Distinct Inhibitory Effects of Tacrolimus and Cyclosporin A on Calcineurin

Phosphatase Activity

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Abbreviations: EC_{50}, drug concentration in effect compartment that gives half-maximum effect; k_{10}, systemic elimination rate constant; k_{e0}, first order rate constant for drug disposition from effect compartment; Vd_1, volume of distribution in central compartment

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Abstract

We have compared the pharmacodynamic properties of calcineurin inhibitors, tacrolimus and cyclosporin A, in rats to clarify the different therapeutic drug monitoring strategy of both drugs in clinical situation. In various tissue extracts, the inhibition of calcineurin activity by cyclosporin A was significantly greater than that by tacrolimus at the same drug concentration (1 µM) in the thymus, heart, liver, spleen, kidney, and testis (p < 0.05). The time profiles of blood concentrations and calcineurin activity in whole blood were examined after single or repeated administration of each drug in rats. A substantial time delay in the inhibition was observed following the single administration of tacrolimus or cyclosporin A, resulting in an anti-clockwise hysteresis in the relationship between blood concentrations and calcineurin inhibition in whole blood. In contrast, such a hysteresis loop diminished after the repeated administration of each drug, and the recovery rate of calcineurin activity was greater for the inhibition induced by cyclosporin A than by tacrolimus. Furthermore, tacrolimus produced a comparable inhibition of calcineurin activity in whole blood at lower blood concentrations than cyclosporin A. The effect compartment model described overall well the time profiles of calcineurin activity in whole blood after the single and repeated administrations of each drug. These findings suggest that the properties of calcineurin inhibition differ between tacrolimus and cyclosporin A. The distinct pharmacodynamics may partly contribute to the therapeutic drug monitoring strategy in transplant patients receiving calcineurin inhibitors.

Introduction

Calcineurin inhibitors, tacrolimus and cyclosporin A, are important immunosuppressive drugs in solid organ transplantations. They are not structurally related and bind distinct intracellular receptors referred to as immunophilins; FK506-binding proteins for tacrolimus and cyclophilins for cyclosporin A (Handschumacher et al., 1984; Siekierka et al., 1989). However, both drugs have a similar mechanism of action involving the formation of a complex with their respective immunophilins followed by binding to a common target, calcineurin, and inhibiting the enzyme activity noncompetitively (Liu et al., 1991). Calcineurin is a Ca^{2+}- and calmodulin-dependent serine-threonine protein phosphatase, also known as protein phosphatase type 2B (Rusnak and Mertz, 2000). Calcineurin is expressed ubiquitously in mammalian tissues including T lymphocytes, and especially abundant in the brain (Fruman et al., 1992; Su et al., 1995). Calcineurin has been recently identified as a key enzyme of the rate-limiting step in the activation of T lymphocytes, and an important regulator of nuclear factor of activated T cells, which activates the transcription of genes for cytokines such as interleukin-2 and interferon-γ (Clipstone and Crabtree, 1992; Batiuk et al., 1997). Therefore, tacrolimus and cyclosporin A inhibit the activation of T lymphocytes, and have been widely used to prevent allograft rejection after solid organ transplantation.

Since tacrolimus has a narrow therapeutic range (10-20 ng/ml) and shows large inter- and intra-individual pharmacokinetic variabilities, therapeutic drug monitoring (TDM) of trough blood concentrations (C_0) is necessary to avoid adverse effects such as

nephrotoxicity and neurotoxicity in transplant patients (Staatz and Tett, 2004). However, C_0 monitoring of tacrolimus is not always sufficient because acute allograft rejection occasionally occurs despite the maintenance of adequate trough blood concentrations. On the other hand, a new monitoring strategy based on 2 h post-dose blood concentrations (C_2) of cyclosporin A has been clinically validated and suggested to be a more reliable predictor of rejection or toxicity than C_0 monitoring (Nashan et al., 2002). Therefore, a more informative TDM of calcineurin inhibitors than traditional monitoring of blood concentrations should be established. Such a monitoring strategy would define an effective and safe therapeutic range for an individual patient, and may provide scientific criteria for the use of immunosuppressive drugs with different mechanisms of action (Yatscoff et al., 1998; Jørgensen et al., 2003).

The therapeutic potential for pharmacodynamic monitoring with a biomarker representing immunosuppressive effects has been extensively examined with mycophenolate mofetil and cyclosporin A (Langman et al., 1996; Halloran et al., 1999). However, limited information is available on the relationship between blood concentrations and pharmacodynamic effects of tacrolimus in clinical transplantations. Koefoed-Nielsen et al. (2002) suggested that no single blood tacrolimus concentration correlates well with overall calcineurin inhibition in whole blood in renal transplant patients. Blanchet et al. (2003) showed a good correlation between calcineurin phosphatase activity in lymphocytes and blood tacrolimus concentrations measured at 2 h after dosing in liver transplant patients. Moreover, little is known about the comparative immunopharmacological effects of tacrolimus and cyclosporin A on calcineurin phosphatase activity in vivo.

In this study, we have investigated the pharmacokinetics and pharmacodynamics of tacrolimus and cyclosporin A in vivo to clarify the different therapeutic drug monitoring strategy of both drugs in clinical situation.   We first evaluated inhibitory effects of tacrolimus and cyclosporin A on calcineurin phosphatase activity in various tissues from rats in vitro.   We next examined the time course of calcineurin inhibition in whole blood after single or repeated administration of tacrolimus and cyclosporin A in rats to investigate the in vivo relationship between blood concentrations and calcineurin activity in whole blood.

Methods

Materials

The tacrolimus injection solution (Prograf® injection, 5 mg/ml) and its placebo formulation were generously supplied by Fujisawa Pharmaceutical Company Ltd. (Osaka, Japan).   The cyclosporin A injection solution (Sandimmun® injection, 50 mg/ml) was obtained from Novartis Pharma KK (Tokyo, Japan).   The RII peptide consisting of 19 amino acids (DLDVPIPGRFDRRVSVAAE), corresponding to the phosphorylation site of the regulatory subunit type II of cAMP-dependent protein kinase, was synthesized by QIAGEN (Tokyo, Japan).   All other chemicals used were of the highest purity available.

