological criteria to distinguish cell death induced by apoptotic and necrotic treatments. *Apoptosis* 2005;10:201-8.

- 1  
2  
3  
4  
5  
6  
7  
8  
9  
10  
11  
12  
13  
14  
15  
16  
17  
18  
19  
20  
21  
22  
23  
24  
25  
26  
27  
28  
29  
30  
31  
32  
33  
34  
35  
36  
37  
38  
39  
40  
41  
42  
43  
44  
45  
46  
47  
48  
49  
50  
51  
52
28. Galluzzi L, Maiuri MC, Vitale I, Zischka H, Castedo M, Zitvogel L, Kroemer G. Cell death modalities: classification and pathophysiological implications. *Cell Death Differr* 2007;14:1237-66.
29. Slee EA, Harte MT, Kluck RM, Wolf BB, Casiano CA, et al. Ordering the cytochrome c-initiated caspase cascade: hierarchical activation of caspases-2, -3, -6, -8, and -10 in a caspase-9-dependent manner. *J Cell Biol* 1999;144:281-92.
30. Kim JS, He L, Lemasters JJ. Mitochondrial permeability transition: a common pathway to necrosis and apoptosis. *Biochem Biophys Res Commun* 2003;304:463-70.
31. Gorlewska K, Mazerska Z, Sowiński P, Konopa J. Products of metabolic activation of the antitumor drug Ledakrin (Nitracrine) in vitro. *Chemical Res Toxicol* 2001;14:1-10.
32. Di X, Shiu RP, Newsham IF, Gewirtz DA. Apoptosis, autophagy, accelerated senescence and reactive oxygen in response of human breast tumor cells to Adriamycin. *Biochem Pharmacol* 2009;77:1139-50.

## 53 **FIGURE LEGENDS**

54 *Figure 1.*

55 Chemical structure of 9-(2'-hydroxyethylamino)-4-methyl-1-nitroacridine derivative,  
56  
57 C-1748.  
58  
59  
60  
61  
62  
63  
64  
65



1  
2 *Figure 2.*

3  
4 Changes in DNA content following treatment with C-1748 at  $EC_{90}$  concentration for  
5  
6 the time indicated. HCT8 (A) and HT29 (B) cells were fixed in ethanol, stained with PI  
7  
8 and their DNA content was measured by FACScan. Representative histograms of  
9  
10 three independent experiments are shown. (C) The percentage of sub-G1 fraction of  
11  
12 HCT8 and HT29 cells treated with C-1748 at  $1xEC_{90}$  and  $4xEC_{90}$  concentrations. The  
13  
14 percentage was calculated by deconvolution of the DNA content histograms.  
15  
16  
17  
18  
19  
20

21 *Figure 3.*

22  
23 Changes in nuclear morphology of HCT8 (A) and HT29 (B) cells following treatment  
24  
25 with C-1748 at  $1xEC_{90}$  and  $2xEC_{90}$  concentrations. Representative pictures of cells  
26  
27 stained with DAPI and examined under a fluorescent microscope (magnification,  
28  
29 x400). Cells with condensed, fragmented chromatin were considered as apoptotic.  
30  
31  
32  
33

34 *Figure 4.*

35  
36 TUNEL assay for HCT8 and HT29 cells exposed to C-1748 at  $EC_{90}$  concentration for  
37  
38 the time indicated. *Left panel*, bivariate flow cytometry histograms of representative  
39  
40 TUNEL assay data. The gated regions are TUNEL positive cells. *Right panel*,  
41  
42 quantitation of the percentage of TUNEL positive cells (mean values from three  
43  
44 experiments  $\pm$  SD).  
45  
46  
47  
48  
49  
50  
51  
52

53 *Figure 5.*

54  
55 Flow cytometric analysis of membrane alteration in HCT8 cells (A) and HT29 cells (B)  
56  
57 treated with C-1748 at  $EC_{90}$  concentration. *Left panel*, representative bivariate flow  
58  
59  
60  
61  
62  
63  
64  
65

1  
2  
3  
4  
5  
6  
7  
8  
9  
10  
11  
12  
13  
14  
15  
16  
17  
18  
19  
20  
21  
22  
23  
24  
25  
26  
27  
28  
29  
30  
31  
32  
33  
34  
35  
36  
37  
38  
39  
40  
41  
42  
43  
44  
45  
46  
47  
48  
49  
50  
51  
52  
53  
54  
55  
56  
57  
58  
59  
60  
61  
62  
63  
64  
65

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.*

1  
2  
3  
4  
5  
6  
7  
8  
9  
10  
11  
12  
13  
14  
15  
16  
17  
18  
19  
20  
21  
22  
23  
24  
25  
26  
27  
28  
29  
30  
31  
32  
33  
34  
35  
36  
37  
38  
39  
40  
41  
42  
43  
44  
45  
46  
47  
48  
49  
50  
51  
52  
53  
54  
55  
56  
57  
58  
59  
60  
61  
62  
63  
64  
65

Characterization of the time course of senescence-associated  $\beta$ -galactosidase (SA- $\beta$ -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 EC<sub>90</sub> concentration, cells were fixed and incubated with X-Gal at pH 6.0 which produced blue coloration in SA- $\beta$ -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 EC<sub>90</sub> 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.*

34  
35  
36  
37  
38  
39  
40  
41  
42  
43  
44  
45  
46  
47  
48  
49  
50  
51  
52  
53  
54  
55  
56  
57  
58  
59  
60  
61  
62  
63  
64  
65

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 EC<sub>90</sub> value for indicated times, or with 0.01% H<sub>2</sub>O<sub>2</sub> for 30 min (positive control). The harvested cells were then incubated with 20  $\mu$ 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-

