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In vivo pharmacokinetic and pharmacodynamic evaluation of the malononitrilamide FK778 in non-human primates

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Abstract The malononitrilamide FK778 is a leflunomide analogue with a shorter half-life than leflunomide. Groups of cynomolgus monkeys were treated orally with various doses of FK778 once daily for 7 days: group A, 10 mg/kg ($n=4$); group B, 5 mg/kg ($n=3$); and group C, one single loading dose of 20 mg/kg followed by 5 mg/kg once daily ($n=2$). Trough plasma concentration of FK778 was measured by HPLC. Lymphocyte proliferation and expression of T-cell activation surface antigens were assessed by flow cytometry. In group A, trough plasma concentration of FK778 reached steady state at 48 h. After 7 days, lymphocyte proliferation was $23 \pm 7.4\%$ (mean ± SEM) and expression of CD71, CD25, CD11a and CD95 on T cells was less than 50% of pre-treatment baseline values. In group B, trough plasma levels of FK778 did not reach steady state, but dropped to near-zero levels after 3 days and on day 7 and lymphocyte proliferation and T-cell surface antigen expression were not different from pre-treatment baseline values. In group C, FK778 trough levels did not reach steady state, but drug exposure was evident over the entire period of treatment, and on day 7, lymphocyte proliferation was $11.4 \pm 8.6\%$ of pre-treatment baseline values. We conclude that FK778 inhibits lymphocyte proliferation and expression of T-cell activation antigens in vivo in non-human primates after 1 week of treatment. These effects are related to the total drug exposure over the time of treatment. At doses lower than 10 mg/kg daily, FK778 is cleared from the circulation between the dosing intervals, thus failing to exert its inhibitory effects on immune functions.

Keywords Malononitrilamides · Leflunomide · Flow cytometry · Non-human primates · Pharmacodynamics · Lymphocyte proliferation

Introduction

FK788 (previously known as HMR 1715, X92 0715, or MNA 715) is structurally similar to A77 1726, the active metabolite of leflunomide. Like A77 1726, FK778 is a malononitrilamide; these compounds are effective immunosuppressants in experimental models of autoimmune diseases and in allotransplantation or xenotransplantation [25]. While leflunomide (Arava™) has been released for clinical use in rheumatoid arthritis [11], the long plasma half-life of A77 1726 in humans (15–18 days) makes the drug undesirable for use in clinical transplantation. FK778 has a shorter plasma half-life in humans (personal communication, H. Lilja,

Fujisawa Healthcare, Deerfield, Ill., USA) and thus holds promise that it might be used in clinical transplantation.

In the past, the effects of FK778 have been studied in vitro in cells from rodents and humans [9, 12, 27], but in vivo studies with this drug have been performed mostly in rodents [17, 18, 20, 21, 22, 23, 24]. Recent studies have shown that FK778 significantly prolongs renal allograft survival in dogs when it is added to cyclosporine [13] or tacrolimus (personal communication, Dr S. Todo, Hokkaido University, Sapporo, Japan). We have recently shown the effects of FK778 on lymphocytes from non-human primates in vitro [2]. The aim of the present study was to evaluate the effect of FK778 on proliferation and various immune functions of lymphocytes from non-human primates in vivo. For this purpose we used whole-blood mitogen-stimulated assays, which we consider to be superior to assays that use isolated T cells or peripheral blood mononuclear cells, because they better approximate components in the circulation and preserve cell–cell interactions. Furthermore, while assays of lymphocyte proliferation measured by tritium-labeled thymidine incorporation have been traditionally employed in the study of cellular immune responses, in the present study we used assays based on flow cytometry, which allowed us to assess not only lymphocyte proliferation, but also expression of various cell surface antigens [1]. We adapted these assays for the use with whole blood from non-human primates and used them for the first time with blood from cynomolgus monkeys treated with various doses of FK778 over 1 week. Furthermore, we measured 24-h trough plasma concentrations of FK778 by HPLC and correlated them with the pharmacodynamic effects with the purpose of expanding our knowledge on the biology of this drug.

