Pharmaceutical Chemistry Journal, Vol. 45, No. 6, September, 2011 (Russian Original Vol. 45, No. 6, June, 2011)

EFFECT OF NITROXYALKYL DERIVATIVES OF FULLERENYLPROLINE ON THE ACTIVITY OF CA^{3+}-ATPASE OF SARCOPLASMIC RETICULUM

L. V. Tat'yanenko,[1] O. V. Dobrokhotova,[1] R. A. Kotel'nikova,[1] D. A. Poletayeva,[1] D. V. Mishchenko,[1] I. Yu. Pikhteleva,[1] G. N. Bogdanov,[1] V. S. Romanova,[2] and A. I. Kotel'nikov[1]

Translated from Khimiko-Farmatsevticheskii Zhurnal, Vol. 45, No. 6, pp. 10 – 13, June, 2011.

Original article submitted May 17, 2010.

The effect of nitroxyalkyl derivatives of fullerenylproline methyl ester on the enzymatic activity of Ca^{2+}-ATPase of sarcoplasmic reticulum (SR) has been studied. It is shown that hybrid derivatives of C_{60} fullerene are capable of inhibiting the activity of Ca^{2+}-ATPase of SR. The mononitrate inhibits the hydrolytic activity of the enzyme with $K_i = 1.92 \times 10^{-6}$ M; active Ca^{2+} transport, with $K_i = 3.79 \times 10^{-6}$ M. The dinitrate inhibits ATP hydrolysis with $K_i = 2.38 \times 10^{-8}$ M; Ca^{2+} transport, with $K_i = 3.08 \times 10^{-8}$ M. Fullerenylproline methyl ester does not affect the enzymatic activity of Ca^{2+}-ATPase. Based on these data it is possible to predict the possible fields of application for hybrid fullerene derivatives as potential drugs.

Key words: Ca^{2+}-Mg^{2+}-ATPase of sarcoplasmic reticulum, phosphodiesterase of cyclic guanosine monophosphate, fullerenylproline nitrates.

Nanotechnological inventions and functional implementation of nanocarbon materials has led to the creation of novel pharmacologically active compounds based on C_{60} fullerene. Amphiphilic derivatives of C_{60} are highly membrane active as a result of the unique nanocarbon spheroid incorporated into them. This determines their pharmacokinetic properties, primarily their permeability through the lipid bilayer of biological membranes and also the altered activity of membrane-bound enzymes. The goal of our work was to study modulation of the activity of Ca^{2+}-ATPase of sarcoplasmic reticulum (SR) as affected by recently synthesized hybrid fullerene derivatives.

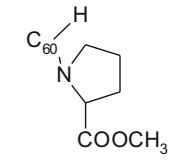
[1] Institute of Problems of Chemical Physics, Russian Academy of Sciences, Moscow Oblast, Chernogolovka, 142432, Russia.
[2] Nesmeyanov Institute of Organoelement Compounds, Russian Academy of Sciences, Moscow, Russia; e-mail: roman@ineos.ac.ru.

The molecular mechanisms of drug resistance [1] and the antimetastatic activity of many cytostatics [2 – 5] are known to involve Ca^{2+}-ATPase.

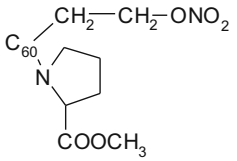
EXPERIMENTAL PART

We used human albumin, imidazole, ouabain, cGMP, nucleotidase (cobra venom), ATP (Sigma), histidine, DMSO, EDTA, trichloroacetic acid (TCA), saccharose, $MgCl_2$, NaCl, KCl, $CaCl_2$, Na oxalate, and ammonium molybdate (Reakhim, Russia) that were purified before use.

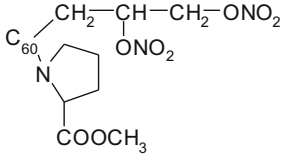
The fullerene derivatives were synthesized stepwise by equimolar nucleophilic addition of proline to fullerene to form fullerenylproline (**I**) and subsequent electrophilic substitution of the hydrofullerenyl proton by nitroxyalkyl halides [6].



fullerenylproline (I)



fullerenyl mononitrate (II)



fullerenyl dinitrate (III)

