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Original article

4-Methyl-1,2,3-selenadiazole-5-carboxylic acid amides: Antitumor action and cytotoxic effect correlation

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Abstract

Synthesis and cytotoxic activity of a series of 4-methyl-1,2,3-selenadiazole-5-carboxylic acid amides on human fibrosarcoma HT-1080, mouse hepatoma MG-22A, and mouse fibroblasts 3T3 cell lines are described. The correlation between compound LD_{50} and 3T3 fibroblast cell line morphology was shown. *In vivo* evaluation of amides on mouse sarcoma S-180 confirms high antitumor activity (58–85%).

Keywords: Selenium; 1,2,3-Selenadiazole; Cytotoxic activity; Morphology

1. Introduction

Selenium has attracted great interest as an essential element and certain diseases have been eradicated by dietary supplementation of this element. Selenium is essential for cell metabolism as a component of glutathione peroxidase and other enzyme systems. Current interest lies in the prevention of certain cancers by supplementation with selenium [1–3]. One proposed mechanism for this activity is a cytotoxic effect of selenium on tumor cells [4,5]. Brief exposure of HeLa cells to micromolar concentrations of selenite resulted in significant inhibition of tumor cell colony formation, indicating that this is an assay for selenite cytotoxicity [6]. However, selenium appears to operate by several mechanisms depending on the chemical form of selenium, the nature of the carcinogenic process, and its dosage. There was no significant difference in the potency of selenate, selenite, selenium dioxide, selenomethionine and selenocysteine to inhibit the development of mammary tumors, drug-resistant and drug non-resistant human ovarian tumor cells [7]. The anti-proliferative effects of selenium have been studied both *in vivo* and *in vitro*. The anti-leukemic effect of sodium selenite is associated with inhibition of DNA replication, transcription and translation. It was shown that 6-phenyl-7(6*H*)-isoselenazolo[4,3-*d*]pyrimidone markedly inhibited the growth of P388 mouse leukemia at dose of 100 μg/mouse per day × 10 with no sign of toxicity [8] and 2-amino-4-substituted-selenazoles exhibit high inhibiting activity on leukemia L1210 cell proliferation ($TD_{50} = 0.2–8$ μmol/ml) [9,10]. Taking into account the importance of the selenium as a trace element in the organism our present investigation [11–15] is connected with the synthesis and antitumor activity studies in a series of 4-methyl-1,2,3-selenadiazole-5-carboxylic acid amides.

2. Results and discussion

2.1. Chemistry

The general synthetic route chosen for preparation of 4-methyl-1,2,3-selenadiazole-5-carboxylic acid amides included amide formation from 4-methyl-5-ethoxycarbonyl-1,2,3-selenadiazole and corresponding amine (Scheme 1). The reaction was carried out in ethanol/water solution at room temperature in darkness to prevent selenadiazole cycle photodegradation. The ^{1}H, ^{13}C and ^{77}Se NMR data confirm

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Scheme 1.

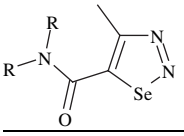
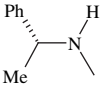
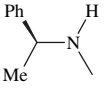
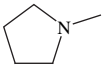
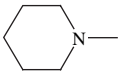
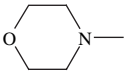
the formation of 4-methyl-1,2,3-selenadiazole-5-carboxylic acid amides. The selenium signal in the ^{77}Se NMR spectra for all compounds appears in 1542.3–1563.9 ppm region. Transamidation of ester proceeds very slowly from 10 to 25 days, however heating is prohibited due to selenadiazole cycle thermolability. 4-Methyl-1,2,3-selenadiazole-5-carboxylic acid ethyl ester reacts with amines to obtain amides in 17–95% yields.

2.2. In vitro

The results of these experiments are summarized in Table 1. The majority of tested 4-methyl-1,2,3-selenadiazole-5-carboxylic acid amides **1**–**7** exhibited slight activity *in vitro* on human fibrosarcoma HT-1080 and mouse hepatoma MG-22A tumor cell lines. The 4-methyl-1,2,3-selenadiazole-5-carboxylic acid dimethylamide (**1**) and di(*iso*-propyl)amide analogue **2** have no cytotoxic activity on HT-1080 cell line. 4-Methyl-1,2,3-selenadiazole-5-carboxylic acid (*S*)-(1-phenyl-ethyl)-amide (**4**, 31 μg/mL) is more active than its (*R*) enantiomer **3** (100 μg/mL). In a series of cyclic amides **5**–7 pyrrolidino derivative **5** shows the highest cytotoxicity (TD_{50} = 19 μg/mL on HT-1080). It should be noted that in the same concentrations 4-methyl-1,2,3-selenadiazole-5-carboxylic acid amides **1**–**7** selectively act against tumor and normal mouse fibroblast (3T3) cells, moreover this series exhibits low LD_{50} toxicity (800–1442 mg/kg).

