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Effect of peritransplant FTY720 alone or in combination with post-transplant tacrolimus in a rat model of cardiac allotransplantation

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Abstract FTY720 is a recently discovered compound that is derived from the fungus *Isaria sinclairii*. Using a DA donor-to-LEW recipient rat combination, we assessed the efficacy of peritransplant FTY720 alone or in combination with post-transplant tacrolimus on the survival of cardiac allografts. Peritransplant FTY720 given orally at a dose of 5 mg/kg on days –1 and 0 prolonged graft survival from 5 to 13 days ($P < 0.05$). Combining peritransplant FTY720 with post-transplant tacrolimus resulted in a further prolongation of allograft survival. The lymphocyte count in transplanted rats decreased within 24 h to 46.6%. Analysis of lymphocyte subsets by FACS revealed that FTY720 affected the total population of CD3-bearing T cells while the ratio of CD4 to CD8 cells remained unchanged. Kidney and liver biochemistry remained elevated for 2 weeks. In conclusion, FTY720 is a powerful immunosuppressive agent when used as induction therapy and may have an additive effect – perhaps a synergistic one – with post-transplant tacrolimus.

Key words FTY720, tacrolimus, heart transplantation · Tacrolimus, FTY720, heart transplantation · Heart transplantation, FTY720, tacrolimus · Rat, FTY720, heart transplantation

Introduction

FTY720, a compound purified from the culture filtrates of the fungus *Isaria sinclairii*, is the product of a chemical modification of ISP-I [4]. Although ISP-I possesses immunosuppressive properties, it has been found to induce severe digestive toxicity resulting in high animal mortality, precluding its use as a therapeutic agent. Additionally, it was revealed that the modification of ISP-I to FTY720 results in lower toxicity and higher activity than the original product ISP-I [1, 4, 5]. FTY720 has a molecular weight of 343.94 and is a 2-amino-2-[2-(4-octylphenyl)ethyl]-1,3-propanediol hydrochloride. Its chemical structure is different from that of cyclosporin A (CyA), tacrolimus and other currently known immunosuppressants. Detailed mechanisms of action are distinct from conventional immunosuppressants in that FTY720 induces apoptosis of lymphocytes and results in profound lymphopenia [13]. Previous studies have demonstrated that FTY720 prolongs survival of free skin allografts in highly allogeneic rodent models, and its effectiveness is 30-fold greater than CyA [2].

We sought to determine, in a strongly allogeneic rodent model, whether a short peritransplant induction treatment with FTY720 could prolong allograft survival. Additionally, we addressed the question whether grafted animals receiving post-transplant tacrolimus immunosuppression might benefit from peritransplant administration of FTY720. Finally, we evaluated drug tolerability following FTY720 administration as well as its in vivo action on peripheral white blood cells, especially lymphocytes.

Materials and methods

Animals

Specific pathogen-free status inbred rats of DA (RTI[a]) and LEW (RTI[l]) strains (200–250 g) were purchased from Harlan and Olac (Bicester, UK) and Charles River (UK) and cared for under the treatment guidelines established by the Home Office in the UK. Rats were housed in filter-top barrier housing cages in light- and temperature-controlled quarters and received food and water ad libitum. All surgical procedures were carried out aseptically; each animal's postoperative condition was monitored a minimum of twice daily. DA rats served as heart donors to LEW recipients.

Transplant surgical model

Heterotopic cardiac transplantation was performed under halothane anaesthesia (for donor animals) using the technique described by Ono and Lindsey [11] and modified by D'Silva et al. [3]. The modification focused on rapid exsanguination of the donor heart, subsequent perfusion and substantially reducing the quantity of bronchus-associated lymphoid tissue with the graft. LEW recipients were anaesthetized with medetomidine hydrochloride (Domitor, Farmos, Finland), 0.5 mg/kg, and ketamine hydrochloride (Vetalar, Parke-Davis Veterinary, England), 75 mg/kg, administered intraperitoneally. LEW recipients were reversed after surgery using atipamezole hydrochloride (medetomidine antidote) (Antiseden, Farmos, Finland) at a dose of 2.5 mg/kg subcutaneously. The cold ischemic time was less than 30 min. Transplanted (TX) hearts that did not beat strongly immediately after reperfusion and for 2 days post-transplant were excluded from the present analysis. Rats were weighed daily and observed clinically for any adverse event.

