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In vitro cytotoxic activity evaluation of phenytoin derivatives against human leukemia cells

Katarzyna Śladowska[1]*, Jadwiga Handzlik[2], Katarzyna Kieć-Kononowicz[2] & Lidia Mazur[1]*

[1]Department of Experimental Hematology, Jagiellonian University in Krakow, Gronostajowa 9, Krakow, Poland

[2]Department of Technology and Biotechnology of Drugs, Medical College, Jagiellonian University in Krakow, Medyczna 9, Krakow, Poland

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Hydantoin derivatives, including phenytoin (5,5-diphenylhydantoin), have recently gained attention as they possess a variety of important biochemical and pharmacological properties. Nevertheless, available information on anticancer activity of hydantoin derivatives is still scarce. Here, we evaluated possible antileukemic potential of four phenytoin analogs, namely: methyl 2-(2,4-dioxo-5,5-diphenylimidazolidin-3-yl)propanoate (1), methyl 2-(1-(3-bromopropyl)-2,4-dioxo-5,5-diphenylimidazolidin-3-yl)propanoate (2), 1-(3-bromopropyl)-3-methyl-5,5-diphenylimidazolidine-2,4-dione (3) and 1-(3-bromobutyl)-3-methyl-5,5-diphenylimidazolidine-2,4-dione (4). The experiments were performed on human acute histiocytic lymphoma U937 cells and human promyelocytic leukemia HL-60 cells. The present study was conducted using spectrophotometric 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl tetrazolium bromide (MTT) assay and the electronic Beckman-Coulter method. We observed temporary changes in the leukemia cell viability, volume and count. The effects of the four 5,5-diphenylhydantoin derivatives on U937 and HL-60 cells depended on the agent tested and its concentration, the time intervals after the compound application, and the leukemia cell line used. HL-60 cells were more sensitive than U937 cells to the action of the phenytoin analogs (1-4). The antileukemic activities of the three bromoalkyl diphenylhydantoin derivatives (2, 3, and 4) were stronger than that of the compound 1 [methyl 2-(2,4-dioxo-5,5-diphenylimidazolidin-3-yl) propanoate]**,** with no bromoalkyl substituent. The structural modifications of 5,5-diphenylhydantoin are responsible for such varied antileukemic potential of its four derivatives.

Keywords: Antileukemic activity, Cancer, Cell viability, 5,5-Diphenylhydantoin derivatives, Human histiocytic lymphoma, Human promyelocytic leukemia, Hydantoin

Compounds possessing hydantoin fragment have been a source of interest in search for new drugs because of their cyclic analogy to natural amino acids, which can be responsible for various pharmacological activities. Phenytoin (5,5-diphenylhydantoin) is one of the most important hydantoin pharmaceutical drugs (Fig. 1), which is used in therapy of epilepsy and cardiac arrhythmias for most than seven decades[1]. Both, antiarrhythmic and anticonvulsant actions of phenytoin are due to its ion channel blocking properties, mainly, sodium channels. Nevertheless, some toxic and undesirable effects, including: carcinogenic properties in animals[2] and an increase in malformations in infants[3] have been notified for phenytoin to limit its therapeutic usage. Phenytoin is also known as a hepatotoxicant used to develop new models of toxicity prediction[4,5].

Taking into account the various biological activities of phenytoin, its structure has been an interesting starting point in search for new potential drugs. Previous studies focused on chemical modifications of 5,5-diphenylhydantoin, gave a number of its derivatives displaying significant biological activity (Fig. 1) as hypotensive, anti-arrhythmic[6-8] and/or G-protein-coupled receptors agents (GPCRs)[9-11] as well as compounds able to inhibit multidrug resistance in cancer[12] or bacterial cells[13]. During 3-step synthesis route of new amine-alkyl derivatives of 5,5-diphenylhydantoin, a number of intermediates (Fig. 1) with alkyl or ester substituent at position 3 of hydantoin ring (R^1) and with alkylating moieties at position 1 (R^2), including alkylbromides[13] or oxiranes were obtained[7-9]. Such substitutions of both NH-groups at the hydantoin ring are known to decrease the anticonvulsant and

