Hydroxamic acids: synthesis and adjuvant activity in combinatorial anticancer therapy

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Mono- and disubstituted *N*-hydroxyamides of dicarboxylic acids were prepared by reaction of dicarboxylic acids or acid anhydrides with hydroxylamine. The use of these compounds in combinatorial cytostatic therapy of implanted tumors with cisplatin or cyclophosphamide totally inhibits metastasis formation in B16 melanoma and Lewis lung carcinoma, and resulted in 100% survival of leukemic animals.

Key words: hydroxamic acids, reaction of maleic anhydride with hydroxylamine, antimetastatic and anticancer activity, chemotherapy of cancer.

Today, combinatorial chemotherapy with different types of cytostatic drugs, alkylating agents, antimetabolites, and platinum complexes is the prevailing approach in chemotherapy of cancer of internal organs.[1,2] However, cytostatics, due to the lack of cancer cell specificity, cause side effects throughout the body, and among them the hematopoietic suppression[2] is the most severe and formidable. This is why the attempts are being made to reduce side effects by using the low toxic adjuvants, which can provide for the decrease of the therapeutic dose of cytostatics while preserving their efficiency.

However, discovery of adjuvants for this purpose shows random nature and cannot be systematized and attributed to specified classes of compounds. In this connection, between the end of the XX and the beginning of the XXI century the approach was developed aimed at the research of so called targeted drugs, which are directed at cell proliferation molecules or DNA replication enzymes. With a view to discover novel compounds with properties listed above we obtained hydroxyamides of dicarboxylic acids. In particular, hydroxyamides of oxalic, tartaric, aspartic, and maleic acids were prepared.[3,4] The experimental data obtained using BDF_1 mice evidenced that these hydroxamic acids showed substantial therapeutic effect when used in combinatorial cytostatic therapy with cisplatin or cyclophosphamide against P388 leukemia, B16 melanoma and Lewis lung carcinoma. In this connection, the research aimed at preparation of biologically active compounds on the basis of metabolically active starting compounds are of great interest. In our publications,[3,4] we explained the presented results by the chelation of the zinc atom with hydroxamic acids thus causing the tumor growth regression and metastasis suppression. Earlier,[5—8] it was shown that, the zinc atom is included into histon deacetylase, which is involved into DNA replication and apoptosis. This observation suggests that the listed above hydroxamic acids can deactivate histon deacetylase and, hence, inhibit the tumor growth and metastasis formation. The present work is aimed at synthesis and study of adjuvant properties of hydroxyamides of aliphatic dicarboxylic acids (hydroxamic acids) in combinatorial cytostatic therapy with known cytostatic drugs cisplatin and cyclophosphamide.

Results and Discussion

Recently, the search for new highly efficient adjuvants has acquired high importance. The development of fundamental research in the field of contemporary medicinal chemistry necessarily includes the detailed mechanistic study of biological action of pharmacologically active compounds or their pharmacophore features. In this connection, the research aimed at synthesis of biologically active compounds on the basis of metabolically active starting compounds generates considerable interest. Earlier,[9—11] we showed that the functional groups (the ligand) play an important and, in some case, a crucial role in pharmacological activity of the complex using the examples of palladium and platinum(IV) complexes with substituted amides of pyridinecarboxylic acids. Thus, we showed in publication[12] that platinum and palladium complexes with substituted amides of pyridinecarboxylic acids interact with sarcoplasmic reticulum Ca^{2+}—Mg^{2+}ATPase and slows down both the active transport of calcium across the cell

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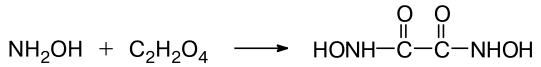
membrane and ATP hydrolysis the membrane-embedded sarcoplasmic reticulum Ca^{2+}—Mg^{2+} ATPase.[12] It was shown, that histon deacetylase with a zinc atom, which plays a catalytic role in metabolism,[5–8] takes part in DNA replication, apoptosis and metastasis formation. In the view of this, we can anticipate that the considered hydroxamic acids deactivate histon deacetylase by chelating the zinc atom with hydroxamic acids, thus causing the tumor growth regression and metastasis suppression.