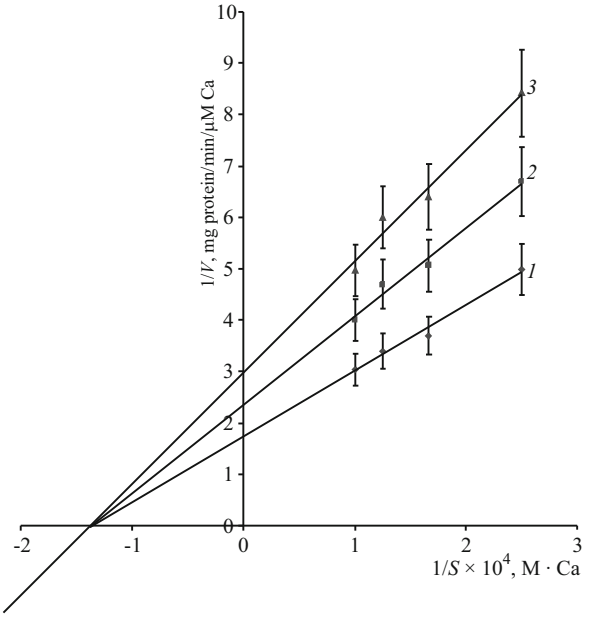


Fig. 1. Change of active Ca^{2+} transport rate as a function of its concentration as affected by **II** and **III** in Lineweaver–Burke coordinates: control (*1*), in the presence of fullerenyl mononitrate, 5×10^{-6} M (*2*), in the presence of fullerenyl dinitrate, 4×10^{-8} M (*3*).

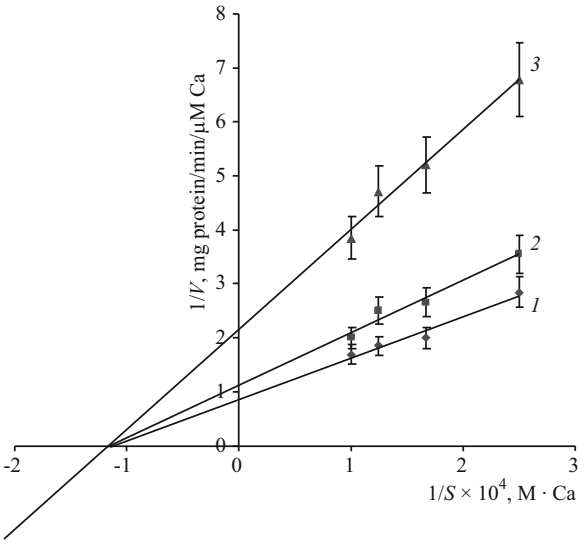


Fig. 2. Change of ATP hydrolysis rate as a function of substrate concentration as affected by **II** and **III** in Lineweaver–Burke coordinates: control (*1*), in the presence of fullerenyl mononitrate, 5×10^{-6} M (*2*), in the presence of fullerenyl dinitrate, 4×10^{-8} M (*3*).

The enzyme Ca^{2+}-ATPase of SR was isolated from hind paw muscle of white rabbits [7]. Muscles were placed in ice-cold physiological solution (0.5 L solution per 100 g muscle) with EDTA (10 mM) at pH 7.5. Muscles were ground, placed in medium containing histidine (10 mM), EDTA (0.1 mM), and saccharose (10%) at pH 7.0, and homogenized in a Potter homogenizer. The resulting homogenate was centrifuged at 10,000 rpm for 20 min. The supernatant liquid was filtered through burlap (six layers). The filtrate was centrifuged at 36,000 rpm for 60 min. The precipitate was suspended in medium containing KCl (0.6 M) and histidine (10 mM) at pH 7.0 – 7.2, treated with human albumin (100 mg), incubated for 8 h at 4 – 8°C with stirring, and centrifuged at 40,000 rpm for 90 min. The middle gelatinous layer was collected from the centrifuge tubes and suspended in medium containing histidine (10 mM), EDTA (0.1 mM), and saccharose (30%) at pH 7.0, The enzyme preparation obtained in this manner was frozen in liquid N_2 and used in the work.

The enzyme activity was determined by the literature method [6]. The reaction medium contained $MgCl_2$ (4 mM), imidazole (2.5 mM), NaCl (100 mM), Na oxalate (5 mM), protein (0.04 mg), and ATP (3 mM) at pH 7.2. The reaction was induced by adding $CaCl_2$ (0.1 mM). The heterolytic activity of Ca^{2+}-ATPase was calculated from the slope of the initial portion of the kinetic curve for ATP hydrolysis. The specific hydrolysis rate of Ca^{2+}-ATPase was 15,000 nM Pi/mg protein/min. The rate of change of $[Ca^{2+}]$ was estimated from Ca^{2+} absorption (from dilute 0.1 mM $CaCl_2$) by SR vesicles during the ATP hydrolysis.