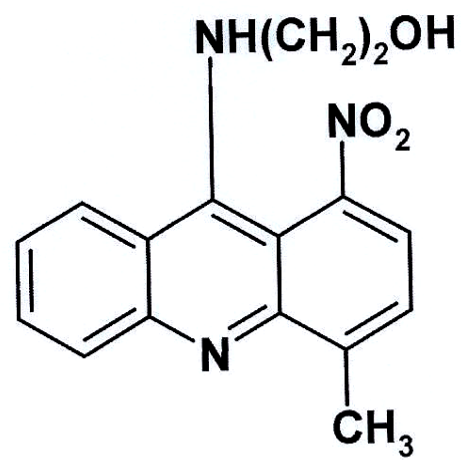
1 1748 (HCT8→0.04  $\mu$ M; HT29→0.21  $\mu$ M) for 72 h. Cell viability was detected by MTT  
2 assay. C, Control-untreated cells; C + 5, cells treated with 5 mM NAC alone; C + 100,  
3 cells treated with 100  $\mu$ M melatonin alone. Data are mean values from three  
4 experiments ( $\pm$  SD). (D), effect of NAC on phosphatidylserine externalization in HCT8  
5 cells following C-1748 exposure. HCT8 cells were pretreated with 5 mM NAC for 1 h  
6 and then exposed to 0.04  $\mu$ M C-1748 for indicated times. After the treatment, cells  
7 were stained with Annexin V and propidium iodide (PI) and then analyzed by flow  
8 cytometry. C + 5, cells treated with 5 mM NAC alone. Bar graphs represent  
9 quantitation of unaffected, early and late apoptotic as well as necrotic cells based on  
10 the flow cytometry analysis. Data are representative of three independent  
11 experiments.  
12  
13  
14  
15  
16  
17  
18  
19  
20  
21  
22  
23  
24  
25  
26  
27  
28  
29  
30  
31  
32  
33  
34  
35  
36  
37  
38  
39  
40  
41  
42  
43  
44  
45  
46  
47  
48  
49  
50  
51  
52  
53  
54  
55  
56  
57  
58  
59  
60  
61  
62  
63  
64  
65

