Pharmaceutical Biology

ISSN: 1388-0209 (Print) 1744-5116 (Online) Journal homepage: https://www.tandfonline.com/loi/iphb20

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To cite this article: Igor A. Parshikov, Bruhaspathy Miriyala, Kannoth M. Muraleedharan, Anuradha Illendula, Mitchell A. Avery & John S. Williamson (2005) Biocatalysis of the Antimalarial Artemisinin by *Mucor ramannianus*. Strains, Pharmaceutical Biology, 43:7, 579-582, DOI: 10.1080/13880200500301597

To link to this article: https://doi.org/10.1080/13880200500301597

Pharmaceutical Biology
2005, Vol. 43, No. 7, pp. 579–582

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Biocatalysis of the Antimalarial Artemisinin by *Mucor ramannianus* Strains

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Abstract

Biocatalysis of artemisinin by different strains of *Mucor ramannianus** illustrates a simple approach to bioengineering for attaining useful biotransformation strains for preparative or downstream development. Three different strains of *M. ramannianus* on the malt/sucrose medium were compared for their native ability to metabolize the antimalarial artemisinin and produce significant yields of hydroxylated artemisinin derivatives. The *M. ramannianus* strains transformed the antimalarial into hydroxylated metabolites, in particular 7β-hydroxyartemisinin at 88% yield and 6β-hydroxyartemisinin at 51% yield.

M. ramannianus strains: *M. ramannianus* 1839, University of Wisconsin, *M. ramannianus* ATCC 9624, American Typical Cultures Collection, *M. ramannianus* ATCC MYA-883 (R-56), Jefferson County, Arkansas (American Typical Cultures Collection).

Keywords: Antimalarial drugs, artemisinin, biocatalysis, *Mucor ramannianus*, transformation.

Introduction

Malaria remains one of the deadliest of all infectious diseases, and with today's widespread chloroquine resistance, the need for alternative, inexpensive antimalarial agents is great. Artemisinin (Fig. 1) has been shown to be an effective drug against malarial parasites, but problems associated with its low water solubility greatly limits its clinical use.

For years, biocatalysis with microorganisms has served as an effective means of preparing derivatives and increasing the water solubility of relatively water-insoluble compounds. Our group and others have used microbial transformations as a means of producing an array of hydroxylated artemisinin derivatives that possess increased water solubility characteristics (Abourashed & Hufford, 1999; Fiaux de Medeiros et al., 2002; Khalifa et al. 1995; Zhan et al., 2002a,b). In addition, we have focused on the preparation of certain hydroxylated artemisinin derivatives that can be used as scaffolds for the production of novel semisynthetic antimalarials with increased water solubility and decreased potential for resistance (Parshikov et al., 2004a,b).

Currently, we are using gram quantities of purified hydroxy-artemisinin derivatives as scaffolds for our semisynthetic syntheses. The increased water solubility and antimalarial characteristics of several of these derivatives have required even larger scale biocatalysis and examination of transformation parameters. As a simple alternative to more sophisticated and time-consuming bioengineering techniques to increase transformation efficiency, we chose to examine strains of *Mucor ramannianus* (Mucoraceae) obtained from different environments for their potential to effectively biotransform artemisinin.

Materials and Methods

M. ramannianus strains *M. ramannianus* 1839 (University of Wisconsin) (Khalifa et al., 1995), *M. ramannianus* ATCC 9624 (American Type Culture Collection), and *M. ramannianus* ATCC MYA-883 (R-56) (Jefferson County, Arkansas, USA) (Parshikov et al., 1999 were used). Artemisinin was obtained from Mediplantex Corp. (Hanoi City, Vietnam). Cultures were grown on a sterile

Accepted: July 15, 2005

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DOI: 10.1080/13880200500301597

