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Antioxidant properties of propargylamine derivatives: assessment of their ability to scavenge peroxynitrite

Stefania Dragoni, Valentina Porcari, Massimo Travagli, Daniele Castagnolo and Massimo Valoti

Abstract

A series of arylpropargylamines, variously substituted in the hydrogen in *p*-position and in the propargyl moiety, were studied as potential peroxynitrite scavengers. The scavenging activity of these compounds was evaluated through peroxynitrite ($ONOO^-$)-mediated oxidation of dichlorofluorescin and linoleic acid by measuring the dichlorofluorescein formation and oxygen consumption, respectively. Among tested compounds, only 1-phenylpropargylamine (AP3) promoted concentration-dependent inhibition of $ONOO^-$-induced dichlorofluorescin and linoleic acid oxidation with IC50 values of 637 and 63 μM, respectively. The AP3 spectral changes in UV-visible absorbance properties in the presence of peroxynitrite suggested the formation of a new compound. This was identified by gas-chromatograph–mass spectrometer analysis as phenylpropargyl alcohol. Structure–activity relationship analysis indicated that the scavenging activity of AP3 was due to the aminopropargyl moiety and availability of the nitrogen electron pair. This data suggested that AP3 could be considered a lead compound for the synthesis of new $ONOO^-$ scavenger derivatives.

Introduction

Propargylamine derivatives are the most extensively studied monoamine oxidase (MAO) inhibitors. An acetylene group in β-position with respect to the amino group confers the property of irreversible MAO inhibitor (Cesura et al 1999). Though many compounds have been synthesized, only L-deprenyl (phenyl-isopropyl-methyl-propargylamine), which has the property of increasing striatal dopamine levels (Gerlach 1996), is currently used as a therapeutic agent in Parkinson's disease. L-Deprenyl has anti-apoptotic activity towards different endogenous and exogenous neurotoxins, such as 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) (Naoi et al 2000; Maruyama et al 2001), nitric oxide and peroxynitrite (Maruyama et al 1998). Many studies have shown also that L-deprenyl could promote a direct antioxidant effect by acting as radical scavenger (Thomasa et al 1996; Mytilineou et al 1998). Since the propargylamine moiety is necessary for the neuroprotective activity of this drug (Maruyama et al 1998, 2000; Naoi et al 2000; Suuronen et al 2000; Szende et al 2000; Lee et al 2002; Ebadi et al 2002; Sowa et al 2004), renewed interest has been expressed in the synthesis of new propargylamine derivatives as neuroprotective compounds with or without MAO-B inhibitory properties (Perez et al 1999; Tatton et al 2002). A recent study showed that one of these derivatives, CGP 3466, without MAO A or B inhibiting properties, had neurorescuing properties 100-times more powerful than L-deprenyl in the same in-vivo and in-vitro paradigms, again emphasizing the importance of the propargylamino group for neuroprotection (Kragten et al 1998).

Nitric oxide (NO), a free radical species produced by several mammalian cell types, is involved in several physiological and pathological conditions. Its undesired effects have been attributed to reactive nitrogen species, such as NOx and peroxynitrite ($ONOO^-$), formed by reaction of NO with oxygen and superoxide anions. While generation of $ONOO^-$ may be beneficial in terms of host defence against invading microorganisms, excess peroxynitrite may be detrimental and entails damage to biomolecules (Muijsers et al 1997). The role of NO and/or $ONOO^-$ in neuronal degeneration has been

Dipartimento di Scienze Biomediche, Sezione di Farmacologia, Università di Siena, Via Aldo Moro 2, 53100 Siena, Italy

Stefania Dragoni, Valentina Porcari, Massimo Valoti

Dipartimento Farmaco Chimico Tecnologico, Università di Siena, Via Aldo Moro 2, 53100 Siena, Italy

Massimo Travagli, Daniele Castagnolo

Correspondence: M. Valoti, Dipartimento di Scienze Biomediche, Via Aldo Moro 2, 53100 Siena, Italy. E-mail: valoti@unisi.it

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highlighted in neuronal nitric oxide synthase (NOS)-deficient mice which were found resistant to stroke, *N*-methyl-D-aspartate neurotoxicity and MPTP-induced Parkinson's disease (Ischiropoulos & Beckman 2003).

In line with these observations, we have tested the protective effects of a series of 1-arylpropargylamines, focusing on their $ONOO^-$-scavenging activity.

