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Structure–activity relationships of 3-substituted-5,5--diphenylhydantoins as potential antiproliferative and antimicrobial agents

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Abstract: A series of twelve 3-substituted-5,5-diphenylhydantoins was synthesized, including some whose anticonvulsant activities have already been reported in the literature. Their antiproliferative activities against HCT-116 human colon carcinoma cells were evaluated to determine structure–activity relationships. Almost all of the compounds exhibited statistically significant antiproliferative effects at a concentration of 100 μM, while the derivative bearing a benzyl group was active even at lower concentrations. Moreover, their *in vitro* antibacterial activities against *Escherichia coli* ATCC 25922, *Staphylococcus aureus* ATCC 25923 and clinical isolates of *Escherichia coli*, *Proteus mirabilis*, *Pseudomonas aeruginosa*, *Enterococcus faecalis* and *Staphylococcus aureus* were evaluated. Only the 3-isopropyl and 3-benzyl derivatives showed weak antibacterial activities against the Gram-positive bacterium *E. faecalis* and the Gram-negative bacteria *E. coli* ATCC 25922 and *E. coli*.

Keywords: phenytoin derivatives; antiproliferative activity; antimicrobial activity; structure–activity relationship.

INTRODUCTION

The derivatives of hydantoin (imidazolidine-2,4-dione) are well known and clinically widely used in the therapy of epilepsy and cardiac arrhythmias. Phenytoin (5,5-diphenylhydantoin, Dilantin®), one of the oldest anticonvulsants, is very effective in controlling a variety of seizure disorders, while impairing neurological function only slightly, if at all.[1] These effects are due to a selective

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block of high frequency neuronal activity. The drug targets the neuronal voltage-sensitive sodium channels (NVSC) to reproduce the normal ion potential and is known to block the release of neurotransmitters, such as serotonin and norepinephrine. At an appropriate level, it inhibits monoamine oxidase activity and tends to alter the membrane potential as well.[2]

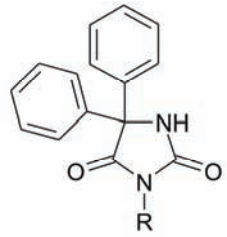
After 70 years of phenytoin application, the precise physiological effects of the drug have not been completely determined and it still remains an important subject for new investigations. The drug and its metabolite 5-(4-hydroxyphenyl)-5-phenylhydantoin were reported to possess significant hypolipidemic activity, which is reflected in the reduction of both serum cholesterol and triglyceride levels.[3] Phenytoin did not show inhibitory effect on the growth of a spectrum of microorganisms (*Mycobacterium smegmatis*, *Candida albicans*, *Staphylococcus aureus*, *Pseudomonas aeruginosa* and *Escherichia coli*).[4] Furthermore, a slight increased occurrence of *E. coli* agglutinins was found in patients on long-term phenytoin therapy.[5] In dermatology, the drug has been investigated for the treatment of ulcers, epidermolysis bullosa and different inflammatory conditions.[6]

Hydantoin derivatives are found in many area of medicinal chemistry (serotonin and fibrinogen receptor antagonists,[7] inhibitors of the glycine binding site of the NMDA receptor,[8] antagonists of leukocyte cell adhesion, acting as allosteric inhibitors of the protein–protein interaction[9]). In particular, several reports present interest in cancer research.[10,11] Cancer is one of the most devastating diseases of today. It is manifested as uncontrolled growth of cells and invasion or intrusion into and the destruction of adjacent tissues. Although the progress is evident in diagnosis, surgical techniques, patient care and adjuvant therapies, most of the deaths from cancer are due to metastases.[12] Spiromustin, a hydantoin-containing nitrogen mustard, rapidly penetrates the blood-brain barrier and directs drug delivery to brain tumors.[13] Carmi *et al.*[14,15] demonstrated that 5--benzylidenehydantoins could function as bioisosters of 4-anilinoquinazolines, which are epidermal growth factor receptor (EGFR) tyrosine kinase inhibitors approved for the treatment of lung cancer. They suggested that the presence of an aromatic unit at position C5 is an important structural feature for interactions with molecular targets. Recently, Ananda Kumar and co-workers investigated the antiproliferative effect of certain diazaspiro bicyclo hydantoin derivatives against human leukemia K562 and CEM cells.[16,17] They reported that the cytotoxic activities of compounds bearing a substituent at the N3 position increase in the order alkene > ester > ether. A similar conclusion was reached in a comparative study of the cytostatic activities of L- and D-amino acid derivatives of hydroxyurea and hydantoins. The best antitumor activities were achieved with lipophilic compounds having cycloalkyl, phenyl and benzhydryl substituents.[18]

