Free Radical Research
Publication details, including instructions for authors and subscription information:
http://www.informaworld.com/smpp/title~content=t713642632

Antioxidant and free radical scavenging properties of N-acetylcysteine amide (NACA) and comparison with N-acetylcysteine (NAC)

Burhan Ates [a]; Linu Abraham [a]; Nuran Ercal [a]
[a] Department of Chemistry, Missouri University of Science and Technology, Rolla, MO, USA

First Published on: 01 April 2008
To cite this Article: Ates, Burhan, Abraham, Linu and Ercal, Nuran (2008) 'Antioxidant and free radical scavenging properties of N-acetylcysteine amide (NACA) and comparison with N-acetylcysteine (NAC)', Free Radical Research, 42:4, 372 - 377
To link to this article: DOI: 10.1080/10715760801998638
URL: http://dx.doi.org/10.1080/10715760801998638

Free Radical Research, April 2008; 42(4): 372–377

informa
healthcare

Antioxidant and free radical scavenging properties of N-acetylcysteine amide (NACA) and comparison with N-acetylcysteine (NAC)

BURHAN ATES, LINU ABRAHAM, & NURAN ERCAL

Department of Chemistry, Missouri University of Science and Technology, Rolla, MO 65409, USA

Accepted by Dr E. Niki

(Received 7 January 2008; in revised form 15 February 2008)

Abstract

The antioxidant potential of N-acetylcysteine amide (NACA), also known as AD4, was assessed by employing different *in vitro* assays. These included reducing power, free radical scavenging capacities, peroxidation inhibiting activity through linoleic acid emulsion system and metal chelating capacity, as compared to NAC and three widely used antioxidants, α-tocopherol, ascorbic acid and butylated hydroxytoluene (BHT). Of the antioxidant properties that were investigated, NACA was shown to possess higher 2,2-diphenyl-1-picryl-hydrazyl-hydrate (DPPH) radical scavenging ability and reducing power than NAC, at all the concentrations, whereas the scavenging ability of H_2O_2 differed with concentration. While NACA had greater H_2O_2 scavenging capacity at the highest concentration, NAC was better than NACA at lower concentrations. NAC and NACA had a 60% and 55% higher ability to prevent β-carotene bleaching, respectively, as compared to control. The chelating activity of NACA was more than 50% that of the metal chelating capacity of EDTA and four and nine times that of BHT and α-tocopherol, respectively. When compared to NACA and NAC; α-tocopherol had higher DPPH scavenging abilities and BHT and α-tocopherol had better β-carotene bleaching power. These findings provide evidence that the novel antioxidant, NACA, has indeed enhanced the antioxidant properties of NAC.

Keywords: *NACA, NAC, antioxidant properties, radical scavenging*

Introduction

Oxidative stress is thought to play a key role in the progression of many diseases ranging from neurodegenerative disorders to cancer [1,2]. A large number of studies have been undertaken to evaluate the ability of synthetic or naturally occurring antioxidants to combat the damaging effects of free radicals and reactive oxygen species. Thiol containing compounds are a class of antioxidants that have gained special attention due to their ability to enhance cellular antioxidant defense mechanisms and act as a precursor to the intracellular antioxidant, glutathione (GSH).

N-acetylcysteine (NAC), a well-known thiol-containing antioxidant, has had multiple uses in clinics for more than 50 years [3–7]. NAC has been used in treating GSH deficiency in a wide range of infections, genetic defects and metabolic disorders, including human immunodefiency virus (HIV) infection and chronic obstructive pulmonary disease (COPD) [8]. The evidence from both *in vitro* and *in vivo* studies indicates that NAC is capable of facilitating intracellular GSH biosynthesis by reducing extracellular cystine to cysteine [9] or by supplying sulphydryl (-SH) groups that can stimulate GSH synthesis and enhance glutathione-S-transferase activity [10,11]. Additionally, NAC is a potent free radical scavenger as a result of its nucleophilic reactions with ROS [12]. However, bioavailability of NAC is very low because its carboxyl group loses its proton at physiological

Correspondence: Nuran Ercal, Department of Chemistry, Missouri University of Science and Technology, 400 West 11th Street, Rolla, MO 65409, USA. Tel: 573-341-6950. Fax: 573-341-6033. Email: nercal@umr.edu

