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Novel seleno-hydantoin palladium(II) complex – antimigratory, cytotoxic and prooxidative potential on human colon HCT-116 and breast MDA-MB-231 cancer cells

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Abstract. Selenium and palladium containing compounds separately exert multifunctional effects on cells. While selenium containing compounds usually exert antioxidative properties, palladium(II) containing compounds are cytotoxic and prooxidative. Here we investigated biological effects of bicyclic seleno-hydantoin *cis*-7a-ethyl-5-methyl-5-phenylselanylmethyl-tetrahydro-pyrrolo[1,2-c] imidazole-1,3-dione (Hid-Se), and its palladium(II) complex, trans-bis-(*cis*-7a-ethyl-5-methyl-5-phenylselanylmethyl-tetrahydro-pyrrolo[1,2-c]imidazole-1,3-dionato) palladium(II) chloride ((Hid-Se)$_2$Pd) on human colon HCT-116 and breast MDA-MB-231 cancer cell lines. Hid-Se and (Hid-Se)$_2$Pd showed prooxidative and cytotoxic character. In all performed experiments (Hid-Se)$_2$Pd proved to be more active, i.e. this substance exerted greater prooxidative effect, cytotoxicity and influence on cell migration potential. Even though Hid-Se and (Hid-Se)$_2$Pd enhanced migration of HCT-116 cells, very important feature of these substances is the strong antimigratory potential on metastatic MDA-MB-231 cells.

Key words: Cancer — Cell Migration — Oxidative stress — Palladium — Selenium

Introduction

Cancer (*malignant neoplasia*) represents a large disease group, which is characterized by unregulated cell differentiation and formation of malignant tumors, which may invade other parts of the body affecting the basic physiological functions (Craig et al. 2010). In the field of cancer research the most of chemotherapeutic protocols are created with the aim of possessing the cytotoxic effects (Blagosklonny and Fojo 1999; Angelis et al. 2013). However, as primary tumors are significantly different from their metastatic analogues, the effect of a chemotherapeutic is not same on these types of tumors. Metastatic cells exhibit increased motility and increased cell migration from tumor stroma to other parts of the body (Polyak and Weinberg 2009). Compared to traditional chemotherapy and radiotherapy methods, there are numerous studies which indicate anti-metastatic effects (Eckhardt et al. 2012; Deb et al. 2014).

Modern medicine is still largely focused on the application of platinum-containing (Kalinowska-Lis et al. 2008; Jevtic et al. 2014) and palladium-containing (Sabo et al. 2004; Matovic et al. 2013) chemotherapeutic cytotoxic agents. On the other hand, selenium has proven to be an important supplement for the suppression of the side effects of chemotherapy (Schroeder et al. 2004; Markovic et al. 2011; Brodin et al. 2015; Mut-Salud et al. 2016). Also, selenium-containing compounds exert inhibition of migration of cancer cells (Zec et al. 2012; Chen et al. 2013; Liu et al. 2015). Combining palladium and selenium in the same structure could provide a novel substance with benefits from the both elements. Substances, presented in this study combine bicyclic hydantoin structure and selenium in ligand, and additionally palladium(II) (Pd(II)) in complex of mentioned ligand. The

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goal of this study was to examine the cumulative effect of a hydantoin structure, selenium and palladium on cancer cells. Hydantoins are widely used compounds in cancer studies (Mudit et al. 2009; Hmuda et al. 2014). Moreover, bicyclic hydantoins are also studied (Ananda Kumar et al. 2009; Basappa et al. 2009). Although seleno-organic compounds (Kosaric et al. 2014; Bugarcic et al. 2015) and Pd(II) complexes of hydantoins are investigated (Kushev et al. 2002; Varbanov et al. 2010), according to our knowledge there are no records regarding antitumor activities of bicyclic selenohydantoins and their Pd(II) complexes.

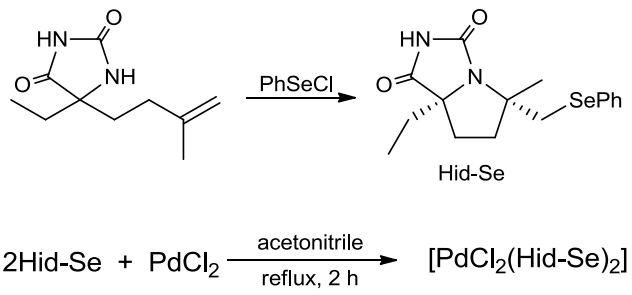
In our investigations we used human adherent colorectal cancer cell line, HCT-116 and human mammary gland breast carcinoma metastatic cells, isolated from lung pleura, MDA-MB-231. Besides the tissue source difference of these two cell lines, one of their most significant differences is metastatic potential. While HCT-116 cells originate from primary tumor, MDA-MB-231 cells are of metastatic origin. Thus, in this study, in addition to investigation of effects of cytotoxicity and impact on redox status, we have chosen also to investigate the effects of tested substances on migratory potential of cells.