Animals

Male Wistar rats weighing approximately 250 g (Japan SLC Co., Hamamatsu, Japan) were used.   Prior to the experiments, rats were housed in a temperature- and humidity-controlled room, and allowed free access to water and standard rat chow throughout the experimental period.   The animal experiments were performed in accordance with the Guidelines for Animal Experiments of Kyoto University.   The experimental protocol was approved by the Animal Research Committee, Graduate School of Medicine, Kyoto University.

In Vitro Studies

To establish assay conditions for calcineurin phosphatase activity and ensure the pharmacological effect of calcineurin inhibitors, we first performed in vitro studies using

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crude tissue extracts from rats. After rats were anesthetized with 50 mg/kg of sodium pentobarbital (Abbott, Chicago, IL), various tissues including brain, thymus, heart, liver, spleen, kidney, and testis were removed. Each snap-frozen tissue was homogenized with a Polytron homogenizer in 8-volumes of ice-cold lysis buffer [50 mM Tris-HCl (pH 7.5), 0.1 mM EGTA, 1 mM EDTA, 0.5 mM dithiothreitol, 0.1% β-mercaptoethanol, 5 mM ascorbic acid, and 0.1% NP-40] containing protease inhibitor cocktails (Nacalai Tesque, Kyoto, Japan). The homogenates were further subjected to sonication on ice, then ultracentrifuged at 100,000 g for 60 min at 4°C. The resulting supernatants were stored at -80°C prior to use. Twenty microliters of the extracts was preincubated with 20 µl of calcineurin assay buffer [50 mM Tris-HCl (pH 7.5), 100 mM NaCl, 2.5 mM $CaCl_2$, 0.5 mM dithiothreitol, 0.1% β-mercaptoethanol, 5 mM ascorbic acid, and 0.1 mg/ml bovine serum albumin] containing tacrolimus or cyclosporin A at a final concentration of 1 µM for 30 min at 30°C in the presence of okadaic acid (Calbiochem, San Diego, CA) at a final concentration of 500 nM to inhibit protein phosphatase type 1 and 2A. Calcineurin activity was measured as described below.

In Vivo Studies

On the day before each experiment, a silastic catheter was implanted in the right external jugular vein under light ether anesthesia to facilitate frequent blood collection. After rats were anesthetized with 50 mg/kg of sodium pentobarbital, the femoral vein was cannulated for intravenous infusion of tacrolimus (0.1 mg/kg and 5 mg/kg) or cyclosporin A (1 mg/kg and 10 mg/kg). The injection solution was diluted to the desired concentration with sterile saline, and administered over a 1-h period (2.2 ml/h) with an

automatic infusion pump (Natsume Seisakusho, Osaka, Japan). At the end of the administration, the cannula was removed from the femoral vein and the opening was aseptically sutured to allow rats to move freely. Blood samples (100 µl) were drawn into heparin-containing tubes (50 IU/ml) via the catheter before and at 1, 2, 4, 8, 24, 48, 72, and 96 h after the administration to measure calcineurin activity in whole blood. At the same time points, blood samples (200 µl) were also drawn into EDTA-containing tubes for the measurement of blood concentrations. Next, we conducted multiple-dose studies to rule out whether the pharmacodynamics differs between single- and multiple-dose groups. Tacrolimus (1 mg/kg) or cyclosporin A (1 mg/kg) was intravenously administered 5 times every 12 h via the catheter under no anesthesia. We selected the dose of 1 mg/kg for the repeated administration to compare the inhibitory effects of both drugs on calcineurin activity at the same dose. Blood samplings were performed before the last dosing and at 0.033, 1, 2, 4, 8, 24, 48, 72, and 96 h after the last dosing for the measurements of blood concentrations and calcineurin activity in whole blood as well as in rats in the single-dose study. As the matrix for measurement of calcineurin activity, we chose to use fresh whole blood, and whole blood extracts were prepared according to previous reports (Halloran et al., 1999; Caruso et al., 2001).

Calcineurin Phosphatase Assay

Calcineurin activity was measured using [γ-^{32}P] RII phosphopeptide as a substrate. Phosphorylation of the serine residue of the RII peptide was carried out according to the procedure described by Hubbard and Klee (1991). Calcineurin phosphatase assay was performed in a final volume of 60 µl as described by Fruman et al. (1996) with minor

modifications.   Briefly, 20 µl of lysate was preincubated with 20 µl of calcineurin assay

buffer or Ca^{2+}-free assay buffer [50 mM Tris-HCl (pH 7.5), 100 mM NaCl, 5 mM EGTA,

0.5 mM dithiothreitol, 0.1% β-mercaptoethanol, 5 mM ascorbic acid, and 0.1 mg/ml

bovine serum albumin] for 30 min at 30°C in the presence of okadaic acid at a final

concentration of 500 nM.   The dephosphorylation was initiated by the addition of 20 µl

of [γ-^{32}P]RII phosphopeptide, and allowed to proceed for 20 min at 30°C.   Each reaction

was terminated by adding 120 µl of ice-cold stop solution containing 0.1 M potassium

phosphate buffer (pH 7) in 5% trichloroacetic acid.   Free inorganic phosphate was

separated by AG 50W-X8 cation-exchange chromatography (200-400 mesh, Bio-Rad,

Hercules, CA).   The radioactivity of ^{32}P released was determined in 5 ml of ACSII

(Amersham Int., Buckinghamshire, UK) by liquid scintillation counting.   The amount of

picomoles of phosphate released was calculated by the specific activity of the substrate on

the day of each assay, and phosphatase activity was expressed as picomoles of phosphate

released per min per mg protein.   Background activity due to protein phosphatase type

2C, obtained by measuring in Ca^{2+}-free assay buffer, was subtracted from total activity to

give a Ca^{2+}-sensitive phosphatase activity, which was taken as calcineurin activity.   The

protein content was determined by the method of Bradford (1976) using the Bio-Rad

Protein Assay kit (Bio-Rad) with bovine γ-globulin as a standard.