End points

TX hearts were graded daily for rejection using a previously validated heart-beat scoring system [ranging from 4 (normal) to 0 (rejection)] described by Morrissey et al. [10]. Based upon daily abdominal palpation, graft survival was defined as the last day of palpable organized contractions. Rejection was defined as complete cessation of heart beat and confirmed at necropsy and by histological examination.

Immunosuppressive drugs

FTY720 (Yoshitomi Pharmaceutical Industries, Osaka, Japan) was provided as a white crystalline powder. For in vivo use, the drug was dissolved in physiological saline. Tacrolimus (Fujisawa Pharmaceuticals, Munich, Germany) was also dissolved in physiological saline. Both drugs were given orally by gavage using a stainless steel cannula in order to deposit the drug directly into the stomach. In this study FTY720 was given on the day before (day −1) and the day of transplantation (day 0) only. When tacrolimus was employed in the treatment schedule, it was administered daily for a 10-day treatment course beginning on the day of transplantation; immunosuppression was withdrawn on postoperative day (POD) 10 and grafts were subsequently observed for rejection.

Hematological parameters

White blood cell counts and differential counts were determined using peripheral blood obtained from tail vein sampling and cardiac puncture in TX ($n = 10$) and non-TX control animals ($n = 10$) before and after two consecutive doses of 5 mg/kg FTY720.

Body weight

To assess drug tolerability, changes in body weight were recorded in TX and non-TX animals that received two doses of 5 mg/kg FTY720 over a period of 2 weeks. Age- and sex-matched normal LEW rats ($n = 8$) served as controls.

Kidney and liver function tests

To further delineate the potential toxicity of FTY720, separate cohorts of animals (non-TX and TX) were sampled for serum on day −1 (predosing) and on days 4 and 13 (postdosing) following administration of two doses of 5 mg/kg FTY720. Kidney function [blood urea nitrogen (BUN), creatinine] and liver function [aspartate transaminase (AST), alanine transaminase (ALT), alkaline phosphatase (ALP), bilirubin] were assessed using an automated system.

Quantitation of lymphocyte subsets by fluorescence-activated cell sorting (FACS)

FACS analysis of peripheral blood mononuclear cells (PBMNC) was performed on cells obtained from control (untreated LEW) rats or from rats 1, 4 or 8 days following administration of two doses of 5 mg/kg FTY720. PBMNCs were separated from whole blood (4–9 ml/animal) using lymphoprep, according to the manufacturer's protocol (Nycomed Pharma, Oslo, Norway). Cells were prepared for FACS analysis as follows:

1. 4 × 50 µl aliquots of cell suspension were dispensed into LP3 round-bottomed tubes containing 50 µl of either (a) buffer alone (no primary antibody control), (b) mouse monoclonal anti-CD3 antibody diluted 1:25, (c) mouse monoclonal anti-CD4 antibody diluted 1:250 or (d) anti-B cell antibody. Incubation was continued for 1 h at room temperature. Monoclonal antibodies were obtained from Sera Labs UK (CD3, CD4, CD8) and Dako, UK (B cell).
2. Cells were washed (x3) in phosphate-buffered saline (PBS) containing 1 % fetal calf serum and 0.1 % w/v sodium azide and resuspended in 100 µl of fluorescence-labeled rabbit antimouse immunoglobulins (Dako, UK) at a dilution of 1:200. Incubation was continued for a further 1 h. The cells were washed thrice and resuspended in 0.5 ml of 1 % paraformaldehyde in PBS.

Experimental design

Six transplant groups were studied according to the administration of peritransplant FTY720 alone or in combination with post-transplant tacrolimus (Table 1).

