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IL FARMACO 59 (2004) 863–868
IL FARMACO
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Azaanalogues of ebselen as antimicrobial and antiviral agents: synthesis and properties

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Received 28 May 2004; accepted 16 July 2004

Available online 11 September 2004

Abstract

The different analogues of ebselen—unsubstituted benzisoselenazol-3(2H)-one (**2a**) 2-pyridylbenzisoselenazol-3(2H)-ones (**2b–h**) and 7-azabenzisoselenazol-3(2H)-ones (**3a–j**) were designed as new selenium-containing antiviral and antimicrobial agents and synthesized. Some of them were found in the antiviral assay in vitro to be strong inhibitors of cythopatic activity of herpes simplex virus type 1—HSV-1 (compounds **2a,b,f,h, 3a–j**) and encephalomyocarditis virus—EMCV (compounds **2a,h, 3a–f,k,l**). The compounds **2a,h** and **3a–e,j** were found to have an appreciable activity against Gram-positive bacteria (*Staphylococcus aureus* and *Bacillus*) in vitro, some of them inhibited growth of pathogenic yeasts (*Candida albicans*) (**3a,b**) and filamentous fungi (**3a–e,f**).

Keywords: Ebselen; Organoselenium compounds; Bactericides; Fungicides; Virucides

1. Introduction

Two decades ago it was revealed that the simple, synthetically available organoselenium compound 2-phenylbenzisoselenazol-3(2H)-one named ebselen (**1**) could act against oxidative stress in a similar way as common enzyme glutathione peroxidase [1,2]. More recently extensive studies on the chemistry and biology of ebselen and its analogues demonstrated their antiinflammatory, antisclerotic and cytoprotective properties [3–6].

In our previous papers we reported that various 2-substituted benzisoselenazol-3(2H)-ones and related diaryl diselenides exhibited high activity as immunostimulants inducing cytokines such as interferons, tumor necrosis factor and interleukin (IL-2) in human peripheral blood leukocytes [7–9]. Moreover some of them were found as potent and selective inhibitors of endothelial nitric oxide synthase [10–12].

In contrast to broad interest focused on the glutathione peroxidase like activity of these compounds as antioxidants a little attention has only been paid for their activity toward pathogenic viruses, bacteria and fungi [6]. In the previous works we reported that some of them were inhibitors of viral cytopathogenicity and replication [13–15] and that ebselen, some other benzisoselenazolones and diaryl diselenides exhibited inhibitory activity against selected strains of bacteria and fungi [15,16]. These findings opened a question: could a replacement of condensed benzene ring of parent benzisoselenazolone system or phenyl substituent by pyridine moiety result in enhancement of biological activity against pathogenic bacteria and fungi in comparison to ebselen and other benzisoselenazol-3(2H)-ones.

In this work ebselen (**1**) and unsubstituted benzisoselenazol-3(2H)-one (**2a**), were obtained as reference compounds. Then, two groups of their azaanalogues were synthesized. There were 2-(2-pyridyl)benzisoselenazol-3(2H)-ones (**2b–f**), also 2-(3-pyridyl)benzisoselenazol-3(2H)-one methiodide (**2h**), 7-azabenzisoselenazol-3(2H)-ones (**3a–j**) and methiodides **3k,l**. All these compounds were screened in the antiviral, antibacterial, and antifungal assay in vitro and most of them exhibited expected high activity depending on the molecular structure.

