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Modulation of Multidrug Efflux Pump Activity by New Hydantoin Derivatives on Colon Adenocarcinoma Cells without Inducing Apoptosis

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Abstract. *Background: Hydantoin derivatives are very promising candidates to improve the efficacy of anticancer chemotherapy. Previously, we demonstrated that eight hydantoin derivatives inhibited the P-glycoprotein (ABCB1) efflux pump of mouse T-lymphoma cells, as well as acting synergistically with the anticancer drug doxorubicin. Materials and Methods: The activity of the hydantoin derivatives were investigated in another MDR cancer model, namely Colo 205/S sensitive and Colo 320/R resistant colon carcinoma cells respectively, having normal or overexpressed ABCB1 systems. Results: Among the hydantoin derivatives evaluated, BS-1, MN-3 and JH-63 were the most effective ABCB1 transporter inhibitors at the concentration of 4 mg/l on the Colo 320/R cells, compared to the positive control, verapamil. Conclusion: The derivatives did not induce apoptosis of Colo 320/R resistant colon carcinoma cells, indicating that these hydantoin compounds are potent efflux pump inhibitors (EPI) without affecting the signalling pathways that regulate apoptosis.*

Hydantoin derivatives possess a variety of biochemical and pharmacological properties. Although hydantoins have been in use for a long time, the anticancer activity of these derivatives has received scant attention in the last decades (1). It has been shown that some 1,5-disubstituted hydantoins inhibit epidermal growth factor receptor (EGFR) autophosphorylation and proliferation of human A431 cells that overexpress EGFR (2, 3). Natural hydantoin compounds are gaining attention as well: phenyl-methylene hydantoins (PMH), guanidine alkaloids derived from Red Sea sponges, have the ability to increase cell-cell adhesion in prostate cancer cells and reduce invasion, furthermore they have potent *in vitro* and *in vivo* anti-growth and anti-invasive properties against PC-3M prostate cancer cells (4, 5). In colon adenocarcinoma cells, 5,5-diphenyl-2-thiohydantoin-N10 (DPTH-N10) inhibited proliferation, but did not cause the death of Colo 205 cells (6). Recent studies have shown that some diversely substituted diazaspiro hydantoins had an antiproliferative effect against MCF-7 breast carcinoma, HepG-2 hepatocellular carcinoma, HeLa cervix carcinoma and HT-29 colon carcinoma cell lines (7).

Previously, we evaluated thirty hydantoin compounds for modulation of the activity of the transporter P-glycoprotein (after the new nomenclature ATP-binding cassette, subfamily B, member 1 transporter or ABCB1) of mouse T-lymphoma cells (8). Among the selected derivatives, some significantly increased the retention of rhodamine 123. Compounds AD-26, AD-29, RW-13, BS-1, MN-3, RW-15b and KF-2 showed synergistic effect with doxorubicin on mouse lymphoma cells. These results indicated the role of chemical modifications within the hydantoin ring for its potential inhibition of the ABCB1 transporter. The most active structures contained aromatic substituents as well as some tertiary amine fragments (8).

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In this current study, the ABCB1 efflux pump modulating effect of the previously selected hydantoin derivatives was investigated in Colo 205/S sensitive colon carcinoma cells with normal ABCB1 expression pattern and Colo 320/R multidrug resistant (MDR) colon carcinoma cells having an overexpressed ABCB1 system. The apoptosis inducing effect of these derivatives was also examined.

Materials and Methods

Compounds. Eight hydantoin derivatives (SZ-7, BS-1, JH-63, MN-3, RW-15b, AD-26, RW-13, AD-29) were tested, kindly provided by Dr. Jadwiga Handzlik and Prof. Dr. Katarzyna Kiec-Kononowicz, Cracow, Poland. The compounds were dissolved in DMSO.

