European Journal of Medicinal Chemistry 127 (2017) 740–747

Contents lists available at ScienceDirect

European Journal of Medicinal Chemistry

journal homepage: http://www.elsevier.com/locate/ejmech

Short communication

Determination of the structures of radicals formed in the reaction of antimalarial drug artemisinin with ferrous ions

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ARTICLE INFO

Article history:
Received 7 July 2016
Received in revised form
21 October 2016
Accepted 22 October 2016
Available online 24 October 2016

Keywords:
Artemisinin
Artemether
Artesunate
Antimalarial action by radical
HPLC-ESR-MS

ABSTRACT

While artemisinin **1** has been widely used to treat malaria in traditional Chinese medicine, its exact antimalarial mechanism remains unclear. To elucidate the mechanisms of the antimalarial action by artemisinin, the reactions of artemisinin, artemether **2** and artesunate **3** with Fe^{2+} were analyzed using an electron spin resonance (ESR), high performance liquid chromatography-electron spin resonance (HPLC-ESR) and high performance liquid chromatography-electron spin resonance-mass spectrometer (HPLC-ESR-MS). α-(4-Pyridyl-1-oxide)-*N*-*tert*-butylnitrone (4-POBN) was used as a spin trap reagent in the reactions. Radicals were detected by ESR and structures of the radicals were determined by HPLC-ESR-MS. Based on the ions, *m*/*z* 478, *m*/*z* 418 and *m*/*z* 238 which were determined by HPLC-ESR-MS, we identified following radicals: artemisinin-derived primary and secondary radicals, **6** and **7**; acetyl radical, **8**; a radical formed through elimination of acetyl group from **6, 10** in the reaction of artemisinin with Fe^{2+}. Radicals, **7** and **8** were also detected in the reaction of artemether and artesunate.

1. Introduction

Malaria is caused by a parasite called *Plasmodium* [1], which is transmitted via the bites of infected mosquitoes. In the human body, the parasites multiply in the liver, and subsequently infect the red blood cells. It is one of the most common infectious diseases in the world. According to the latest estimates from the World Health Organization (WHO), there were 214 million new cases of malaria worldwide in 2015 which led to an estimated 438,000 malaria deaths worldwide [2]. WHO recommends artemisinin-based combination therapies (ACT) for the treatment of uncomplicated malaria due to *Plasmodium falciparum* [3,4]. ACT combine an artemisinin **1** derivative such as artemether **2** or artesunate **3** (Fig. 1) with an effective antimalarial medicine [3,5–8].

Artemisinin is extracted from the leaves of the plant *Artemisia annua* (or sweet wormwood), also known as *qinghaosu*. It has been used for over a thousand years in traditional Chinese medicine to treat fever and malaria. Artemisinin has a sesquiterpene lactone bearing an endoperoxide group [9] which is necessary for biological activity [10–12].

The mechanism for the antimalarial activity of artemisinin has been examined using artemisinin and its model compounds, 1,2,4,5-tetraoxane and 1,2,4-trioxolane derivatives [13–17]. The artemisinin, 1,2,4-trioxolane and 1,2,4,5-tetraoxane derivatives contain an endoperoxide bridge, key pharmacophore of artemisinin. Using an oxygen-18-labeled 1,2,4-trioxane derivatives and 4-substituted analogs, it was showed that a C-centered radical forms from an oxy radical via an intermolecular 1,5-hydrogen atom shift in the antimalarial mechanism study [13–15]. On the other hand, heme/artemisinin [16], heme/1,2,4-trioxolanes [17] and heme/1,2,4,5-tetraoxanes [17] adducts were reported, suggesting that artemisinin, 1,2,4-trioxolanes and 1,2,4,5-tetraoxanes form radicals. In addition to heme, 2,2,6,6-tetramethylpiperidine 1-oxyl (TEMPO)/1,2,4,5-tetraoxanes adducts were also reported [17].