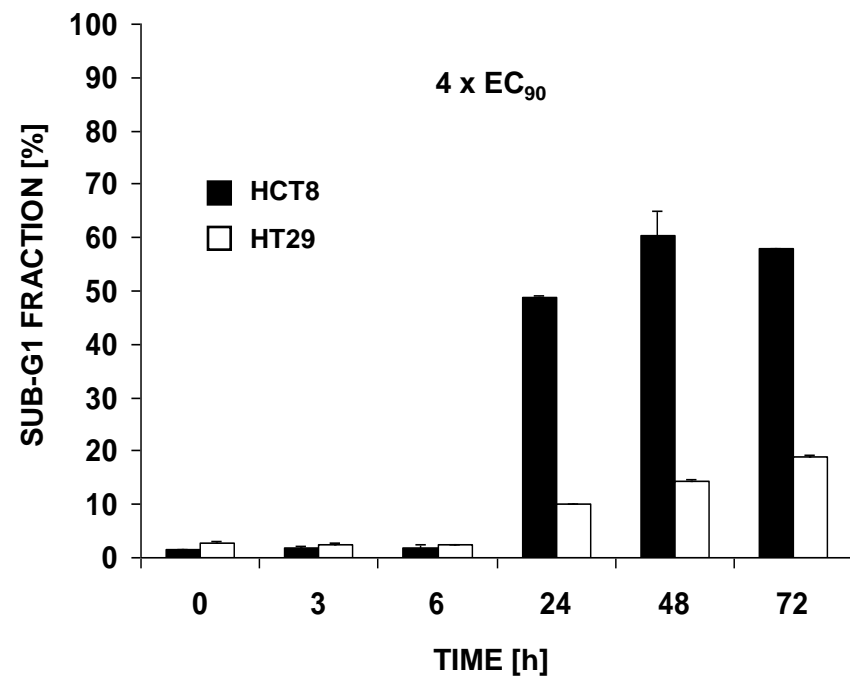
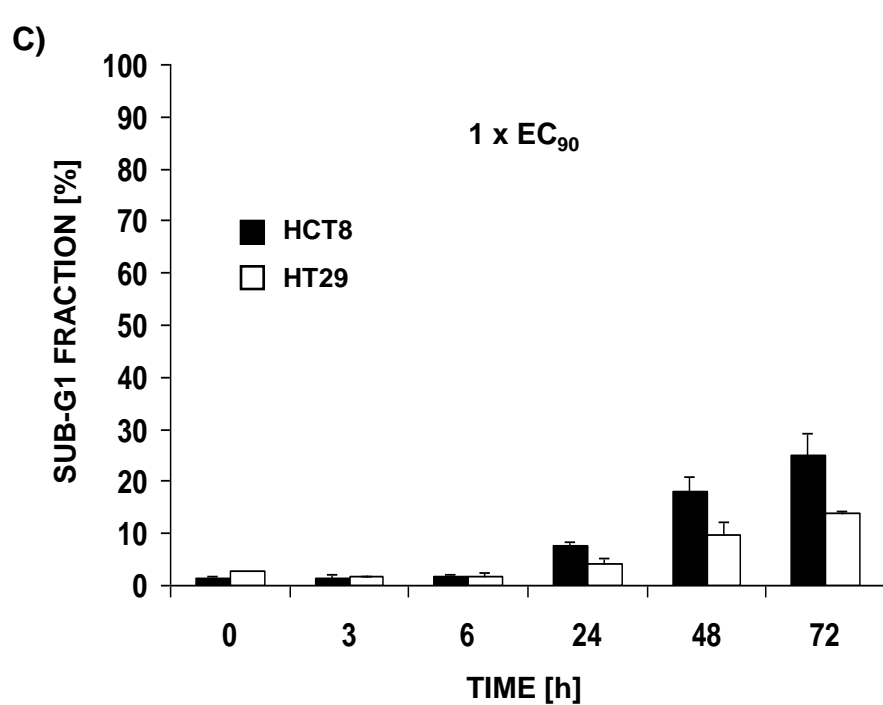
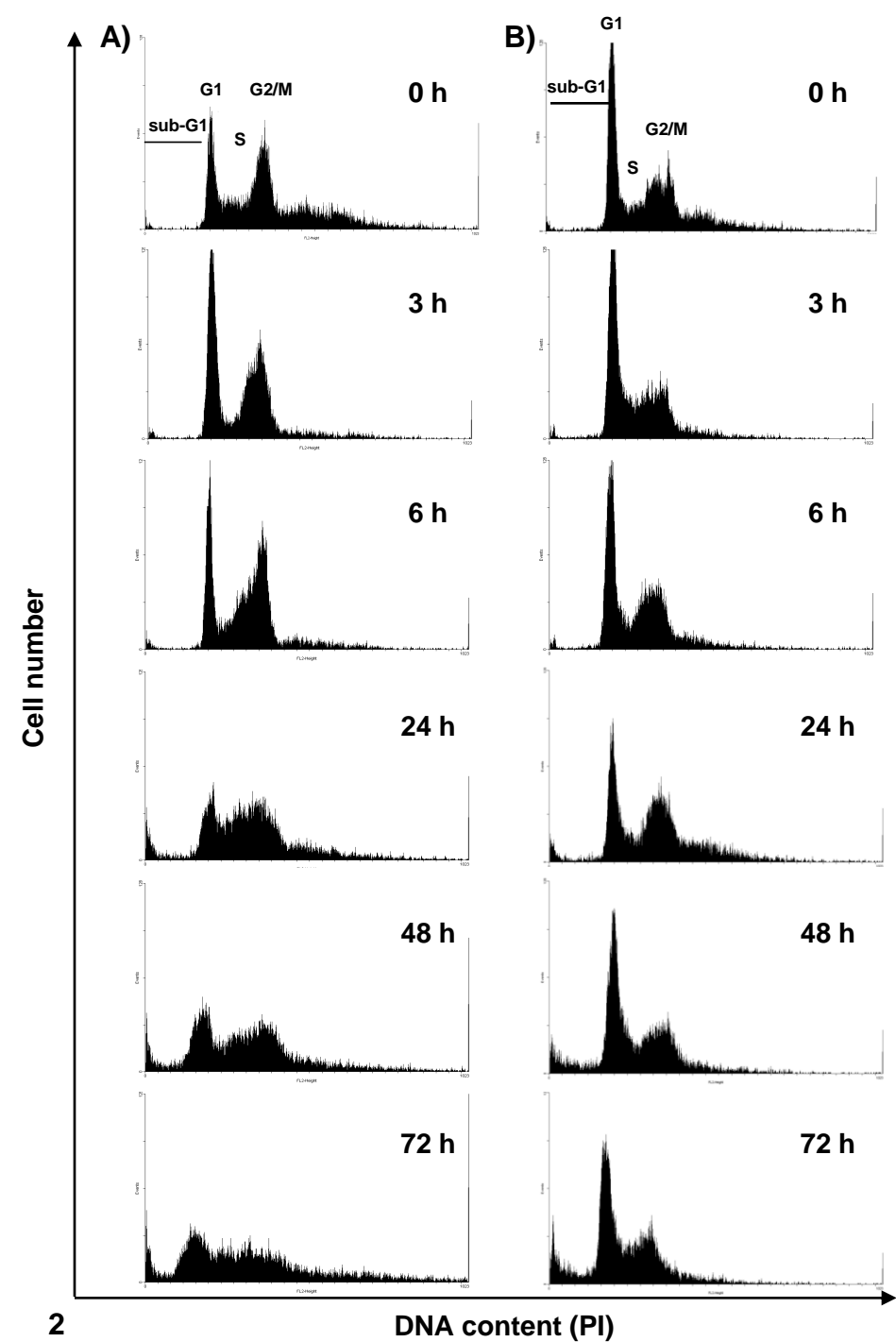
Table 1. Percentage of sub-G1 and cell cycle distribution of HCT8 and HT29 cells treated with 0.04  $\mu\text{M}$  ( $1 \times \text{EC}_{90}$ ) or 0,21  $\mu\text{M}$  ( $1 \times \text{EC}_{90}$ ) C-1748 respectively, for the time indicated. Data are presented as mean  $\pm$  SD of triplicate experiments.