Drug Assay

    The concentrations of tacrolimus and cyclosporin A in whole blood were measured

by a microparticle enzyme immunoassay method using an IMx® analyzer (Dinabot Co.

Ltd., Tokyo, Japan) and by a fluorescence polarization immunoassay method using a

TDx® analyzer (Dinabot Co. Ltd.), respectively.

Data Analysis

The area under the concentration-time curve over 24 h after single administration or

after the last dosing during repeated administration ($AUC_{0-24\,h}$) was calculated according

to the trapezoidal rule. The highest observed concentration and associated time point

were defined as the maximum concentration (C_{max}) and corresponding time (T_{max}),

respectively. The inhibitory effects of tacrolimus and cyclosporin A on calcineurin

activity in whole blood were calculated using the following equation:

Calcineurin inhibition (%) = $(1 - CaN/CaN_0) * 100$

where CaN_0 is the baseline calcineurin activity before drug administration and CaN

represents the observed calcineurin activity at each time point after the administration.

The incremental area under the pharmacodynamic effect-time curve over 96 h ($AUE_{0-96\,h}$),

corresponding to the time course of calcineurin inhibition, after single administration or

after the last dosing during repeated administration was calculated according to the

trapezoidal rule. The highest observed calcineurin inhibition and associated time point

were defined as the maximum inhibitory effect (E_{max}) and corresponding time (T_{nadir}),

respectively.

Effect Compartment Model Analysis

Since an anti-clockwise hysteresis was observed between blood concentrations and

calcineurin inhibition after the single administration of tacrolimus or cyclosporin A, we

analyzed the concentration-effect relationship with an effect compartment model (Sheiner

et al., 1979). The blood concentration-time profile was described using a

one-compartment model, and the relationship between the concentrations in a

hypothetical effect compartment and calcineurin activity was analyzed with the maximum

effect (E_{max}) model. Data for all individual rats in the different treatment groups for each

drug were fitted simultaneously using the nonlinear mixed effect modeling program

NONMEM (Beal et al., 1992). The individual parameter estimates of each rat were

calculated with the Bayesian post hoc method.

Statistical Analysis

All data were expressed as the mean ± S.E. The statistical significance of

differences in mean values was analyzed by the paired or unpaired *t*-test. For multiple

comparisons, Dunnett's test was used to calculate the statistical significance of differences

in calcineurin activity between before and after drug administration. A value of $p < 0.05$

were considered statistically significant.

Results

Inhibitory Effects of Tacrolimus and Cyclosporin A on Calcineurin Phosphatase Activity in Various Tissues in Vitro

A specific calcineurin activity was measured in crude tissue extracts from normal rats (Fig. 1). The highest level of calcineurin activity was observed in the brain and the lowest level was in the liver, and the specific activities in other tissues were comparable. Table 1 shows the in vitro inhibitory effects of tacrolimus and cyclosporin A on calcineurin activity in various tissues. At the same concentrations (1 µM) of tacrolimus and cyclosporin A in incubation buffer, the inhibition of calcineurin activity by cyclosporin A was significantly greater than that by tacrolimus in all the tissues examined except for the brain ($p < 0.05$).

Measurement of Calcineurin Phosphatase Activity in Whole Blood

The Ca^{2+}-dependent release of phosphate from the $[\gamma-^{32}P]RII$ phosphopeptide increased linearly with incubation time until 30 min (Fig. 2). Therefore, the assay was performed at 20 min of incubation time for the measurement of calcineurin activity in whole blood. The assay precision (CV%) was less than 5% when assayed in triplicate. When blood samples were stored at room temperature after blood collection, no remarkable change in calcineurin activity was observed for up to 4 h in blood samples after blood collection.

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Calcineurin Phosphatase Activity in Whole Blood After Single Administration of Tacrolimus or Cyclosporin A in Vivo.

We examined the time course of calcineurin activity in whole blood after single intravenous continuous administration of tacrolimus and cyclosporin A in rats. Both tacrolimus and cyclosporin A inhibited calcineurin activity in a dose-dependent manner in vivo (Figs. 3 and 4). The pharmacokinetic and pharmacodynamic parameters in single-dose groups are shown in Table 2. Even at a higher dose, calcineurin activity in whole blood was only partially inhibited by tacrolimus and cyclosporin A. Although the peak blood concentrations were reached at 1 h after the single administration of tacrolimus or cyclosporin A, the maximum inhibition of calcineurin activity was observed later (Table 2). After the single administration of the lower dose of each drug, the inhibited calcineurin activity recovered completely at 96 h (Figs. 3A and 4A). On the other hand, the enzyme activity remained significantly inhibited in rats treated with the higher dose of tacrolimus or cyclosporin A even when the blood concentration was undetectable (Figs. 3B and 4B). Notably, an anti-clockwise hysteresis was observed in the relationship between blood concentrations of tacrolimus or cyclosporin A and calcineurin inhibition in whole blood (Figs. 3C and 4C). Although the C_{max} and $AUC_{0-24 h}$ showed direct dose-dependent increases among the two dose groups for tacrolimus and cyclosporin A, the $AUE_{0-96 h}$ showed much less of an increase with increasing dose. To determine possible effects of the vehicle (HCO-60), anesthesia, and serial sampling on calcineurin activity, placebo (200 mg/kg of HCO-60, the maximum dose used in the in vivo studies) was administered to control rats, and blood sampling was performed as well

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as in rats treated with the active drugs. No significant change in calcineurin activity in whole blood was observed in the control rats.

Calcineurin Phosphatase Activity in Whole Blood After Repeated Administration of Tacrolimus or Cyclosporin A in Vivo.