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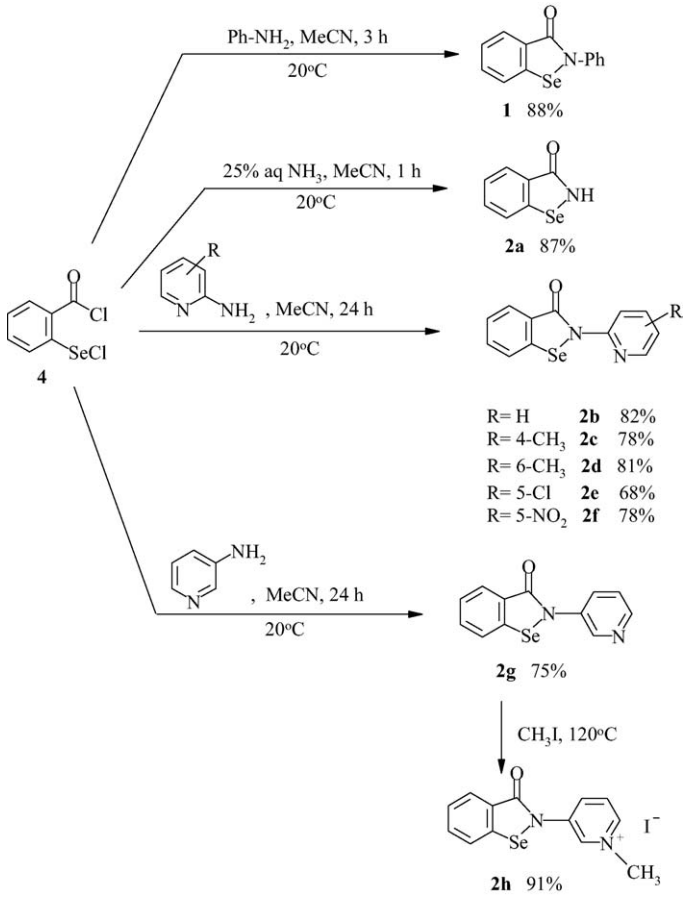
doi:10.1016/j.farmac.2004.07.003

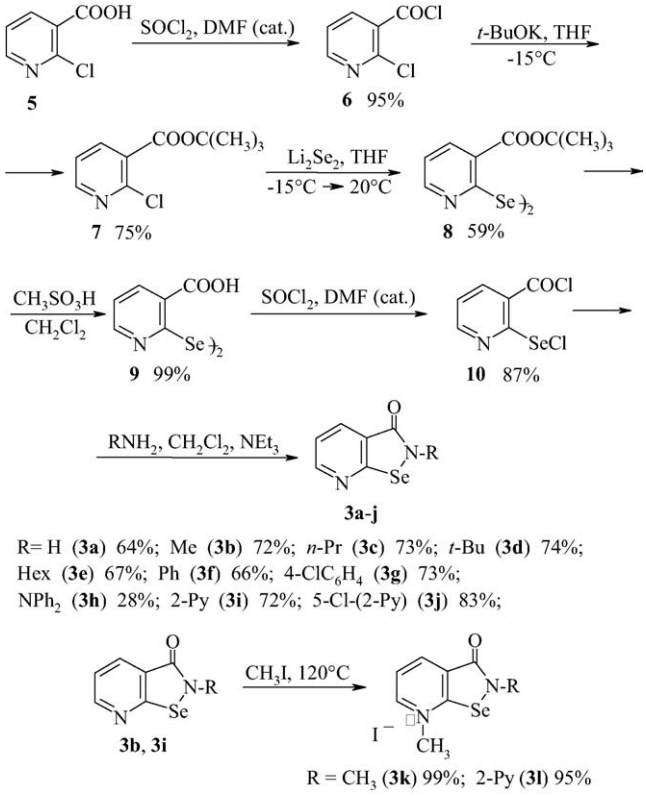


Scheme 1.

2. Chemistry

Ebselen (**1**) and unsubstituted benzisoselenazol-3(2H)-one (**2a**) were prepared by treatment of 2-chloroselenobenzoyl chloride (**4**), obtained in four-step synthesis starting from anthranilic acid, with aniline or aqueous ammonia, respectively, in the same way as reported in our earlier works [8,12]. The same chloride **4** reacting with various 2-aminopyridines produced 2-(2-pyridyl)benzisoselenazol-3(2H)-ones (**2b–f**) and with 3-aminopyridine gave 2-(3-pyridyl)benzisoselenazol-3(2H)-one (**2g**) as it is shown in Scheme 1. The compound **2g** heated with iodomethane, used in excess, gave methiodide **2h**. *N*-Methylation of its 2-pyridyl analogue **2b** was unsuccessful, most probably because of steric hindrance of the lone electron pair of the nitrogen atom. Possible methylation of selenium atom was not observed. 4-Amino pyridine reacted with chloride in another way then its 2- and 3-isomer. In this case instead of tandem acylation-selenenylation of the primary amino group both of the electrophilic centers, localized on the carbonyl carbon and selenium, reacted separately and *N*-pyridin-4-yl-2-(*N*-pyridin-4-yl-aminoseleno)benzamide reported in our earlier work [8] was produced.