Cell lines. The human colon adenocarcinoma cell lines (Colo 205 doxorubicin sensitive parent and Colo 320/MDR-LRP resistant to anticancer agents overexpressing ABCB1(MDR1)-LRP), ATCC-CCL-220.1 (Colo 320) and CCL-222 (Colo 205), were purchased from LGC Promochem, Teddington, England.

The cells were cultured in RPMI 1640 medium supplemented with 10% heat-inactivated fetal bovine serum, 2 mM L-glutamine, 1 mM Na-pyruvate and 100 mM Hepes. The cells were incubated in a humidified atmosphere (5% CO_2, 95% air) at 37˚C. The semi-adherent cancer cells were detached with 0.25% trypsin and 0.02% EDTA for 5 min at 37˚C.

Assay for antiproliferative and cytotoxic effect. The effects of increasing concentrations of the drugs alone on cell growth were tested in 96-well flat-bottomed microtitre plates. The compounds were diluted in a volume of 100 μl medium. Then, 6×10^3 (for antiproliferative assay) or 2×10^4 cells (for cytotoxic assay) in 50 μl of medium, respectively, were added to each well, with the exception of the medium control wells. The culture plates were further incubated at 37˚C for 24 and 72 h, respectively; at the end of the incubation period, 15 μl of MTT (thiazolyl blue tetrazolium bromide, Sigma-Aldrich Chemie GmbH, Steinheim) solution (from a 5 mg/ml stock) was added to each well. After incubation at 37˚C for 4 h, 100 μl of sodium dodecyl sulfate (SDS) (Sigma) solution (10% in 0.01 M HCl) was added to each well and the plates were further incubated at 37˚C overnight. Cell growth was determined by measuring the optical density (OD) at 550 nm (ref. 630 nm) with a Multiscan EX ELISA reader (Thermo Labsystems, Cheshire, WA, USA).
Inhibition of the cell growth was determined according to the formula:

$$IC_{50} = 100 - \left[\frac{OD\,sample - OD\,medium\,control}{OD\,cell\,control - OD\,medium\,control}\right] x100$$

Where IC_{50} is defined as the inhibitory dose that reduces the growth of the compound-exposed cells by 50%.

Flow cytometry assay. Functional analysis of the ABCB1 transporter can be accomplished using flow cytometric accumulation assays with fluorescent dyes (9); for this purpose the intracellular accumulation of the non-toxic fluorescent ABCB1 (P-glycoprotein) substrate rhodamine 123 by the Colo 320 cells with an overexpressed ABCB1 efflux system was studied. This assay has been fully described previously (10). Briefly, the cells were adjusted to a density of 2×10^6/ml, re-suspended in serum-free RPMI 1640 medium and distributed in 0.5 ml aliquots into Eppendorf centrifuge tubes. The test compounds (10 μl) were added at various concentrations and the samples were incubated for 10 min at room temperature. Next, 10 μl (5.2 mM final concentration) of rhodamine 123 was added to the samples and the cells were incubated for a further 20 min at 37˚C, washed twice and re-suspended in 0.5 ml phosphate-buffered saline (PBS) for analysis. The fluorescence uptake of the cell population was measured with a FACStar Plus flow cytometer (Beckton, Dickinson and Company, Franklin Lakes, NJ, USA). Verapamil was used as a positive control in the rhodamine 123 exclusion experiments. The percentage mean fluorescence intensity was calculated for the treated MDR Colo 320 and sensitive Colo 205 cells as compared to untreated cells. A fluorescence activity ratio (FAR) was calculated *via* the following equation, on the basis of the measured fluorescence values.

$$FAR = \frac{\text{Colo320 treated / Colo320 control}}{\text{Colo205 treated / Colo205 control}}$$

The results obtained from a representative flow cytometry experiment in which 10,000 individual cells of the population were evaluated for the quantity of rhodamine 123 retained with the aid of the Beckton Dickinson FACStar flow cytometer, were first presented as histograms and the data was converted to FAR units that define fluorescence intensity, standard deviation, peak channel in the total- and in the gated- populations.

