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Effects of Covalent Conjugates of Fullerene Derivatives with Xanthene Dyes on Activity of Ca^{2+}-ATPase of the Sarcoplasmic Reticulum

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The effects of the newly synthesized covalent conjugates of water-soluble fullerene derivatives (WSFD) with xanthene dyes: polyanionic WSFD—fluorescein (**1**), polycationic WSFD—fluorescein (**2**), polyanionic WSFD—eosin (**3**), and polyanionic WSFD (**4**), polycationic WSFD (**5**), fluorescein (**6**) and eosin (**7**), on activity of the membrane-bound Ca^{2+}-ATPase of the sarcoplasmic reticulum (SR Ca^{2+}-ATPase) were studied. Compounds **1**, **3**, **4**, **6,** and **7** inhibit the hydrolytic function of the enzyme, the inhibition constants for these compounds are $K_i=1.3\times10^{-5}$ M (**1**), $K_i=4.7\times10^{-6}$ M (**3**), $K_i=2.5\times10^{-6}$ M (**4**), $K_i=6.1\times10^{-5}$ M (**6**), and $K_i=5.8\times10^{-6}$ M (**7**). The effects of compounds **3**, **6**, and **7** on the hydrolytic function of the enzyme is competitive; compounds **1** and **4** are noncompetitive. Polycationic WSFD fluorescein (**2**) and polycationic WSFD (**5**) do not affect ATP hydrolysis, but inhibit active Ca^{2+} transport in a concentration of 0.01 mM by 100±10 and 40±4%, respectively. Conjugates **1** and **3** completely inhibit the hydrolytic and transport functions of the enzyme in a concentration of 0.01 mM, and in a concentration of 0.001 mM inhibit active Ca^{2+} transport by 60±6 and 55±6% uncoupling the hydrolytic and transport functions of SR Ca^{2+}-ATPases. The obtained results demonstrate a significant effect of the studied compounds on the active transmembrane transfer of Ca^{2+} and make it possible to predict the presence of antimetastatic and antiaggregatory activities of the studied compounds.

Key Words: *Ca^{2+}-ATPase of the sarcoplasmic reticulum; xanthene dyes; fullerene derivatives; inhibition*

Nanotechnological developments and advances in the functionalization of nanocarbon materials led to the creation of new pharmacologically active compounds based on fullerene C_{60}. Water-soluble derivatives of fullerene C_{60} (WSFD) have membranotropic properties due to the presence of a unique nanocarbon spheroid in their composition, which determines their pharmacological features, first of all, the permeability of the lipid bilayer of biological membranes for them, as well as the effect on activity of membrane-bound enzymes [3,5,8,9].

Systematic studies are conducted at the Institute of Problems of Chemical Physics in the field of creation

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of a new type of photodynamic antitumor compounds and microbicides based on complexes of WSFD with dyes or covalent WSFD—dye structures [2,10,14]. The combination of fullerene and dye in one hybrid structure due to effective excitation of the dye and transfer of excitation or electron from the excited dye to fullerene allows significantly increasing the efficiency of ROS generation, which is necessary for suppression of the growth of tumor cells. It also allows using not only triplet excited dyes for photodynamic therapy, but also dyes characterized by a low yield to the triplet state, *e.g.* fluorescein [2,10]. In this context, it seems necessary to analyze the various types of biological activity of new hybrid WSFD—dye compounds, the mechanisms of their action on the molecular targets associated with the development of cancer and other diseases.

It is known that Ca^{2+}-ATPase of the sarcoplasmic reticulum (SR Ca^{2+}-ATPase) catalyzes active transport of Ca^{2+} ions by using energy of ATP hydrolysis [8]. It was previously shown that SR Ca^{2+}-ATPase is involved in the molecular mechanisms underlying drug resistance and antimetastatic action of many cytostatics [12,13]. Inhibition of SR Ca^{2+}-ATPase-catalyzed active Ca^{2+} transport through the membrane changes the ratio of intracellular and extracellular Ca^{2+}, which prevents the formation of blood thrombus and adhesion of metastatic cells to the endothelium of capillaries and prevents the growth of metastases [1,6,7,11].