Materials and methods

Animals, drugs, treatment, and routine animal care

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 US 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., USA). 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., USA, 1996).

FK778 was a gift from Fujisawa Healthcare, (Deerfield, Ill., USA). The drug was provided in powder form and stored at room temperature in the dark. For treatment, FK778 was suspended in 0.2% hydroxypropylmethylcellulose (HPMC, Sigma, St. Louis, Mo., USA) with the use of a hand-held homogenizing device (Biospec Products, Racine, Wis., USA). The suspension was stored in the dark at 4 °C for no longer than 72 h after preparation. Immediately before administration, the FK778 suspension was homogenized again via a vortex device; each vial of FK778 suspension was used for one dosing only. The calculated volume of FK778 suspension was aspirated into the smallest appropriate syringe and administered without any dilution through a nasogastric tube directly into the stomach. The tube was then rinsed with 20 ml of water.

Vital signs, attitude, fluid intake, urine output, appetite, and amount and consistency of feces were recorded daily during the entire study period. After initial visual assessment, animals were sedated with ketamine at a dose range of 5–10 mg/kg i.m. for treatments and/or blood collections. During the entire study period, animals were fed biscuits, fruit, water, and oral electrolyte solution (Gatorade™).

Experiment design

Groups of cynomolgus monkeys were treated orally with FK778 once daily over 7 days. The treatment groups were as follows: group A ($n=4$), 10 mg/kg FK778 daily; group B ($n=3$), 5 mg/kg FK778 daily; group C ($n=2$), one single loading dose of 20 mg/kg FK778 on the first day of treatment followed by daily doses of 5 mg/kg FK778 on the subsequent 6 days. Different monkeys were used for different study groups. Together with the animals in groups A and B, one additional cynomolgus monkey (negative control), which did not receive any immunosuppressive treatment, was sedated and had blood collected at the same time as the animals treated with FK778. These control animals were sedated daily even when no manipulation or blood collection was scheduled, to ensure that administration of ketamine was identical in all animals. Thus, any immunosuppressive effects in drug-treated animals could be attributed FK778 and not to differences in exposure to ketamine.

Pharmacokinetic and pharmacodynamic assays

Twenty-four-hour trough drug concentrations of FK778 were determined by reverse phase HPLC in plasma samples that were collected daily, immediately before drug administration.

For assessment of lymphocyte proliferation and T-cell activation surface antigen expression, blood was collected at various times before and during treatment. Before we started immunosuppressive drug treatment, blood was collected on two separate days. At inception of the study, blood was also collected immediately before administration of the first drug dose. A mean of the values obtained on these three occasions was used to quantify the baseline of lymphocyte proliferation and T-cell activation surface antigen expression after mitogen stimulation without immunosuppressive treatment. During the study, blood was collected on days 1, 4, and 7, in the morning, immediately before administration of the daily drug dose.

Immediately after collection, whole blood anti-coagulated with heparin was stimulated with concanavalin A (Vector Laboratories, Burlingame, Calif., USA), 7.5 μg/100 μl whole blood) and incubated at 37 °C. After 72 h of incubation, the samples were analyzed via an Epics XL-MCL flow cytometer equipped with an air-cooled argon-ion laser (488 nm) using System II Coulter software (Coulter, Miami, Fla. USA).

Assessment of proliferating cell nuclear antigen/DNA content

After red blood cell lysis, leukocytes were suspended in PBS containing 1% formaldehyde for 5 min. Fixed cells were re-suspended in methanol at 4 °C for 10 min. The cell pellet was then re-suspended in a staining mixture containing 102 μl permeabilizing