ISSN 1071-5762 print/ISSN 1029-2470 online
DOI: 10.1080/10715760801998638

pH, making the compound negatively charged [13, 14]. This renders its passage through biological membranes difficult. N-acetylcysteine amide (NACA), also known as AD4, is the modified form of N-acetylcysteine that contains an amide group in place of the carboxyl group of NAC. It was designed and synthesized with the possibility that neutralizing the carboxyl group would aid in its passage through cell membranes. One recent study provided evidence that NACA had more efficient membrane permeation than NAC and could replenish intracellular GSH in red blood cells, possibly by disulphide exchange with oxidized glutathione (GSSG) [13,14]. Further, NACA can be hydrolysed to give cysteine that can boost the production of endogenous glutathione. This compound was also shown to cross the blood–brain barrier, scavenge free-radicals, chelate copper, protect red blood cells from oxidative stress and attenuate myelin oligodendrocyte glycoprotein (MOG)-induced experimental autoimmune encephalomyelitis in a multiple sclerosis mouse model [15,16].

The present study was undertaken to evaluate the *in vitro* antioxidant potential of the newly synthesized thiol NACA, as compared to NAC and other well known antioxidants, using different *in vitro* models: (i) DPPH radical scavenging; (ii) reducing power; (iii) β-carotene bleaching; (iv) H_2O_2 scavenging; (v) $OH^{\bullet}$-scavenging; and (vi) metal chelating activity.

Materials and methods

Chemicals

All chemicals used for analytical purposes were obtained from Sigma (St.Louis, MO) and Fisher Scientific (Fair Lawn, NJ). N-acetylcysteine amide (NACA) was provided by Dr Glenn Goldstein (David Pharmaceuticals, New York).

Radical scavenging power

Radical scavenging power of NACA, NAC and standard antioxidants were assessed by the method of Shimada et al. [16] with slight modifications. The reaction mixture was a total volume of 3 ml, which included 2.9 ml of DPPH (1×10^{-4} M DPPH) and 0.1 ml of the corresponding sample at various concentrations. The solutions were left in the dark at room temperature for 30 min and the resulting colour was measured spectrophotometrically at 520 nm against blanks. A decreasing intensity of the colour purple was related to a higher radical scavenging power percentage, which was calculated using the following equation;

$$\text{Radical scavenging power} = \left[1 - \left(\frac{A_{S:30}}{A_{B:30}}\right)\right] \times 100$$

where, A_{S30} is absorbance of sample and A_{B30} is absorbance of blank at 30 min reaction time.

Reducing power

Reducing power of samples was determined according to the method of Oyaizu [17]; 2.5 ml 0.2 M phosphate buffer (pH 6.6) and 2.5 ml 1% potassium ferricyanide were added to 1 ml sample solution and mixed gently. The mixtures were incubated at 50°C in a water bath for 20 min. Reaction was stopped by adding 2.5 ml of 10% trichloroacetic acid (TCA) and the mixtures were centrifuged at 6000 rpm for 10 min. From the top layer, 2.5 ml was transferred into tubes containing 2.5 ml distilled water and 0.5 ml of 0.1% ferric chloride ($FeCl_3.6H_2O$). The resulting solutions were mixed well and, after 5 min, the colour intensity read at 700 nm against blanks.

β-carotene bleaching

Antioxidant activity of samples was determined using the β-carotene bleaching method of Miller [18]. Crystalline β-carotene (2 mg) was dissolved in 10 ml of chloroform and 1 ml of this solution was taken in a round-bottom flask. To this, 20 μg of linoleic acid and 200 μl of Tween-20 (Merck) were added. The chloroform was removed, using a rotary evaporator under vacuum at 40°C for 5 min and 50 ml distilled water was added to the residue, with vigorous stirring in order to form an emulsion. Five millilitres of this emulsion were added to each tube containing samples (50 μg/ml). Tubes were placed in a water bath at 50°C and absorbance at 470 nm was measured at intervals, for up to 90 min.