The structure of SR Ca^{2+}-ATPase has been well studied by modern physical methods, including X-ray structural analysis with a resolution of 2.6 Å [15]. In different protein domains, nucleotide, inorganic phosphate, and Ca^{2+} binding sites were distinguished. The binding sites of the ATP polyphosphate fragment are predominantly positively charged (cationic center) and bind anionic molecules, while Ca^{2+} binding sites are

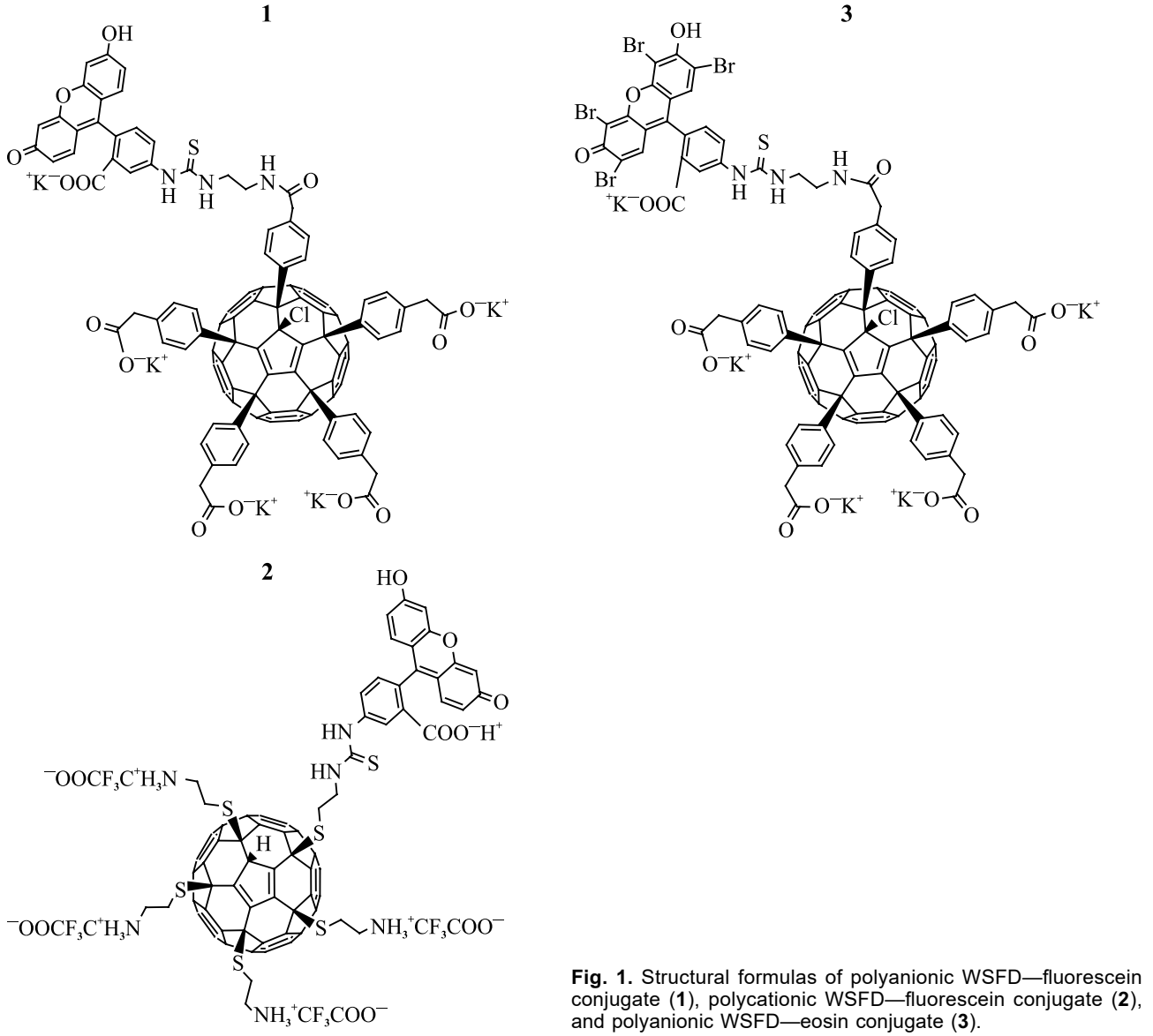

Fig. 1. Structural formulas of polyanionic WSFD—fluorescein conjugate (**1**), polycationic WSFD—fluorescein conjugate (**2**), and polyanionic WSFD—eosin conjugate (**3**).