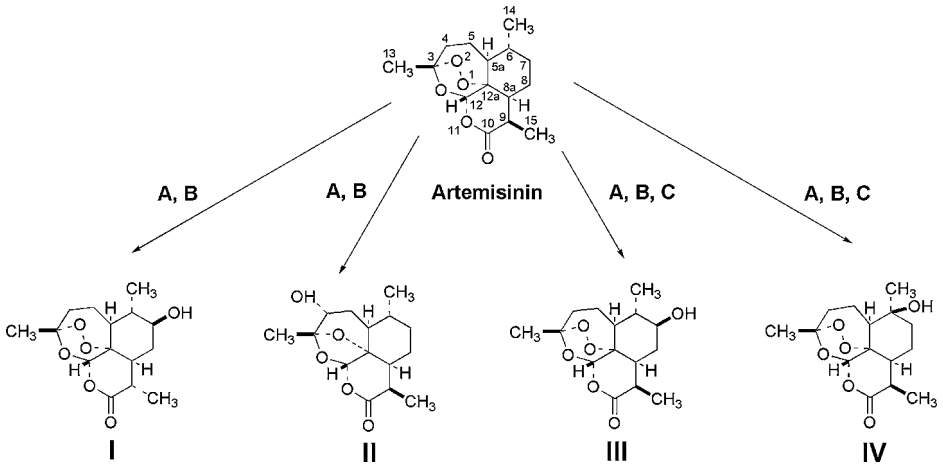

Figure 1. Pathways for the metabolism of artemisinin by *M. ramannianus* ATCC MYA-883 (pathway A), *M. ramannianus* ATCC 9624 (pathway B), and *M. ramannianus* 1839 (pathway C). **I**, 7α-hydroxyartemisinin; **II**, 4α-hydroxy-1-deoxoartemisinin; **III**, 7β-hydroxyartemisinin; **IV**, 6β-hydroxyartemisinin.

complex medium (20 g malt extract, 15 g sucrose, 10 g peptone, and 1000 ml microfiltered, deionized water, pH 6.5) for 48 h, at which point artemisinin (500 mg/l) was added (Parshikov et al., 2004b). Incubation of the artemisinin-fed cultures continued for 14 days or until the substrate was consumed, as evidenced by high performance liquid chromatography (HPLC) analysis. Various control cultures including noninoculated media and cultures that did not receive artemisinin dosing were also examined.

The crude extract was obtained by exhaustive extraction of the fermentation broths with ethyl acetate (EtOAc) and subsequent evaporation. Small samples of the crude extracts of the cultures and controls, extracted, were dissolved in methanol and analyzed by HPLC using evaporative light scattering (PL-ELS1000 Polymer Laboratories, Amherst, MA, USA) as the detection method (Parshikov et al., 2004b). The crude extract bulk containing the transformation products were purified over silica gel (Parshikov et al., 2004a). High-resolution mass spectral (HR-MS) analysis of the resulting metabolites was performed using electrospray positive ionization and nuclear magnetic resonance (NMR) spectrometry, which has been described earlier (Parshikov et al., 2004b).

Results

HPLC analysis of the EtOAc extract of the culture medium from *M. ramannianus* ATCC MYA-883 (R-56) elicited a signal corresponding to a major metabolite

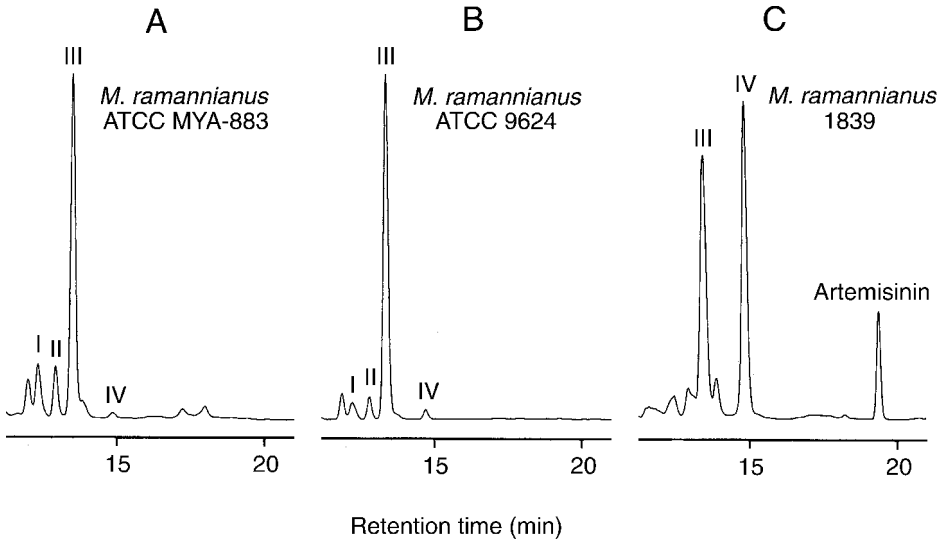

Figure 2. HPLC elution profile showing the biotransformation of artemisinin by: (**A**) *M. ramannianus* ATCC MYA-883, (**B**) *M. ramannianus* ATCC 9624, and (**C**) *M. ramannianus* 1839. **I**, 7α-hydroxyartemisinin; **II**, 4α-hydroxy-1-deoxoartemisinin; **III**, 7β-hydroxyartemisinin; **IV**, 6β-hydroxyartemisinin.

eluting at 13.2 min (78% total peak area as determined by ELSD) and three minor metabolites eluting at 14.6 min (1% total peak area as determined by evaporative light scattering detector (ELSD)), 12.2 min (11% total peak area as determined by ELSD) and 12.6 min (10% total peak area as determined by ELSD; all of the original artemisinin was completely exhausted) (Fig. 2A).