H_2O_2 scavenging activity assay

H_2O_2 scavenging activity was measured according to the literature procedure of Zhao et al. [19] with minor modifications. H_2O_2 (1.0 ml, 0.1 mM) and 1.0 ml of various concentrations of the samples were mixed, followed by addition of 100 μl of 3% ammonium molybdate, 10 ml of H_2SO_4 (2 M) and 7.0 ml of KI (1.8 M). The mixed solutions were titrated with $Na_2S_2O_3$ (5 mM) until the yellow colour disappeared. The percentage scavenging effect was calculated as

$$\text{Scavenging rate} = ((V_0 - V_1)/V_0) \times 100\%$$

where V_0 was the volume of the $Na_2S_2O_3$ solution used to titrate the control sample in the presence of hydrogen peroxide (without sample) and V_1 was the volume of the $Na_2S_2O_3$ solution used in the presence of the sample.

$OH^{\bullet}$-scavenging activity assay

Hydroxyl ($OH^{\bullet}$) radical scavenging ability was measured according to the literature procedure of Smirnoff and Cumbes [20] with a few modifications. The reaction mixture (3 ml) contained 1 ml of $FeSO_4$ (1.5 mM), 0.7 ml of H_2O_2 (6 mM), 0.3 ml of sodium salicylate (20 mM) and varying concentrations of

NAC, NACA and standard antioxidants. This mixture was incubated at 37°C for 1 h, after which the absorbance of the hydroxylated salicylate complex was measured at 562 nm. The percentage scavenging effect was calculated as

$$\text{Scavenging rate} = [1 - (A_1 - A_2)/A_0] \times 100\%$$

where A_0 was the absorbance of the control (without sample) and A_1 was the absorbance in the presence of the sample, A_2 was the absorbance without sodium salicylate.

Metal chelating capacity

Ferrous ions chelating activity of samples was determined by the method of Dinis et al. [21]. A solution containing 20 μl of the sample and 0.38 ml of water or ethanol was added to 0.05 ml of 2 mM $FeCl_2$. The mixture was shaken vigorously for 1 min and initiated by the addition of 5 mM of ferrozine [3-(2-pyridyl)-5,6-diphenyl-1,2,4-triazine-4′,4″-disulphonic acid sodium salt] (0.2 mL) and ethanol (3.35 mL). Final colour was monitored at 562 nm after 10 min incubation. The metal chelating efficiency of samples was determined by comparing with the chelating activity of ethylene diamine tetraacetic acid (EDTA, disodium salt). The Fe^{2+} chelating activity of the samples was calculated as

$$\%\ \text{chelating} = [(A_0 - A_1)/A_0] \times 100$$

where, A_0 indicates the absorbance of the control and A_1 the absorbance in the presence of the samples or EDTA.

Results and discussion

Radical scavenging power

Free radical scavenging is a very important property of an antioxidant, due to the many deleterious effects of radicals in biological systems. The DPPH scavenging method is a simple method of assessing the radical scavenging power of a compound [22]. This method is based on the ability of antioxidants to reduce the DPPH radical to a more stable DPPHH form. The DPPH radical has an absorption maxima at 520 nm. In the presence of a free radical scavenger, the DPPH gets reduced with a corresponding decrease in absorbance.

The radical scavenging powers of NAC and NACA were compared to two standard antioxidants, BHT and α-tocopherol. The DPPH scavenging activities of NAC, NACA, α-tocopherol and BHT are summarized in Figure 1. In order to determine the effect of concentration on radical scavenging power, we used three different concentration (25, 50 and 75 μg/ml) of samples. Results indicated that at a lower concentration, the radical scavenging characteristics of NACA and NAC were similar (25.9% and 21.8%, respectively). However, at higher concentrations,

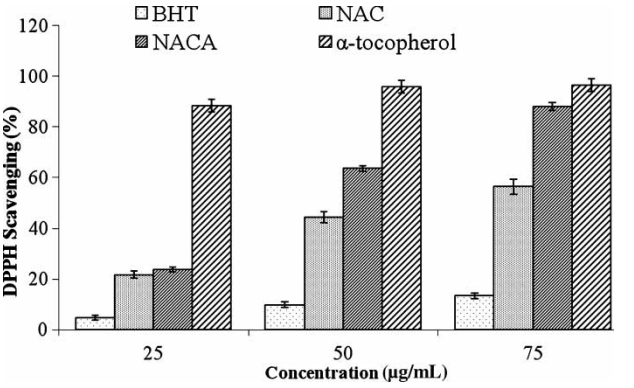

Figure 1. Radical scavenging power of NACA, NAC, BHT and α-tocopherol at different concentrations. Radical scavenging ability of different compounds was evaluated by their ability to scavenge DPPH radicals, as measured by a decrease in absorbance at 520 nm. Molar concentrations of each compound used were, as follows: NAC (153.1, 306.2 and 459.3 μM), NACA (154.1, 308.2 and 462.3 μM), BHT (113.4, 226.8, 340.2 μM) and α-tocopherol (58, 116, 174 μM). Each value is the average of triplicates with error bars indicating STDEVs (σ_{n-1}).

