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Preliminary in vivo pharmacokinetic and pharmacodynamic evaluation of a novel calcineurin-independent inhibitor of NFAT

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Abstract A-285222 (A-285) is a bis-trifluoromethyl-pyrazole (BTP), a novel class of immunosuppressive agents that inhibit NFAT activity in vitro in human and non-human primate cells through a calcineurin-independent mechanism. In this preliminary study, we treated cynomolgus monkeys with different doses of A-285 for several days. Blood was collected from all animals at different times during the study. From these samples, plasma concentrations of A-285 were measured by liquid chromatography/mass spectrometry (LC/MS), and intracellular T-cell production of the cytokines IL-2, IFN-γ, and TNF-α was quantified by flow cytometry using a mitogen-stimulated whole blood assay. Marked inhibition of cytokine production occurred after administration of the first dose of A-285, and this effect was comparable to that of cyclosporine. While neurological toxic side effects were seen when the plasma concentration of A-285 exceeded 4 μg/ml, at lower plasma levels the drug was well tolerated over 2 weeks and its pharmacodynamic effects were sustained throughout this time.

Keywords Calcineurin · Cyclosporine · Flow cytometry · NFAT · Non-human primates · Pharmacodynamics

Introduction

The majority of solid organ transplant recipients are currently treated with one of the two calcineurin inhibitors, cyclosporine or tacrolimus, as life-long maintenance immunosuppression. Although structurally different, the modes of action of these drugs are similar: they inhibit the calcium-dependent serine/threonine phosphatase calcineurin and its substrate, the transcription factor nuclear factor of activated T cells (NFAT) [1]. NFAT is essential for the expression of interleukin-2 (IL-2), an autocrine growth factor expressed by activated T cells upon their interaction with antigens and which is crucial for the complex cascade of events resulting in clonal expansion of T cells and, ultimately, in allograft rejection. While cyclosporine and tacrolimus are effective immunosuppressants, their use can cause serious side effects (nephrotoxicity, neurotoxicity, dyslipidemia, diabetogenesis). Although the precise mechanisms responsible for these toxic side effects are unknown, it is likely

that many are caused by the pleiotropic metabolic effects these agents exert through inhibition of calcineurin in non-immune cells [2, 3, 4].

Calcineurin is activated by the rise in intracellular calcium which occurs when T cells are stimulated through receptors coupled to phospholipase C, and it dephosphorylates NFAT. Dephosphorylation of NFAT by calcineurin is followed by translocation of NFAT to the nucleus, where it is incorporated into a transcriptionally active enhanceosome complex at the promoter/enhancer regions of NFAT-dependent genes [5]. NFAT plays an essential role in the expression of IL-2, but is also involved in the expression of other T-cell cytokines (IL-4, IL-5, IL-6, IL-8, IL-13, IFN-γ, TNF-α) essential for the immune response initiated by the recognition of alloantigens [6]. Since NFAT activity is indirectly hindered by two clinically important immunosuppressants, direct interference with NFAT regulation might yield new, more selective means of immunosuppression.

A-285222 (A-285) is a 3,5-bis-trifluoromethyl pyrazole (BTP). BTP derivatives cause NFAT to be maintained in the cytosol in a phosphorylated state and block the nuclear translocation of NFAT and, thus, the NFAT-dependent cytokine gene transcription by a mechanism other than direct inhibition of calcineurin phosphatase activity [7]. In vitro, these substances inhibit production of various TH1 and TH2 cytokines in normal human peripheral blood T cells, suggesting that these agents might be useful for treating autoimmune diseases or preventing organ transplant rejection [8]. Since BTPs inhibit T-cell cytokine production in vitro in human cells far more potently than in rodent cells [9], they could not be evaluated for in vivo efficacy in traditional rodent allotransplantation models. We chose to evaluate the efficacy of A-285 in vivo in non-human primates because the ability of orally administered BTP to block cytokine production of circulating T cells in this species had already been evidenced [10] and because of the close relationship between non-human primate and human biology. As information about pharmacokinetics and toxicity of the drug was limited and non-human primate transplantation models are much more expensive than other transplantation models, we searched for a means to evaluate the pharmacodynamic effects of the drug without the employment of such definitive measures as allotransplantation. In this preliminary study we treated cynomolgus monkeys with A-285 and used flow cytometry to assess the effects of treatment with this drug on intracellular T-cell cytokine production in vivo. The aim of our study was to determine what treatment doses would be tolerated by the animals without major side effects and to verify whether the pharmacodynamic effect of the BTP (i.e., inhibition of T-cell cytokine production) would be maintained for the entire duration of the administration of the drug. In non-human primates that do not receive any immunosuppression, allografts fail at 1 week after transplantation [6, 11, 12]. We decided to treat cynomolgus monkeys for 2 weeks with A-285 since in the eventuality of subsequent experiments with organ transplants in this species, graft survival superior to 1 week in animals treated with A-285 would prove the immunosuppressive value of the BTP.