Next, we examined the time course of calcineurin activity in whole blood after repeated intravenous administration of tacrolimus and cyclosporin A in rats. A significant inhibition of calcineurin activity in whole blood was evident at time 0 before the last dosing of tacrolimus or cyclosporin A compared to the pre-treatment level (baseline) (Fig. 5). In addition, a significant inhibition persisted until 24 h and 8 h after the last dosing of tacrolimus and cyclosporin A, respectively (Figs. 5A and 5B). The pharmacokinetic and pharmacodynamic parameters in the multiple-dose groups are shown in Table 3. The inhibition of calcineurin activity induced by tacrolimus was of a similar magnitude from 0 to 24 h after the last dosing (Fig. 5A). In contrast, cyclosporin A showed a more dynamic change in its inhibition than tacrolimus, and the rate of recovery from the inhibition by cyclosporin A was faster. Calcineurin activity in whole blood correlated well with the blood concentrations of cyclosporin A rather than tacrolimus (Fig. 5C). Although the maximum inhibitory effect of cyclosporin A on the enzyme activity tended to be greater than that of tacrolimus of the same dose, the difference did not reach a level of statistical significance (Table 3). The $AUC_{0-24 h}$ of cyclosporin A was 6.9-fold greater than that of tacrolimus, although the $AUE_{0-96 h}$ was not significantly different between tacrolimus and cyclosporin A (Table 3). Notably, the

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pronounced anti-clockwise hysteresis loop observed in the single-dose groups of

tacrolimus and cyclosporin A was minimized in the multiple-dose groups (Fig. 5C).

Effect Compartment Model Analysis.

To investigate the time delay in the calcineurin inhibition by tacrolimus and

cyclosporin A, we analyzed the concentration-effect relationship with an effect

compartment model.   The pharmacokinetic and pharmacodynamic parameters obtained

by fitting all data from single- and multiple-dose studies using the effect compartment

model are shown in Table 4.   Good agreement was demonstrated between

model-predicted values and observed measurements of blood concentrations and

calcineurin activity for each drug, respectively (Figs. 6 and 7).   The k_{e0} values of

tacrolimus were smaller by one-order of magnitude than those of cyclosporin A.   The

maximum inhibitory effects of tacrolimus and cyclosporin A were comparable (E_{max} =

54.5% *vs.* 59.1%).   In addition, the EC_{50} values of tacrolimus were markedly smaller than

those of cyclosporin A in the single- and multiple-dose groups.   Notably, the EC_{50} value

of tacrolimus after the repeated administration was increased by approximately 10 times

compared to that in the single-dose study.   The hysteresis loop diminished in the

relationship between the concentrations in the effect compartment and the inhibition of

calcineurin activity in whole blood.

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Discussion

Despite extensive studies on the pharmacokinetics of tacrolimus and cyclosporin A in animals and humans, little information is available about the comparative immunopharmacological effects of these drugs on calcineurin phosphatase activity in vivo. In this study, we have performed a pharmacokinetic and pharmacodynamic analysis in rats to clarify the properties of calcineurin inhibition by tacrolimus and cyclosporin A.

Using various tissue extracts from rats, specific calcineurin activity was observed and the degree of inhibition by a high concentration of tacrolimus or cyclosporin A (1 µM) varied considerably among the tissues (Fig. 1 and Table 1). The sensitivity of calcineurin in rat tissues to each drug was generally comparable to the previous observations in murines (Su et al., 1995; Kung et al., 2001). Therefore, the validity of assay conditions for the calcineurin phosphatase assay was confirmed. Notably, cyclosporin A inhibited more potently calcineurin activity in most tissues than tacrolimus, except for the brain (Table 1). There are some indications that immunophilins may limit calcineurin inhibition by tacrolimus and cyclosporin A (Kung et al., 2000, 2001). The difference in the inhibition of calcineurin activity between tacrolimus and cyclosporin A may be partially explained by the consideration that cyclophilins are more abundant than FK506-binding proteins in the tissues. While the mechanism of organ toxicity induced by calcineurin inhibitors is not fully understood, cyclosporin A might cause more extensive tissue-specific adverse reactions than tacrolimus during high-dose therapy. Further studies are necessary to clarify whether the organ toxicities of tacrolimus and cyclosporin A are based on the calcineurin inhibition.

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Following the single administration of each drug, the inhibition of calcineurin activity was delay compared to the blood concentrations, and persisted for hours after the concentrations became undetectable (Figs. 3 and 4).   Even at high blood concentrations, calcineurin activity in whole blood was only partially inhibited by tacrolimus and cyclosporin A (Table 2).   Limited amounts of immuniphilins in whole blood may be responsible for the incomplete inhibition of the enzyme activity (Kung and Halloran, 2000).   The time delay in calcineurin inhibition may be due to the equilibration delay of the drugs to peripheral blood lymphocytes and/or the time required for subsequent intracellular events including formation of the drug-immunophilin-calcineurin complex. During the repeated administration of tacrolimus, a similar degree of calcineurin inhibition in whole blood was maintained overall for hours after the last dosing (Fig. 5A). On the other hand, the enzyme activity correlated well with the blood concentrations of cyclosporin A, and returned to the pre-dose levels within 24 h after the last dosing of cyclosporin A (Fig. 5B).   Interestingly, the pattern of hysteresis in the relationship between blood concentrations and calcineurin inhibition differed between the single- and multiple-dose groups for each drug (Figs. 3C, 4C, and 5C).   These changes might be due to the shorter delay in the distribution of the drugs to the effect compartment on the repeated administration of each drug.   Moreover, the recovery rate of calcineurin activity from the inhibition induced by cyclosporin A was greater than that from the inhibition induced by tacrolimus, indicating that cyclosporin A is eliminated from the target site faster than tacrolimus.

