Vol. 202, No. 1, 1994 BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS
July 15, 1994 Pages 437-443

INTERACTION OF TACROLIMUS(FK506) AND ITS METABOLITES WITH FKBP AND CALCINEURIN

K.Tamura, T.Fujimura, K.Iwasaki, S.Sakuma, T.Fujitsu, K.Nakamura, K.Shimomura, T.Kuno *, C.Tanaka * and M.Kobayashi

Research Laboratories, Fujisawa Pharmaceutical Co., Ltd., 2-1-6 Kashima, Yodogawa-Ku, Osaka 532, Japan

* Kobe University, School of Medicine, Kusunoki-Cho, Chuo-Ku, Kobe 655, Japan

Received May 28, 1994

Summary. Tacrolimus(FK506) is a strong immuno-suppressant and shows its activity through inhibiting IL-2 mRNA transcription by forming pentameric complex with intracellular receptor(FK506 binding protein 12 KDa or FKBP12), Ca^{2+}, calmodulin, and calcineurin. Here, we report the binding activity to FKBP12, the pentameric complex formation and Con-A response inhibiting activities of 7 metabolites. C15-demethylated metabolite(M-3) needed higher quantity to compete in Con-A assay and in pentamer formation assay, although it binds more strongly to FKBP12. The result suggests that the ability to form a pentameric complex is not a two step reaction with the first binding to FKBP12, but a single step reaction by components for the pentamer formation. © 1994 Academic Press, Inc.

Tacrolimus, FK506, is a 23-membered ring macrolide with a strong immuno-suppressive activity(1) and has been widely used for suppression of transplanted organ rejection and of GVHD, and for treatment of auto-immunediseases. By the intensive work on the functional mechanism of FK506 by Schreiber and Crabtree(2), it has become clear that FK506 shows its immuno-suppressive activity by first binding to its intracellular receptor, FKBP12, one of FK506 binding proteins, which gains new functional activity of binding Ca^{2+}/calmodulin dependent serine-threonine phosphatase, calcineurin, and inhibits its enzyme activity, essential to form active NFAT(nuclear factor of activated T-cells) leading to IL-2 mRNA transcription(2).

From the three dimensional structure of FK506/FKBP12 complex, C15-OMe and C21-allyl groups of FK506 are considered as the binding sites for calcineurin(3). Recently, 7 FK506 metabolites have been isolated and chemically identified(4, 5) in in-vitro metabolizing system, whose concentration in blood and its relation to patients' condition have not yet been reported. They also reported the biological and immuno-cross reactivities of those metabolites.

0006-291X/94 $5.00
Copyright © 1994 by Academic Press, Inc.
All rights of reproduction in any form reserved.

We were interested in looking at how this difference in biological activities could be attributed; at the step of binding to FKBP12 or at the step of pentamer formation with calcineurin? We found that the pentamer formation is not a two step reaction first with FKBP12 followed by calcineurin association, but a single step reaction by all participating components of FK506/FKBP12/Ca^{2+} /calmodulin and calcineurin.

Materials and Methods

Materials. Bovine calcineurin was purified according to the method by Tallant et al(6) by Dr. Okuhara of Fujisawa Pharmaceutocal Co. Exploratory Res. Labs. and kindly provided for this research work. Preparations of FK506 metabolites(4, 5) and rabbit polyclonal antibody specific to bovine calcineurin A α (7) have been reported. Alkalinephosphatase (ALP)-labelled anti-rabbit IgG was purchased from VECTOR Labs.(Burlingame, CA, USA), and 4-methylumberiferyl phosphate, calmodulin, and Con-A were from Sigma Chemicals(St. Lous, MO, USA). ^{3}H-FK506 was an order-made and has a specific radioactivity of 1.86 TBq/mmole. ^{3}H-thymidine(3.0 TBq/mmole) was purchased from Amersham Japan (Tokyo,Japan). Other reagents are of the best quality available on the market. FK506 metabolites tested in this report are C13-demethyl-FK506(M-1), C31-demethyl-FK506(M-2), C15-demethyl-FK506(M-3), C12-hydroxyl-recyclized-FK506(M-4), C13,C31-didemethyl-FK506(M-5), C13,C15-didemethyl-FK506(M-6), and C15,C31-didemethyl-FK506(M-7). The concentration of those metabolites was calculated from HPLC-UV method assuming that the molar absorption coefficient of those metabolites is the same as FK506.