buffer (1% heat-inactivated fetal calf serum [HyClone, Logan, Utah, USA], 0.1% saponin and 0.1% sodium azide in PBS), 10 μl RNAse (100 mg/ml in water), 5 μl propidium iodide (1 mg/ml in water, both reagents from Sigma) and 2.5 μl FITC-labeled anti-proliferating cell nuclear antigen (PCNA) monoclonal antibodies (Clone PC10, Dako, Carpinteria, Calif., USA). Cells were incubated in the staining mixture for 25 min at 37 °C. Before analysis, leukocytes were re-suspended in PBS containing 10 μg/ml propidium iodide. Two-color analysis was performed by collection of the FITC signal (PCNA) through a 525-nm band-pass filter and the propidium iodide fluorescence emission through a 635-nm band-pass filter. Total lymphocytes were gated via forward and side light scatter. We set the lymphocyte gate conservatively, to exclude as many dead cells and as much debris as possible. Proliferating cells were identified and enumerated in two-parameter DNA/PCNA distributions as PCNA positive cells with S/G_2M-phase DNA content. Five thousand gated lymphocytes were collected per sample. For simplicity, we refer to these data as percent S/G_2M cells.

Assessment of T-cell surface activation antigen expression

Expression of the following surface activation antigens on T cells was quantified by flow cytometry: transferrin receptor (CD71), IL-2 receptor α chain (CD25), LFA-1 α chain (CD11a), Fas (CD95). To perform three-color flow cytometric analysis, we used the following monoclonal antibodies (all from PharMingen, San Diego, Calif., USA):

- FITC-labeled anti-CD71 + PE-labeled anti-CD3ε + Cy-Chrome-labeled anti-CD25.
- FITC-labeled anti-CD11a + PE-labeled anti-CD95 + PerCP-CY5.5-labeled anti-CD3ε.

After incubation of the blood with the monoclonal antibodies in the dark for 30 min, red blood cells were lysed. Leukocytes were pelleted, washed with PBS, and then re-suspended in 500 μl PBS containing 0.5% formaldehyde.

Emitted light of the fluorochromes was collected through 525-nm (FITC), 575-nm (PE) and 675-nm (PerCP-CY5.5 and Cy-Chrome) 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 IgG1,κ, PharMingen) and used as specificity controls. To distinguish between fluorescence positive and negative cell populations, we set analysis regions, using isotype controls to achieve non-specific binding of < 1% within the positive event regions. For all studied activation antigens, the entire population of lymphocytes was light-scatter gated, and CD3 positive cells were subsequently subgated. The percentages of $CD3^+$ lymphocytes expressing CD71, CD25, CD11a, and CD95 were calculated thereafter.

Calculation of drug effects

Lymphocyte proliferation and T-cell activation surface antigen expression under immunosuppressive drug treatment was expressed as percent of pre-treatment baseline values (i.e., without immunosuppression) using the formula:

$$\text{Immune function}(\%) = (\text{treated}/\text{untreated}) \times 100$$

For each value, "treated" is the percentage of PCNA positive S/G_2M cells or surface antigen positive $CD3^+$ cells at a certain time during the study, while "not treated" is the baseline percentage of the same population of cells (mean of three values obtained before inception of immunosuppressive drug therapy). The same formula was used to express the immune functions as percentage of baseline values obtained before the inception of the study for the two untreated control animals. Although immune functions of these two animals were not assessed simultaneously, we will show the mean values of data obtained from them.

Results

In the animals treated with 10 mg/kg of FK778 (group A), drug plasma concentrations reached steady-state after 2 days of treatment (Fig. 1). Thereafter, mean plasma concentration was 99.4 μg/ml. However, we saw a considerable inter-individual variability in drug exposure (SEM: 27.4).

