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Organoammonium hydroselenites: antitumor action through radical balance regulation

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Abstract

Organoammonium hydroselenites were synthesized and investigated as potential selective, anticancer prodrugs. These compounds were studied in vitro on human fibrosarcoma (HT-1080), hamster kidney endothelial (BHK 21) and normal mouse embryonic fibroblasts (NIH 3T3). Most of them were very active against HT-1080 (0.6–5.3 g/ml). Amino acid hydroselenites readily increased the nitric oxide (NO) concentration in the culture medium of HT-1080 cells (up to $TG_{100} = 1500\%$); however, 4-amidohydroximinomethylpyridinium hydroselenite ($TG_{100} = 24\%$) and *o*-phenanthrolinium hydroselenite ($TG_{100} = 50\%$) were free radical inhibitors. All compounds were glutathione peroxidase inhibitors; some of them could also prevent hydrogen peroxide degradation by inhibition of catalase. The influence of the investigated ammonium hydroselenites on tumor cell (HT-1080) morphology was examined. The substances studied were also active in vivo against sarcoma S-180. The role of organoammonium hydroselenites as free radical regulators and their therapeutic antitumor are discussed.

Keywords: Antitumor activity; Catalase; Hydroselenite; Glutathione peroxidase; Morphology; Nitric oxide (NO); Selenium

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 (Hill and Meat, 2002; Moyad, 2002; Spallholz, 2001). One proposed mechanism for this activity is a cytotoxic effect of selenium on tumor cells (Mugesh et al., 2001; Alaejos et al., 2000). 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 (Caffrey and Frenkel, 1991). 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 nonresistant human ovarian tumor cells (Caffrey and Frenkel, 1997). The antiproliferative effects of selenium have been studied both in vivo and in vitro. The antileukemic effect of sodium selenite is associated with inhibition of DNA replication, transcription and translation. Selenocystine and sodium selenite have antitumor activity and these are also the only compounds, which demonstrate significant redox chemistry, including depletion of cellular glutathione, stimulation of glutathione reductase and stimulation of oxygen consumption. The interaction of these two compounds with glutathione suggests an intriguing potential role for them in cancer therapy (Batist et al., 1986). Significant chemopreventive effects have been produced with sodium selenite in the following rodent model carcinogenesis systems: mouse skin papillomas, rat mammary adenocarcinoma, hamster tracheal squamous cell carcinoma and mouse bladder carcinoma, mouse colon and lung adenocarcinoma, rat and mouse colon adenocarcinoma (Boone et al., 1992). Sodium selenite may provide protection against *cis*-diaminedichloroplatinum(II) nephrotoxicity when it is given before *cis*-

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diaminedichloroplatinum(II). Moreover, selenium has antineoplastic activity against several tumors. Administration of sodium selenite reduces cisplatin toxicity without inhibiting the antitumor activity of cisplatin. The combination of these qualities may open new perspectives in cancer chemotherapy (Baldew et al., 1989; Olas and Wachowicz, 1997).

The majority of tested organic base hydroselenites exhibit high activity in vitro in investigated tumor cell lines. *o*-Phenanthrolinium and imidazolinium selenites exhibit the highest cytotoxic effect on HT-1080, MG-22A, B16 and Neuro 2A cell lines. In addition, that most synthesized selenites are very active (0.5–0.6 μg/ml) against mouse melanoma B16. Of a series of ethanolamine derivatives, *N*-methylethanolammonium selenite is more active in experiments with Neuro 2A cells (TD_{50} = 1 μg/ml), while triethanolammonium selenite is toxic against HT-1080 cells (TD_{50} = 2.3 μg/ml), being less toxic against normal cells 3T3 (TD_{50} = 47.7 μg/ml) (Lukevics et al., 2002a). Previously, we reported that hydroselenites are able to activate nitric oxide generation in various tumor cell cultures (Lukevics et al., 2002b). The activation of nitric oxide production is especially prolonged in HT-1080 and MG-22A cell lines. The amount of nitric oxide produced depends on the type of tumor and the cation structure. According to the literature and our results, we propose that the antitumor effect of hydroselenites stems from the fact that they are able to regulate the free radical balance in vitro and in vivo.