The role of NO in biosystems has attracted considerable attention in the last decade. NO is formed by enzymatic and non-enzymatic mechanisms. Because of its molecular weight and high lipophilicity, NO has good diffusion properties. It may act not only in the cell where it is produced, but also in nearby tissues. Biologically produced NO originates from oxygen and L-arginine in the reaction catalyzed by NO synthase. NO, a long-lived radical with a wide range of actions, is known as a regulator of a variety of biological processes [16]. The NO level was determined accordingly [17], NO release was defined using the Greyss reagent (by NO_2 concentration in the cultural medium). The yield of nitrite was expressed as NO_2 nmol/200 μL of cultural medium in testing plates for 100% alive cells after CV coloration assay (selenadiazole's concentration 100 μg/mL). It was shown (Table 1) that dimethylamide **1** is a NO radical protector on MG-22A cell line, but di(*iso*-propyl)amide analogue **2** exhibits an opposite effect. (*R*) Enantiomer **3** is a free radical protector on human fibrosarcoma cell line, besides (*S*) analogue **4** which readily increase NO concentration in the cultural medium (TG_{100} = 200–300%). (4-Methyl-1,2,3-selenadiazol-5-yl)-pyrrolidin-1-yl-methanone (**5**) and piperidine derivative **6** are NO inducers (TG_{100} = 100-400%).

Table 1
In vitro cytotoxicity in monolayer tumor cell lines [HT-1080 (human fibrosarcoma), MG-22A (mice hepatoma), NIH 3T3 (normal mouse fibroblasts) and inhibition of tumor growth (sarcoma S-180)] caused by 1,2,3-selenadiazoles **1**–**7**[a]

		HT-1080		MG-22A		NIH 3T3 TD_{50}	LD_{50} (mg/kg)	S-180 Inhibition (%)	Toxic effect (%)
		TD_{50}[a]	NO 100%[b]	TD_{50}	NO 100%				
1	Me_2N	[c]	10	35	63	351	1134	58	17
2	$(i\text{-Pr})_2N$	[c]	29	31	200	718	1755	−32	0
3		100	17	29	400	195	1029	20	3
4		31	300	32	200	407	1442	−23	0
5		19	350	33	350	214	977	85	23
6		31	100	27	400	123	800	71	8
7		[c]	11	34	250	280	1145	[c]	8

[a] TD_{50} – concentration (μg/mL) providing 50% cell killing effect [(CV + MTT)/2].
[b] No concentration (%) (CV: coloration).
[c] No cytotoxic effect.

2.3. Morphology

The influence of the studied 4-methyl-1,2,3-selenadiazole-5-carboxylic acid amides **1**–**7** on the phenotype of mouse fibroblasts, human fibrosarcoma HT-1080, and mouse hepatoma MG-22A cells was examined (Fig. 1). Figures in the table show the morphological changes after 72 h at 30 °C (visualization by acridine orange). Our experimental data shows that morphology of selenadiazoles **1**–**7** on 3T3 cells correlates with cytotoxic data on 3T3 cell line. Derivatives **1**, **3**, **5**, and **6** are more toxic than **2**, **4** and **7**. Dimethylamide derivative **1** induced apoptosis and polyploidy of fibrosarcoma cells (Table 2), but di(*iso*-propyl) analogue **2** increases the amount of tumor cells. (*S*)-(1-Phenyl-ethyl)-amide **4** changed the cell phenotype and mitotic mechanism, however, its (*R*) analogue **3** has no influence on the fibrosarcoma cell phenotype. The introduction of piperidinyl fragment into the molecule (**6**) leads to the increase in cell volume and formation of more than one apoptotic nucleus. The inspection of selenadiazole amides **1**–**7** influence on mouse hepatoma MG-22A phenotype shows that only dimethylamide **1** and pyrrolidine derivative **5** evoke apoptosis.

