Turkish Journal of Biology

Volume 37 | Number 6 Article 14

1-1-2013

Evaluation of antioxidative effects of twelve 3-substituted-5,5-diphenylhydantoins on human colon cancer cell line HCT-116

ANA OBRADOVIC

JOVANA ZIZIC

NEMANJA TRISOVIC

BOJAN BOZIC

GORDANA USCUMLIC

See next page for additional authors

Follow this and additional works at: https://journals.tubitak.gov.tr/biology

 Part of the Biology Commons

Recommended Citation

OBRADOVIC, ANA; ZIZIC, JOVANA; TRISOVIC, NEMANJA; BOZIC, BOJAN; USCUMLIC, GORDANA; BOZIC, BILJANA; and MARKOVIC, SNEZANA (2013) "Evaluation of antioxidative effects of twelve 3-substituted-5,5-diphenylhydantoins on human colon cancer cell line HCT-116," *Turkish Journal of Biology*: Vol. 37: No. 6, Article 14. https://doi.org/10.3906/biy-1302-15
Available at: https://journals.tubitak.gov.tr/biology/vol37/iss6/14

This Article is brought to you for free and open access by TÜBİTAK Academic Journals. It has been accepted for inclusion in Turkish Journal of Biology by an authorized editor of TÜBİTAK Academic Journals. For more information, please contact academic.publications@tubitak.gov.tr.

Evaluation of antioxidative effects of twelve 3-substituted-5,5-diphenylhydantoins on human colon cancer cell line HCT-116

Authors

ANA OBRADOVIC, JOVANA ZIZIC, NEMANJA TRISOVIC, BOJAN BOZIC, GORDANA USCUMLIC, BILJANA BOZIC, and SNEZANA MARKOVIC

This article is available in Turkish Journal of Biology: https://journals.tubitak.gov.tr/biology/vol37/iss6/14

TÜBİTAK

Turkish Journal of Biology

http://journals.tubitak.gov.tr/biology/

Research Article

Turk J Biol
(2013) 37: 741-747
© TÜBİTAK
doi:10.3906/biy-1302-15

Evaluation of antioxidative effects of twelve 3-substituted-5,5-diphenylhydantoins on human colon cancer cell line HCT-116

Ana OBRADOVIĆ[1,*], Jovana ŽIŽIĆ[1], Nemanja TRIŠOVIĆ[2], Bojan BOŽIĆ[2], Gordana UŠĆUMLIĆ[2], Biljana BOŽIĆ[3,], Snežana MARKOVIĆ[1,**]**

[1]Department of Biology and Ecology, Faculty of Science, University of Kragujevac, Kragujevac, Serbia
[2]Department of Organic Chemistry, Faculty of Technology and Metallurgy, University of Belgrade, Belgrade, Serbia
[3]Institute for Physiology and Biochemistry, Faculty of Biology, University of Belgrade, Belgrade, Serbia

Received: 04.02.2013 • **Accepted:** 02.08.2013 • **Published Online:** 08.10.2013 • **Printed:** 04.11.2013

Abstract: Antioxidant effects of twelve 3-substituted-5,5-diphenylhydantoins in concentrations of 0.01, 0.1, 1, 10, and 100 µM on human colon cancer cell line HCT-116 were determined 24 h after treatment. The concentrations of superoxide anion radical ($O_2^{\cdot-}$), nitrites (NO_2^-), and total glutathione were determined spectrophotometrically. Results indicated that treatment with all compounds induced a decrease in $O_2^{\cdot-}$ and an increase in NO_2^- concentrations in HCT-116 cells. Treatment with all compounds resulted in an increase in glutathione, and this indicates changes in redox homeostasis. Based on the obtained results, the investigated hydantoins act as antioxidants because they decrease the production of superoxide anion radical and increase concentrations of glutathione, but they also induce an increase in nitrite production.

Key words: Antioxidants, phenytoin derivatives, free radicals, redox homeostasis

1. Introduction

Hydantoins (imidazolidine-2,4-diones) are clinically widely used for epilepsy therapy and for cardiac arrhythmias. Phenytoin (5,5-diphenylhydantoin) is one of the oldest anticonvulsants and is very effective in controlling a variety of seizure disorders while impairing neurological function only slightly (Yaari et al., 1986). 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 of new investigations. The drug and its metabolite, 5-(4-hydroxyphenyl)-5-phenylhydantoin, have been reported to possess significant hypolipidemic activity, which is reflected in the reduction of both serum cholesterol and triglyceride levels (Maguire et al., 1985).