Assay for apoptosis induction. The assay was carried out using an Annexin V-FITC Apoptosis Detection Kit Cat. No. PF 032 from Calbiochem (EMD Biosciences, Inc. La Jolla, CA) according to the manufacturer's instructions.

The concentration of the cell suspension was adjusted to approximately 1×10^6 cells/ml. The cell suspension was distributed into 0.5 ml aliquots (5×10^5 cells) into a 24-well microplate and incubated overnight, at 37˚C, 5% CO_2. On the following day the medium was removed and fresh medium was added to the cells. The cells were incubated in the presence of the hydantoin derivatives for 3 hours at 37˚C; the concentration for the apoptosis induction (4 or 40 mg/l) were selected based on previous results (8).12H-benzo[α]phenothiazine was used as positive control at a final concentration of 50 μg/ml. The culture medium was removed, the cells were washed with PBS and fresh medium was added to the cells. The 24-well plates were incubated overnight at 37˚C, 5% CO_2.

After the incubation the supernatant was collected in a microfuge tube and 200 μl of 0.25 trypsin (Trypsin-Versen) was added to the wells until the cells detached from the surfaces of the wells. After the addition of culture medium, the cells were centrifuged at 2000 × g for 2 min at room temperature, the supernatant was removed and the cells were re-suspended in fresh serum-free medium.

After this procedure, the apoptosis assay was carried out according to the rapid protocol of the kit. The other conditions including AnnexinV-FITC and propidium iodide controls were applied as described elsewhere (11). The fluorescence was analysed immediately using a Becton Dickinson FACStar flow cytometer.

Results

As shown in Table I, after determination of the IC_{50} values, the hydantoin derivatives had more potent anti-proliferative

Table I. *Antiproliferative and cytotoxic effects of hydantoin derivatives on Colo 205/S and Colo 320/R colon adenocarcinoma cells.*

	Antiproliferative effect µg/ml		Cytotoxic effect µg/ml	
	Colo 205	Colo 320	Colo 205	Colo 320
SZ-7	14.9	8.8	28.3	33.6
BS-1	22.4	21.2	44.7	67.2
JH-63	36.6	22.9	39.9	84.8
MN-3	20.9	13.5	31.5	36.5
RW-15b	32.4	17.3	43.9	42.5
AD-26	17.3	8.3	45.9	35.8
RW-13	26.3	11.2	50.4	48.3
AD-29	23.9	14.5	52.5	31.6
DMSO	8.4%	9.7%	3.1%	0.8%

Table II. *Effect of hydantoin derivatives on rhodamine 123 retention by Colo 320/R colon adenocarcinoma cells.*

Samples	µg/ml	FSC	SSC	FL-1	FAR
Colo 205	-	456.93	255.18	1061.97	-
Colo 205	-	451.93	258.98	1051.21	-
Colo 320	-	462.79	273.89	98.83	
Colo 320	-	445.59	274.50	61.77	-
Colo 320 (mean)	80.3				
Verapamil	10	456.49	267.55	298.20	3.71
SZ-7	4	451.37	261.31	246.40	3.07
SZ-7	40	433.56	286.43	565.24	7.04
BS-1	4	436.49	267.53	1073.13	13.36
BS-1	40	418.93	265.36	1079.71	13.44
JH-63	4	441.80	273.48	850.05	10.59
JH-63	40	431.41	272.49	792.07	9.86
MN-3	4	433.35	264.46	676.06	8.41
MN-3	40	425.09	257.94	1011.00	12.59
RW-15b	4	434.72	273.80	79.41	0.98
RW-15b	40	427.51	271.01	133.30	1.66
AD-26	4	435.91	270.97	239.68	2.98
AD-26	40	431.00	269.75	709.54	8.83
RW-13	4	447.03	278.66	316.33	3.93
RW-13	40	438.97	269.12	562.99	7.01
AD-29	4	448.88	282.65	145.85	1.81
AD-29	40	443.51	269.72	406.22	5.06
DMSO	4%	433.16	270.89	65.94	0.82

FSC: Forward scatter count of cells in the samples (cell size ratio); SSC: side scatter count of cells in the samples; FL-1: mean fluorescence intensity of the cells. FAR: Fluorescence activity ratio.

effect on the MDR Colo 320 cells than on the sensitive Colo 205 cells. The compounds AD-26 and AD-29 were more cytotoxic on the MDR Colo 320 cells compared to the sensitive Colo 205 cells.

