MODIFICATION OF THE ANTITUMOR EFFECT OF DOXORUBICIN BY PHOSPHORYLATED RETINOIDS CONJUGATED TO α-FETOPROTEIN

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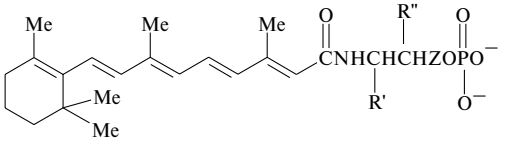
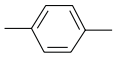
Retinoids are known to possess a broad spectrum of biological activity, which is related to the ability of these compounds to interact with RAR and RXR – the intrisic receptors containing transcription factors belonging to the steroid – thyroid superfamily of nuclear hormonal receptors [1]. Exhibiting strong antiproliferative and cytodifferentiating effects, retinoids are of interest for the chemotherapy of oncologic disorders [2, 3].

N-(4-hydroxyphenyl)-*all-trans*-retinoylamide (fenretinide) represents a group of retinoids synthesized in attempts at obtaining a compound of this type possessing minimum toxicity [4]. Fenretinide reduced the incidence of cancer of the mammary glands, skin, lung, bladder, and prostate in experimental animals treated with carcinogens [5]. It was also found that fenretinide inhibited the growth of human ovarian carcinoma transplanted in mice and potentiated the antitumor activity of cisplatin [6, 7]. Treatment of the neuroblastoma cells with fenretinide prior to introducing cisplatin, etoposide, or carboplatin into the culture medium led to apoptosis with evidence of a pronounced synergism in the drug action [8].

Below we report on the synthesis of new retinoids representing *all-trans*-retinoic acid (RA) amides with O-phosphates of ethanolamine, *L*-serine, *L*-threonine, and *L*-tyrosine. We have studied the influence of these retinoids conjugated to α-fetoprotein (α-FP) on the antitumor action of the antibiotic doxorubicin with respect to inoculated Ehrlich ascitic carcinoma. The synthesized compounds are structurally analogous to N-palmitoyl-O-phospho-*L*-serine (NP-Ser-PA) and N-palmitoyl-O-phospho-*L*-tyrosine (NP-Tyr-PA), which are antagonists of the receptors of lysophosphatidic acid (an intercell messenger inducing cell proliferation and cancer cell invasion) [9].

It should be noted that recently described synthesis of NP-Ser-PA and NP-Tyr-PA [10, 11] involves hydrogenolysis on the phosphorylation stage of and, hence, is applicable only to the synthesis of unsaturated acid derivatives.



	R′	R″	Z
NR-Ser-PA	COOH	H	—
NR-Thr-PA	COOH	CH_3	—
NR-Tyr-PA	COOH	H	
NR-EA-PA	H	H	—

The problem of acylating and phosphorylating serine, threonine, and tyrosine presents certain difficulty because amino acid molecules contain three reactive functional groups. Additional difficulty is related to the fact that free serine, threonine, and tyrosine are soluble only in water: this circumstance hinders using acylating agents based on fatty acids. We suggest using the initial substrates (serine, threonine, and tyrosine) in the form of methyl ester hydrochlorides. The acylation is performed with a mixed anhydride of RA and *n*-butylcarbonic acid. For tyrosine, the reaction at room temperature leads to the formation of a considerable amount of bis-O,N-acyl derivative. Conducting the acylation of tyrosine at –18°C for 1 h yields a virtually pure methyl ester (NR-Tyr-PA).