In the current study, we report the cytotoxic activity of selected organoammonium hydroselenites in vitro against HT-1080, BHK 21 and NIH 3T3 cell lines, their activity against glutathione peroxidase and catalase, and in vivo against sarcoma S-180 and their effect on cell morphology. The role of organoammonium hydroselenites as free radical regulators and the therapeutic antitumor effect of a balanced generation of free radicals are discussed.

2. Materials and methods

2.1. Synthesis of hydroselenites

To a solution of the amine (4-hydroximinomethylpyride, 4-amidohydroximinomethylpyride, benzimidazole, 2-mercaptobenzimidazole, *o*-phenanthroline, triethanolamine, β-Ala, L-Ser, L-Pro, Gly–Gly, Gly–Gly–Gly, L-Ala-Gly-Pro-ONb) (0.02 mol) in 50 ml of water or a mixture of water/ethanol (1:1), the equimolar amount of selenium dioxide was added. The reaction mixture was stirred 1 h at room temperature. The residue was recrystallized from ethanol or purified on silica gel. The structure was confirmed by the ^{1}H, ^{13}C, ^{77}Se nuclear magnetic resonance (NMR) data, and in the case of triethanolammonium hydroselenite [$HN^{+}(CH_2CH_2OH)_3HSeO_3^{-}$] by X-ray analysis (Lukevics et al., 2002a,b).

2.2. In vitro cytotoxicity assay

Monolayer tumor cell lines, HT-1080 (human fibrosarcoma), NIH 3T3 (normal mouse fibroblasts) and BHK21 (hamster kidney endothelial) cells, were cultured in standard medium Dulbecco's modified Eagle's medium (DMEM) 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 were added to the wells. The control cells without test compounds were cultured on separate plate. The plates were incubated for 72 h, 37 °C, 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 Lukevics et al. (2002a). Glutathione peroxidase activity and catalase activity were determined according to Eventoff (1976).

2.3. 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 (6 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 9 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 two times in the case of flat tumor shape).

2.4. Morphology assay

The change in cell morphology caused by hydroselenites was investigated on Nikon ECLIPSE TE 300 microscope slides. Crystal violet and acridine orange stains were used. The adherent cells were stained in the plate wells following culture of the cells with hydroselenites. Chromatin condensation in apoptotic cells was visualized by staining the cellular DNA with the dye acridine orange (Loweth and Morgan, 1998). Living cells stained green, apoptotic cells orange or yellow and necrotic cells red (Fig. 2).

3. Results

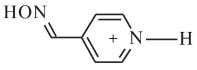
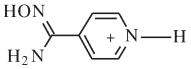
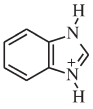
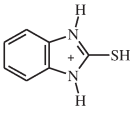
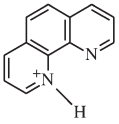
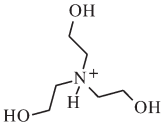
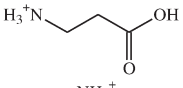
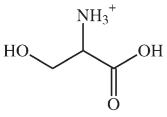
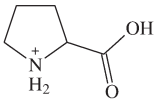
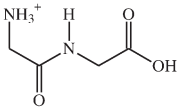
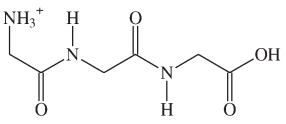
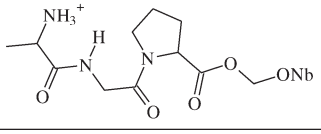
The results of cytotoxic activity studies of organoammonium hydroselenites **1**–**12** in vitro on HT-1080, BHK 21

and NIH 3T3 cells are summarized in Table 1. The majority of the compounds tested, such as 4-amidohydroximinomethylpyridinium hydroselenite (**2**) (3.6 μg/ml), o-phenanthrolinium hydroselenite (**5**) (0.6 μg/ml), triethanolammonium hydroselenite (**6**) (3.15 μg/ml) and prolinium hydroselenite (**9**) (1.7 μg/ml), exhibited a high activity in vitro on human fibrosarcoma HT-1080 cells.

Glutathione peroxidase and catalase inhibition data for the organoammonium hydroselenites **1**–**12** are given in Table 1. All compounds studied were glutathione peroxidase inhibitors. Some derivatives also prevented hydrogen peroxide degradation by inhibiting catalase.