Cancer is one of the most devastating diseases of today (Lee et al., 2002). It manifests as uncontrolled growth of cells and invasion or intrusion into and destruction of adjacent tissues. Although progress is evident in diagnosis, surgical techniques, patient care, and adjuvant therapies, most of the deaths from cancer are due to metastases (Fidler, 2002). Colon cancer provides a suitable model for studying the genetic basis of cancer development and progression. Most colon cancers are the outcome of multiple processes in which benign adenomas that occur because of inherited and/or acquired mutations lead to invasive malignant tumors (Henderson and Fagotto, 2002).

Cancer chemotherapy started with the discovery of the cytostatic effect of drugs, and this observation opened the way for the synthesis of various drugs with antitumor activities against several human malignancies. The considerable toxicity of these drugs, however, limited their application, and the majority of human malignant tumors proved to be chemoresistant. Consequently, there is still an urgent need for less toxic compounds that possess a broader antitumor spectrum. Better understanding of cellular metabolism, due to the revolution in molecular biology, yielded new targets for cancer chemotherapeutic agents (Eckhardt, 2002). Hydantoins are important in cancer research. Carmi et al. (2006) and Cavazzoni et al. (2008) demonstrated that 5-benzylidenehydantoins can function as bioisosteres of 4-anilinoquinazolines, which are epidermal growth factor receptor tyrosine kinase inhibitors approved for the treatment of lung cancer. The best antitumor activities have been achieved with lipophilic

* Correspondence: anaobradovic@kg.ac.rs
** These authors contributed equally to this article.

compounds having cycloalkyl, phenyl, and benzhydryl substituents (Kumar et al., 2009). Hydantoin derivatives were obtained by modifying the structure of phenytoin derivatives that exhibit different degrees of anticonvulsant activity. Based on the structure of 3-substituted-5,5-diphenylhydantoins, which are biologically active derivatives, we focused on the biological activity of these compounds.

Oxidative stress is caused by the production of reactive oxygen species (ROS), which may cause carcinogenesis via genetic and epigenetic mechanisms. Elevated levels of ROS have been noted in many tumors, strongly implicating oxidative DNA damage in the etiology of cancer. A deeper understanding of hydantoins and the modeling of new derivatives with potential antitumor and antioxidant activity can be facilitated by accumulation of detailed structural and pharmacological data. In this context, a set of 12 phenytoin derivatives bearing different alkyl (methyl, ethyl, *n*-propyl, isopropyl, *n*-butyl, isobutyl, and benzyl), alkenyl (allyl), ether (ethoxymethyl and benzyloxymethyl), ester (acetoxymethyl), and alkanoyl (benzoyl) substituents in position 3 have been synthesized by our group. Their antitumor effect on the inhibition of human colon cancer cell line HCT-116 proliferation has been investigated, and our previous results showed that these compounds have significant cytotoxic effects (Trišović et al., 2011). The purpose of this study was to evaluate their antioxidant activities on the human colon cancer HCT-116 cell line.

2. Materials and methods

2.1. Chemicals

Dulbecco's modified Eagle medium (DMEM) and phosphate-buffered saline were obtained from GIBCO, Invitrogen, USA. Fetal bovine serum (FBS) and trypsin-EDTA were from PAA (The Cell Culture Company), Austria. Nitro blue tetrazolium (NBT) and nicotinamide adenine dinucleotide phosphate-oxidase (NADPH) were obtained from SERVA, Germany. Sodium nitrite, phosphoric acid, sulfanilamide, sulfanilic acid, sulfosalicylic acid, and 5,5′-dithio-bis (2-nitrobenzoic acid) (DTNB) were from Sigma-Aldrich, USA. All other solvents and chemicals were of analytical grade.

2.2. Preparation of drug solutions

Stock solutions of the compounds (Figure 1), prepared as previously described (Trišović et al., 2011), were made in dimethyl sulfoxide (DMSO) at the concentration of 1 M and diluted with DMEM to various working concentrations. The concentration of DMSO in the most concentrated working solutions was 0.5% (v/v).

2.3. Cell preparation and culturing

The human colon cancer adenocarcinoma cell line HCT-116 was obtained from the American Tissue Culture Collection. These cells were propagated and maintained in DMEM and supplemented with 10% FBS and antibiotics (100 IU/mL of penicillin and 100 µg/mL of streptomycin). The cells were grown in 75-cm^2 culture bottles and supplied with 15 mL of DMEM at a confluence of 70%–80%. After a few passages the cells were seeded in a 96-well plate and cultured in a humidified atmosphere with 5% CO_2 at 37 °C.