Materials and Methods

Chemicals

2′,7′-Dichlorofluorescin diacetate, 2′,7′-dichlorofluorescein and linoleic acid were obtained from Sigma (St Louis, MO). (*R, S*)-1-Phenylpropargylamine (AP3), (*R, S*)-1-phenyl-*N*-acetyl-propargylamine (AP2), (*S*)-1-(4-chlorophenyl)propargylamine (AP39), (*R*)-1-(4-chlorophenyl)propargylamine (AP41), (*S*)-1-(4-fluorophenyl)propargylamine (FM100), (*R*)-1-(4-fluorophenyl)propargylamine (FM103), (*R, S*)-1-phenylpropenylamine (DAN2) and (*R, S*)-1-phenylpropylamine (DAN10) were synthesized as reported by Messina et al (1999). Their structures are shown in Table 1. The F and Cl derivatives were enantiomers, while the other compounds were an enantiomeric mixture. All other chemicals and solvents were of the highest grade available from common commercial sources.

Peroxynitrite synthesis

Peroxynitrite was synthesized in a quenched flow reactor and stored in 1.5 M NaOH at −70°C (Beckmann et al 1994). Its concentration was determined spectrophotometrically by measuring absorbance at 302 nm ($\epsilon_{nM} = 1670\,mol^{-1}\,cm^{-1}$) as described by Kooy et al (1997).

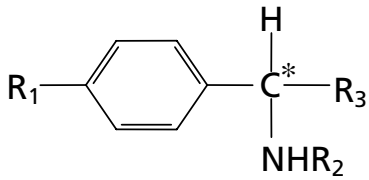

Table 1 Chemical structure of synthesized compounds

Compound	R_1	R_2	R_3
AP3 (*R,S*)	H	H	$-C\equiv CH$
AP2 (*R,S*)	H	$COCH_3$	$-C\equiv CH$
AP39 (*S*)	Cl	H	$-C\equiv CH$
AP41 (*R*)	Cl	H	$-C\equiv CH$
FM100 (*S*)	F	H	$-C\equiv CH$
FM103 (*R*)	F	H	$-C\equiv CH$
DAN2 (*R,S*)	H	H	$-CH=CH_2$
DAN10 (*R,S*)	H	H	$-CH_2$-CH_3

(*R,S*)-1-Phenylpropargylamine (AP3), (*R,S*)-1-phenyl-*N*-acetyl-propargylamine (AP2), (*S*)-1-(4-chlorophenyl)propargylamine (AP39), (*R*)-1-(4-chlorophenyl)propargylamine (AP41), (*S*)-1-(4-fluorophenyl)propargylamine (FM100), (*R*)-1-(4-fluorophenyl)propargylamine (FM103), (*R, S*)-1-phenylpropenylamine (DAN2), (*R,S*)-1-phenylpropylamine (DAN10).

Effects of arylpropargylamine derivatives on peroxynitrite-mediated oxidation of dichlorofluorescin and linoleic acid

The peroxynitrite scavenging activity of the propargylamine compounds was measured by following peroxynitrite-mediated oxidation of dichlorofluorescin ($DFCH_2$) to the fluorescent dichlorofluorescein (DCF) (Kooy et al 1997). Briefly, 5 μM peroxynitrite was added under vigorous stirring to 10 μM $DCFH_2$ with different concentrations of propargylamines (10–1000 μM) in 50 mM phosphate buffer, pH 7.4, containing 100 μM DTPA (diethylenetriaminepentaacetic acid), for 3 min at 37°C to completely oxidize $DCFH_2$. Dichlorofluorescein content was determined spectrofluorimetrically at excitation and emission wavelengths of 502 and 523 nm, respectively.

In a second series of experiments peroxynitrite scavenging activity was assayed by following $ONOO^-$-promoted oxidation of linoleic acid through oxygen consumption measured with a Clark oxygen electrode (Patel & Darley-Usmar 1996). The electrode chamber was filled with 33 mM linoleic acid in 50 mM phosphate buffer, pH 7.4, containing 100 μM DTPA, with variable concentrations (10–1000 μM) of propargylamine compounds, 3 mL final volume, and oxygen consumption was measured at 37°C for 3 min. The reaction was started by adding 500 μM peroxynitrite to the assay mixture and oxygen consumption was monitored for 10 min.

All experiments were performed in triplicate and controls were obtained by measuring $DCFH_2$ or linoleic acid oxidation in the presence of $ONOO^-$ alone. All solutions were made using high quality de-ionized water.

