Accepted Manuscript

New glutathione peroxidase mimetics – insights into antioxidant and cytotoxic activity

Agata J. Pacuła, Katarzyna B. Kaczor, Angelika Wojtowicz, Jędrzej Antosiewicz, Anna Janecka, Angelika Długosz, Tomasz Janecki, Jacek Ścianowski

PII: S0968-0896(16)30995-6
DOI: http://dx.doi.org/10.1016/j.bmc.2016.10.018
Reference: BMC 13345

To appear in: *Bioorganic & Medicinal Chemistry*

Received Date: 12 July 2016
Revised Date: 11 October 2016
Accepted Date: 15 October 2016

Please cite this article as: Pacuła, A.J., Kaczor, K.B., Wojtowicz, A., Antosiewicz, J., Janecka, A., Długosz, A., Janecki, T., Ścianowski, J., New glutathione peroxidase mimetics – insights into antioxidant and cytotoxic activity, *Bioorganic & Medicinal Chemistry* (2016), doi: http://dx.doi.org/10.1016/j.bmc.2016.10.018

New glutathione peroxidase mimetics – insights into antioxidant and cytotoxic activity

Agata J. Paculła[a], Katarzyna B. Kaczor[b], Angelika Wojtowicz[b], Jędrzej Antosiewicz[b], Anna Janecka[c], Angelika Długosz[c], Tomasz Janecki[d] and Jacek Ścianowski[a*]

[a]*Department of Organic Chemistry, Faculty of Chemistry, Nicolaus Copernicus University, 7 Gagarin Street, 87-100 Torun, Poland;* [b]*Department of Bioenergetics and Physiology of Exercise, Medical University of Gdansk, 1 Debinki Street, 80-211 Gdansk, Poland;* [c]*Department of Biomolecular Chemistry, Faculty of Medicine, Medical University of Lodz, Mazowiecka 6/8, 92-215 Lodz, Poland;* [d]*Institute of Organic Chemistry, Lodz University of Technology, Zeromskiego 116, 90-924 Lodz, Poland; *e-mail: jsch@chem.umk.pl*

Abstract: A series of *N*-alkyl benzisoselenazol-3(2*H*)-ones has been obtained and transformed to corresponding diselenides by the reduction with sodium borohydride. Additionally, efficient methodology for the oxidative Se-N bond formation by potassium iodate has been presented, new conversion of diselenide to benzisoselenazolone was observed. The GPx-like activity of all synthetized derivatives has been evaluated by NMR. *N*-allyl diselenide was up to five times better antioxidant than ebselen. Anticancer capacity towards MCF7 and DU145 cancer cells has been also tested. The highest antiproliferative activity was obtained for *N*-cyclohexyl benzisoselenazolone.

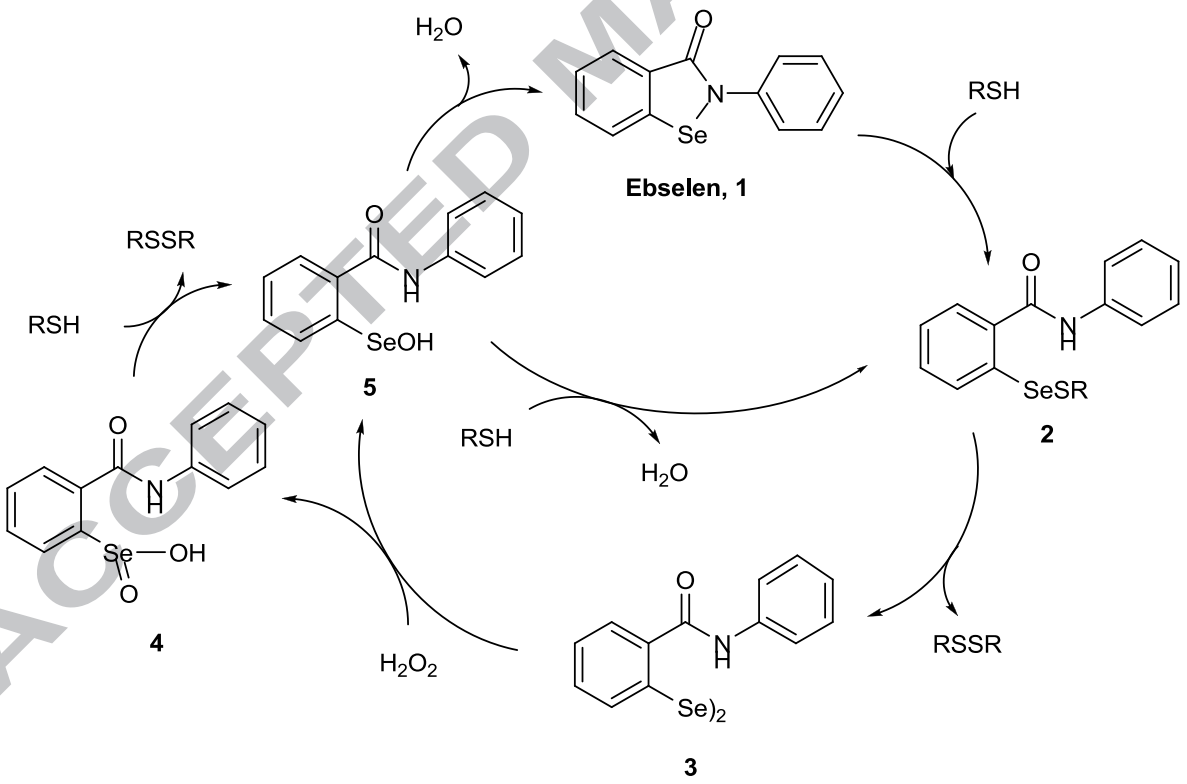
Key words: benzisoselenazolones/ diselenides / antioxidant activity / cytotoxicity