Compounds **1**–**12** mostly increased the NO concentration in the culture medium. This effect was especially expressed in the case of compounds **7**–**11** (TG_{100} = 600–1500%) incubated with HT-1080 cells. The effect on NO generation of compounds **2** (TG_{100} = 24%) and **5** (TG_{100} = 50%) showed that they were free radical inhibitors.

Table 1
In vitro cytotoxicity in monolayer tumor cell lines [HT-1080 (human fibrosarcoma), NIH 3T3 (normal mouse fibroblasts) and BHK21 (hamster kidney endothelial)] and inhibition of tumor growth (sarcoma S-180) and enzyme activity (glutathione peroxidase and catalase) caused by hydroselenites **1**–**12**[a]

No.	(Amine)H^+ $HSeO_3^-$	HT-1080 TD_{50}	NO, 100%	NIH 3T3 TD_{50}	BHK21 TD_{50}	S-180 Inhibition, %	GSH-Px Inhibition, %	Catalase Inhibition, %
1		>50	5	37	22	25	26 ± 2	17 ± 3
2		3.6	24	6.6	3.4	27	50 ± 5	15 ± 3
3		5.3	200	4.0	>10	51	46 ± 6	5 ± 3
4		40.0	250	22.5	22	−37	36 ± 2	12 ± 7
5		0.6	50	0.9	>10	39	52 ± 10	20 ± 1
6		3.15	150	48.5	>50	81	60 ± 3	−2 ± 1
7		3.6	800	1.1	3.1	−4	62 ± 3	99 ± 1
8		10	1150	2.5	31.5	51	26 ± 8	100 ± 3
9		1.7	1500	1.5	2.5	1	21 ± 1	12 ± 7
10		20	800	47	40	33	35 ± 6	99 ± 1
11		10	600	21	5	−46	42 ± 6	−11 ± 2
12		2	80	7.9	56.2	36	46 ± 2	13 ± 3

[a] TD_{50} concentration (μg/ml) providing 50% cell killing effect [(CV + MTT)/2]; NO concentration (%) (CV: coloration); " – " tumor growth activation.

The influence of the studied ammonium hydroselenites **1**–**12** on tumor cell (fibrosarcoma HT-1080) morphology was examined. Figs. 2 and 3 present the changes in morphology after 72 h (visualization by acridine orange and crystal violet).

The antitumor activity of hydroselenites **1**–**12** was tested against sarcoma S-180 in male ICR mice (18–20 g). 4-Hydroximinomethylpyridinium hydroselenite (**1**) exhibited antitumor activity, without having a cytotoxic effect. Pyridinium hydroselenites **1** and **2** inhibited tumor growth by 25% and 27%, respectively (Table 1). Inspection of the tumor inhibition activity of compounds **7**–**12** showed that β-alanine hydroselenite (**7**) and prolinium hydroselenite (**9**) had no influence on sarcoma growth.

4. Discussion

The cytotoxic activity results showed that the studied hydroselenites are cytotoxic against human fibrosarcoma HT-1080 cells. It should be noted that, at the same concentrations, many hydroselenites were toxic against both fibrosarcoma and normal cells (NIH 3T3 and BHK 21). Benzimidazolinium hydroselenite (**3**) and 2-mercaptobenzimidazolinium hydroselenite (**4**) had comparable NO generating activity, but the insertion of a mercapto substituent into the benzimidazole ring (**4**) considerably decreased cytotoxicity against fibrosarcoma HT-1080. In a series of compounds (**7**–**12**), **9** was the most active in the experiments with HT-1080, while tripeptide derivative **12** had the highest selectivity (TD_{50} = 2.0 μg/ml on HT-1080), being less toxic against normal cells 3T3 (TD_{50} = 7.9 μg/ml on NIH 3T3).

The regulation of the glutathione peroxidase/glutathione reductase system may affect free radical balance in tumors. Glutathione peroxidase may scavenge various peroxides. Overprotection of this enzyme was observed to suppress reactive oxygen species inducing apoptotic death. The extent of enzymes inhibition strongly depended on the nature of the cation structure. Compounds **6** and **7** demonstrated the greatest inhibition glutathione peroxidase (60–62%). However, they showed opposite activity on catalase: hydroselenite **6** has no influence (− 2%), but **7** completely inhibited catalase activity (99%). Also, serine (**8**) and glycylglycine (**10**) hydroselenites even at a concentration 1×10^{-4} M completely inhibited catalase (99–100%).