HR-MS and NMR analysis confirmed the major metabolite as 7β-hydroxyartemisinin (**III**) and three minor metabolites as 6β-hydroxyartemisinin (**IV**), 7α-hydroxyartemisinin (**I**) and 4α-hydroxy-1-deoxoartemisinin (**II**) (Fig. 1).

HPLC analysis of the EtOAc extract of the biotransformation medium from *M. ramannianus* ATCC 9624 elicited a signal corresponding to a major metabolite eluting at 13.2 min (88% total peak area as determined by ELSD) and three minor metabolites eluting at 14.6 min (1% total peak area as determined by ELSD), 12.2 min (5% total peak area as determined by ELSD), and 12.6 min (6% total peak area as determined by ELSD; all of the original artemisinin was completely exhausted) (Fig. 2B). HR-MS and NMR analysis confirmed the structure of the major metabolite to be 7β-hydroxyartemisinin (**III**) and three minor metabolites to be 6β-hydroxyartemisinin (**IV**), 7α-hydroxyartemisinin (**I**), and 4α-hydroxy-1-deoxoartemisinin (**II**) (Fig. 1).

HPLC analysis of the EtOAc extract of the biotransformation medium from *M. ramannianus* 1839 elicited a signal corresponding to a major metabolite eluting at 14.6 min (51% total peak area as determined by ELSD) and a minor metabolite eluting at 13.2 min (51% total peak area as determined by ELSD; only 14% [total peak area as determined by ELSD] corresponded to the original artemisinin substrate eluting at 19.1 min) (Fig. 2C). HR-MS and NMR analysis confirmed the structure of the major metabolite (**IV**) to be 6β-hydroxyartemisinin and the minor metabolite to be 7β-hydroxyartemisinin (**III**) (Fig. 1). Comprehensive HR-MS and NMR data have been described previously (Parshikov et al., 2004b).

Discussion

Our increased need for gram quantities of purified hydroxy-artemisinin derivatives, which are being used as scaffolds for syntheses of semisynthetic antimalarial agents, has indicated the need of more effective production methods. As a simple alternative to more sophisticated and time-consuming bioengineering methods, we have chosen to examine three different *M. ramannianus* species to provide us with more effective product yields.

Our selection of *M. ramanninanus* as a model for the variation of catalysis of artemisinin stemmed from our previous experience with the transformation of 10-deoxoartemisinin by *M. ramannianus* 1839 (Fiaux de Medeiros et al., 2002). *M. ramannianus* 1839, *M. ramannianus* ATCC 9624, or *M. ramannianus* ATCC MYA-883 (R-56) had never been reported to transform artemisinin. Previous experiments examining the biocatalytic yields of artemisinin have shown it to be converted to 6β-hydroxyartemisinin by *Cunninghamella echinulata* AS 3.3400 (China General Microbiological Culture Collection Center) (Zhan et al., 2002a) and *Cunninghamella elegans* ATCC 9245 (American Typical Cultures Collection) (Parshikov et al., 2004b); to 4α-hydroxyartemisinin (Zhan et al., 2002b) by *Mucor polymorphosporus* AS 3.3443 (China General Microbiological Culture Collection Center); to 4α-hydroxy-1-deoxoartemisinin by *M. polymorphosporus* (Zhan et al., 2002b) and *Cunninghamella elegans* (Parshikov et al., 2004b); and by *M. polymorphosporus* (Zhan et al., 2002b) and *Cunninghamella elegans* ATCC 9245 (American Typical Cultures Collection) (Parshikov et al., 2004b) to 7β-hydroxyartemisinin.

We report here a yield of 6β-hydroxyartemisinin (51%) with *M. ramannianus* 1839, a yield comparable to that reported earlier (50%) with *C. echinulata* (Zhan et al., 2002a). Nevertheless, our yield of 7β-hydroxyartemisinin using *M. ramannianus* ATCC 9624 (88%) greatly exceeds the previously reported yield of 22% using *M. polymorphosporus* (Zhan et al., 2002b), and even exceeds the 79% yield using *C. elegans* (Parshikov et al., 2004b).

In this investigation, strains of *M. ramannianus* showed their activities at malt/sucrose medium instead of Sabouroad-dextrose broth, where they did not show any activities for artemisinin (Parshikov et al., 2004b).

We have now illustrated that in the case of *M. ramannianus* and the transformation of artemisinin, examination of a strain provides a simple, inexpensive, yet useful approach to increasing biocatalytic efficiencies prior to microbial bioengineering development methods. Using our ability to manipulate different strains of *M. ramannianus*, we are now able to efficiently direct the site of hydroxylation to the 6β and 7β positions on the artemisinin molecule in significant yield. These transformations have allowed us to produce several novel semisynthetic artemisinin derivatives with attractive antimalarial profiles in quantities sufficient to begin pharmacokinetic analyses.

Acknowledgments

This work was supported by the Centers for Disease Control cooperative agreements U50/CCU418839 and UR3/CCU418652.

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