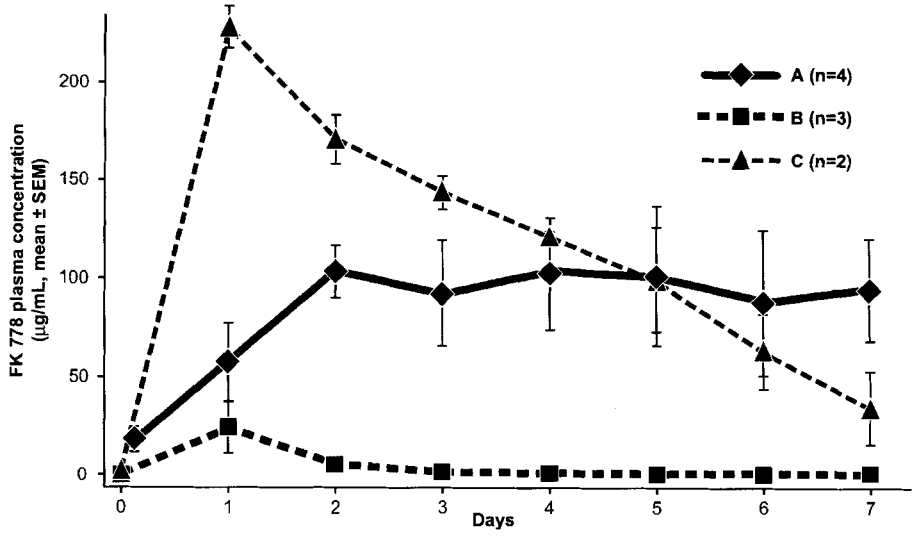

Fig. 1 Trough plasma concentration of FK778. Values are mean ± SEM

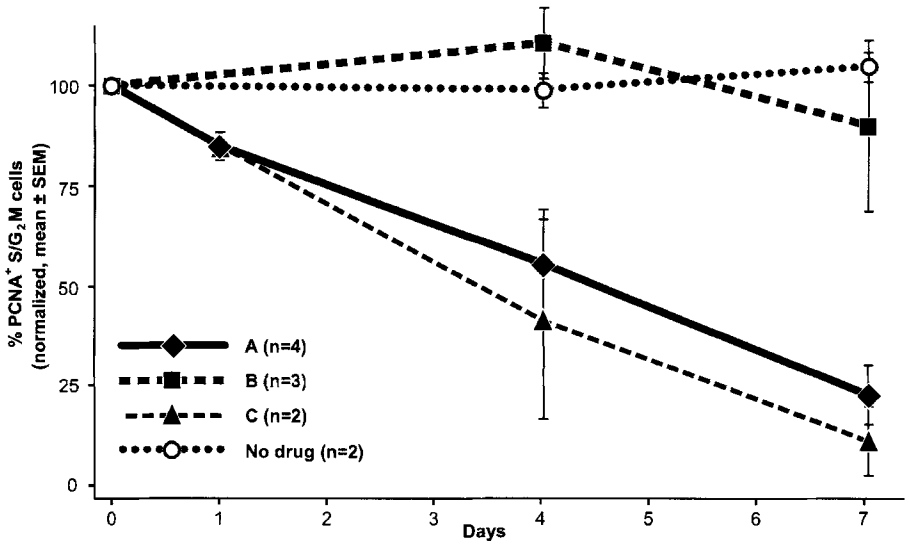

Fig. 2 Inhibition of lymphocyte proliferation by FK778 in vivo. Inhibition of proliferation is expressed normalized to 100% (baseline). At baseline, the absolute percentage of PCNA-positive S/G_2M phase cells was 32.4 ± 2.0. Values are mean ± SEM

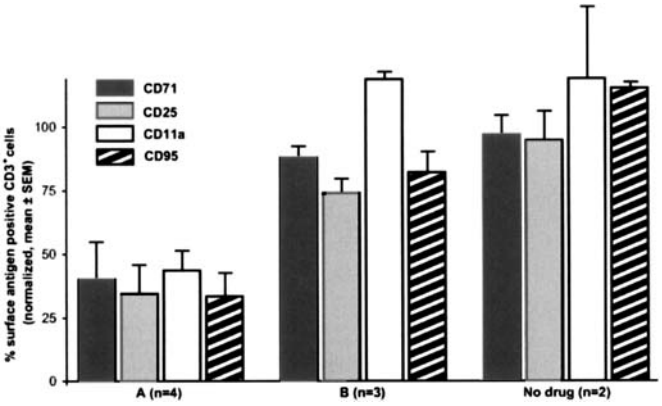

Fig. 3 Inhibition of expression of T cell activation surface antigens in vivo after 7 days of treatment with FK778. Bars represent the percentage of T cells expressing activation surface antigens after 7 days of treatment with FK778 as normalized to 100% (i.e., surface antigen expression before inception of immunosuppressive drug treatment). At baseline, the absolute percentages of T cells positive for CD71, CD25, CD11a, and CD95 were 62.4 ± 6.8, 47.2 ± 5.9, 15.9 ± 1.4, and 43.3 ± 3.3, respectively. Values are mean ± SEM