Statistical analysis

Allograft survival data were represented as median (range) in days and compared by K-wallis and Mann-Whitney U-tests. Statistical analysis for quantitation of lymphocyte subsets by FACS was also

Table 1 Experimental design. Effect of FTY720 on cardiac allograft survival in the presence and absence of tacrolimus

Groups	Treatment and duration		Graft survival (days)	Median (range, days)	*P* value
	FTY720	Tacrolimus			
1. $n = 5$	–	–	4, 5, 5, 5, 5	5 [4–5]	
2. $n = 6$	5 mg/kg, day-1 and 0	–	11, 11, 13, 13, 16, 16	13 [11–16]	< 0.05 vs 1
3. $n = 10$	–	1 mg/kg, days 0–9	6, 6, 7, 11, 11, 12, 15, 20, 24, 29	11.5 [6–29]	NS vs 2
4. $n = 6$	5 mg/kg, day-1 and 0	1 mg/kg, days 0–9	15, 20, 20, 21, 21, 21	20.5 [15–21]	0.09 vs 3; 0.0007 vs 2
5. $n = 6$	–	2 mg/kg, days 0–9	18, 19, 22, 23, 24, 26	22.5 [18–26]	0.003 vs 2; NS vs 4
6. $n = 6$	5 mg/kg, day-1 and 0	2 mg/kg, days 0–9	20, 26, 26, 28, 38, 38	27 [20–38]	0.003 vs 2; 0.02 vs 4 0.03 vs 5

performed using the Mann-Whitney U-test. Serum biochemistry was analyzed using the one-way ANOVA and *t*-test. Body weight data were examined using linear regression analysis and the *F*-test for variances. A two-tailed *P* value below 0.05 was considered significant.

Results

Effect of FTY720 on cardiac allograft survival (Table 1)

Median graft survival time in nonimmunosuppressed control animals was 5 days (range 4–5 days), indicating that the DA-to-LEW strain combination is strongly allogeneic and that rejection proceeds vigorously in the absence of immunosuppression. A short induction protocol treatment with FTY720 significantly prolonged cardiac allograft survival from 5 to 13 days (range, 11–16 days, $P < 0.05$). Peritransplant treatment with FTY720 proved to be more efficacious in prolonging allograft survival (median graft survival 13 days) compared to the outcome obtained in recipients who received a 10-day course of tacrolimus, 1 mg/kg post-transplant (median graft survival 11.5 days, $P > 0.05$). However, a 10-day post-transplant treatment with 2 mg/kg tacrolimus was more efficient than peritransplant FTY720 (median graft survival 22.5 days vs 13 days, respectively, $P < 0.05$).

Effect of FTY720 on cardiac allograft survival in the presence of tacrolimus (Table 1)

Combining peritransplant FTY720 with post-transplant tacrolimus, 1 mg/kg, resulted in further allograft survival as compared to FTY720 treatment alone (median graft survival 20.5 days vs 13 days, respectively, $P = 0.0007$). Although strong numerical differences in median graft survival were observed between recipients of combination therapy versus recipients treated with tacrolimus alone (median graft survival 20.5 days vs 11.5 days, respectively), this did not achieve statistical significance but rather demonstrated a trend ($P = 0.09$). Likewise, combining peritransplant FTY720 with post-transplant tacrolimus, 2 mg/kg, resulted in even further allograft survival as compared to either treatment alone (median graft survival 27 days vs 13 days of FTY720 monotherapy, $P = 0.003$, and 22.5 days of tacrolimus, 2 mg/kg, monotherapy, $P = 0.03$, respectively). Moreover, the combination of FTY720 and tacrolimus, 2 mg/kg, prolonged graft survival beyond that observed for the combination that employed tacrolimus, 1 mg/kg ($P = 0.02$).

White blood cell count and lymphocyte count

Following administration of FTY720, the white cell count decreased dramatically in both TX and non-TX animals. The nadir was achieved on day 3 post-treatment and gradually recovered thereafter, albeit to a slower extent in non-TX animals (Fig. 1). Mean lymphocyte count reached a minimal value on day 1 post-treatment (46.6% and 53% in TX and non-TX rats, respectively), remained low until day 3 and gradually recovered thereafter. Lymphocyte count returned to pretreatment levels by day 13 (Fig. 2).