The role of NO in biosystems has attracted considerable attention in the last decade. NO is formed by enzymatic and nonenzymatic 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. NO and oxygen anion (O_2^-) have a similar number of electrons and compete in biosystems. The understanding the role of NO, NO derivatives and NO inhibitors in the therapy of cancer is difficult because the data in the literature are contradictory. The multitude of actions and dynamics of NO suggest that regulatory compounds and NO-dependent processes lead to the transformation of cells into tumor cells under pathological conditions and to the normal action of NO under normal conditions (Fig. 1). We present a model with the four vectors pointing towards different biomolecules (RSNO, NO_x, [M···NO], $ONOO^-$). The bottom of the cone represents the normal level of NO-dependent system. Any biochemical or photochemical impulse from outside the system leads to the activation of NO system. The top of the cone represents the maximum activation of the system without pathological process occurring. If the impulse is too strong, the process by which a normal cell is converted into a tumor starts. The addition of selenium-containing derivatives **1**–**12** reduced the activation level to the inactivated state. It is necessary to regulate the entire system, not just one of the components. Our results indicate that the investigated organoammonium hydroselenites may be promising antitumor agents with an action mediated by the regulation of radical formation. The hydroselenite compounds **1**–**12** disrupt the different radical-dependent systems and slowly normalize the biochemical processes in the cell.

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 (Bauer et al., 2000). In the case of hydroselenites **1**–**12**, the role of thiol is played by glutathione. Moreover, hydroselenites may regulate the level of glutathione in the cell because selenium-containing derivatives are glutathione peroxidase inhibitors.

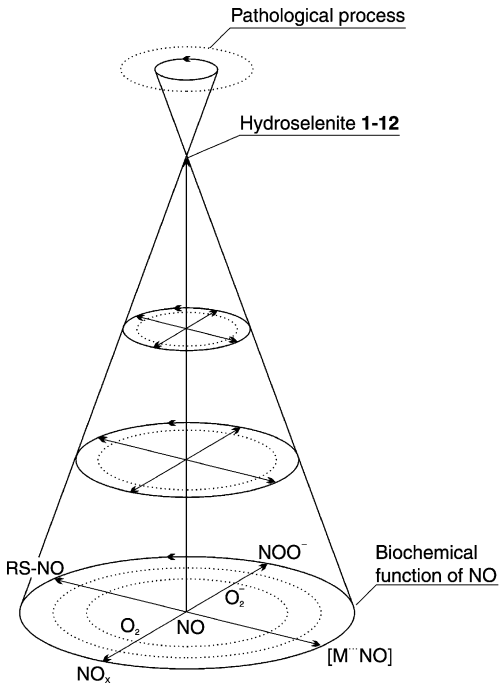


Fig. 1. The major reactive pathways of NO under physiological conditions. The extent and dynamics of NO action suggest the formation of regulatory compounds and NO-dependent processes to transform the tumor cell under pathological conditions to a normal state of NO action [the four vectors toward different biomolecules (RSNO, NO_x, [M···NO], NOO^-)].

According to our results, there is no correlation between the ability to inhibit glutathione peroxidase and the efficacy to generate NO. The increase in NO depends on the type of tissue, prodrug dose, etc. According to the literature, the conversion of L-arginine to L-citrulline with production of NO is catalyzed by heme iron (Pufahl and Marletta, 1993). The NO-inhibiting activity of compounds **2** and **5** may stem from their ability to make complexes with iron by amidoxime and two nitrogen fragments, respectively. Compounds **3**, **4** and especially amino acid hydroselenites **7**–**11**, unlike L-arginine and L-ornithine derivatives (Mayer et al., 1991; Stuehr et al., 1991), readily increased the NO concentration in the culture medium of HT-1080 cells (TG_{100} = 600–1500%). The amino acid derivatives studied nonspecifically activated NO generation in the cell. There was no correlation between NO generation and the cytotoxic effect of the salts studied that related to specific features of the cell lines.