1. Introduction

Reactive oxygen species (ROS) are important factors both in health and disease. At physiological concentrations ROS act as modulators of the cells signaling pathways and immune system responses.[1] When the formation of ROS increases, exceeding the compensatory antioxidant processes, oxidative stress is generated. This state can be explained as the imbalance between the formation and elimination of ROS leading to the disruption of the redox homeostasis of cells. This can induce various pathological processes leading to cancer, diabetes, neurodegenerative diseases and cardiovascular system dysfunctions.[2] Conversely, ROS can stimulate proliferation and activate pro survival signaling pathways in cancer cells. [3,4] The redox homeostasis in the cell can be maintained by a natural antioxidant defense system in which several enzymes take part including the selenoenzyme glutathione peroxidase (GPx). The presence of selenocysteine in the active site of GPx is crucial for its

peroxide scavenging capacity. In recent years organoselenium compounds had emerged as promising antioxidants able to act as an artificial selenocysteine and mimic the activity of GPx.[5] Ebselen (*N*-phenylbenzisoselenazol-3(2*H*)-one) **1** is currently one of the most thoroughly studied organoselenium molecules that exhibit high therapeutic potential.[6] It was proven to be active in the treatment of Alzheimer's disease,[7] retinopathies,[8] diabetes [9] and inflammation [10] and is also at clinical trial for bipolar disorder [11] and hearing loss.[12]

Recent mechanistic investigations performed by Mugesh and co-workers resulted in new conclusions concerning the catalytic cycle for the antioxidant activity of ebselen. Compound **1** reacts with a thiol to produce an unstable selenenyl sulfide **2** which, through a disproportionation reaction, forms the diselenide **3**. Compound **3** reduces hydrogen peroxide, producing seleninic **4** and selenenic acid **5**. In the presence of an excess of thiol, compound **4** and **5** react with RSH to form selenenyl sulfide **2**. Ebselen is regenerated by the elimination of a water molecule from selenenic acid **5** (Scheme 1).[13]



Scheme 1. Catalytic cycle for the elimination of hydrogen peroxide by ebselen

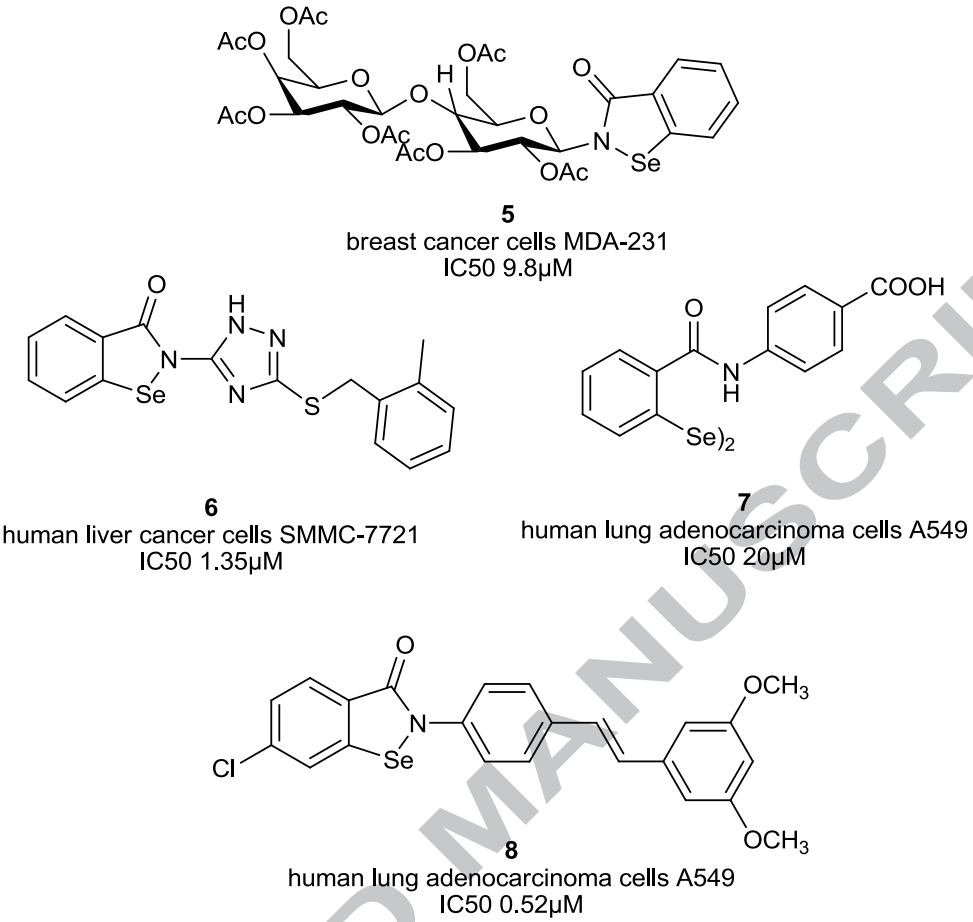
According to Mugesh the rate limiting step of the cycle is the disproportionation reaction to diselenide **3**, which depends not on the structure of the reduced peroxide but on the

type of the thiol used as cofactor. In the assay mixture with the excess of thiol and peroxide the predominant form is the seleninic acid **4** and the selenenyl sulfide **2**. The presence of seleninic acid was established using ^{77}Se NMR spectroscopy and the structure was additionally confirmed by X-ray analyses[14,15] However, current investigations, applying DFT techniques, indicate that after the formation of selenenyl sulfide **2**, the preferred path is the reaction with a second molecule of RSH to form a selenol which is able to reduce hydrogen peroxide with the lowest activation barrier giving the selenenic acid **5**.[16]

As suggested in the known ebselen catalytic cycle the benzisoselenazolone reduces hydrogen peroxide after being converted to a diselenide. To investigate if this conversion takes place, and formation of the diselenide is the rate determining step of the cycle, we decided to test in an NMR antioxidant activity assay both the benzisoselenazoles and diselenides to evaluate if applying directly the diselenide would increase the rate of the reaction proving this activity-structure relation.