Materials and methods

Animals and routine husbandry

The study was approved by the Institutional Animal Care and Use Committee at Stanford University, a facility that is fully accredited by the Association for Assessment and Accreditation of Laboratory Animal Care and registered with the U.S. Department of Agriculture. Male, wild caught cynomolgus monkeys (*Macaca fascicularis*) with a weight between 5 and 8 kg were purchased from Charles River Biomedical Research Foundation (Houston, Tex.). The animals received humane care in compliance with the Guide for the Care and Use of Laboratory Animals prepared by the National Research Council and published by the National Academy of Sciences (National Academy Press, Washington, D.C., 1996).

Drugs and treatments

A-285 was a gift from Abbott Laboratories (Abbott Park, Ill.). For treatment, a suspension of A-285 in 0.2% hydroxypropylmethylcellulose was administered without any dilution through a naso-gastric tube. Liquid microemulsion formulation of cyclosporine (Neoral™) for oral administration (100 mg/ml) was purchased from Novartis Pharma (East Hannover, N.J.) and administered without any dilution via a naso-gastric tube.

Experiment design

Cynomolgus monkeys were treated with A-285 orally twice daily (7 a.m. and 7 p.m.) under ketamine sedation over several days. One additional cynomolgus monkey (as a positive control) was treated with 100 mg/kg of cyclosporine orally once daily for the same duration. This dose had proven to be immunosuppressive in cynomolgus monkeys in previous studies performed at our laboratory [13]. One further cynomolgus monkey (negative, untreated control) did not receive any immunosuppressive treatment, but was sedated and had blood collected at the same time as the animals treated with A-285 or cyclosporine. The latter two animals were sedated twice daily, even when no manipulation or blood drawing was done, to ensure that administration of ketamine was identical in all five animals. Thus, any immunosuppressive effects in drug-treated animals could be attributed to the immunosuppressive drugs and not to differences in exposure to ketamine.

After evaluation of computer-generated pharmacokinetic models by the manufacturer of A-285, a single initial loading dose of 60 mg/kg followed by 30 mg/kg twice daily was initially chosen. This dosage regimen had to be reduced twice during our experiments due to neurotoxic side effects.

Pharmacokinetic analysis

Pre-dose drug concentrations of A-285 and cyclosporine were measured once daily, immediately before drug administration. Cyclosporine concentrations were determined in whole blood samples by liquid chromatography/mass spectrometry (LC/MS) [14]. Plasma concentrations of A-285 were determined using a validated LC/MS method (K. Marsh, personal communication).

Pharmacodynamic assays

Blood sampling

For assessment of T-cell cytokine production, blood was collected at different time-points before and during treatment. Before starting immunosuppressive drug treatment, blood was collected on two separate days. At inception of the study, blood was also collected immediately prior to administration of the first drug dose. A mean of the values obtained on these three occasions was used to quantify the baseline T-cell cytokine production without immunosuppressive treatment.