A deeper understanding of the SARs and modeling of new derivatives with potential antitumor activity can be facilitated by accumulation of detailed struc-

tural and pharmacological data. In this context, a set of twelve phenytoin derivatives bearing different alkyl (methyl, ethyl, *n*-propyl, isopropyl, *n*-butyl, isobutyl and benzyl), alkenyl (allyl), ether (ethoxymethyl, benzyloxymethyl), ester (acetoxymethyl) and alkanoyl (benzoyl) substituents at the N3 position was synthesized (Table I). The antiproliferative activity was evaluated against the HCT--116 human colon carcinoma cell line. These structural modifications of the phenytoin molecule led to several derivatives exhibiting different degrees of anticonvulsant activity (Table I). Poupaert *et al*.[20] observed that the anti-electroshock activity was decreased when the hydantoin ring was N-methylated. The 3-alkoxymethyl derivatives were reported to be active against electrically as well as chemically induced seizures.[19,22] On the other hand, 3-(acetoxymethyl)-5,5-diphenylhydantoin resembled the parent compound showing good activity against maximal electroshock seizures, but was inactive against pentylenetetrazole.[23] In previous papers,[24,25] the hypothesis that the ability to form hydrogen bonds plays the determining role in the anticonvulsant action of these compounds was confirmed.

TABLE I. Structures and anticonvulsant potencies of the investigated compounds



Compound	R	ED_{50}[a] / mg kg^{-1}
1	H	≈7.5[19]
2	CH_3	39.6[20]
3	C_2H_5	–
4	*n*-C_3H_7	–
5	*i*-C_3H_7	–
6	*n*-C_4H_9	–
7	*i*-C_4H_9	–
8	$C_6H_5CH_2$	>200[19]
9	CH_2=$CHCH_2$	30.4[21]
10	$C_2H_5OCH_2$	–
11	$C_6H_5CH_2OCH_2$	>25[22]
12	$CH_3C(=O)OCH_2$	<12.5[19]
13	C_6H_5CO	–

[a]The effective dose required to protect mice against spasms induced by the maximum electric shock (MES)

Certain derivatives of hydantoin, which contained aromatic or heterocyclic substituents at the nitrogen, were reported to exhibit antimicrobial effects.[26]

Hence, the *in vitro* antimicrobial activities of the investigated compounds were additionally evaluated against *E. coli* ATCC 25922, *S. aureus* ATCC 25923 and clinical isolates of *E. coli*, *Proteus mirabilis*, *P. aeruginosa*, *Enterococcus faecalis* and *S. aureus*.

RESULTS AND DISCUSSION

Antiproliferative screening

The investigation of the antiproliferative activities of the phenytoin derivatives on the HCT-116 cell line at a concentration of 100 μM showed that almost all of the compounds, except **2**, **3** and **10**, exhibited statistically significant antiproliferative effects, as is shown in Fig. 1. Furthermore, **8** at concentrations of 0.01, 0.1, 1, 10 and 100 μM showed significant antiproliferative effects (Fig. 2), while compound **9** demonstrated a statistically significant antiproliferative effect at a concentration of 10 μM. Interestingly, valproate (valproic acid) manifested a similar dose-dependent inhibition of proliferation of gastrointestinal neuroendocrine[27] and carcinoid cancer cells.[28] Valproic acid is a simple branched-chain fatty acid, the anticonvulsant efficacy of which is comparable to that of phenytoin.[29] The other compounds showed no significant inhibition of HCT-116 proliferation at lower concentrations.

