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Antioxidant effect of FeAOX-6 on free radical species produced during iron catalyzed breakdown of *tert*-butyl hydroperoxide

CRISTINA ROTA[1], ALDO TOMASI[2], PAOLA PALOZZA[3], STEFANO MANFREDINI[4], & ANNA IANNONE[1]

[1]*Department of Biomedical Sciences, University of Modena and Reggio Emilia, Modena, Italy,* [2]*Department of Diagnostic and Laboratory Services, University of Modena and Reggio Emilia, Modena, Italy,* [3]*Institute of General Pathology, Catholic University, Rome, Italy, and* [4]*Department of Pharmaceutical Sciences, Ferrara University, Ferrara, Italy*

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Abstract

There is a body of evidences demonstrating, in biological systems, a cooperative interaction between tocopherols and carotenoids. FeAOX-6 is a novel antioxidant that combines the chroman head of α-tocopherol and a fragment of the isoprenyl chain of lycopene. We have tested its antioxidant effect on different radical species generated in a chemical system, where peroxyl, alkoxyl and methyl radicals are generated by the ferrous ion-mediated decomposition of *tert*-butyl hydroperoxide. We found that FeAOX-6 has the same effectiveness of α-tocopherol in quenching peroxyl radical with no contribution by lycopene. The antioxidant activity of FeAOX-6 on alkoxyl and methyl radicals is comparable to that of the equimolar mixture of the parent compounds. Lycopene is able to quench alkoxyl radical, while it has no effect on peroxyl radical, showing a different antioxidant activity compared to other carotenoids, such as β-carotene and lutein.

Keywords: *Free radicals, FeAOX-6, lycopene, vitamin E, ESR spectroscopy*

Introduction

A growing body of evidences suggests that the combinations of antioxidants with different chemical characteristics may result in a better defense against oxidative stress compared with the individual usage of the same compound. In particular, it has been demonstrated that the existence in biological systems of a cooperative interaction between tocopherols and carotenoids [1–4].

Recently, the novel antioxidant (±)-(E/Z)-2,5,7,8-tetramethyl-2(4,8,12-trimethyl-trideca-1,3,5,7,11-pentaenyl)chroman-6-ol (FeAOX-6), has been synthesized (Figure 1). This compound combines, in the same molecule, the chroman head of α-tocopherol and a fragment of the isoprenyl chain of lycopene, the linear molecule from which all carotenoids derive [5]. It is well known that the chroman moiety of tocopherols is responsible for their antioxidant activity and that the polyunsaturated chain confers to carotenoids their antioxidant characteristics and also has an important role in their distribution in biological systems [5].

FeAOX-6 has a excellent antioxidant effect in biological systems, as demonstrated in experiments where the malondialdehyde production was assayed; also the inhibition of ROS production was tested in chemical and biological systems by using fluorescent probes [5,6].

The aim of this work was to test the ability of FeAOX-6 in quenching different radical species, using ESR spectroscopy combined with the spin trapping technique. This is the only technique that guarantees the direct measurement and identification of free radical species. We have compared the antioxidant effect of FeAOX-6 with those of lycopene and α-tocopherol in a chemical system where the decomposition of *tert*-butyl hydroperoxide (*t*BOOH) was

Correspondence: A. Iannone, Department of Biomedical Sciences, University of Modena and Reggio Emilia, Modena, Italy. Tel: 39 059 2055420. Fax: 39 059 2055426. E-mail: iannone@unimore.it

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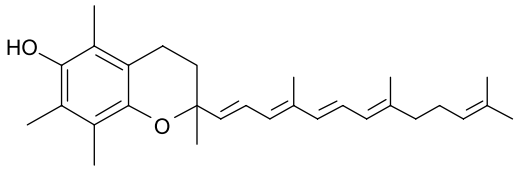

Figure 1. Chemical structure of FeAOX-6, (±)-(E/Z)-2,5,7,8-tetramethyl-2(4,8,12-trimethyl-trideca-1,3,5,7,11-pentaenyl) chroman-6-ol).

catalyzed by ferrous ions. This reaction produces peroxyl radical in the presence of 1 M *t*BOOH in the reaction mixture, while in the presence of 1 mM *t*BOOH the species generated are the alkoxyl and methyl radicals [7].