FACS analysis of lymphocyte subsets

PBMNCs from untreated control animals were shown to contain on average 29.4% CD3-positive cells by FACS analysis. Following administration of two doses of FTY720, the percentage of CD3-bearing cells had fallen to 9.1%, a value that was statistically lower than in control animals ($P < 0.0006$). The reduced CD3 positivity persisted and had not recovered by the last sampling time point (day 8), remaining at 7.9% on average. The ratio of CD4 to CD8-positive cells remained constant, as did the percentage of B cells. These data are summarised in Table 2.

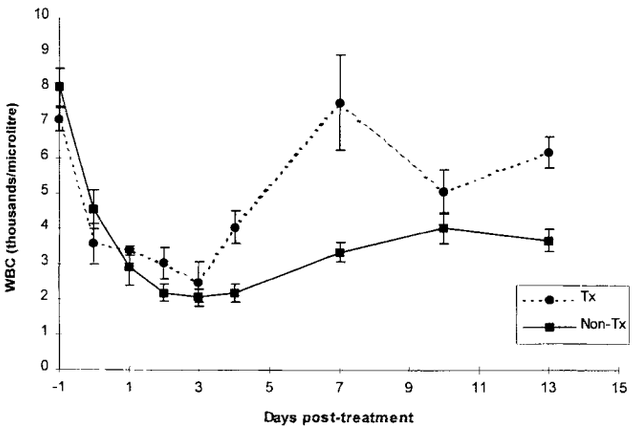

Fig. 1 The effect of FTY720 on white cell count in peripheral blood. Peripheral white cell counts (mean ± SD) following administration of 5 mg/kg FTY720 for 2 consecutive days (day –1 and day 0), were examined at days –1, 0, 1, 2, 3, 4, 7, 10 and 13, respectively. Lowest values were recorded on post-treatment day 3

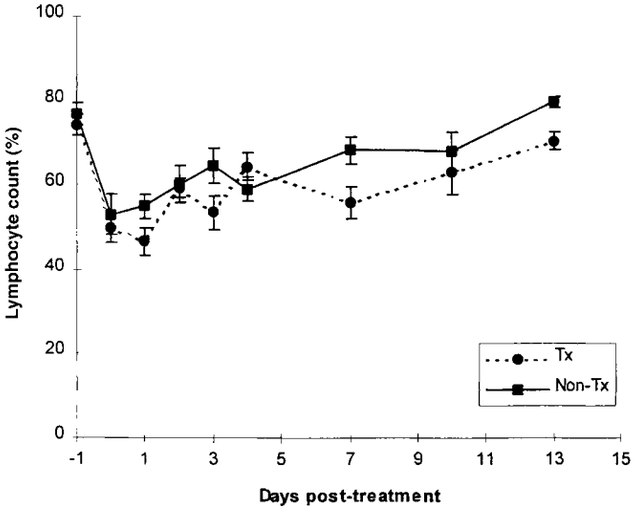

Fig. 2 The effect of FTY720 on lymphocyte count in peripheral blood. Lymphocyte counts (mean ± SD) following administration of 5 mg/kg FTY720 for 2 consecutive days (day –1 and day 0), were examined at days –1, 0, 1, 2, 3, 4, 7, 10 and 13, respectively. Lymphocyte counts in TX and non-TX animals dropped to 46.6% and 54% at post-treatment day 1, respectively

Body weight (Fig. 3)

Linear regression analysis of the growth curves demonstrated that non-TX and TX animals receiving FTY720 weighed significantly less than an age- and sex-matched cohort of LEW rats ($P < 0.05$, $r^2 = 0.83$). Furthermore, TX animals who received FTY720 weighed much less than animals that received FTY720 only ($P < 0.05$, $r^2 = 0.85$). This difference in growth weight curves persisted for the first 8 days post-transplantation after which, nearer rejection, a significant decrease in body weight was again evident ($P < 0.05$, $r^2 = 0.66$). FTY720 at the dose of 5 mg/kg was well tolerated according to the induction schedule adopted in this study. During a 2-week observation period, non-TX and TX rats treated with two doses of 5 mg/kg FTY720 gained 8% and 5% of their preoperative body weight, respectively.