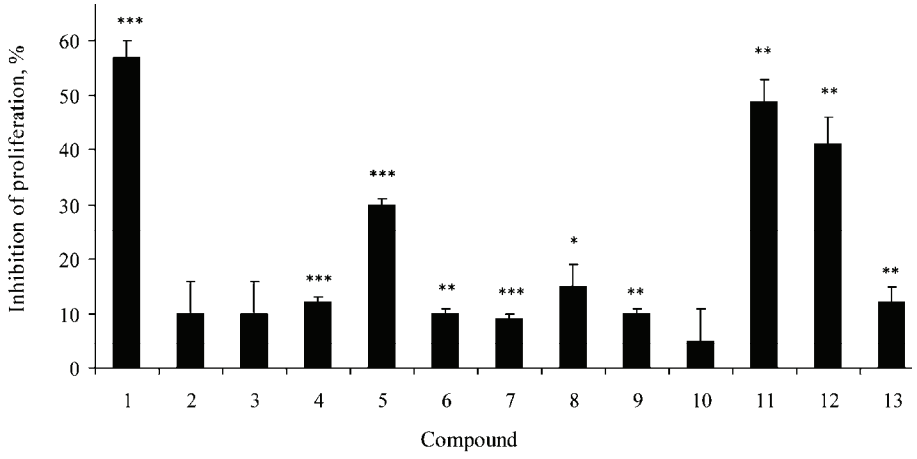


Fig. 1. The antiproliferative effect of 3-substituted-5,5-diphenylhydantoins on the HCT-116 cell line. The cells were treated with a 100 μM concentration of the drugs during a 24 h exposure. The antiproliferative effect was measured by the MTT assay. The results are expressed as the means ± *SD* from cells cultured in triplicate (*$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, compound *vs.* the control).

N-Alkylation of the phenytoin molecule resulted in a diminished ability to form hydrogen bonds and also a decreased antiproliferative activity of compounds **2**–**7** at a concentration of 100 μM. A net stepwise increase in the size of

the alkyl substituent resulted in a slight decrease in the potencies of the compounds with the exception of the isopropyl group. Furthermore, compounds **11** and **12**, potent anticonvulsants, manifested a cytotoxic activity to the cancer cells similar to that of the parent compound **1**. The unexpected activity of compound **8** in low concentrations implies that the relative activity of these compounds is not determined only by the physico–chemical properties of the substituent at the N3 position. It might only be assumed that compounds bearing a benzyl unit (**8** and **11**) are well located in the molecular target, while the derivative with a rigid benzoyl group (**13**) is not well tolerated.

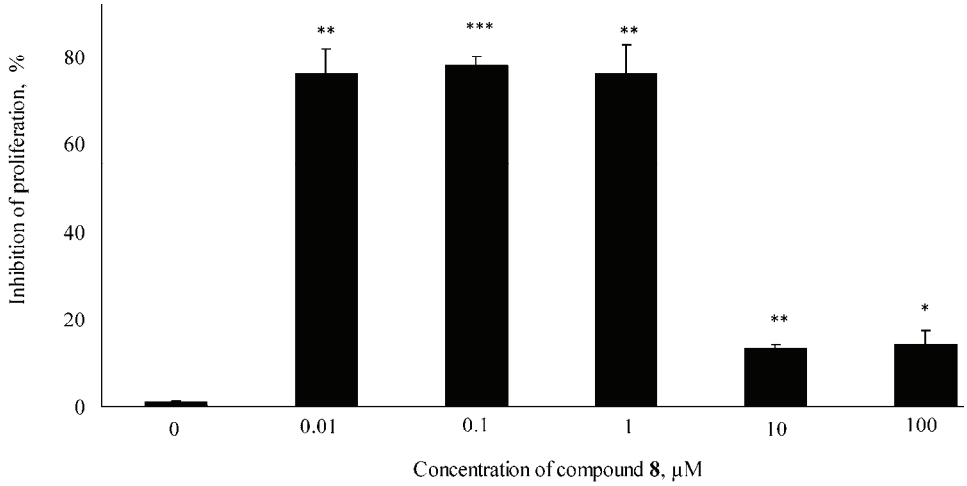


Fig. 2. The antiproliferative effect of **8** on HCT-116 cell line. The cells were treated with various concentrations of drugs during a 24 h exposure. The antiproliferative effect was measured by the MTT assay. The results are expressed as the means ± SD from cells cultured in triplicate. (*p < 0.05, **p < 0.01, ***p < 0.001, different concentrations *vs.* control).

Antibacterial screening

The investigated phenytoin derivatives were additionally screened for their *in vitro* antibacterial activities against three Gram-positive and four Gram-negative bacteria using the well-diffusion method[30] and the microdilution method with resazurin.[31] Overnight cultures of standard strains of *E. coli* ATCC 25922, *S. aureus* ATCC 25923 and clinical isolates of *E. coli*, *P. mirabilis*, *P. aeruginosa*, *E. faecalis* and *S. aureus* were used for the preparation of the bacterial suspensions. The *in vitro* antibacterial activities of the new compounds against both Gram-positive and Gram-negative bacteria are listed in Table II. Among the tested compounds, only **5** and **8** showed significant antibacterial activity against the Gram-positive bacterium *E. faecalis* and the Gram-negative bacteria *E. coli* ATCC 25922 and *E. coli* (clinical isolate). The active compounds showed the best effects against *E. coli.*