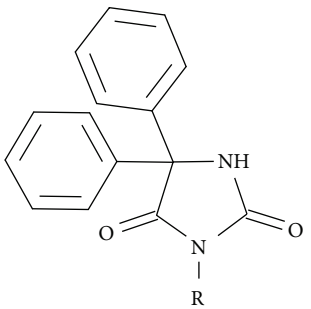


Figure 1. Structures of the investigated 3-substituted-5,5-diphenylhydantoins.
R- different substituents at N3 position: **1**- H, **2**- CH_3, **3**- C_2H_5, **4**- *n*-C_3H_7, **5**- *i*-C_3H_7, **6**- *n*-C_4H_9, **7**- *i*-C_4H_9, **8**- $C_6H_5CH_2$, **9**- CH_2=$CHCH_2$, **10**- $C_2H_5OCH_2$, **11**- $C_6H_5CH_2OCH_2$, **12**- CH_3OCOCH_2, and **13**- C_6H_5CO.

2.4. Determination of superoxide anion radical (NBT assay)

The concentration of superoxide anion radical ($O_2^{\cdot-}$) in the sample was determined by spectrophotometric method (Auclair and Voisin, 1985) and is based on the reduction of NBT to nitroblue-formazan in the presence of $O_2^{\cdot-}$. HCT-116 cells were seeded in triplicate in a 96-well plate (100,000 cells/well). After 24 h of cell incubation, in each well 100 µL of medium containing various doses of the investigated compounds at different concentrations (0.01, 0.1, 1, 10, and 100 µM) was added, and cells were incubated for 24 h. An assay was performed by adding 20 µL of 5 mg/mL NBT to each well, and then the cells were incubated for 45 min at 37 °C in 5% CO_2. To quantify the formazan product, formazan was solubilized in 20 µL of SDS-HCl (10% SDS in 0.01 M HCl), and the resulting color reaction was measured spectrophotometrically on a microplate reader at 550 nm (ELISA 2100C). The concentrations of superoxide anion radical are expressed as nanomoles per milliliter.

2.5. Determination of nitrites (Griess assay)

The spectrophotometric determination of nitrites (NO_2^-), an indicator of nitric oxide (NO) level, was performed by using the Griess method (Griess, 1879). Briefly, the Griess reaction is a diazotization reaction in which the NO-derived nitrosating agent (e.g., N_2O_3), generated from the acid-catalyzed formation of nitrous acid from nitrite (or the interaction of NO with oxygen), reacts with sulfanilic acid to produce a diazonium ion that is then coupled to N-(1-naphthyl) ethylenediamine to form a chromophoric azo product that absorbs strongly at 550 nm. Typically, a

nitrite standard solution was serially diluted in a range from 125 μM to 1.953 μM in triplicate in a 96-well, flat-bottomed microtiter plate. HCT-116 cells were seeded in triplicate in a 96-well plate (100,000 cells/well). After 24 h of cell incubation, 100 μL of medium containing various doses of the investigated compounds in different concentrations (0.01, 0.1, 1, 10, and 100 μM) was added to each well for 24 h. Equal volumes of 0.1% (1 mg/mL) N-1-naphthylethylenediamine dihydrochloride and 1% (10 mg/mL) sulfanilamide solution (in 5% phosphoric acid) to form the Griess reagent were mixed together immediately prior to application to the plate. After incubation in the dark for 10 min the absorbance was measured at 550 nm by using a microplate reader (ELISA 2100C). The concentrations of nitrite were calculated from the appropriate standard curves for nitrite and are expressed as nanomoles per milliliter.

2.6. Determination of total glutathione

This assay is based on oxidation of a reduced form of glutathione (GSH) with a reagent with an active thiol group, i.e. DTNB, which forms a yellow product of 5′-thio-2-nitrobenzoic acid (Baker et al., 1990). All samples were seeded in triplicate in a 96-well microtiter plate (100,000 cells/well). The treatment was performed with 100 μL of the various doses of the investigated compounds at different concentrations (0.01, 0.1, 1, 10, and 100 μM) for 24 h. In order to lyse the cells, cells were dissolved in 5% sulfosalicylic acid and centrifuged at 1000 rpm for 10 min at 4 °C, and the supernatant was taken for further analysis. Clear supernatant (50 μL) was combined with phosphate-EDTA buffer (pH 7.4, 150 μL) and 0.7 U GSH reductase, 1 mM NADPH, and 1 mM DTNB in DMSO (1%). Color reaction was measured spectrophotometrically on a microplate reader at 405 nm (ELISA 2100C) following incubation for 5 min. The formed glutathione disulfide is immediately reduced to GSH with glutathione reductase. Concentrations of GSH were calculated from a standard curve constructed with known concentrations of reduced GSH, and the results are expressed as nanomoles per milliliter from a standard curve established in each test.

2.7. Statistical analysis

All values are expressed as mean ± standard error (SE). Statistical evaluation was calculated by paired samples t-test and one-way analysis of variance with least significant difference post hoc test (SPSS 17 for Windows). The magnitude of correlation between variables was measured using SPSS. For all comparisons $P < 0.05$ for control versus compound was considered significant. Biological activity is the result of 2 individual experiments performed in triplicate for each dose.