Table 2 Effect of FTY720 on lymphocyte subsets – FACS analysis of peripheral blood mononuclear cells

	Control	Day 1	Day 4	Day 8
CD3	29.4 ± 6.4	9.1 ± 2.4*	7.7 ± 3.1*	7.9 ± 2.8*
CD4 : CD8	1.7 ± 0.2	1.7 ± 0.5	1.6 ± 0.6	1.5 ± 0.4
B cells	8.9 ± 1.0	6.3 ± 1.1	5.4 ± 2.2	7.8 ± 1.3
n	8	7	7	7

CD3 and B cell data represent the positive staining cells for untreated control animals versus treated animals at the four time points following FTY720 administration. The ratio of CD4 : CD8 positive cells is also shown. All values represent mean ± SD, * $P < 0.0006$ for control vs post-treatment days 1, 4 and 8

Kidney and liver function tests

After two doses of 5 mg/kg of FTY720, BUN and creatinine remained elevated at both POD 4 and POD 13, irrespective of whether the animals were grafted or not (Table 3a).

Treatment with two consecutive doses of FTY720 to non-TX and TX animals elevated AST, ALT and ALP levels to above normal by post-treatment day 4, showing a statistically significant increase. In addition, these biochemical abnormalities persisted until post-treatment day 13 compared to controls and demonstrated a statistical trend compared to post-treatment day 4 levels for AST ($P = 0.07$); ALT ($P = 0.05$) and ALP ($P = 0.08$). Serum bilirubin remained within the normal range in both non-TX and TX animals (Table 3b).

Discussion

The present success of organ transplantation is largely due to advances in immunosuppressive therapy. The strategy to combat allograft rejection is to enhance therapeutic efficacy while reducing the toxicity of individual drugs in the regimen. FTY720, a recently synthesized compound, possesses unique immunosuppressive properties and mechanisms that are distinct from those of CyA and tacrolimus. Various in vivo experimental studies on FTY720 have demonstrated its powerful immunosuppressive properties which are said to be 30-fold

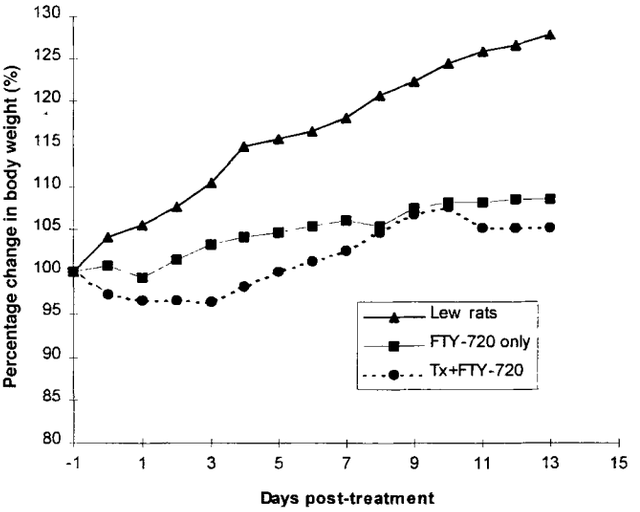

Fig. 3 Percentage change in body weight (as a percentage of the preoperative weight). Percentage change in body weight was recorded for 15 days. FTY720, 5 mg/kg, was given orally for 2 consecutive days (day −1 and day 0). Each group comprised eight animals. Body weight change after administration of FTY720 in non-TX and TX animals was significantly lower than in an age- and sex-matched cohort of LEW rats ($P < 0.05$, $r^2 = 0.83$). TX animals who received FTY720 weighed much less than those receiving FTY720 only ($P < 0.05$, $r^2 = 0.85$). Values of percentage change in body weight are expressed as mean

greater than those of CyA [2, 8]. Notably, no significant drug-related toxicity was detected. These studies also suggest that this agent does not intercept antigen-induced IL-2 production but instead leads to lymphopenia mediated by apoptotic cell death [2, 8, 13].