The obtained N-acylamino acid methylates were phosphorylated using a β-cyanoethyl method, which was originally proposed for the nucleotide synthesis [12]. However, the phosphorylation of N-acyltyrosine methylate under the originally proposed conditions (with pyridine as a solvent) proceeds with a low yield, which is probably related to the formation of an ionic bond between a phenolic hydroxy group and pyridine. A more satisfactory yield of the phosphorylated diester is achieved when the reaction is con-

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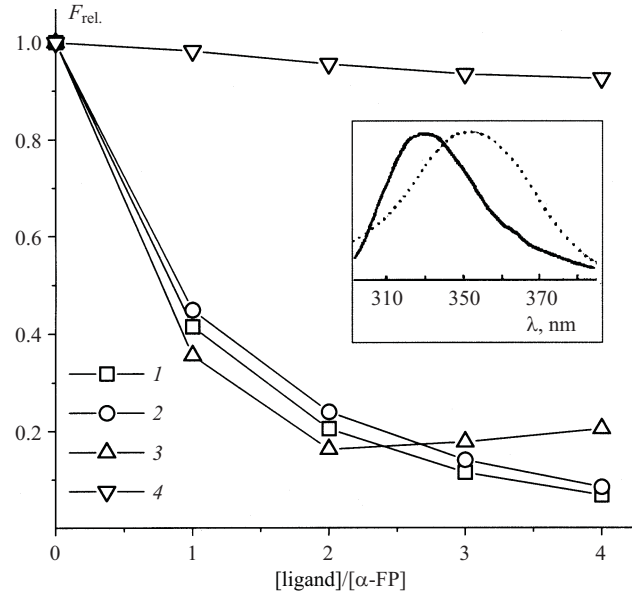

Fig. 1. The intrinsic fluorescence of α-FP quenched as a result of adding ligands: (*1*) NR-Ser-PA; (*2*) NR-Tyr-PA; (*3*) *all-trans*-retinoic acid; (*4*) docosahexaenoic acid. The inset shows the fluorescence spectrum of α-FP excited at a wavelength of 290 nm (solid curve) and a fragment of the absorption spectrum of retinoids (dashed curve).

ducted in acetonitrile for 5 days at 20°C (Table 1). This modified method is convenient because both protective moieties (methyl ester and β-cyanoethyl group) can be simultaneously removed with a virtually quantitative yield under mild alkaline conditions. During the hydrolysis, relatively labile amide and phosphate bonds are fully retained, and the RA polyene chain is neither subject to isomerization or oxidation, nor exhibits polymerization.

The targeted delivery of the synthesized retinoids to a tumor tissue can be provided by α-FP. Selecting this molecule as a vector is explained by the fact that receptors of this protein are expressed in considerable amounts in the tumor cells [13]. At the same time, RA is capable of specifically binding to α-FP [14]. However, the synthesized compounds possess a more complicated structure as compared to that of free RA, which may hinder their binding to α-FP. We have estimated the possibility of complexation between α-FP and the synthesized retinoids by measuring the quenching of fluorescence from tyrosine and tryptophan-162 residues as a result of the induction-resonance energy transfer from an excited state of the donor to the retinoyl acceptor. Owing to an overlap of the fluorescence spectrum of the donor (λ_{max} = 328 nm) and the absorption spectrum of the acceptor (λ_{max} = 345 nm), an effective fluorescence quenching is observed when the protein fluorophore is excited at a wavelength of 290 nm. It was found that the efficiency of NP-Ser-PA and NP-Tyr-PA binding to the oncofetal protein differs but little from their binding to free retinoic acid (Fig. 1). Adding a different ligand (docosahexaenoic acid incapable of quenching fluorescence in this spectral interval [14]) to the α-FP solution did not lead to any significant change in the fluorescence intensity. Therefore, the fluorescence quenching is related to an induction-resonance energy transfer realized within a region bounded by the Forster radius (usually, on the order of 30 – 50 Å) that is within the boundaries of the ligand protein complex studied.