Materials and methods

Chemicals

Iron(II) sulfate heptahydrate, *t*BOOH (70% aqueous solution), (+) α-tocopherol (VE) and lycopene (Lyc) were purchased from Sigma-Aldrich (Milan, Italy). N-*t*-butyl-α-phenylnitrone (PBN) was purchased from Alexis Italia (Vinci, Florence, Italy). Methylene chloride was purchased from Fluka (Buchs, Switzerland). Hydrochloric acid was purchased from Carlo Erba (Rodano, Milan, Italy). FeAOX-6 was synthetized as previously described [5] and dissolved in DMSO.

Experimental procedures

Reactions were carried out under anaerobic conditions as previously reported [8]: in a eppendorf tube kept under a nitrogen stream; methylene chloride was added to the required amount of *t*BOOH (1.57 M or 1 mM); vitamin E, lycopene or FeAOX-6 were then added at the indicated concentrations. Eighty-five millimolar PBN was added to the system, mixed and the reaction started by adding 0.2 mM $FeSO_4$. Samples were incubated for 1 min, then transferred into a glass capillary tubing (1 mm diameter), that was sealed at both ends with Critoseal, inserted in a 3 mm i.d. quartz tube and immediately run by ESR.

All stock solutions were prepared in methylene chloride, beside $FeSO_4$ solution that was prepared in 0.2 mM HCl. Samples contained DMSO in a percentage that matched that of the corresponding samples containing FeAOX-6: 1% when using 1 mM and 0.015% when using 1.57 M *t*BOOH. The presence of such amounts of DMSO in the reaction mixtures did not show any effect on free radical formation (data not shown).

Electron spin resonance measurements

ESR spectra were recorded using a Bruker EMX spectrometer (Billerica, MA, USA) operating at 9.3 GHz with a modulation frequency of 100 kHz and a TM_{110} cavity. Spectrometer conditions were: modulation amplitude, 1 G; microwave power, 20 mW; time constant, 41 ms; conversion time, 164 ms; scan time, 168 s; scan range, 50 G; and receiver gain, 1×10^5. Spectra were recorded on an IBM-compatible computer, interfaced to the spectrometer. Free radical intermediates were identified by the resulting hyperfine coupling constants of their PBN adduct. Computer simulation was performed using a program that is available through the internet (http://epr.niehs.nih.gov/). The details of the program have been published [9].

Results

When 1.57 M *t*BOOH was reacted with 0.2 mM $FeSO_4$ in the presence of PBN, a mixture of three radical adducts was detected. The corresponding ESR spectrum is shown in Figure 2A; computer simulation (dashed line) is superimposed on the experimental signal. Calculated hyperfine coupling constants of two of the detected PBN radical adducts: *tert*-butylperoxyl, PBN/·$OOC(CH_3)_3$ ($a^N = 13.84$ and $a_\beta^H = 1.52$) (72%) and ·PBNOx ($a^N = 8.12$) (8%), were consistent with values previously obtained using 1 M *t*BOOH [7]. Under present conditions, we have detected an extra radical species that accounts for the 20% of the radical concentration. This radical adduct is characterized by the following hyperfine coupling constants: $a^N = 14.45$, $a_\beta^H = 2.30$, that are consistent with previously obtained values for the methoxyl PBN/·OCH_3 radical adduct [10]. When iron sulfate or *t*BOOH was not present in the reaction mixture only a weak background signal was detected (Figure 2 spectra B and C).

In Figure 3, the antioxidant effect of FeAOX-6 on iron sulfate catalyzed breakdown of 1.57 M *t*BOOH is compared with the effect of α-tochopherol and lycopene. FeAOX-6 shows an inhibitory effect comparable with the one of vitamin E (Figure 3 spectra B and D): 15 μM vitamin E or FeAOX-6 resulted in the detection of a background signal. When using 12 μM vitamin E or FeAOX-6 only a weak signal deriving from the peroxyl radical was detected; the addition of 5 μM vitamin E or FeAOX-6 resulted in almost the 75% reduction of the peroxyl radical signal (data not shown). This demonstrates a dose dependency of the scavenger activity of the two antioxidants. Lycopene, on the other hand, showed a much weaker antioxidant effect (Figure 3 spectrum C), with a still very weak efficacy, even when using concentrations as high as 100 μM (data not shown).