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

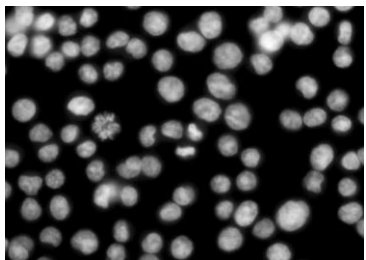
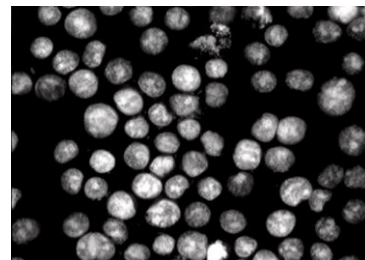
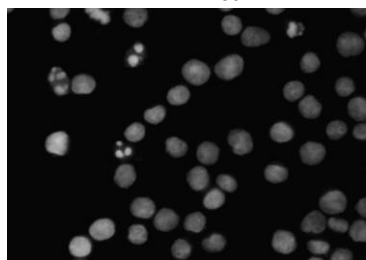
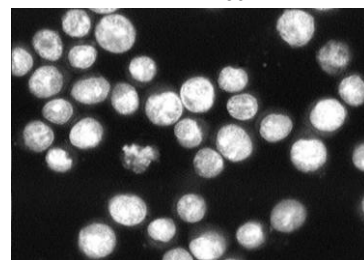
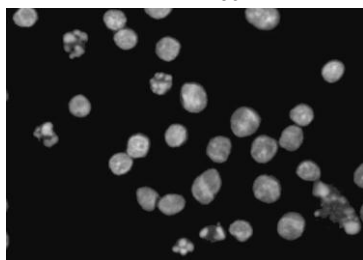
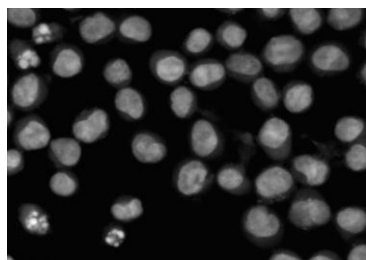
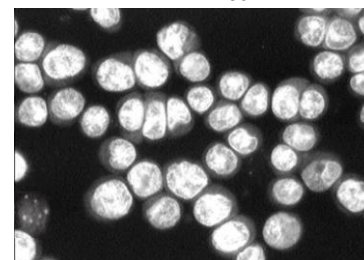
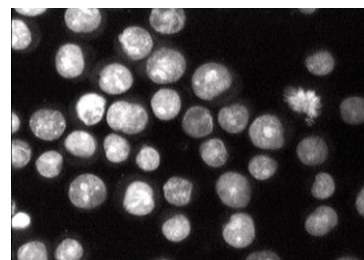
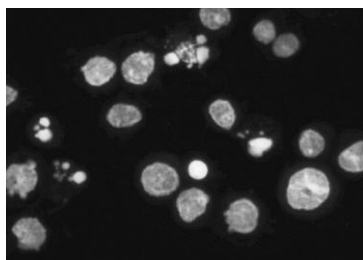
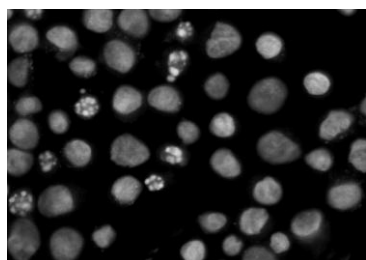
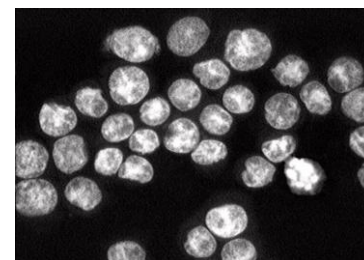
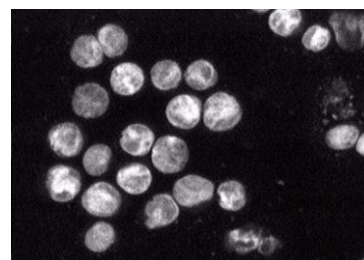
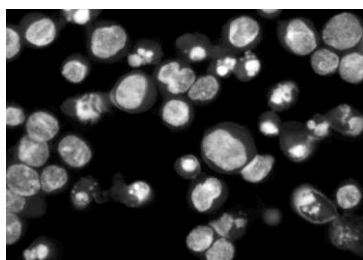
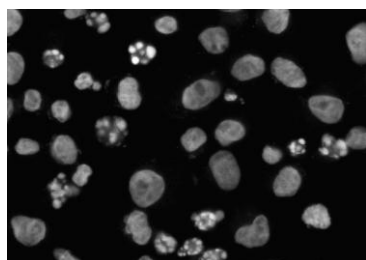
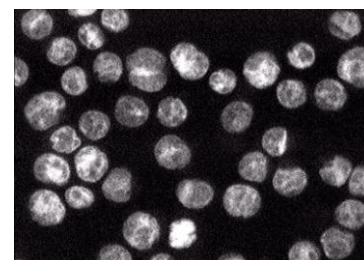
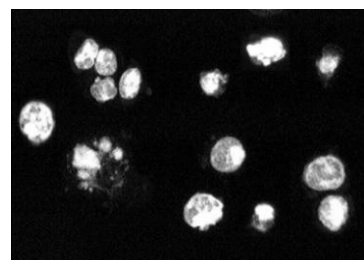
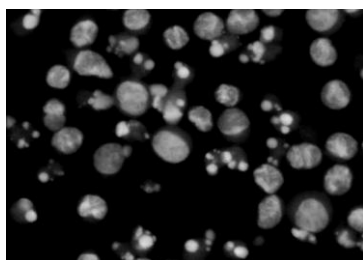
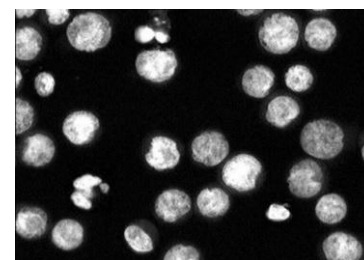
Table 2. Percentage of sub-G1 and cell cycle distribution of HCT8 and HT29 cells treated with 0.16  $\mu\text{M}$  ( $4x\text{EC}_{90}$ ) or 0,84  $\mu\text{M}$  ( $4x\text{EC}_{90}$ ) C-1748 respectively, for the time indicated. Data are presented as mean  $\pm$  SD of triplicate experiments.

