Antioxidant properties of water-soluble amino acid derivatives of fullerenes and their role in the inhibition of herpes virus infection

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The antioxidant properties of water-soluble amino acid derivatives of fullerene C_{60} (ADF) were studied. It was shown by the change in the concentration of malonic dialdehyde in rat brain mitochondria that the ADF are antioxidants. They were shown by the change in the luminol glow to possess antiradical activity, which is determined by the acceptor properties of the fullerene spheroid and is independent of the structure of the attached addends. Stereoselectivity of the antioxidant properties of the enantiomers of amino acid derivatives of fullerene C_{60} was found. The L-isomers of ADF inhibit lipid peroxidation, whereas the D-isomers do not inhibit. A reliable correlation between the development of cytomegalovirus (CMV) infection and the process of lipid peroxidation in the cell culture was established. An efficient inhibitor of the CMV infection from the class of amino acid derivatives of fullerene with antioxidant activity was obtained.

Key words: water-soluble fullerene derivatives, lipid peroxidation, antioxidant activity, malonic dialdehyde, chemiluminescence, stereoselectivity.

Due to the unique structure of the carbon skeleton, fullerenes possess donor-withdrawing, lipophilic, and membranotropic properties, and the ability to transform oxygen into the singlet state and exhibit antiradical activity, which maintains permanent interest of researcher in the synthesis of a new class of drugs on the basis of fullerenes. It is known that the free radical mechanism of lipid peroxidation (LPO) and its inhibition by bioantioxidants play an important role in the development of many pathological processes that occur in living organisms,[1] in particular, in the regulation of processes of cell proliferation and multiplication of bacteria and some viruses.[2]

The ability of fullerenes and their derivatives to inactivate free radicals of oxygen was described in 1991 in the work,[3] where fullerene C_{60} was characterized as a "sponge soaking up free radicals." It was shown[4] that fullerenes can efficiently capture and inactivate both the superoxide radical anion and hydroxyl radicals *in vivo* and *in vitro*.

The present work is devoted to the study of the antioxidant properties of water-soluble amino acid derivatives of fullerene C_{60}.

Experimental

Water-soluble derivatives of fullerene C_{60} (ADF) were synthesized according to a known procedure.[5] The antioxidant and antiradical activities of ADF were studied at concentrations of $0.2 \cdot 10^{-5}$, $1 \cdot 10^{-5}$, and $2 \cdot 10^{-5}$ mol L^{-1}.

The antioxidant activity (AOA) was determined by the content of malonic dialdehyde (MDA) in rat brain mitochondria. The MDA content was determined spectrophotometrically by the color reaction with thiobarbituric acid at $\lambda = 535$ nm.[6]

Lipids in the culture of human embryonic fibroblast (HEF) were isolated according to the method described.[7] The protein concentration in cells was determined by the Lowry method[8] and recalculated to million of cells.

The antiradical activity of the compounds was estimated by a change in the luminescence kinetics of luminol in samples of the rat brain homogenate in the presence of oxygen radicals according to the published work[9] on a Luminometr-1250 LKB Wallak chemiluminometer. The homogenate was prepared in the Potter homogenizer from the rat brain tissue: 8 mL of the buffer was used per 1 g of the brain tissue. A luminol (Sigma) solution with a concentration of $5 \cdot 10^{-4}$ mol L^{-1} was introduced into the samples. The initiation of LPO in the homogenate was

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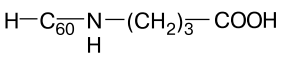
carried out with an aqueous solution of *tert*-butyl hydroperoxide (TBHP) with a concentration of $7.3 \cdot 10^{-2}$ mol L^{-1}. The amount of oxygen radicals and the effect of the ADF on their content in the homogenates were evaluated from a change in the surface area under the kinetic curve of luminol luminescence.[10] The experimental kinetic curves were analyzed by the computer program of the instrument, and averaging over 512 points was performed for plotting. The surface areas and constants were calculated by the Microcal Origin 7.0 and Microsoft Office Excel programs.

Mitochondria were isolated according to a described procedure.[11] Cells of HEF are available from the cell culture bank of the D. I. Ivanovsky Research Institute of Virusology. The HEF cells were infected by cytomegalovirus (reference strain AD169 was kindly presented by Prof. D. Emanuel, Memorial Sloan—Kettering Cancer Center, New York, USA). The antiviral effect of C_{60}-AMNa was evaluated according to a published procedure.[12]

Results and Discussion

In the present work, we studied the antioxidant properties of the following water-soluble amino acid derivatives of fullerene C_{60}: sodium salts of fullerenylaminobutyric acid (C_{60}-γ-ABNa, **1**) and fullerenylaminocaproic acid (C_{60}-ω-ACNa, **2**), as well as a hybrid structure based on fullerenylproline and natural antioxidant carnosine attached to the latter to enhance the antioxidant properties (C_{60}-Pro-carnosine, **3**).