NACA possessed a higher radical scavenging ability, as compared to NAC (88.2% and 56.5%, respectively). In fact, there was a linear relationship between DPPH's scavenging ability and the concentration of NACA ($R^2 = 0.98$). Of the four compounds used in this experiment, α-tocopherol was shown to have maximal DPPH scavenging power, while BHT had minimal radical scavenging properties. A possible explanation of the lowered ability of NAC to scavenge radicals at a higher concentration may be due to the saturation kinetics of the reaction [23].

Reducing power

Reducing power of a compound is another supporting feature for its antioxidant activity. The reducing properties are generally associated with the presence of reductones, which have been shown to exhibit antioxidant action by breaking the chain reactions by donating a hydrogen atom. Reductones are also reported to react with certain precursors of peroxide, thus preventing peroxide formation [24].

Reducing power characteristics of NACA, NAC, α-tocopherol and BHT are summarized in Figure 2. Two different concentrations of compounds were used for this study. The concentration-dependent reducing power of samples followed the order: NACA > NAC > BHT > α-tocopherol. The reducing abilities of NACA and NAC, at both concentrations used, were far superior to those of BHT and α-tocopherol. Specifically, at a concentration of 50 μg/ml, the ratio of reducing powers of NAC to BHT and α-tocopherol were 1.64 and 2.02, respectively. These ratios for NACA were 1.73 and 2.12, thus showing the slightly higher reducing ability of NACA, as compared to NAC.

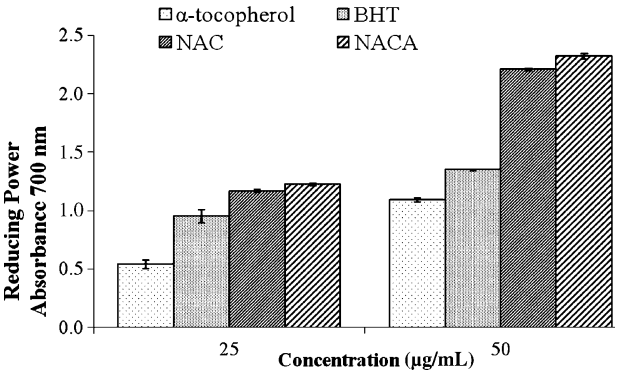

Figure 2. Reducing power of NACA, NAC, BHT and α-tocopherol at different concentrations. Reducing ability was assessed by the capacity of compounds to reduce potassium ferricyanide. Increased absorbance of the final reaction mixture at 700 nm indicates a greater reducing ability of the compounds. Molar concentrations of each compound used were, as follows: NAC (153.1 and 306.2 μM), NACA (154.1 and 308.2 μM), BHT (113.4 and 226.8 μM) and α-tocopherol (58 and 116 μM). Each value is the average of triplicates with error bars indicating STDEVs (σ_{n-1}).

β-carotene bleaching

The β-carotene bleaching method is based on the ability of radicals of oxidized linoleic acid to react with β-carotene and lead to its discolouration. The presence of an antioxidant can prevent the discolouration of β-carotene. This method is of significance because β-carotene is a physiologically important compound with strong biological activity [25]. The ability of samples to prevent β-carotene bleaching was studied by monitoring the colour intensity of emulsions at 470 nm, at various time intervals, for 90 min (Figure 3). The β-carotene bleaching ability of BHT, α-tocopherol, NAC, NACA and ascorbic acid were 95.9%, 88.5%, 74.8%, 63.7% and 43.5%, respectively. The greater efficacy of BHT and α-tocopherol to prevent β-carotene bleaching can be attributed to their greater solubility in the linoleic acid system. Therefore, the β-carotene bleaching abilities of NAC, NACA and ascorbic acid were compared since these are water-soluble. In the first 15 min of incubation, NAC and NACA had 60% and 55% higher ability to prevent β-carotene bleaching, as compared to the control.