3. Results

3.1. Determination of superoxide anion radical (NBT assay)

The data presented in Table 1 show $O_2^{\cdot-}$ levels after 24 h of incubation with various concentrations of phenytoin derivatives. Treatment with all compounds at different concentrations, except **3**, showed a significant inhibitory

Table 1. Effect of 3-substituted-5,5-diphenylhydantoins on superoxide anion radical ($O_2^{\cdot-}$) production in the HCT-116 cell line. The cells were treated with various concentrations of drugs during 24 h of exposure. The effect was measured by the NBT assay, and $O_2^{\cdot-}$ concentrations are expressed as nanomoles per milliliter.

	0 μM	0.01 μM	0.1 μM	1 μM	10 μM	100 μM
1	102.40 ± 7.13	79.71 ± 9.23 *	77.44 ± 6.48 *	76.24 ± 8.01 *	83.60 ± 7.51 *	70.69 ± 2.82 *
2	102.40 ± 7.13	73.64 ± 3.81 *	69.97 ± 4.60 *	68.72 ± 2.41 *	80.47 ± 10.26 *	71.12 ± 6.78 *
3	102.40 ± 7.13	85.00 ± 9.81	90.04 ± 10.17	88.61 ± 9.49	87.87 ± 10.82	91.01 ± 9.46
4	102.40 ± 7.13	90.05 ± 3.49	87.71 ± 4.15 *	88.30 ± 2.41	95.01 ± 7.58	82.84 ± 3.62 *
5	102.40 ± 7.13	89.92 ± 6.74 *	95.81 ± 7.46	89.63 ± 5.36	97.33 ± 7.72	82.45 ± 6.60 *
6	102.40 ± 7.13	71.65 ± 5.02 *	90.43 ± 9.13	90.12 ± 8.74	81.44 ± 6.66 *	77.17 ± 7.20 *
7	102.40 ± 7.13	81.15 ± 4.99 *	81.41 ± 5.23 *	74.89 ± 5.25 *	78.67 ± 7.44 *	74.32 ± 2.74 *
8	102.40 ± 7.13	81.39 ± 4.59 *	75.25 ± 6.22 *	70.72 ± 2.29 *	84.67 ± 10.35 *	79.33 ± 4.65 *
9	102.40 ± 7.13	77.07 ± 4.10 *	77.63 ± 7.85 *	94.91 ± 7.08	81.04 ± 9.55 *	67.13 ± 2.66 *
10	102.40 ± 7.13	76.99 ± 7.99 *	73.01 ± 6.65 *	76.45 ± 7.33 *	74.56 ± 6.16 *	74.21 ± 4.73 *
11	102.40 ± 7.13	66.21 ± 5.95 *	75.15 ± 4.54 *	80.32 ± 3.93 *	70.99 ± 6.39 *	68.35 ± 5.64 *
12	102.40 ± 7.13	75.68 ± 3.22 *	72.75 ± 3.51 *	77.65 ± 6.37 *	71.12 ± 3.23 *	60.77 ± 4.26 *
13	102.40 ± 7.13	73.20 ± 4.43 *	65.71 ± 4.52 *	64.93 ± 5.66 *	63.81 ± 2.90 *	65.33 ± 3.82 *

The results are expressed as the means ± SE from 2 experiments performed in triplicate. *: $P < 0.05$ for different concentrations of compound versus control.

effect on $O_2^{\cdot-}$ production by HCT-116 cells compared to the control. After 24 h of treatment at a concentration of 100 μM all compounds showed a statistically significant decrease in concentration of $O_2^{\cdot-}$, except compound **3**. The effects of substances **1**, **3**, **4**, **5**, **7**, **9**, and **12** on the HCT-116 cell line show a dose-dependent decrease in $O_2^{\cdot-}$ levels compared to control. The lowest $O_2^{\cdot-}$ level, compared to the other compounds, was found in supernatants of HCT-116 cells treated with compound **12** at a 100 μM concentration. Compound **12** shows a significant dose-dependent decrease in $O_2^{\cdot-}$ levels (P = 0.016).

3.2. Determination of nitrites (Griess assay)

The data shown in Table 2 present nitrite concentrations in the HCT-116 cell line after 24 h of incubation with various concentrations of phenytoin derivatives. Treatment with all compounds showed significant increase in the production of nitrite by HCT-116 cells compared to the control. After 24 h of treatment at a concentration of 100 μM, all compounds showed a statistically significant increase in production of nitrite, except compounds **1**, **3**, **5**, and **6**. The effects of substances **2**, **3**, **4**, **5**, **6**, **7**, **8**, **12**, and **13** on the HCT-116 cells show a dose-dependent increase in the production of nitrite compared to the control. The effect of compound **7** at a concentration of 100 μM was the highest compared to the other compounds. Compounds **4** (P = 0.024) and **7** (P = 0.04) showed significant dose-dependent increases in production of nitrite.