When 1 mM *t*BOOH was reacted with 0.2 mM $FeSO_4$ in the presence of PBN, a mixture of two radical adducts was detected. The corresponding ESR spectrum is shown in Figure 4A, computer simulation (dashed line) is superimposed on the experimental

signal. Calculated hyperfine coupling constants of the detected PBN radical adducts: *tert*-butoxyl, PBN/·OC$(CH_3)_3$ ($a^N = 13.91$ and $a^H_\beta = 2.33$) (27%) and methyl, PBN/·CH_3 ($a^N = 15.18$ and $a^H_\beta = 3.40$) (73%), are consistent with previously obtained values under similar conditions [7].

In Figure 5, the antioxidant effect of FeAOX-6 on iron sulfate catalyzed breakdown of 1 mM *t*BOOH is compared with the effect of α-tocopherol and lycopene. FeAOX-6 showed an inhibitory effect comparable with the one of vitamin E and lycopene (Figure 5 spectra B, D and E): 1 mM vitamin E, lycopene or FeAOX-6 resulted in the disappearance of the alkoyl and the detection of only methyl radical adduct. Also a mixture of 0.5 mM lycopene and 0.5 mM vitamin E abolished the alkoyl free radical (Figure 5 spectrum C), while using 0.5 mM lycopene or 0.5 mM vitamin E alone demonstrated only a partial effect (Figure 5 spectra F and G).

Discussion

In this paper, we have employed the ESR spin trapping technique to compare the antioxidant potential of FeAOX-6 that derives from the assembly of the chroman head of α-tocopherol and a fragment of the polyisoprenyl chain of lycopene.

The antioxidant activity of these compounds has been tested in a chemical model system, where different free radical species are detected when high (1.57 M) or low (1 mM) concentrations of *t*BOOH are reacted with iron sulfate in methylene chloride [7].

When 1.57 M *t*BOOH was reacted with iron sulfate we have detected the *tert*-butylperoxyl $(CH_3)_3COO\cdot$ as major radical species (Figure 2A). This radical species derives from the following reactions:

$$(CH_3)_3COOH + Fe^{++} \rightarrow (CH_3)_3CO\cdot + Fe^{+++} + {}^{-}OH \quad (1)$$

$$(CH_3)_3CO\cdot \rightarrow (CH_3)_2CO + \cdot CH_3 \quad (2)$$

$$(CH_3)_3CO\cdot + (CH_3)_3COOH \rightarrow (CH_3)_3COH + (\mathbf{CH_3})_3\mathbf{COO}\cdot \quad (3)$$

$$\cdot CH_3 + (CH_3)_3COOH \rightarrow CH_4 + (\mathbf{CH_3})_3\mathbf{COO}\cdot \quad (4)$$

Under these conditions we have also detected the methoxyl $\cdot OCH_3$ free radical as minor species (Figure 2A). As reported by Jones and Burkitt [11] *tert*-butylperoxyl radicals easily decompose to *tert*-butoxyl