Binding Assay to FKBP12. Modified binding assay to FKBP12 was established, where bound/free separation was made by dextran charcoal adsorption rather than using gel-filtration over Sephadex LH-20(8, 9). Briefly, serial dilution of FK506 or the metabolites in 50 mM phosphate buffer(pH 7.5) containing 0.5 % BSA(the assay buffer A; 100 μ l) was mixed in siliconized polypropylene tube with ^{3}H-FK506(55 TBq/ml x 100 μ l), 100 μ l of recombinant human FKBP12(400 ng/ml in the assay buffer A) and 200 μ l of the assay buffer A, and incubated at room termperature for 4 hr. After the incubation, 0.5 ml of dextran charcol solution was added, mixed and kept for 10 min at room temperature. Bound ^{3}H-FK506 to human FKBP12 and free ^{3}H-FK506 were separated by centrifugation(3000 rpm x 10 min). Radio-activity in 200 μ l of the supernatant(bound form) was counted in Aquasol solution by scintilation counter. Relative binding activity of the metabolites to FK506 was calculated.

Pentamer Formation Assay. Pentamer formation assay by Asami et al(10) was modified for ELISA. Briefly, recombinant human FKBP12(rh-FKBP12) was coated to the solid surface of immuno-microtiter plate(Nunc Maxisorp) by incubating recombinant human FKBP12 solution in PBS(50 μ g/ml, 100 μ l) at room temperature for 2 hr. The residual binding sites were blocked by incubating the plate with 300 μ l of 50 mM Tris · HCl(pH 7.5) containing 0.5 % BSA, 0.1 % Triton X-100, 0.5 mM DTT and 1 mM $CaCl_2$(the assay buffer B) for 30 min at room temperature, and the solution was removed by aspiration. The plate was incubated at 37 ℃ for 1 hr with a serial dilution of FK506 or the metabolites(0 ~ 1000 ng/ml, 100 μ l), 50 μ l of calcineurin(40 μ g/ml) and 50 μ l of calmodulin(6 μ g/ml) per well. All preparations were made in the assay buffer B. The plate was washed with the BSA free assay buffer B 4 times followed by incubation with polyclonal anti-calcineurin A α antibody solution(100 μ l of the 10^4 fold diluted in the assay buffer B) at room temperature for 1 hr. The plate was washed 4 times with the BSA free assay buffer B, and 100 μ l of the ALP-labelled polyclonal goat anti-rabbit IgG solution(2,000 fold diluted with the assay buffer B) was added and incubated at room temperature for 1 hr. After washing the plate 4 times with the BSA free assay buffer B, 200 μ l of 1 mM 4-methylumberiferyl phosphate in 10 mM

diethanolamine, 0.5 mM $MgCl_2$(pH 10) was added. The plate was incubated at room temperature for 30 min, and the fluorescent intensity was read on Millipore Cytofluor TM 2350(excitation at 360 nm, emmision at 460 nm).

Competitive activity of C15-demethylated metabolite(M-3) in FK506 pentamer forming activity. All procedure was the same as described in the Pentamer Formation Assay(see above) except 50 μ l of FK506(40 ng/ml) and 50 μ l of serially diluted M-3(0 ~ 3 x 10^4 ng/ml) were used.

Con-A Response Assay. Con-A Assay was performed as usual. Briefly, 1 x 10^5 spleen cells from Balb/c mouse was cultured in 10 % FCS-RPMI 1640 with Con-A(0.2 μ g/well) in the presence of FK506(0, 0.25, 0.5 and 1 ng/ml) and C15-demethylated matabolite(M-3, 0 ~ 32 ng/ml) for 2 days at 37 ℃ in 5 % CO_2 humidified chamber. ^{3}H-Thymidine(10 KBq/well) was pulsed for the last 6 hr before cells were harvested on the filter paper by Labo-Mash. The radioactivity on the filter was counted by scintilation counter as usual.