The strategy for synthesis of 7-azabenzisoselenazol-3(2H)-ones (**3**), presented in Scheme 2, was based on the conversion of 2-chloronicotinic acid (**5**) into 2-(chloro-



Scheme 2.

Table 1
Virucidal activity of organoselenium compounds **1–3**, acyclovir and dideoxycytidine

Compound	Cytotoxicity [a]	EMCV		HSV-1		VSV	
		MIC [b]	Index [c]	MIC [b]	Index [c]	MIC [b]	Index [c]
1	15.0	10.0	1.5	4.0	0.8	>1000	<0.01
2a	3.0	0.4	7.5	1.0	3.0	600.0	0.005
2b	120.0	>1000	<0.12	2.0	60.0	>1000	<0.12
2c	200.0	>1000	<0.20	>1000	<0.20	>1000	<0.20
2d	230.0	>1000	<0.23	>1000	<0.23	>1000	<0.23
2e	100,0	>1000	<0.10	>1000	<0.10	>1000	<0.10
2f	40.0	500.0	0.08	5.0	8.0	500.0	0.08
2g	3.0	>1000	<0.03	>1000	<0.03	>1000	<0.03
2h	80.0	20.0	4.00	20.0	4.0	80.0	1.00
3a	25.0	6.0	4.02	0.4	62.5	>1000	<0.02
3b	8.5	4.0	2.10	0.6	14.0	>1000	<0.08
3c	5.0	4.0	1.30	0.4	12.5	400.0	0.01
3d	10.0	4.0	2.50	1.0	10.0	>1000	<0.01
3e	6.0	4.0	1.50	0.8	7.5	>1000	<0.06
3f	20.0	4.0	5.00	0.6	33.5	>1000	<0.02
3g	50.0	500.0	0.10	1.0	50.0	500.0	0.10
3h	7.5	500.0	0.01	1.0	7.5	500.0	0.01
3i	70.0	500.0	0.14	2.0	35.0	500.0	0.14
3j	80.0	100.0	0.80	1.0	80.0	>1000	<0.08
3k	15.6	2.0	7.80	800.0	<0.02	>1000	<0.02
3l	31.2	2.0	15.60	400.0	<0.02	>1000	0.03
Acyclovir	>2500	>1000	NA[e]	>1000	NA[e]	>1000	NA[e]
DdC[d]	4000	>1000	<4.0	>1000	<4.0	>1000	<4.0

[a] Cytotoxicity on human A549 cells (μg/ml).
[b] Minimal virus inhibiting concentration (μg/ml).
[c] Index = cytotoxicity/MIC.
[d] Dideoxycytidine.
[e] Not applicable.

seleno)nicotinoyl chloride (**10**) and finally on the tandem acylation-selenenylation of the primary amino group of aminoalkanes and aminoarenes with this reagent. For this purpose acid **5** was converted into chloride **6** which treated with potassium *tert*-butoxylate in dry tetrahydrofurane gave ester **7**. In the next step diselenide **8** was obtained by substitution of chlorine atom in ester **7** by diselenide group with dilithium diselenide generated in situ from elemental lithium and selenium. Acid hydrolysis of carboxyester group in diselenide **8** lead to 2,2′-diselenobisnicotinic acid **9** which treated with thionyl chloride in the presence of catalytic amounts of dimethylformamide gave 2-(chloroseleno)nicotinoyl chloride (**10**). The reaction of chloride **10** and ammonia or corresponding primary amines in dry dichloromethane at ca –15 °C resulted in formation of desired 7-azabenzisoselenazol-3(2H)-ones (**3**). For this synthesis the procedures elaborated recently in our laboratory were applied [17]. From **3b** and **3i** methiodides **3k** and **3l** were obtained by heating with iodomethane.