During the study, blood was collected on different days, immediately prior to the morning drug dose administration. The same assays were also performed with whole blood collected 4 days after administration of the last drug dose to assess the recovery of T-cell cytokine production after cessation of immunosuppressive drug treatment. The pre-dose time-point was chosen for evaluation of the pharmacodynamic effects of A-285 for two reasons: to avoid additional ketamine sedation of animals; and to verify the existence of a pharmacodynamic effect pre-dose, as this feature is common for other immunosuppressive agents used in clinical transplantation.

Assessment of intracellular T-cell cytokine production

For the detection of intracellular T cell cytokines, 100 µl of undiluted whole blood was incubated for 5 h at 37°C with phorbol 12-myristate 13-acetate (PMA) (30 ng/ml) and ionomycin (750 ng/ml) in the presence of brefeldin A (10 µg/ml, added 30 min after the mitogens). After incubation, 50 µl of whole blood was incubated with 3 µl of peridinin-chlorophyll a complex-Cy-Chrome™ (PerCP-CY5.5)-labeled monoclonal anti-human CD3 antibodies for 15 min at room temperature in the dark. Subsequently, the cells were fixed and permeabilized using a standard fixation-permeabilization reagent kit and the manufacturer's immunofluorescence staining protocol (IntraPrep, Immunotech, Marseille, France). For staining of intracellular cytokines, 2 µl of either fluorescein isothiocyanate (FITC)-labeled anti-IL-2 or FITC-labeled anti-IFN-γ and phycoerythrin (PE)-labeled anti-TNF-α anti-human monoclonal antibodies (all from PharMingen, San Diego, Calif.) were used. Samples were analyzed by flow cytometry within 6 h of preparation. Emitted light of the fluorochromes was collected through 525-nm (FITC), 575-nm (PE), and 675-nm (PerCP-CY5.5) band-pass filters, respectively. Forward and side scatter were used to differentiate lymphocytes from debris, dead cells, and other leukocytes. Five thousand light scatter gated lymphocytes were analyzed per sample. Unstimulated and stimulated diluted whole blood cultures were incubated with isotype control antibodies (PE-labeled mouse $IgG_{1,\kappa}$) and used as specificity controls. To distinguish between fluorescence-positive and -negative cell populations, analysis regions were set using isotype controls to achieve non-specific binding of less than 1% within the positive event regions. For all studied activation antigens, the entire population of lymphocytes was light scatter gated and $CD3^+$ cells were subsequently subgated. The percentages of $CD3^+$ lymphocytes positive for IL-2, IFN-γ, and TNF-α were calculated thereafter.

Calculation of drug effects

T-cell cytokine production under immunosuppressive drug treatment was expressed as percent of baseline values (i.e., without immunosuppression) using the formula:

$$\text{Cytokine production (\%)} = (\text{"treated"}/\text{"not treated"}) \times 100$$

where for each value "treated" is the percentage of cytokine-positive $CD3^+$ cells at a certain time-point during the study, while "not treated" is the baseline percentage of cytokine-positive $CD3^+$ cells (mean of three values obtained before inception of immunosuppressive drug therapy).

Results

Preliminary experiments

In an initial experiment, two cynomolgus monkeys received a single loading dose of 60 mg/kg A-285, followed by 30 mg/kg twice daily. After 2 days of treatment with this regimen, the experiment was stopped due to side effects (depression, anorexia, ataxia) in one animal, which was found to have a pre-dose plasma concentration of A-285 of 4.1 µg/ml. The other animal tolerated the drug well, but his pre-dose plasma levels of A-285 never exceeded 1.5 µg/ml.

In a second experiment, three animals received a single loading dose of 40 mg/kg of A-285 followed by 20 mg/kg twice daily. Although this regimen was better tolerated initially (no anorexia or depression), on the fourth day of treatment one animal developed seizures. The study drug had achieved plasma levels in excess of 4 µg/ml (mean pre-dose plasma concentration 6.78 µg/ml after 3 days of treatment) in all animals at the time when the neurological symptoms occurred. The seizures remitted spontaneously, but the experiment was stopped.

In both experiments, T-cell cytokine production at 12 h pre-dose was found to be less than 20% of pre-treatment baseline values after 2 days of treatment with A-285, and this effect was comparable to that seen in the cyclosporine-treated animal (data not shown).

