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ORIGINAL ARTICLE

Takuji Nakashima · Yoko Kato · Kenichi Yamaguchi
Tatsuya Oda

Evaluation of the anti-*Trichophyton* activity of a prodigiosin analogue produced by γ-*proteobacterium*, using stratum corneum epidermis of the Yucatan micropig

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Abstract Prodigiosins (PGs) are known to be a family of natural red pigments, characterized by a common pyrrolydipyrrolylmethane skeleton structure with a C-4 methoxy group, and some of these pigments have been isolated from some microorganisms. Members of the PG family have been reported to show several biological activities, such as immunosuppressive and cytotoxic activities. Recently, we discovered a bacterial strain (MS-02-063), from our microbial library, that produces large amounts of a PG analogue (PG-L-1). In this study, we examined the anti-*Trichophyton* activity of PG-L-1 (produced by strain MS-02-063) against clinically isolated *Trichophyton* spp., by a method using stratum corneum epidermis (SCE) of the Yucatan micropig, which is suitable for estimating the antifungal activity of drugs in vitro. In the National Committee for Clinical Laboratory Standards (NCCLS) method, PG-L-1 showed potent antifungal activity against nine clinically isolated strains of *Trichophyton* spp., although the minimum inhibitory concentration (MIC) values were slightly higher than those of bifonazole. In spite of the lower efficiency of PG-L-1 transfer into SCE from medium than that of bifonazole, PG-L-1 transferred into SCE showed more potent antifungal activity than bifonazole, at lower concentrations.

Key words Prodigiosin · Antifungal activity · Susceptibility · Stratum corneum · γ-*proteobacterium*

T. Nakashima · K. Yamaguchi · T. Oda
Division of Biochemistry, Faculty of Fisheries, Nagasaki University, Nagasaki, Japan

T. Nakashima (✉) · Y. Kato
Nagasaki Prefecture Collaboration of Regional Entities for the Advancement of Technological Excellence, JST, 2-1308-8 Ikeda Omura, Nagasaki 856-0026, Japan
Tel. +81-95-819-2831; Fax +81-95-819-2831
e-mail: takuji.nakashima@nifty.com

Introduction

A number of topical antifungal agents are used in the clinical treatment of superficial cutaneous mycoses. Especially, azole antifungal agents (e.g., bifonazole)[1] and allylamine antifungals (e.g., terbinafine)[2] are very widely used as therapeutic agents for dermatophyte infections. The efficacy of antifungal agents is primarily evaluated by in vitro antifungal susceptibility tests. However, the minimum inhibitory concentration (MIC) is influenced by testing conditions such as pH, inoculum size, incubation time, incubation temperature, and the composition of the medium.[3–6] Therefore, the clinical efficacy of certain antifungal agents cannot be predicted from the results of the antifungal susceptibility tests against dermatophytes. To estimate the therapeutic efficacy of an agent, its pharmacokinetics and antifungal activities in the stratum corneum are especially important.[7,8]

To further evaluate the therapeutic efficacy of topical antifungal agents, experimental animal models, such as guinea pigs infected with *Trichophyton mentagrophytes* on the back and foot, have been widely used.[9–12] In contrast to the in vitro susceptibility tests, these model systems are thought to be useful to assess clinical efficacy, and are assumed to be suitable for experiments related to treatment with antifungal agents.[13] We have recently established a new susceptibility method, in which the stratum corneum of the Yucatan micropig was used. This method is simpler and easier than methods involving experimentally infected animal model systems, and the results are in agreement with the results of the antifungal activities of certain agents in vivo.[14]

Recently, from our marine bacteria library including approximately 20000 isolates, we found a bacterial strain that produced large amounts of a red pigment with antifungal activity. In this study, we report that this red pigment showed potent antifungal activity, when examined by a method using the stratum corneum of the Yucatan micropig noted above.

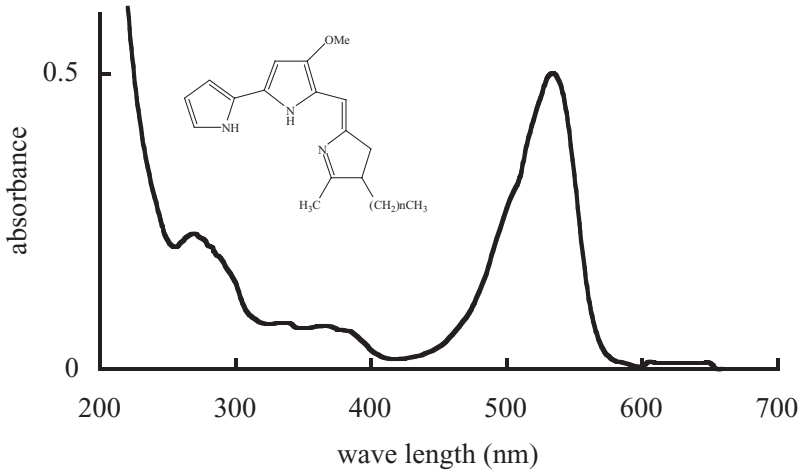

Fig. 1. Absorption spectrum of the purified preparation of prodigiosin analogue (PG)-L-1, and basal backbone structure of prodigiosin (prodigiosin; $n = 4$). The pigment showed maximal absorption at 533 nm