It should be clarified that in this publication we consider dihydroxyamides of oxalic (**1**) and tartaric acids (**2**), and monohydroxyamides of aspartic (**3**) and maleic acids (**4**).

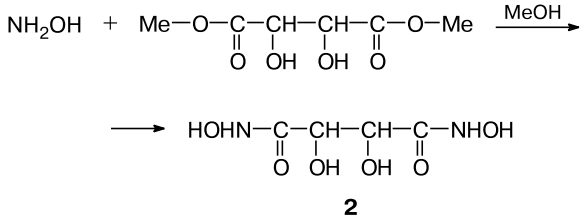
N,*N*′-Dihydroxyamide of oxalic acid (**1**) was synthesized as shown in Scheme 1.

Scheme 1



N,*N*′-Dihydroxyamide of tartaric acid (**2**) was prepared as shown in Scheme 2.

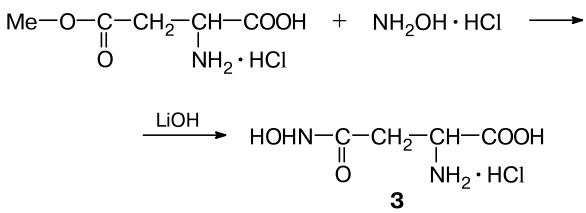
Scheme 2



However, solubility of compounds **1** and **2** in water proved to be insufficient for conduction of anti-cancer study. This is why aspartic and maleic acids were converted to monohydroxyamides leaving one of the carboxyl groups intact.

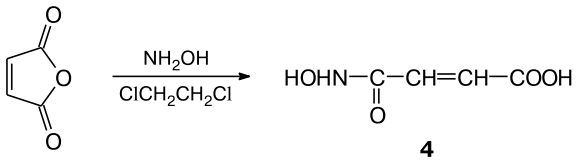
N-Hydroxyamide of aspartic acid (compound **3**) was obtained by interaction of β-methyl ester of aspartic acid with hydroxylamine hydrochloride in the presence of lithium hydroxide (Scheme 3).

Scheme 3



Lithium hydroxide was used because lithium chloride, which arises from the neutralization reaction, is readily soluble in ethyl acetate and can be removed from the reaction mixture by washing with ethyl acetate. On the contrary, sodium chloride and potassium chloride, which are formed in the reaction of hydroxylamine hydrochloride and, accordingly, sodium and potassium hydroxides, are insoluble in ethyl acetate, and additional purification of the monohydroxamic acid from metal chloride by multiple recrystallizations is needed.

Scheme 4



N-Hydroxyamide of maleic acid (**4**) was prepared by interaction of maleic anhydride with hydroxylamine in 1,2-dichloroethane in the presence of triethyl amine. The synthesized compounds were further transferred to the lab-

