Biological Activity of Hydantoin Derivatives on P-Glycoprotein (ABCB1) of Mouse Lymphoma Cells

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Abstract. *Background: Hydantoin derivatives possess a variety of biochemical and pharmacological properties. Although hydantoin compounds are studied extensively, there are not many studies that investigate their anticancer properties. Materials and Methods: Thirty hydantoin compounds were evaluated for their efflux modulating effects in cancer cells using a rhodamine 123 accumulation assay and real-time fluorometry based on the intracellular accumulation of ethidium bromide. Results: The 30 derivatives were screened by real-time fluorometry for rhodamine 123 accumulation. Among the selected derivatives, compounds SZ-7, LL-9, BS-1, MN-3, P3, RW-15b, AD-26, RW-13, AD-29 and KF-2 significantly increased the retention of rhodamine 123. Compounds AD-26, AD-29, RW-13, KF-2, BS-1, MN-3, RW-15b and JH-63 showed synergistic effect with doxorubicin on mouse lymphoma cells. Furthermore, compound SZ-7 had indifferent effect with doxorubicin. Conclusion: 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.*

Multidrug resistance plays a crucial role in the failure of treatment of infectious diseases and cancer (1). Micro-organisms have developed various ways to resist the toxic effects of antibiotics and other drugs (2, 3). One such mechanism involves the cytoplasmic membrane-localised transport system, which also takes part in normal physiological functions (4). In cancer patients, the main reason for treatment failure is the presence of resistance to the chemotherapy. The major mechanism of multidrug resistance is the elevated expression of ATP-dependent drug-efflux pumps, which reduce the accumulation of the anticancer agents (5). In many tumour cell lines, multidrug resistance is often associated with the overexpression of ABC drug-transporter P-glycoprotein (Pgp), also known as ABCB1 (6).

Hydantoin derivatives possess a variety of biochemical and pharmacological properties and are used to treat many human diseases. They possess good anticonvulsant properties and depending on the nature of substitution on the hydantoin ring, a wide range of other pharmacological properties, including fungicidal, herbicidal, antitumour, anti-inflammatory, anti-HIV, hypolipidemic, antiarrhythmic and antihypertensive activities (7, 8). Although hydantoin compounds are studied extensively, there are not many studies about their anticancer properties. Recently, the cytotoxic activity of spirohydantoin derivatives was tested in ovarian and breast cancer cells (9). It has been shown that a spirohydantoin derivative induces growth inhibition and apoptosis in leukemic cells (10). Former studies demonstrated that 5-arylidene-2-thiohydantoins have *in vitro* antimycobacterial activity (11).

Materials and Methods

Compounds. Thirty hydantoin derivatives (SZ-2, SZ-7, LL-9, BS-1, JH-63, MN-3, TD-7k, GG-5k, P3, P7, P10, P11, RW-15b, AD-26, RW-13, AD-29, KF-2, PDPH-3, Mor-1, KK-XV, Thioam-1, JHF-1, JHC-2, JHP-1, Fur-2, GL-1, GL-7, GL-14, GL-16, GL-18) were tested, kindly provided by Dr. Jadwiga Handzlik and Prof. Dr. Katarzyna Kieć-Kononowicz, Cracow, Poland). The compounds were dissolved in DMSO.

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Other chemicals used in the study were: doxorubicin hydrochloride (Wako Pure Chem. Ind., Osaka, Japan), rhodamine 123 (R123; Sigma, St. Louis, MO, USA), verapamil (EGIS Hungarian Pharmaceutical Company, Budapest, Hungary), 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT; Sigma, St Louis, MO, USA), sodium dodecylsulfate (SDS; Sigma), dimethyl sulfoxide (DMSO; Sigma) and ethidium bromide (EB; Sigma, St. Louis, MO, USA). Stock solutions of R123 and verapamil were prepared in water. All solutions were prepared on day of assay.

Cell lines. L5178Y mouse T-cell lymphoma cells (ECACC cat. no. 87111908; U.S. FDA, Silver Spring, MD, USA) were transfected with pHa MDR1/A retrovirus, as described previously (12, 13). The *ABCB1*-expressing cell line was selected by culturing the infected cells with 60 ng/ml of colchicine (Sigma-Aldrich Chemie GmbH, Steinheim, Germany) to maintain the MDR phenotype. L5178 mouse T-cell lymphoma cells (parental, PAR) and the human *ABCB1*-gene transfected sub-line (MDR) were cultured in McCoy's 5A medium supplemented with 10% heat-inactivated horse serum, L-glutamine (Lonza BioWhittaker, Verviers, Belgium) and antibiotics (penicillin, streptomycin) at 37˚C and in a 5% CO_2 atmosphere.