TABLE II. Antibacterial activity of the tested 3-alkyl-5,5-diphenylhydantoins

Compds.	*E. coli* ATCC 25922			*E. coli*			*P. mirabilis*			*P. aeruginosa*		
	IZ[a]	*MIC*[b]	*MBC*[c]	*IZ*	*MIC*	*MBC*	*IZ*	*MIC*	*MBC*	*IZ*	*MIC*	*MBC*
1–**4**	/	>10^3	>10^3	–	>10^3	>10^3	–	>10^3	>10^3	–	>10^3	>10^3
5	21.25±1.50	250	>500	23.00±0.00	125	125						
6 and **7**	–	>10^3	>10^3		>10^3	>10^3						
8	20.05±2.28	500	>500	20.50±0.71	250	250						
9–**13**	–	>10^3	>10^3		>10^3	>10^3						

	E. faecalis			*S. aureus* ATCC 25923			*S. aureus*		
	IZ[a]	*MIC*[b]	*MBC*[c]	*IZ*	*MIC*	*MBC*	*IZ*	*MIC*	*MBC*
1–**4**	–	>10^3	>10^3	–	>10^3	>10^3	–	>10^3	>10^3
5	21.00±0.00	250	500						
6 and **7**	–	>10^3	>10^3						
8	18.75±1.50	500	500						
9–**13**	–	>10^3	>10^3						

[a]Inhibition zone, mm; [b]minimum inhibitory concentration, μM; [c]minimum bactericidal concentration, μM

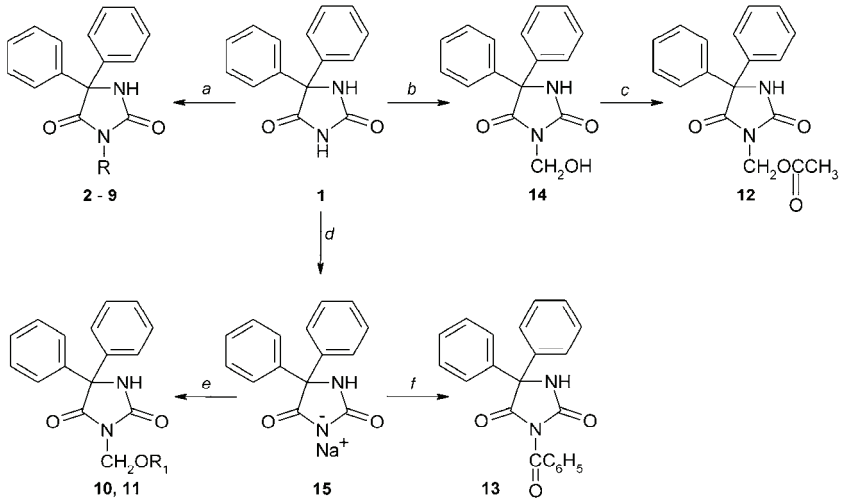
Compound **8** demonstrated weak antibacterial activity as an additional biological effect. Compound **5**, which was shown to be an exception from the group of 3-alkyl substituted derivatives, exhibited a similar effect. Since cytochrome P450 enzymes are responsible for the metabolism of an ever-expanding array of drugs, Suzuki and co-workers[32] tested, among others, the inhibitor potencies of **5** and **8** against recombinant CYP2C19 and CYP2C9 to probe their interactions with their active sites. Metabolic profiling and homology modeling studies suggested that the 3-benzyl substituted derivative was preferentially bound in the active site of CYP2C19 with its C5 phenyl group oriented towards the active oxygen and the benzyl group bound within a lipophilic pocket of the receptor. We are of the opinion that these structural features might be responsible for the biological activity of compound **8**. Several examples from the literature indicate that a benzyl group attached to the nitrogen atom is a promising pharmacophore for antiproliferative activity to be exhibited.[33–35] Since the molecular basis of the biological activity of **8** remains to be determined, further experiments aimed at defining the modes of actions are currently in progress.