*Correspondence:
Phone: +48 12 6645237/30; Fax: +48 12 6645101
E-mail: lidia.mazur@uj.edu.pl (LM);
sladowska.katarzyna@gmail.com (KS)

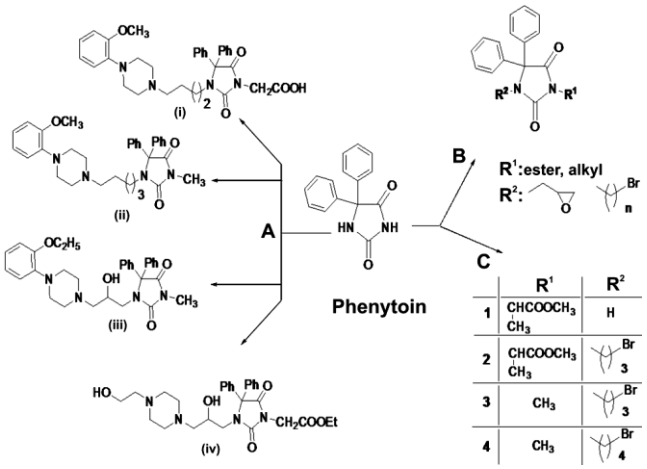

Fig. 1—Phenytoin and its derivatives. (A) Biologically active amine derivatives: (i) a modulator of bacterial multidrug resistance[13], (ii) a modulator of cancer multidrug resistance[12], (iii) an α_1-adrenoceptor blocker with antiarrhythmic properties[9], (iv) an antiarrhythmic agent[7]; (B) General structures of the phenytoin derivatives that were found as potential cytotoxic agents using toxicity simulation in silico (OSIRIS); and (C) The tested phenytoin derivatives 1, 2, 3 and 4.

antiarrhythmic action of phenytoin, related to its blocking sodium channels properties[6-8,11]. It is low probability that the alkylating hydantoins (1-4, Fig. 1) act on the mentioned GPCRs[9-11] or as bacterial multidrug resistance modulators because they do not contain either arylpiperazine[8-11] or amine-alkyl moieties in their structures[13]. Thus, the new compounds, being used only as alkylating intermediates in synthesis, have not been investigated in any experimental pharmacological test till now. Their structures were involved in toxicology training *in silico* to evaluate toxicity risk among various derivatives of hydantoin. The training was performed using OSIRIS bioinformatics tools (http://www.organic-chemistry.org/prog/peo/), and the obtained results indicated that the 3-(2-methyl) acetate derivative 1 and the three bromoalkyl derivatives of diphenylhydantoin, compounds 2-4 (Fig. 1), displayed high risk of mutagenic and tumorigenic action and/or moderate risk of irritant and reproductive effects. Although results of the OSIRIS simulation had only general and qualitative character, they suggested potential cytotoxic properties for the four structures (1-4) which could be available in various aspects of battle against cancer diseases. The results of *in silico* studies together with those of newest works, describing anticancer abilities of some hydantoin derivatives[12,14], suggested that the new group of alkylating phenytoin derivatives should be investigated experimentally on their cytotoxic activities. In the present investigation, the antileukemic activity of the compounds 1-4 mentioned above, were analyzed.

Materials and Methods

Cells

Human histiocytic lymphoma U937 cells and human promyelocytic leukemia HL-60 cells were obtained from American Type Culture Collection (Rockville, MD, USA). U937 and HL-60 cells were cultured in RPMI 1640 medium supplemented with 10% fetal calf serum (both from Gibco BRL Life Technologies, Warsaw, Poland), 2 mM L-glutamine and antibiotic antimycotic solution (AAS) (both from Sigma Aldrich, Poznań, Poland). AAS contained 20 units of penicillin, 20 µg streptomycin and 0.05 µg amphotericin B. Every 3rd day, the cells were passaged. U937 and HL-60 cells grew at 37°C in an atmosphere of 5% CO_2 in air (HERAcell incubator, KendroLab, Warsaw, Poland).

