A New Assay Method for Immunosuppressants with a Tacrolimus (FK506)-Like Mode of Action

KAZUTOSHI SAKAMOTO, SHIZUE IZUMI, MICHIYO MIYAUCHI and MASAKUNI OKUHARA

Exploratory Research Laboratories, Fujisawa Pharmaceutical Co., Ltd., 5-2-3 Tokodai, Tsukuba, Ibaraki 300-26, Japan

(Received for publication February 27, 1995)

In March 1984 we isolated a potent immunosuppressant, tacrolimus (formerly designated as FK506), from the culture broth of an actinomycete, *Streptomyces tukubaensis* No.9993[1,2]. Since its discovery, tacrolimus has attracted considerable interest because of its unique profile of action and remarkable efficacy in animal models of transplantation and autoimmune disease. Tacrolimus has recently been marketed clinically for suppression of tissue rejection following organ transplantation in Japan (liver and bone marrow), the United States (liver) and the United Kingdom (liver and kidney).

As the clinical importance of tacrolimus became clear, the discovery of new immunosuppressants with a similar immunological profile has been important. With the aim of establishing new screening assays for such microbial products, we extensively investigated the biological effects of tacrolimus on microbial cells. During this study, we made an interesting observation: Tacrolimus showed a characteristic effect on the morphology of several tacrolimus-sensitive fungi, such as *Aspergillus niger*, *Fusarium oxyporum*, and *Geotrichum candium*. Surprisingly, cyclosporin A (CsA), a fungal product with a immunosuppressive profile similar to tacrolimus, caused similar morphological effects which included deformation and branching of the hyphal tips. However, rapamycin (RAP), a macrolide immunosuppressant which has a similar chemical structure but different mode of action from tacrolimus, was inactive. Furthermore, other antifungal antibiotics devoid of immunosuppressive activity such as cycloheximide and amphotericin B did not exhibit activity (Fig. 1). Since tacrolimus analogs, including FR900520 and FR900512, also share this common trait of causing a characteristic fungal shape change (data not shown), we hypothesized that the morphological response caused by tacrolimus and CsA might be closely associated with their common immunosuppressive mechanism, involving complex formation with their respective binding proteins (immunophilins) which lead to inhibition of calcineurin[3]. Based on this working hypothesis, we developed a screening assay to examine immunosuppressants with a similar mechanisms of action as tacrolimus and CsA.

Preparation of Agar Plates for Screening

A loopful conidia of *Aspergillus niger* IFO 4417, grown on an agar slant, was inoculated on CM agar slants (1.5% soluble starch, 0.5% glucose, 0.5% yeast extract, 0.1% KH_2PO_4, 0.2% $NaNO_3$, 0.02% $MgSO_4 \cdot 7H_2O$, 0.02%, KCl and 2% agar, pH 6.5). After 4 days growth at 25°C, conidia were harvested with 4.5 ml of 0.9% saline and filtered through sterile filter paper. One half ml of the conidial solution containing approximately 1×10^7 conidia were suspended in 100 ml of Eiken sensitivity test agar medium (Eiken agar medium, Eiken Chemical Co., Ltd.) which had been previously prepared as follow: Nineteen grams of Eiken agar medium was dissolved in 1 liter of distilled water and autoclaved at 121°C for 15 minutes, then was cooled to and kept at 43°C. The agar plates for screening were prepared by dispensing 10 ml of the Eiken agar medium containing conidia into a 150-mm diameter petri dish.