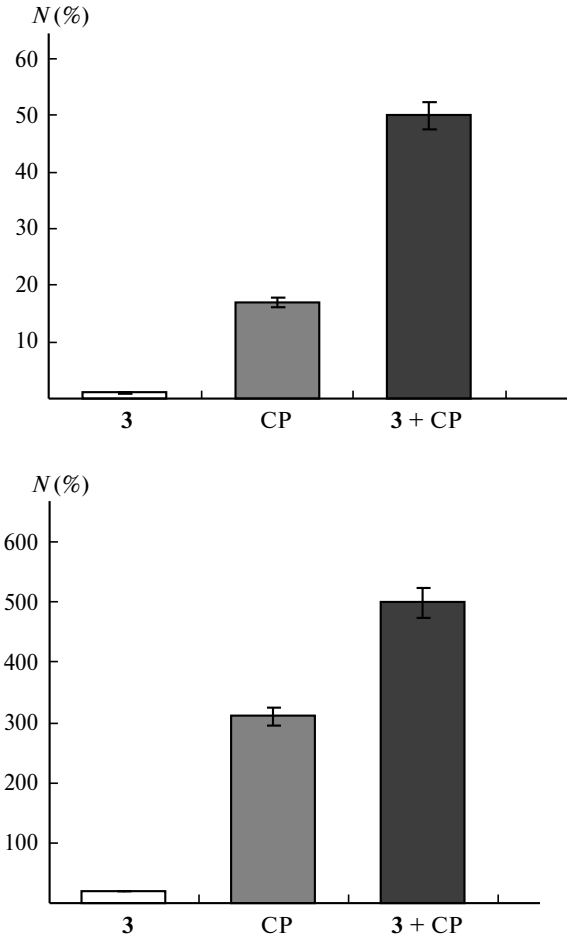


Fig 1. Increase of antitumor efficiency against P388) leukemia upon combination of cyclophosphamide (CP) with aspartic acid *N*-hydroxyamide (**3**): **3** (50 mg kg^{-1}), CP (60 mg kg^{-1}), **3** + CP (50 + 60 mg kg^{-1}); *N* is survival rate.

oratory of experimental chemotherapy of tumors of the Institute of Problems of Chemical Physics of Russian Academy of Sciences for examination of antitumor and antimetastatic activity using BDF_1 mice. The experimental data showed 70—100% inhibition of metastasis formation in implanted B16 melanoma and Lewis lung carcinoma, when hydroxyamides of aspartic and maleic acids (**3** and **4**) were used in combinatorial cytotoxic therapy by cyclophosphamide or cisplatin (Tables 1—3). Also, the experiments on animals evidenced that the use of monohydroxyamide of aspartic acid **3** in combinatorial cytostatic therapy with cyclophosphamide provided 50% survival and the life span of the other 50% of animals increased 5 times (Fig. 1). At the same time, the use of monohydroxyamide of maleic acid (**4**) in combinatorial cytostatic therapy with cisplatin totally inhibited metastasis formation in implanted B16 melanoma and Lewis lung carcinoma, and provided 100% animal survival in leuke-

Table 1. The effect of compounds **1**—**4** on the average lifespan (LS) of male BDF_1 mice with implanted P-388 lymphleukemia

Compound	Single dose/mg kg^{-1}	N_{60}	n	N (%)	LS/day	ILS (%)
Control	—	0	6	0	10.7	—
cPt	1.2	3	3	50	22.3	109.4
	2	3	3	50	27.3	156.3
1	1	0	6	0	10.8	1.6
	10	0	6	0	10.8	1.6
2	1	5	1	83	10.7	0.0
	10	0	6	0	10.7	0.0
3	10	0	6	0	10.7	0.0
4	10	0	6	0	—	—
cPt+**1**	1.2 + 1	6	0	100	—	—
	2 + 1	6	0	100	—	—
	1.2 + 10	6	0	100	—	—
	2 + 10	5	1	83	57.0	434.4
cPt+**2**	1.2 + 1	5	1	83	30.0	180.0
	2 + 1	6	0	100	—	—
	1.2 + 10	6	0	100	—	—
	2 + 10	6	0	100	—	—
cPt+**3**	2 + 10	6	0	100	—	—
cPt+**4**	2 + 10	6	0	100	—	—

Note. Inoculum: 10^6 tumor cells in 0.2 mL; intraperitoneal injection. The number of experimental animals is six. Dosage regimen/days: 1, 3, 5, 7. N_{60} is the number of survived animals by the 60 day. n is the number of fallen animals. N is the number of survived animals.