Materials and Methods

Synthesis of Hid-Se and its Pd(II) complex (Hid-Se)$_2$Pd

Synthesis of ligand, a seleno derivate of fused bicyclic hydantoin (*cis*-7a-ethyl-5-methyl-5-phenylselanylmethyl-tetrahydro-pyrrolo[1,2-c]imidazole-1,3-dione; Hid-Se), is shown in Scheme 1 and reported recently (Šmit and Pavlović 2015). In the reaction of the ligand with $PdCl_2$, a complex with $[PdCl_2(ligand)_2]$ stoichiometry was obtained.

A solution of 17.7 mg (0.1 mM) $PdCl_2$ in acetonitrile (15 ml) was heated under reflux for 2 hours. Yellow colored solution was cooled to room temperature and 70.3 mg (0.2 mM) of ligand (Hid-Se) dissolved in acetonitrile (2.5 ml) was added dropwise. Reaction mixture was stirred during 24 hours. Resulting yellow powder was filtered off and dried. Yield 60.9 mg (69%).



Scheme 1. Synthesis of Hid-Se and (Hid-Se)$_2$Pd

The structure of the complex was confirmed on the basis of elemental analyses and spectral data.

IR (KBr) ν_{max}: 3419, 3160, 3055, 2970, 1767, 1701, 1576, 1403, 1385, 1241, 1097, 922, 791, 740, 687, 647 cm^{-1}. ^{1}H NMR (200 MHz, DMSO-d_6) δ 0.78 (t, J = 7.3 Hz, 3H), 1.41 (s, 3H), 1.58-1.69 (m, 1H), 1.73 (q, J = 7.3 Hz, 2H), 1.97-2.13 (m, 3H), 3.54 (d, J = 12.5 Hz, 1H), 3.75 (d, J = 12.5 Hz, 1H), 7.20-7.41 (m, 3H), 7.47-7.65 (m, 2H), 10.77 (bs, 1H). ^{13}C (50 MHz, DMSO-d_6) δ 7.5, 29.2, 30.0, 30.8, 35.8, 39.6, 64.2, 74.9, 127.2, 129.5, 130.6, 131.8, 156.8, 176.5. Anal. calc. for $C_{32}H_{40}Cl_2N_4O_4PdSe_2$ C: 43.68, H: 4.58, N: 6.41%; found C: 43.36, H: 4.40, N: 6.76%.

Chemicals

Dulbecco`s modified eagle medium (DMEM) and phosphate-buffered saline (PBS) were obtained from GIBCO, Invitrogen, USA. Foetal bovine serum (FBS) and trypsin-EDTA were from PAA (The Cell Culture Company, Pasching, Austria). Dimethyl sulfoxide (DMSO), bovine serum albumin (BSA), 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT), and nitro blue tetrazolium (NBT) were obtained from SERVA, Heidelberg, Germany. Polyvinyl alcohol mounting medium, N-1-napthylethylenediamine dihydrochloride were purchased from Fluka chemie GMBH, Buchs, Switzerland. Sulfanilamide and sulphosalicylic acid were purchased from MP Hemija Belgrade, Serbia. 5,5′-dithio-bis(2-nitrobenzoic acid) was purchased from Sigma Chemicals Co., St Louis, MO, USA. Paraformaldehyde originated from Merck, Germany. All solvents and chemicals were of analytical grade. The stock solutions were prepared in 100% DMSO at final concentration of 100 mM. Subsequent dilution was performed with DMEM cell growing medium, where in the highest treatment concentration (500 μM) DMSO concentration was at 0.05%, which is non-toxic to investigated cells.

Cell preparation and cultivation

Human colorectal HCT-116 and breast MDA-MB-231 cancer cell lines were purchased from the American Tissue Culture Collection (Manassas, VA, USA). The cells were cultivated in 75 cm^2 culture flasks under controlled conditions in a humidified atmosphere with 5% CO_2 at 37°C in DMEM serum supplemented with 10% FBS and 100 IU/ml penicillin and 100 μg/ml streptomycin until reaching a confluency of about 80%. For MTT, NBT and Griess assays, 10^4 cells were seeded in 96-well plates in triplicates for each treatment concentration, while for GSH assay it was seeded 5×10^4 cells per well. For immunofluorescence determination of expression of iNOS protein 7×10^4 cells

were seeded in triplicates in 6-well plates on glass coverslips (Thermo Scientific), while for migratory potential inhibition assay 10^5 cells *per* well were seeded in transwell cell culture chamber.

Determination of cell viability and redox status parameters

Cell viability was determined by MTT (Mosmann 1983) assay. Redox parameters: superoxide anion radical determined by NBT (Auclair and Voisin 1985), nitrites by Griess (Griess 1879) and glutathione by GSH assay (Baker et al. 1990). For investigation of influence of tested substances on cell viability and redox status we used standardized procedures briefly described in our previous papers (Kosaric et al. 2014; Petrovic et al. 2014, 2015). For the purposes of redox status assays we used concentrations of 1, 10, 50 and 100 μM, while IC_{50} values are determined as a plot of % cytotoxicity *versus* sample concentrations. All results were expressed as μM.