Medium. McCoy's 5A medium (Lonza BioWhittaker) supplemented with 10% heat-inactivated horse serum (Sigma-Aldrich Química SA, Madrid, Spain), L-glutamine (Lonza BioWhittaker) and antibiotics.

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) 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. The cell growth was determined by measuring the optical density (OD) at 550 nm (ref. 630 nm) with Multiscan EX ELISA reader (Thermo Labsystems, Cheshire, WA, USA). Inhibition of the cell growth was determined according to the formula:

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

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

Real-time fluorimetry was performed using a previously developed semi-automated method (14, 15). Briefly, the cells were adjusted to a density of 2×10^6 cells/ml, centrifuged at 2000 × *g* for 2 min and re-suspended in phosphate-buffered saline (PBS) pH 7.4. The cell suspension was distributed in 90 μl aliquots into 0.2 ml tubes. The tested compounds were individually added at different concentrations (4 and 40 mg/l) in 5 μl volumes of their stock solutions and the samples incubated for 10 min at 25˚C. Verapamil was used as a positive control. After this incubation, 5 μl (1 μg/ml final concentration) of EB (20 μg/ml stock solution) were added to the samples and the tubes were placed into a Rotor-Gene 3000™ thermocycler with real-time analysis software (Corbett Research, Sidney, Australia) and the fluorescence monitored on a real-time basis. The results were evaluated by Rotor-Gene Analysis Software 6.1 (Build 93) provided by Corbett Research.

Flow cytometry assay for evaluation of a compound on the retention of rhodamine 123 by MDR in tumour cells. This assay has been fully described previously (16). Briefly, the cells were adjusted to a density of 2×10^6/ml, re-suspended in serum-free McCoy's 5A medium and distributed in 0.5 ml aliquots into Eppendorf centrifuge tubes. 10 μl of test compounds were added at various concentrations (4 and 40 mg/l), 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 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 and parental cell lines as compared to untreated cells. A fluorescence activity ratio (FAR) was calculated *via* the following equation, on the basis of the measured fluorescence values:

$$FAR = \frac{MDR\,treated\,/\,MDR\,control}{parental\,treated\,/\,parental\,control}$$

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

Checkerboard microplate method. The checkerboard microplate method was applied to study the drug interactions between resistance modifiers and anticancer drugs on cancer cells, as previously described (17). The interaction of the anticancer drug doxorubicin and the resistance modifiers hydantoins was studied in combination on MDR mouse T-lymphoma cells. The serial dilutions of doxorubicin (A) were made in 100 μl, horizontally, furthermore the dilutions of hydantoin derivatives (B) vertically, in 50 μl volume in the microtiter plate (the dilutions were prepared separately in Eppendorf tubes, using tissue culture medium). An aliquot of 50 μl of the cell suspension in tissue culture medium containing 2×10^4 cells was distributed to each well and the plates incubated for 48 h at 37˚C in a CO_2 incubator. The cell growth rate was determined after MTT staining and the intensity of the blue colour was measured with micro ELISA reader. Drug interactions were evaluated according to the following protocol, where ID_{50} is defined as the inhibitory dose that reduces the growth of the compound-exposed cells by 50%.