2.4. In vivo

Antitumor activity of 4-methyl-1,2,3-selenadiazole-5-carboxylic acid amides **1**–**7** against sarcoma S-180 was determined for male ICR mice (18–20 g). The 4-methyl-1,2,3-selenadiazole-5-carboxylic acid dimethylamide (**1**) inhibits sarcoma growing by 58% in dose 2 mg/kg in two days, but negatively influences on mice respiratory system due to its toxic effect (17%). Di(*iso*-propyl)amide analogue **2** undesirably increases the tumor growth by 32%. The introduction of optical center in the core molecule [**3** – (*R*)-isomer; **4** – (*S*)-isomer] shows an opposite activity: (*R*) analogue slightly inhibits sarcoma growing, however, (*S*)-isomer increases the tumor growth by 23% in 8 days. Experimental data for cyclic amides **5**–**7** show mice weight decrease by 8–23%. Pyrrolidine amide **5** possesses the sarcoma S-180 inhibition ability by 85% with simultaneous loss of mice weight by 23%.

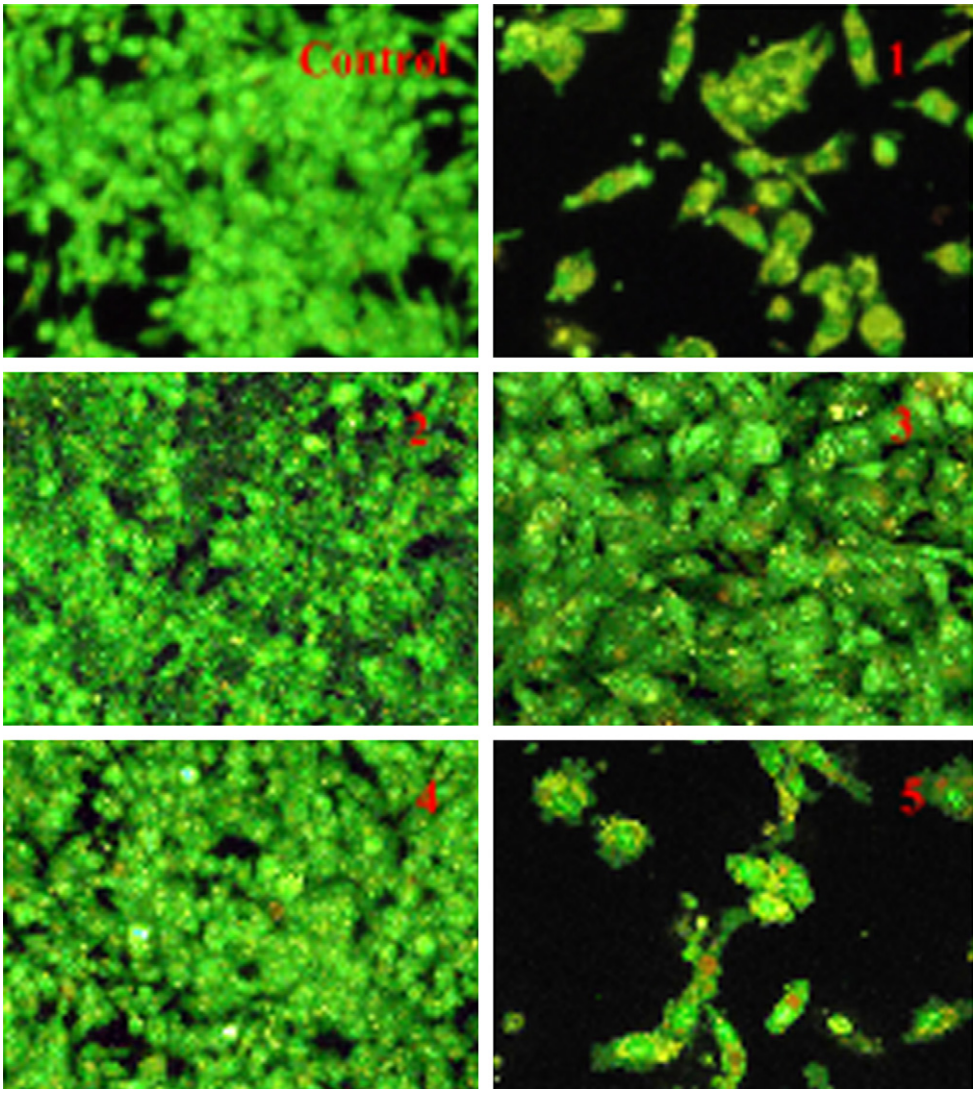


Fig. 1. 4-Methyl-1,2,3-selenadiazole-5-carboxylic acid amides **1**–**5** morphology data (HT-1080 cell line).