P-glycoprotein, which is an efflux pump involved in multidrug resistance in tumor cells, is expressed in peripheral blood lymphocytes at relatively low levels (Ford J et al.,

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2003).   Since tacrolimus and cyclosporin A are substrates of this transporter,

P-glycoprotein may be a limiting factor for the distribution of calcineurin inhibitors to T

lymphocytes.   However, Batiuk et al. (1995) have shown that inhibitors of

P-glycoprotein did not prevent the recovery of calcineurin activity in peripheral blood

lymphocytes from inhibition by cyclosporin A under conditions similar to the in vivo

environment.   Therefore, it is necessary to clarify the pharmacological role of

P-glycoprotein in peripheral blood lymphocytes during the onset and offset of calcineurin

inhibition by tacrolimus and cyclosporin A.

Because both drugs are high lipophilic and primarily associated with red blood cells,

red blood cells may partly contribute to the regulation of the cell distribution of

calcineurin inhibitors (Atkinson et al., 1984; Takada et al., 1993).   A longer time would

be necessary to reach high enough drug concentrations in T lymphocytes to cause

maximum inhibition of calcineurin activity.   In addition, tacrolimus and cyclosporin A

also show high affinity for their intracellular binding sites, immunophilins (Liu et al.,

1992).   It has been reported that tacrolimus remains stable in human Jurkat T-cell

lymphoma for at least 6 h upon incubation in vitro after being taken up by the cells

(Dumont et al., 1994).   These findings may indicate that FK506-binding proteins in

peripheral blood lymphocytes contribute to the persistence of calcineurin inhibition by

tacrolimus, through tight binding to tacrolimus within the cells.

The effect compartment model described overall well the time profiles of

calcineurin activity in whole blood after single and repeated administrations of tacrolimus

or cyclosporin A (Figs. 6 and 7).   Although the fitting of blood concentrations of

tacrolimus was not good enough with the one-compartment model, no further

improvement was observed using the two-compartment model (data not shown).

Therefore, the application of the one-compartment model with an effect compartment

appeared to be suitable for the present pharmacokinetic and pharmacodynamic analysis.

Interestingly, the EC_{50} value of tacrolimus after the repeated administration was increased

by approximately 10 times compared to that in the single-dose group.   This change in the

EC_{50} value may be caused by the development of a counterregulatory phenomenon or by

the desensitization of intracellular receptors, FK506-binding proteins.   Recently,

Ramakrishnan et al. (2002) have reported an indirect response model of time-dependent

transduction systems for corticosteroid pharmacodynamics, one of other

immunosuppressive drugs.   Therefore, it is of interest to develop a mechanism-based

pharmacodynamic model to describe more precisely the concentration-effect relationship

of calcineurin inhibitors.   Further investigation is necessary to identify regulatory and/or

limiting factors in calcineurin inhibition and recovery, and to clarify the functional

relevance in vivo.

As shown in Fig 5B, the calcineurin activity in whole blood changed parallel with

blood cyclosporin A concentrations after the repeated administration, and that is

consistent with the previous observations in renal transplant patients treated with

cyclosporin A (Halloran et al., 1999; Caruso et al., 2001).   In addition, the maximum

inhibitory effect of cyclosporin A on the enzyme activity was observed near the peak

blood concentrations.   Therefore, the peak monitoring of blood cyclosporin A

concentrations may provide useful information to predict the maximum calcineurin

inhibition in patients receiving cyclosporin A.   On the other hand, the inhibited

calcineurin activity in whole blood was not further inhibited after the last dosing of

tacrolimus during the repeated administration (Fig. 5A).   In renal transplant patients, the

calcineurin activity in whole blood was partially inhibited after tacrolimus dosing and

showed less dynamic changes (Koefoed-Nielsen et al., 2002).   Taking these findings into

consideration, the trough monitoring of blood tacrolimus concentrations would be

sufficient in patients receiving tacrolimus, because similar magnitude of calcineurin

inhibition may be maintained during a dosing interval.   However, clinical studies in

transplant patients should be carried out to clarify whether the measurement of calcineurin

phosphatase activity is useful to avoid allograft rejection and adverse effects in addition to

the classical therapeutic drug monitoring of tacrolimus and cyclosporin A.

In conclusion, we found that tacrolimus produced a comparable inhibition of

calcineurin activity in whole blood in vivo at lower blood concentrations than cyclosporin

A during the dosing interval.   We also clarified that calcineurin inhibition in whole blood

relates well with blood concentrations after repeated administration of cyclosporin A

rather than tacrolimus.   Furthermore, the recovery rate of calcineurin activity was greater

for the inhibition induced by cyclosporin A than by tacrolimus.   These different

pharmacodynamic properties may at least in part contribute to the therapeutic drug

monitoring strategy in transplant patients receiving calcineurin inhibitors.

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Footnotes

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Legends for figures

Fig. 1. Calcineurin phosphatase activity in various tissues from rats. Each column represents the mean ± S.E. of four to five rats.

Fig. 2. Time course of dephosphorylation of [γ-^{32}P] RII phosphopeptide by whole blood extracts from normal rats. Broken lines show the mean Ca^{2+}-dependent dephosphorylation of [γ-^{32}P] RII phosphopeptide by subtracting the nonspecific dephosphorylation measured in the Ca^{2+}-free assay buffer (closed circles) from the total dephosphorylation measured in the calcineurin assay buffer (open circles). Each point represents the mean ± S.E. of three rats.

Fig. 3. Time profiles of blood tacrolimus concentrations (open columns) and calcineurin activity in whole blood (closed circles) in rats after single administration of tacrolimus at 0.1 mg/kg (A) and 5 mg/kg (B). Each point or column represents the mean ± S.E. of four rats. *p < 0.05, significantly different from the basal calcineurin activity before the administration (baseline). (C) Relationship between blood concentrations and calcineurin inhibition after single administration of tacrolimus. Open and closed circles represent the mean data from rats treated with 0.1 mg/kg and 5 mg/kg of tacrolimus, respectively, without S.E. for clarity. Each arrow indicates an elapse of time.