Iron ions cause the generation of free radicals from artemisinin which can be directly seen by electron spin resonance (ESR) using the spin trap 5,5-dimethyl-1-pyrroline-*N*-oxide (DMPO) [18]. Furthermore artemisinin (or artemether)-derived primary [19,20] and secondary [21,22] C-centered radicals were directly detected using spin traps 3,5-dibromo-4-nitrosobenzene sulfonate (DBNBS) and 2-methyl-2-nitrosopropane (MNP).

In this paper, several radicals which formed in the reaction of artemisinin (artemether or artesunate) with ferrous ion were detected using spin trapping by ESR. Moreover, the radicals were separated and identified using high performance liquid chromatography-electron spin resonance-mass spectrometer

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http://dx.doi.org/10.1016/j.ejmech.2016.10.053
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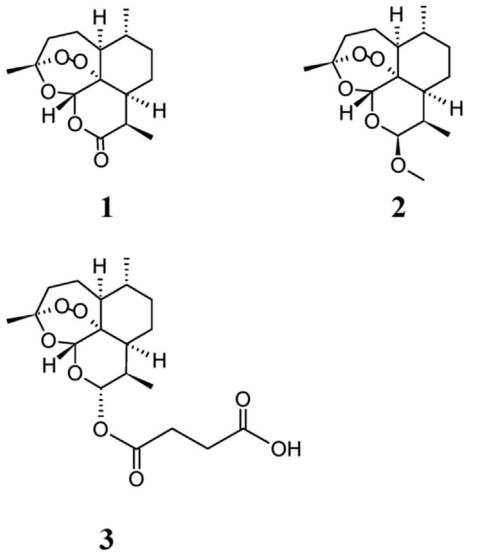

Fig. 1. The structures of artemisinin **1**, artemether **2** and artesunate **3**.

(HPLC-ESR-MS).

2. Results

2.1. ESR measurements of the standard reaction mixture

ESR measurements of the standard reaction mixtures were performed as described in the Experimental section (Fig. 2). Prominent ESR signals were observed for the standard reaction mixture of artemisinin (Fig. 2A). Peak heights of the ESR signals markedly decreased for the standard reaction mixture of artemisinin without Fe^{2+} (Fig. 2B) (6.1% ± 0.5% of the standard reaction mixture of artemisinin, n = 3). Similarly, the ESR signals were hardly observed for the standard reaction mixture without artemisinin (Fig. 2C) (2.6% ± 1.0% of the standard reaction mixture of artemisinin, n = 3). Standard reaction mixtures of artemether and artesunate showed salient ESR signals (Fig. 2D and 2E). Without Fe^{2+}, weak ESR signals were observed for the standard of the reaction mixtures of artemether (11.9% ± 1.2% of the standard reaction mixture of artemether, n = 3) and artesunate (8.3% ± 1.3% of the standard reaction mixture of artesunate, n = 3) (date not shown). The ESR peak heights of the standard reaction mixtures of artemisinin increased in tandem with reaction time (Fig. 3).

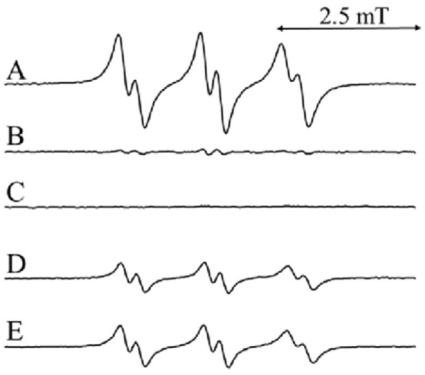

Fig. 2. ESR spectra were measured for the standard reaction mixtures. (A) Standard reaction mixture of artemisinin, (B) standard reaction mixture of artemisinin without Fe^{2+}, (C) standard reaction mixture without artemisinin, (D) standard reaction mixture of artemether and (E) standard reaction mixture of artesunate. The reaction and ESR conditions are described in the Experimental section.