The influence of the complexes between α-FP and retinoids was studied on a model of Ehrlich ascitic carcinoma in mice. The results presented in Table 2 shows that doxorubicin intravenously injected in a dose of 3 × 2 mg/kg produces no significant effect upon the dense ascites fraction, but reliably inhibits (28%, $p < 0.05$) the cell growth in the ascitic form of Ehrlich carcinoma. The compound NR-Tyr-PA (as well as α-FP) introduced by the same scheme in the corresponding dose produces no significant change in the tumor development either. Nor does the antitumor action (Ehrlich ascitic tumor growth inhibition) of the doxorubicin complex with NR-Tyr-PA reach a level characteristic of free anthracycline. Only adding α-FP (250 μg/kg) to the complex increases the model tumor growth inhibition up to 38% ($p < 0.05$), although the decrease in the ascites residue volume amounts only to 21% (which is statistically insignificant). On the whole, the antitumor action of this retinoid – α-FP complex is not superior to the doxorubicin effect. Nevertheless, the experimental results reliably indicate that α-FP is a significant factor accounting the the positive effect of the composition studied. A certain specific protein content in this composition not only eliminates the nega-

TABLE 1. Yields and ^{1}H NMR Spectra of Phosphorylated Retinoids

Compound	Yield, %	Empirical	^{1}H NMR spectrum (in CD_3SOCD_3): δ, ppm
NR-Ser-PA	24	$C_{23}H_{34}NO_7P$	1.0 (s, 6H); 1.4 – 2.4 (m, 15H); 3.9 – 4.0 (bs, 2H); 4.3 – 4.4 (m, 1H); 5.8 – 7.0 (m, 6H); 8.2 – 8.4 (m, 3H)
NR-Thr-PA	27	$C_{24}H_{36}NO_7P$	1.0 (s, 6H); 1.2 – 1.4 (d, 3H); 1.4 – 2.4 (m, 15H); 4.1 – 4.3 (m, 2H); 5.8 – 7.0 (m, 6H); 8.2 – 8.4 (m, 3H)
NR-Tyr-PA	19	$C_{29}H_{38}NO_7P$	1.0 (s, 6H); 1.4 – 2.4 (m, 15H); 2.9 – 3.1 (m, 2H); 4.3 – 4.4 (m, 1H); 5.8 – 7.0 (m, 6H); 7.0 – 7.2 (q, 4H); 8.2 – 8.4 (m, 3H)
NR-EA-PA	28	$C_{22}H_{34}NO_5P$	1.0 (s, 6H); 1.4 – 2.4 (m, 15H); 3.4 – 3.5 (bs, 2H); 3.9 – 4.0 (bs, 2H); 5.8 – 7.0 (m, 6H); 8.2 – 8.4 (m, 3H)

TABLE 2. Effect of Complexation with Retinoic Acid Derivatives and α-Fetoprotein on the Antitumor Activity of Doxorubicin with Respect to the Ehrlich Ascitic Carcinoma in C3H Mice

Preparation (dose)	Tumor process parameters			
	Ascites residue volume	Number of tumor cells, $\times 10^6$	Tumor growth inhibition, %	p
First series				
Control	1.9 ± 0.62	601.5 ± 42.87	–	–
Doxorubicin (2 mg/kg)	1.7 ± 0.72	433.2 ± 30.87	28	< 0.05
NR-Tyr-PA (6.8 mg/kg)	2.1 ± 1.36	523.9 ± 147.39	13	> 0.05
α-FP (0.25 mg/kg)	1.8 ± 0.29	470.5 ± 50.61	22	> 0.05
Doxorubicin + NR-Tyr-PA (2.0 + 6.8 mg/kg)	1.9 ± 0.62	498.2 ± 87.67	17	> 0.05
Doxorubicin + NR-Tyr-PA + α-FP[a)]	1.5 ± 0.94	373.2 ± 97.50	38	< 0.05
Second series				
Control	1.3 ± 0.46	499.4 ± 70.15	–	–
Doxorubicin (3 mg/kg)	0.7 ± 0.37	249.3 ± 41.38	50[b)]	< 0.02
Doxorubicin + NR-Ser-PA + α-FP	1.1 ± 0.26	407.9 ± 60.53	– 64[c)]	< 0.05
Doxorubicin + NR-Thr-PA + α-FP	1.0 ± 0.58	315.4 ± 47.66	37[b)]	< 0.05
Doxorubicin + NR-Tyr-PA + α-FP	0.6 ± 0.19	212.1 ± 71.97	58[b)]	< 0.02
Doxorubicin + NR-EA-PA + α-FP	1.2 ± 0.44	367.1 ± 36.08	– 47[c)]	< 0.05