Inhibition of the enzyme hydrolytic activity was calculated using the formula:

$$I = 100(A_0 - A)/A_0,$$

where I is the inhibition index in percent; A_0, the specific content of inorganic phosphate in the control; and A, the specific content of inorganic phosphate in the test sample.

The protein concentration was determined by a modified Lowry method.

The kinetics of Ca^{2+}-ATPase of SR inhibition were studied using the rate of the enzymatic reaction as a function of substrate (ATP) concentration in the presence and absence of the mono- and dinitrates at concentrations of 5×10^{-6} M and 4×10^{-8} M, respectively.

The reversibility of the action of the studied compounds was determined by dialysis of an aqueous solution of Ca^{2+}-ATPase of SR containing **II** or **III** (1 μM). The dialysis was carried out against a 100-fold excess of incubation medium without the complexes for 24 h at 4 – 5°C.

RESULTS AND DISCUSSION

Table 1 shows that fullerenyl mononitrate **II** and fullerenyl dinitrate **III** without starting fullerenylproline **I** had pronounced inhibitory effects on the functioning of Ca^{2+}-ATPase of SR. Thus, **III** at a concentration of 1 μM almost completely (97%) inhibited active Ca^{2+} transport and ATP hydrolysis (87%); at a concentration of 0.01 μM, by 60 and 50%, respectively. Compound **II** at a concentration of 1 μM inhibited the hydrolytic and transport functions of the enzyme by 56 and 44%, respectively.

TABLE 1. Effect of Fullerene Derivatives on Activity of Ca^{2+}-ATPase of Rabbit Muscle Sarcoplasmic Reticulum

Compound	Activity of Ca^{2+}-ATPase of SR, % of control					
	Compound concentration:					
	1 μM		0.1 μM		0.01 μM	
	Active Ca^{2+} transport	ATP hydrolysis	Active Ca^{2+} transport	ATP hydrolysis	Active Ca^{2+} transport	ATP hydrolysis
I (n = 6)	100	100	100	100	100	100
II (n = 6)	44 ± 4*	56 ± 5*	60 ± 6*	69 ± 5*	67 ± 6*	83 ± 7*
III (n = 6)	3 ± 0.3*	13 ± 1*	23 ± 2*	38 ± 4*	40 ± 4*	52 ± 5*

* $p < 0.01$ compared with control.

An important characteristic of the inhibition mechanism of the studied compounds is the reversibility of their effect on Ca^{2+}-ATPase activity.

Table 2 indicates that the enzyme transport function after dialysis as affected by **II** and **III** was partially restored. This indicated that these compounds were partially reversible inhibitors of Ca^{2+}-ATPase functioning. This was consistent with their non-covalent binding to the enzyme active site.

A kinetic method for studying enzymatic reactions that could suggest the nature of the enzyme–inhibitor binding gave a more complete description of the Ca^{2+}-ATPase inhibition mechanism. The effect of the inhibitor on the enzyme activity is determined from the inverse rate of the enzymatic reaction (1/V) as a function of the inverse substrate concentration (1/S) in the presence of the inhibitor.

The numerical values of the maximum ATP hydrolysis rate and active Ca^{2+} transmembrane transport rate were used to calculate the corresponding inhibition constants (K_i) as affected by **II** and **III**. The calculations used the slopes in Lineweaver–Burke coordinates (Figs. 1 and 2), which were $(1 + [\mathbf{I}]/K_i)$-times greater with inhibition than without inhibitor [8]. For ATP hydrolysis, $K_i = 1.92 \times 10^{-6}$ M; for active Ca^{2+} transport, $K_i = 3.79 \times 10^{-6}$ M.

Figures 1 and 2 show that **II** was a non-competitive inhibitor of the hydrolytic and transport functions.