Pharmacokinetic/pharmacodynamic correlation study

The study was conducted in three cynomolgus monkeys, using an initial loading dose of 20 mg/kg A-285 followed by 10 mg/kg twice daily. The dose was further adjusted during the study according to premonitory signs for neurological symptoms (lack of appetite, reduced activity, depressive attitude) and/or plasma levels. Because in our preliminary experiments neurological symptoms occurred when pre-dose plasma concentration of A-285 exceeded 4 µg/ml, we attempted to maintain levels lower than 3.5 µg/ml. After 3 days of treatment with 10 mg/kg twice daily, 12-h pre-dose plasma concentration of the drug still showed a tendency to increase, suggesting an accumulation of drug in the circulation. The daily dose was reduced to 5 mg/kg and no drug accumulation was noted thereafter. Finally, steady state of 12-h pre-dose plasma concentration of A-285 at levels between 1 and 2 µg/ml was finally achieved by treating the animals with 7.5 mg/kg twice

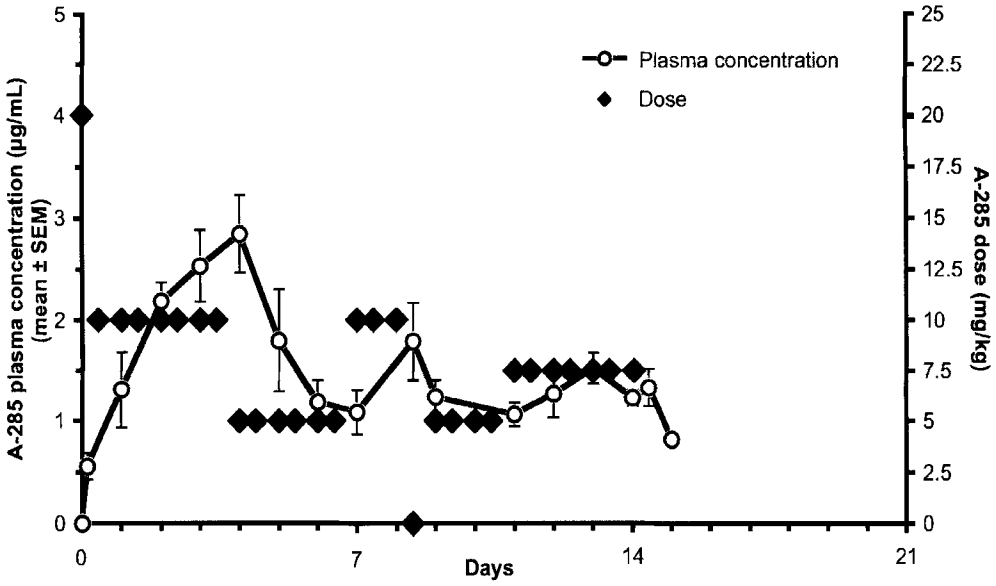

Fig. 1 Plasma concentrations and treatment doses of A-285. Animals ($n = 3$) received one oral loading dose of 20 mg/kg, followed by 10 mg/kg twice daily. After the third day of treatment, the dose was adjusted in all animals to avoid the occurrence of adverse effects. The dose (in mg/kg) is represented by *diamonds*. Blood was collected every day before administration of the morning dose of A-285 (12-h pre-dose), and plasma concentrations of A-285 were measured by liquid chromatography/mass spectrometry. Values (represented by *circles*) are mean ± SEM

daily. No side effects were seen during the 14-day study period. The relation between dosage of A-285 and pre-dose plasma concentration is summarized in Fig. 1.

In the cyclosporine-treated control animal, steady-state 24-h trough levels were attained after 4 days of treatment. Thereafter, the average cyclosporine trough concentration was 390.3 ± 42.1 ng/ml (as measured every other day until day 14). Except for a moderate rise in serum creatinine, which resolved completely after the study was ended, no toxic side effects were seen in this animal.