Chemicals

The four phenytoin derivatives, methyl 2-(2,4-dioxo-5,5-diphenylimidazolidin-3-yl)propanoate (1), methyl 2-(1-(3-bromopropyl)-2,4-dioxo-5,5-diphenyl-imidazolidin-3-yl)propanoate (2), 1-(3-bromopropyl)-3-methyl-5,5-diphenylimidazolidine-2,4-dione (3) and 1-(3-bromobutyl)-3-methyl-5,5-diphenylimidazolidine-2,4-dione (4) were examined (Fig. 1). Synthesis of the compounds, performed basing on earlier methods[8,9] described elsewhere[13]. Purity and identity of the compounds were confirmed by TLC, spectral methods (^{1}H-NMR and IR), elemental analysis and melting points measurement. The stability of the compounds in DMSO solution in ambient temperature was confirmed using TLC (toluene-acetone 40:3) and ^{1}H-NMR (Varian Mercury VX, 300 MHz PFG instrument, in DMSO, by using the solvent signal as an internal standard).

Cell treatment with phenytoin derivatives

After a dilution of the cell suspension to a density of 15×10^4 cells/mL medium, U937 and HL-60 cells were subjected to the exposure of four phenytoin derivatives. The cells were exposed to the action of these agents at the concentrations of 25, 50, 100 and 200 µM. All tested phenytoin derivatives (1-4) were dissolved in dimethyl sulfoxide (DMSO, Sigma Aldrich) and the solutions were freshly prepared

before their application. The control materials consisted of untreated U937 and HL-60 cells and those treated only with DMSO.

Analyses of leukemia cells exposed to phenytoin derivatives

The temporary changes occurring in U937 and HL-60 cells were observed at 24 and 48 h after their exposure to the phenytoin derivatives. At these two time intervals, the cell viability, volume and count were assessed. The spectrophotometric MTT (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl tetrazolium bromide) test and the electronic Beckman-Coulter method were used.

In viable, metabolically active cells, the tetrazolium ring of MTT is cleaved, yielding formazan crystals. Changes in the metabolic activity of cell populations result in a concomitant change in the amount of formazan formed[15-17]. According to the *in vitro* MTT assay, the IC_{50} values for the diphenylhydantoin derivatives were determined. The IC_{50} value represents the concentration of the tested compound required for 50% inhibition of the cell viability.

The Beckman-Coulter method of cell sizing and counting is based on the detection of an electrical pulse which results from the passage of each cell through an aperture. The amplitude of the produced electrical pulse depends on the cell volume. The number of pulses indicates the cell count[18].

***In vitro* spectrophotometric MTT assay**

MTT (Sigma-Aldrich) was dissolved in RPMI 1640 medium, at a concentration of 5 mg/mL, and filtered through a 0.2 μM filter. Subsequently, 100 μL of the yellow MTT solution was added to each well of a 24-well plate, containing 1 mL of the cell suspension. The cells were then incubated at 37°C with 5% CO_2. A blank solution was prepared according to the above procedure using complete medium without cells. After a 3 h incubation period, the resulting formazan crystals were dissolved with 1 mL of acidified isopropanol (0.05 N HCl in absolute isopropanol), and the absorbance of the obtained solution was measured at a wavelength of 570 nm using a Pharmacia Ultrospec III spectrophotometer (Pharmacia LKB Biotechnology).

IC_{50} value determination

Based on the obtained data using the *in vitro* MTT assay, the half maximal inhibitory concentration IC_{50} values for the phenytoin derivatives 1-4, were calculated separately, at 48 h after the human leukemia cells exposure to the action of these compounds. To determine the IC_{50} values, the concentration range of the 5,5-diphenylhydantoin analogs was from 25-200 μM.