Table 2. Antimetastatic activity of compounds **1**—**3** administered in combination with cisplatin (cPt) in Lewis lung carcinoma

Compound	Single dose /mg kg^{-1}	The number of animals		The number of metastases per mouse	MII (%)	Total tumor weight	Tumor growth retention (%)
		in the experiment	with metastases				
Control	—	8	8	14.7±1.36	—	10.9	—
cPt	2	7*	7	5.7±0.99	61.0	8.8±0.25	19.0
1	1	8	8	11.1±1.99	24.0	9.7±0.45	11.0
	10	6*	6	7.3±1.33	50.0	9.7±0.26	11.0
2	1	7*	7	14.3±0.68	3.0	10.4±0.3	4.0
	10	6*	6	6.2±0.87	58.0	9.5±0.51	13.0
3	10	7	7	7.0±1.96	35.0	8.5±0.48	22.0
cPt+**1**	2 + 1	6*	6	9.8±2.9	33.0	9.1±0.42	17.0
	2 + 10	7*	7	6.7±2.4	54.0	8.5±0.37	22.0
cPt+**2**	2 + 1	8	8	3±0.34	80.0	7.7±0.51	29.0
	2 + 10	8	8	2.8±0.53	81.0	8.9±0.41	18.0
cPt+**3**	2 + 10	8	8	6.3±1.2	100.0	9.0±0.47	13.0

Note. Inoculum 10^6 tumor cells in 0.3 mL; subcutaneous injection. Dosage regimen/days: 2, 4, 6, 8. The animals were sacrificed on the 20 day after tumor implantation. All groups consisted of eight animals.

* In these groups some animals died before the 20th day after injection.

Table 3. Antimetastatic activity of compounds **1**, **2**, and **4** administered in combination with cyclophosphamide (CP) in Lewis lung carcinoma

Compound	Single dose /mg kg^{-1}	The number of animals		The number of metastases per mouse	MII (%)	Total tumor weight	Tumor growth retention (%)
		in the experiment	with metastases				
Control	—	8	8	10.4±0.43	—	9.1±0.3	—
CP	60	7*	7	4.4±0.89	57.0	4.6±0.54	49.0
1	1	7*	7	9.7±0.75	7.0	10.2±0.37	0.0
	10	8	8	11.3±0.75	0.0	9.3±0.3	0.0
2	1	6*	6	9.3±1.5	11.0	9.1±0.52	0.0
	10	8	8	6±0.75	42.0	9.1±0.6	0.0
4	10	8	8	7.0±2.2	13.0	7.1±1.76	24.0
CP+**1**	60 + 1	7*	7	4.3±0.75	59.0	5.3±0.43	42.0
	60 + 10	8	8	3.4±0.94	68.0	4.5±0.28	21.0
CP+**2**	60 + 1	8	7	3.6±0.73	69.0	5.1±0.2	44.0
	60 + 10	8	5	1.5±0.57	91.0	5.9±0.27	35.0
CP+**4**	60 + 10	8	4	1.7±1.33	88.0	4.1±1.22	56.0

Note. Inoculum 10^6 tumor cells in 0.3 mL; subcutaneous injection. Dosage regimen/days: 2, 7. The animals were sacrificed on the 20th day after tumor implantation. All groups consisted of eight animals.
* In these groups animals died before the 20 day after injection.

mia P-388. Comparison of the results and, in particular, the adjuvant properties of hydroxyamides of dicarboxyl acids under consideration showed that monohydroxyamide of maleic acid (**4**) was the most efficient. Notably, the results were obtained using low doses of cisplatin (from 1 to 1.2 mg kg^{-1}), which are not therapeutically effective if cisplatin is used alone. Hence, we can resume, that hydroxamic acids **1**—**4** show pronounced adjuvant (chemosenstizing) effect.

The obtained results have thus confirmed the validity of our approach, which suggests using metabolically active compounds as the basis for physiologically active groups. Besides, the obtained results show that the design of antitumor cytostatics and adjuvants on the basis of metabolically active compounds is an appropriate strategy. At the same time, the obtained data demonstrated that the effectivity of search for new biogenic adjuvants and targeted drugs has promising prospects in experimental chemotherapy of cancer.