Inducible nitric oxide synthase (iNOS) protein expression (immunofluorescence microscopy)

Inducible nitric oxide synthase (iNOS) protein expression in HCT-116 and MDA-MB-231 cells was detected by immunofluorescence method (Javois 1999). Cells were cultured in 6-well plates on glass coverslips. When cells were at about 80% confluence the supporting medium was removed and the cells were treated with medium containing tested substances at final concentration of 50 μM. After 24 h medium was removed and cells were triple washed with PBS (pH 7.2). Next, the cells were fixed with 4% *p*-formaldehyde in PBS for 20 min at 37°C. After the fixation, the cells were washed three times with PBS and then permeabilized with methanol at −20°C for 2 min, washed with PBS three times and blocked on non-specific binding sites using 1% BSA for 20 min. The fixed cells were incubated with 20 μg/ml anti-iNOS specific primary antibody (RD Systems) for 1 h at 37°C. Sample coverslips were then washed twice and incubated with anti-mouse secondary antibody conjugated with Alexa448 (Thermo Scientific) at a 1:200 dilution in PBS. DAPI was used to stain the cell nuclei (blue) at 1:1000 dilutions. Sample coverslips were washed twice and mounted on glass slide by polyvinyl alcohol mounting medium. The cell visualization was performed at Nikon inverted fluorescent microscope (Ti-Eclipse) at 600× magnification by using Nikon NIS-Elements Advanced Research software.

Migratory potential inhibition assay

Principle of migratory assay is based on potential of investigated substance to inhibit or promote cell migration. Cells are placed on the upper layer of a cell permeable membrane with the medium containing tested substance. After incubation period, the cells which are migrated through the membrane were stained and counted. Migration of HCT-116 and MDA-MB-231 cancer cells through 8 μm filter pores was followed using Transwell cell culture chamber (ThinCert with translucent membranes, Greiner Bio-One GmbH, Germany) (Entschladen et al. 2005). Firstly, cells that are cultured in DMEM medium with 10% FBS were twice washed with PBS and resuspended in the serum free DMEM medium. After centrifugation at 800 rpm for 3 min the 10^5 cells *per* well were resuspended in 500 μl of serum free DMEM medium with addition of Hid-Se and $(Hid\text{-}Se)_2Pd$ and plated in 24-well plates onto the upper compartment of the chamber. The final concentrations of substances were 10 and 100 μM, respectively. In the lower compartment of the chamber 750 μl of complete medium (DMEM with 10% FBS as chemoattractant) was added. The control cells have not been treated with the substances. After 24 h of incubation at 37°C and 5% CO_2 cells were washed three times with PBS and fixed with 4% *p*-formaldehyde for 20 minutes at room temperature, followed by washing three times with PBS. After fixation, the cells were carefully and thoroughly removed with cotton swab. Remains of cells at the lower part of the Transwell membrane were stained with 0.1% of Crystal Violet in 200 mM MES buffer, pH 6.0 for 10 minutes at room temperature. After washing, and drying at room temperature overnight, membranes were cut and placed in another 24-well plate. Crystal Violet stained membranes were resuspended in 100 μl 10% acetic acid and 80 μl of stained acetic acid solution was moved to a 96-well microtiter plate with subsequent reading of optical density at 595 nm at Microplate reader.

Statistics

All experimental data were expressed as mean ± standard error (SE). Biological activity assays are performed in triplicate for each dose. Statistical significance was determined using the one-way ANOVA test for multiple comparisons. $p < 0.05$ was considered as significant. The magnitude of correlation between variables was done using SPSS (Chicago, IL) statistical software package (SPSS for Windows, version 17, 2008). The IC_{50} values were calculated from the dose curves by a computer program (CalcuSyn).

Results

Cytotoxic effects

The cytotoxicity of investigated substances was determined by MTT assay. The results were expressed graphically in

Table 1. Growth inhibitory effects – IC_{50} values of Hid-Se and $(Hid\text{-}Se)_2Pd$ on HCT-116 and MDA-MB-231 cell lines after 24 and 72 h of exposure

Tested substance	IC_{50} (μM)			
	HCT-116		MDA-MB-231	
	24 h	72 h	24 h	72 h
Hid-Se	> 500	92.1 ± 3.1	> 500	288.5 ± 13.8
$(Hid\text{-}Se)_2Pd$	105.7 ± 8.4	130.7 ± 7.6	> 500	81.6 ± 7.2

All values are mean ± SE.

Figure 1 and as IC_{50} values presented in Table 1. Evaluation on HCT-116 cells revealed that investigated substances exhibit cytotoxic character. Hid-Se induced decreasing of cell viability in dose and time depended manner, with significant toxicity 72 h from treatment. On the other hand, $(Hid\text{-}Se)_2Pd$ exhibits more significant cytotoxic effect acutely, i.e. after 24 h. Based on the obtained results, $(Hid\text{-}Se)_2Pd$ is more cytotoxic to HCT-116 cells than Hid-Se. On MDA-MB-231 cells, investigated substances showed cytotoxic character exhibited in dose and time dependent manner, i.e. with increasing the substance concentration cell viability decreased which is more evident 72 h from treatment. After 24 h from treatment the both substances exhibited no cytotoxic effect, while after 72 h the cytotoxic effect was obvious. The more significant effect was obtained with $(Hid\text{-}Se)_2Pd$. From the results explaining cytotoxic character one could observe that HCT-116 cells are more sensitive to the investigated substances than MDA-MB-231 cells.

