Analytical Biochemistry 482 (2015) 25–31

Contents lists available at ScienceDirect

Analytical Biochemistry

journal homepage: www.elsevier.com/locate/yabio

Biotinylated probes of artemisinin with labeling affinity toward *Trypanosoma brucei brucei* target proteins

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ARTICLE INFO

Article history:
Received 18 January 2015
Received in revised form 18 March 2015
Accepted 8 April 2015
Available online 23 April 2015

Keywords:
Biotinylated probes
Synthesis
Affinity labeling
Trypanosoma
Artemisinin
Target proteins

ABSTRACT

We studied the target proteins of artemisinin in *Trypanosoma brucei brucei* using the affinity-labeling method. We designed and synthesized four biotinylated probes of artemisinin for use as molecular tools. Their in vitro trypanocidal activities (data not shown) proved that they mimicked the biological action of artemisinin. We assessed the chemical stability for all of the probes in the parasite culture medium and lysate using reversed-phase high-performance liquid chromatography (HPLC). After 3-h incubations, the probes remained undecomposed in a range of 40 to 65% in the parasite culture medium, whereas approximately 80% of the probes remained stable in the parasite lysate. Using liquid chromatography mass spectrometry (LC–MS), we demonstrated that, with respect to all of the probes, uptakes into the parasite ranging from 81 to 96% occurred after 30-min incubations. In a competitive binding assay between artemisinin and the four biotinylated probes, we searched for the trypanosomal target protein of artemisinin. Consequently, we observed that only the diazirine-free probe **5** could provide the desired result with high affinity-labeling efficiency. Using the horseradish peroxidase-tagged streptavidin–biotin method, we showed that artemisinin could specifically bind to candidate target proteins of approximately 60, 40, and 39 kDa.

Trypanosomiasis is a reemerging tropical infectious disease that affects both humans and animals. Specialists emphatically point to it as one of the world's most neglected infections [1–4]. In the singular case of the human African trypanosomiasis (nicknamed "sleeping sickness"), medical sources note that 60 million people are afflicted in sub-Saharan countries of high endemicity and/or epidemicity; the incidence rate is approximately 300,000 to 500,000 cases annually, and only 3 to 4 million patients are under medical surveillance [1,2]. In 2009, the World Health Organization (WHO)[2] estimated the prevalence of this disease to be 30,000 to 40,000 cases, but the number of reported cases was less than 10,000. Many cases remain undiagnosed or unreported because of the remoteness and instability of the affected areas, which prevent access by mobile epidemiological surveillance teams [3,5]. The latest WHO report [6] notes that by 2012 the number of reported cases was less than 8000, although the actual number was estimated to be 20,000 because of incomplete surveillance. The causal agent, transmitted through the bite of the tsetse fly, is an obligatory extracellular protozoan parasite of the *Trypanosoma* genus. The most dominant subspecies are *Trypanosoma brucei gambiense*, *Trypanosoma brucei rhodesiense*, and *Trypanosoma brucei brucei*. The three subspecies are morphologically and biochemically indistinguishable [1–7]. Clinically, sleeping sickness develops in the human body following two main stages of infection: the early stage (blood) and the late stage (brain). If it remains untreated, the disease can be fatal. Currently, only four drugs (suramin, pentamidin, melarsoprol, and difluoromethyl ornithine) are available for chemotherapy [1–5] (Fig. 1). A combination therapy of nifurtimox–difluoromethyl ornithine is also being used, although nifurtimox is registered for Chagas disease [3]. However, emerging drug resistance, serious side effects, high toxicity levels, and lengthy parenteral administrations restrict their clinical use [1–5]. Those problems, combined with the lack of a vaccine, prompted us to seek new trypanocidal leads against the parasite from Central African medicinal plants. In the search for naturally occurring trypanocidal principles, we isolated artemisinin [1] (Fig. 2), an endoperoxide-containing sesquiterpene lactone, as a trypanocidal lead compound from the medicinal plant *Artemisia annua* [8]. During the 1970s, the naturally occurring artemisinin was isolated as a promising antimalarial lead compound

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[2] *Abbreviations used:* WHO, World Health Organization; *T. b. brucei*, *Trypanosoma brucei brucei*; HRP, horseradish peroxidase; NMR, nuclear magnetic resonance; HPLC, high-performance liquid chromatography; LC–MS, liquid chromatography–mass spectrometry; UV, ultraviolet; AcOH, acetic acid; EtOH, ethanol; DMSO, dimethyl sulfoxide; MeOH, methanol; CH_3CN, acetonitrile; NP–TBS, Nonidet-P40 Tris-buffered saline; ECL, enhanced chemiluminescence.