Fig. 4. Time profiles of blood cyclosporin A concentrations (open columns) and calcineurin activity in whole blood (closed circles) in rats after single administration of

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cyclosporin A at 1 mg/kg (A) and 10 mg/kg (B).   Each point or column represents the

mean ± S.E. of four to six rats.   *p < 0.05, significantly different from the basal

calcineurin activity before the administration (baseline).   (C) Relationship between blood

concentrations and calcineurin inhibition after single administration of cyclosporin A.

Open and closed circles represent the mean data from rats treated with 1 mg/kg and 10

mg/kg of cyclosporin A, respectively, without S.E. for clarity.   Each arrow indicates an

elapse of time.

Fig. 5.   Time profiles of blood concentrations (open columns) and calcineurin activity in

whole blood (closed circles) in rats after 5 administrations of tacrolimus (A) and

cyclosporin A (B) at 1 mg/kg every 12 h.   Each point or column represents the mean ±

S.E. of four rats.   *p < 0.05, significantly different from the basal calcineurin activity

before the administration (baseline).   (C) Relationship between blood concentrations and

calcineurin inhibition after repeated administration of tacrolimus or cyclosporin A.   Open

and closed circles represent the mean data from rats treated with tacrolimus and

cyclosporin A, respectively, without S.E. for clarity.   Each arrow indicates an elapse of

time.

Fig. 6.   Effect compartment model analysis for blood concentrations (left panels) and

calcineurin activity in whole blood (right panels) after single administrations of

tacrolimus at a lower dose (A) and at a higher dose (B), and repeated administration of

tacrolimus (C) in rats.   The lines are simulations using the averaged individual Bayesian

post hoc parameter estimates and the mean dosage given in each group.

Fig. 7. Effect compartment model analysis for blood concentrations (left panels) and calcineurin activity in whole blood (right panels) after single administrations of cyclosporin A at a lower dose (A) and at a higher dose (B), and repeated administration of cyclosporin A (C) in rats. The lines are simulations using the averaged individual Bayesian post hoc parameter estimates and the mean dosage given in each group.

TABLE 1.   Inhibitory effects of tacrolimus and cyclosporin A on calcineurin

phosphatase activity in rat tissues

Tissues	Inhibition (%)	
	Tacrolimus	Cyclosporin A
Brain	47.0 ± 2.8	48.2 ± 4.6
Thymus	23.0 ± 3.6	72.6 ± 5.1*
Heart	31.2 ± 1.8	60.7 ± 2.2*
Liver	80.8 ± 2.6	94.7 ± 0.8*
Spleen	26.6 ± 5.3	81.5 ± 2.5*
Kidney	18.7 ± 2.1	82.0 ± 2.6*
Testis	30.6 ± 1.6	85.6 ± 0.3*

Tissue homogenates were preincubated with 1 μM of tacrolimus or cyclosporin A at

30°C for 30 min.   Data are shown as a percentage of inhibition compared with the

control activity measured in the absence of tacrolimus and cyclosporin A.   Each value

represents the mean ± S.E. of four to five rats.

*p < 0.05, significantly different from the inhibition by tacrolimus.

TABLE 2.   Pharmacokinetic and pharmacodynamic parameters of tacrolimus and cyclosporin A after single administration in rats.

Parameters	Tacrolimus		Cyclosporin A	
	0.1 mg/kg	*5 mg/kg*	*1 mg/kg*	*10 mg/kg*
PK parameters				
T_{max} (h)	1	1	1	1
C_{max} (ng/ml)	26.9 ± 1.2	$1,790 \pm 67$	$1,570 \pm 57$	$10,800 \pm 181$
$AUC_{0-24\,h}$ (ng · h/ml)	44.6 ± 0.9	$2,930 \pm 142$	$5,080 \pm 219$	$44,300 \pm 1,640$
PD parameters				
T_{nadir} (h)	7.0 ± 1.0	30.0 ± 6.0	6.3 ± 1.8	22.7 ± 6.0
E_{max} (%)	22.2 ± 3.6	46.0 ± 3.1	30.4 ± 11.3	53.1 ± 1.5
$AUE_{0-96\,h}$ (% · h)	$1,330 \pm 615$	$3,290 \pm 451$	$1,260 \pm 522$	$3,760 \pm 266$

Tacrolimus or cyclosporin A was intravenously administered for 1 h at two different

doses.   Data are presented as the time or the mean ± S.E. of four to six rats.

TABLE 3.   Pharmacokinetic and pharmacodynamic parameters of tacrolimus and cyclosporin A after repeated administration in rats.

Parameters	Tacrolimus	Cyclosporin A
PK parameters		
T_{max} (h)	0.033	0.033
C_{max} (ng/ml)	$1,120 \pm 67$	$4,360 \pm 421$*
$AUC_{0-24\,h}$ (ng · h/ml)	$1,050 \pm 25$	$7,260 \pm 776$*
PD parameters		
T_{nadir} (h)	6.0 ± 2.0	1.0 ± 0.4*
E_{max} (%)	35.3 ± 3.3	49.2 ± 4.7
$AUE_{0-96\,h}$ (% · h)	$1,780 \pm 476$	917 ± 217

Tacrolimus or cyclosporin A was intravenously administered 5 times at a dose of 1 mg/kg every 12 h.   Data are represented as the time or the mean ± S.E. of four rats.

*$p < 0.05$, significantly different from the mean value in rats treated with tacrolimus.

TABLE 4. Pharmacokinetic and pharmacodynamic parameters of tacrolimus and cyclosporin A in rats according to the effect compartment model.