Table 2
^{1}H, ^{13}C and ^{77}Se NMR spectra and elemental analysis data of 4-methyl-1,2,3-selenadiazole-5-carboxylic acid amides **1–7**

Compound	Yield (%)	M.p. (°C)	^{1}H NMR, δ, ppm		^{13}C NMR, δ, ppm				^{77}Se NMR, δ, ppm	Elemental analysis					
			Me (s, 3H)	$-NR_2$	Me	Diazolyl cycle	C=O	$-NR_2$		Calculated			Found		
										H	C	N	H	C	N
1	67	82–84	2.75	2.92 and 3.15 (2s, 3H, NMe)	13.6	149.4, 155.8	164.2	35.3, 38.6 (NCH_3)	1552.7	4.16	33.04	19.26	4.12	32.99	19.27
2	35	140	2.95	1.34 (d, 12H, $J = 6$ Hz, 4Me), 3.32 (septet, 2H, $J = 6$ Hz, 2CH)	14.3	156.9, 157.5	166.4	18.9 ($NCCH_3$), 46.4 (CH)	1554.1	6.25	43.80	15.32	6.23	43.78	15.33
3	21	168	2.73	1.53 (d, 3H, $J = 6.8$ Hz, Me), 4.20 (q, 1H, $J = 6.8$ Hz, CH), 7.22 (s, 5H, Ph), 8.46 (bs, 1H, NH)	14.3	150.6, 157.8	168.1	21.4 (CH*Me*), 51.4 (C*Me*), 126.0, 128.8 (*p*-), 129.0, 138.3 (*i*-)	1563.7	4.45	48.99	14.28	4.43	49.00	14.27
4	22	152	2.73	1.53 (d, 3H, $J = 6.8$ Hz, Me), 4.20 (q, 1H, $J = 6.8$ Hz, CH), 7.22 (s, 5H, Ph), 8.63 (bs, 1H, NH)	14.3	154.8, 157.8	168.2	21.3 (CH*Me*), 51.4 (C*Me*), 126.0, 128.8 (*p*-), 129.0, 138.3 (*i*-),	1563.9	4.45	48.99	14.28	4.44	49.0	14.29
5	95	Oil	2.65	1.81–1.90 (m, 4H, H-3, H-4), 3.10 (t, 2H, $J = 6$Hz, NCH_2), 3.51 (t, 2H, $J = 6$Hz, NCH_2)	13.5	149.7, 156.1	162.1	24.0 and 25.6 (C–C), 46.0 and 48.5 (N–C)	1542.3	4.54	39.36	17.21	4.54	39.32	17.24
6	71	84–85	2.75	1.51 (m, 2H, *p*-CH_2), 1.67 (m, 4H, 2*m*-CH_2), 3.24 and 3.73 (2m, 2H, NCH_2)	13.4	149.5, 155.4	162.3	24.0, 25.3 and 26.2 (C–C), 43.1 and 48.2 (N–C)	1553.8	5.08	41.87	16.28	5.06	41.85	16.33
7	17	122–123	2.77	3.30 and 3.64–3.76 (2m, 8H)	13.7	148.4, 155.9	163.0	42.7 and 47.4 (N–C), 66.56 (O–C)	1560.5	4.26	36.93	16.15	4.25	36.92	16.13

Piperidino analogue **6** shows high inhibition level (71%) and relatively slight influence on mice weight. Besides, introduction of the oxygen atom in the amide cycle (**7**) leads to disappearance of the tumor growth inhibition ability.

2.5. Conclusions

4-Methyl-1,2,3-selenadiazole-5-carboxylic acid amides were prepared by convenient synthetic way. The correlation between compound LD_{50} and 3T3 fibroblast cell line morphology was shown. 4-Methyl-1,2,3-selenadiazole-5-carboxylic acid amides show a potency *in vitro* against HT-1080 and MG-22A tumor cell lines. *In vivo* evaluation of amides **1**, **3**, and **6** also confirms high antitumor activity against sarcoma S-180 (58–85%).