Redox status

Superoxide anion radical ($O_2^{\bullet-}$) concentration

Superoxide anion radical, $O_2^{\bullet-}$ is an important indicator of reactive oxygen species (ROS) level (Hancock et al. 2001). $O_2^{\bullet-}$ was determined by spectrophotometric NBT assay, and results of measurement are depicted in Table 2. Measurement on HCT-116 cells revealed that Hid-Se and $(Hid\text{-}Se)_2Pd$ induced significant change in $O_2^{\bullet-}$ content.

After 24 h Hid-Se induced decreasing of $O_2^{\bullet-}$ in low concentrations, while higher concentrations significantly increased $O_2^{\bullet-}$. On the other hand, 72 h from treatment Hid-Se decreased $O_2^{\bullet-}$ content. Similar decreasing was

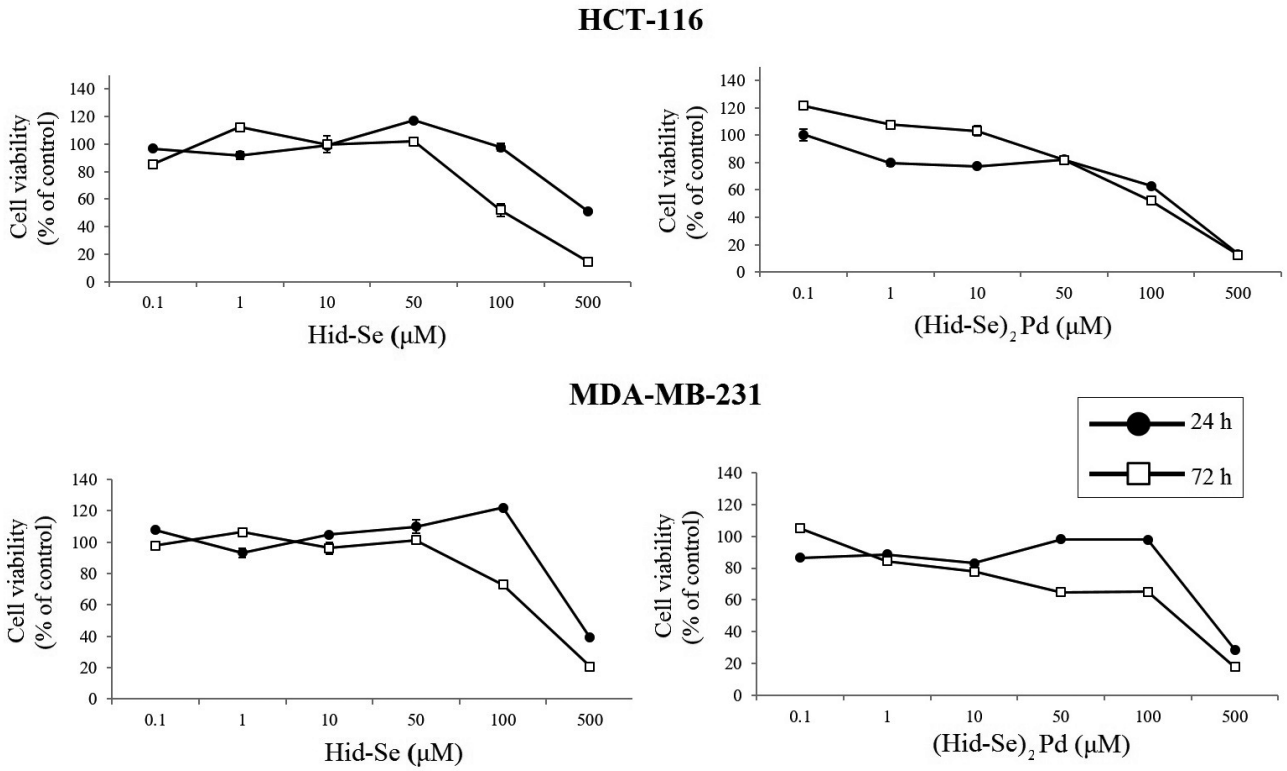


Figure 1. The dose response curve of effect of Hid-Se and $(Hid\text{-}Se)_2Pd$ on HCT-116 and MDA-MB-231 cell lines after 24 and 72 h of exposure. All values are mean ± SE.