Based on the flow cytometric evaluation, all of the compounds inhibited the activity of the ABCB1 transporter

Table III. *Apoptosis inducing activity of selected hydantoin derivatives, after 3 hours of incubation on Colo 320/R colon adenocarcinoma cells. A+: Annexin V-FITC staining, A–: without Annexin V-FITC, I+: propidium iodide staining, I–: without propidium iodide*

		Gated events %		
	Conc. µg/ml	Early apoptosis %	Late apoptosis and necrosis %	Cell death %
Control A–I–		0	0	0.40
Control A–I+		0	0	3.24
Control A+I–		0.52	0	0.19
Control A+I+		5.36	1.31	0.34
DMSO	1%	8.02	1.64	0.40
12H-benzo[α]phenothiazine	50	18.57	18.25	7.70
SZ-7	4	1.56	0.67	1.38
BS-1	4	4.39	1.86	0.37
JH-63	4	0.42	0.26	1.65
MN-3	4	0.44	0.12	0.72
RW-15b	40	1.83	0.77	0.73
AD-26	40	2.24	1.04	0.85
RW-13	40	1.36	0.81	0.57
AD-29	40	0.70	0.36	0.52
Control A–I–	0	0	0.02	

Note. Compounds that had maximum activity on the efflux pump (Table II) at concentration of 4 µg/ml tested only at this concentration for apoptosis.

(Table II). The derivatives BS-1, MN-3 and JH-63 were the most effective inhibitors at the concentration of 4 mg/l on the Colo 320/R cells compared to the positive control verapamil. As demonstrated in Table III, the selected hydantoin derivatives did not induce apoptosis in the Colo 320/R cells compared to the positive control 12H-benzo[α]phenothiazine. The proportion of early and late apoptotic cells was not changed after incubation with the hydantoin derivatives, indicating that these derivatives did not posses apoptosis inducing activity.

Discussion

Hydantoin analogs have been reported as potent anti-tumour agents (1-8), furthermore the apoptosis inducing activity of some hydantoin derivatives has been demonstrated (6, 12, 13). Previously, a series of novel 5-arylidene-2-arylaminothiazol-4(5H)-ones and 2-aryl(benzyl)amino-1H-imidazol-4(5H)-ones were evaluated for anticancer properties and the majority of these compounds showed significant antitumour cytotoxic effects at the micromolar and submicromolar level (14). Increasing evidence suggests that some hydantoin derivatives can induce apoptosis in various tumour cell lines (6, 12, 13). Previously, the selected eight derivatives were shown not to induce apoptosis of L5178Y

mouse T-cell lymphoma cells over-expressing the ABCB1 system compared to the positive control 12H-benzo[α]phenothiazine (unpublished results), even though the hydantoin derivatives inhibited the ABCB1 multidrug transporter system of the mouse T-lymphoma cells (8).These derivatives increased the intracellular accumulation of rhodamine 123 in the MDR Colo 320 cells, they were potent ABCB1 inhibitors. Furthermore, the studied hydantoin analogs did not induce the apoptosis of the Colo 320 cells after 180 minutes of incubation, indicating that these hydantoin compounds are potent efflux pump inhibitors (EPI) without affecting the signalling pathways that regulate apoptosis. Taking into consideration that hydantoin analogs SZ-7, BS-1, JH-63, MN-3, RW-15b, AD-26, RW-13 and AD-29 can inhibit the ABCB1 transporter, the mode of action of these derivatives should be clarified by further studies.

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