In vivo both ebselen and diselenides can be easily metabolized. After intravenous administration the extracellular pool of ebselen is bounded to the cysteine residue of albumin and transported to cells. Inside the cell the favored target organelle are the endoplasmic reticulum and mitochondria.[17] The metabolic pathway includes isoselenazolone ring opening with Se-N bond cleavage, further methylation to 2-methyl selenobenzanilide or glucuronidation to 2-glucuronyl selenobenzanilide released with bile.[6] For the diaryl diselenides a convenient way of excretion includes a reaction with GSH to form a selenol-GSH adduct, further transformation to mercapturic acid derivatives which can be released with urine.[18]

Selenium atom incorporated in the structure of ebselen is not bioavailable, thus not toxic. The ability to form Se-S bonds with the thiol group of cysteine residues in proteins is associated with significant pharmacological functions, including the cytotoxic activity.[19] Several research groups had modified the structure of ebselen to increase its antitumor activity. Various substitutions on the nitrogen atom, with a saccharide moiety **5**,[20] a triazole scaffold **6**,[21] conversion to a diselenide **7**[22] or combining with the structure of resveratrol **8**[23] enabled to obtain promising results revealing the potential of this field of research in the search for new organoselenium chemotherapeutics (Scheme 2).



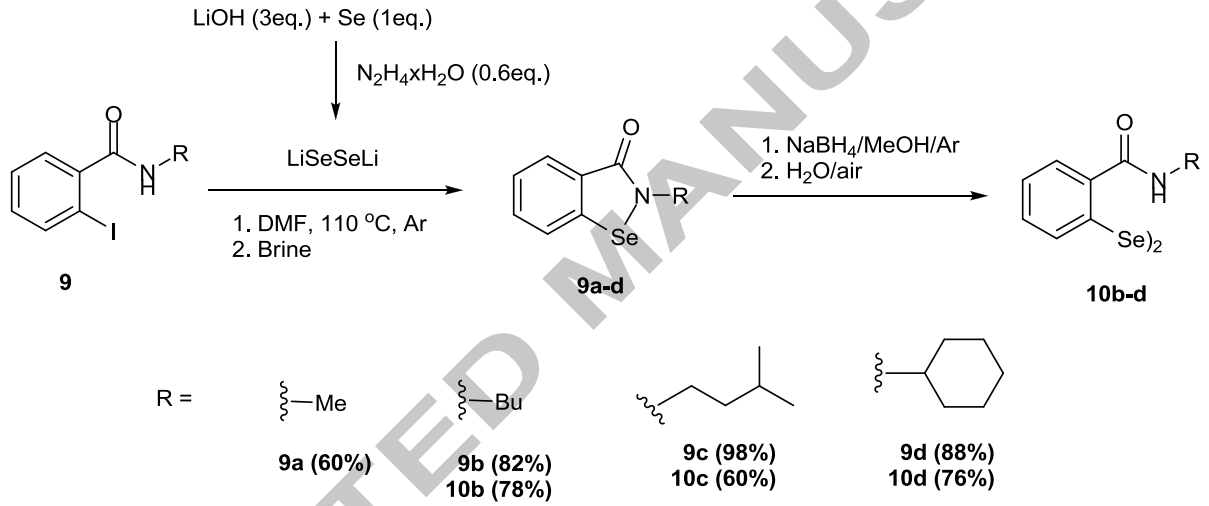
Scheme 2. Examples of ebselen derivatives with antiproliferative potential

During our previous investigations we have observed a significant decrease in solubility when an aryl moiety was introduced on the nitrogen atom. In contrary, *N*-alkyl derivatives were easily dissolved in the media used for the performed antioxidant activity assays. As good solubility is an important feature when choosing the right candidate for screening and preliminary results were promising we have decided to evaluate further the biological potential of the *N*-alkyl benzisoselenazolones. Moderate GPx-like activity of ebselen is also associated with its low solubility. Derivatives presented in this paper bear an *N*-alkyl moiety which improves solubility while maintaining the stable arylseleno core. This arylseleno structure is less susceptible to converse to more toxic inorganic forms than alkylseleno compounds. The aim of this work was to synthesize a series of *N*-substituted benzisoselenazolones, transform them to corresponding diselenides and compare their antioxidant and cytotoxic activity *in vitro*.

2. Results and discussion

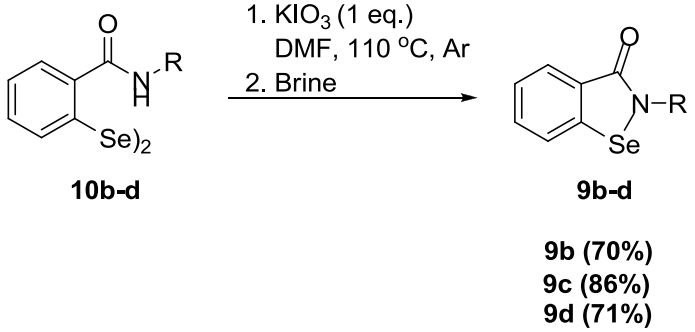
2.1. Chemistry

We have used our previously published methodology in which lithium diselenide, obtained *in situ* from elemental selenium and lithium hydroxide in the presence of hydrazine hydrate, reacts with *N*-substituted *o*-iodobenzamides **9** to form *N*-substituted benzisoselenazolones **9a-d** (Scheme 3).[24] *N*-methyl **9a**, *N*-butyl **9b**, *N*-3-methylbutyl **9c** and *N*-cyclohexyl **9d** derivatives were further converted to corresponding diselenides **10b-d** by the reduction with sodium borohydride and oxidation with air.



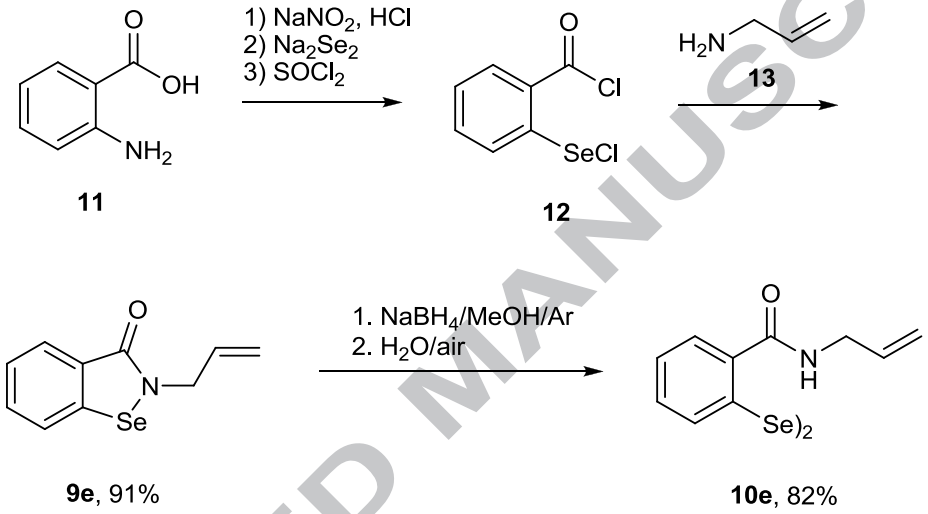
Scheme 3. Synthesis of *N*-substituted benzisoselenazolones **9a-d** and diselenides **10b-d**

We also noticed that obtained diselenides **10b-d** can be transformed into starting benzisoselenazolones **9b-d** by the oxidative formation of the Se-N bond using potassium iodate. Cyclization of the diselenides proceeded in good yields (Scheme 4).