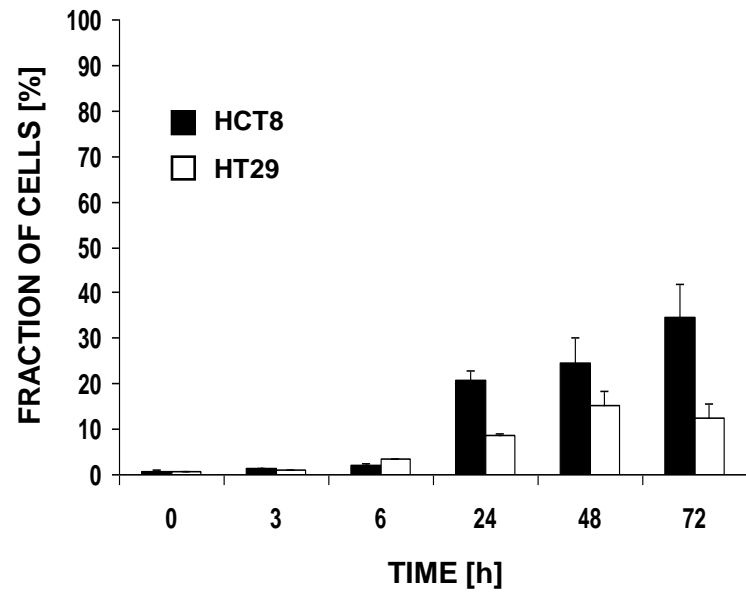
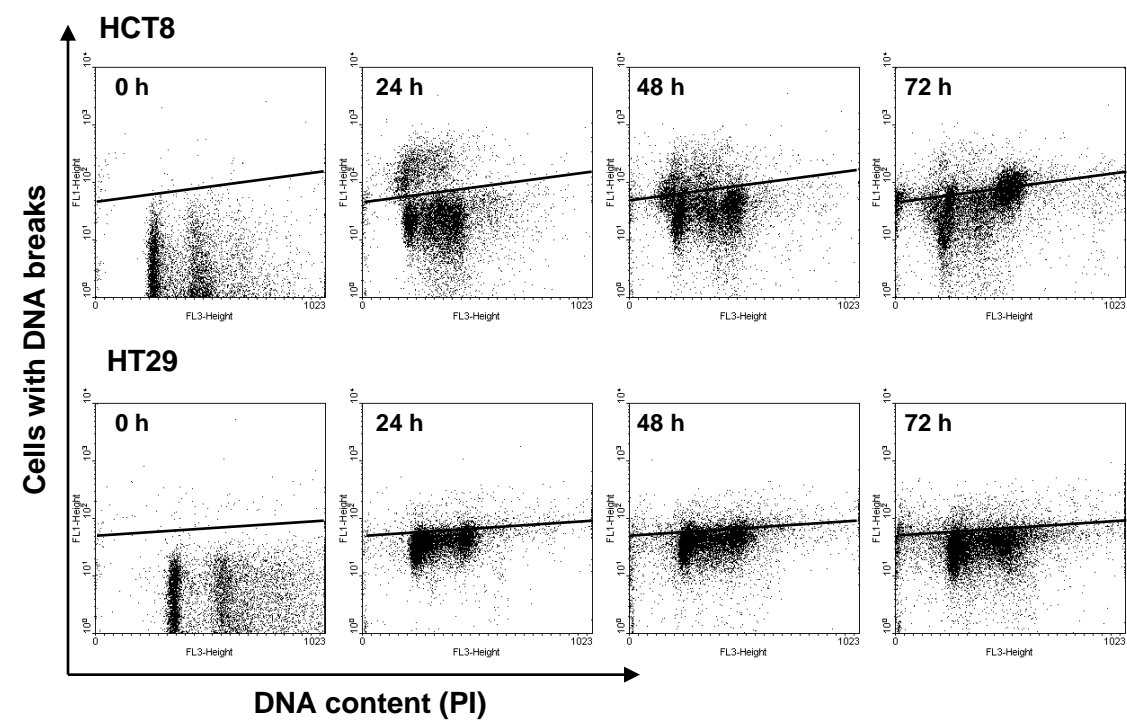
Cell line	Time of treatment [h]	Sub-G1	G1	S	G2/M	> 4n
HCT8	0	1.5 $\pm$ 0.10	35.6 $\pm$ 0.30	23.5 $\pm$ 0.50	39.4 $\pm$ 1.2	4.3 $\pm$ 0.50
	3	2.0 $\pm$ 0.04	31.3 $\pm$ 0.50	12.5 $\pm$ 2.86	31.7 $\pm$ 5.69	21.2 $\pm$ 2.8
	6	2.0 $\pm$ 0,45	23.7 $\pm$ 0.37	13.2 $\pm$ 2.93	35.8 $\pm$ 2.31	24.0 $\pm$ 0.90
	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