Screening Procedure

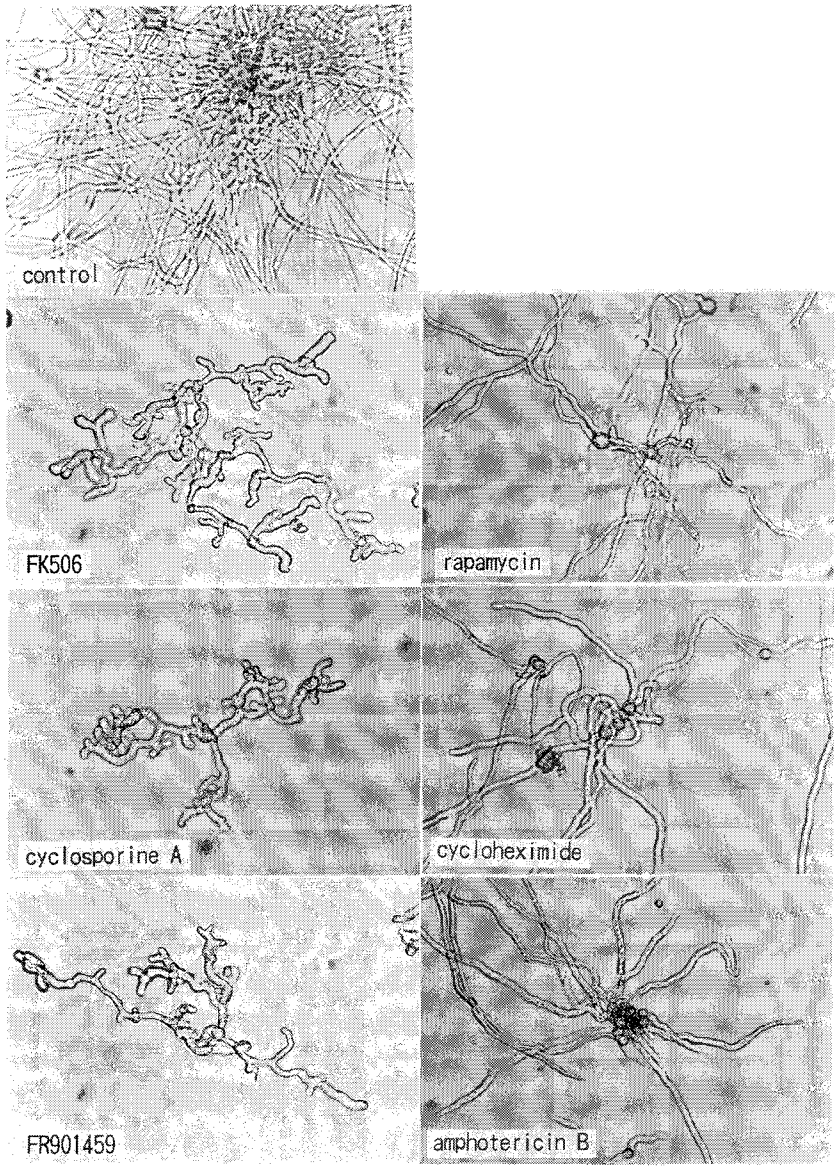
Paper discs dipped in screening samples prepared from microbial extracts were applied to an Eiken agar plate seeded with the assay fungus, and was incubated at 30°C for 16 hours. The cell morphology of the fungus in the inhibitory zones which appeared around the paper discs was examined under a light microscope at magnification of $\times 400$. Microbial extracts which produced morphological change in the fungal strain were scored as active.

Results and Discussion

Using this assay method, which is suitable for high throughput natural product screening, several thousand microbial extracts were screened, and as a result, a fungal extract was found to have the above mentioned activity. The active principle FR901459, from a strain which was identified as *Stachybotorys chartarum*, was isolated[4]. The chemical structure of FR901459 was determined and found to be a member of the cyclosporin family. A major goal of this screening program has been to detect immunosuppressants more potent and less toxic than CsA or tacrolimus. However, FR901459 was approximately one third the potency of CsA, though its nephrotoxicity was also 3 times less toxic than CsA. Screens which rely on the induction of morphological changes in test organisms, such as formation of protoplasts, bulges or swellings, have been extensively performed in the area of antimicrobial product discovery[5,6]. Because of its mechanism-based screening, bacterial or fungal protoplasting screens are rational and have been successful. The rationale for this immunosuppressant screen based on the alterations in fungal morphology is purely hypothetical at present. However, the fact that this screening program resulted in the discovery of a new

This paper is dedicated to Professor SATOSHI ŌMURA in honor of his 60th birthday.

Fig. 1. Morphological changes of *Aspergillus niger* induced by various anti-fungal agents.



immunosuppressant, FR901459, along with the fact that known immunosuppressants, such as tacrolimus and CsA were detected at a high rate while other antifungals were not might suggest its rationality.