The influence of the studied ammonium hydroselenites **1**–**12** on the phenotype of fibrosarcoma HT-1080 cells was examined. Figs. 2 and 3 show the morphological changes after 72 h (visualization by acridine orange and crystal violet). Figs. 2A and 3A show the morphological structure

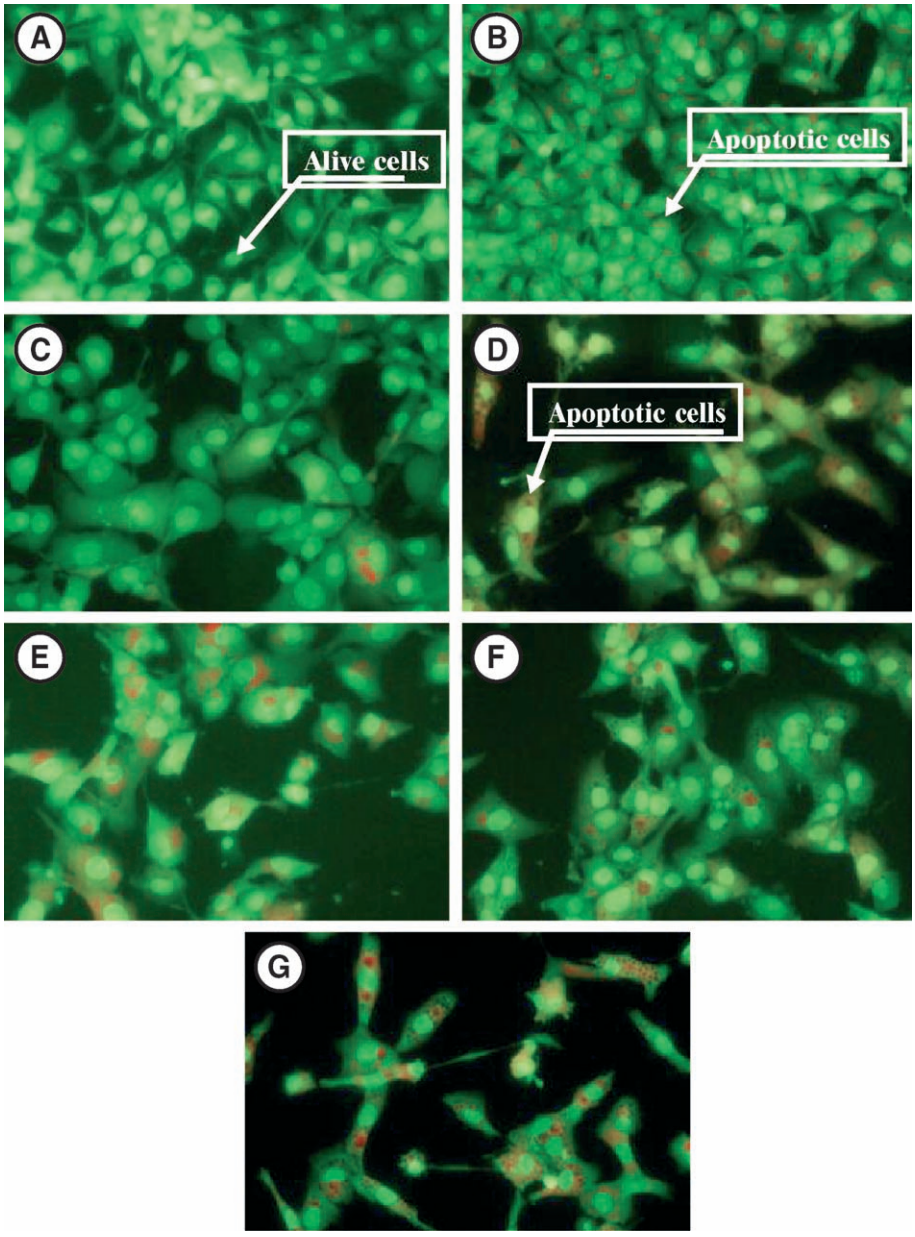


Fig. 2. (A) View of human fibrosarcoma HT-1080 cells (24 °C, 72 h) visualized by staining the cellular DNA with the dye acridine orange. Living cells exhibit a green color, cells with orange or yellow color are apoptotics, and cells with a red color are necrotics; (B) view of human fibrosarcoma HT-1080 cell phenotype with benzimidazolinium hydroselenite **3**; (C) with 2-mercaptobenzimidazolinium hydroselenite **4**; (D) with phenantrolinium hydroselenite **5**; (E) with serine hydroselenite **8** in dose 0.3 μg/ml; (F) with serine hydroselenite **8** in dose 3.0 μg/ml; (G) with tripeptide hydroselenite **12**.