Parameters	Tacrolimus	Cyclosporin A
Single administration at lower dose (mg/kg)	*0.1*	*1*
k_{10} (h^{-1})	0.225 [a]	0.167 ± 0.002
k_{e0} (h^{-1})	0.0192 ± 0.0107	0.124 ± 0.080
Vd_1 (l)	1.30 [a]	0.265 [a]
E_{max} (%)	54.5 [a]	59.1 [a]
EC_{50} (ng/ml)	7.50 ± 4.97	275 ± 88
Single administration at higher dose (mg/kg)	*5*	*10*
k_{10} (h^{-1})	0.225 [a]	0.167 ± 0.003
k_{e0} (h^{-1})	0.0015 ± 0.0002	0.024 ± 0.003
Vd_1 (l)	1.30 [a]	0.264 [a]
E_{max} (%)	54.5 [a]	59.1 [a]
EC_{50} (ng/ml)	6.87 ± 3.05	266 ± 41
Repeated administration (mg/kg)	*1*	*1*
k_{10} (h^{-1})	0.225 [a]	0.162 ± 0.009
k_{e0} (h^{-1})	0.0305 ± 0.0131	0.116 ± 0.026
Vd_1 (l)	1.30 [a]	0.264 [a]
E_{max} (%)	54.5	59.1 [a]
EC_{50} (ng/ml)	63.2 ± 22.2	354 ± 94

Data of blood concentrations and calcineurin activity from single- and multiple-dose groups were analyzed by nonlinear mixed effect modeling.   The individual Bayesian post hoc parameter estimates are represented as the mean $\pm$ S.E. of four to six rats.

[a] S.E. was not obtainable because of small inter-individual variability.