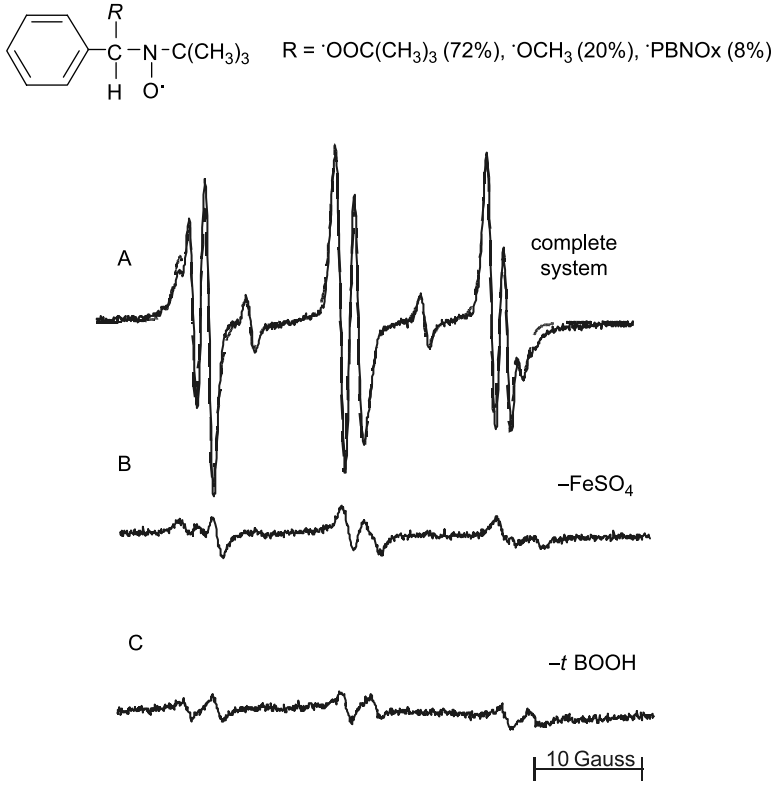

Figure 2. ESR spectra of PBN radical adducts detected during the reaction of 1.57 M *t*BOOH with $FeSO_4$. Spectrum A is the ESR spectrum obtained from a reaction mixture containing 1.57 M *t*BOOH, 85 mM PBN and 0.2 mM $FeSO_4$; computer simulation (dashed line) is superimposed on the experimental signal. Spectrum B was obtained in the absence of iron and Spectrum C was obtained in the absence of *t*BOOH.

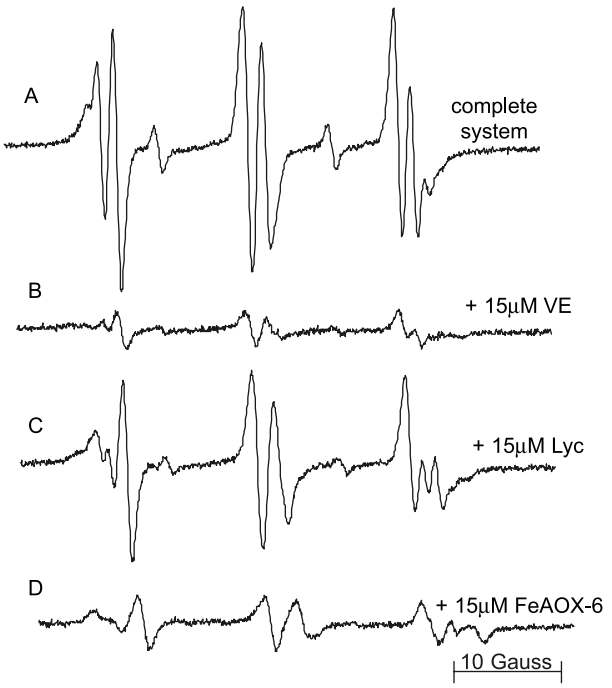

Figure 3. Effect of FeAOX-6, α-tocopherol (VE) and lycopen (Lyc) on the free radical species detected during the reaction of 1.57 M *t*BOOH with $FeSO_4$. Spectrum A is the ESR spectrum obtained from a reaction mixture containing 1.57 M *t*BOOH, 85 mM PBN and 0.2 mM $FeSO_4$. Spectrum B was obtained upon addition of 15 μM VE. Spectrum C derives from the addition of 15 μM Lyc. Spectrum D results from the addition of 15 μM FeAOX.