TABLE 2. Effect of Fullerene Derivatives on Hydrolytic and Transport Functions of Ca^{2+}-ATPase of SR Before and After Dialysis

Compound	Activity of Ca^{2+}-ATPase, % of control			
	Before dialysis		After dialysis	
	Active Ca^{2+} transport	ATP hydrolysis	Active Ca^{2+} transport	ATP hydrolysis
II (n = 6)	44 ± 4	46 ± 2	75 ± 7 *	80 ± 8 *
III (n = 6)	3 ± 0.3	13 ± 1	60 ± 6*	44 ± 5*

* $p < 0.01$ compared with control, before dialysis.

Fullerenyl dinitrate **III** differed from **II** by K_i values that were two orders of magnitude smaller although it was a close analog of **II** and, like it, inhibited non-competitively both enzyme functions. The values were $K_i = 3.08 \times 10^{-8}$ M for ATP hydrolysis; 2.38×10^{-8} M, for transmembrane Ca^{2+} transport. This indicated that the enzyme hydrolytic function had increased sensitivity to the action of **III** and may have indicated that the Ca^{2+}-ATPase functions were partially decoupled. Therefore, attention should be paid to the noticeable reduction of the $[Ca^{2+}]/[ATP]$ ratio compared with the control, which is theoretically equal to 2. This ratio without the studied nitrates (control) was acceptable for $[Ca^{2+}]/[ATP]$? 1.8, which corresponds with Table 3. This parameter was

TABLE 3. Effect of Fullerene Derivatives on $[Ca^{2+}]/[ATP]$ Ratio

Compound	Activity of Ca^{2+}-ATPase of SR in specific activity units								Compound concentration, M			
	$[Ca^{2+}]/[ATP]$ ratio											
	10^{-6}				10^{-7}							
	Active Ca^{2+} transport	ATP hydrolysis	Active Ca^{2+} transport	ATP hydrolysis	Active Ca^{2+} transport	ATP hydrolysis	Active Ca^{2+} transport	ATP hydrolysis	Control	10^{-6}	10 – 7	10^{-8}
Control (n = 6)	5.6	3.0	–	–	–	–	–	–	1.86	–	–	–
I (n = 6)	–	–	5.7	3.2	5.6	3.1	5.65	3.18	–	1.78	1.8	1.8
II (n = 6)	–	–	2.5 *	1.7 *	3.4 *	2.1 *	3.7 *	2.5 *	–	1.47	1.55	1.5
III (n = 6)	–	–	0.17 *	0.39 *	1.3 *	1.14 *	2.2 *	1.6 *	–	0.44	1.14	1.4

* $p < 0.01$ compared with control.

markedly decreased by fullerenyl mononitrate **II** whereas fullerenylproline (**I**) had no effect on the enzyme activity. The effect was practically constant for **II** at concentrations in the range $10^{-6} - 10^{-8}$ M. In contrast with this, the $[Ca^{2+}]/[ATP]$ ratio decreased smoothly as the concentration of **III** increased from 10^{-8} to 10^{-6} M.

Thus, **II** and **III**, although not bonded to the active site of Ca^{2+}-ATPase, could induce certain structural and functional changes in the enzyme that affected the hydrolytic and transport functions of Ca^{2+}-ATPase.

The results on the induced change of Ca^{2+}-ATPase activity that was related to a change in the ratio of extra- and intracellular Ca^{2+} suggested that the studied C_{60} fullerene derivatives may exhibit antimetastatic properties.

REFERENCES

1. N. P. Konovalova, *Tekhnol. Zhivykh Sist.*, **1**(3), 42 – 48 (2004).
2. V. P. Nifontov, L. V. Tat'yanenko, V. A. Chernov, et al., *Khim.-farm. Zh.*, **22**(5), 522 – 526 (1988).
3. N. P. Konovalova, L. M. Volkova, and L. V. Tatyanenko, *News Lett.*, **4**(2), 3 – 6 (1997).
4. N. P. Konovalova, L. M. Volkova, and L. V. Tatyanenko, *Neoplasma*, **44**(6), 361 – 365 (1997).
5. L. V. Tat'yanenko, N. P. Konovalova, G. N. Bogdanov, et al., *Zh. Biomed. Khim.*, **52**(1), 52 – 61 (2006).
6. A. I. Kotel'nikov, V. S. Romanova, G. N. Bogdanov, et al., Intl. Pat. No. PST / RU 2007 / 000337 (2007).
7. V. B. Ritov, V. M. Mel?gunov, and P. G. Komarov, *Dokl. Akad. Nauk SSSR*, **233**, 720 – 733 (1977).
8. A. L. Lehninger, *Biochemistry*, Worth Publ., New York (1970).