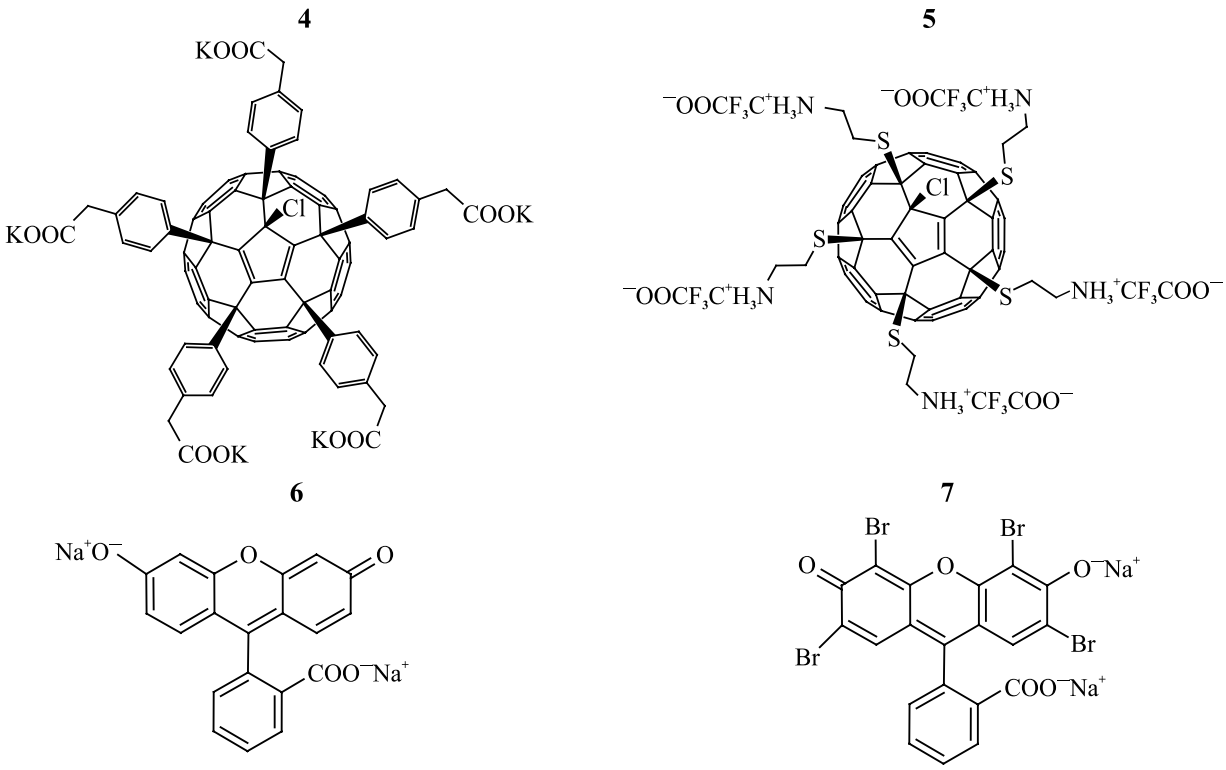

Fig. 2. Structural formulas of polyanionic WSFD (**4**), polycationic WSFD (**5**), fluorescein (**6**), and eosin (**7**).

negatively charged (anionic center) and bind cationic molecules. The binding center of the nucleotide fragment of ATP has a generally neutral charge. Therefore, it can be expected that the studied water-soluble complexes of fullerenes with different charges will interact with different centers of the enzyme and inhibit its activity to varying degrees.

Earlier, we studied the mechanisms of the influence of various water-soluble fullerene derivatives on enzymatic activity of SR Ca^{2+}-ATPase [5,8,9]. In most cases, they inhibited the hydrolytic function of SR Ca^{2+}-ATPase in a concentration of 10^{-5}-10^{-6} M. In parallel, uncoupling of hydrolytic and transport functions occurred, which led to a decrease in active Ca^{2+} transport through the membrane.

The purpose of this work was to study the effects of hybrid compounds: polyanionic WSFD—fluorescein conjugate (**1**), polycationic WSFD—fluorescein conjugate (**2**), and polyanionic WSFD—eosin conjugate (**3**), and also reference drugs polyanionic WSFD (**4**), polycationic WSFD (**5**), and individual dyes fluorescein (**6**) and eosin (**7**) (Figs. 1, 2) on the enzymatic functions of SR Ca^{2+}-ATPase.