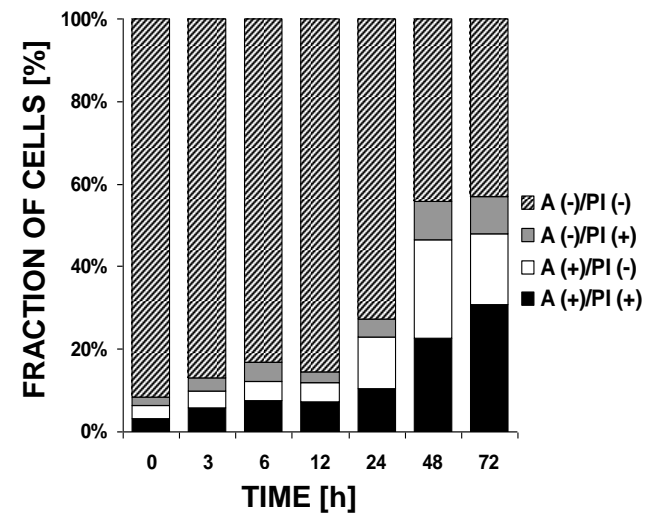
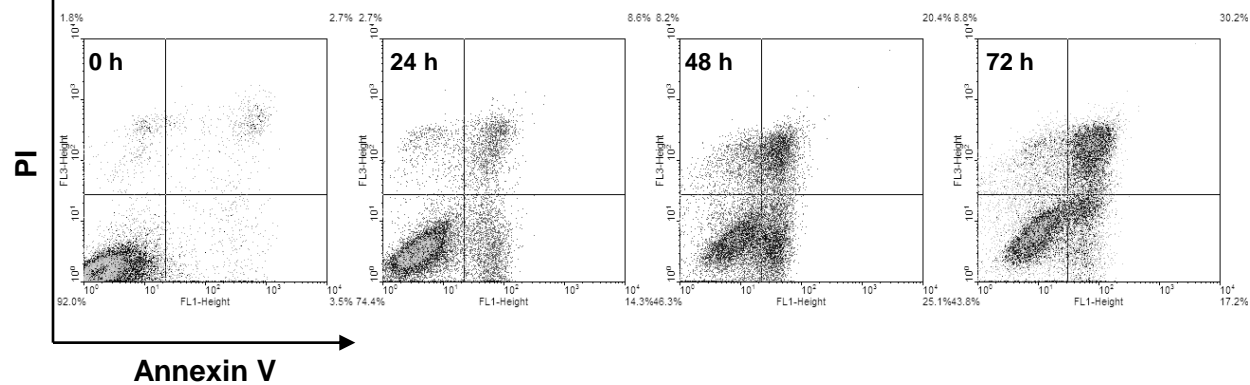




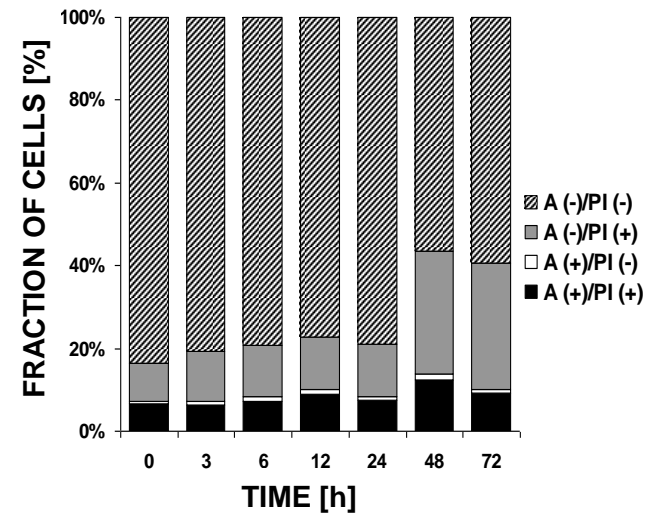
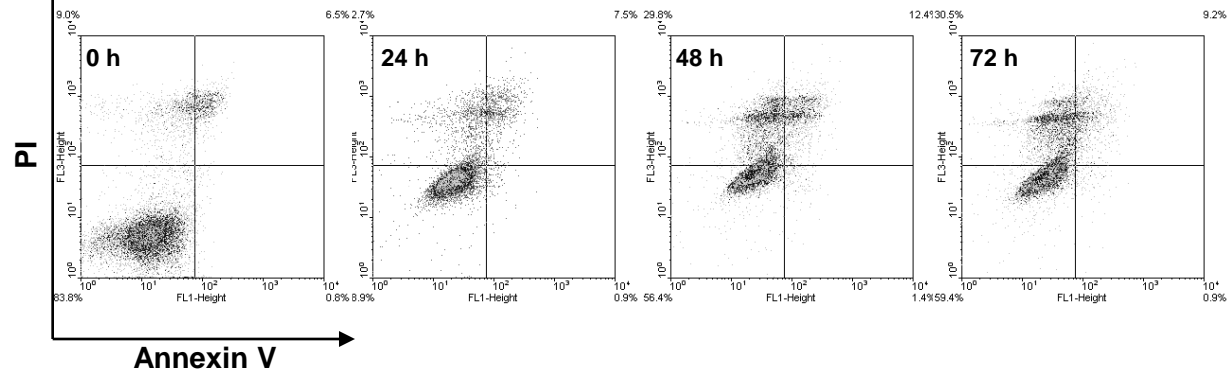
**A)****1 x EC<sub>90</sub>****0 h****2 x EC<sub>90</sub>****B)****1 x EC<sub>90</sub>****0 h****2 x EC<sub>90</sub>****12 h****12 h****24 h****24 h****48 h****48 h****72 h****72 h**