At 24 h after the first dose of FK778, lymphocyte proliferation was not affected (Fig. 2), but after the animals had been treated for 4 days, lymphocyte proliferation was less than 50% of pre-treatment baseline values, and it was $23 \pm 7.4\%$ (mean ± SEM) of these values after 7 days of treatment. Furthermore, after the animals had been treated with FK778 for 7 days, expression of activation surface antigens on T cells was less than 50% of pre-treatment baseline values (Fig. 3). In the animals treated with 5 mg/kg of FK778 (group B), 24-h trough levels were 24.25 ± 12.97 μg/ml (mean ± SEM). The trough levels dropped over the following 2 days to levels below 1 μg/ml (Fig. 1) and remained at this low level for the rest of the study period. Lymphocyte proliferation and T-cell activation surface antigen expression stayed within 20% of pre-treatment baseline values (Figs. 2 and 3).

When the animals were given an initial loading dose of 20 mg/kg FK778 (group C), 24-h trough levels were 227.5 ± 10.5 μg/ml (mean ± SEM). With the subsequent daily doses of 5 mg/kg FK778, trough plasma drug levels steadily decreased to 34.5 ± 18.5 μg/ml on day 7, without reaching steady-state (Fig. 1). Nevertheless, drug exposure was evident over the entire period of treatment. On day 7, lymphocyte proliferation was $11.4 \pm 8.6\%$ of pre-treatment baseline values in this group (Fig. 2). There was no evidence of a reduction in the absolute number of T cells during treatment with FK778 in either group.

Discussion

This is the first in vivo pharmacokinetic/pharmacodynamic study of FK778 in non-human primates. FK778 inhibits lymphocyte proliferation and T-cell activation in vivo in non-human primates after 1 week of treatment when drug exposure is present throughout the entire treatment period. At doses lower than 10 mg/kg daily, FK778 is cleared from circulation between the dosing intervals, thus failing to exert its inhibitory effect on immune functions.

While it is known that A77 1726, the active metabolite of leflunomide, is extensively protein bound and cleared via several metabolic pathways, including biliary and urinary excretion in humans [15], little is known about the pharmacokinetic characteristics of FK778. We assume that in cynomolgus monkeys at doses less than 10 mg/kg the drug is not capable of saturating its metabolism and is thus cleared from the circulation between the dosing intervals. The fact that steady-state

trough plasma concentrations of FK778 are achieved only when the animals are treated with doses higher than 10 mg/kg is supported by other experiments: when we treated animals with 20 mg/kg over 1 week, FK778 trough plasma concentrations reached steady state after 3 days (mean 240 μg/ml), whereas, when animals were treated with daily doses of 2.5 and 1.0 mg/kg, trough plasma concentrations of the drug, initially evident at 24 h after the first drug dose (average of 16 and 2 μg/ml, respectively), dropped to near-zero values after 3 days (data not shown). It still needs to be clarified whether induction of metabolic enzymes plays a role. Furthermore, data from phase I clinical trials will show whether this pharmacological characteristic is also evident in humans or is specific for non-human primates.

Surprisingly, a pronounced effect on lymphocyte proliferation was found only after 1 week of treatment with 10 mg/kg of FK778, while no effect could be seen at 24 h after the first drug dose. Lymphocyte proliferation was already inhibited after 4 days of treatment, but the inhibitory effect continued to progress until day 7. This finding is in accordance with results of other experiments we performed: when animals were treated with single doses of FK778 ranging from 40 to 150 mg/kg, lymphocyte proliferation did not differ from pre-treatment baseline values, either at 24, or at 48 h, in spite of the presence of drug in the circulation over that period (range of plasma concentration of FK778 at 24 h: 60–370 μg/ml; data not shown). One possible explanation could be that pyrimidines contained in the laboratory diet fed to the animals (Monkey Diet 5038, PMI Nutrition International, Richmond, Ind., USA) were degraded to uridine, which would counteract the effect of FK778.