Results and Discussion

Ascomycin is a FK506 analogue with ethyl substitution to allyl group at C21 of FK506 and often refered to FK520. When Ascomycin is demethylated at C15, it loses the calcineurin inhibiting activity, while it retains binding activity to FKBP12(3), similar to rapamycin. In order for FK506 to show immuno-suppressive activity, FK506 needs to bind to intracellular receptor, FKBP12, followed by further pentamer complex formation together with Ca^{2+}, calmodulin and calcineurin. We established a simple pentamer formation assay utilizing anti-

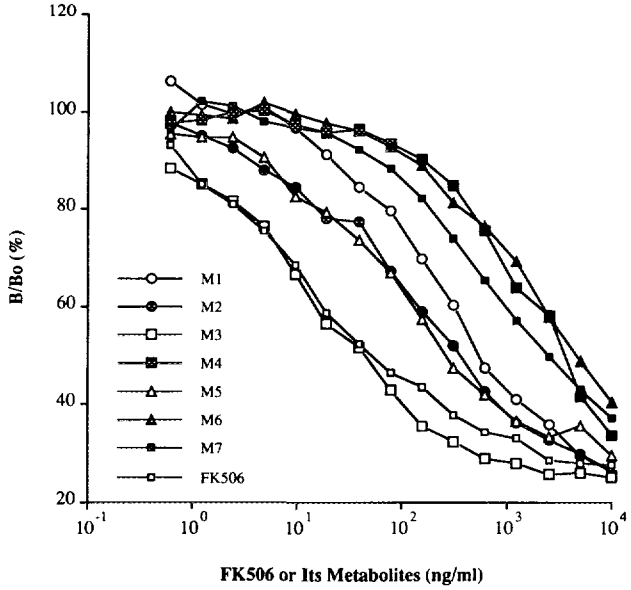

Figure 1. FKBP12 binding activity of FK506 and its metabolites.
Serially diluted FK506 or its metabolite was incubated with 5.5 TBq ^{3}H-FK506 and 40ng FKBP12 in 500 μ l of 0.5% BSA-50mM phosphate buffer for 4 hr at room temperature. Free ^{3}H-FK506 was removed by dextran-charcoal method. Relative binding activity to FK506 was calculated and shown in Table 1.

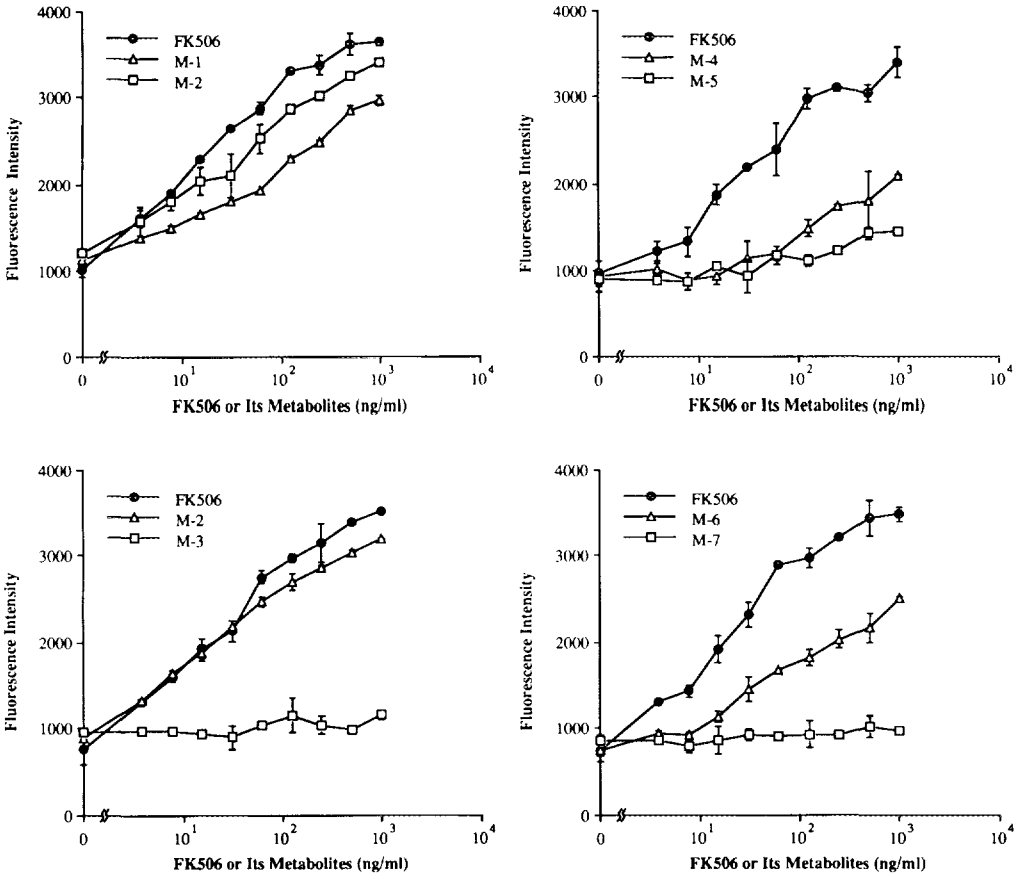

Figure 2. Pentamer forming activity of FK506 and its metabolites.
Serially diluted FK506 or its metabolite in the assay buffer was incubated in the recombinant human FKBP12 coated well at 37℃ for 1 hr in the presence of calcineurin(40 μg/ml, 50 μl) and calmodulin(6 μg/ml, 50 μl). Bound calcineurin was detected by ALP-labelled polyclonal anti-rabbit IgG via rabbit anti-calcineurin A α polyclonal antibody(7) using 4-methylumberiferyl phosphate as a substrate.