On day 1, after administration of the first two doses of A-285, (pre-dose) T-cell cytokine production was inhibited by about 70% when compared to pretreatment baseline values, as shown by the average percentage of $CD3^+$ cells positive for IL-2, IFN-γ or TNF-α (Fig. 2). T-cell cytokine production remained below 30% of pretreatment baseline values throughout the entire period of treatment with A-285, and it slowly recovered when the treatment was stopped. The average percentage of $CD3^+$ cells positive for IL-2, IFN-γ, and TNF-α reached 56%, 48%, and 38% of pretreatment baseline values, respectively, 4 days after administration of the last dose of A-285. The inhibitory effect of A-285 on T-cell cytokine production was similar to that of cyclosporine.

Discussion

The BTP compounds were identified based on their ability to block IL-2 gene transcription. While IL-2 gene transcription is dependent upon several transcription factors (nuclear factor κB [NFκB], activated protein-1 [AP-1] complex, NFAT), BTPs selectively block the activation-dependent nuclear localization of NFAT, without affecting other transcription factors [7]. BTPs do not inhibit calcineurin activity in vitro, and it is unlikely that they exert their effect on NFAT by regulating intracellular free calcium ion levels. It has recently been reported that the binding of the peptidyl prolyl cis-trans isomerase Pin1 to NFAT inhibits dephosphorylation of NFAT by calcineurin in vitro and that overexpression of Pin1 in T cells blocks calcium-dependent activation of NFAT in vivo [15]. BTPs might block calcineurin-dependent dephosphorylation of NFAT by enhancing the binding of NFAT to Pin1, but this hypothesis has not been verified yet.

BTPs act on NFAT more selectively than cyclosporine and, unlike calcineurin inhibitors, do not affect activation, nuclear localization, or binding of NFκB or AP-1 to their respective IL-2 enhancer elements [7]. Furthermore, certain cellular effects of cyclosporine and tacrolimus may not involve calcineurin or NFAT at all: blocking of the peptidyl-prolyl isomerase activity of Cyp-40 [16], potentiation of transcription driven by ligand-activated glucocorticoid receptors [17]. Although it is unclear how much these effects contribute to the overall immunosuppressive efficacy of calcineurin inhibitors, it is improbable that NFAT-based immunosuppressants would exert these functions in immune cells.

In our study, marked inhibition of T-cell cytokine production was seen when pre-dose plasma levels of A-285 were between 1 and 2 µg/ml. A surprising and disappointing finding was the neurotoxicity seen at plasma levels of A-285 exceeding 4 µg/ml. This finding might as well suggest that neurotoxic side effects of A-285 occurred when animals were dosed excessively, as the degree of inhibition of the T cell cytokines we