We interpret the time necessary to register an effect on lymphocyte proliferation to be in relation to the mechanism of action of the drug. Malononitrilamides exert their biological effects through at least two distinct biochemical mechanisms: inhibition of various tyrosine kinases and reversible inhibition of dehydro-orotate dehydrogenase (DHODH), the fourth enzyme in the de novo pyrimidine biosynthesis pathway [4, 5, 6, 7, 8, 19, 31]. Pyrimidines are essential for T-cell proliferation [10]. If the failure of FK778 to inhibit immune functions early in the course of treatment were due to uridine from exogenous sources (metabolism of dietary pyrimidines), this effect should have been ongoing throughout the entire period of treatment, as food intake did not change remarkably during the study. Under treatment with FK778, a certain time may be necessary to deplete existing tissue uridine pools, and this would explain the time lag between inception of treatment and full-scale pharmacodynamic effect. Our findings might confirm the assumption that inhibition of DHODH is the predominant mechanism of action in vivo [11]. However, this interpretation remains speculative, as our study design did not include the measurement of intracellular uridine or pyrimidine levels in lymphocytes during the animals' treatment with FK778.

When FK778 is added to cynomolgus monkey whole blood in vitro, the dose/effect curve is sigmoidal, and both lymphocyte proliferation and T-cell surface antigen expression are in relation to the drug dose [2]. In vivo, the inhibitory effect of FK778 on lymphocyte proliferation after 7 days of treatment is in relation to continuous drug exposure and not to the trough plasma concentration on the 7th day: in group C, an initial loading dose of 20 mg/kg followed by daily doses of 5 mg/kg failed to produce steady state, but ensured continuous drug exposure. Inhibition of lymphocyte proliferation on day 7 was similar to that observed in group A (10 mg/kg), despite lower trough plasma concentrations on day 7 and a lower total drug dose. Furthermore, although in group A trough plasma concentrations showed high inter-individual (but not intra-individual) variability, the degree of inhibition of lymphocyte proliferation was similar for all four animals.

We previously found that, together with lymphocyte proliferation, FK778 inhibits expression of different T-cell activation surface antigens in blood from non-human primates in vitro [2]. Our present results show that in vivo too, FK778 exerts an inhibitory effect, not only on lymphocyte proliferation, but also on various important T-cell activation surface antigens. Clonal expansion of activated T cells depends upon expression of CD25 [14, 28]. CD71 induction is required for DNA synthesis and cell division and is regulated by IL-2 in mitogen stimulated T cells [16]. CD11a and its counter receptor CD54 (ICAM-1) are bi-directionally expressed on antigen-presenting cells and T cells, and their binding provides a co-stimulatory signal for T-cell receptor-mediated activation of T cells [29, 30]. CD95 is expressed together with its ligand on activated T cells, inducing their apoptosis [3]. All these functions were suppressed by FK778 together with lymphocyte proliferation. These effects are probably due to depletion of pyrimidine nucleotides: in previously reported experiments the addition of uridine to rat blood at the same time as malononitrilamides antagonized not only the inhibitory effects of the drugs on lymphocyte proliferation, but also inhibition of T-cell surface antigen expression [26].

Our study proves the value of flow-cytometric whole-blood assays to describe the in vivo effects of an experimental drug on lymphocytes from non-human primates. We show for the first time that in vivo FK778 inhibits not only lymphocyte proliferation, but also T-cell activation surface antigen expression, and that these effects are in relation to total drug exposure. These findings should help us to understand better the biology of malononitrilamides in non-human primates. Furthermore, they underscore the value of pharmacokinetic/

pharmacodynamic evaluation of new drugs before they are tested in expensive large animal transplant models. Finally, our flow-cytometric whole-blood assays may also be used to assess the effects of FK778 in clinical trials.

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