3. Results and discussion

All compounds **2** and **3** were tested as inhibitors of cytopathic activity of encephalomyocarditis virus (EMCV, non-enveloped RNA virus), herpes simplex virus type 1 (HSV-1 enveloped DNA virus) and vesicular stomatitis virus (VSV, enveloped RNA virus). The results are presented in Table 1.

Contrary to previously tested alkyl and aryl diselenides which mostly were inactive or only moderately active against HSV-1 virus [15], activity in a range of 0.4–2.0 μg/ml, several times higher than activity of ebselen (**1**), was observed for unsubstituted benzisoselenazol-3(2H)-one (**2a**) and different 7-azabenzisoselenazol-3(2H)-ones (**3a–j**). Among 2-(pyridyl)benzisoselenazol-3(2H)-ones high activity was observed for the **2b,f** substituted with 2-pyridyl. Presence of 3-pyridyl as a substituent made the compound **2g** inactive although its methiodide **2b** was active. Methiodides **3k** and **3l** derived from 7-azabenzisoselenazol-3(2H)-ones, were inactive toward HSV-1 virus. Strong anti-EMCV activity, higher that activity of ebselen (**1**) was found for unsubstituted benzisoselenazol-3(2H)-one (**2a**), its 7-azaanalogue **3a**, 7-azabenzisoselenazol-3(2H)-ones substituted with alkyl groups **3b–f**, and methiodides **3k** and **3l**.

To our knowledge the compounds **2a,h** and **3a–j** are the most potent selenium-containing compounds active toward both these viruses. VSV virus remained resistant toward tested organoselenium compounds, except active methiodide **2h**.

To compare with organoselenium compounds two antiviral drugs (acyclovir and dideoxycytidine) were tested in similar conditions. The drugs were inactive against viruses used in the virocidal assays (Table 1). Dideoxycytidine is an anti-HIV drug, specific inhibitor of retroviral reverse transcriptase only. Although acyclovir is widely used as a potent

Table 2
Antimicrobial activity of organoselenium compounds **1–3** against bacteria, yeasts and filamentous fungi characterized by values of MIC (μg/ml)

Compound	Bacteria					Yeasts	Filamentous fungi		
	E. coli	*S. marcescens*	*S. aureus* [a]	*B. subtilis*	*B. cereus*	*C. albicans*	*A. niger*	*P. chrysogenum*	*P. citrinum*
1	n.a. [b]	512.0	128.0	**32.0**	**32.0**	128.0	256.0	256.0	128.0
2a	**32.0**	**64.0**	**16.0**	**64.0**	**64.0**	256.0	256.0	256.0	256.0
2b	n.a.	n.a.	n.a.	128	n.a.	n.a.	n.a.	n.a.	n.a.
2c	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.
2d	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.
2e	n.a.	n.a.	n.a.	128.0	n.a.	n.a.	n.a.	n.a.	n.a.
2f	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.
2g	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.
2h	**16.0**	n.a.	**8.0**	**64.0**	**64.0**	n.a.	n.a.	n.a.	n.a.
3a	256.0	**64.0**	**16.0**	**8.0**	**8.0**	128.0	**32.0**	**32.0**	**32.0**
3b	**32.0**	**32.0**	**16.0**	**4.0**	**4.0**	**64.0**	**2.0**	**2.0**	**2.0**
3c	512.0	n.a.	128.0	**4.0**	**4.0**	512.0	**2.0**	**4.0**	**4.0**
3d	n.a.	n.a.	n.a.	**4.0**	**4.0**	n.a.	**4.0**	**8.0**	**8.0**
3e	n.a.	n.a.	**64.0**	**2.0**	**4.0**	n.a.	**32.0**	**32.0**	**32.0**
3f	n.a.	n.a.	512.0	256.0	128.0	n.a.	n.a.	n.a.	n.a.
3g	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.
3h	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.
3i	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.
3j	n.a.	n.a.	**64.0**	**16.0**	**16.0**	n.a.	**32.0**	**32.0**	**32.0**
3k	256.0	256.0	128.0	256.0	256.0	n.a.	n.a.	n.a.	n.a.
3l	n.a.	n.a.	**32.0**	**16.0**	128.0	128.0	n.a.	n.a.	n.a.
3m	n.a.	n.a.	128.0	512	n.a.	n.a.	n.a.	n.a.	n.a.