MATERIALS AND METHODS

We used ATP (Sigma), human albumin, imidazole, sodium oxalate, EDTA, sucrose, $MgCl_2$, NaCl, KCl, $CaCl_2$, imidazole, DMSO, trichloroacetic acid, ammonium molybdate $(NH_4)_2MoO_4$, eosin and fluorescein sodium salt (Sigma).

Synthesis of polyanionic conjugates WSFD—fluorescein (**1**) and polycationic WSFD—fluorescein (**2**) was previously described in detail [14]. Synthesis of polyanionic WSFD—eosin (**3**) was carried out according to the method similar to compound (**1**) [14]. The composition and structure of conjugate **3** are proved using electrospray mass spectrometry and IR spectrometry. For compound **3** (in the form of an acid, $C_{123}H_{48}Br_4ClN_3O_{14}S$): ESI MS: m/z=1088 $[M-2H]^{2-}$. IR (KBr tablet, ν, cm^{-1}): 3424 (VS), 3386 (VS), 3302 (S), 2954 (S), 2924 (VS), 2854 (S), 1708 (S), 1638 (S), 1614 (S), 1562 (S), 1548 (S), 1510 (S), 1460 (S), 1416 (S), 1386 (S), 1344 (S), 1314 (S), 1288 (S), 1228 (S), 1160 (S), 1122 (S), 1082 (S), 1060 (S), 1020 (S).

SR Ca^{2+}-ATPase was isolated from the white muscles of rabbit hindlimbs and its hydrolytic and transport functions were studied as described elsewhere [4]. Specific activity of the enzyme was 15,000 nM/mg protein/min.

The reaction medium contained 4 mM $MgCl_2$, 2.5 mM imidazole, 100 mM NaCl, 5 mM Na oxalate, 0.04 mg of protein, 3 mM ATP (pH 7.2). The reaction was initiated by the addition of 0.1 mM $CaCl_2$.

The effects of the studied compounds on SR Ca^{2+}-ATPase activity was studied after a 3-min preincu-

bation of the preparations with the enzyme. Activity of Ca^{2+}-ATPase was determined by the kinetics of sample pH, because phosphate ion formation during this reaction leads to acidification of the medium. The hydrolytic activity of Ca^{2+}-ATPase was calculated as the tangent of the slope of the initial part of curve describing the kinetics of accumulation of inorganic phosphate as a result of ATP hydrolysis. The rate of changes in Ca^{2+} ions concentration was estimated by the time of their complete uptake by the SR vesicles, which leads to stopping of the ATP hydrolysis reaction. Relative activity of the enzyme was calculated by the formula: $J=100(A_0-A)/A_0$, where I is relative enzyme activity and A_0 and A are specific content of inorganic phosphate in the control and test (containing the analyzed compound) samples, respectively.

The mechanism of inhibition of SR Ca^{2+}-ATPase by compounds was analyzed by the dependence of the rate of enzymatic reaction on the substrate concentration (ATP) in the presence or absence of the test compounds.

Statistical processing of the results was performed by calculating the arithmetic mean and error of the mean; significance of differences was determined using the Student's *t* test. The differences were considered significant at 5% significance level.

RESULTS

All the studied compounds in varying degrees modulated the catalytic functions of SR Ca^{2+}-ATPase. Polyanionic conjugates **1** and **3** in a concentration of 0.01 mM completely inhibited the hydrolytic and transport functions of Ca^{2+}-ATPase (Table 1). Compound **2** (cationic WSFD—fluorescein), unlike compound **1** (anionic WSFD—fluorescein), did not affect ATP hydrolysis and completely inhibited active transport of Ca^{2+} in a concentration of 0.01 mM. It can be assumed that compound **1** binds to the cationic center of the enzyme and compound **2** binds to the binding site of Ca^{2+} ions. Reference compounds fluorescein **6** and eosin **7** in a concentration of 0.01 mM inhibited Ca^{2+} transport by 62±6 and 21±2%, respectively, and ATP hydrolysis by 82±8 and 65±6%, respectively, *i.e.* less potently than their conjugates with polyanionic WSFD (compounds **1** and **3**). The reference compound **4** (polyanionic WSFD) completely inhibits the transport function of SR Ca^{2+}-ATPase and by 88±8% hydrolytic function in a concentration of 0.01 mM, which is close to the inhibitory effects of polyanion conjugates **1** and **3**. At the same time, polycationic WSFD (**5**) does not affect ATP hydrolysis and inhibits by only 40±4% active transport of Ca^{2+} at a concentration of 0.01 mM, which is noticeably weaker than the inhibitory effect of the polycation conjugate **2**.