EXPERIMENTAL

Chemistry

The methods for the preparations of the investigated compounds are presented in Scheme 1. In the synthesis of **2**–**9**, commercially obtained 5,5-diphenylhydantoin (**1**, Fluka) was alkylated at the N3 position using the corresponding alkyl halide in K_2CO_3/dimethylformamide.[32] The alkoxymethyl substituent was introduced by the reaction of phenytoin sodium salt (**15**) in dimethylformamide with the appropriate chloromethyl alkyl ether (**10** and **11**).[19] The process for preparing **15** was described in the literature.[36] 3-Acetoxymethyl-5,5-diphenylhydantoin (**12**) was prepared by the reaction of 3-(hydroxymethyl)-5,5-diphenylhydantoin and acetic anhydride, and 3-(hydroxymethyl)-5,5-diphenylhydantoin (**14**) was initially synthesized

by the addition of **1** to formaldehyde in the presence of sodium hydroxide in ethanol.[23] 3-Benzoyl-5,5-diphenylhydantoin (**13**) was synthesized in the reaction of phenytoin sodium salt and benzoyl chloride in dry benzene.[37] The structures of the compounds were determined by their melting points, and IR, ^{1}H- and ^{13}C-NMR spectra, which were in agreement with literature data. The ^{1}H- and ^{13}C-NMR spectral measurements were performed on a Bruker AC 250 spectrometer at 250 MHz for the ^{1}H-NMR and 62.89 MHz for the ^{13}C-NMR spectra. FT-IR spectra were recorded on a Bomem MB 100 spectrophotometer. The spectra were recorded at room temperature in DMSO-d_6. The chemical shifts are expressed in ppm values referenced to TMS (δ_H = 0 ppm) in ^{1}H-NMR spectra, and the residual solvent signal (δ_C = 39.5 ppm,) in the ^{13}C-NMR spectra. Elemental analysis was realized using an Elemental Vario EL III micro-analyzer. The yield and chemical characterization of the newly synthesized compound **7** are given here.



Scheme 1. Synthesis of the investigated derivatives of phenytoin (**1**). Reagents and conditions: a) alkyl halide (1.1 eq.), K_2CO_3, DMF, r. t., 24 h; b) formalin, NaOH, EtOH, r. t., 30 min; c) Ac_2O, r. t. 24 h; d) NaOH, benzene, reflux, 6 h; e) chlormethyl alkyl ether (1.1 eq.), DMF, r. t., 24 h; f) benzoyl chloride, benzene, reflux, 6 h.

Characterization

3-Isobutyl-5,5-diphenylhydantoin (7). White crystalline solid; yield 72 %; mp: 119–122 °C; Anal. Calcd. for $C_{19}H_{20}N_2O_2$: C, 74.00; H, 9.08; N, 6.54 %. Found C 74.08, H, 9.10, N 6.57 %. IR (KBr, cm^{-1}): 3293 (N–H), 1705, 1775 (C=O). ^{1}H-NMR (200 MHz, DMSO-d_6, δ / / ppm): 9.66 (1H, *s*, NH), 7.26–7.44 (10H, *m*, 2Ph–H), 3.25–3.29 (2H, *m*, CH_2–N), 1.89–2.03 (1H, *m*, C**H**–CH_2N), 0.78 (6H, *d*, *J* = 7.0 Hz, 2CH_3). ^{13}C-NMR (50 MHz, DMSO-d_6, δ / / ppm): 173.60 (C4), 155.85 (C2), 140.03 (Ph–C), 128.81 (Ph–CH), 128.37 (Ph–CH), 126.82 (Ph–CH), 69.22 (C5), 45.40 (CH_2–N), 27.12 (CH), 19.91 (CH_3).

In vitro *antiproliferative screening*

The antiproliferative potential of the investigated phenytoin derivatives was determined using the MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] assay for the HCT-116 human colon cancer cell line. The relative antiproliferative potency is expressed as

the percentage of proliferation inhibition of the control HCT cells cultured without compounds in the cell cultivation medium. The HCT-116 cells were maintained in Dulbecco-modified Eagle medium (DMEM) supplemented with 10 % fetal bovine serum. The cells were grown in 75 ml culture bottles supplied with 12 ml DMEM, and after a few passages, the cells were seeded in a 96-well plate. The cells were cultured in a humidified atmosphere of 5 % CO_2 at 37 °C. The HCT-116 cells were treated with 0.01, 0.1, 1, 10 and 100 μM concentrations of the investigated compounds for 24 h. Untreated cells served as the control. After 24 h of treatment, the cell proliferation was determined by the MTT assay. This test is based on the color reaction of mitochondrial dehydrogenase from living cells with MTT. Briefly, 10 ml of MTT solution (5 mg ml^{-1}) was added to each well after 24 h of culture and the cultures were incubated for an additional 3 h at 37 °C. The produced formazan was dissolved by overnight incubation in SDS–HCl (10 % SDS (sodium dodecyl sulfate) in 0.01 M HCl) and absorbance was measured at the dual wavelengths of 570/650 nm with an ELISA 96-well plate reader. The percentage of viable cells was calculated as the ratio between the absorbance at each dose of the compounds and the absorbance of the untreated control×100.