Notes. [a] The three-component mixture in the first series of experiments was composed of doxorubicin (2 mg/kg), retinoid (6.8 mg/kg), and α-FP (0.25 mg/kg); in the second series, doxorubicin (3 mg/kg), retinoid (6.8 mg/kg), and α-FP (0.25 mg.kg); [b] relative to control; [c] relative to doxorubicin.

tive influence of NR-Tyr-PA upon the antitumor activity of doxorubicin but even renders the inhibiting effect of the complex more pronounced in comparison to that of the antibiotic component alone.

The above data provided a base for the second series of experiments, aimed at a comparative study of the modifying action of four RA derivatives in compositions of the doxorubicin – ligand – α-FP type. In this series, only RA derivatives were replaced in the complex. Taking into account the relatively low activity of doxorubicin in the experiments described above, the drug dose was increased from 2 to 3 mg/kg.

It was established that the increase in the doxorubicin dose led to the antitumor effect being more pronounced, reaching 50% with respect to the tumor cell growth inhibition; the ascites residue volume was found to decrease as well (Table 2). The complex of doxorubicin with NR-Thr-PA in the presence of α-FP exhibited a lower antitumor activity as compared to that of free doxorubicin: the Ehrlich carcinoma cell growth was inhibited by 37% ($p < 0.05$). The RA complex containing only NR-Tyr-PA produced a more pronounced cytotoxic action upon the tumor as compared to both the aforementioned complex and free doxorubicin.

The two other complexes, involving doxorubicin conjugated to NR-Ser-PA and NR-EA-PA, produced no significant cytotoxic action upon Ehrlich ascitic carcinoma in comparison to mice in the untreated control group. Moreover, the presence of these retinoids reduced the cytotoxic activity of doxorubicin (the corresponding tumor growth inhibition values are 64 and 47% lower as compared to the effect of free doxorubicin). Based on these data, we may suggest that (i) an optimum composition is offered by the doxorubicin complex with NR-Tyr-PA and (ii) α-FP produces a positive influence on the antitumor properties of this complex. We may also suggest that the absence of a reliable effect for the mixture of doxorubicin with NR-Tyr-PA is explained by the formation of an ion complex between the components, related to the interaction between the amino group of daunosamine and the phosphate moiety of NR-Tyr-PA. The presence of α-FP seems to destroy this ion complex, binds NR-Tyr-PA, and delivers it to the target tumor cells.

Thus, adding α-FP and synthetic N-*all-trans*-retinoyl-*L*-tyrosine-O-phosphate to doxorubicin reliable increases the chemotherapeutic effect of antibiotic, while the other synthesized retinoids were found ineffective with respect to the tumor model studied.

EXPERIMENTAL CHEMICAL PART

The experiments were performed with *all-trans*-retinoic acid, *n*-butylchloroformate, β-cyanoethylphosphate (Ba salt), N,N′-dicyclohexylcarbodiimide, and amino acids from Sigma (USA) and doxorubicin from Pharmacia AB (Sweden). α-FP was isolated from retroplacental serum of parturient women as described in [15]. The analysis for phosphorus was performed using the method described in [16].