3.3. Determination of total GSH

The data presented in Table 3 show the concentration of total GSH after 24 h of incubation with various concentrations of phenytoin derivatives. All compounds at some investigated concentrations showed a significant increase in total GSH levels compared to the control. After 24 h of treatment at a concentration of 100 μM, all compounds showed statistically significant increases in total GSH levels, except compounds **4**, **9**, and **12**. The effects of substances **2**, **4**, **5**, **6**, and **11** on the HCT-116 cells show dose-dependent increases in total GSH levels compared to the control. The highest GSH level, compared to the other compounds, was detected in HCT-116 cells treated with compound **5** at a concentration of 100 μM. Compound **5** (P = 0.034) showed a significant dose-dependent increase in total GSH levels.

3.4. Redox status for compound 8

The data presented in Figure 2 show the concentrations of superoxide anion radical, nitrites, and total GSH production in the HCT-116 cell line after 24 h of incubation with various concentrations of compound **8** (phenytoin derivatives). The effect of compound **8** in all tested concentrations in the HCT-116 cell line was reflected in a statistically significant reduction in the production of superoxide anion radical as compared to untreated cells. Additionally, compound **8** led to a statistically significant increase in the levels of nitrite at

Table 2. Effect of the 3-substituted-5,5-diphenylhydantoins on the nitrite production in the HCT-116 cell line. The cells were treated with various concentrations of drugs during 24 h of exposure. The effect was measured by the Griess assay, and nitrite concentrations are expressed as nanomoles per milliliter.

	0 μM	0.01 μM	0.1 μM	1 μM	10 μM	100 μM
1	23.61 ± 0.61	38.09 ± 1.83 *	21.02 ± 1.73	27.56 ± 0.71 *	53.44 ± 4.91 *	15.75 ± 1.59 *
2	23.61 ± 0.61	17.38 ± 1.57 *	23.10 ± 2.06 *	26.34 ± 0.29 *	29.23 ± 0.16 *	39.16 ± 0.76 *
3	23.61 ± 0.61	40.83 ± 2.98 *	26.65 ± 2.06	25.23 ± 1.05 *	15.30 ± 0.41 *	8.51 ± 0.17 *
4	23.61 ± 0.61	16.92 ± 1.14 *	16.46 ± 0.86 *	18.54 ± 1.54 *	28.37 ± 0.93 *	40.63 ± 0.97 *
5	23.61 ± 0.61	40.27 ± 2.13 *	27.25 ± 0.75 *	35.51 ± 0.41 *	31.21 ± 1.23 *	14.44 ± 1.85 *
6	23.61 ± 0.61	42.55 ± 0.65 *	42.65 ± 0.99 *	36.04 ± 0.41 *	57.34 ± 0.45 *	18.54 ± 1.56 *
7	23.61 ± 0.61	26.29 ± 0.55 *	36.98 ± 3.42 *	36.73 ± 3.67 *	40.98 ± 1.38 *	56.64 ± 2.58 *
8	23.61 ± 0.61	34.04 ± 1.47 *	32.88 ± 1.57 *	38.30 ± 1.61 *	33.84 ± 3.46 *	39.82 ± 3.89 *
9	23.61 ± 0.61	49.80 ± 2.62 *	44.07 ± 1.41 *	41.13 ± 0.83 *	49.44 ± 1.70 *	41.79 ± 2.46 *
10	23.61 ± 0.61	55.17 ± 3.27 *	58.76 ± 6.64 *	35.76 ± 2.82 *	22.95 ± 1.21	28.47 ± 0.36 *
11	23.61 ± 0.61	39.46 ± 1.29 *	37.28 ± 2.29 *	23.80 ± 1.99	44.78 ± 3.16 *	45.34 ± 1.82 *
12	23.61 ± 0.61	57.40 ± 3.53 *	62.16 ± 1.26 *	64.79 ± 2.66	66.16 ± 3.09 *	48.02 ± 1.65 *
13	23.61 ± 0.61	67.07 ± 2.31 *	61.90 ± 2.75 *	60.99 ± 5.20 *	38.91 ± 3.24 *	28.11 ± 0.40 *

The results are expressed as the means ± SE from 2 experiments performed in triplicate. *: P < 0.05 for different concentrations of compound versus control.

Table 3. Effect of 3-substituted-5,5-diphenylhydantoins on the total GSH level in the HCT-116 cell line. The cells were treated with various concentrations of drugs during a 24 h exposure. The effect was measured by the GSH assay, and concentrations are expressed as nanomoles per milliliter.