Rats receiving a single oral dose of 10 mg/kg FTY720 showed a profound and lasting decrease in peripheral lymphocyte counts [13]. Initial experiments by our group demonstrated that 5 mg/kg FTY720 (as a 2-day course) also induced a profound drop in mean lymphocyte counts from 80% to 46.6% within 24 h. With no further administration of FTY720, these counts remained significantly depressed but returned to pretreatment levels within 14 days. An identical pattern was observed in TX animals under the same schedule of FTY720. The lymphopenic effect of FTY720 is profound and lasting, but reversible. More detailed knowledge of the nature of the lymphopenic effect of FTY720 was gained by FACS analysis of the lymphocyte subsets in the PBMNC fraction of leucocytes. These data indicated that FTY720 treatment results in a marked reduction in CD3-positive lymphocytes (predominantly T cells) that broadly reflects the drop in lymphocyte counts (Table 2, Fig. 2). The ratio of CD4 to CD8 cells remained constant and within a range that is considered normal for humans. In addition, the percentage of B cells within the PBMNC fraction remained unchanged. Together these data suggest that FTY720 exerts its immunosuppressive effect by causing a substantial and specific depletion in the T-cell population.

We used FTY720 as a short induction treatment (in lieu of maintenance therapy) in order to avoid the potentially negative impact of sustained lymphopenia. Furthermore, continuous dosing with FTY720 may not be essential in light of its lymphopenic effect persisting for nearly 14 days. Recent clinical observations prove that prolonged treatment with lymphocyte-targeted therapies (e.g. antithymocyte globulin) may not be essential in preventing kidney transplant rejection in humans [14].

In vivo experiments were designed using a strongly histoincompatible system (DA x LEW). FTY720 proved effective when used as induction therapy in a mode of rejection prophylaxis; these data support previous observations. DA heart graft survival was significantly prolonged from 5 (control) to 13 days after receiving only two doses of peritransplant FTY720. Although the magnitude of this prolongation was not dramatic in this stringent model, this phenomenon is exceedingly difficult to achieve, based on our previous experience. Different compounds, e.g. CyA and tacrolimus, have failed to produce extended graft survival or tolerance [3]. In comparison, graft survival after a 2-day course of FTY720, 5 mg/kg, was equivalent to a full 10-day post-transplant course with tacrolimus, 1 mg/kg (13 days vs 11 days, $P > 0.05$). Using an identical immuno-prophylaxis regimen, Suzuki et al. proved that graft survival of the liver was greater than that of the heart (11.5 vs 28.5 days, respectively) [13]. This differential effect of FTY720 on heart versus liver allografts probably reflects the known tolerogenic nature of the liver and the greater ease of its engraftment.

We then determined the effect of a short induction course with FTY720, 5 mg/kg, on cardiac allograft survival in the presence of tacrolimus (1 mg/kg or 2 mg/kg). This mode of dual therapy resulted in further prolongation of allograft survival, suggesting the possibility that both drugs may have an additive, and perhaps synergistic, effect. Other investigators have also convincingly demonstrated a similar phenomenon using FTY720 in combination with CyA in rat skin [2] and heart [6] allografts and in canine kidney [7, 12] transplants. The results of these later investigations ruled out the possibility that the observed synergy was due to altered pharmacokinetic profiles [7, 13]. It follows, therefore, that the respective doses of each drug used in combination can be reduced, consequently reducing the potential for detrimental side effects. The relevance of these important observations needs to be elucidated with additional studies using longer term dosing (for agents such as CyA and tacrolimus) so as to verify the relevance of these findings to the human situation.

Table 3a Kidney function biochemical data (mean ± SD)

Group	Post-treatment day 4		Post-treatment day 13	
	BUN (mg/dl)	Creatinine (mg/dl)	BUN (mg/dl)	Creatinine (mg/dl)
1. Control LEW	16.3 ± 2.4	0.13 ± 0.04		
2. FTY720 (5 mg/kg)	33.2 ± 5.9*	0.3 ± 0**	35 ± 2*	0.38 ± 0.04**
3. TX + FTY720 (5 mg/kg)	32 ± 6.7*	0.45 ± 0.05**, ***	37.2 ± 0.5*	0.4 ± 0**