General method for the synthesis of retinoids. To a solution of RA (152 mg, 0.5 mmole) and triethylamine (52 mg, 0.51 mmole) in 3 ml of a THF – acetonitrile (1 : 2) mixture, cooled to – 18°C, was added *n*-butylchloroformate (70 mg, 0.51 mmole) and the reaction mixture was allowed to stand at – 18°C for 20 min. Then the precipitate of triethylamine hydrochloride was separated and the remaining mixed anhydride solution was added to a solution of ethanolamine or amino acid methylate (1 mmole) in 1 ml of methanol (also cooled to – 18°C). The reaction mixture was kept for 20 min at – 18°C and stirred for 2 h at room temperature (for tyrosine, the acylation was conducted for 1 h at – 18°C). Then 10 ml of 0.5 M HCl cooled to – 5°C was added and the mixture was extracted with 20 ml of diethyl ether. The ether extract was washed with 10 ml of water, dried over anhydrous Na_2SO_4, and evaporated. The residue was dissolved in chloroform (2 ml) and applied onto a chromatographic column filled with alumina (alkaline, activity degree II according to Brockman). The column was eluted with 10 ml of a chloroform – methanol (9 : 1, v/v) mixture. Finally, the eluate was evaporated to obtain N-retinoyl derivatives of ethanolamine or amino acid methylate (yield, 95%). TLC on silica gel reveals a single yellow spot at $R_f = 0.4 – 0.5$ (benzene – dioxane – acetic acid, 25 : 5 : 1, v/v).

To this product were added a solution of β-cyanoethylphosphate (pyridinium salt, 2 mmole) in 3 ml of anhydrous pyridine and N,N′-dicyclohexylcarbodiimide (413 mg, 2 mmole) and the reaction mixture was stirred for 20 h at 20°C. Then the mixture was cooled to 0°C and 0.5 ml of water was added, after which stirring was continued for 30 min at room temperature (for tyrosine derivative, the phosphorylation was performed in acetonitrile at 20°C for 5 days). The precipitate of N,N′-dicyclohexylurea was separated by filtration. The filtrate was evaporated to dryness and the residue was purified by chromatography on a silica gel column eluted with chloroform – methanol (70 – 60 : 30 – 40, v/v) mixtures. Fractions containing the target product were evaporated and the residue was dissolved in 1 ml of THF. The solution was added dropwise with stirring to 1.5 M NaOH (4 ml) at 0°C and allowed to stand for 25 min. Then the reaction mixture was acidified to pH 1.5 with 1 M HCl and extracted with a chloroform – methanol (2 : 1, v/v) mixture. The extract was washed with a chloroform – water (10 : 9) mixture, evaporated, and purified by chromatography on a silica gel column. The target phosphates were eluted with chloroform – methanol (30 – 20 : 70 – 80, v/v) mixtures. Fractions containing the target product were combined and evaporated. TLC on silica gel: $R_f = 0.05 – 0.10$ (chloroform – methanol – 28% ammonia, 9 : 7 : 2, v/v). Yields and some analytical data are listed in Table 1.

EXPERIMENTAL BIOLOGICAL PART

The Ehrlich ascitic carcinoma was modeled by a conventional method [17] in inbred C3H female mice weighing 20 – 24 g. The animals were kept on a standard laboratory diet with free access to water. The tumor was taken from the given strain mice and intraperitoneally implanted into test mice (2×10^6 carcinoma cells). On the next day, the test mice were randomized to form experimental groups, each of seven mice, and the untreated control group of 15 mice.

Doxorubicin (3 mg/kg) and its complexes with retinoids were injected into test mice according to an interrupted schedule, on the second, fourth, and sixths day after tumor implantation. The substances were injected into a lateral tail vein with 0.2 ml of an isotonic NaCl solution. The compositions of doxorubicin complexes are indicated in Table 2.

The results were evaluated on the eighth day in terms of the tumor growth inhibition, ascites residue volume, and number of tumor cells. The tumor growth inhibition, expressed as percentage relative to control, was calculated by the formula [(control – test)/control] × 100%. The results were statistically processed; activities of the standard drug and the complexes tested were compared in terms of the Student *t*-criterion.

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