Bioorganic & Medicinal Chemistry Letters 22 (2012) 2424–2427

Contents lists available at SciVerse ScienceDirect

Bioorganic & Medicinal Chemistry Letters

journal homepage: www.elsevier.com/locate/bmcl

Radical-regulating and antiviral properties of ascorbic acid and its derivatives

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ARTICLE INFO

Article history:
Received 5 January 2012
Revised 6 February 2012
Accepted 7 February 2012
Available online 16 February 2012

Keywords:
Ascorbic acid
2-*O*-Glycosylascorbic acid
Herpes simplex type I
Steady state radiolysis
α-Hydroxyl-containing carbon-centered radicals

ABSTRACT

The ability of ascorbic acid and a number of its derivatives to suppress replication of *Herpes simplex* virus type I was investigated in human rhabdomyosarcoma cell line. In parallel, interaction of the test compounds with carbon- and oxygen-centered radicals formed on radiolysis of hydroxyl-containing organic compounds was studied using the steady state radiolysis method. It has been shown that 2-*O*-glycoside of ascorbic acid, displaying marked antiviral properties against *Herpes simplex* virus type I, is also capable of inhibiting fragmentation and recombination reactions of α-hydroxyl-containing carbon-centered radicals while not affecting processes involving oxygen-centered radicals.

The basic antiherpetic medicines used to-date are synthetic purine- and pyrimidine nucleosides able to suppress replication of the polynucleotide sequences of viral particles.[1] Narrow specificity of these agents and the problems associated with the development of viral resistance dictate the necessity of finding-out new antiviral agents with an essentially different mechanism of action. Pharmacological agents with relatively versatile mechanisms of antiviral action may be less liable to provoke rapid development of resistant strains. For example, such agents may be compounds capable of regulating free-radical processes in biosystems. Virological studies have shown that a number of infections including those caused by rhino-, cytomegalo- and influenza viruses, HIV and various neuroviruses are associated with hyperproduction of reactive oxygen species (ROS) and activation of free-radical processes in a human organism.[2–7] Under conditions of a viral infection, ROS are generated by activated cells of the immune system—in order to destroy viral particles, as well as by infected cells—as a result of changes in the free-radical homeostasis.[8]

L-ascorbic acid (AA) acts as a co-factor for at least eight hydroxylases and monooxygenases involved in synthesis of collagen, noradrenalin, serotonin and carnitine, as well as in detoxication of xenobiotics.[9] AA is a key water-soluble antioxidant due to its ability to interact with ROS and to reduce the radicals formed from α-tocopherol and β-carotene.[10,11] The participation in various free-radical processes is a likely cause of the known immunomodulating and antiviral properties of AA.[12–14] It is widely used as vitaminous, regenerative and antiviral medication in the treatment of various respiratory viral infections including influenza, herpetic infections, viral hepatitis and other infectious diseases.[9,15,16]

The main goal of the present study was finding-out a relationship between antiviral activity and radical-inhibiting properties of AA derivatives. For this purpose, the ability of AA, 6-*O*-palmitoyl-L-ascorbic (**PAA**), 2-*O*-glucopyranosyl-L-ascorbic (**I**), 6-*O*-palmitoyl-2-*O*-glucopyranosyl-L-ascorbic (**Ia**), 5,6-*O*-isopropylidene-2,3-*O*-dimethyl-L-ascorbic (**II**) and 2,3-*O*-dimethyl-L-ascorbic (**IIa**) acids (Fig. 1) to suppress replication of *Herpes simplex* virus type I (HSV-1) in human rhabdomyosarcoma (RD) cell culture was investigated.

To evaluate radical-regulating properties of AA and its water soluble derivatives **I** and **II** their reactions with various carbon- and oxygen-centered radicals of hydroxyl-containing organic compounds in aqueous solutions were studied by the steady state radiolysis method.