Measurement of cell size and count

Samples of the leukemia cell suspension were taken from flasks and immediately diluted in ISOTON II (Beckman-Coulter filtered electrolyte solution based on 0.9% saline). After the dilution of the leukemia cell suspension in ISOTON II (1:9), individual cells were measured using a Z2 Coulter counter (Beckman-Coulter, Miami, FL, USA). The volume and count distribution of leukemia cells was obtained using a counter equipped with a 100 μM diameter orifice. The flow rate was 500 μL/12.5 s. The range for U937 and HL-60 cell measurement was determined as 268-7238 fL. The volume and count of U937 and HL-60 cells were analyzed at 688-7238 fL and 717-7238 fL, respectively. The instrument was calibrated using 10 μM diameter latex beads (Beckman-Coulter CC size standard). The mean cell volume and the cell count were calculated using Z2 AccuComp software (Beckman-Coulter, Miami, FL, USA).

Statistical evaluation

All experiments were repeated thrice with duplicate or triplicate determinations. The data are presented as the mean values ± standard deviation. Statistical analyses were performed using STATISTICA 10 (StatSoft, Poland). Data were analyzed by one-way analysis of variance (ANOVA) followed by Tukey's honestly significant differences (HSD) multiple range test. A difference with $P < 0.05$ was considered statistically significant.

Results

In the present study, the *in vitro* cytotoxic activities of the derivatives of 5,5-diphenylhydantoin (1-4) against U937 and HL-60 cells were assessed. Different patterns of temporary changes in the viability (Fig. 2A, Table 1), size (Figs. 2B and 3) and

Table 1—The half inhibitory concentration (IC_{50}) values determined at 48 h after the exposure of U937 and HL-60 cells to the action of the phenytoin derivatives 1, 2, 3 and 4.

Phenytoin derivative	Cell line	
	U937	HL-60
	IC_{50} ± SD [μM]	
1	>200	162.0 ± 7.8
2	50.0 ± 2.9	42.5 ± 2.5
3	71.0 ± 5.2	66.0 ± 9.7
4	49.0 ± 4.3	46.0 ± 1.9