[a] The literature data for: oxacillin MIC ≤0.03 μg/ml [18].
[b] Non-active MIC >500 μg/ml.

antiherpetic drug, it has no direct virocidal activity against HSV-1.

Cytotoxicity of the compounds reported in this work, determined on human A 549 cells, presented in Table 1, falls in a broad range from 3.0 μg/ml for **2a,g** to 230 μg/ml for the lowest toxic compound **2d**. For EMCV, because of cytotoxicity, only ebselen (**1**) and compounds **2a,h,3a–f** have appreciable indices >1.0 (inhibitory dose is lower than cytotoxic dose). Among these compounds unsubstituted benzisoselenazol-3(2H)-one (**2a**) and azaanalogue of ebselen **3f** having index values 7.5 and 5.0 are most interesting.

Most of the compounds tested against HSV-1 have index values substantially higher than ebselen. The indices for **2b, 3a,f,g,i,j** falls in a range 30.0–80.0.

Cytotoxicity of acyclovir and dideoxycytidine were found to be considerably lower than that of the organoselenium compounds.

All azaanalogues of ebselen (**2b–g, 3a–j**) and methiodides (**2h, 3k** and **3l**) were tested against pathogenic bacteria, yeasts and filamentous fungi. The results, expressed as minimum inhibitory dose (MIC) values, are shown in Table 2, together with those obtained for the same strains for ebselen (**1**) and unsubstituted benzisoselenazol-3(2H)-one (**2a**). The broadest spectrum of activity against tested microorganisms was observed for 2-methyl-7-azabenzisoselenazol-3(2H)-one (**3b**) having MIC values in the range 2.0–32.0 μg/ml. The biological response for the Gram-positive and Gram-negative bacteria, and yeasts *Candida albicans* was substantially stronger to that established for ebselen (**1**) and its unsubstituted analogue **2a**. The same compound **3b** was active toward filamentous fungi strains *Aspergillus niger, Penicillium chrysogenum* and *Penicillium citrinum* more resistant toward ebselen.

Generally, antibacterial activity of the tested compounds is far from being interesting (for example activities against *Staphylococcus aureus* of two antibiotics Oxacillin and Penicillin G, taken from Ref. [18], are given in Table 2) contrary to their appreciable activity against filamentous fungi (Table 2).

According to known hypothesis saying that mechanism of biological action of benzisoselenazol-3(2H)-ones involves interactions of selenenamide Se–N moiety with sulfhydryl groups of biomolecules present in the living cells [3,4,6] it seems to be possible that biological action of all tested compounds on the molecular level should be similar. Probably observed differences in the biological response result from different polarity and shape of the inhibitor molecule.

It may be concluded that the structural modifications of parent ebselen structure lead to substantial enhancement of the antiviral and antimicrobial activity of its azaanalogues. Some of them, particularly 7-azabenzisoselenazol-3(2H)-ones (**3**) were found to be strong inhibitors of cytopathic activity of encephalomyocarditis virus EMCV (compounds **3a–f,k,l**) and herpes simplex virus HSV-1 (compounds **3a–j**), and to have appreciable activity against broad spectrum of microorganisms (particularly compound **3b**). These results make evidence that replacement of condensed benzene ring in benzisoselenazol-3(2H)-one by pyridine ring and introduction of small non-polar alkyl group in 2-position plays a crucial role in strong enhancement of antiviral and

antimicrobial activity. On the contrary, introduction of pyridine moiety as a substituent generally does not enhance of the biological response or even made the compound inactive.