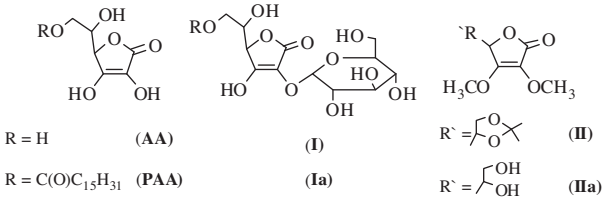

Figure 1. Structures of compounds studied.

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doi:10.1016/j.bmcl.2012.02.022

2-O-substituted derivatives of AA possess antioxidant, radioprotector and immunomodulatory properties.[9,17,18] Therefore 2-*O*-glycosides (**I** and **Ia**) have been chosen as the study objects. 2,3-*O*-Dialkylated derivatives (**II** and **IIa**) were synthesized according to[19] in order to determine the role of 2,3-enediol moiety conjugated with the carbonyl group in manifestation of radical-regulatory and antiviral properties by the analogs of AA. 6-*O*-Acylated compounds (**PAA** and **Ia**) were used to find out a relationship between lipophilicity of the AA derivatives and their antiviral properties/toxicity.

Experimental details of radiation experiment are described in.[20] Antiviral properties were studied in human RD cell line infected with HSV-1. The investigation was performed using the method based on evaluation of cytopathic effect produced by the virus.[21,22] Virus titer reduction in the presence of the test compounds as compared with control was taken as a criterion of antiviral activity. Based on the data obtained, concentrations of the test compounds were calculated that suppressed virus replication by 50% and 90% (EC_{50} and EC_{90}, respectively) (Table 1). Maximum non-toxic concentrations (MNTC) of the substances were determined in non-infected cell culture after incubation for 72 h.

Antiviral properties of AA, including those against HSV-1, influenza A and poliovirus 1, have been described earlier[13,23] and characterized as weak. In our experiments, AA did not prevent replication of HSV-1 in RD cell culture (Table 1). The 6-*O*-acylated derivative of AA, **PAA**, as well as 2,3-*O*-dimethylated analogs of AA, **II** and **IIa**, also manifested the absence of antiviral activity, whereas 2-*O*-α-D-glucopyranosyl-L-ascorbic acids, **I** and **Ia**, proved to be effective inhibitors of HSV-1 replication. The somewhat higher toxicity and lower EC_{50} value of compound **Ia** as compared to **I** are probably due to higher lipophilicity caused by the presence of palmitic acid moiety at 6-O-position in the structure of the former. To characterize radical-regulating properties of AA and its derivatives we have studied interaction of AA, **I** and **II** with various carbon- and oxygen-centered radicals of hydroxyl-containing organic compounds in aqueous solutions using the steady state radiolysis method.

In order to find out possible causes of the differences in antiviral activity observed for compounds having closely related structures, we investigated radical-regulating properties of AA and its water-soluble derivatives, the 2-*O*-glycoside **I** and its fully substituted analog **II**, using the steady state radiolysis method. The study of influence produced by the test compounds on the processes occurring during radiolysis of ethanol and aqueous solutions of some other organic compounds opens up the possibility to assess reactivity of the test compounds towards oxygen- and carbon-centered radicals of various structures.

The major radical product formed during radiolysis of ethanol is α-hydroxyethyl radical (α-HER) resulting from reactions (1) and (2). Under deaerated conditions, it is consumed in subsequent recombination and disproportionation (3) reactions with about equal probability to form 2,3-butanediol and acetaldehyde, respectively.[24]

$$C_2H_5OH \xrightarrow{\gamma} C_2H_5OH^{\oplus\bullet} + e^{\ominus}_{solv} \quad (1)$$

$$C_2H_5OH^{\oplus\bullet} + C_2H_5OH \rightarrow C_2H_5OH_2^{\oplus} + CH_3\dot{C}HOH \quad (2)$$

$$CH_3\dot{C}HOH + CH_3\dot{C}HOH \rightarrow \begin{cases} CH_3CH(OH)CH(OH)CH_3 \\ CH_3CHO + C_2H_5OH \end{cases} \quad (3)$$