3. Experimental section

3.1. General

^{1}H and ^{77}Se NMR spectra were recorded on a Varian 200 Mercury spectrometer at 200 and 39.74 MHz correspondingly at 303 K in $CDCl_3$/TMS solution. The ^{1}H chemical shifts are given relative to TMS, ^{13}C – relative to chloroform, and ^{77}Se – relative to dimethyl selenide. The melting points were determined on a ''digital melting point analyser'' (Fisher) and the results are given without correction.

3.2. Synthesis of 4-methyl-1,2,3-selenadiazole-5-carboxylic acid amides

The mixture of 4-Methyl-1,2,3-selenadiazole-5-carboxylic acid ethyl ester (0.02 mol) and amine (0.04 mol) in 50% ethanol solution in water was stirred in darkness at room temperature (TLC control). After reaction completion the solvent was evaporated under reduced pressure. Crude product was purified on silica gel using ethyl acetate/ethanol mixture as an eluent with the following recrystallization from ethyl acetate. ^{1}H, ^{13}C, and ^{77}Se NMR data and elemental analysis data are presented in Table 2.

3.3. In vitro cytotoxicity assay

Monolayer tumor cell line: HT-1080 (human fibrosarcoma), MG-22A (mice hepatoma), and NIH 3T3 (normal mouse fibroblasts) were cultured in standard medium DMEM (Dulbecco's modified Eagle's medium) without an indicator (''Sigma'') supplemented with 10% heat-inactivated fetal bovine serum (''Sigma''). After the ampoule was thawed the cells from one to four passages were used. About $2–5 \times 10^4$ cells/ml (depending on line nature) were placed in 96-well plates immediately after compounds added to the wells. The control cells without test compounds were cultured on separate plate. The plates were incubated for 72 h at 37 °C in 5% CO_2. The number of surviving cells was determined using tri(4-dimethylaminophenyl)methyl chloride (crystal violet) or 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolinium bromide (MTT), and the concentration of nitric oxide (NO) was determined according to Ref. [11].

MTT-test: After incubating with preparations culture medium was removed and 200 μl fresh medium with 10 mM HEPES was added in each well of the plate, then 20 μl MTT (2 mg/ml in HBSS) was added. After incubation (3 h, 37 °C, 5% CO_2) the medium with MTT was removed and 200 μl DMSO and 25 μl glycine buffer pH 10.5 were added once to each sample. The samples were tested at 540 nm on Anthos HT II photometer.

CV-test: After incubating with preparations cell culture was removed and 100 ml of 1% glutaraldehid in HBSS was added to each well. After incubation (15 min) the HBSS with glutaraldehid samples washed off H_2O (1 time) and 0.05% crystal violet was added. After incubation with dye (15 min) the samples washed off H_2O (3 times) and citrate buffers pH 4.2 and ethanol (1:1) were added. The samples were tested at 540 nm.

3.4. In vivo activity assay

The compounds were tested *in vivo* against sarcoma S-180 cells. Sarcoma S-180 (5×10^6) cells were inoculated s.c. into male ICR mice (six weeks old, 18–20 g) on day 0. Drugs were administered i.p.; the treatment was started 24 h after tumor transplantation. The number of mice used in each group was between 6 and 10. The daily dose was 10 mg/kg; duration of treatment was nine days. The efficacy of the treatment was estimated by the ellipsoid formula, V of control group was taken in calculations for 100%. The tumor volume (V) was calculated from equation: $V = 4\pi ab^2/3$, where a and b are ellipsoid maximum and minimum diameters (calculated volume was reduced by 2 times in the case of flat tumor shape).

3.5. Morphology assay

The change in cell morphology caused by selenadiazole amides **1–7** was investigated under inverted fluorescence microscope Nikon ECLIPSE TE 300. Acridine orange stain (Sigma) was used. Stain solution: 10 μg/ml acridine orange in phosphate buffered saline (PBS) pH 7.4. After incubating with selenadiazole amides for 72 h at 37 °C in 5% CO_2 the adherent cells were stained in 96-wells cell culture plate (Sarsted AG). Cell culture mediums were removed and 40 μl stain solution was added in each well. After 2 min the dye was removed and samples were once washed with PBS. Then 40 μl PBS was added to the samples and they were investigated under microscope control. Chromatin condensation in apoptotic cells was visualized by staining the cellular DNA with the dye acridine orange [18,19]. Living cells stained green, apoptotic cells orange or yellow, and necrotic cells red (Fig. 1). (For interpretation of the references to colors in the text, the reader is referred to the web version of this article.)

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