H_2O_2 scavenging capacity

Hydrogen peroxide is not a direct reactive oxygen species, but due to its high membrane permeability, it enters cells and leads to the production of hydroxyl radicals and superoxide radicals in the presence of metal ions. Hydrogen peroxide's scavenging ability is, thus, an important measure of the antioxidant activity of a compound. The ability of NAC and NACA to scavenge hydrogen peroxide, in comparison with ascorbic acid, is shown in Figure 4. Three concentrations of NACA, NAC and ascorbic acid were used. Of

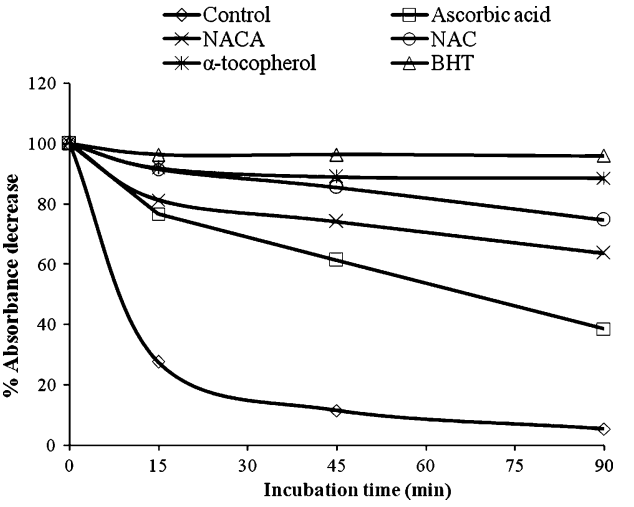

Figure 3. Antioxidant properties of NACA, NAC, BHT, α-tocopherol and ascorbic acid (50 μg/mL) by the β-carotene bleaching method. Relative changes in absorbance of β-carotene emulsions containing NAC and NACA or artificial antioxidants were measured at 470 nm. The molar concentrations of NAC, NACA, BHT, α-tocopherol and ascorbic acid used were 306.2, 308.2, 226.8, 116 and 282.8 μM, respectively. For clarity, STDEVs are not shown but they are mostly smaller than 5% of average value. Each value is the average of triplicates.

the three compounds, ascorbic acid exhibited the lowest scavenging activity. NAC showed a non-linear relation between scavenging activity and concentration. At lower concentrations of 125 and 250 μg/ml, NAC was shown to have the best H_2O_2 scavenging among all three compounds. However, at the highest concentration used, NACA showed superior scavenging activity to NAC. The H_2O_2 scavenging activity of NACA showed a high linear ($R^2 = 0.99$) dependence on concentration. The non-linearity in the H_2O_2 scavenging ability of NAC could be attributed to its auto-oxidation in the presence of oxygen and/or its pro-oxidant effects in the presence of trace amounts of transition metals in the reaction mixture [26,27].

$OH^{\bullet}$-scavenging capacity

Hydroxyl radicals are short lived free radicals that can be highly deleterious to cell membranes and other biomolecules. $OH^{\bullet}$ scavenging is therefore necessary to protect cells from oxidative damage. Of the many different ways by which $OH^{\bullet}$ hydroxyl radicals can be produced, the most important is the Fenton reaction. This reaction involves the transition metal catalysed decomposition of hydrogen peroxide to produce hydroxyl radicals [28]. The $OH^{\bullet}$-scavenging capacity of various compounds was measured using the method of Smirnoff and Cumbes [20]. $OH^{\bullet}$ radicals were generated using a system containing $FeSO_4$ and H_2O_2 and detected by their ability to hydroxylate salicylate. Initial experiments with α-tocopherol and BHT were performed, but results obtained are not shown due to possible interference created by