Spectrum changes of propargylamine derivatives in the presence of peroxynitrite

The reaction of propargylamine derivatives and $ONOO^-$ were followed spectrophotometrically at 500–220 nm with a double beam UV-vis spectrophotometer (Shimadzu UV-1601). The reaction mixture contained 50 mM phosphate buffer, pH 7.4, 100 μM propargyl compounds and 100 μM $ONOO^-$, added under vigorous stirring. Absorbances of UV-vis spectra were recorded every 60 s. A blank cuvette contained reaction mixture without $ONOO^-$.

Analysis of propargylamine oxidation by gas chromatography–mass spectroscopy

The reaction mixture containing 50 mM phosphate buffer, pH 7.4, 100 μM AP3 and 100 μM $ONOO^-$ was extracted twice with ethylacetate. The organic phase was dried under N_2 stream at 25°C and the residue was derivatized with pentafluoropropionic anhydride for 30 min at 90°C. The samples were dried under a nitrogen stream and resuspended in methanol. The samples were then injected in a Varian CP-3800 gas chromatograph with mass selective

detector (Varian Saturn 2000). The chromatography column was an RTx-5MS (Restek), 30 m × 0.25 mm i.d. × 0.2 film thickness. The oven temperature was programmed from 60°C (4 min) to 270°C at 20°C min^{-1}. Helium was used as carrier gas at a flow of 1 mL min^{-1}. The mass range monitored was m/z = 40–650 and the ionization energy was set at 70 eV.

Statistical analysis

Results were expressed as mean ± s.e. Statistical analysis of the data was performed by one-way analysis of variance followed by Dunnet's post-test analysis (Prism 3.02 Graphpad Software, Inc., San Diego, CA, USA). $P < 0.05$ was considered significant. IC50 values were calculated by non-linear regression analysis (Prism 3.02 GraphPad Software, Inc., San Diego, CA, USA).

Results

Effects of arylpropargylamine derivatives on peroxynitrite-mediated oxidation of dichlorofluorescin and linoleic acid

Chemical structures of the compounds are shown in Table 1. The hydrogen in *p*-position of the aromatic ring (AP2 and AP3) was variously substituted with a Cl or F atom (AP 39, 41 and FM 100, 103). To assay the contribution of the propinyl group to peroxynitrite scavenging properties, the moiety was substituted with a propylene and propyl group (DAN2 and DAN10, respectively). To study the role of the amino group, AP3 was converted to the corresponding acetylamide (AP2) with acetic acid.

Among the compounds tested, only AP3 promoted concentration-dependent inhibition of $ONOO^-$-induced $DCFH_2$ oxidation (IC50 637 μM) as shown in Figure 1.

Table 2 Inhibition of peroxynitrite-mediated formation of dichlorofluoroscein by 1-arylpropargylamine derivatives

	Fluorescence (arbitrary units)	Relative fluorescence (%)
Control	627.1 ± 20.8	100.0
AP2	598.4 ± 15.3	95.4
AP3	221.2 ± 5.9*	35.2
AP39	572.8 ± 18.9	91.3
AP41	668.7 ± 22.1	106.6
FM100	709.4 ± 78.3	113.1
FM103	843.6 ± 12.3*	134.5
DAN2	533.6 ± 34.5	85.1
DAN10	605.3 ± 30.5	96.52

Reactions were carried out at 37 °C for 3 min, incubating 50 mM phosphate buffer (pH 7.4) containing 100 μM DTPA and 10 μM dichlorofluorescin in the presence of 5 μM peroxynitrite with or without (control) 1000 μM propargylamino derivatives. Each value is the mean ± s.e. of three separate experiments. *$P < 0.05$ compared with control.

At the highest concentration (1000 μM), AP3 promoted 70% inhibition. In similar experimental conditions, the IC50 value of cysteine, a strong inhibitor of $ONOO^-$-dependent $DCFH_2$ oxidation, was 47 μM, one order of magnitude lower than AP3. The other compounds showed no activity, and with FM103 a significant increase in dichlorofluorescein formation was observed (see Table 2).