Scheme 4. Synthesis of *N*-substituted benzisoselenazolones **9b-d**

In turn, *N*-allyl benzisoselenazolone **9e** was obtained by transformation of anthranilic acid **11** into 2-chloroselenobenzoyl chloride **12**, followed by the reaction with allyl amine **13**[25] (Scheme 5). Procedure previously described for the synthesis of **9a-d** did not work in this case. Benzisoselenazolone **9e** was also reduced to corresponding diselenide **10e**. Interestingly, the formation of Se-N bond by treatment with KIO_3 did not occur leading to the assumption that due to the electronic effect of the allylic moiety, the Se-N bond can be rapidly cleaved to form a more stable diselenide **10e** (Scheme 4).



Scheme 5. Synthesis of *N*-allyl derivatives **9e** and **10e**

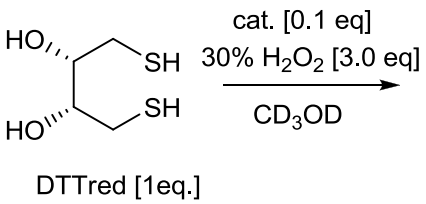
2.2. Antioxidant activity

Second part of our research involved evaluation of the antioxidant activity of the synthesized benzisoselenazolones and diselenides using the methodology presented by Iwaoka and co-workers. Dithiothreitol (DTT^{red}) was oxidized to dithiane (DTT^{ox}) by hydrogen peroxide in the presence of 10% of the Se-catalyst. Rate of the reaction was evaluated on the basis of ^1H NMR spectra. Decay of the substrate and increase of the product concentration were observed (Table 1, Fig. 1).[26]

The best peroxide scavenging capacity was obtained for *N*-allyl diselenide **10e**, a total conversion of the substrate was assessed after 30 min of the reaction.

All *N*-alkyl benzisoselenazolones showed better antioxidant activity than ebselen. In case of diselenides **10 b-d** we did not observe any increase of antioxidant activity. Only diselenide

10e demonstrated a satisfying result. Probably, the ease of formation of ebselen derivatives from diselenides influences the speed of this reaction. When the diselenide is not converted to ebselen in the reaction conditions (see scheme 1) the antioxidant properties are better (see also reaction with KIO_3, only diselenide **10e** did not form the ebselen **9e**). In our opinion the stability of Se-N bond in ebselen derivatives influences the speed of this reaction. Strong Se-N bond gives lower antioxidant activity.



DTTred [1eq.] DTTox

	Remaining Dithiothreitol [%]				
Catalyst [0.1 equiv.]	3 min	5 min	15 min	30 min	60 min
9a	78	65	35	11	0
9b	81	59	41	32	29
9c	77	58	42	28	13
9d	75	69	62	55	44
9e	76	69	58	53	41
Ebselen	84	75	64	58	52
10b	93	88	82	77	73
10c	65	49	38	31	21
10d	92	85	75	65	54
10e	59	36	12	0	0

Table 1. Antioxidant activity of the tested Se-catalysts

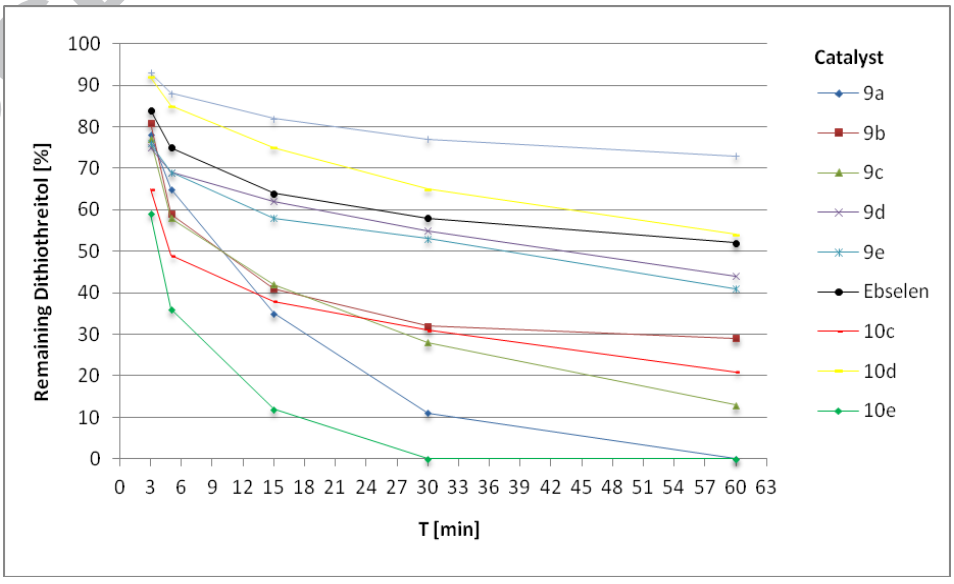


Fig 1. Graphical presentation of antioxidant activities

2.3. Cytotoxic activity

Cytotoxic activity of the obtained derivatives was evaluated by the MTT assay using breast carcinoma MCF-7 cell line. SRB viability assay was performed on prostate cancer cell line DU-145 and noncancerous epithelial cell line PNT1A. The IC_{50} values are presented in Table 2.