* $P < 0.05$ vs group 1 at POD 4 and POD 13; P = NS between groups 2 and 3 at POD 4 and POD 13 (BUN); ** $P < 0.05$ vs group 1 at POD 4 and POD 13; *** $P < 0.05$ vs group 2; P = NS between groups 2 and 3 at POD 13 (creatinine)

Table 3b Liver function biochemical data (mean ± SD)

Group	Post-treatment day 4			Post-treatment day 13		
	AST*[1] (IU/ml)	ALT*[1] (IU/ml)	ALP*[1] (IU/ml)	AST*[2] (IU/ml)	ALT*[2] (IU/ml)	ALP*[2] (IU/ml)
1. Control LEW	39.5 ± 18.8	16.8 ± 6.6	24.4 ± 0.6			
2. FTY720	44 ± 6.6	29.5 ± 7.3	111.1 ± 22.4	54.2 ± 9.9*[3]	37.6 ± 3.7*[4]	134.6 ± 15.9*[5]
3. TX + FTY720	59.5 ± 42.6	26 ± 6.1	113.5 ± 25.4	148.2 ± 141.4	31.5 ± 4.7	103.7 ± 8.3

*[1] $P < 0.05$ for control vs groups 2 and 3 post-treatment day 4. P = NS for groups 2 vs 3 for AST, ALT and ALP; *[2] $P < 0.05$ for control vs groups 2 and 3 at post-treatment day 13; *[3] $P = 0.07$; *[4] $P = 0.05$; *[5] $P = 0.08$ vs post-treatment day 4 values for group 2 AST, ALT and ALP, respectively

Drug tolerability is pivotal in the development of a novel agent for transplantation. There was no animal morbidity or mortality related to FTY720 administration in our study. Analyses of growth curves indicated that FTY720 did not adversely reduce body weight, although the velocity of growth in treated cohorts was retarded compared to matched normal cohorts. Others have also reported significant inhibition of body weight, but no mortality, when normal or skin- and heart-grafted rats received 14 consecutive daily doses of FTY720, 10 mg/kg, [2, 6, 13]. However, one dose of FTY720, 10 mg/kg, was found to be lethal when given pretransplant to liver recipients. Studies in dogs indicate that doses up to 5 mg/kg were well tolerated for periods up to 90 days [2, 7].

Importantly, we found that kidney and liver function tests remained abnormally elevated for as long as 2 weeks following FTY720 treatment. This finding was unexpected in view of the fact that animals only received a 2-day course of FTY720. Although other small and large animal studies indicate that FTY720 has few side effects and is generally well tolerated, more detailed toxicological studies, particularly in higher species, are obligatory before FTY720 can be used in humans.

In view of FTY720's performance in rejection prophylaxis, further studies were performed in our laboratory that demonstrated that delayed administration of FTY720 postponed ongoing cardiac allograft rejection, suggesting an important role of FTY720 as a rescue therapy agent (data not shown). Others have shown this property of FTY720 in rat liver allografts [12]. A recent study demonstrated the potential of FTY720 to combat the vigorous alloimmune response elicited by intestinal allografts [9].

Further studies should address not only the role of FTY720 in controlling rejection of highly immunogenic organs (pancreas, small bowel) but also the assessment of its tolerogenic potential. Newly developing lymphocytes appear after FTY720 administration [2]. These immature lymphocytes may not only have the potential to undergo negative selection upon adequate exposure to donor antigen, but may also lack some accessory receptors required for full recipient T-cell activation [2]. Whether these mechanisms account for the immunosuppressive activity of the compound, and to what extent they can be used to promote long-term graft acceptance, warrants further investigations.

In summary, these results indicate that FTY720 is a powerful immunosuppressant with a marked antirejection effect when used as induction therapy and that it may have an additive and perhaps synergistic, effect with post-transplant tacrolimus. FTY720 induces a rapid, substantial and reversible lymphopenia and acts specifically on T lymphocytes. Although FTY720 did not result in any mortality in our study, its adverse effects on kidney and liver function tests are of concern and encourage further studies in large animals before it can be used clinically.

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