In ethanol saturated with oxygen, α-HER interact with oxygen at a diffusion-limited rate to form peroxyl radicals $CH_3CH(OO^{\cdot})OH$, which decompose to form acetaldehyde and hydroperoxyl radicals, $HO_2^{\bullet}$:

$$CH_3\dot{C}HOH \xrightarrow{O_2} CH_3CH(OO^{\bullet})OH \rightarrow CH_3CHO + HO_2^{\bullet} \quad (4)$$

$$HO_2^{\bullet} + CH_3CH_2OH \rightarrow H_2O_2 + CH_3\dot{C}HOH \quad (5)$$

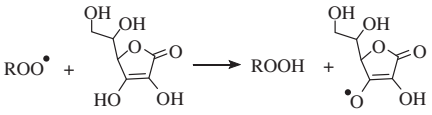
$$2HO_2^{\bullet} \rightarrow H_2O_2 + O_2 \quad (6)$$

On irradiation of aqueous ethanol solutions, α-HER are formed in reaction of C_2H_5OH with the radical products of water radiolysis:

$$H_2O \xrightarrow{\gamma} H^{\bullet}, {}^{\bullet}OH, e^{\ominus}_{aq}, H_3O^{\oplus} \quad (7)$$

$$CH_3CH_2OH \xrightarrow[-H_2O,\ (-H_2)]{{}^{\bullet}OH,\ (^{\bullet}H)} CH_3\dot{C}HOH \quad (8)$$

On irradiation of oxygenated ethanol or its aqueous solutions in the presence of AA, a significant decomposition of the additive is observed, as well as a decrease in radiation-chemical yields of acetaldehyde and H_2O_2, with a slight change in the main molecular product yield ratio in favor of hydrogen peroxide (Table 2). The obtained data evidence the ability of AA to interact with oxygen-centered radicals formed in reaction (4) according to the general scheme shown below:



(9)

Compound **I** produces virtually no effect on yields of the major products formed on radiolysis of oxygenated ethanol and its aqueous solution. The additive itself shows low radiation-chemical decomposition yield. These facts indicate poor reactivity of **I** towards oxygen-centered radicals.

The increase of radiation-chemical yields of acetaldehyde on irradiation of ethanol or its aqueous solutions, saturated with oxygen, in the presence of **II** may be the evidence of α-HER oxidation by the test compound according to reaction (10). In this case, the decrease in radiation-chemical yields of H_2O_2 is probably caused by suppression of reaction (4) in the presence of the additive. The low radiation-chemical yields of decomposition observed in

Table 1
Antiviral properties and toxicity of the test compounds

Compound	Maximum non-toxic concentrations (MNTC) (μM)	EC_{50} (I_{95}) (μM)	EC_{90} (I_{95}) (μM)	MNTC/EC_{50}	MNTC/EC_{90}
AA	2270	>2270	—	<1	—
PAA	240	>240	—	<1	—
I	1183	16,3 (17,46 ÷ 15,09)	26,9 (28,99 ÷ 24,85)	72,7	43,9
Ia	86,81	4,5 (4,86 ÷ 4,34)	6,2 (6,60 ÷ 5,90)	19,2	13,9
II	1639	>1639	—	<1	—
IIa	1961	1809 (2284 ÷ 1456)	3284 (4080 ÷ 2643)	1,1	0,6

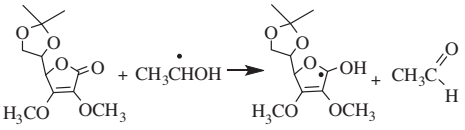
I_{95} is confidence interval at 95% probability.