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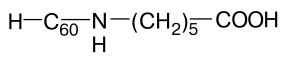

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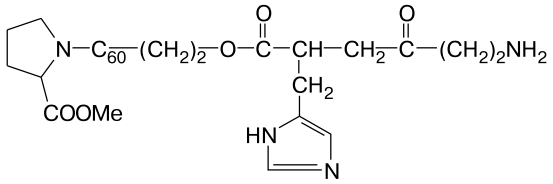

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Amino acid derivatives of fullerene C_{60} **1**—**3** possess antioxidant activity, as shown from the change in the concentration of malonic dialdehyde (MDA) in the rat brain mitochondria (Table 1). It is known that MDA is one of the final products of lipid peroxidation and is formed upon the cleavage of fatty acids. Activation or inhibition of LPO can be judged from the rate of MDA formation.[6]

As can be seen from the data in Table 1, all studied water-soluble derivatives of fullerene C_{60} decrease the rate of MDA accumulation in mitochondria, indicating their ability to inhibit the LPO process. The most efficient antioxidant is C_{60}-Pro-carnosine **3**. Note that the carnosine itself is less efficient than C_{60}-Pro-carnosine, which can be explained by the fact that C_{60}-Pro-carnosine **3**, unlike carnosine, is an antioxidant of the binary effect and inhibits LPO due to both the acceptor properties of the fullerene spheroid and the antioxidant action of carnosine.

Table 1. Rate of MDA accumulation in rat brain mitochondria in the presence of the water-soluble derivatives of fullerene C_{60} and carnosine

Entry	Compound	[MDA]	
		/mol L^{-1}	(% to reference)
1	Reference	1.42±0.02	100
2	C_{60}-γ-ABNa	0.89±0.02	63
3	C_{60}-γ-ACNa	0.75±0.03	53
4	C_{60}-Pro-carnosine	0.37±0.03	26
5	Carnosine	0.62±0.03	44

Note. The concentration of ADF and carnosine in the mitochondria was $1 \cdot 10^{-5}$ mol L^{-1}, and the incubation duration is 30 min. The reference is the MDA content in the mitochondria in the absence of additives. The average values of five measurements are presented.

The antioxidant activity of ADF was estimated by their influence on the kinetics of TBHP-induced chemiluminescence (CL) of luminol in the rat brain homogenate. It is known that, in the presence of active oxygen species, luminol (5-amino-2,3-dehydro-4-phthalazinedione) is oxidized and generates electron-excited carbonyl chromophores in high quantum yield. The addition of substances with the antiradical activity to this model system decreases the number of light quanta emitted by the chromophore.[10]

As already mentioned above, several authors showed the antiradical activity of fullerenes and their derivatives and their ability to capture and inactivate free radicals of oxygen.[3—4]

The content of free radicals in the system was estimated by a change in the light sum: the surface area under the kinetic curve of luminol luminescence (S) upon the interaction of luminol with free radicals (Fig. 1). The surface area S corresponds to the number of light quanta emitter by the chromophore, which, in turn, is proportional to the concentration of the luminescing chromophore and, correspondingly, to the concentration of free oxygen radicals interacting with luminol during the all time of luminescence. In the presence of antioxidants, the number of free radicals decreases and, correspondingly, the CL of luminol and, hence, the value of light sum decrease. Based on the aforesaid, the antiradical activity of compounds can be estimated by the change in the light sum.

As can be seen from the data in Table 2 and Fig. 1, the ADF studied have a weak antiradical activity, which is determined by the acceptor properties of the fullerene spheroid only and is independent of the structure of the attached addends. So, unlike the total antioxidant activity (see Table 1), the antiradical activity of the hybrid mole-

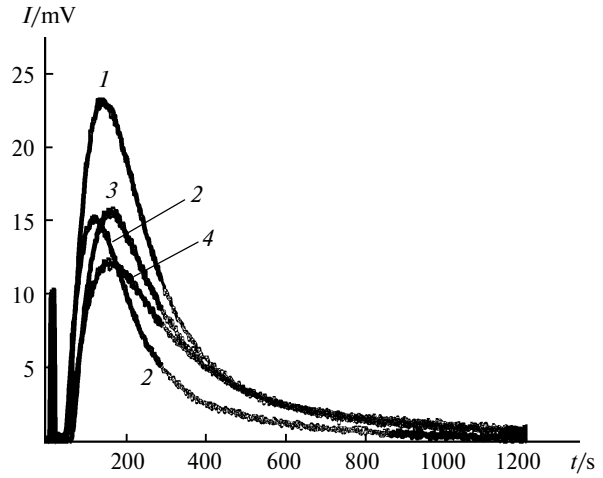

Fig. 1. Kinetics of the TBHP-induced CL of luminol in the rat brain homogenate before (*1*) and after the addition of C_{60}-ω-ACNa (*2*), C_{60}-γ-ABNa (*3*), and C_{60}-Pro-carnosine (*4*). The protein concentration in the homogenate is 1 mg mL^{-1}, $T = 20$ °C.