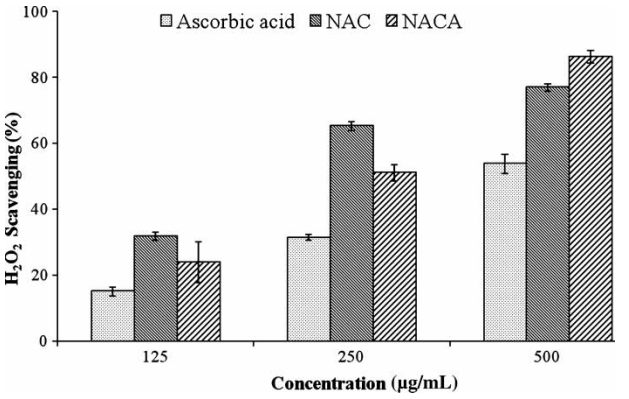

Figure 4. H_2O_2 scavenging activities of NACA, NAC and ascorbic acid at various concentrations. These concentrations are as follows: NAC (0.765, 1.53 and 3.06 mM), NACA (0.77, 1.54 and 3.08 mM) and ascorbic acid (0.705, 1.41 and 2.82 mM). The absorbance values were converted to scavenging effects (%) and data plotted as the mean of replicate scavenging effect (%) values ± STDEVs (σ_{n-1}) vs antioxidant concentration in µg/ml reaction volume.

solubility problems. Ascorbic acid was, therefore, used as a standard antioxidant for comparison. The results are summarized in Figure 5. Three concentrations (50, 100 and 200 µg/ml) were used in this experiment and the hydroxyl radical scavenging capacity of samples followed the order: NAC > NACA > ascorbic acid.

Fe^{2+} chelating capacity

Metal chelating property is especially important because of the ability of transition metal ions like Fe^{2+} to catalyse a number of free radical generating reactions such as the Fenton reaction. Hydroxyl radicals produced as a result of this reaction can accelerate lipid peroxidation and damage cell membranes [29]. Shown below is the Fenton reaction:

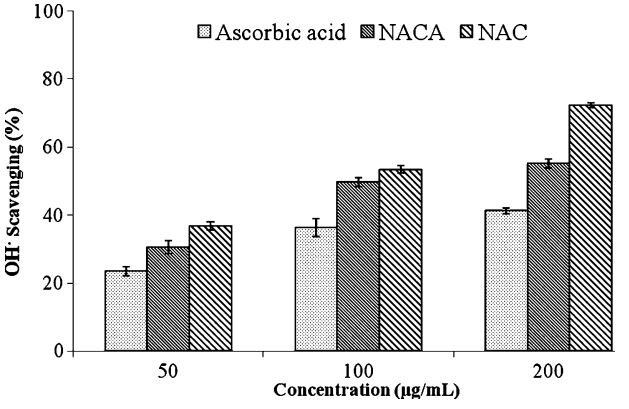

Figure 5. $OH^{\cdot}$ scavenging activities of NACA, NAC and ascorbic acid solutions at different concentrations. The method is based on measurement of hydroxylated salicylate complex produced in a hydroxyl radical generating system. Molar concentrations of each compound used were, as follows: NAC (0.306, 0.612 and 1.12 mM), NACA (0.308, 0.616 and 1.232 mM) and ascorbic acid (0.282, 0.564 and 1.128 mM). Each value is the average of triplicates with error bars indicating STDEVs (σ_{n-1}).

$$Fe^{2} + H_2O_2 \rightarrow Fe^{3} + OH^{-} + OH^{\cdot}$$

Metal chelating activity is, therefore, an important indicator of antioxidant capacity of a compound. The method used here is based on the formation of a coloured Fe^{2+}-Ferrozine complex that absorbs maximally at 562 nm. In the presence of a chelating compound, Fe^{2+} is no longer available to form a coloured complex, as reflected by a decrease in absorbance. Results indicate that NACA has very high Fe^{2+} chelating capacity. At a concentration of 5 µg/ml, the chelating activity of NACA was more than 50% that of the metal chelating capacity of EDTA. NACA had about 4-times and 9-times the metal chelating activity of BHT and α-tocopherol, respectively (Figure 6). The metal chelating capability of NAC and ascorbic acid could not be analysed using this method because the ability of NAC to reduce Fe^{3+} (possibly produced by auto-oxidation) to Fe^{2+} interferes with the measurement of its chelating activity. It is well known that ascorbic acid reduces Fe^{3+} to Fe^{2+}. NAC reduces Fe^{3+} to the same extent as ascorbic acid, as was confirmed by a separate experiment (data not shown) in which incubation of NAC and ascorbic acid with $FeCl_3$ showed a significant increase in absorbance in a reaction mixture containing ferrozine.