4. Experimental

4.1. Chemistry

All reagents and solvents were purchased from Aldrich and Fluka. Melting points were determined with a digital melting point apparatus Electrothermal IA 9100. IR spectra were measured on a Perkin-Elmer 200 FT spectrometer in KBr pellets. ^{1}H NMR spectra were recorded in DMSO-d_6 on a Bruker DRX spectrometer 300 MHz. Chemical shifts are reported in ppm relative to TMS. Reaction progress was monitored by thin layer chromatography (TLC) on silica gel $60F_{254}$ coated aluminium TLC plates from Merck. Elemental analyses were performed in our analytical laboratory and agreed with theoretical values to within 0.3%.

*4.1.1. Ebselen (**1**) and benzisoselenazol-3(2H)-one (**2a**)*

These compounds were prepared from 2-(chloroseleno) benzoyl chloride (**4**) following the synthetic route described in our previous work [8].

*4.1.2. 2-Pyridylbenzisoselenazol-3(2H)-ones (**2b–g**). General procedure*

The solution of 2-(chloroseleno)benzoyl chloride (**4**) (1.27 g, 5 mmol) in acetonitrile (25 ml) was added dropwise at room temperature with stirring to the solution of the corresponding aminopyridine (16.5 mmol) in acetonitrile (25 ml) during 30 min and the reaction was continued for 24 h (**2b–d,f,g**) or 2 h (**2e**). After the reaction was finished the solvent was evaporated in vacuo. To the crystalline residue water (100 ml) was added and the mixture was stirred for 24 h at room temperature. Unsoluble product was filtered off, washed with water, dried and recrystallized from methanol.

*4.1.2.1. 2-(2-Pyridyl)benzisoselenazol-3(2H)-one (**2b**) (82%).* m.p. 233–234 °C (decomp.): IR ν 3053 cm^{-1} (C–H_{ar}), 1617, 1568 cm^{-1} (C=O amide), 1586, 1461 cm^{-1} (C–C_{ar}), 1290, 743 cm^{-1} (C–N amide), 678 cm^{-1} (Py): ^{1}H NMR δ 7.21 (t, 1H, J = 6.0 Hz, PyH), 7.45 (t, 1H, J = 7.5 Hz, ArH), 7.68 (t, 1H, 7.5 Hz, ArH), 7.87 (t, 1H, J = 6.9 Hz, PyH), 7.90 (d, 1H, J = 7.4 Hz, ArH), 8.06 (d, 1H, J = 7.9 Hz, ArH), 8.41 (d, 1H, J = 3.4 Hz, PyH), 8.64 (d, 1H, J = 8.5 Hz, PyH).

*4.1.2.2. 2-[4-Methyl(2-pyridyl)]benzisoselenazol-3(2H)-one (**2c**) (78%).* m.p. 268–269 °C (decomp.): IR ν 3065 cm^{-1} (C–H_{ar}), 2920 cm^{-1} (C–H_{aliph}), 1622, 1557 cm^{-1} (C=O amide), 1589, 1445 cm^{-1} (C–C_{ar}), 1286, 737 cm^{-1} (C–N amide), 676 cm^{-1} (Py): ^{1}H NMR δ 2.48 (s, 3H, CH_3), 7.05 (d, 1H, J = 4.7 Hz, PyH), 7.44 (t, 1H, J = 7.4 Hz, ArH), 7.67 (t, 1H, J = 7.5 Hz, ArH), 7.9 (d, 1H, J = 7.4 Hz, ArH), 8.05 (d, 1H, J = 7.9 Hz, ArH), 8.25 (d, 1H, J = 5.1 Hz, PyH), 8.45 (s, 1H, PyH).

*4.1.2.3. 2-[6-Methyl(2-pyridyl)]benzisoselenazol-3(2H)-one (**2d**) (81%).* m.p. 238–239 °C: IR ν 3060 cm^{-1} (C–H_{ar}), 2959 cm^{-1} (C–H_{aliph}), 1627, 1573 cm^{-1} (C=O amide), 1590, 1445 cm^{-1} (C–C_{ar}), 1308, 739 cm^{-1} (C–N amide), 675 cm^{-1} (Py): ^{1}H NMR δ 2.42 (s, 3H, CH_3), 7.07 (d, 1H, J = 7.4 Hz, PyH), 7.43 (t, 1H, J = 7.4 Hz, ArH), 7.66 (t, 1H, J = 7.3 Hz, ArH), 7.78 (t, 1H, J = 7.9 Hz, PyH), 7.88 (d, 1H, J = 7.6 Hz, ArH), 8.03 (d, 1H, J = 7.6 Hz, ArH), 8.37 (d, 1H, J = 8.1 Hz, PyH):