Table 2. Cytotoxic activity evaluated *in vitro* [IC_{50}]

Entry	Compound	MCF-7 (MTT assay)	DU-145 (SRB assay)	PNT1A (SRB assay)
		IC_{50}, µM		
1	**9a**	32.67 ± 1.15	30.18±0.09	>40
2	**9b**	13.77 ± 0.29	20.76±0.36	>40
3	**9c**	29.0 ± 1.90	30.39± 0.13	>40
4	**9d**	**7.31 ± 0.14**	**5.71±0.39**	**>40**
5	**9e**	25.70 ± 3.40	10.30 ±0.15	>40
7	**10b**	> 500	>40	-
8	**10c**	> 500	>40	-
9	**10d**	90 ± 0.0	>40	-
10	**10e**	125 ± 15	20.41±0.12	-

The cytotoxic activity was correlated with the structure of the tested compounds. For both cancer cell lines the *N*-cyclohexyl benzisoselenazolone **9d** was the most active derivative. *N*-allylic diselenide **10e** exhibited a moderate antiproliferative activity in comparison to the high antioxidant activity obtained for the oxidation of DTT^{red}. When we compare the antioxidant and cytotoxic properties of *N*-alkyl ebselen derivatives we can see that the best cytotoxicity was observed for ebselen **9d** with the lowest antioxidant activity. Stable S-N bond gives better cytotoxic properties. In normal prostate cell line all ebselen derivatives did not reveal high cytotoxivity and antiproliferative activity. The IC50 values were above 40 µM in all of the tested compunds.

Moreover, to get insight to the mechanism of antiproliferative activity of the studied compounds, active form of Akt kinase (p-Akt) has been measured. Akt is a serine threonine kinase which is often up regulated in many cancer types including breast and prostate [27,28] It has been shown to stimulate proliferation and survival of cancer cells and its activity can be increased by ROS.[3] Additionally, most of anticancer drugs efficacy depends on their ability to target Akt kinase.[29] Interestingly, both compounds **9d** and **9c** significantly reduced p-Akt level in DU145 cell line (Figure 1). Moreover, compound **9d** had no effect on Akt activity in normal epithelial cell line (PNT1A).

3. Conclusions

We have obtained a series of *N*-alkyl benzisoselenazolones and developed an efficient methodology for their conversion to diselenides with sodium borohydride and the reverse reaction to form the Se-N bond with the use of potassium iodate as an oxidant. All prepared compounds were evaluated as antioxidants and anticancer agents. *N*-allyl diselenide **10e** was the most reactive antioxidant. The highest antiproliferative activity was obtained for *N*-cyclohexyl benzisoselenazolone **9d**. We have shown that antioxidant and cytotoxic ativity strongly depends on the stability of the Se-N bond present in the 'ebselen-like' structures. The highest cytotoxic activity was observed for compounds with the lowest antioxidant activity. In addition, our preliminary data indicate that compounds with antiproliferative activity **9d**

and **9c** target Akt kinase in cancer cells but not in PNT1A normal human prostate epithelial cells. This suggests that they can have potential anticancer activity in *in vivo* conditions.

The newly obtained compounds were selected based on their antiproliferative activity and are currently under further investigation. They are evaluated for the molecular mechanism of action on cancer cell lines with different genetic background.

4. Experimental

^{1}H NMR spectra were obtained at 400 or 700 MHz and chemical shifts were recorded relative to $SiMe_4$ (δ 0.00) or solvent resonance ($CDCl_3$ δ7.26, CD_3OD δ3.31). Multiplicities were given as: s (singlet), d (doublet), dd (double doublet), ddd (double double doublet), t (triplet), td (triple doublet), dt (double triplet) and m (multiplet). The number of protons (n) for a given resonance was indicated by *n*H. Coupling constants were reported as a *J* value in Hz. ^{13}C NMR spectra were acquired at 100.6Hz and chemical shifts were recorded relative to solvent resonance ($CDCl_3$ δ77.25). ^{77}Se NMR spectra were recorded on Bruker Avance III / 400 or Bruker Avance III / 700 with diphenyl diselenide as an external standard. Commercially available solvents DMF, DCM and MeOH (Aldrich) and chemicals were used without further purification. Column chromatography was performed using Merck 40-63D 60Å silica gel.

4.1. Synthesis of compound 9e.[25]

4.1.1. *N*-allyl-1,2-benzisoselenazol-3(2*H*)-one 9e

Yield: 91%, mp 119-121^{o}C, ^{1}H NMR (400 MHz, $CDCl_3$) δ = 4.49 (dt, *J*=1.2, 6.0 Hz, 2H), 5.32 (ddd, *J*=1.2, 2.4, 10.0 Hz, 1H), 5.38 (ddd, *J*=1.2, 2.4, 16.8 Hz, 1H),), 5.93-6.03 (m, 1H), 7.43 (ddd, *J*=1.2, 7.2, 8.0 Hz, 1H_{ar}), 7.57-7.67 (m, 2H_{ar}), 8.07 (ddd, *J*=0.4, 1.2, 7.6 Hz, 1H_{ar}) ppm; ^{13}C NMR (100.6 Hz, $CDCl_3$) δ = 47.1 (CH_2), 119.2 (CH_2), 124.0 (CH), 126.1 (CH_{ar}), 127.6 (C_{ar}), 128.8 (CH_{ar}), 131.9 (CH_{ar}), 133.5 (CH_{ar}), 137.9 (C_{ar}), 166.9 (C=O) ppm; ^{77}Se (76.3 MHz, $CDCl_3$), δ = 887.32 ppm, IR 3270, 2956, 2925, 2865, 1634, 1601, 1585, 1257 cm^{-1}. Elemental Anal. Calcd for $C_{10}H_9NOSe$ (238.14): C, 50.43; H, 3.81. Found: C, 50.12; H, 3.89.

4.2. Synthesis of diselenides

General procedure for the synthesis of compounds 10b-10d: To a solution of benzisoselenazolone **9b-9d** (1.0 mmol) in methanol (10 ml) cooled to 0^{o}C, sodium

borohydride (1.0 mmol) was added and the mixture was stirred for 1h. Water (15ml) was added and the mixture was oxidized with air for 1h. Formed precipitate was filtered and dried in air.