Table 2. Effects of Hid-Se and $(Hid\text{-}Se)_2Pd$ on HCT-116 and MDA-MB-231 cell lines, expressed as the $O_2^{\bullet-}$ concentration after 24 h and 72 h of exposure

Concentration (μM)	Superoxide anion radical, $O_2^{\bullet-}$ (μM)							
	HCT-116				MDA-MB-231			
	Hid-Se		$(Hid\text{-}Se)_2Pd$		Hid-Se		$(Hid\text{-}Se)_2Pd$	
	24 h	72 h	24 h	72 h	24 h	72 h	24 h	72 h
0	39.20 ± 0.20	29.04 ± 0.54	39.20 ± 0.20	29.04 ± 0.54	25.12 ± 0.12	24.88 ± 0.38	25.12 ± 0.12	24.88 ± 0.38
1	35.28 ± 1.78*	28.76 ± 0.14	33.93 ± 1.58*	25.23 ± 0.23*	27.91 ± 0.06*	26.04 ± 0.30*	30.68 ± 2.22*	25.40 ± 0.43
10	31.91 ± 0.17*	29.57 ± 0.38	33.75 ± 2.00*	26.08 ± 0.27*	27.05 ± 0.17*	24.03 ± 1.09	27.69 ± 0.30*	29.11 ± 1.35*
50	38.88 ± 0.66	26.53 ± 0.10*	31.65 ± 1.71*	25.40 ± 0.05*	28.32 ± 1.70*	22.37 ± 0.10*	27.75 ± 0.21*	30.00 ± 1.35*
100	44.29 ± 1.59*	25.40 ± 1.05*	36.57 ± 0.49	30.52 ± 1.81	28.28 ± 1.59*	26.08 ± 1.42	26.75 ± 0.34*	36.31 ± 1.59*

* $p < 0.05$ as compared to the control cells.

observed with $(Hid\text{-}Se)_2Pd$ in both treatment periods. $(Hid\text{-}Se)_2Pd$ exerted stronger reduction of $O_2^{\bullet-}$ content than Hid-Se on HCT-116 cells. While tested substances, in general, decreased $O_2^{\bullet-}$ in HCT-116 cells, on MDA-MB-231 cells they acted as prooxidants, i.e. induced significant increasing in $O_2^{\bullet-}$ content 24 and 72 h from treatment. This effect was stronger after 24 h, where $(Hid\text{-}Se)_2Pd$ again showed greater influence. Comparison of these two cell lines showed that $O_2^{\bullet-}$ production was greater on MDA-MB-231 cells.

Nitrites (NO_2^-) concentration

Concentration of nitrites indicates quantity of nitrosonium ions (NO^+) in anaerobic conditions. In water, the final product of aerobic phase reaction between nitrogen oxide (NO) and O_2 is nitrogen dioxide (NO_2), which quickly combines with excess of NO, forming nitrogen trioxide (N_2O_3) that is hydrolyzed to nitrites. So, nitrite concentration may indicate level of NO and other reactive nitrogen species (RNS) in cells (Lancaster 2006). Results of measurement of nitrite level are presented in Table 3. On HCT-116 cells in treatment with Hid-Se it was observed decreasing of nitrites, except in the highest concentration which induced significant increasing. $(Hid\text{-}Se)_2Pd$ significantly induced increasing of nitrites. On MDA-MB-231 cells after 24 h Hid-Se in lower concentrations induced decreasing, while higher concentrations induced increasing of nitrite concentration. After 72 h change of nitrites was not significant as after 24 h. $(Hid\text{-}Se)_2Pd$ predominantly induced significant increasing of nitrite level. Similarly to NBT test, measurement of nitrite concentration indicated MDA-MB-231 cells as more sensitive to investigated substances when compared to HCT-116 cells.

Reduced glutathione (GSH) concentration

Glutathione is an important tripeptide responsible for neutralizing of ROS/RNS and thus for maintaining the redox equilibrium in eukaryotic cell (Pompella et al. 2003). Table 4 represents the effects of investigated substances on GSH level in HCT-116 and MDA-MB-231 cells. On HCT-116 we

Table 3. Effects of Hid-Se and $(Hid\text{-}Se)_2Pd$ on HCT-116 and MDA-MB-231 cell lines, expressed as the NO_2^- concentration after 24 h and 72 h of exposure

Concentration (μM)	Nitrites, NO_2^- (μM)							
	HCT-116				MDA-MB-231			
	Hid-Se		$(Hid\text{-}Se)_2Pd$		Hid-Se		$(Hid\text{-}Se)_2Pd$	
	24 h	72 h	24 h	72 h	24 h	72 h	24 h	72 h
0	33.85 ± 0.15	45.77 ± 0.77	33.85 ± 0.15	45.77 ± 0.77	43.65 ± 0.65	50.77 ± 0.47	43.65 ± 0.65	50.77 ± 0.47
1	18.28 ± 0.25*	55.77 ± 2.78*	41.81 ± 2.60*	47.80 ± 3.25	29.08 ± 1.73*	55.31 ± 1.12*	42.50 ± 2.43	58.55 ± 0.53*
10	24.35 ± 0.89*	51.60 ± 2.99*	45.49 ± 2.75*	44.35 ± 0.79	36.03 ± 1.48*	50.13 ± 2.62	65.78 ± 9.00*	53.02 ± 0.12*
50	23.96 ± 1.03*	54.32 ± 0.40*	53.67 ± 0.20*	51.25 ± 0.68*	78.65 ± 8.25*	48.64 ± 0.17	91.23 ± 5.93*	48.17 ± 2.63
100	60.12 ± 1.26*	32.95 ± 4.94*	57.58 ± 0.69*	45.78 ± 3.46	66.38 ± 9.50*	48.45 ± 2.59	84.20 ± 2.83*	52.95 ± 0.53

* $p < 0.05$ as compared to the control cells.