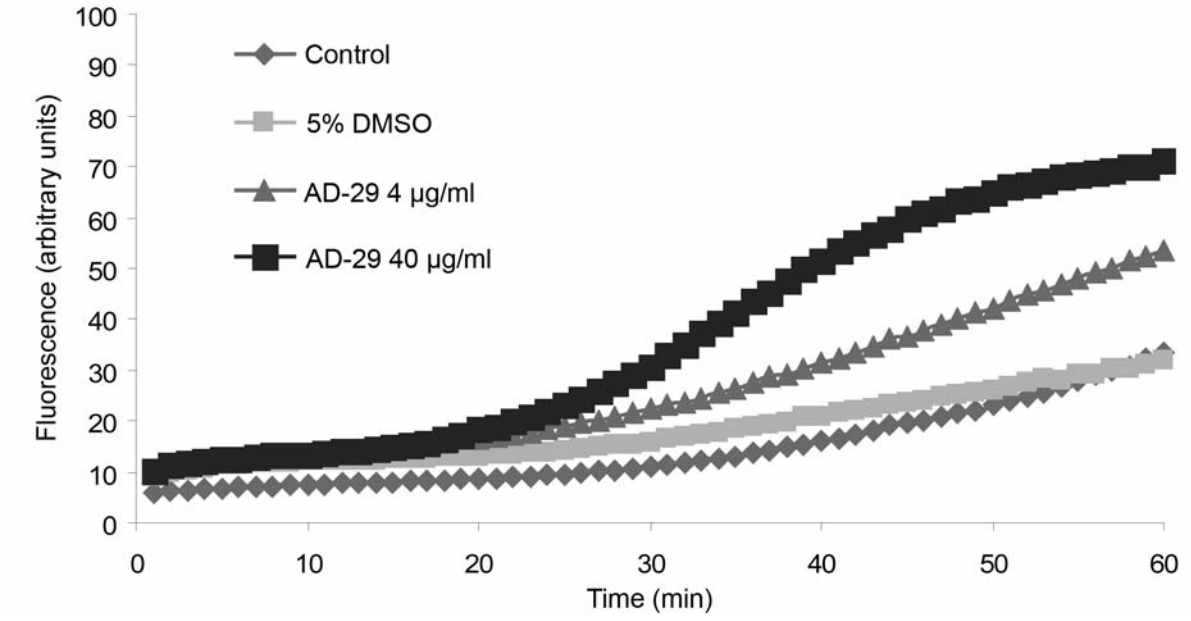

Figure 1. *Accumulation of EB (1 mg/l) by human MDR1 (ABCB1) gene-transfected mouse lymphoma cells in the presence of 4 and 40 µg/ml of AD-29.*

ID_{50}=50% inhibitory dose.
FIC=fractional inhibitory concentration
FIX=fractional inhibitory index
$FIC_A=ID_{50A \text{ in combination}}/ID_{50A \text{ alone}}$
$FIC_B=ID_{50B \text{ in combination}}/ID_{50B \text{ alone}}$

$FIX=FIC_A+FIC_B$

FIX=0.51-1	Additive effect
FIX<0.5	Synergism
1<FIX<2	Indifferent effect
FIX>2	Antagonism

Results

After determination of ID_{50} values of the hydantoin derivatives in antiproliferative and cytotoxicity assays, all of the compounds at varying concentrations were evaluated for effects on the real-time accumulation of EB by human *MDR1* (*ABCB1*) gene transfected mouse lymphoma cells. Using this method (15, 18), a large number of compounds can be screened and selected for further studies. Because of the extremely large number of graphs that result from the evaluation of each compound at two concentrations, Figure 1 serves as an example of the type of data that results from the assay and which afforded the selection of compounds that presented with significant activity. The following compounds at concentrations that were not toxic (Table I) were selected for rhodamine 123 accumulation studies: SZ-2, SZ-7, LL-9, BS-1, JH-63, MN-3, GG-5k, P3, RW-15b, AD-26, RW-13, AD-29, KF-2, PDPH-3 and KK-XV. The inhibition of ABCB1 transporter is evident when FAR >1. As demonstrated by Table II, the compounds SZ-7, LL-9, BS-1, MN-3, P3, RW-15b, AD-26, RW-13, AD-29 and KF-2, significantly increase retention of rhodamine 123. Compound BS-1 was the most potent inhibitor in as much as very low concentrations produced the greatest retention of the fluorescent substrate.

From the most effective compounds presenting high FAR values, nine compounds were chosen to determine their interaction with the anticancer drug doxorubicin. Compounds AD-26, AD-29, RW–13, KF-2, BS-1, MN-3, RW-15b, JH-63 and SZ-7 were combined with doxorubicin using *MDR1* (*ABCB1*) gene transfected mouse lymphoma cells. Compounds AD-26, AD-29, RW–13, KF-2, BS-1, MN-3, RW-15b and JH-63 showed synergistic effect with doxorubicin on mouse lymphoma cells; compound SZ-7 had an indifferent effect with doxorubicin (Table III).

Discussion

Various aspects of the biochemical and pharmacological properties of hydantoin derivatives have been studied (7, 8, 11). However, the anticancer activity of these compounds has received little attention. Among those few studies, two novel spirohydanto in compounds, 8-(3,4-difluorobenzyl)-1'-(pent-4-enyl)-8-azaspiro[bicyclo[3.2.1] octane-3,4'-imidazolidine]-2',5'-dione (DFH) and 8-(3,4-dichlorobenzyl)-1'-(pent-4-enyl)-

Table I. *Antiproliferative and cytotoxic effects of hydantoin derivatives on multidrug resistant (MDR) human MDR1 (ABCB1) gene-transfected mouse lymphoma cells. The non-toxic compounds used for further studies are in grey.*