4.2.1. 2,2'-Diselenobis(*N*-butylbenzamide) 10b

Yield: 78%, mp 175-177°C (lit.[30] mp 181-183°C), ^{1}H NMR (400 MHz, $CDCl_3$) δ = 0.99 (t, *J*=7.2 Hz, 3H, CH_3), 1.40-1.51 (m, 2H), 1.60-1.70 (m, 2H), 3.53 (dt, *J*=6.0, 7.2 Hz, 2H), 6.81 (bs, 1H, NH), 7.23 (td, J=1.2, 7.2 Hz, 1H$_{ar}$), 7.28-7.32 (m, 1H$_{ar}$), 7.49 (dd, *J*=1.6, 7.6 Hz, 1H$_{ar}$), 7.91 (dd, *J*=1.2, 8.0 Hz, 1H$_{ar}$) ppm; ^{13}C NMR (100.6 Hz, $CDCl_3$) δ = 13.7 (CH_3), 20.18 (CH_2), 31.7 (CH_2), 40.0 (CH_2), 126.1 (CH_{ar}), 126.5 (CH_{ar}), 131.4 (CH_{ar}), 131.6 (CH_{ar}), 132.9 (C_{ar}), 133.4 (C_{ar}), 168.2 (C=O) ppm; ^{77}Se (76.3 MHz, $CDCl_3$), δ = 451.98 ppm, IR 3301, 2954, 1609, 1584, 1541, 1433, 1317, 1026 cm^{-1}.

4.2.2. 2,2'-Diselenobis[*N*-(3-methylbutyl)benzamide) 10c

Yield: 60%, mp 160-162°C, ^{1}H NMR (700 MHz, $CDCl_3$) δ = 0.98 (d, *J*=0.7 Hz, 6H, 2xCH_3), 1.53-1.57 (m, 2H), 1.70-1.75 (m, 1H), 3.50-3.54 (m, 2H), 6.12 (bs, 1H, NH), 7.22 (td, *J*=1.4, 7.7 Hz, 1H$_{ar}$), 7.28 (td, J=1.4, 7.7 Hz, 1H$_{ar}$), 7.45 (dd, *J*=1.4, 7.7 Hz, 1H$_{ar}$), 7.89 (dd, *J*=0.7, 7.7 Hz, 1H$_{ar}$) ppm; ^{13}C NMR (100.6 Hz, $CDCl_3$) δ = 22.1 (2xCH_3), 25.6 (CH), 38.06 (CH_2), 38.2 (CH_2), 125.6 (CH_{ar}), 126.1 (CH_{ar}), 131.0 (CH_{ar}), 131.2 (CH_{ar}), 132.5 (C_{ar}), 132.9 (C_{ar}), 167.7 (C=O) ppm; ^{77}Se (76.3 MHz, $CDCl_3$), δ = 451.67 ppm, IR 3292, 2953, 2925, 1620, 1541, 1450, 1309, 1282 cm^{-1}. Elemental Anal. Calcd for $C_{24}H_{32}N_2O_2Se_2$ (538.44): C, 53.54; H, 5.99. Found: C, 53.42; H, 5.93.

4.2.3. 2,2'-Diselenobis(*N*-cyclohexylbenzamide) 10d

Yield: 76%, mp 261-263°C, ^{1}H NMR (400 MHz, $CDCl_3$) δ = 1.18-1.36 (m, 4H), 1.40-1.45 (m, 1H), 1.66-1.75 (m, 1H), 1.77-1.85 (m, 2H), 2.10 (dd, *J*=4.0, 12.8 Hz, 2H),), 4.00-4.09 (m, 1H), 6.03 (d, *J*=6.8 Hz, 1H, NH), 7.23-7.26 (m, 1H$_{ar}$), 7.48 (dd, *J*=1.6, 7.6 Hz, 1H$_{ar}$), 7.61-7.65 (m, 1H$_{ar}$), 7.9 (d, *J*=8.0 Hz, 1H$_{ar}$) ppm; ^{13}C NMR (100.6 Hz, DMSO) δ = 25.3 (2xCH_2), 25.6 (CH_2), 32.7 (2xCH_2), 49.1 (CH), 126.5 (CH_{ar}), 128.4 (CH_{ar}), 129.8 (C_{ar}), 131.8 (CH_{ar}), 132.3 (CH_{ar}), 134.0 (C_{ar}), 166.9 (C=O) ppm; ^{77}Se (76.3 MHz, DMSO), δ = 443.47 ppm, IR 3275, 2924, 2851, 1614, 1583, 1536, 1450, 1432, 1372, 1281, 1149, 1080, 1026 cm^{-1}.

4.2.4. 2,2'-Diselenobis(*N*-allylbenzamide) 10e

Yield: 82%, mp 201-203°C, ^{1}H NMR (700 MHz, $CDCl_3$) δ = 4.14 (tt, *J*=1.4, 5.6 Hz, 2H), 5.23 (ddd, *J*=1.4, 2.8, 10.5 Hz, 1H), 5.32 (ddd, *J*=1.4, 2.8, 10.5 Hz, 1H),), 5.90-6.01 (m, 1H), 6.23 (s, 1H, NH), 7.23 (td, *J*=1.4, 7.7 Hz, $1H_{ar}$), 7.30 (td, J=1.4, 7.7 Hz, $1H_{ar}$), 7.51 (dd, *J*=1.4, 7.7 Hz, $1H_{ar}$), 7.91 (dd, *J*=1.4, 7.7 Hz, $1H_{ar}$) ppm; ^{13}C NMR (100.6 Hz, $CDCl_3$) δ = 42.6 (CH_2), 117.2 (CH_2), 126.1 (CH), 126.6 (CH_{ar}), 131.5 (CH_{ar}), 131.8 (CH_{ar}), 133.0 (C_{ar}), 133.2 (C_{ar}), 133.8 (CH_{ar}), 168.0 (C=O) ppm; ^{77}Se (76.3 MHz, $CDCl_3$), δ = 453.62 ppm, IR 3301, 2954, 2924, 1609, 1539, 1317, 1260, 1163 cm^{-1}. Elemental Anal. Calcd for $C_{20}H_{20}N_2O_2Se_2$ (478.30): C, 50.22; H, 4.21. Found: C, 49.90; H, 4.27.