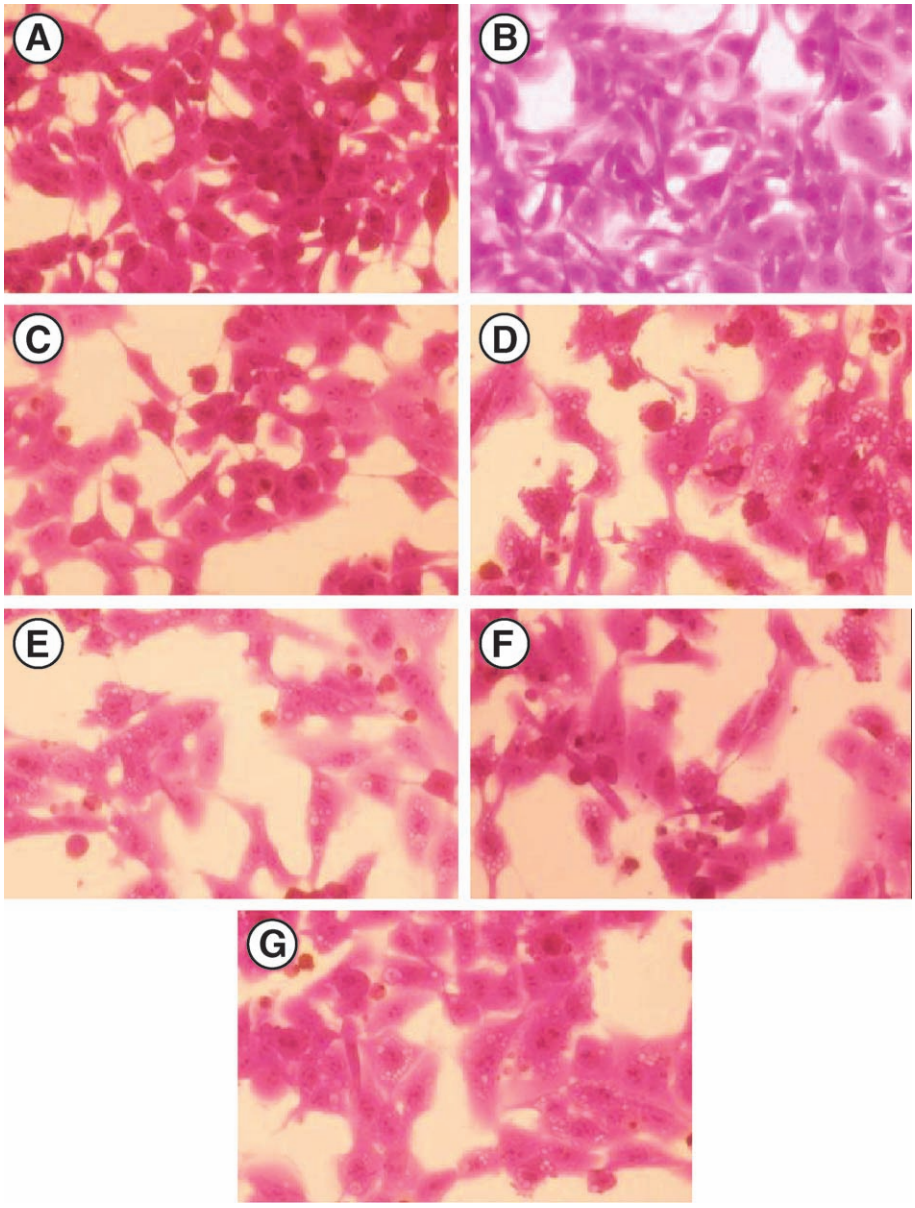


Fig. 3. (A) View of human fibrosarcoma HT-1080 cells (24 °C, 72 h) visualized with crystal violet; (B) view of human fibrosarcoma HT-1080 cell phenotype with benzimidazolinium hydroselenite **3**; (C) with 2-mercaptobenzimidazolinium hydroselenite **4**; (D) with phenantrolinium hydroselenite **5**; (E) with serine hydroselenite **8** in dose 0.3 μg/ml; (F) with serine hydroselenite **8** in dose 3.0 μg/ml; (G) with tripeptide hydroselenite **12**.