Conclusion

Oxidative stress is known to cause a number of diseases. This underlines the need for the synthesis of novel and effective antioxidants. Thiol containing compounds are a class of antioxidants that are especially relevant due to their ability to increase intracellular glutathione levels. NAC is a widely used thiol antioxidant. Its use, however, has been limited

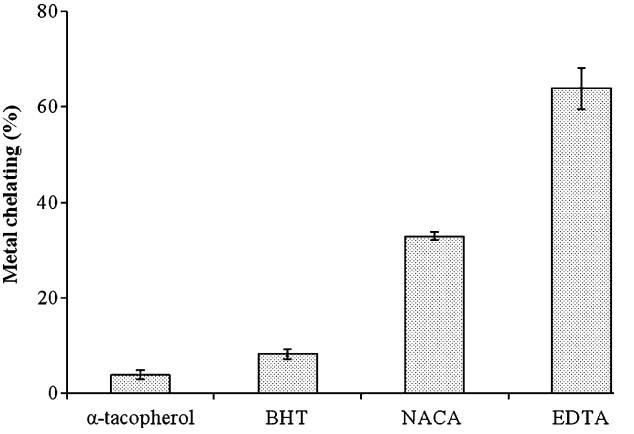

Figure 6. Metal chelating properties of NACA, BHT and α-tocopherol (5 µg/mL) as compared to EDTA. This method is based on the formation of a coloured Fe^{2+}-ferrozine complex that absorbs maximally at 562 nm. Molar concentrations of NACA, BHT, α-tocopherol and EDTA used were 30.82, 22.68, 10.16 and 12 µM, respectively. Each value is the average of triplicates with error bars indicating STDEVs (σ_{n-1}).

by several drawbacks including low membrane penetration and pro-oxidant activity at high concentrations. NACA is a newly synthesized amide form of NAC, designed to be more cell permeable than NAC. In our study, antioxidant and free radical scavenging properties of NACA were very similar or even better compared to those of NAC. Furthermore, this study reveals that NACA has potent metal chelating activity and can be used at high concentrations without pro-oxidant drawback. These findings provide evidence that the novel antioxidant, NACA, has overcome the disadvantages of NAC and could possibly replace NAC in future studies.

Acknowledgements

The authors would like to thank Barbara Harris for carefully editing this manuscript. B. Ates was supported by the Turkish Scientific Technical Research Council. This study was supported by NIH 1 R15 DA023409-01A2.

***Declaration of interest*:** The authors report no conflicts of interest. The authors alone are responsible for the content and writing of the paper.

References

[1] Hayes JD, McLellan LI. Glutathione and glutathione-dependent enzymes represent a co-ordinately regulated defence against oxidative stress. Free Radic Res 1999;31:273–300.

[2] Nakabeppu Y, Tsuchimoto D, Furuichi M, Sakumi K. Review: the defense mechanisms in mammalian cells against oxidative damage in nucleic acids and their involvement in the suppression of mutagenesis and cell seath. Free Radic Res 2004;38:423–429.

[3] Kelly GS. Clinical applications of N-acetylcysteine. Altern Med Rev 1998;3:114–127.

[4] Holdiness MR. Clinical pharmacokinetics of N-acetylcysteine. Clin Pharmacokinetics 1991;20:123–134.

[5] Parcell S. Sulfur in human nutrition and applications in medicine. Altern Med Rev 2002;7:22–44.

[6] Ziment I. Acetylcysteine: a drug that is much more than a mucokinetic. Biomed Pharmacother 1988;42:513–519.

[7] Flanagan RJ, Meredith TJ. Use of N-acetylcysteine in clinical toxicology. Am J Med 1991;91:131–139.

[8] Atkuri KR, Mantovani JJ, Herzenberg LA, Herzenberg LA. N-Acetylcysteine—a safe antidote for cysteine/glutathione deficiency. Curr Opin Pharmacol 2007;7:355–359.

[9] Issels RD, Nagele A, Eckert KG, Wilmanns W. Promotion of cystine uptake and its utilization for glutathione biosynthesis induced by cysteamine and N-acetylcysteine. Biochem Pharmacol 1988;37:881–888.