4.3. Conversion of diselenides to benzisoselenazolones.

To a solution of diselenide **10b-10d** (1.0 mmol) in DMF (3 ml), under argon atmosphere, potassium iodate (1.0 mmol) was added and the mixture was stirred at 120°C for 24h. Mixture was cooled to ambient temperature and brine (5ml) was added. Mixture was stirred for 3h and the formed precipitate was filtered and dried in air. Yield: 70% (**9b**), 86% (**9c**), 71% (**9d**).

4.4. Evaluation of antioxidant activity[26].

To a solution of compounds **9a-9e** and **10b-10e** (0.015mmol) and dithiothreitole DTT^{red} (0.15mmol) in 1.1mL of CD_3OD 30% H_2O_2 (0.15mmol) was added. ^{1}H NMR spectra were measuared right after addition of hydrogen peroxide and then in specific time intervals. The concentarion of the substrate was determined according to the changes in the integration on the ^{1}H NMR spectra.

4.5. Cell viability assays

4.5.1. MTT viability assay

Cell culture. The MCF-7 cell line was purchased from the European Collection of Cell Cultures (ECACC). The MCF-7 cells were cultured in Minimum Essential Medium Eagle (MEME, Sigma-Aldrich) with glutamine (2 mM) (Sigma-Aldrich), MEM Non-essential amino acid solution 100x (Sigma-Aldrich), gentamycin (5 lg/mL) and 10% heatinactivated fetal bovine serum (FBS) (both from Biological Industries, Beit-Haemek, Israel). Cells were maintained at 37°C in a 5% CO_2 atmosphere and were grown until 80% confluent.

MTT assay. The MTT assay was performed according to the known procedure.[31] The cells were grown to sub-confluent levels in the culture medium and then plated onto 24-well plates (10^4 cells/well) in the final volume of 1 ml of the culture medium. After 24 h, vehicle

(0.1% DMSO) or compounds in various concentrations were added and the plates were incubated for 24 h. Then, the cells were incubated for 2 h at 37^{o}C with 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide [MTT, 5 mg/ml in phosphate buffered saline (GIBCO, Invitrogen, Carlsbad, CA, USA)]. The absorbance of the blue formazan product was measured at 540 nm using an automated plate reader (iMark Bio-Rad, Hercules, CA, USA) and compared with the control (untreated cells). All experiments were performed in triplicate.

4.5.2. SRB viability assay

Cell culture. The prostate cancer cell line DU-145 and noncancerous human prostate cell line PNT1A were purchased from the American Type Culture Collection (ATTC, Manassas, VA). DU 145 cells were cultured in MEME medium supplemented with 10 % fetal bovine serum, 1% penicillin/streptomycin, 2 mM glutamine and 1 mM sodium pyruvate at 37°C under 5% CO_2 incubator. Stock solutions of *N*-substituted ebselen derivatives were prepared in (0,1%) DMSO. The PNT1A cells were cultured in RPMI 1640 supplemented with serum, L-glutamine and antibiotics.

SRB assay. The prostate cancer cell line DU-145 and noncancerous human prostate epithelial cells PNT1A were used in this study. Cell viability was measured by Sulphorhodamine B (SRB) assay. The cells were grown to sub-confluent levels at the certain culture medium and then seeded into 96-well plates at 6.0 x 10^{3} cells/ well in the final volume of 200 µl in the culture medium for 24 h. Then, they were treated with various concentrations (2,5, 5, 10, 20, 30, 40 µl) of *N*-substituted ebelsen derivatives for the next 24h. After incubation, the cells were fixed in 20% trichloroacetic acid for an 1h. The plates were washed with distilled water and 0,4% SRB (Sigma Aldrich) in 1% acetic acid solution was added to the plates for 15 minutes. The SRB solution was washed with 1% acetic acid. SRB was then solubilized in 10 mM Trisma-base solution and the absorbance was measured at 570 nm using an automated microplate reader. The experiments were done in triplicate and the IC_{50} values were calculated.

4.5.3 Western blotting

The cells were treated with 40µM *N*-cyclohexyl-1,2-benzisoselenazol-3(2*H*)-one and 40 µM and *N*-(3-methyl)-butyl-1,2-benzisoselenazol-3(2*H*)-one at different time points. Both floating and attached cells were collected : washed in PBS; resuspended in a lysis solution containing 50 mmol/L Tris–HCl (pH 7.5), 150 mmol/L NaCl, 1% Triton X-100, 0.1% SDS; and

incubated for 40 min on ice with gentle shaking. The cell lysate was cleared by the centrifugation at16.000g for 20 min. Lysate proteins were resolved in 10–12% SDS-PAGE and transferred onto polyvinyli-dene difluoride membrane. The membrane was incubated with a solution containing Tris-buffered saline, 0.05% Tween 20, and 5–10% (w/v) nonfat dry milk and then exposed to the desired primary antibody for 1 h at room temperature. Following treatment with the appropriate secondary anti-body, the bands were visualized using enhanced chemiluminescence method. Changes in protein level was assessed by densitometric scanning of the bands and cor-rected for β-actin loading control.

Acknowledgments

This work was supported by the National Science Centre, Poland, grant no. UMO-2015/17 /B/ NZ7/03058.