TABLE 1. Effects of WSFD on Activity of SR Ca^{2+}-ATPase (% of control; *M*±*m*)

Compound	Compound concentration in samples						K_i, M	Type of inhibition of ATP hydrolysis
	10^{-5} M		10^{-6} M		10^{-7} M			
	active Ca^{2+} transport	ATP hydrolysis	active Ca^{2+} transport	ATP hydrolysis	active Ca^{2+} transport	ATP hydrolysis		
Polyanionic WSFD—fluorescein (**1**)	100±10	100±10	60±6	17±2	45±5	11±1	1.3×10^{-5}	Non-competitive
Polycationic WSFD—fluorescein (**2**)	100±10	9±1	40±4	0	33±3	0	-	-
Polyanionic WSFD—eosin (**3**)	100±10	100±10	55±6	17±2	28±3	0	4.7×10^{-6}	Competitive
Polyanionic WSFD (**4**)	100±10	88±8	23±2	18±2	12±1	0	2.5×10^{-6}	Non-competitive
Polycationic WSFD (**5**)	40±4	0	30±3	0	0	0	—	—
Fluorescein (**6**)	62±6	21±2	46±2	10±1	20±2	0	6.1×10^{-5}	Competitive
Eosin (**7**)	82±8	65±6	75±8	30±3	32±3	10±1	5.8×10^{-6}	Competitive

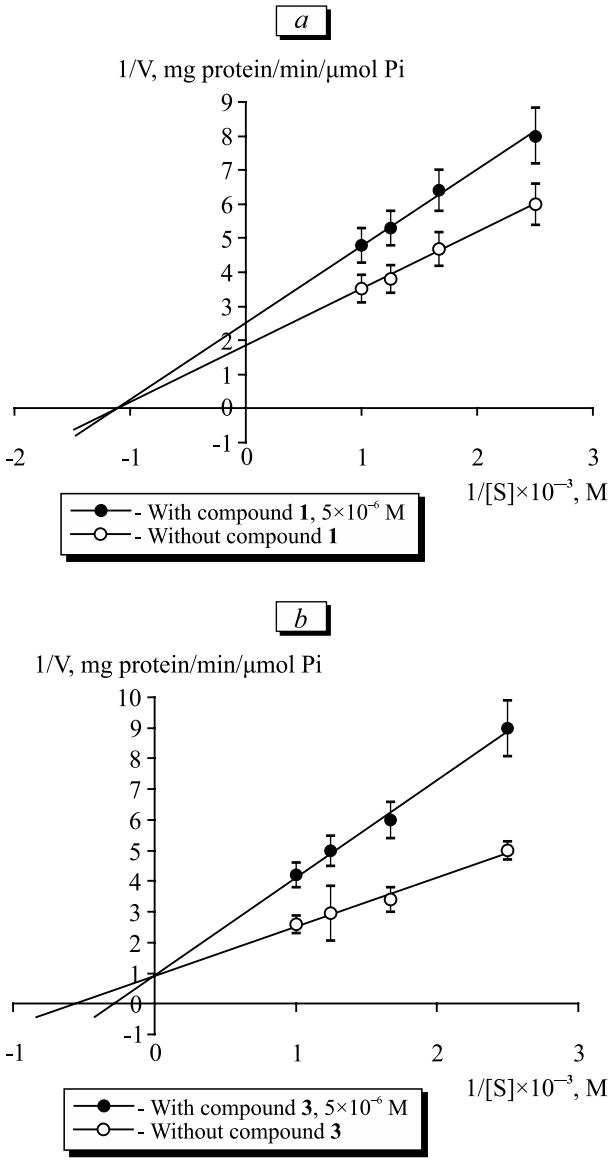

Fig. 3. Changes in the rate of ATP hydrolysis at different substrate concentrations in Lineweaver—Burk coordinates: *a*) in the presence and absence of compound (**1**); *b*) in the presence and absence of compound (**3**).