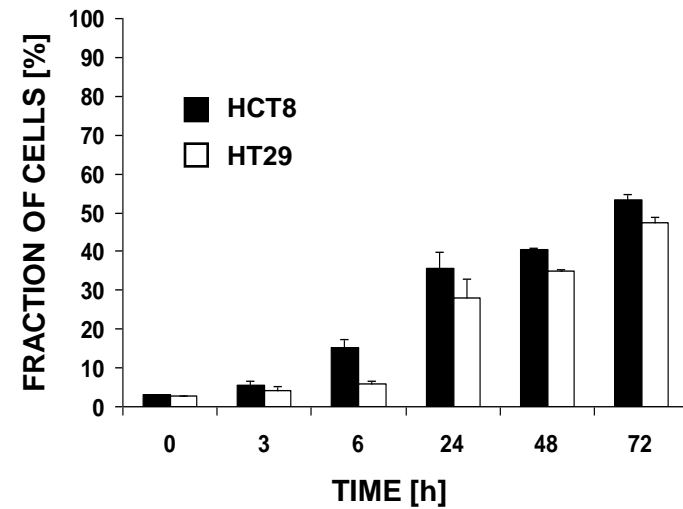
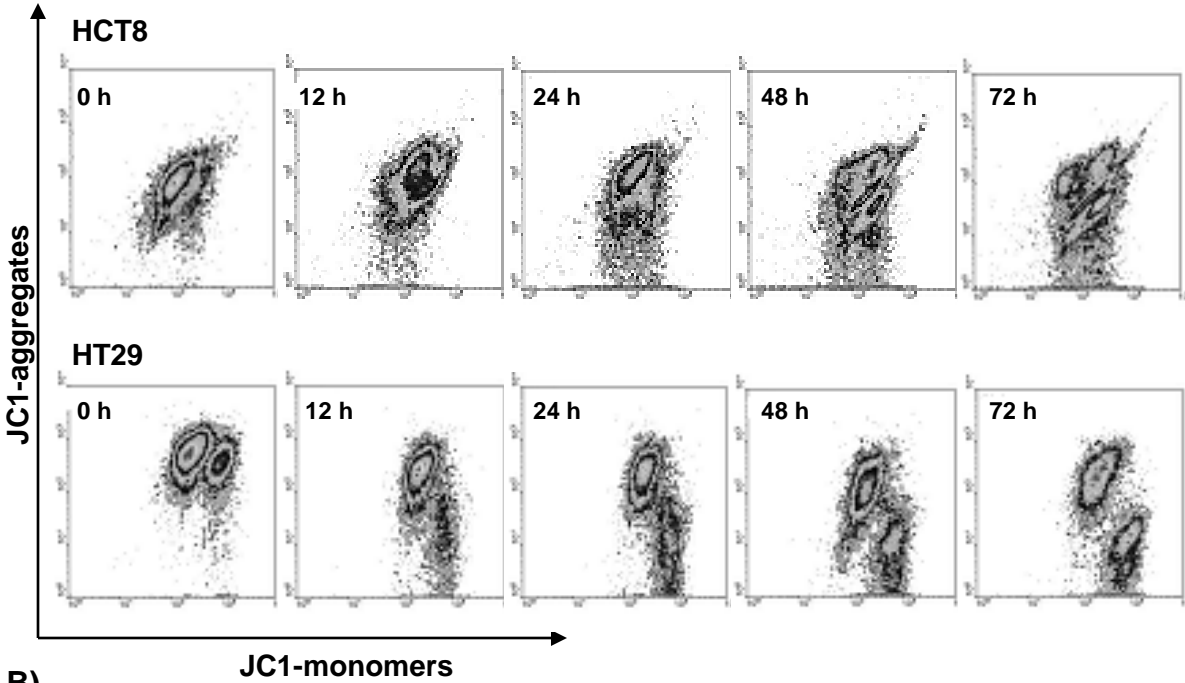
A)



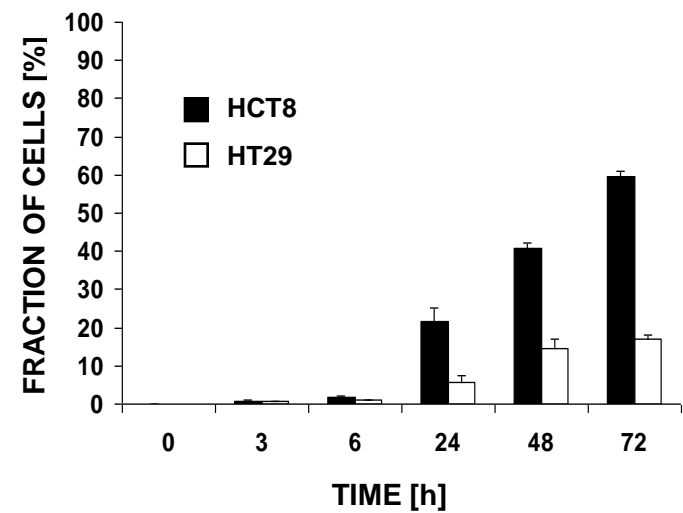
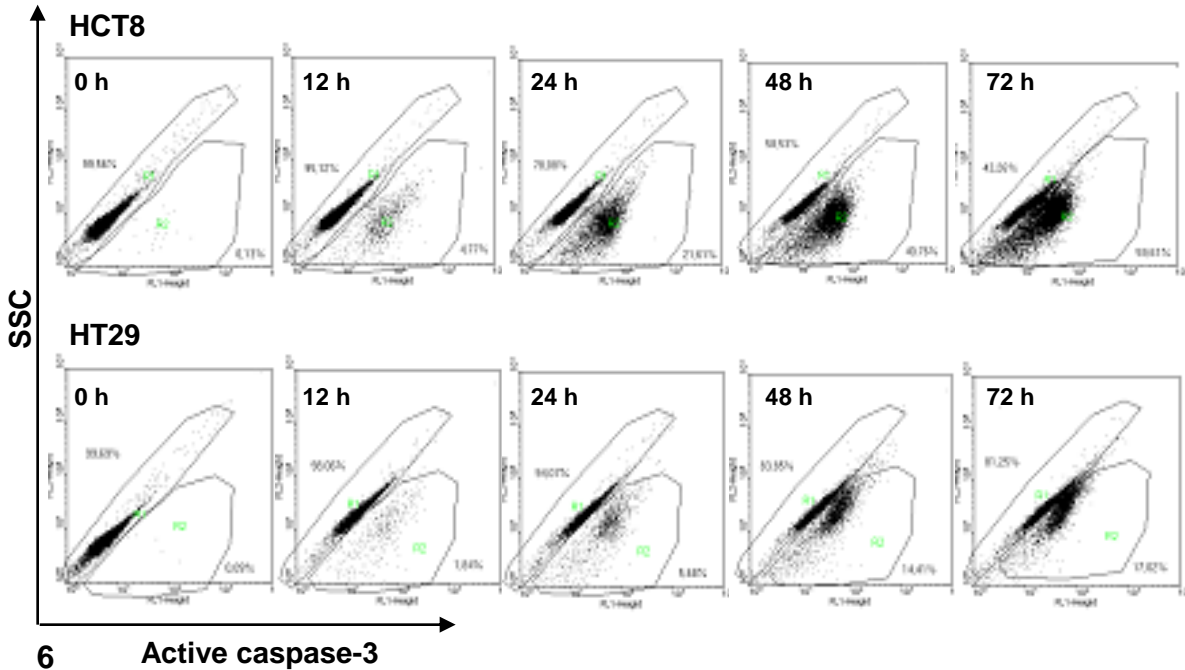
B)