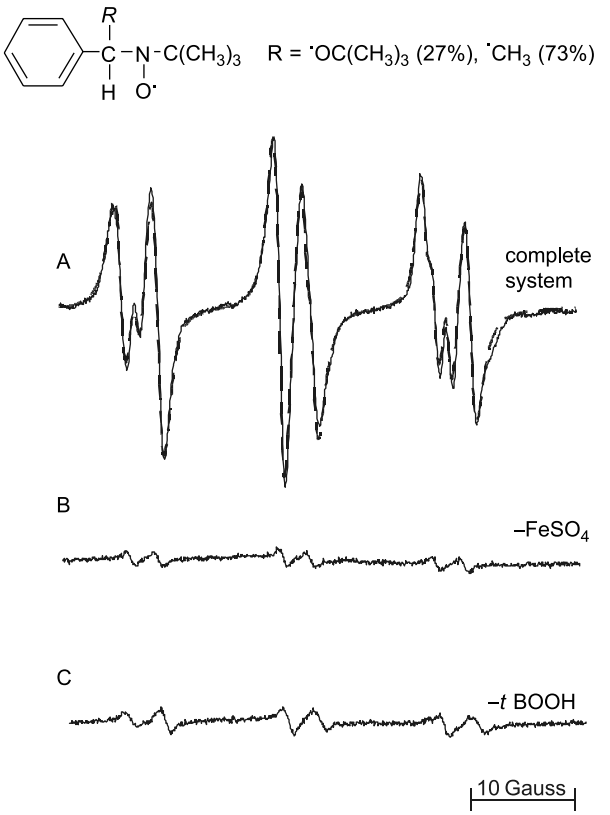

Figure 4. ESR spectra of PBN radical adducts detected during the reaction of 1 mM *t*BOOH with $FeSO_4$. Spectrum A is the ESR spectrum obtained from a reaction mixture containing 1 mM *t*BOOH, 85 mM PBN and 0.2 mM $FeSO_4$; computer simulation (dashed line) is superimposed on the experimental signal. Spectrum B was obtained in the absence of iron and Spectrum C was obtained in the absence of *t*BOOH.

with the release of oxygen:

$$2(CH_3)_3COO\cdot \rightarrow 2(CH_3)_3CO\cdot + O_2 \qquad (5)$$

The oxidation of the methyl radical will produce methylperoxyl radicals:

$$O_2 + \cdot CH_3 \rightarrow H_3COO\cdot \qquad (6)$$

The combination of two methylperoxyl free radicals will then form a tetraoxide which subsequently decomposes producing methoxyl radical species:

$$2H_3COO\cdot \rightarrow H_3COOOOCH_3 \qquad (7)$$

$$H_3COOOOCH_3 \rightarrow 2\cdot\mathbf{OCH_3} + O_2 \qquad (8)$$

FeAOX-6 was able to abolish the formation of both *tert*-butylperoxyl and methoxyl free radicals at the same concentration at which α-tocopherol is effective (15 μM) (Figure 3). Lycopene did not show any effect even when used at very high concentration (see above). These data demonstrate virtually no contribution of lycopene on the antioxidant activity in the present reaction system.

When using a much lower concentration of the hydroperoxide (1 mM), we have detected *tert*-butoxyl, and methyl radical species that are formed during the reactions (1) and (2). FeAOX-6, vitamin E and lycopene showed the same level of efficiency in abolishing the arising of the alkoxyl radical signal in the spectrum: 1 mM concentrations of each of these three antioxidants were effective against the appearance of this radical species (Figure 5), while part of it was still converted to methyl radical as shown in reaction (2). In this case, the combination of 0.5 mM vitamin E and 0.5 mM lycopene was effective as well (Figure 5).

Under our conditions we found that FeAOX-6 is characterized by an antioxidant efficacy comparable with that of α-tocopherol, when in the system under exam is produced mainly peroxyl. Under these conditions lycopene demonstrated almost no effect (Figure 3). On the other hand, when the major species produced is an alkoxyl free radical (Figure 5), the antioxidant efficacy is much lower and comparable for all the three antioxidants examined and also for the equimolar mixture of vitamin E and lycopene. In this work, we have also found that lycopene behaves differently in respect to other carotenoids. In fact, we had previously reported a good antioxidant efficacy of lutein and β-carotene against peroxyl free radical,