2.2. HPLC-ESR analyses of the standard reaction mixtures of artemisinin, artemether and artesunate using acid solvent systems

Using acid solvent systems, HPLC-ESR analyses of the standard reaction mixture of artemisinin revealed several peaks with retention times of 31.3 min (peak 1), 41.7 min (peak 2), 42.8 min (peak 3), 43.7 min (peak 4) and 45.1 min (peak 5) (Fig. 4A). These peaks (peaks 1–5) were not observed in standard reaction mixtures of artemisinin without Fe^{2+} (Fig. 4B) or without artemisinin (Fig. 4C). Three peaks with retention times of 31.1 min (peak 1), 47.8 min (peak 6) and 48.9 min (peak 7) were observed for the standard reaction mixture of artemether (Fig. 4D). Standard reaction mixture of artesunate showed three HPLC-ESR peaks with retention times of 31.7 min (peak 1), 41.9 min (peak 8) and 42.9 min (peak 9) (Fig. 4E).

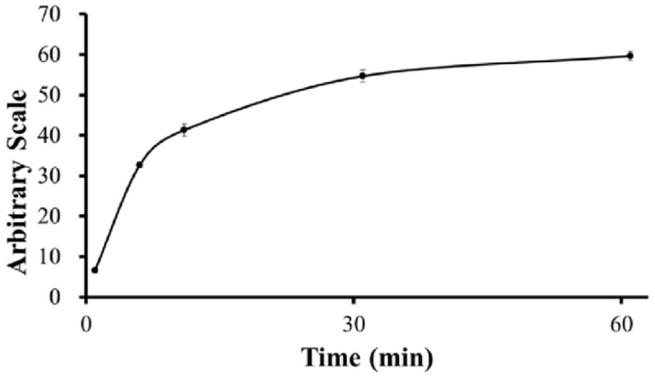

Fig. 3. A time course of the ESR peak heights. ESR spectra were measured for the standard reaction mixture of artemisinin. These were incubated for 1, 6, 11, 31 and 61 min, respectively. The data represent the mean ± SD of independent three measurements. The reaction and ESR conditions are described in the Experimental section except for time constant which was 0.03 s.

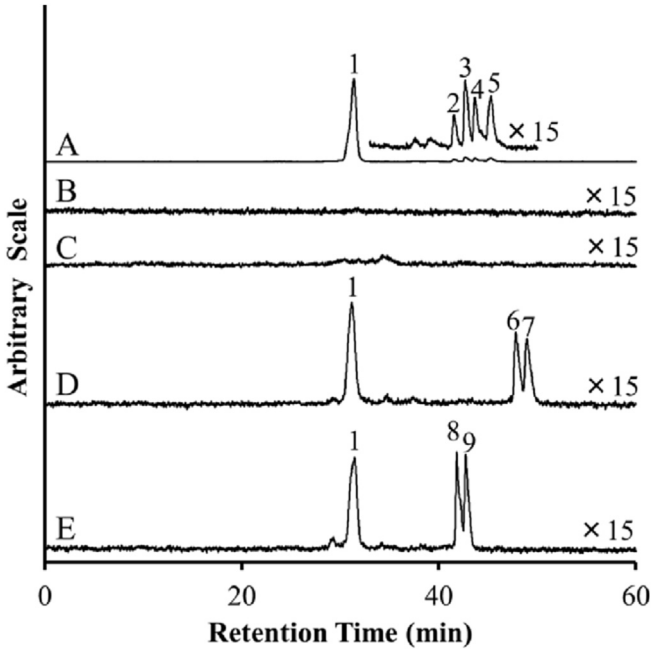

Fig. 4. HPLC-ESR analyses of the standard reaction mixtures using acid solvent systems. HPLC-ESR analyses were performed for (A) standard reaction mixture of artemisinin, (B) standard reaction mixture of artemisinin without Fe^{2+}, (C) standard reaction mixture without artemisinin, (D) standard reaction mixture of artemether and (E) standard reaction mixture of artesunate.