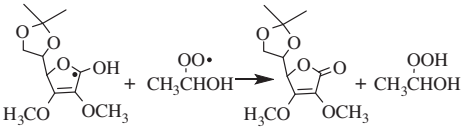
Table 2
Effects of AA, **I** and **II** ($C = 1 \times 10^{-3}$ mol/l) on yields (G) of the major products formed during radiolysis of ethanol and some hydroxyl-containing organic compounds in aqueous solutions

Initial system	Products	$G \times 10^7$ (mol/J)			
		Without additive	AA	**I**	**II**
Oxygenated solutions					
Ethanol	Acetaldehyde	7.06 ± 0.28	4.69 ± 0.26	7.64 ± 0.29	9.43 ± 0.30
	Hydrogen peroxide	7.54 ± 0.27	5.69 ± 0.14	7.81 ± 0.25	6.42 ± 0.28
	Additive decomposition		−2.91 ± 0.14	−0.78 ± 0.05	−0.15 ± 0.02
1 M Ethanol	Acetaldehyde	4.31 ± 0.23	4.09 ± 0.30	4.12 ± 0.23	4.45 ± 0.11
	Hydrogen peroxide	2.73 ± 0.18	1.98 ± 0.20	2.72 ± 0.20	2.36 ± 0.18
	Additive decomposition		−3.48 ± 0.26	−0.17 ± 0.01	−0.32 ± 0.04
Deaerated solutions					
Ethanol	Acetaldehyde	1.94 ± 0.17	2.37 ± 0.14	1.48 ± 0.05	2.65 ± 0.20
	2,3-Butanediol	2.05 ± 0.09	0.55 ± 0.15	1.36 ± 0.09	0.88 ± 0.11
	Additive decomposition		−0.48 ± 0.11	−1.13 ± 0.29	−0.99 ± 0.13
1 M Ethanol	Acetaldehyde	0.18 ± 0.04	0.54 ± 0.08	0.53 ± 0.08	1.82 ± 0.09
	2,3-Butanediol	1.93 ± 0.15	0.05 ± 0.01	1.28 ± 0.08	0.34 ± 0.01
	Additive decomposition		−1.15 ± 0.14	−1.70 ± 0.16	−2.24 ± 0.05
3 M Ethylene glycol	Acetaldehyde	9.05 ± 1.03	3.26 ± 0.07	6.01 ± 0.35	5.42 ± 0.09[a]
	Additive decomposition		−1.91 ± 0.03	−0.57 ± 0.05	−1.91 ± 0.05
1 M Ethylene glycol	Acetaldehyde	3.24 ± 0.19	1.64 ± 0.15	2.04 ± 0.22	2.20 ± 0.37[a]
	Additive decomposition		−1.84 ± 0.05	−0.64 ± 0.05	−2.06 ± 0.06
0.1 M α-Glycerophosphate	Inorganic phosphate	3.44 ± 0.12	2.48 ± 0.04	3.15 ± 0.10	2.53 ± 0.07
	Additive decomposition		−2.25 ± 0.05	−0.67 ± 0.25	−1.76 ± 0.13
0.1 M α-Glucosophosphate	Inorganic phosphate	2.63 ± 0.05	1.78 ± 0.03	2.50 ± 0.04	2.12 ± 0.06
	Additive decomposition		−1.59 ± 0.08	−0.34 ± 0.15	−1.92 ± 0.07

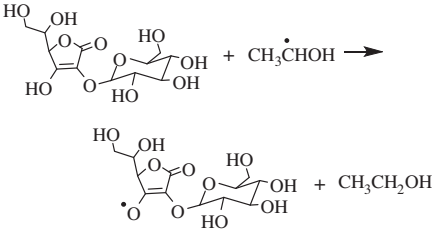
[a] The concentration of compound **II** in aqueous solutions was 5×10^{-4} mol/l.

the case of **II** suggest the possibility of realization of processes leading to regeneration of the additive, for example, according to reaction (11).