	0 μM	0.01 μM	0.1 μM	1 μM	10 μM	100 μM
1	8.23 ± 0.41	12.78 ± 1.04 *	12.40 ± 0.62 *	12.29 ± 0.61 *	8.88 ± 0.64	14.28 ± 0.51 *
2	8.23 ± 0.41	15.10 ± 1.80 *	15.24 ± 1.75 *	15.54 ± 3.16 *	16.16 ± 1.82 *	16.87 ± 1.43 *
3	8.23 ± 0.41	12.06 ± 0.87 *	11.90 ± 1.10 *	12.08 ± 0.86 *	17.14 ± 1.65 *	14.33 ± 0.31 *
4	8.23 ± 0.41	15.56 ± 1.29 *	12.38 ± 1.63 *	12.69 ± 1.09 *	11.65 ± 2.18	9.63 ± 0.46
5	8.23 ± 0.41	9.69 ± 0.34	13.33 ± 1.61 *	10.64 ± 1.57	13.78 ± 1.08 *	19.13 ± 1.30 *
6	8.23 ± 0.41	11.09 ± 0.54 *	11.95 ± 0.99 *	12.89 ± 0.62 *	12.73 ± 0.64 *	13.43 ± 1.81 *
7	8.23 ± 0.41	9.96 ± 0.38 *	9.70 ± 1.04	8.62 ± 0.37	9.41 ± 0.28 *	10.17 ± 0.68 *
8	8.23 ± 0.41	10.11 ± 0.65 *	12.52 ± 0.94 *	9.99 ± 0.48 *	8.59 ± 0.19	8.42 ± 0.31
9	8.23 ± 0.41	11.62 ± 0.88 *	9.71 ± 0.25	9.63 ± 0.45 *	10.71 ± 0.77 *	9.18 ± 0.57
10	8.23 ± 0.41	10.05 ± 0.99	16.19 ± 1.45 *	8.70 ± 0.68	12.38 ± 1.38 *	11.02 ± 1.36 *
11	8.23 ± 0.41	12.78 ± 0.44 *	9.75 ± 0.42 *	11.38 ± 0.74 *	11.52 ± 1.04 *	14.91 ± 1.08 *
12	8.23 ± 0.41	8.78 ± 0.35	10.69 ± 0.77	10.92 ± 1.02 *	12.26 ± 1.67 *	9.18 ± 0.38
13	8.23 ± 0.41	10.97 ± 0.45 *	15.23 ± 1.07 *	13.59 ± 0.81 *	12.69 ± 1.09 *	12.82 ± 0.85 *

The results are expressed as the means ± SE from 2 experiments performed in triplicate. *: $P < 0.05$ different concentrations of compound versus control.

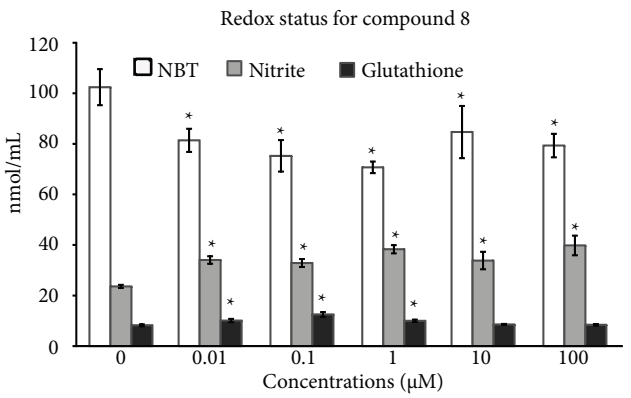


Figure 2. Effect of compound **8** on superoxide anion radical, nitrite, and total GSH levels in the HCT-116 cell line. The cells were treated with various concentrations of drugs during 24 h of exposure. The effect was measured and concentrations are expressed as nanomoles per milliliter.
The results are expressed as the means ± SE from 2 experiments performed in triplicate. *: $P < 0.05$ for different concentrations of compound versus control.

all concentrations compared to the control. The effects of compound **8** showed a statistically significant increase in the total production of GSH at concentrations of 0.01, 0.1, and 1 μM compared to untreated control cells. Compound **8** should be the subject of further research and synthesis of new phenytoin derivatives, due to the high percentage of proliferation inhibition produced at low concentrations (Trišović et al., 2011).

4. Discussion

Colorectal cancer, one of the most common human malignancies, is a major public health problem in the developed world (Yang et al., 2013). Hydantoins have become a focus of interest since they are increasingly used in various fields of medicine. Therefore, many studies target the synthesis and investigation of derivatives of these compounds that show biological activity (Basappa et al., 2009).