Experimental

Identification of obtained hydroxamic acids **1**—**4** was conducted using the elemental analysis and NMR ^{1}H spectroscopy data, which was obtained on an AVANCE III 500 MHz Bruker Biospin instrument. For compounds **1** and **2** mass spectra were obtained on a LC/MS 20-20 Shimadzu instrument (Japan) equipped with electrospray (ESI) ionization source, with a scan range 10—2000 *m/z*. Mass resolution (FWHM) was 0.6. Aqueous solutions of compounds **1** and **2** were used as analytes. The mass spectrometer was operated in the positive ion mode in the range from *m/z* 10 (150)—600. Compound **1** was detected as the molecular ion *m/z* 120 Da in good agreement with the calculated molecular mass of **1** with molecular formula $C_2H_4N_2O_4$.

MS analysis of compound **2** showed the base peak at *m/z* 179. This observation evidences the formation of $[M - H]^+$ ion, where M is a molecular mass of **2**, $[180 - H]^+$. It is in good agreement with the molecular mass 180 calculated for **2** with a molecular formula $C_4H_8N_2O_6$.

Animal experiments. Examination of anticancer activity of new compounds was conducted according to Guidelines[13] on male BDF_1 hybrid mice (22—24 g) with P388 lymphleukemia, B16 melanoma and mouse Lewis lung cancer LL. Mice were kept in cages with free access to food and tap water. Tumors were implanted according to standard procedure intraperitoneally (P388 leukemia), subcutaneously (B16 melanoma) or intramuscularly (LL carcinoma) (10^6 tumor cells in isotonic solution of NaCl in 0.2 mL (P388 leukemia), $5 \cdot 10^6$ cells in 0.3 mL (B16 melanoma and LL carcinoma) inoculum). New chemical compounds and known cytostatics were injected intraperitoneally as aqueous solutions. Dosage regimen of injection (a day after implantation of the tumor) are listed in Tables. In each experiment animals with implanted tumor, which did not receive medication, were used as a control group. Each group consisted of 6—10 mice. To validate the therapeutic effect of anticancer therapy we used the number of survived animals for the 60-day observation period after the beginning of the therapy, average life span (LS), and increase of average lifespan (ILS) as the criteria of efficiency.

$$\text{ILS}\ (\%) = 100\ (T_o/T_к - 1),$$

where T_o and $T_к$ are the experimental average lifespan (days) observed for animals of the experimental and the control groups, respectively, and the number of cured animals (which survived to the 60-th day of the observation period).

The tumor growth inhibition rate was calculated on the basis of the regression of tumor growth (TGR) which is calculated as

$$\text{TGR}\ (\%) = [(V_c - V_e)/V_c] \cdot 100\%,$$

where V_e and V_c are the average volumes of the tumor (mm^3) observed in the experimental and the control groups, respectively.

Metastasis inhibition index (MII, %), which shows the frequency of metastasis, was calculated as

$$MII\ (\%) = [((A_c \cdot B_c) - (A \cdot B))/A_c \cdot B_c] \cdot 100\%,$$

where A_c and A are the frequency of lung metastases in mice of experimental and control groups; B_c and B are the average number of lung metastases in mice of experimental and control groups. Experimental values of MII found for compound **4** in Lewis lung carcinoma and B16 melanoma comprised 100%.