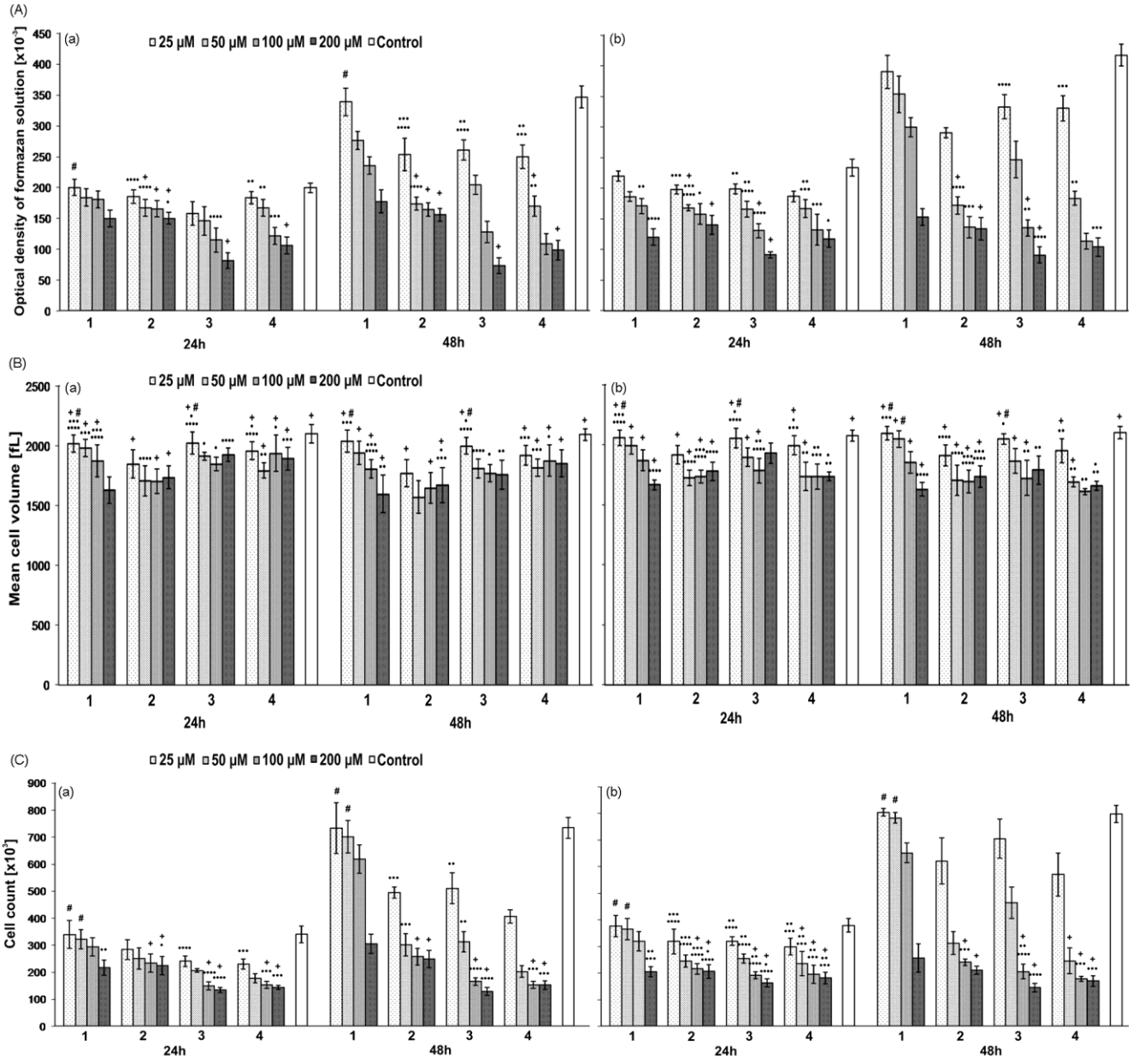

Fig. 2—Effects of the phenytoin derivatives 1, 2, 3 and 4 on the (A) viability; (B) mean volume; and (C) count of U937 (a) and HL-60 cells (b). [The extent of 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl tetrazolium bromide (MTT) conversion to formazan in the leukemia cells reflects their viability. Values not significantly different at P <0.05 according to the Tukey's multiple range test: •, ••, •••, •••• between the groups of leukemia cells treated with the phenytoin derivatives; # compared to control; + between the time points]

count (Fig. 2C) of the leukemia cells were observed at 24 and 48 h after their exposure to the action of the phenytoin derivatives. The U937 and HL-60 cell viability and count decreased after the phenytoin derivatives application. However, compound 1 affected the viability and count of both cell lines to a lesser degree than did the agents 2, 3 and 4 (Fig. 2 A and C). IC_{50} values, calculated 48 h after exposure of U937 and HL-60 cells to the action of the derivatives 2, 3, and 4, were distinctly lower than the IC_{50} value calculated for the compound 1 (Table 1). The mean cell volume of the leukemia cells decreased when the phenytoin derivatives were applied. The lowest value of the mean cell volume were observed 48 h after