calcineurin A α antibody(7) by modification of the radio-ligand pentamer formation assay by Asami et al(10) who claims the method to assay FKBP12 and calcineurin. We also modified radio receptor assay with FKBP12(8, 9). By these methods, we investigated the characteristics of 7 FK506 metabolites(4, 5) in FKBP12 binding and in pentamer formation activites. The results of FKBP12 binding and pentamer formation assays were shown in Fig. 1 and 2, and was summarized in Table 1 together with the immuno-suppressive and immuno-cross reactivities to the anti-FK506 monoclonal antibody being used for the current therapeutic drug monitoring(11). When methoxy group at C13 of FK506 is demethylated, pyranose ring was recyclized to furanose ring(4). As a result, FKBP12 binding activity, thus a pentamer formation activity, was lost to lead to the loss of immuno-suppressive activity. However,

Table 1. Characterization of FK506 Metabolites in FKBP12 binding, Pentamer Complex Foramation, MLR Suppression and Immuno-cross reactivity to anti-FK506 monoclonal antibody #60-46-1

	Characterization of FK506 Metabolites (%)			
	FKBP12 Binding Activity	Complex Formation Assay	MLR Suppression	Reactivity to FK506 McAb
M-1	9.6	13.1	6.4	0
M-2	14.2	79.7	100	70-109
M-3	116.0	0	0	90.5
M-4	1.6	6.4	3.5	0
M-5	20.0	0	0	92.3
M-6	1.3	7.7	1.3	0
M-7	2.3	0	0	0
FK506	100	100	100	100
		r=0.336	r=0.988	
		r=0.302		

Values are calculated as relative activities of FK506.
For FKBP12 binding activity, displacing concentration of FK506 and its metabolites to ^{3}H-FK506 was compared. Pentamer forming activity was assessed by measuring bound calcineurin to the solid phase by anti-calcineurin A α antibody(7). MLR suppressive and immuno-cross reactivity data were taken from ref. 4 and 5.

when C15-methoxy group was demethylated, it lost immuno-suppresive activity while it retained almost comparable binding activity toward FKBP12 as is the case with C15-demethyl-ascomycin(6). This loss of immuno-suppressive activity is easily explained by an unfavorable interaction to calcineurin of C15-OH group on the complex with FKBP12. For C31-demethyl-FK506 (M-2), we anticipated a comparable binding of FKBP12 as well as a pentamer formation to FK506, since it retained almost equal immuno-suppressive activity. However, it was found that C31-demethyl-FK506(M-2) retained only 14 % FKBP12 binding activity of FK506. This discrepancy was easily explained and confirmed by high pentamer formation activity of M-2, which suggested that the pentamer formation is not a two step reaction with the first binding of FK506 to FKBP12, but a single reaction by components participating in the pentamer formation. Thus, newly formed hydroxyl group at C31 by demethylation can