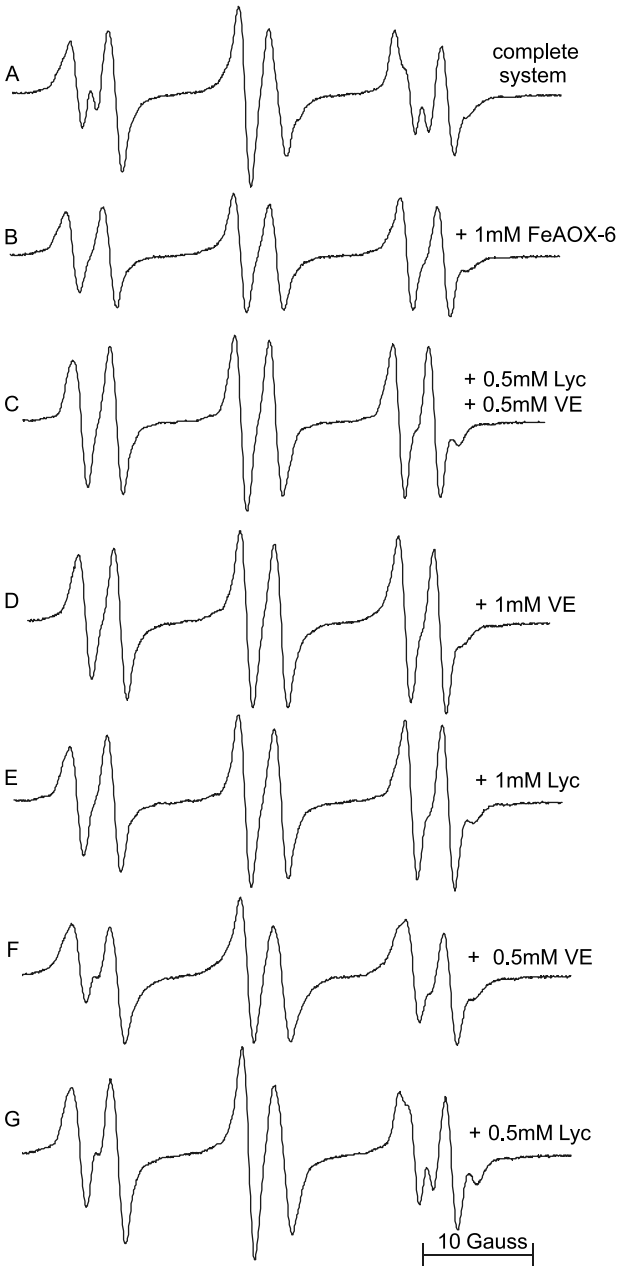

Figure 5. Effect of FeAOX-6, α-tocopherol (VE) and lycopen (Lyc) on the free radical species detected during the reaction of 1 mM *t*BOOH with $FeSO_4$. Spectrum A is the ESR spectrum obtained from a reaction mixture containing 1 mM *t*BOOH, 85 mM PBN and 0.2 mM $FeSO_4$. Spectrum B was obtained upon addition of 1 mM FeAOX-6. Spectrum C derives from the simultaneous addition of 0.5 mM VE and 0.5 mM Lyc. Spectrum D results from the addition of 1 mM VE. Spectrum E derives from the addition of 1 mM Lyc. Spectra F and G were detected after the addition of 0.5 mM VE or Lyc.

generated in the same chemical system here used, and almost no effect against alkoxyl free radical [8]. The differences observed in our chemical system among the antioxidant action of lycopene as compared to lutein and β-carotene are, so far unexplained, but might be reasonably due to the difference in structures. Indeed the linear chain of lycopene, as compared to the cyclic structure of lutein and β-carotene might be responsible of the differences observed in the efficiency of the radical scavenging. In a previous paper, Naguib [12] demonstrated that in the presence of air the relative peroxyl radical scavenging activities of lycopene, beta-carotene and lutein was different, being the lycopene the more effective. Here, we found that lycopene antioxidant activity is dependent on the presence of oxygen, since our experiments have been performed under anaerobic conditions, or in the presence of very low concentrations of oxygen deriving from its release through reactions (5) and (8) (see above).

In conclusion, our results imply that the antioxidant activity of the novel compound FeAOX-6 is mostly due to its ability in quenching peroxyl radicals and that the antioxidant activity of the parent compounds is improved compared to the antioxidant efficacy of lycopene. On the other hand, FeAOx-6 was ineffective in improving the antioxidant activity of the parent compounds against other type of radical species. Lycopene contribution on FeAOX-6 activity might be probably associated with a better distribution in tissues and organs due to its more lipophilic structure. We suppose that the specificity, here demonstrated, of this novel antioxidant in reacting with peroxyl radical consent to get better insight into the mechanistic aspect of these class of antioxidants and offer a better protection against free radical mediated damage in biological systems, compared to other antioxidant molecules. It has been previously demonstrated [5] that FeAOX-6 has a good antioxidant activity in normal mice thymocytes, which is higher than that of α-tocopherol and lycopene. These data suggest the possibility of an enhanced synergistic antioxidant action of the two native antioxidants when incorporated in cellular membranes as FeAOX-6. Toxicity, safety and drug delivery of this novel compound *in vivo*, are currently under investigation.

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