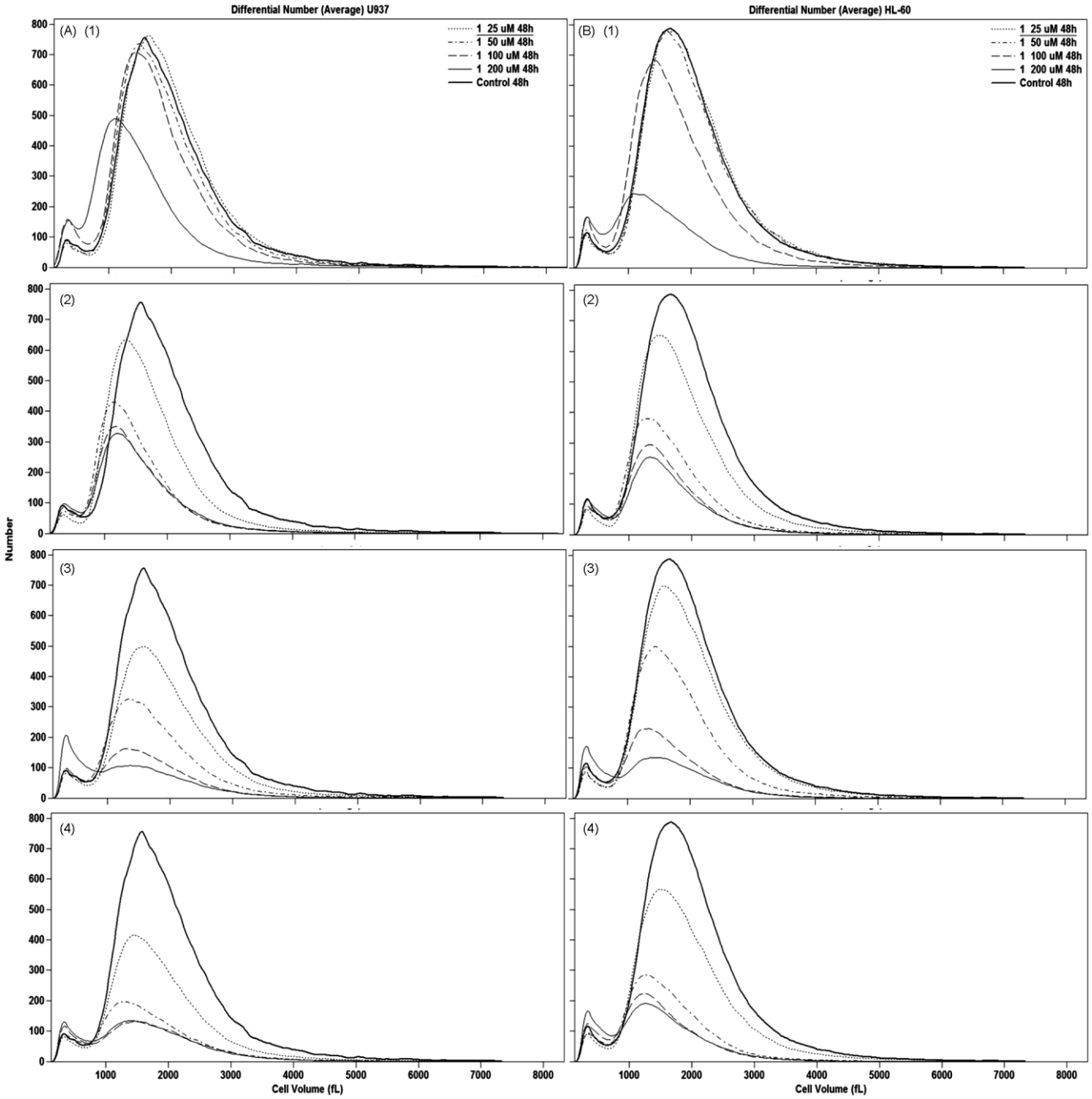

Fig. 3—The mean volume distribution curves of U937 (A) and HL-60 cells (B) recorded 48 h after exposure to the action of phenytoin derivatives 1, 2, 3 and 4. [The peaks on the left represent cellular debris, presumably apoptotic bodies and cell fragments, which were excluded from the analysis of the U937 and HL-60 cell volume]

U937 cells exposure to the action of the agent 2, while in HL-60 cells after application of the agent 4 (Figs. 2B and 3). The cytotoxic effects of the four phenytoin analogs depended on the agent given and its concentration, the time intervals after the compound application, and the cell line used (Fig. 2 and Table 1).