[10] Nakata K, Kawase M, Ogino S, Kinoshita C, Murata H, Sakaue T, Ogata K, Ohmori S. Effects of age on levels of cysteine, glutathione and related enzyme activities in livers of mice and rats and an attempt to replenish hepatic glutathione levels of mouse with cysteine derivatives. Mech Age Dev 1996;90:195–207.

[11] De Vries N, De Flora S. N-acetyl-l-cysteine. J Cell Biochem Suppl 1993;17:270–277.

[12] Aruoma OI, Halliwell B, Hoey BM, Butler J. The antioxidant action of N-acetylcysteine: its reaction with hydrogen peroxide, hydroxyl radical, superoxide, and hypochlorous acid. Free Radic Biol Med 1989;6:593–597.

[13] Grinberg L, Fibach E, Amer J, Atlas D. N-Acetylcysteine amide, a novel cell-permeating thiol, restores cellular glutathione and protects human red blood cells from oxidative stress. Free Radic Biol Med 2005;38:136–145.

[14] Atlas D, Melaned E, Offen D. Brain targeted low molecular weight hydrophobic antioxidant compounds. US patent No. 5,874,468; 1999.

[15] Offen D, Gilgun-Sherki Y, Barhum Y, Benhar M, Grinberg L, Reich R, Melamed E, Atlas D. A low molecular weight copper chelator crosses the blood–brain barrier and attenuates experimental autoimmune encephalomyelitis. J Neurochem 2004;89:1241–1251.

[16] Shimada K, Fujikawa K, Yahara K, Nakamura T. Antioxidative properties of xanthan on the autoxidation of soybean oil in cyclodextrin emulsion. J Agric Food Chem 1992;40:945–948.

[17] Oyaizu M. Antioxidaive activities of browning products of glucosamine fractionated by organic solvent and thin-layer chromatography. Nippon Shokuhin Kogyo Gakkaishi 1988;35:771–775.

[18] Miller HE. A simplified method for the evaluation of antioxidants. J Am Oil Chem Soc 1971;48:91–97.

[19] Zhao GR, Xiang ZJ, Ye TX, Yuan YJ, Guo ZX. Antioxidant activities of Salvia miltiorrhiza and Panax notoginseng. Food Chem 2006;99:767–774.

[20] Smirnoff N, Cumbes QJ. Hydroxyl radical scavenging activity of compatible solutes. Phytochemistry 1989;28:1057–1060.

[21] Dinis TCP, Madeira VMC, Almeida LM. Action of phenolic derivatives (acetaminophen, salicylate, and 5-aminosalicylate) as inhibitors of membrane lipid peroxidation and as peroxyl radical scavengers. Arch Biochem Biophys 1994;315:161–169.

[22] Wang BJ, Lien YH, Yu ZR. Supercritical fluid extractive fractionation—study of the antioxidant activities of propolis. Food Chem 2004;86:237–243.

[23] Dangles O, Dufour C, Fargeix G. Inhibition of lipid peroxidation by quercetin and quercetin derivatives: antioxidant and prooxidant effects. J Chem Soc, Perkin Trans 2000;2:1215–1222.

[24] Matsushige K, Basnet P, Kadota S, Namba T. Potent free radical scavenging activity of dicaffeoyl quinic acid derivatives from propolis. J Trad Med 1996;13:217–228.

[25] Sakanaka S, Tachibana Y, Okada Y. Preparation and antioxidant properties of extracts of Japanese persimmon leaf tea (kakinoha-cha). J Food Chem 2005;89:569–575.

[26] Sagristá ML, García AF, De Madariaga MA, Mora M. Antioxidant and pro-oxidant effect of the thiolic compounds N-acetyl-l-cysteine and glutathione against free radical-induced lipid peroxidation. Free Radic Res 2002;36:329–340.

[27] Yen GC, Duhb PD, Tsaia HL. Antioxidant and pro-oxidant properties of ascorbic acid and gallic acid.Food Chem 2002;79:307–313.

[28] Stohs SJ, Bagchi D. Oxidative mechanism in the toxicity of metal ions. Free Radic Biol Med 1995;18:321–336.

[29] Miller DD. Mineral. In: Fennema OR, editor. Food chemistry. New York: Marcel Dekker; 1996. p 618–649.