http://dx.doi.org/10.1016/j.ab.2015.04.020
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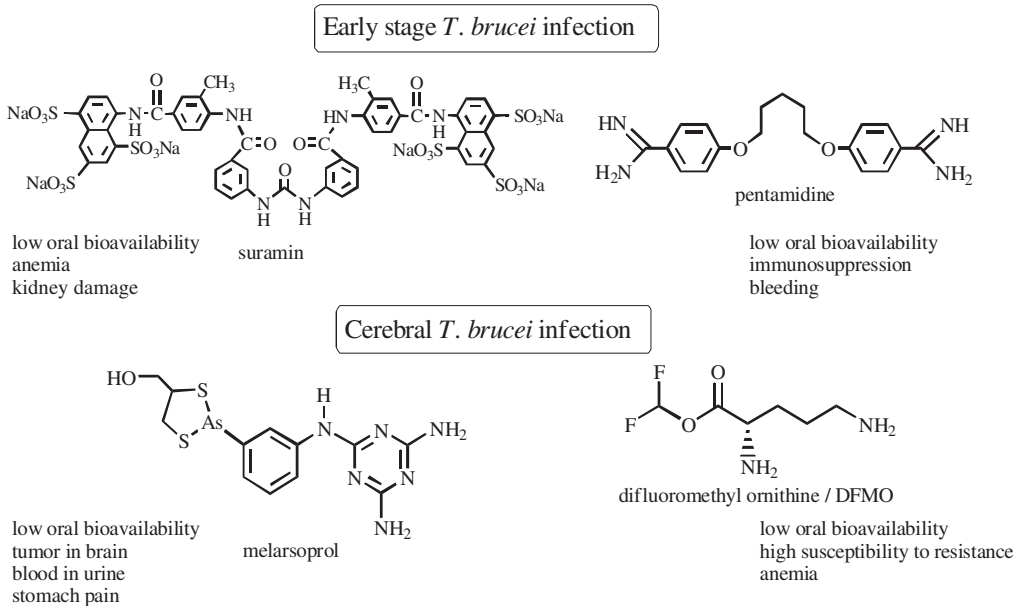

Fig.1. Clinically available trypanocides and relevant problems.

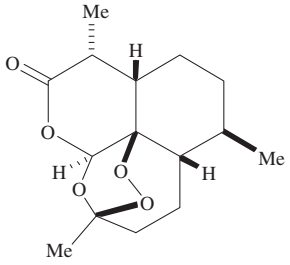

Fig.2. Chemical structure of artemisinin (**1**) from *Artemisia annua*.

from *A. annua* [9,10]. Since then, several of its synthetic congeners have been successfully developed and used in the treatment of malaria cases. However, despite their extensive clinical use for several decades, the precise mechanism through which artemisinin and/or its congeners kills the parasite has remained obscure and controversial. Several years ago, some authors [11–16] demonstrated that artemisinin could specifically recognize a target receptor molecule within the malarial parasite *Plasmodium falciparum*. In line with that novel approach, we explored the mechanism of action of artemisinin within the trypanosomal parasite *Trypanosoma brucei brucei* (*T. b. brucei*) to unravel its target receptor proteins. As a key method for reaching our goals, we designed and synthesized four types of biotinylated probes of artemisinin that were used in the affinity-labeling exploration. Detection via the horseradish peroxidase (HRP)-tagged streptavidin–biotin method allowed us to determine that the three diazirine-tagged probes **2**, **3**, and **4** did not compete with artemisinin for binding to the target proteins, and we were able to prove that the diazirine photophore-free probe **5** and artemisinin competed to specifically bind to the 60-, 40-, and 39-kDa target proteins. This report demonstrates that the mechanism of action of artemisinin in *T. b. brucei* involves its binding to specific target receptor proteins.

Materials and methods

General

^{1}H NMR (nuclear magnetic resonance) and ^{13}C NMR spectra in $CDCl_3$ or CD_3OD with tetramethylsilane (TMS) as the internal standard were recorded using a JNM-GX-500 or Lambda 500 NMR spectrometer (JEOL, Tokyo, Japan) operating at 500 or 125 MHz, respectively. Two-dimensional NMR data in $CDCl_3$ were recorded using a Varian Inova 600 NMR spectrometer (Varian, Tokyo, Japan) operating at 600 MHz. Fast atom bombardment (FAB) and high-resolution fast atom bombardment (HR–FAB) mass spectra were recorded with a JMS SX-102 spectrometer (JEOL) in positive ion mode using magic bullet (5:1 dithiothreitol/dithioerythritol, Tokyo Kasei Kogyo) or *m*-nitrobenzyl alcohol as the matrix. For column chromatography, silica gel (Fuji Sylisia BW-200 or Merck 60-230 mesh) and octadecyl silane (ODS, Cosmosil 75C_{18} OPN, Nacalai Tesque) were used. An HPLC Pump (model 576, GL Sciences, Tokyo, Japan) and a Shodex RI-71 refractive index detector (Shoko, Tokyo, Japan) were used for performing normal-phase high-performance liquid chromatography (HPLC). A JASCO MD-2010 Plus Multiwavelength Detector, a JASCO PU-2080 Plus Intelligent HPLC Pump, and a JASCO AS-2057 Plus Intelligent Sampler (JASCO, Tokyo, Japan) were used for performing reversed-phase HPLC. A Waters 2695 Separations Module, a Waters 2996 Photodiode Array Detector, and a MICROMASS Quattro Micro API mass spectrometer (Nihon Waters, Osaka, Japan) were used to perform liquid chromatography–mass spectrometry (LC–MS). Chemical reactions were performed under Ar gas unless otherwise indicated. Thin-layer chromatography analyses were performed using normal-phase precoated plates (Kiesel gel 60F_{254}, Merck) and reversed-phase high-performance thin-layer chromatography (HPTLC) plates (RP-18 WF_{254S}, Merck). The spots on the thin-layer chromatograms were detected under ultraviolet (UV) light at 254 and 366 nm and were visualized with either *p*-anisaldehyde/H_2SO_4 (5 ml of acetic acid (AcOH), 25 ml of *c*-H_2SO_4, 425 ml of ethanol (EtOH), and 25 ml of water) or phosphomolybdic acid (5 g in 100 ml of EtOH) spraying reagents and subsequent heating. For in vitro experiments, stock solutions of samples were prepared in dimethyl sulfoxide (DMSO), the concentration of which never exceeded 1% in culture medium unless otherwise noted. Pentamidine (Sigma–Aldrich) was used as a positive control.

Trypanosome stocks

A culture suspension of *T. b. brucei* was obtained from the Research Institute for Microbial Diseases (Osaka University,