Fig. 1
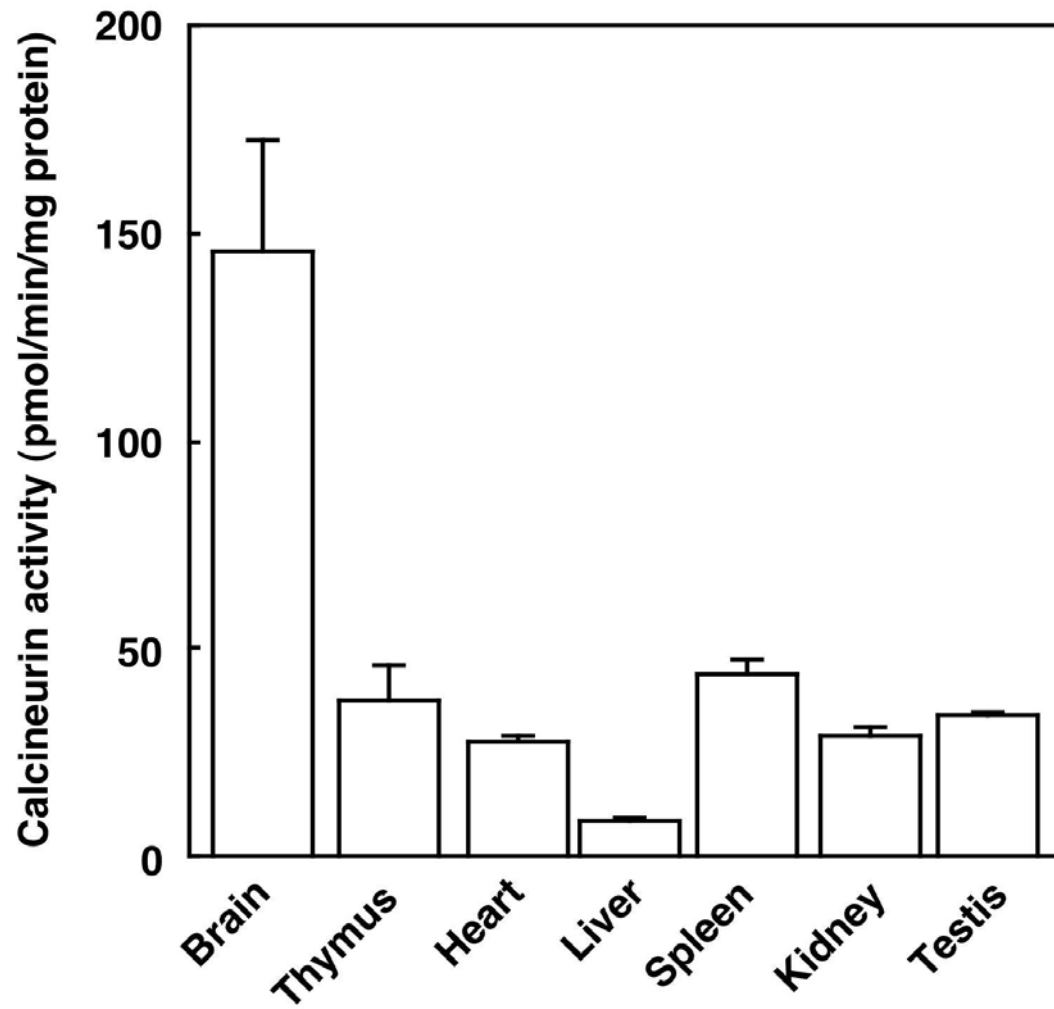
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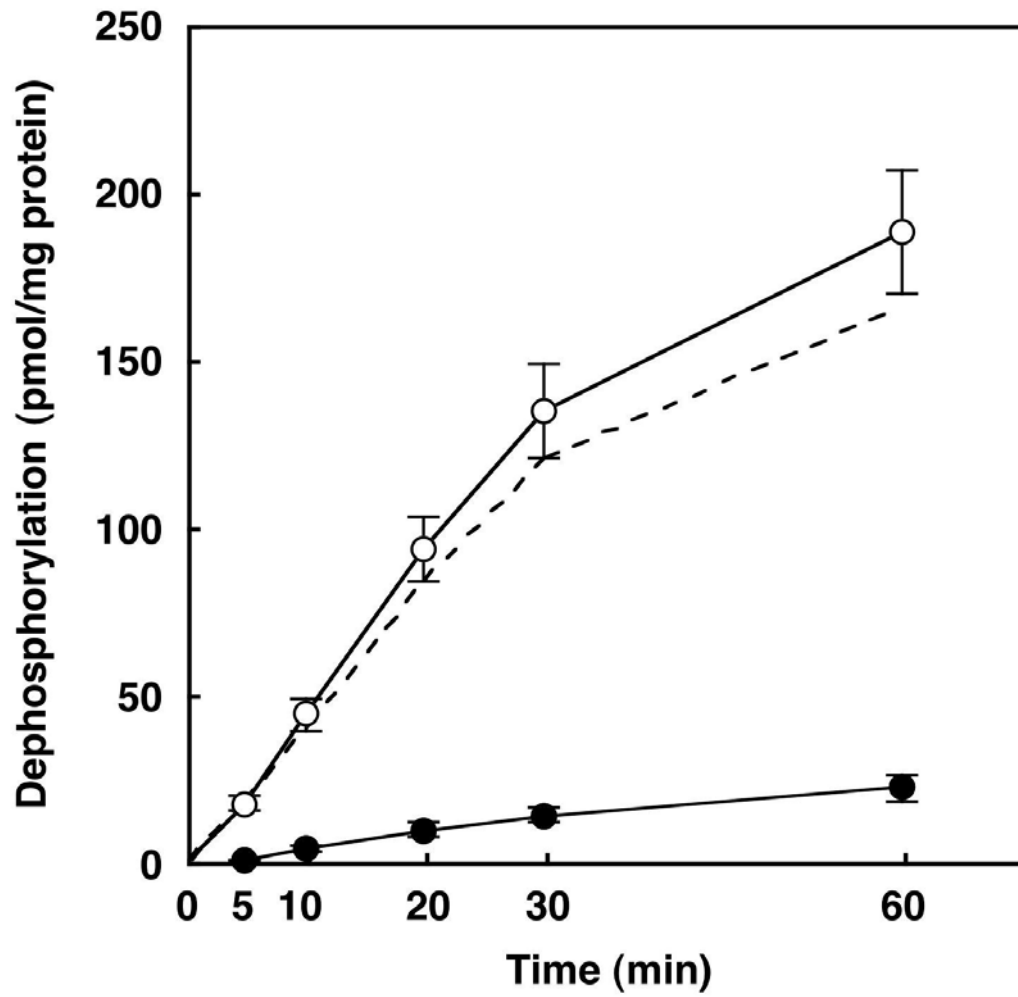
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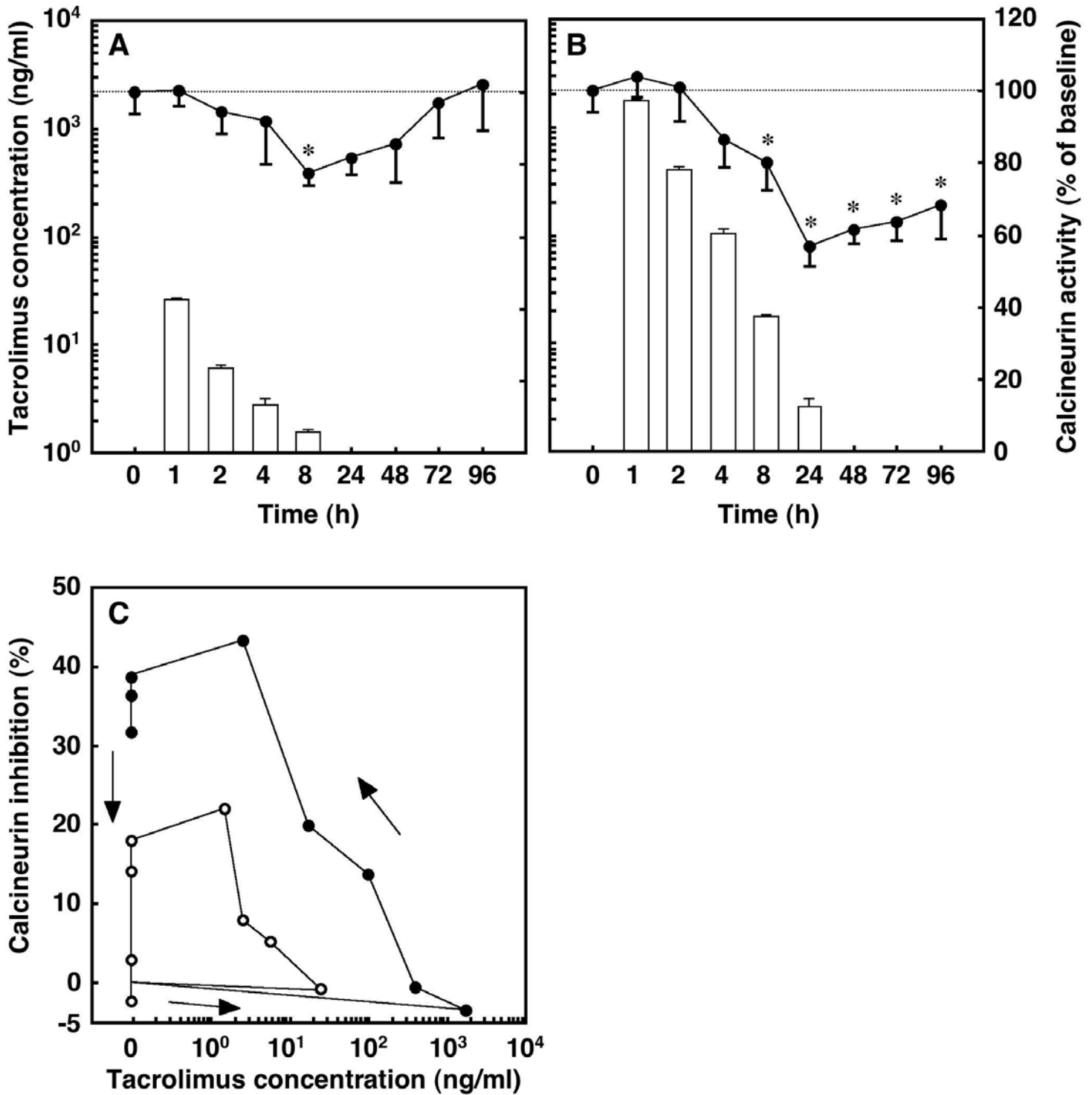
Fig. 4

Fig. 5

Fig. 6

(A)

(B)

(C)

Fig. 7

(A)

(B)

(C)