	ID_{50} (μg/ml)		
Hydantoin derivative	Cytotoxic effect	Antiproliferative effect	Precipitation (crystal type)
SZ-2	48.33	25.61	Amorphous
SZ-7	3.84	4.54	Amorphous
LL-9	54.12	54.19	-
BS-1	60.07	15.21	Needle-like
JH-63	28.09	15.24	Amorphous
MN-3	16.97	10.55	Amorphous
TD-7k	237.94	107.73	-
GG-5k	151.23	72.66	-
P3	28.09	19.33	-
P7	18.15	87.13	Oval
P10	286.50	112.09	Pine-leaf-like
P11	115.71	118.77	-
RW-15b	37.97	53.42	-
AD-26	31.33	33.19	Amorphous
RW-13	19.12	11.95	Amorphous
AD-29	19.48	39.42	Needle-like
KF-2	82.31	71.99	-
PDPH-3	99.36	90.10	-
Mor-1	240.24	76.80	-
KK-XV	245.56	78.67	-
Thioam-1	3.92	5.272	Amorphous
JHF-1	225.71	111.59	-
JHC-2	143.80	84.78	-
JHP-1*	N.D.	N.D.	-
Fur-2	426.01	94.64	-
GL-1	187.74	92.48	-
GL-7	313.42	47.34	-
GL-14	276.72	78.78	-
GL-16	208.35	100.99	-
GL-18	443.95	86.69	-
DMSO	2.39%	1.09%	-

*Complex formation with MTT in McCoy's 5A medium, ID_{50} non-determinable.

Table II. *Effect of compounds SZ-2, SZ-7, LL-9, BS-1, JH-63, MN-3, TD-7k, GG-5k and P3 on rhodamine 123 retention by human MDR1 (ABCB1) gene-transfected mouse lymphoma cells. The most promising derivatives are in grey.*

Sample	μg/ml	FAR
Verapamil	10	13.19
SZ-7	0.4	1.68
SZ-7	1	7.45
SZ-7	4	44.12
SZ-7	40	55.98
BS-1	0.4	46.71
BS-1	1	74.78
BS-1	4	77.68
BS-1	40	174.35
JH-63	0.4	9.60
JH-63	1	23.11
JH-63	4	57.05
JH-63	40	107.62
MN-3	0.4	4.64
MN-3	1	17.65
MN-3	4	59.95
MN-3	40	196.42
AD-26	0.4	1.33
AD-26	1	4.05
AD-26	4	28.12
AD-26	40	144.86
AD-29	0.4	2.01
AD-29	1	3.05
AD-29	4	8.65
AD-29	40	180.77
KF-2	4	7.63
KF-2	40	57.38
RW-15b	4	1.68
RW-15b	40	32.69
RW-13	4	8.56
RW-13	40	25.21
LL-9	4	2.00
LL-9	40	23.42
P-3	4	1.16
P-3	40	12.13
Thioam-1	4	1.28
Thioam-1	40	9.49
DMSO	4%	0.77

FAR: Fluorescence activity ratio.

8-azaspiro[bicyclo[3.2.1]octane-3,4'-imidazolidine]-2',5'-dione (DCH) produce dose- and time-dependent cytotoxic effects on human leukemic, K562, Reh, CEM and 8E5 cell lines and induce apoptosis. This has previously been further confirmed and quantified both by fluorescence-activated cell sorting (FACS) and confocal microscopy following annexin V-FITC/propidium iodide staining (10). Furthermore, naturally-occurring or synthetic phenyl-methylene hydantoin (PMH) and *S*-ethyl PMH (S-PMH) reportedly augment cell–cell adherence and reduce invasion and growth of prostate cancer cells (19).

The mouse lymphoma cell line transfected with the human *MDR1* gene that codes for the ABC transporter Pgp that is responsible for multidrug resistance of this cell line to cytotoxic agents is a very useful model for evaluating agents that inhibit the activity of the transporter. Among the hydantoin derivatives evaluated for potential inhibition of the P-gp1 transporter, BS-1, MN-3 and JH-63 at the lowest concentration of 4 mg/l, were the most effective inhibitors. All of the most active structures contained aromatic substituents as well as some tertiary amine fragments.

Based on the results obtained, compounds AD-26, AD-29, RW-13, KF-2, BS-1, MN-3, RW-15b and JH-63 should be further studied for *in vitro* capability of reversing or reducing

Table III. *Interaction between selected hydantoin derivatives and doxorubicin on MDR1 (ABCB1)-gene transfected mouse lymphoma cells.*

Compound	FIX values	Interaction
AD-26	0.032	Synergism
AD-29	0.076	Synergism
RW-13	0.16	Synergism
KF-2	0.18	Synergism
BS-1	0.217	Synergism
MN-3	0.311	Synergism
RW-15b	0.24	Synergism
JH-63	0.493	Synergism
SZ-7	1.252	Indifferent

resistance of the mouse lymphoma *MDR1 (ABCB1)* gene transfected cell to cytotoxic agents to which they are initially resistant. Active compounds should then be examined in the mouse model for ability to shrink solid transplanted tumours in order to assess their suitability to progress to clinical trial.

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