Materials and methods

Strains

A bacterial strain (MS-02-063) producing an antifungal compound was isolated from the soil of a coastal area of Omura Bay, Nagasaki, Japan. The 16S rDNA gene sequence of strain MS-02-063 showed 99.6% similarity with γ-*proteobacteria Hahella* sp. MBIC 3957.[15] *T. mentagrophytes* and *T. rubrum* isolated from patients with tinea pedis were kindly provided by Honma Clinic, Nagasaki, Japan, and used for susceptibility tests.

Antifungal agents

Purification of the antifungal compound from strain MS-02-063 was conducted by the following procedures; strain MS-02-063 was cultivated in 100-ml yeast extract-peptone-glucose (YPG) broth (which consisted of 12.5 g yeast extract, 12.5 g peptone, and 30 g glucose in 1 l of 50% artificial seawater) at 28°C for 48 h. An equal volume of methanol was added to the culture broth, and the mixture was vigorously stirred, and then centrifuged at 10 000 *g* for 30 min. The supernatant was evaporated under a vacuum. The dried residue was extracted with chloroform. The residue was washed several times with the same solvent and combined with the first extract. The extracted fraction was centrifuged at 10 000 *g* for 10 min to remove insoluble materials. The supernatant was evaporated under a vacuum, and then the dried residue was dissolved in methanol again. The methanol solution was applied to a prepared silica-gel thin layer chromatograph (TLC), and then developed with methanol/chloroform/ethyl acetate (5:30:65). The red-colored band with antifungal activity (TLC-Rf = 0.61) was collected and extracted with methanol. The extracted sample was then applied to gel filtration on Sephadex LH-20 (Pharmacia, Uppsala, Sweden), using methanol as the eluting solvent. The absorption spectrum of the purified antifungal agent from strain MS-02-063 was measured on a U-2001 spectrophotometer (Hitachi Instruments, Tokyo, Japan) over the range of 200 nm to 700 nm. Based on the absorption spectrum of the red pigment with antifungal activity, it was suggested that the red pigment might be a prodigiosin (PG) analogue, with maximal absorbance at 533 nm (Fig. 1).[16–18] Thus, we named it PG-L-1. Because bifonazole is frequently used as the reference standard drug in double-blind comparison studies to develop clinically effective antifungal agents, bifonazole, purchased from Sigma-Aldrich (St. Louis, MO, USA) was used as the reference antifungal agent.

Susceptibility tests

The minimum inhibitory concentrations (MICs) of antifungal agents were determined for nine clinical isolates of *Trichophyton* spp. by a National Committee for Clinical Laboratory Standards (NCCLS) standard method, as previously described.[19] These *Trichophyton* spp. were grown on a Sabouraud's dextrose agar (Becton Dickinson, Franklin Lakes, NJ, USA) slant at 28°C for 2 weeks, and a conidial suspension of the dermatophytes was prepared in sterile physiological saline containing 0.05% (w/v) Tween 80. Following filtration through sterilized gauze to remove hyphal fragments and agar blocks, experimental conidial suspensions for susceptibility testing and the stratum corneum epidermis (SCE) study were adjusted to concentrations of 2.5×10^4 and 10^8 conidia/ml, respectively. Dilutions of conidial suspensions were made in RPMI 1640 medium containing 10 mM 3-morpholinopropanesulfonic acid (MOPS). The MIC for *Trichophyton* spp. isolates in clinical samples was determined by a microdilution technique, with RPMI 1640 medium, in 96-well flat-bottomed microdilution plates (Sumitomo Bakelite, Tokyo, Japan). Twofold concentrations were dispensed in 100 µl-volumes, using a multichannel pipette, into each well of rows 1 to 15 of the plate. That is, 10-µl volumes of PG-L-1 and bifonazole, at 0.12–2000 µg/ml, dissolved with dimethyl sulfoxide (DMSO), were added to 90 µl of RPMI 1640 medium containing MOPS. Row-1 wells received 100-µl volumes of antifungal agent at the highest concentration, and row 15 wells