Table 4. Effects of Hid-Se and $(Hid\text{-}Se)_2Pd$ on HCT-116 and MDA-MB-231 cell lines, expressed as the GSH concentration after 24 h and 72 h of exposure

Concentration (μM)	GSH (μM)							
	HCT-116				MDA-MB-231			
	Hid-Se		$(Hid\text{-}Se)_2Pd$		Hid-Se		$(Hid\text{-}Se)_2Pd$	
	24 h	72 h	24 h	72 h	24 h	72 h	24 h	72 h
0	19.04 ± 0.14	15.61 ± 0.11	19.04 ± 0.14	15.61 ± 0.11	16.94 ± 0.44	19.55 ± 0.45	16.94 ± 0.44	19.55 ± 0.45
1	18.35 ± 0.49	14.52 ± 0.34	18.60 ± 0.32	15.23 ± 0.53	18.56 ± 1.51	27.51 ± 1.51*	18.63 ± 0.26*	16.88 ± 0.30
10	18.30 ± 0.21	14.06 ± 0.24	21.77 ± 0.21*	16.91 ± 0.15*	19.54 ± 1.36	23.45 ± 0.97*	21.61 ± 0.98*	25.03 ± 2.05*
50	18.09 ± 0.21	16.07 ± 0.30	20.75 ± 0.93	16.32 ± 0.38	20.14 ± 0.22*	23.28 ± 2.50*	16.95 ± 0.15	20.69 ± 0.88
100	19.97 ± 0.29	16.56 ± 0.73	18.45 ± 0.74	16.52 ± 0.19	20.14 ± 1.58*	22.73 ± 1.19*	17.09 ± 0.34	18.78 ± 0.94

* $p < 0.05$ as compared to the control cells. GSH, reduced glutathione.

observed a moderate effect on GSH level change, i.e. Hid-Se did not induced any statistically significant changing of GSH concentration, while cells treated with $(Hid\text{-}Se)_2Pd$ slightly increased GSH level. This increasing was slightly higher after 24 h. On MDA-MB-231 cells it was observed significant increasing in GSH level with both substances. It could be concluded that greater increasing in GSH for $(Hid\text{-}Se)_2Pd$ is followed by greater increasing in ROS/RNS induced with this substance. Thus, cells respond to changing in redox equilibrium by lesser or greater induction in GSH production.

Inducible nitric oxide synthase (iNOS) protein expression

Nitric oxide synthase is a protein which induces the synthesis of NO from L-arginine (Liu and Gross 1996). In order to investigate the possible origin of the increase of nitrites we considered examination of the expression of iNOS protein. On HCT-116 cells 24 h from treatment Hid-Se in concentration of 50 μM decreased expression of iNOS protein (Figure 2). Similarly, in the same measuring conditions Hid-Se significantly decreased nitrite content (Table 3). On the other hand, $(Hid\text{-}Se)_2Pd$ significantly increased

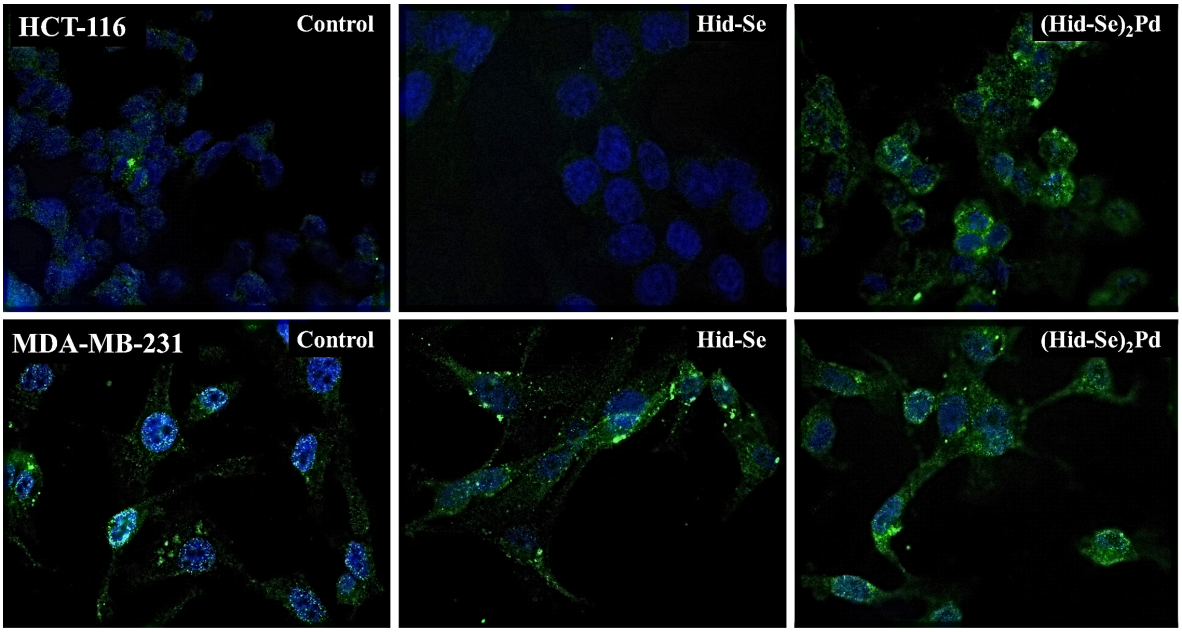


Figure 2. Detection of iNOS protein expression in HCT-116 and MDA-MB-231 cells by immunofluorescence staining. Cells are treated with 50 μM substances and iNOS protein expression was examined 24 h from treatment. Cell nuclei are DAPI stained to blue, while iNOS proteins are green (NIS-Elements Advanced Research Captures merged). All sections were examined by Nikon inverted fluorescent microscope (Ti-eclipse) at 600× magnification.