The complex of tacrolimus or CsA with their respective binding proteins, FKBP or cyclophilin (CyP), inhibits the Ca^{2+}- and calmodulin-dependent protein phosphatase, calcineurin. The inhibition of calcineurin results in blocking the intracellular signal transduction in T cells. The formation of a complex of these immunosuppressants and their binding proteins has been shown to be closely associated with growth inhibition of certain fungi. TROPSCHUG and coworkers isolated CsA-resistant mutants in strains of *Neurospora crassa* and *Saccharomyces cerevisiae*, and demonstrated that these resistant mutants either completely lost CyP or contained an altered CyP lacking its CsA-binding ability[7]. Based on these data, they speculated that the CsA-CyP complex must interact with another cellular component and that this interaction resulted in lethal consequences for the organisms. More recently, a mutant of *Saccharomyces cerevisiae* which was hypersensitive to tacrolimus and CsA has been described[8]. The mutant fks1 is considered to require calcineurin for growth because simultaneous disruption of fks1's two genes that encode calcineurin, CNA1 and CNA2, resulted in the loss of hypersensitivity to tacrolimus and CsA. The minimum concentrations of tacrolimus and CsA which caused the morphological changes in *Aspergillus* were 10 ng/ml and 10 μg/ml, respectively. This is almost the same as the minimum inhibitory concentrations (MICs) of these drugs against fks1 and is similar to the nanomoler concentrations which inhibit T cells. This fact strongly suggests that tacrolimus-FKBP or CsA-CyP complexes formed in *Aspergillus* caused the morphological effects.

References

1) KINO, T.; H. HATANAKA, M. HASHIMOTO, M. NISHIYAMA, T. GOTO, M. OKUHARA, M. KOHSAKA, H. AOKI & H. IMANAKA: FK-506, a novel immunosuppressant isolated from a *Streptomyces*. I. Fermentation, isolation, and physico-chemical and biological characteristics. J. Antibiotics 40: 1249～1255, 1987
2) KINO, T.; H. HATANAKA, S. MIYATA, N. INAMURA, M. NISHIYAMA, T. YAJIMA, T. GOTO, M. OKUHARA, M. KOHSAKA, H. AOKI & T. OCHIAI: FK-506, a novel immunosuppressant isolated from a *Streptomyces*. II. Immunosuppressive effect of FK-506 *in vitro*. J. Antibiotics 40: 1256～1265, 1987
3) LIU, J.; J. D. FARMER, Jr., W. S. LANE, J. FRIEDMAN, I. WEISSMAN & S. L. SCHREIBER: Calcineurin is a common target of cyclophilin-cyclosporin A and FKBP-FK506 complexes. Cell 66: 807～815, 1991
4) SAKAMOTO, K.; E. TSUJII, M. MIYAUCHI, T. NAKANISHI, M. YAMASHITA, N. SHIGEMATSU, T. TADA, S. IZUMI & M. OKUHARA: FR901459, a novel immunosuppressant isolated from *Stachybotrys chartarum* No. 19392. Taxonomy of the producing organism, fermentation, isolation, physico-chemical properties and biological activities. J. Antibiotics 46: 1788～1798, 1993
5) OKUHARA, M. & T. GOTO: New phosphonic acid antibiotics produced by strains of *Streptomyces*. Drugs Exptl. Clin. Res. 7: 559～564, 1981
6) GUNJI, S.; K. ARIMA & T. BEPPU: Screening of antifungal antibiotics according to activities inducing morphological abnormalities. Agric. Biol. Chem. 47: 2061～2069, 1983
7) TROPSCHUG, M.; I. B. BARTHELMESS & W. NEUPERT: Sensitivity to cyclosporin A is mediated by cyclophilin in *Neurospora crassa* and *Saccharomyces cerevisiae*. Nature 342: 953～955, 1989
8) PARENT, S. A.; J. B. NIELSEN, N. MORIN, G. CHREBET, N. RAMADAN, A. M. DAHL, M. HSU, K. A. BOSTIAN & F. FOOR: Calcineurin-dependent growth of an FK506- and CsA-hypersensitive mutant of *Saccharomyces cerevisiae*. J. Gen. Microbiol. 139: 2973～2984, 1993