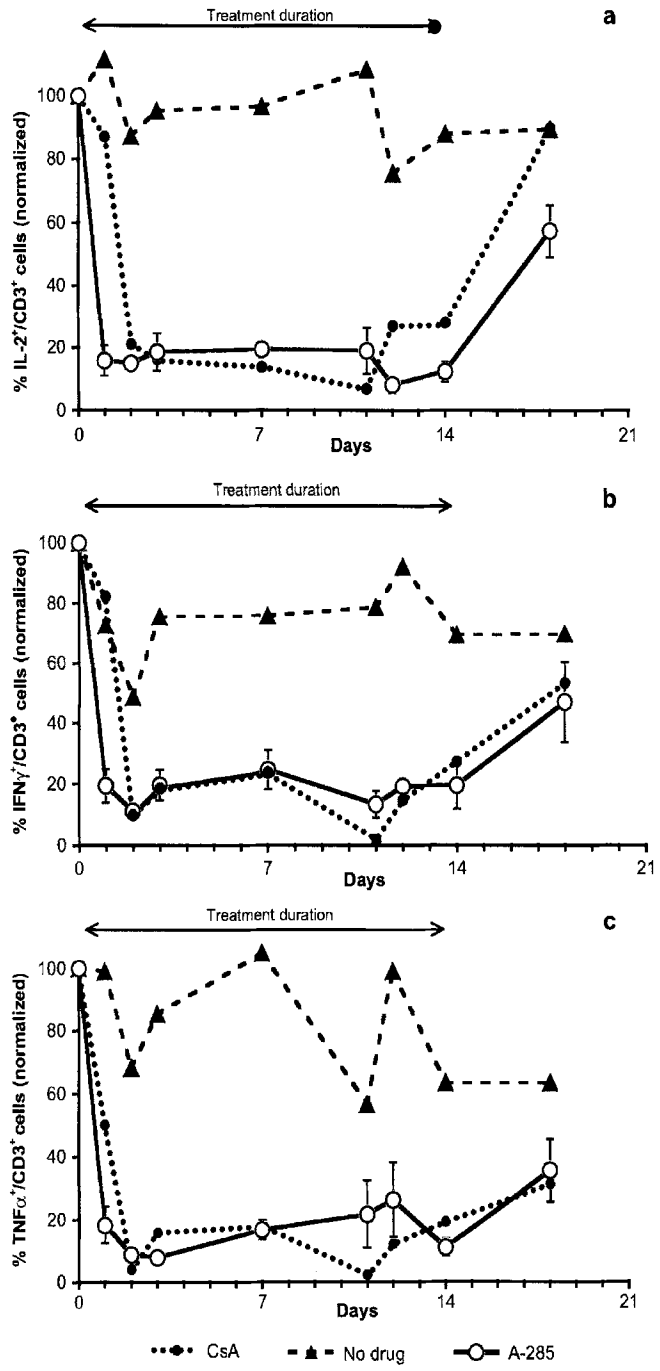

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Fig. 2 Inhibition of intracellular T-cell cytokine production by A-285 in vivo. Three cynomolgus monkeys were treated with A-28522 orally twice daily for 14 days (A-285, *open circles*). One further animal (*filled circles*) was treated with cyclosporine (*CsA*, positive control), a fifth animal (no drug, *triangles*) received no treatment, but was sedated and had blood collected at the same times as the other four treated animals (negative control). Blood samples were obtained at different time-points during the study. Whole blood was stimulated with phorbol 12-myristate 13-acetate and ionomycin in the presence of brefeldin. Flow cytometry identified the percentage of $CD3^+$ cells positive for (**a**) IL-2, (**b**) IFN-γ, or (**c**) TNF-α. Values for the percentage of cytokine-positive $CD3^+$ cells were normalized to 100% (i.e., baseline production of cytokine after mitogen stimulation without immunosuppressive drug treatment). At baseline, the absolute percentages of $CD3^+$ cells positive for IL-2, IFN-γ, or TNF-α were 17.31 ± 1.70, 16.36 ± 2.30, and 20.17 ± 2.62, respectively. Values are mean ± SEM

evaluated was not higher at plasma levels at which toxic side effects occurred (data not shown). Avoidance of toxic side effects is one of the driving forces behind the quest for more selective immunosuppressants than the calcineurin inhibitors. The direct protein target of BTPs is still unknown, and neurotoxicity seen in our experiment may as well be related to the effect of A-285 on an intracellular target other than NFAT. Furthermore, little is known about tissue distribution of BTPs in vivo. Brain penetration of A-285 may be greater than that of cyclosporine, thus leading to neurotoxicity due to its pharmacokinetic properties. If this were true, neurotoxic side effects could be avoided by the development of new BTPs with diminished ability to cross the blood-brain barrier.

Although our preliminary experiments show that A-285 inhibits intracellular T-cell cytokine production in a manner comparable to immunosuppressive concentrations of cyclosporine, its value in preventing allograft rejection needs further investigation. Due to the low potency of BTPs in rodent cells, their value as immunosuppressive agents of the future will need more extensive testing in non-human primates. Our study shows that treatment with BTPs is possible over longer periods of time. Potential toxic side effects correlate with drug plasma concentration and can be avoided by adjustments of the drug dose. At plasma concentrations that are well tolerated, A-285 inhibits T-cell cytokine production in vivo in a manner that parallels cyclosporine. Furthermore, this study illustrates the value of flow-cytometric assays for intracellular T-cell cytokine production in evaluating the pharmacodynamic effects of novel drugs in vivo.

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