iNOS protein expression, which also could be related to the nitrite production (Table 3). On MDA-MB-231 cells both substances induced significant increasing of iNOS protein expression.

Migratory potential inhibition

To evaluate influence of tested substances on migratory potential we examined the migration of cells through Transwell membrane assembled in 24-well plate. The size of pores on Transwell membrane is 8.0 μm, i.e. small enough compared to cancer cells. Thus, investigated cells influenced by substances, could only actively migrate through the pores in greater or smaller extent. Influence of tested substances on migratory potential of HCT-116 cells is less significant when compared to the results obtained with MDA-MB-231 cells (Figure 3). Investigated substances induced increasing of HCT-116 motility. Hid-Se in concentration of 10 μM showed no effect, while concentration of 100 μM increased migration for about 15%. On the other hand $(Hid\text{-}Se)_2Pd$ increased cell migration in concentrations of 10 μM and 100 μM for 60 and 80%, respectively. MDA-MB-231 cells are influenced in a great extent, especially in concentration of 100 μM. Hid-Se induced no statistically significant change in concentration of 10 μM, but 100 μM reduced migration for about 65%. $(Hid\text{-}Se)_2Pd$ in concentration of 10 μM increased migration for 20%, while 100 μM reduced migration for 60%. All results presented in Figure 3 are recalculated in relation with the number of survived cells estimated in MTT assay.

Discussion

Results presented in this paper are the first investigation of biological activity of such fused bicyclic seleno-hydantoin and its Pd(II) complex on cancer cell lines. Cytotoxic character of Pd(II) complexes, hydantoins and seleno-derivatives is proved and well explained in numerous articles (Kushev et al. 2002; Sabo et al. 2004; Ananda Kumar et al. 2009; Basappa et al. 2009; Mudit et al. 2009; Varbanov et al. 2010; Matovic et al. 2013; Hmuda et al. 2014; Kosaric et al. 2014; Bugarcic et al. 2015). Comparing with results obtained in this article, many of tested Pd(II) complexes, seleno-organic compounds, hydantoins and their combinations exerted considerable cytotoxicity on cancer cell lines. Such pronounced cytotoxicity in most cases is closely related to their prooxidative character. This point of view is important, but one of the most important features that also could be followed is the influence on potential of cancer cells to migrate. Our results suggest that these compounds possess potential to reduce migration of metastatic MDA-MB-231 cells.

At the beginning it was considered that Pd(II) complexes possess no anti-tumor properties. Compared to cisplatin, cispalladium, *cis*-$[Pd(NH_3)_2Cl_2]$ complex does not show anti-tumor activity. Coordination chemistry of Pd(II) and Pt(II) is very similar whereas their structural and equilibrium behavior of the solutions are very similar (Bugarčić et al. 2015), but introduction of specific ligands in coordination with Pd(II) can result with great anti-tumor activities (Divsalar et al. 2011; Ulukaya et al. 2011). Compared to action of the cisplatin presented in our previous article (Petrovic et al. 2015), our results showed that Hid-Se exerts lower cytotoxic effects, while $(Hid\text{-}Se)_2Pd$ showed greater effect 24 h after treatment on HCT-116 cells and lower but comparable effect on MDA-MB-231 cells. From the presented results it could be concluded that Hid-Se and especially $(Hid\text{-}Se)_2Pd$ exert cytotoxic character. More significant effect showed $(Hid\text{-}Se)_2Pd$, especially on HCT-116 cells. Cytotoxicity is closely related to enhanced production of ROS/RNS, i.e. superoxide anion radical and nitrites. The produced $O_2^{\bullet -}$ could be from many possible origins. One is that structures of investigated