5. References

1. Zuo, L.; Zhou, T.; Pannell, K. B.; Ziegler A. C. Best, T. M. *Acta. Physiol.*, **2015**, *214*, 329–348.
2. (a) Sies, H. *Redox Biol*, **2015**, *4*, 180–183. (b) Steinbrenner, H.; Sies, H. *Bio. et Biophy. Acta.*, **2009**, *1790*, 1478–1485.
3. Borkowska, A.; Sielicka-Dudzin, A.; Herman-Antosiewicz, A.; Wozniak, M.; Fedeli, D.; Falcioni, G.; Antosiewicz, J. *Eur. J. Nutr.*, **2012**, *51*, 817–825.
4. Luo, L.; Kaur Kumar, J.; Clement, M. V. *Free Radic. Biol. Med*. **2012**, *53*, 1697–1707.
5. (a) Pacuła, A. J.; Mangiavacchi, F.; Sancineto, L.; Lenardao, E. J.; Ścianowski J.; Santi, C. *Curr. Chem. Biol.*, **2015**, *9*, 97–112. (b) Weekley, C.M.; Harris, H. H. *Chem. Soc. Rev.*, **2013**, *42*, 8870–8894. (c) Nogueira, C. W.; Rocha, J. B. T. in Organic Selenium and Tellurium Compounds; Rappoport, Z., Ed.; Wiley: Chichester, 2012; Volume 3, Part II, Chapter 21. (d) Back, T. G. in "Biochalcogen Chemistry: The Biological Chemistry of Sulfur, Selenium and Tellurium", eds. Bayse, C. and Brumaghin, J., ACS Symposium Series, American Chemical Society, 2013, Ch. 7. (e) Day, B. J. *Biochem. Pharmacol*. **2009**, *77*, 285–296. (f) Bhabak, K. P.; Mugesh, G. *Acc. Chem. Res.* **2010**, *43*, 1408–1419. (g) Mugesh, G.; Singh, H. B. *Chem. Soc. Rev.* 2000, 29, 347–357. (h) Mugesh, G.; du Mont, W.-W.; Sies, H. *Chem. Rev*. 2001, *101*, 2125–2179.
6. Parnham J. M.; Sies, H. *Biochem. Pharm.*, **2013**, *86*, 1248–1253.
7. Martini, F.; Bruning, C. A.; Soares, S. M.; Naguiera C. W.; Zeni, G. *Current Pharmaceutical Design*, **2015**, *21*, 920–924.
8. Tan, S. M.; Deliyanti, D.; Figget, W. A.; Talia, D. M.; de Haan, J. B.; Wilkinson-Berka, J. L. *Experimental Eye Research*, **2015**, *136*, 1–8.
9. Park, S.; Kang, S.; Kim, D. S.; Shin, B. K.; Moon, N. R.; Daily III, J. W. *Free Radical Research*, **2014**, *48*, 864–874.
10. Schewe, C.; Schewe, T.; Wendel, A. *Biochem. Pharmacol.* **1994**, *48*, 65–74.

11. N. Singh, N.; Sharpley, A. L.; Emir, U. E.; Masaki, C.; Herzallah, M. M.; Gluck, M. A.; Sharp, T.; Hamer, C. J.; Vasudevan, S. R.; Coven, P. J.; Churchill, G. C. *Neuropsychopharmacology* **2016**, *41*, 1768–1778.
12. Lynch, E. D.; Kill, J. *Drug Discovery Today*, **2005**, *10*, 1291–1298.
13. Mugesh, G. *Curr. Chem. Biol.*, **2013**, *7*, 47–56.
14. Sarma, K. B.; Mugesh, G. *Chem. Eur. J.* **2008**, *14*, 10603 – 10614.
15. Bhabak, K. P.; Mugesh, G. *Acc. Chem. Res.*, **2010**, *43*, 1408–1419.
16. Orian, L.; Toppo, S. *Free Radic. Biol. Med.*, **2014**, *66*, 65–74.
17. Kumar Azad, G.; Tomar, R. S. *Mol. Bio. Rep*., **2014**, *41*, 4865-4879.
18. Prigol, M.; Nogueira, C. W.; Zeni, G.; Bronze, M. R.; Constantino, L. *Chem. Biol. Interact.*, **2012**, *200*, 65–72.
19. Kumar Azad, G.; Singh, V.; Mandal, P; Singh, P.; Golla, U.; Barnawal, S. Chauhan, S.; Tomar, R. S. *FEBS Open Bio.*, **2014**, *4*, 77–89.
20. Bijain, K.; Zhang, Z.; Xu, B.; Jie, S.; Chen, B.; Wan, Sh.; Wu, J.; Jiang, T.; Alaoui-Jamali, A. M. *Eur. J. Med. Chem.*, **2012**, *48*, 143–152.
21. (a) Li, B.-L.; Li, B.; Zhang, R.-L.; Zhao, J.-J.; Wang, X.-F.; Liu, Y.-M.; Shi, Y.-P.; Liu, J.-B.; Chen, B.-Q. *Bioorg. Med. Chem. Lett.*, **2016**, *26*, 1279–1281. (b) Zhao, J.; Xuan, L; Zhao, H.; Cheng, J.; Fu, X.; Li, S.; Jing, F.; Liu, Y.; Chen, B. *Chem. Res. Chin. Univ.*, **2014**, *30*, 764–769.
22. Młochowski, J.; Kloc, K.; Syper, L.; Inglot, A. D.; Piasecki, E. *Liebigs Ann. Chem*., **1993**, 1239–1244.
23. Yan, J.; Guo, Y.; Wang, Y.; Mao, F.; Huang, L.; Li, X. *Eur. J. Med. Chem.*, **2015**, *95*, 220–229.
24. Pacuła, A. J.; Ścianowski, J.; Aleksandrzak, K. B. *RSC Adv*, **2014**, *4*, 48959–48962.
25. Welter, A.; Christiaens, L.; Ferdinand, W.-P. Eur. Pat. Appl. EP 44453, **1982**, *Chem. Abstr.*, **1982**, *96*, 199699v.
26. Kumakura, F.; Mishra, B.; Priyadarsini, K. I.; Iwaoka, M. *Eur. J. Org. Chem*., **2010**, 440–444.
27. Altomare, D. A.; Testa, J. R. *Oncogene*, **2005**, *24*, 7455–7464.
28. Schmidt, M.; Voelker, H. U.; Kapp, M.; Krockenberger, M.; Dietl, J.; Kammerer, U. *J. Cancer. Res. Clin. Oncol.*, **2010**, *136*, 219–225.
29. Cheng, J. Q.; Lindsley, C. W.; Cheng, G. Z.; Yang, H.; Nicosia, S. V. *Oncogene*, **2005**, *24*, 7482–7492.
30. Pietka-Ottlik, M.; Potaczek, P.; Piasecki, E.; Młochowski, J. *Molecules*, **2010**, *15*, 8214–8228.
31. Mosmann, T. *J. Immunol. Methods*, **1983**, *65*, 55–63.

Graphical abstract