It is known that the hydrolytic and transport functions of SR Ca^{2+}-ATPase are linked, namely hydrolysis of one ATP molecule leads to active transmembrane transfer of two Ca^{2+} ions [4]. In case of enzyme malfunction, uncoupling of these functions leads to a decrease in the efficiency of the transfer of Ca^{2+} ions. All studied conjugates, WSFD and dyes, uncoupled the hydrolytic and transport functions of the enzyme: polycationic conjugate **2** and polycationic WSFD **5** in a concentration of 0.01 mM and other compounds in concentrations of 0.001 and 0.0001 mM (Table 1). The obtained data suggest the antimetastatic and antiaggregatory effects of the studied compounds, most pronounced for conjugates **1** and **3**, as was shown earlier for compounds of other classes [1,6,7,11].

More complete understanding of the mechanism of SR Ca^{2+}-ATPase inhibition gives a kinetic method for the study of enzymatic reactions, which allows evaluating the nature of enzyme binding with the inhibitors. The effects of the inhibitor on enzyme activity is determined by the dependence reciprocal of the enzymatic reaction velocity (1/V) on the inverse values of the substrate concentration (1/S) in the presence of the inhibitor. Numerical values for the maximum rates of ATP hydrolysis were used to calculate the inhibition constant (K_i) under the influence of the studied compounds **1**, **3**, **4**, **6** and **7**. The calculation was performed according to the values of the slope of the Lineweaver—Burk plot (Fig. 3), which was by (1 [V]/Ki) times higher in the presence of the inhibitor than in the control (without inhibitor).

Compounds **3**, **6**, **7** competitively inhibited SR Ca^{2+}-ATPase with inhibition constants of $K_i=4.7\times10^{-6}$, $K_i=6.1\times10^{-5}$, and $K_i=5.8\times10^{-6}$ M, respectively, which implied their binding to the active center of the enzyme. Compounds **1** and **4** noncompetitively inhibited the hydrolytic function of the enzyme (Table 1, Fig. 3), which implied their interaction with the enzyme beyond the hydrolytic center or the influence on SR membrane with $Ki=1.3\times10^{-5}$ M (**1**), $Ki=2.5\times10^{-6}$ M (**4**). Compounds **2** and **5** did not affect ATP hydrolysis.

Thus, all studied compounds, including the covalent structures of WSFD—dye, individual WSFD and xanthene dyes fluorescein and eosin, are effective inhibitors of active transmembrane transport of Ca^{2+} ions. At the same time, uncoupling of the transport and hydrolytic functions of SR Ca^{2+}-ATPase is observed. The most effective inhibitors of the hydrolytic function of SR Ca^{2+}-ATPase are compounds of anionic nature, these include negatively charged dyes fluorescein (**6)** and eosin (**7**), polyanionic WSFD (**4**) and polyanionic conjugates of WSFD—fluorescein (**1**) and WSFD—eosin (**3**). It should be noted that for negatively charged dyes eosin and fluorescein competitive inhibition is observed, an order of magnitude more effective for eosin than for fluorescein. Polyanionic WSFD (**4**) inhibits ATP hydrolysis more efficiently than eosin, but the mechanism of inhibition is noncompetitive. It should be noted that previously studied water-soluble fullerene derivatives also demonstrated noncompetitive inhibition [5,8,9].

Compounds of cationic nature, polycationic WSFD and covalent conjugate polycationic WSFD—fluorescein, inhibit only active transport of Ca^{2+} without affecting the hydrolytic function.

As already mentioned, inhibition of active transport of Ca^{2+} by biologically active compounds violates the ratio of the content of Ca^{2+} ions inside and outside

the cells, which leads to antimetastatic and antiaggregatory effects [1,6,7,11-13].

Thus, it can be concluded that the studied compounds covalent conjugates **1** and **3**, as well as polyanionic WSFD (**4)** and xanthene dyes fluorescein (**6)** and eosin (**7)** are effective inhibitors of Ca^{2+}-ATPase and can be recommended for further study of their antimetastatic and antiaggregatory activity.