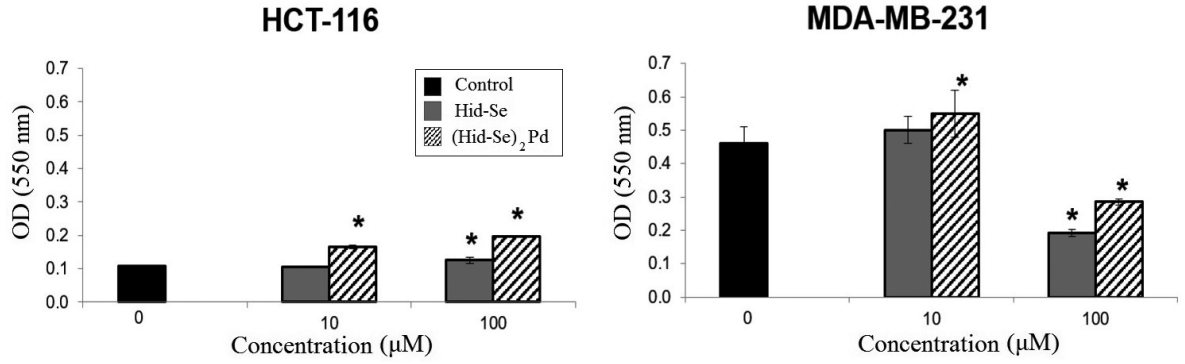


Figure 3. The influence of Hid-Se and $(Hid\text{-}Se)_2Pd$ on migratory potential of HCT-116 and MDA-MB-231 cells, after 24 h of treatment related to the number of viable cells. The cells were treated with substances with concentrations of 10 and 100 μM. All values are mean ± SE, $n = 2$, * $p < 0.05$ as compared to control. OD, optical density measured spectrophotometrically on 595 nm.

substances possess asymmetric electron densities that could provide electron(s) that could subsequently start radical chain reaction. Another source certainly could be explained by Fenton reaction inside the cells. H_2O_2 created in cells easily reacts with iron yielding $O_2^{\bullet-}$. Besides iron, also many transition metals could catalyze Fenton reaction, such as palladium, platinum and rhodium (Halliwell and Gutteridge 2007). Nitrites, and thus the NO in cancer cell, also could be originated from a wide range of sources. One of possibilities, we intended to investigate was iNOS. This specific protein from the family of nitric oxide synthases (NOS) could be responsible for statistically significant formation of nitrites and also according to Xu et al. (2002) for the cytotoxicity in tumor cells. Our results follow strong relationship between iNOS synthesis induction/suppression and nitrite level in tested cells. According to showed analogy, we could suggest that these substances significantly influenced iNOS protein expression, and thus the increasing or decreasing of level of nitrites. On the other hand, NO (and thus nitrite) level greatly depends on $O_2^{\bullet-}$ production. This could be explained by fact that $O_2^{\bullet-}$ possess great affinity towards NO, forming peroxynitrites (Ferrer-Sueta and Radi 2009). Also, the fact that investigated substances contain a lot of nitrogen atoms in their imidazole structure could offer a possibility that substances were metabolized by cells. As the human cell is very complex system, every source that impacts at least one of the intracellular parameters greatly influences the redox equilibria (Cordero and deMiguel 2012). Greater production of free radical species influenced greater production of glutathione, which is in agreement with the nature of GSH and its cellular defense role against disruption of redox equilibria. Hid-Se does not possess such a denominated prooxidative and cytotoxic character as $(Hid\text{-}Se)_2Pd$ does, thus the changing in GSH level influenced by Hid-Se is not as significant as with $(Hid\text{-}Se)_2Pd$.

In addition to examination of the influence of tested substances on the viability and redox status of cells, testing the impact on migratory potential is also very important. A certain substance does not need to possess significant cytotoxic character, but if given substance significantly reduces the migratory potential then it may be considered as potentially interesting for further studies towards the synthesis the appropriate drug. Our results showed that Hid-Se and $(Hid\text{-}Se)_2Pd$ induced increasing in migration potential of HCT-116 cells. On the other hand, these substances greatly decreased the migration potential of metastatic MDA-MB-231 breast cancer cells. The occurrence that MDA-MB-231 cells migrate more intensive than HCT-116 cells is previously described (Wu et al. 2011), and the result indicating that MDA-MB-231 cells are migratory limited is promising. Also, it has been found that significant increase in ROS/RNS has direct impact on antimigratory effect on cancer cells (Urbich et al. 2002). It was found that moderate production of ROS/RNS promotes cancer migration (Nishikawa 2008), while overproduction possesses an opposite effect, i.e. significantly reduces cancer cell migration (Fini et al. 2008). Considering the cell viability MTT assay, one could observe that HCT-116 cells are more sensitive than MDA-MB-231 cells, which is in agreement with our earlier findings (Kosaric et al. 2014; Petrovic et al. 2014, 2015). But, redox status parameters measured and presented in this paper suggest that MDA-MB-231 cells are more susceptible to ROS/RNS increasing in treatment with investigated substances. We observed moderate ROS/RNS increasing in HCT-116 cells, and thus increasing in cell migration. In MDA-MB-231 cells lower concentration (10 μM) predominantly increased ROS/RNS in less significant extent when it is compared to higher concentration (100 μM). According to Nishikawa (2008) and Fini et al. (2008) we consider that moderate increasing of ROS/RNS induced with 10 μM of substances promotes cell migration, while higher 100 μM treatment induced great oxidative stress and thus decreasing in cell migration potential.

Evidence that these substances possess cytotoxic and antimigratory character (especially on metastatic MDA-MB-231 cells) appears to be promising in terms of considering the further serious examination *in vitro* and/or *in vivo*. The exact mechanism of this action still remains unclear and many studies is needed to be done to reveal influence of superoxide anion radical and other radical species on migration processes in origin-different cancer cells.

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