In vitro *antibacterial screening*

An overnight culture of standard strains of *E. coli* ATCC 25922, *S. aureus* ATCC 25923 and clinical isolates of *E. coli*, *P. mirabilis*, *P. aeruginosa*, *E. faecalis* and *S. aureus* were used for the preparation of bacterial suspensions. The turbidity of the initial bacterial suspensions was adjusted by comparing with a 0.5 McFarland standard and then diluted 1:100 in sterile 0.85 % saline.

Antibacterial assay was realized by the well-diffusion method[30] and the microdilution method with resazurin.[31] The diffusion method is a qualitative test which allows the classification of microorganisms as susceptible or resistant to the test substance according to diameter of the zone of inhibition. Petri plates with Mueller–Hinton agar were inoculated with adequate bacterial suspensions. The surface of the media was allowed to dry for 3–5 min at room temperature. Subsequently, wells (7 mm) were made in the plate with a sterile metal cylinder, which were filled with 100 μl of solutions of the tested compounds; concentration of 1000 μM. The antibacterial activity was evaluated by measuring the diameters of the zones of inhibition. All tests were performed in triplicate and the results are expressed as mean ± standard deviation. A negative control was prepared with the same solvent used to dissolve the tested substances (5 % DMSO) to ensure that the solvent had no effect on bacterial growth. Each test also included a growth control and a sterility control. The minimum inhibitory concentration (*MIC*) and the minimum bactericidal concentration (*MBC*) were determined using the microdilution plate method. Two-fold, serial dilutions of the tested compounds were performed in Mueller-Hinton broth. The obtained concentration range was from 1000 to 7.81 μM. The diluted bacterial suspensions (10 μl) were added to each well to give a final concentration of 5×10^5 CFU mL^{-1}. Finally, 10 μl resazurin indicator solution was added. Resazurin is an oxidation–reduction indicator used for the evaluation of microbial growth. It is a blue non-fluorescent dye that becomes pink and fluorescent when reduced to resorufin by the oxidoreductases within viable cells. The inoculated plates were incubated at 37 °C for 24 h. The *MIC* is defined as the lowest concentration of a tested substance that prevented the resazurin color change from blue to pink. Each test included a growth control and a sterility control. All tests were performed in triplicate and the *MIC* values were constant. The *MBC* was determined by plating 10 μl of samples from wells where no indicator color change was recorded onto nutrient agar medium. At the end of the incubation period, the lowest concentration with no visible growth was defined as the *MBC*.

CONCLUSIONS

In summary, an evaluation of 3-substituted-5,5-diphenylhydantoins as potential antiproliferative and antimicrobial agents was reported. The trend of the changes in the biological effects produced by substituents at position N3 was studied. Compound **8** showed significant antiproliferative activity even in low concentrations. In addition, it exhibited weak antibacterial activity. Since the molecular basis of its biological activity remains to be determined, further experiments aimed at defining the modes of its actions are currently in progress.

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ИЗВОД

УТИЦАЈ СТРУКТУРЕ НА АНТИПРОЛИФЕРАТИВНУ И АНТИБАКТЕРИЈСКУ АКТИВНОСТ 3-СУПСТИТУИСАНИХ-5,5-ДИФЕНИЛХИДАНТОИНА

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Синтетисана је серија од дванаест 3-супституисаних-5,5-дифенилхидантоина, која обухвата неке од деривата чије су антиконвулзивне активности познате у литератури. Одређена је њихова антипролиферативна активност према ћелијској линији хуманог карцинома колона, како би се утврдио утицај структуре на активност. Скоро сва једињења испољавају антипролиферативан ефекат у концентрацији од 100 μM, док је дериват са бензил групом активан и у нижим концентрацијама. Додатно је одређена и антибактеријска активност проучаваних једињења према *Escherichia coli* ATCC 25922, *Staphylococcus aureus* ATCC 25923 и клиничким изолатима *Escherichia coli*, *Proteus mirabilis*, *Pseudomonas aeruginosa*, *Enterococcus faecalis* и *Staphylococcus aureus*. 3-Изопропил и 3-бензил деривати показују слабу активност према грам-позитивној бактерији *E. faecalis* и грам-негативним бактеријама *E. coli* ATCC 25922 и *E. coli*.

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