(10)


(11)

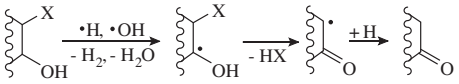
On radiolysis of deaerated ethanol and aqueous ethanol solutions in the presence of AA and its derivatives, formation of 2,3-butanediol, the product of α-HER recombination, was suppressed. This fact evidences the ability of the test compounds to interact with the carbon-centered radicals of the named type. In the case of compound **I**, a simultaneous decrease in yields of both acetaldehyde and 2,3-butanediol was observed. This might occur if α-HER were reduced by the additive as shown below:


(12)

In the presence of **II**, a change in favor of acetaldehyde, the α-HER oxidation product, was noted in the radiation-chemical yield ratio for the major radiolysis products. This fact points to realization of reaction (10) on irradiation of deaerated ethanol or its aqueous solutions containing **II**.

The character of changes in radiation-chemical yields of acetaldehyde and 2,3-butanediol in the presence of AA provides good reasons to believe that this additive may either reduce α-HER in deaerated aqueous solutions, like **I**, or oxidize α-HER in ethanol, like **II**. Possible factors influencing the dependence of interaction mechanisms between α-HER and AA on the form of its presence in solution were discussed in.[25]

Earlier,[26] we have shown that β-substituted analogs of α-HER being formed on radiolysis of aqueous solutions of hydroxyl-containing organic compounds are able to undergo free radical fragmentation reactions according to the scheme depicted below:


(13)

X = OH, OPO_3^{2-} etc.

Realization of reaction (13) on radiolysis of aqueous solutions of ethylene glycol leads to its dehydration. Moreover this process takes place according to a chain mechanism at high concentrations of the starting compound. Reactions of similar type ensure modification of carbohydrates[27] and transformation of ribonucleotides into deoxyribonucleotides.[28] Irradiation of organic phosphates leads to their quantitative dephosphorylation due to a high value of the rate constant for the phosphate anion elimination from the radicals of the starting compounds. For example, in the case of C-2 radicals formed from glycerol-1-phosphate, this value amounts to about 10^7 s^{-1}.[29] In the case of RNA, reactions of such type lead to rupture of phosphodiester bonds,[30] while degradation of phospholipids according to this scheme results in formation of phosphatidic acids playing the role of signaling molecules.[31,32]

The data presented in Table 2 demonstrate that AA and **II** decrease the radiation-chemical yields of compounds being formed according to reaction (13) on radiolysis of aqueous solutions of hydroxyl-containing organic compounds. Although to a lesser extent, the 2-*O*-glycosylated derivative **I** also suppressed formation of free-radical fragmentation products. Thus, AA, **I** and **II** are capable of interacting with α-hydroxyl-containing carbon-centered radicals of various structures and hence blocking the reactions that cause damage to hydroxyl-containing biomolecules.

The results obtained in this study show that AA suppresses oxidation and free radical fragmentation of hydroxyl-containing organic

compounds in aqueous solutions due to reduction of oxygen- and carbon-centered radicals. The fully substituted AA derivative, **II**, is capable of oxidizing α-hydroxyl-containing carbon-centered radicals; consequently, it suppresses radiation-induced transformations of hydroxyl-containing organic compounds. Investigation of antiviral properties of these compounds has shown that AA, its 6-*O*-palmitate (**PAA**) and 2,3-*O*-dialkylated derivatives (**II** and **IIa**) produce no effect on replication of HSV-1 in human RD cell culture. At the same time, 2-*O*-glycosylated derivatives of AA (**I** and **Ia**) display marked antiviral properties against HSV-1.

We believe that the antiviral activity of compound **I** may be due to the presence of the following combination of properties in this compound:

(a) 2-*O*-Glycoside of ascorbic acid (**I**) is capable of inhibiting fragmentation and recombination reactions of hydroxyl-containing carbon-centered radicals, which may ensure protection of vitally important components of uninfected cells from injuries caused by ROS.

(b) However, unlike AA and its 2,3-disubstituted analog (**II**), **I** does not influence the processes involving oxygen-centered radicals, and hence it does not interfere with realization of physiological response of the organism to a viral infection.

The experimental facts and relationships revealed in this study may be used to perform targeted search for new antiviral agents among the compounds capable of regulating free radical processes.

Acknowledgment

The study was performed under support from the International Science and Technology Center (ISTC), Grant B-1746.

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