The scavenging property of AP3, assayed by measuring peroxynitrite-induced linoleic acid oxidation, showed concentration-dependent inhibition of oxygen consumption, with an IC50 value of 63 μM (Figure 2). The other propargylamine compounds did not show any antioxidant activity. To clarify if AP3 was a chain-reaction breaker of the linoleic acid oxidation, the compound was added to the reaction mixture 3 min after $ONOO^-$. In these

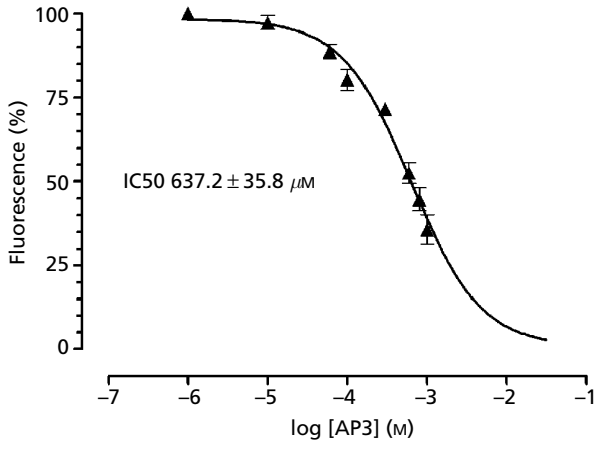

Figure 1 Peroxynitrite-mediated dichlorofluorescein formation in the presence of different concentrations of AP3. $ONOO^-$ (5 μM) was added under vigorous stirring to 10 μM $DCFH_2$, in the presence of different concentration of AP3. Values are means of three separate experiments. Bars indicate s.e.

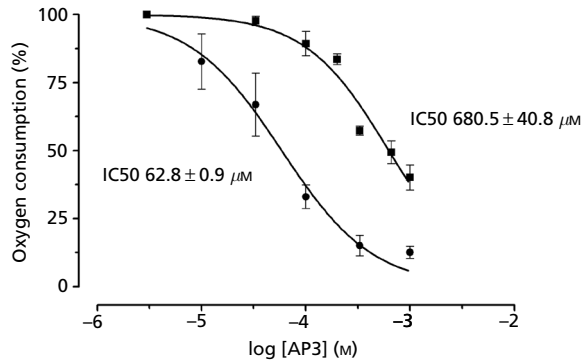

Figure 2 Peroxynitrite-mediated linoleic acid oxidation in the presence of different concentrations of AP3. The reaction was started by adding 500 μM $ONOO^-$ to the assay mixture; AP3 was added before (●) or 3 min after (■) $ONOO^-$. Oxygen consumption was monitored for 10 min. Values are means of three separate experiments. Bars indicate s.e.

experiments, AP3 showed weaker antioxidant properties, with an IC50 value of 680 μM, one order of magnitude greater than that seen in the previous experiments (Figure 2).

Spectrum changes of AP3 in the presence of peroxynitrite

The reaction of α-arylpropargylamine compounds with 100 μM $ONOO^-$ was monitored by UV-vis analysis. In the case of AP3 (100 μM), no spectral changes were recorded during the 10-min period in which the compound was maintained in control conditions. The addition of 100 μM $ONOO^-$ caused a time-dependent increase in absorbance at 370 nm and a decrease at 249 nm. The absorbance properties of the other compounds did not change in the presence of $ONOO^-$.

Analysis of AP3 oxidation by gas chromatography mass spectroscopy

The GC profile of the analytes detected after the reaction between AP3 and $ONOO^-$ included an unknown peak. The MS spectrum of the peak was different from that observed for authentic AP3 (Figure 3A) and had a molecular ion with m/z = 132, one mass unit greater than the molecular mass of AP3. The MS spectrum had a base peak at m/z = 105 and lower intensity peaks at m/z = 131 and 115 (Figure 3B). The MS results suggested formation of 1-phenylpropargyl alcohol with m/z = 132. The fragments at m/z = 131 and 115 could have been due to the loss of H and OH groups, respectively, and the base fragment at m/z = 105 could be ascribed to the benzyloxy ion $[C_6H_5CO]^+$.

Discussion

The inhibition studies of peroxynitrite-mediated $DCFH_2$ oxidation clearly indicated that of the 1-arylpropargylamine derivatives tested, only AP3 had peroxynitrite scavenging properties. Spectral changes in UV-vis absorbance properties of AP3 in the presence of $ONOO^-$ indicated formation of a new compound which was identified by GC-MS analysis. These observations suggested the following pathway for the reaction between AP3 and $ONOO^-$: AP3 reacts with peroxynitrite to form the nitramine derivative which is rapidly deaminated to the corresponding 1-phenylpropargyl alcohol.