Discussion

The chemical structure of the 5,5-diphenyl-hydantoin analogs was surely responsible for their cytotoxic activity against U937 and HL-60 cells. The compounds 1-4 differ within two substitution places at hydantoin ring, a substituent at position 3 (R^1) and length of the alkyl chain,

at position 1 (R^2) of hydantoin ring. The derivatives 2, 3 and 4 have a bromoalkyl substituent at position 1 (R^2) at hydantoin ring, while the compound 1 has only a hydrogen atom. Taking into account the cell viability and count, and the values of IC_{50}, it can be generally stated that the compound 1, lacking bromoalkyl substituent, was less active than the agents 2, 3 and 4 (Fig. 2 A and C, Table 1). Chemical structure of the active derivatives of phenytoin (2-4) underlines their alkylating properties. Particularly, these compounds were active in N-alkylation processes, including an alkylation of amines and amides[11,13]. Organic synthesis works confirmed successful alkylation attack of the compounds 2-4 on various primary and secondary amines[11,13]. Although the present study does not explain the exact pathways of the observed cytotoxic actions of the tested derivatives at the molecular level, it indicates that a main pathway is based on probable alkylations of nucleobases within the leukemia cells. This is also supported by the fact that the compound 1, devoid of alkylating properties, displayed a significant decrease of cytotoxic action when compared to that of compounds 2-4.

Among the bromoalkyl analogs with methyl substituent at position 3 (R^1), the highest antileukemic activity was found for the compound 4 with bromobutyl moiety, whereas the cytotoxic activity was decreased in the case of a shorter propyl chain of the agent 3 (Fig. 2 A and C, Table 1). The bromobutyl derivative 4 possibly facilitate the alkylation process better than the bromopropyl one as the terminated bromide is placed in some distance from aromatic-hydantoin core. Position of bromide enables an efficacious alkylating attack. In the case of the propyl derivative 3, the distance is smaller and some resistant effects of 5,5-diaromatic hydantoin may decrease the N-alkylation action causing a bit weaker cytotoxic effect. A different profile of cytotoxic action among the two propyl derivatives (2 and 3) which corresponds to the type of a substituent at position 3-hydantoin (Fig. 2 A and C, Table 1) was also observed. The compound 2 has a metylpropanoate substituent at position 3 (R^1), while the analog 3 has only the methyl group. The obtained results indicated that the methyl substituent, at lower concentrations, 25 and 50 μM, is less profitable for the cytotoxic action than the ester (methylpropanoate) fragment (Fig. 2 A and C, Table 1).

Conclusion

To summarize, the structural modifications of 5,5-diphenylhydantoin were reflected in the different leukemia cell response to the action of its four derivatives methyl 2-(2,4-dioxo-5,5-diphenylimidazolidin-3-yl)propanoate (1), methyl 2-(1-(3-bromopropyl)-2,4-dioxo-5,5-diphenylimidazolidin-3-yl)propanoate (2), 1-(3-bromopropyl)-3-methyl-5, 5-diphenylimidazolidine-2,4-dione (3) and 1-(3-bromobutyl)-3-methyl-5,5-diphenylimidazolidine-2,4-dione (4). These are the first data comparing the antileukemic activities of the phenytoin analogs which differ within the two substitution places, 1 and 3 (R^1 and R^2) at hydantoin ring. The precise mechanisms of their action on pathological hematopoietic cells remain still unclear and require further studies.

Conflict of interest statement

Authors disclose that there is no conflict of interest.

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References

1 Southam E, Stratton SC & Davies CH, Anticonvulsant mechanisms for today and tomorrow. *Drug News Perspect,* 18 (2005) 483.

2 Diwan BA, Henneman JR & Nims RW, Tumor promotion by an anticonvulsant agent, phenytoin, in mouse liver: correlation with CYP2B induction. *Carcinogenesis*, 14 (1993) 2227.

3 Kindig D, Garriott ML, Parton JW, Brunny JD & Beyers JE, Diphenylhydantoin is not genotoxic in a battery of short-term cytogenetic assays. *Teratog Carcinog Mutagen*, 12 (1992) 43.

4 Flynn TJ & Ferguson MS, Multiendpoint mechanistic profiling of hepatotoxicants in HepG2/C3A human hepatoma cells and novel statistical approaches for development of a prediction model for acute hepatotoxicity. *Toxicol In Vitro*, 22 (2008) 1618.