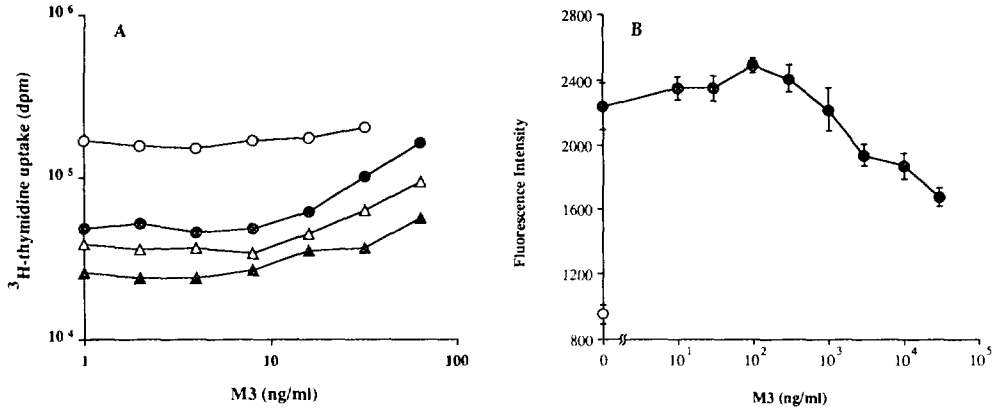

Figure 3. Competitive activity by C15-demethylated metabolite(M-3) in Con-A stimulation inhibition by FK506 and competitive activity in FK506 pentamer forming activity by C15-demethylated metabolite(M-3).
(A) Inhibition of Con-A stimulation by FK506 was measured in the presence of serially diluted M-3 in the assay buffer. ○, FK506 0 ng/ml; ● FK506 0.25 ng/ml; △, FK506 0.5 ng/ml, ▲, FK506 1.0 ng/ml. (B) Inhibition of pentamer formation by FK506(40 ng/ml) was measured in the presence of serially diluted M-3 in the assay buffer.

compensate the loss of affinity to FKBP12 for the pentamer formation. The same is true for di-demethylated metabolites, M-5, M-6 and M-7.

Next, we examined whether C15-demethyl-metabolite(M-3) could compete to FK506 in inhibiting lymphocyte proliferation by Con-A. As is shown in Fig. 3A, 150 fold higher concentration of M-3 was required to compete to FK506(0.25 ng/ml), although M-3 retains higher binding affinity to FKBP12 than FK506. Less competitive activity of M-3 to FK506 in Con-A assay was supported by the higher concentration of M-3 required in competing FK506 in the pentamer formation(Fig. 3B), which is explained by an idea of one step reaction by participating components, thus displacing a pentamer nonformable derivative from its FKBP12 complex, when pentamer formable derivatives are present, forming FKBP12 complex followed by a tighter pentamer formation.

The result of correlation analysis among the several assay methods examined(Table 1) indicates that MLR suppressive activity correlates well with pentamer formation activity, while that with FKBP12 binding activity(8, 9) or with monoclonal antibody binding activity(4, 5, 11) does not. Thinking of the concern in the clinical field on cross-reactivity in ELISA of the metabolites and their immuno-suppressive activity, the pentamer formation assay may provide an alternative method to assess immuno-suppressive state of the patients.

Acknowledgment

The authors are thankful to Dr. M. Okuhara of Fujisawa Pharmacutical Co. Exploratory Res. Labs. for providing us with purified bovine calcineurin.

References

1. Kino, T., Hashimoto, M., M. Nishiyama, Goto, T., Okuhara, M., Kohsaka, M., Aoki, H. and Imanaka, H.(1987) J.Antibiot. 40, 1249-1255.
2. Schreiber, S.L. and Crabtree, G.R.(1992) Immunol.Today 13, 136-142.
3. Yang. D., Rosen, M. and Schreiber, S.L.(1993) J.Am.Chem.Soc. 115, 819-820.
4. Iwasaki, K., Shiraga, T., Nagase, K., Tozuka, Z., Noda, K., Sakuma, S., Fujitsu, T., Shimatani, K., Sato, A. and Fujioka, M.(1993) Drug Metab. Dispos. 21(6), 971-977.
5. Iwasaki, K., Shiraga, T., Nagase, K., Tozuka, Z., Noda, K., Sakuma, S., Fujitsu, T., Shimatani, K., Sato, A. and Fujioka, in preparation.
6. Tallant, E.A., Wallace, R.W., and Cheung, W.Y.(1983) Methods Enzymol. 102, 244-256.
7. Kuno, T., Mukai, H., Ito, A., Chang, C-D., Kishima, K., Saito, N., and Tanaka, C. (1992), J.Neurochem. 58(5), 1643-1651.
8. Murthy, J.N., Chen, Y. Warty, V.S. et al(1992), Clin. Chem. 38(7), 1307-1310.
9. Soldin S.J., Murthy J.M., Donelly J.G. Chen Y., and Goodyear N.(1993) Therapetuic Drug Monitoring 16, 468-471.
10. Asami, M., T.Kuno, T., H.Mukai, H. and C.Tanaka, C.(1993) Biochem.Biophys.Res. Commun. 192(3), 1388-1394.
11. Tamura, K., Kobayashi, M., Hashimoto, K., Kojima, K., Iwasaka, K., Kaizu, T., Tanaka, H. and Niwa, M.(1987) Transpl. Proc. 19(5), Suppl. 6, 23-29.