cule C_{60}-Pro-carnosine **3** does not almost differ from the activity of ADF **1** and **2**. Carnosine exerts no effect on the content of oxygen radicals in the studied samples, because it catalyzes the decomposition of peroxides and has no intrinsic antiradical activity (see Table 2).

Thus, it is shown that the studied water-soluble amino acid derivatives of fullerene C_{60} are antioxidants and possess a weak antiradical activity, which is determined by the acceptor properties of the fullerene spheroid and is independent of the attached addends.

The stereoselectivity of the antioxidant properties of C_{60}-L-Arg and C_{60}-D-Arg has been found for the first time in Ref. 13. In the present work, we studied the AOA of the amino acid derivatives of fullerene: C_{60}-L-Ser, C_{60}-D-Ser, C_{60}-L-Ala, C_{60}-D-Ala, C_{60}-L-Ala-Ala, and C_{60}-D-Ala-Ala. Analogously to Ref. 13, it was shown that the L-enantiomers of all amino acid derivatives decrease the MDA concentration in mitochondrial lipids, whereas the D-enantiomers exert no effect on the kinetics of MDA accumulation (Table 3). The result obtained indicates the antioxidant activity of L-enantiomers of the amino acid derivatives of fullerene C_{60} and no AOA in the D-isomers.

It was shown[13] that the L-isomers incorporate into the hydrophobic sites of phospholipid membranes, whereas the D-isomers do not incorporate into the sites. Perhaps, the stereoselectivity of the antioxidant properties of the amino acids derivatives of fullerenes C_{60} is explained by homochirality of phospholipids forming mitochondrial membranes. As known, the oxidative transformation of polyunsaturated fatty acids is predominantly observed in the hydrophobic sites of phospholipid membranes containing unsaturated lipids: in mitochondrial membranes, endoplasmic reticulum, lysosomes, and plasmatic membranes.[14] The L-enantiomers penetrate through the homochiral bilayer of membranes to the area of nonpolar fatty acid residues of phospholipids and inhibit LPO, whereas the D-enantiomers cannot surmount the stereospecific "filter" of membranes and, therefore, they exert no effect on the LPO in phospholipid membranes.

As known, the reaction of chain oxidation of lipids plays the exclusive role in cell pathology.[15] As a rule, the sharp activation of LPO followed by the suppression of the antioxidant activity of plasma occurs already at the initial stage of any disease.

It is proved in numerous recent studies that the active species of oxygen play the key role in the molecular mechanisms of pathogenesis of many frequently met diseases: in the regulation of processes of cell proliferation and multiplication of bacteria and some viruses.[15,16] It is known that almost all classes of fullerenes, as well as fullerene C_{60} itself, inhibit the development of many viral infections.[1—4] The fast wide expansion of herpes virus infections is ob-

Table 2. Change in the light sum of luminol in the rat brain homogenate in the presence of ADF and carnosine

Entry	Compound	Light sum (% to reference)
1	C_{60}-γ-ABNa	82
2	C_{60}-γ-ACNa	78
3	C_{60}-Pro-carnozine	76
4	Carnosine	100

Note. The concentration of ADF and carnosine in the homogenate was $1 \cdot 10^{-5}$ mol L^{-1}. The reference (100%) was the light sum of the TBHP-induced CL of luminol in the absence of additives. The average values of five measurements are presented.

Table 3. Content of MDA in the rat brain mitochondria in the presence of the ADF enantiomers

Entry	ADF	[MDA]	
		/mmol L^{-1}	(% to reference)
1	Reference	1.42±0.02	100
2	C_{60}-L-Arg	1.02±0.03	72
3	C_{60}-D-Arg	1.42±0.03	100
4	C_{60}-L-Ser	0.82±0.02	58
5	C_{60}-D-Ser	1.42±0.03	100
6	C_{60}-L-Ala	0.74±0.03	52
7	C_{60}-D-Ala	1.42±0.04	100
8	C_{60}-L-Ala-Ala	0.90±0.02	63
9	C_{60}-D-Ala-Ala	1.42±0.02	100