Oxalic acid *N,N´*-dihydroxyamide (1). To a stirred solution of hydroxylamine hydrochloride (6.95 g, 100 mmol) in methanol (70 mL) a solution of NaOH (4 g, 100 mmol) in methanol (30 mL) was added. The reaction mixture was stirred for 20 min, and the precipitated solid NaCl was filtered off. To the resulting methanolic solution anhydrous oxalic acid (4.25 g, 47 mmol) of was added. The reaction mixture was stirred for 1 h, white crystalline residue was filtered off, washed with methanol (5 mL) and dried in the air flow for 24 h and for 2 days in a vacuum desiccator over NaOH. Compound **1** (5 r, 89%) as colorless crystals, m.p. 185—186.5 °C (decomposes). Found (%): C, 20.21; H, 3.14; N, 23.40. $C_2H_4N_2O_4$. Calculated (%): C, 20.0; H, 3.33; N, 23.3. ^{1}H NMR (DMSO-d_6), δ: 4.13 (s, 1 H, OH); 8.03 (br.s, 2 H, NH). Found: *m/z* 120 $[M]^+$. $C_2H_4N_2O_4$. Calculated: M = 120.

(+)-Tartaric acid *N,N´*-dihydroxyamide (2). To 45 mL of 1 *M* solution of hydroxylamine in dry methanol a solution of methyl tartrate (2.69 g, 15 mmol) in dry methanol (10mL) was added upon stirring at ambient temperature, the reaction mixture was kept for two days, cooled to 0—5 °C, and the white crystalline residue was filtered off, washed with ethyl acetate (2×10 mL) and diethyl ether, and dried in air. Compound **2** (1.33 g, 73.8%) was obtained as a colorless powder, m.p. 170 °C (decomposes). Found (%): C, 26.1; H, 4.0; N, 16.1. $C_4H_8N_2O_6$. Calculated (%): C, 26.66; H, 4.44; N, 15.55. ^{1}H NMR (DMSO-d_6), δ: 4.22 (d, 2 H, CH, *J* = 6.5 Hz); 5.33 (d, 2 H, CHOH, *J* = 5.6 Hz); 8.75 (br.s, 2 H, NH); 10.38 (br.s, 1 H, COOH). Found: *m/z* 179 $[M - H]^+$. $C_4H_8N_2O_6$. Calculated: M = 180.

Aspartic acid *N*-hydroxyamide hyrdochloride (3). Aspartic acid β-methyl ester hydrochloride (3 g, 16.5 mmol) and hydroxylamine hydrochloride (2.3 g, 33 mmol) were added to methanol (50 mL), followed by lithium hydroxide (2 g, 83.5 mmol). After 15 min colorless solid residue of aspartic acid monooxamide and lithium chloride was filtered off, washed with ethyl acetate and dried in air at room temperature and over P_2O_5. Compound **3** (2.6 g, 86%) was obtained as hydrochloride, m. p. 175 °C (decomposes). Found (%): C, 26.44; H, 4.67; Cl, 19.02; N, 15.74. $C_4H_9N_2O_4Cl$. Calculated (%): C, 26.01; H, 4.98; Cl, 19.24; N, 15.18. ^{1}H NMR (DMSO-d_6), δ: 8.35 (br.s, 6 H, COOH, NH_2, HCl, NHOH); 3.96 (m, 1 H, CH); 2.71 (m, 2 H, CH_2).

Maleic acid *N*-hydroxyamide (4). Finely powdered hydroxylamine hydrochloride 8.34 g (120 mmol) was added to a solution of maleic anhydride (9.8 g, 100 mmol) in 1,2-dichloroethane (150 mL) at room temperature, the mixture was cooled to 10—20 °C, and triethylamine (30.4 mL, 220 mmol) was added. The reaction mixture was heated to the boiling point, kept for 1 h, cooled to 0—5 °C, and CF_3COOH (28.5 g, 250 mmol) was added. The mixture was stirred at 0—5 °C for 30 min, and colorless crystals were filtered off, washed with dichloroethane and dried in air. Compound **4** (12.4 g, 92%) was obtained as a solid, m.p. 92—93 °C. Found (%): C, 32.45; H, 3.48; N, 19.64. $C_4H_6N_2O_4$. Calculated (%): C, 32.87; H, 3.87; N, 19.18. ^{1}H NMR (DMSO-d_6), δ: 11.0 (br.s, 3 H, COOH, NHOH); 6.24 (br.s, 2 H, CH=CH).

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