*4.1.2.4. 2-[5-Chloro(2-pyridyl)]benzisoselenazol-3(2H)-one (**2e**) (68%).* m.p. 269–271 °C: IR ν 3073 cm^{-1} (C–H_{ar}), 1667, 1579 cm^{-1} (C=O amide), 1598, 1446 cm^{-1} (C–C_{ar}), 1282, 731 cm^{-1} (C–N amide), 671 cm^{-1} (Py): ^{1}H NMR δ 7.42 (t, 1H, J = 7.5 Hz, ArH) 7.65 (t, 1H, J = 7.6 Hz, ArH), 7.87 (d, 1H, J = 7.1 Hz, ArH) 7.98 (dd, 1H, J = 9.0 and J = 2.5 Hz, PyH) 8.02 (d, 1H, J = 8.0 Hz, ArH) 8.46 (d, 1H, J = 2.0 Hz, PyH) 8.59 (d, 1H, J = 9.2 Hz, PyH).

*4.1.2.5. 2-[5-Nitro(2-pyridyl)]benzisoselenazol-3(2H)-one (**2f**) (78%).* m.p. 337–340 °C: IR ν 3081 cm^{-1} (C–H_{ar}), 1670, 1577 cm^{-1} (C=O amide), 1593, 1460 cm^{-1} (C–C_{ar}), 1502, 1340, 844 cm^{-1} (NO_2), 1272, 732 cm^{-1} (C–N amide), 671 cm^{-1} (Py): ^{1}H NMR δ 7.45 (t, 1H, J = 7.4 Hz, ArH), 7.70 (t, 1H, J = 7.8 Hz, ArH), 7.93 (d, 1H, J = 7.7 Hz, ArH), 8.05 (d, 1H, J = 7.9 Hz, ArH), 8.65 (dd, 1H, J = 9.3 and 2.7 Hz, PyH), 8.74 (d, 1H, J_1 = 9.3 Hz, PyH), 9.2 (d, 1H, J_2 = 2.6 Hz, PyH).

*4.1.2.6. 2-(3-Pyridyl)benzisoselenazol-3(2H)-one (**2g**) (88%).* m.p. 274–275 °C: IR ν 3064 cm^{-1} (C–H_{ar}), 1630, 1578 cm^{-1} (C=O amide), 1477, 1446 cm^{-1} (C–C_{ar}), 1314, 739 cm^{-1} (C–N amide), 674 cm^{-1} (Py): ^{1}H NMR δ 7.42–7.51 (m, 2H, ArH, PyH), 7.70 (t, 1H, J = 7.2 Hz, ArH) 7.91 (d, 1H, J = 7.7 Hz, ArH), 8.05–8.11 (m, 2H, ArH, PyH), 8.45 (d, 1H, J = 4.6 Hz, PyH), 8.87 (s, 1H, PyH).

*4.1.3. 7–Azabenzisoselenazol-3(2H)-ones (**3a–j**). General procedure*

Were prepared from 2-(chloroseleno)nicotinoyl chloride and ammonia (**3a**), alkyl amine (**3b,c**), phenylamine (**3f–h**) or aminopyridine (**3i,j**), respectively, following the synthetic route described in our recent work [17].

*4.1.4. Methiodides **2h, 3k** and **3l***

A mixture of 2-(3-pyridyl)benzisoselenazol-3(2H)-one (**2g**) or 2-substituted 7–azabenzisoselenazol-3(2H)-ones (**3b,i**) (2.5 mmol) and methyl iodide (5 ml, 80 mmol) was heated at 120 °C for during 3 h for 2 g or 24 h **3b** and **i** in hermetically closed tube. After this time the yellow solid precipitated. It was filtered, washed with dichloromethane and dried in the air. Compound **2h** (91%): m.p. 268–269 °C: IR ν 3073 cm^{-1} (C–H_{ar}), 2996 cm^{-1} C–H_{aliph}), 1640 cm^{-1} (C=O amide), 1608, 1498 cm^{-1} (C–C_{ar}), 1280, 748 cm^{-1} (C–N amide), 664 cm^{-1} (Py): ^{1}H NMR δ 4.41 (s, 3H, CH_3) 7.52 (t, 1H, J = 7.5 Hz, PyH), 7.74 (t, 1H, J = 7.7 Hz, ArH),