5 Santos NAG, Medina WSG, Martins NM, Mingatto FE, Curti C & Santos AC, Aromatic antiepileptic drugs and mitochondrial toxicity: Effects on mitochondria isolated from rat liver. *Toxicol In Vitro*, 22 (2008) 1143.

6 Ciechanowicz-Rutkowska M, Stadnicka K, Kieć-Kononowicz K, Byrtus H, Filipek B, Zygmunt M & Maciąg D, Structure-activity relationship of some new anti-arrhythmic phenytoin derivatives. *Arch Pharm* (*Weinheim*), 333 (2000) 357.

7 Kieć-Kononowicz K, Stadnicka K, Mitka A, Pekala E, Filipek B, Sapa J & Zygmunt M, Synthesis, structure and antiarrhythmic properties evaluation of new basic derivatives of 5,5-diphenylhydantoin. *Eur J Med Chem*, 38 (2003) 555.

8 Dyląg T, Zygmunt M, Maciąg D, Handzlik J, Bednarski M, Filipek B & Kieć-Kononowicz K, Synthesis and evaluation of *in vivo* activity of diphenylhydantoin basic derivatives. *Eur J Med Chem*, 39 (2004) 1013.

9 Handzlik J, Maciąg D, Kubacka M, Mogilski S, Filipek B, Stadnicka K & Kieć-Kononowicz K, Synthesis, α_1-adrenoceptor antagonist activity and SAR-study for novel arylpiperazine derivatives of phenytoin. *Bioorg Med Chem*, 16 (2008) 5982.

10 Handzlik J, Pertz HH, Görnemann T, Jähnichen S & Kieć-Kononowicz K, Search for influence of spatial properties on affinity at α_1-adrenoceptor subtypes for phenylpiperazine derivatives of phenytoin. *Bioorg Med Chem Lett*, 20 (2010) 6152.

11 Handzlik J, Bajda M, Zygmunt M, Maciąg D, Dybała M, Bednarski M, Filipek B, Malawska B & Kieć-Kononowicz K, Antiarrhythmic properties of phenylpiperazine derivatives of phenytoin with α_1-adrenoreceptor affinities. *Bioorg Med Chem*, 20 (2012) 2290.

12 Spengler G, Evaristo M, Handzlik J, Serly J, Molnár J, Viveiros M, Kieć-Kononowicz K & Amaral L, Biological activity of hydantoin derivatives on P-glycoprotein (ABCB1) of mouse lymphoma cells. *Anticancer Res*, 30 (2010) 4867.

13 Handzlik J, Szymańska E, Chevalier J, Otrębska E, Kieć-Kononowicz K, Pagès JM & Alibert S, Amine-alkyl derivatives of hydantoin: new tool to combat resistant bacteria. *Eur J Med Chem*, 46 (2011) 5807.

14 Kavitha CV, Nambiar M, Ananda Kumar CS, Choudhary B, Muniyappa K, Rangappa, KS & Raghavan SC, Novel derivatives of spirohydantoin induce growth inhibition followed by apoptosis in leukemia cells. *Biochem Pharmacol*, 77 (2009) 348.

15 Berridge MV & Tan AS, Characterization of the cellular reduction of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT): subcellular localization, substrate dependence, and involvement of mitochondrial electron transport in MTT reduction. *Arch Biochem Biophys*, 303 (1993) 474.

16 Marshall NJ, Goodwin CJ & Holt SJ, A critical assessment of the use of microculture tetrazolium assays to measure cell growth and function. *Growth Regul*, 5 (1995) 69.

17 Berridge M, Tan A, McCoy K & Wang R, The biochemical and cellular basis of cell proliferation assays that use tetrazolium salts. *Biochemica*, 4 (1996) 14.

18 Peng L, Wang W & Bai L, Performance evaluation of the Z2 coulter counter for WBC and RBC counting. *Int J Lab Hematol*, 29 (2007) 361.