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REFERENCES

1. Konovalova NP, Tat'yanenko LV, Volkova LM. Inhibitory effect of radiosensitizen AK-2123 on experimental liver metastases and active transport of calcium ions. Vopr. Onkol. 1997;43(3):309-312. Russian.
2. Kotel'nikov AI, Rybkin AY, Goryachev NS, Belik AY, Troshin PA. Spectral Properties and Photodynamic Activity of Complexes of Polycationic Derivative of Fullerene C60 with Xanthene Dye Fluorescein. Optics and Spectroscopy. 2016;120(3):379-385.
3. Kotelnikova RA, Smolina AV, Zhilenkov AV, Soldatova YV, Faingold II, Kotelnikov AI, Troshin PA. Interaction of water-soluble pentaamino acid fullerene derivatives with membranes of phosphatidylcholine liposomes. Russ. Chem. Bull. 2018;67(2):366-370.
4. Ritov VB, Mel'gunov VI, Komarov PG, Alekseeva OM, Akimova EI.. Integral proteins of the sarcoplasmic reticulum of rabbit and carp skeletal muscles. Dokl. Akad. Nauk SSSR. 1977;233(4):730-733.
5. Tat'yanenko LV, Dobrokhotova OV, Kotel'nikova RA, Poletayeva DA, Mishchenko DV, Pikhteleva IYu, Bogdanov GN, Kotel'nikov AI, Romanova VS. Effect of nitroxyalkyl derivatives of fullerenylproline on the activity of Ca^{2+}-ATPase of sarcoplasmic reticulum. Pharm. Chem. J. 2011;45(6):329-332.
6. Tatyanenko LV, Konovalova NP, Bogdanov GN, Dobrokhotova OV, Fedorov BS. Inhibition of active transport of calcium ions by PT(IV) and PD(II) metal complexes. Correlation between the process and the inhibition of growth of experimental metastases. Biomed. Khimiya. 2006;52(1):52-59. Russian.
7. Tat'yanenko LV, Konovalova NP, Dobrokhotova OV, Pikhteleva IYu, Mishchenko DV, Fedorov BS, Vystorop IV. Effect of cyclic hydroxamic acids derived from glycine and D,L-alanine on activity of Ca2+ Mg2+-ATPase hydrolases of sarcoplasmic reticulum and cyclic guanosine monophosphate phosphodiesterase. Pharm. Chem. J. 2013;47(5):235-238.
8. Tat'yanenko LV, Kotel'Nikova RA, Poletaeva DA, Dobrokhotova OV, Pikhteleva IYu, Kornev AB, Khakina EA, Troshin PA, Kotel'Nikov AI. Effects of water-soluble polysubstituted fullerene derivatives on sarcoplasmic reticulum Ca2+-ATPase and cyclic guanosine monophosphate phosphodiesterase activities. Pharm. Chem. J. 2013;47(8):405-408.
9. Tat'yanenko LV, Khakina EA, Zhilenkov AV, Troshin PA, Dobrokhotova OV, Pikhteleva IY, Kotel'nikov AI. Effects of Fullerene Derivatives on Activity of Ca2+-ATPase of the Sarcoplasmic Reticulum and cGMP Phosphodiesterase. Bull. Exp. Biol. Med. 2017;163(3):321-325. doi: 10.1007/s10517-017-3794-y
10. Belik AY, Rybkin AY, Voronov II, Goryachev NS, Troshin PA, Kotelnikov AI, Volyniuk D, Grazulevicius JV. Non-covalent complexes of polycationic fullerene C60 derivative with xanthene dyes — spectral and photochemical properties in water and in liposomes. Dyes Pigments. 2017;139:65-72.
11. Konovalova NP, Volkova LM, Tatyanenko LV, Kotelnikova RA, Yakushchenko TN, Kagiya TV. Inhibitory effect of radiosensitizen AK-2123 on experimental hepatic metastases and Ca2+-active transport. Neoplasma. 1997;44(6):361-365.
12. Mason RP. Calcium channel blockers, apoptosis and cancer: is there a biologic relationship? J. Am. Coll. Cardiol. 1999;34(7):1857-1866.
13. Mason RP. Effects of calcium channel blockers on cellular apoptosis Implications for carcinogenic potential. Cancer. 1999;85(10):2093-2102.
14. Rybkin AY, Belik AY, Kraevaya OA, Khakina EA, Zhilenkov AV, Goryachev NS, Troshin PA, Kotelnikov AI, Volyniuk D, Grazulevicius JV. Covalently linked water-soluble fullerene—fluorescein dyads as highly efficient photosensitizers: Synthesis, photophysical properties and photochemical action. Dyes Pigments. 2019;160:457-466.
15. Toyoshima C, Nakasako M, Nomura H, Ogawa H. Crystal structure of the calcium pump of sarcoplasmic reticulum at 2.6 A resolution. Nature. 2000;405:647-655.