Note. The concentration of ADF in mitochondria was $1 \cdot 10^{-5}$ mol L^{-1}, and the incubation duration was 30 min. The reference was content of MDA in mitochondria in the absence of ADF. The average values of three measurements are presented.

served in the recent years. These infections result in heavy complications, especially for children, and are often lead to child disablement and even death.[12] The use of the modern patented clinical drugs produce considerable side effects and stimulate the fast development of resistance of the organism to them. Therefore, it was necessary to create a new class of efficient drugs: inhibitors of herpes virus infections, in particular, cytomegalovirus infection (CMVI). In the present work, we studied the mechanisms of the development of the CMVI in the culture of human embryonic fibroblast (HEF). It was shown from the first time that the process of CMVI development correlates with the LPO process with the high reliability (Pearson coefficient 0.8) (Fig. 2).

It is shown above that the amino acid derivatives of fullerene C_{60} are pronounced antioxidants. They are not cytotoxic (IC_{50} 1000 and 1200 μg mL^{-1} (mln. cells) for C_{60}-ω-ACNa and C_{60}-γ-ABNa, respectively).[12] In the present work, the antioxidants from the class of ADF were studied as inhibitors of CMVI for the first time. It is shown that the introduction of C_{60}-γ-ABNa into infected HEF decreases the concentration of virus proteins in the cells to values approaching the protein concentration in the non-infected cell culture (Table 4). The MDA concentration in the infected HEF culture also decreased to the concentration in intact HEF. The action of the patented clinic drug ganciclovir on the HEF culture also decreased the LPO level, but the efficiency of the antiviral effect of this drug on CMVI and its AOA were significantly lower that those of C_{60}-γ-ABNa (see Table 4). The results obtained indicate that the antoxidant activity of the derivatives of fullerene C_{60} play a substantial role in their antiviral effect against CMVI. Compound C_{60}-γ-ABNa is a more efficient inhibitor of CMVI compared to the patented drug ganciclovir and can be considered as a chemiotherapeutical drug against CMVI. Its chemiotherapeutical index (CTI) is 5000, which fivefold exceeds the CTI of ganciclovir, being 1000 (see Ref. 12).

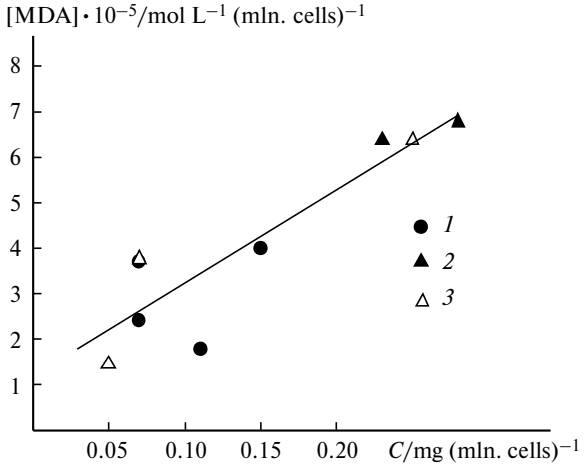

Fig. 2. Linear correlation between the contents of the protein (*C*) and MDA in the human embryonic fibroblast culture: *1*, intact culture; *2*, CMV-infected culture; and *3*, the culture infected with CMV after C_{60}-γ-ABNa was introduced. The Pearson linear correlation coefficient is 0.83.

Table 4. Contents of the protein and MDA in the HEF cultures

Sample	Protein content /mg (mln. cells)$^{-1}$	[MDA]·10^{-5} /mol L^{-1} (mln. cells)$^{-1}$
HEF	0.07±0.02	3.75±0.03
HEF—CMV	0.284±0.03	6.8±0.01
HEF—CMV—ganciclovir	0.22±0.04	5.4±0.03
HEF—CMV—C_{60}-γ-ABNa	0.072±0.02	3.8±0.03

Note. The concentration of ganciclovir and C_{60}-γ-ABNa in the cell culture was $2 \cdot 10^{-5}$ mol L^{-1} (mln. cells)$^{-1}$. The average values of five measurements are presented.

Thus, in the present work, we established the antioxidant and antiradical activities of the water-soluble amino acid derivatives of fullerene C_{60}. It was shown that the antiradical activity is independent of the type of the attached addend but is determined only by the acceptor properties of the fullerene spheroid. The stereoselectivity of the antioxidant properties of ADF enantiomers was revealed. The correlation between the process of lipid oxidation in the cell culture and the development of cytomegalovirus infection was established for the first time. The efficient inhibitor of CMVI, an antioxidant from the class of amino acid derivatives of fullerene C_{60}, was synthesized. Its high chemiotherapeutical index makes it possible to recommend it for studies as a potential drug for the treatment of cytomegalovirus infection.

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