Our previous results show that these compounds have significant cytotoxic effects on HCT-116 cell lines of human colon cancer. According to the results, compound **8** showed significant cytotoxic effects at concentrations of 0.01, 0.1, and 1 μM. The other compounds showed no significant inhibition of HCT-116 proliferation at lower concentrations. The unexpected activity of compound **8** at low concentrations implies that the relative activity of these compounds is not determined only by the physicochemical 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 (Trišović et al., 2011).

It is known that oxidative stress and high production of ROS and nitrogen can cause carcinogenesis by genetic and epigenetic mechanisms. Elevated levels of ROS in many tumors strongly implicate oxidative DNA damage in the etiology of cancer. The understanding and synthesis of novel hydantoin derivatives with potential anticancer and antioxidant action can be facilitated through a detailed structural and pharmacological database (Addabbo et al., 2009), and hence there is a need for the design of new hydantoin derivatives with better or different pharmacological profiles.

The introduction of substituents in position 3 produces a trend of changes in antiproliferative activity, which is similar to the changes in anticonvulsant activity. *N*-alkylation reduces the ability of compounds **2**–**7** to form hydrogen bonds and consequently reduces their cytotoxic activity as well at a concentration of 100 μM (Trišović et al., 2011). One feature of cancer cells is the dysfunction of protecting mechanisms against reactive species, because cancer cells are resistant both to the enhanced constitutive oxidative stress and to ROS-generating therapies (Manda et al., 2009). The scavenging potential of the investigated compounds might be in correlation with their structure, because a decrease in $O_2^{\cdot-}$ levels is noticed. On the other hand, a great increase in the $NO^{\cdot}$ content is evident. This can be partly explained by the fact that $NO^{\cdot}$ has a half-life of only several seconds in an environment rich in superoxide anion radicals; surrounded by low levels of superoxide anion radicals, $NO^{\cdot}$ has much greater stability (Valko et al., 2006). Nitric oxide is an important signaling molecule in numerous physiological and pathological conditions (Liu et al., 2003; Rigas and Williams, 2008). $NO^{\cdot}$ has been reported to have antitumor activities as well as protumor properties. Its effect may depend on timing, concentration, and tissue type (Wink et al., 1998). Low concentrations of $NO^{\cdot}$ can stimulate cell growth and protect many cell types from apoptosis, while high concentrations of $NO^{\cdot}$ can inhibit cell growth and induce apoptosis (Kim et al., 2001; Cook et al., 2004). All investigated compounds decrease the oxidative stress to a certain extent by decreasing the $O_2^{\cdot-}$ concentration, and that is one of the reasons why the concentration of nitrite increases. There is no free oxygen radical that can transform nitrite into peroxynitrite. GSH plays an important role in modulating cytotoxicity (Peklak-Scott et al., 2008). In HCT-116 cells, treatment with all compounds causes an increase in the total GSH content, which is suggestive of a GSH-mediated antioxidant response (Chew et al., 2006).

This conclusion can serve as a basis for the design of novel, potent antitumor agents. Further studies should provide a key answer for evaluation of biological effects, specifically those concerning the structure of hydantoin compounds. In conclusion, hydantoins may be potential antioxidants, but it is necessary to perform new research to correlate the structure and biological activity of these compounds in order to confirm this hypothesis. The limitation of this finding is the tumor cell line model system, because further analysis of antioxidative activity of these compounds requires experiments with a normal primary cell. All investigated compounds can reduce the concentration of superoxide anion radical to a certain extent, and this may also be one of the reasons why they induce an increase in concentrations of nitrite. There are no free oxygen radicals that can transform nitric oxide to peroxynitrite. All investigated hydantoins have antioxidant potential, which can be correlated with a decrease in superoxide anion radicals and an increase in concentrations of GSH in our model system.

Acknowledgments

This investigation was supported by the Ministry of Education, Science and Technological Development of the Republic of Serbia (III41010 and 172013).

References

Addabbo F, Montagnani M, Goligorsky MS (2009). Mitochondria and reactive oxygen species. Hypertension 53: 885–892.

Auclair C, Voisin E (1985). Nitroblue tetrazolium reduction. In: Greenwald RA, editor. Handbook of Methods for Oxygen Radical Research. Boca Raton, FL, USA: CRC Press, Inc., pp. 123–132.

Baker MA, Cerniglia GJ, Zaman A (1990). Microtiter plate assay for the measurement of glutathione and glutathione disulfide in large numbers of biological samples. Anal Biochem 190: 360–365.

Basappa, Ananda Kumar CS, Nanjunda Swamy S, Sugahara K, Rangappa KS (2009). Anti-tumor and anti-angiogenic activity of novel hydantoin derivatives: inhibition of VEGF secretion in liver metastatic osteosarcoma cells. Bioorg Med Chem 17: 4928–4934.

Carmi C, Cavazzoni A, Zuliani V, Lodola A, Bordi F, Plazzi PV, Mor M (2006). 5-Benzylidene-hydantoins as new EGFR inhibitors with antiproliferative activity. Bioorg Med Chem Lett 16: 4021–4025.