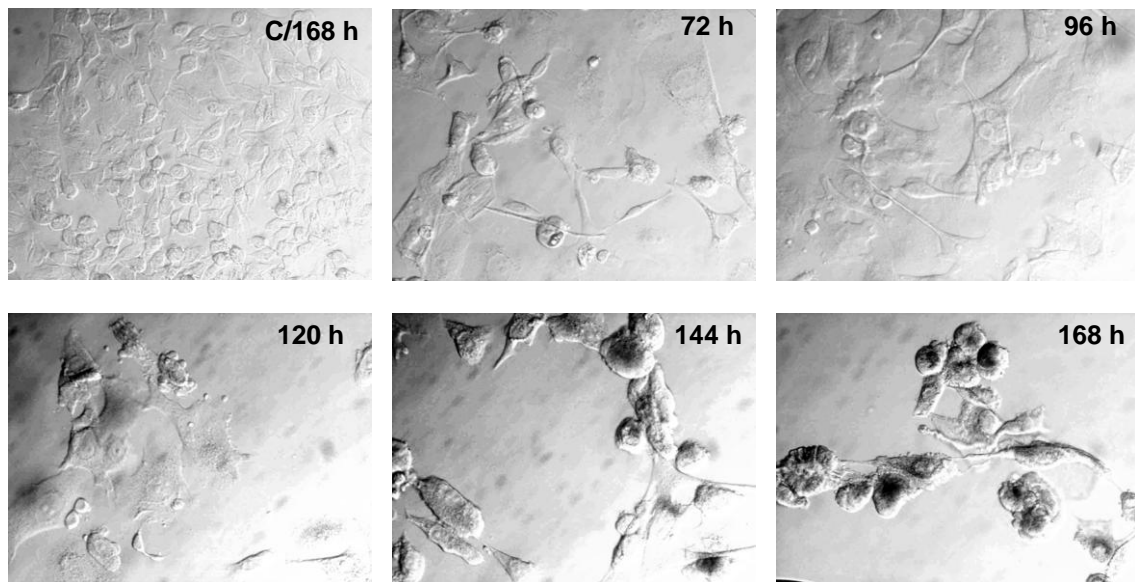
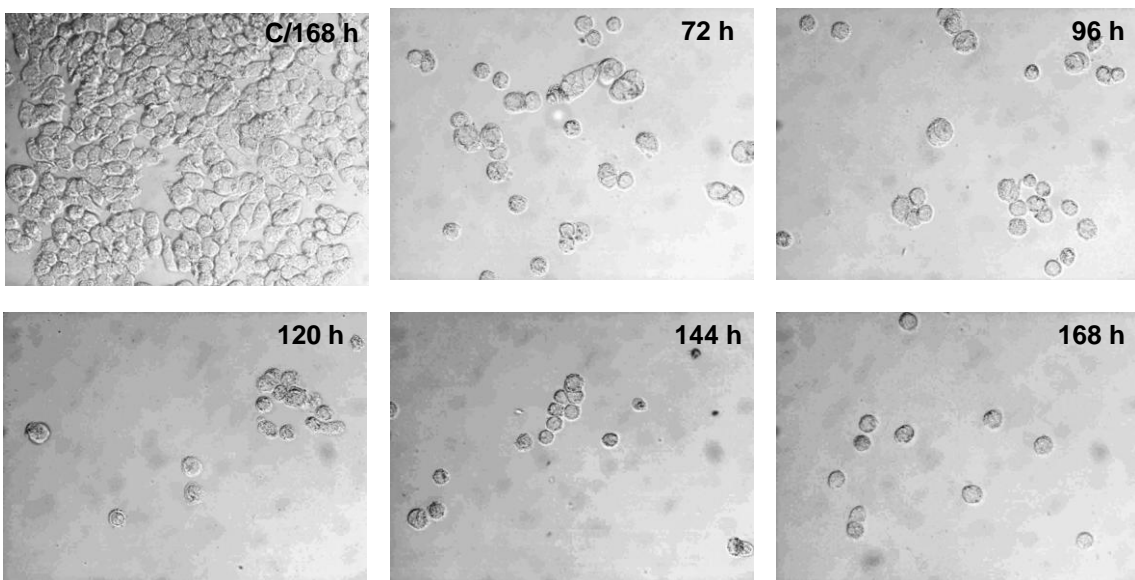


A)



B)



**A)****B)****C)**