of human fibrosarcoma HT-1080 cells at 30 °C (control). The majority of tumor cells were alive and the cell nucleus was a dark color. Compound **3** induced apoptosis of fibrosarcoma cells (Figs. 2B and 3B). Derivative **3** changed the cell phenotype and mitotic mechanism. The cells increased in volume and contained more than one apoptotic nucleus. The insertion of a thiol substituent into the benzimidazole ring (**4**) significantly changed the type of action (Figs. 2C and 3C), with only few tumor cells undergoing apoptosis, as in the control. The action of hydroselenite **5** (Figs. 2D and 3D) was similar to that of hydroselenite **3** with more extensive cell polyploidy and high level of dispersity. To establish the dose-apoptotic activity relationship, the effect on cell morphology of serine hydroselenite (**8**) was studied at a dose of 0.3 (Figs. 2E and 3E) and 3 μg/ml (Figs. 2F and 3F). With the low dose, the tumor cells became friable, with two to five apoptotic nuclei. Increasing the dose of compound **8** by 10 times led to full apoptosis: the cells started to round off and their volume decreased. The increase in apoptosis depending on serine hydroselenite (**8**) concentration could be due to the high level of NO generation (TG_{100}=1150%). The tripeptide hydroselenite (**12**) strongly induced the extensive apoptosis, polyploidy and cell nucleus fragmentation (Figs. 2G and 3G). It should be noted that pyridinium hydroselenites (**1** and **2**), triethanolammonium hydroselenite (**6**) and amino acid hydroselenites (**7**, **9**, **10** and **11**) had no effect on tumor cell

morphology. An increase in NO level leads to apoptosis, whereas an increase in the oxygen radical level leads to necrosis. Fig. 3 shows that, in general, tumor cells die by apoptosis (Blanco et al., 1995). So, the way a tumor cell dies reflects the radical balance in the system. In our case, NO radical regulation has a dominant role.

Pyridinium hydroselenites **1** and **2** inhibited tumor growth by 25% and 27%, respectively; however, compound **1** had no cytotoxic effect (Table 1). Hydroselenites **3** and **4** had a different effect on cell morphology and on opposite influence on the speed of sarcoma S-180 growth. Tumor growth was inhibited by 51% after 9 days of hydroselenite **3** treatment. The insertion of a mercapto substituent into the benzimidazole ring (**4**) led to a stimulation of cancer growth by 37%. The most active compound in vitro, hydroselenite **5** and the most selective compound **6** were toxic (LD_{50} = 12.9 mg/kg for hydroselenite **5** and 25.8 mg/kg for compound **6**, i.p.). Selenite **5** at a dose of 10 mg/kg had undesirable effects and decreased tonus; however, a 39% inhibition of tumor growth was observed. The same concentration of compound **6** inhibited the tumor growth by 81%. At a dose of 8 mg/kg, the inhibition of tumor growth by compound **6** was slightly decreased (73%); however, at this dose, no undesirable side effects were detected. Inspection of the tumor inhibition ability of the compounds **7**–**12** shows that hydroselenites **7** and **9** had no influence on sarcoma growth. Compound **10** (dipeptide with a slight cytotoxicity) inhibited S-180 growth by 33%. However, the addition of one more glycine fragment (tripeptide **11**) led to an undesirable effect, namely stimulation of tumor growth by 46%. Of the derivatives **7**–**12**, **8** caused the greatest inhibition of tumor growth (51%).

We report the cytotoxic activity of a series of organoammonium hydroselenites. All the compounds studied are glutathione peroxidase inhibitors. Some derivatives may also prevent hydrogen peroxide degradation by inhibiting catalase. The investigated organoammonium hydroselenites are potential antitumor agents and act by regulating the free radical balance. In general, tumor cells die by apoptosis under the action of selenium derivatives **1**–**12**. Nitric oxide production plays a major role. A correlation between the cell morphology changes and antitumor activity was found. Human fibrosarcoma HT-1080 cell apoptosis caused by hydroselenite testifies that the compound is able to inhibit tumor growth.

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