Cavazzoni A, Alfieri RR, Carmi C, Zuliani V, Galetti M, Fumarola C, Frazzi R, Bonelli M, Bordi F, Lodola A et al. (2008). Dual mechanisms of action of the 5-benzylidene-hydantoin UPR1024 on lung cancer cell lines. Mol Cancer Ther 7: 361–370.

Chew E, Matthews CS, Zhang J, McCarroll AJ, Hagen T, Stevens FGM, Westwell AD, Bradshaw TD (2006). Antitumor quinols: role of glutathione in modulating quinol-induced apoptosis and identification of putative cellular protein targets. Biochem Biophys Res Comun 346: 242–251.

Cook T, Wang Z, Alber S, Liu K, Watkins SC, Vodovotz Y, Billiar TR, Blumberg D (2004). Nitric oxide and ionizing radiation synergistically promote apoptosis and growth inhibition of cancer by activating p53. Cancer Res 64: 8015–8021.

Eckhardt S (2002). Recent progress in the development of anticancer agents. Curr Med Chem Anti-Cancer Agents 2: 419–439.

Fidler IJ (2002). Critical determinants of metastasis. Semin Cancer Biol 12: 89–96.

Griess P (1879). Bemerkungen zu der Abhandlung der HH. Weselsky und Benedikt "Ueber einige Azoverbindungen". Ber Dtsch Chem Ges 12: 426–428 (in German).

Henderson BR, Fagotto F (2002). The ins and outs of APC and beta-catenin nuclear transport. EMBO Rep 3: 834–839.

Kim PK, Zamora R, Petrosko P, Billiar TR (2001). The regulatory role of nitric oxide in apoptosis. Int Immunopharmacol 1: 1421–1441.

Kumar ACS, Kavitha CV, Vinaya K, Benaka PSB, Thimmegoweda NR, Chandrappa S, Raghavan SC, Rangappa KS (2009). Synthesis and in vitro cytotoxic evaluation of novel diazaspiro bicyclo hydantoin derivatives in human leukemia cells: a SAR study. Invest New Drug 27: 327–337.

Lee CW, Hong DH, Han SB, Jong SH, Kim HC, Fine RL, Lee SH, Kim HM (2002). A novel stereo-selective sulfonylurea, 1-[1-(4-aminobenzoyl)-2,3-dihydro-1*H*-indol-6-sulfonyl]-4-phenyl-imidazolidin-2-one, has antitumor efficacy in in vitro and in vivo tumor models. Bio Pharmacol 64: 473–480.

Liu Q, Chan STF, Mahendran R (2003). Nitric oxide induces cyclooxygenase expression and inhibits cell growth in colon cancer cell lines. Carcinogenesis 24: 637–642.

Maguire JH, Murthy AR, Hall IH (1985). Hypolipidemic activity of antiepileptic 5-phenylhydantoins in mice. Eur J Pharmacol 117: 135–138.

Manda G, Nechifor MT, Neagu TM (2009). Reactive oxygen species, cancer and anti-cancer therapies. Curr Chem Biol 3: 342–366.

Peklak-Scott C, Smitherman PK, Townsend AJ, Morrow CS (2008). The role of glutathione *S*-transferase P1-1 (GSTP1-1) in the cellular detoxification of cisplatin. Mol Cancer Ther 7: 3247–3255.

Rigas B, Williams JL (2008). NO-donating NSAIDs and cancer: an overview with a note on whether NO is required for their action. Nitric Oxide 19: 199–204.

Trišović N, Božić B, Obradović A, Stefanović O, Marković S, Čomić Lj, Božić B, Ušćumlić G (2011). Structure-activity relationships of 3-substituted-5,5-diphenylhydantoins as potential antiproliferative and antimicrobial agents. J Serb Chem Soc 76: 1597–1606.

Valko M, Rhodes CJ, Mancol J, Izakovic M, Mazur M (2006). Free radicals, metals and antioxidants in oxidative stress-induced cancer. Chem Biol Interact 160: 1–40.

Wink DA, Vodovotz Y, Laval J, Laval F, Dewhirst MW, Mitchell JB (1998). The multifaceted roles of nitric oxide in cancer. Carcinogenesis 19: 711–721.

Yaari Y, Selzer ME, Pincus JH (1986). Phenytoin: mechanisms of its anticonvulsant action. Ann Neurol 20: 171–184.

Yang J, Li TZ, Xu GH, Luo BB, Chen YX, Zhang T (2013). Low-concentration capsaicin promotes colorectal cancer metastasis by triggering ROS production and modulating Akt/mTOR and STAT-3 pathways. Neoplasma 60: 364–372.