7.79 (d, 1H, J = 7.7 Hz, ArH) 8.16 (dd, 1H, J = 6.0 and 2.58 Hz, PyH), 8.76 (t, 2H, J = 6.2 Hz, PyH), 9.15 (s, 1H, PyH): compound **3k** (99%): m.p. 192–194 °C: IR ν 3008 cm^{-1} (C–H_{ar}), 2925 cm^{-1} C–H_{aliph}), 1668, 1615 cm^{-1} (C=O amide), 1584, 1451 cm^{-1} (C–C_{ar}), 1312, 742 cm^{-1} (C–N amide), 668 cm^{-1} (Py): ^{1}H NMR δ 3.46 (s, 3H, CH_3), 4.37 (s, 3H, CH_3), 8.14 (dd, 1H, J = 7.6 and 6.2 Hz, PyH), 8.81 (d, 1H, J = 7.8 Hz, PyH), 9.18 (d, 1H, J = 6.0 Hz, PyH): compound **3l** (95%): m.p. 309–310 °C: IR ν 3063 cm^{-1} (C–H_{ar}), 2965 cm^{-1} (C–H_{aliph}), 1676, 1585 cm^{-1} (C=O amide), 1569, 1430 cm^{-1} (C–C_{ar}), 1308, 743 cm^{-1} (C–N amide), 1086, 667 cm^{-1} (Py): ^{1}H NMR δ 4.41 (s, 3H, CH_3), 7.41 (dd, 1 H, J = 6.9 and J = 1.5 Hz, PyH), 8.08 (dt, J = 7.6 and J = 1.6 Hz, PyH), 8.18 (dd, 1H, J = 7.9 and J = 1.7 Hz, PyH), 8.45 (d, 1H, J = 8.3 Hz, PyH), 8.50 (1H, J = 4.84 Hz, PyH), 8.89 (d, 1H, J = 7.4 Hz, PyH), 9.34 (d, 1H, J = 5.9 Hz, PyH).

4.2. Biological activity

4.2.1. Cytotoxicity

Cytotoxicity of the compounds was determined in human lung adenocarcinoma cell line A549 (ATCC 185). The experiment was performed in 96-cells microplates. The cells were treated with various doses of the compounds for 48 h at 37 °C in the atmosphere of 5% CO_2 in air. Then the cultures were examined under microscope and next stained with MTT. The minimal concentration which was toxic to approximately 50% of the cells was taken as $TCCD_{50}$ [9].

4.2.2. Antiviral assay

The compounds in various concentrations were incubated with following viruses: EMCV (encephalomyocarditis virus, Picornaviridae, naked virus), HSV-1 (herpes simplex virus type 1, Herpesviridae, enveloped virus) and VSV (vesicular stomatis virus, Rhabdoviridae, enveloped virus). The viruses were used at dose 10^5 $TCID_{50}$/ml. After 2 h incubation at room temperature, the virus titer was measured in human A549 cells and minimal virus inhibiting concentration (MIC) was determined. Acyclovir (Sigma) and dideoxycytidine (Sigma) were used as control antiviral drugs.

4.2.3. Antimicrobial assay

The antimicrobial activities of tested compounds were evaluated by the agar dilution method [19]. Nutrient agar and mycological Agar were used for bacteria and fungi, respectively. Gram-positive bacterial species; *S. aureus* PCM 1944, *Bacillus subtilis* PCM 1949, *Bacillus cereus* PCM 1951, Gram-negative bacterial species; *Escherichia coli* PCM 2057, *Serratia marcescens* PCM 549 and fungal strains; *C. albicans, A. niger, P. chrysogenum and P. citrinum* were used for the test.

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