A similar reaction was observed when guanine reacted with peroxynitrite to form 8-hydroxydeoxyguanosine (Inoue & Kawanishi 1995). Other authors have demonstrated *N*-nitrosamine formation by reaction of a series of secondary amines with peroxynitrite derivatives (Masuda et al 2000). In this study nitro and nitroso compounds were not detected by GC-MS analysis, suggesting that under our experimental conditions the primary nitro/nitroso-amine derivatives were unstable.

Protection against peroxynitrite is an important defence of normal tissues, especially under pathological conditions. Biological protection against peroxynitrite is organized in three categories: prevention by compounds controlling formation of peroxynitrite precursor, interception by substances that react directly with peroxynitrite, and repair by chemicals that remove damaged cell products (Arteel et al 1999). The evidence that AP3 belongs to the second category of compounds was confirmed by experiments with linoleic acid. The reaction of peroxynitrite with linoleic acid was rather complex and led to the formation of oxidized and nitrated products (O'Donnell et al 1999) that could

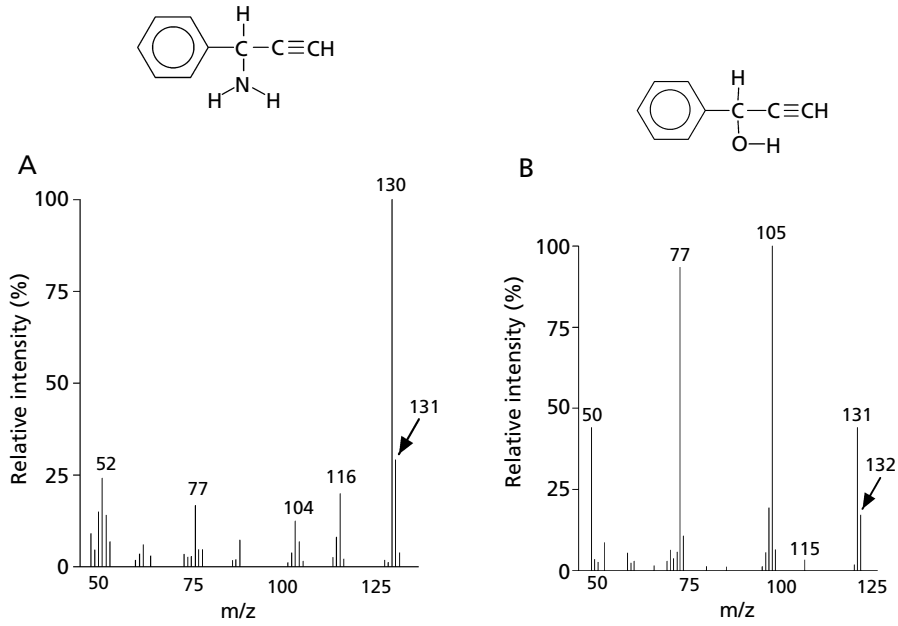

Figure 3 Electron impact mass spectra of authentic AP3 (A) and an AP3-oxidation compound obtained after reaction with peroxynitrite (B).

contribute to oxygen consumption. The evidence that AP3 powerfully inhibited oxygen consumption when present in the reaction mixture before $ONOO^-$ addition suggested that AP3 prevented oxidation of linoleic acid by scavenging $ONOO^-$. On the contrary, the higher IC50 observed when AP3 was added after $ONOO^-$ suggested that the compound was less active as a free radical quencher.

The $ONOO^-$ scavenging property of AP3 seemed to be associated with the propargyl group in the molecule. In fact, DAN2 and DAN10, in which the propargyl group was substituted with propenyl and propyl groups, respectively, did not have $ONOO^-$ scavenging properties. Introduction of a halogen (F in FM100, FM103 and Cl in AP39, AP41) in *para*-position on the aromatic ring produced compounds devoid of scavenging activity. This could have been due to reduced availability of the nitrogen electron pair. The role of nitrogen electron pair availability in antioxidant properties was illustrated by the observation that AP2, the amide derivative of AP3, had no activity.

Although further experiments are required to clarify the protective role of the present compound against oxidative stress promoted by $ONOO^-$, it is to outline that AP3 is more efficient at inhibiting $ONOO^-$-induced $DCFH_2$ oxidation than desferrioxamine and urate (Kooy et al 1997), which are useful compounds to counteract the oxidative stress in-vivo. For these reasons AP3 could be considered a lead compound for the synthesis of new substances that could prevent $ONOO^-$-induced cell damage.

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