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Prevention of graft rejection and graft-versus-host reaction by a novel immunosuppressant, FTY 720, in rat small bowel transplantation

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Abstract In the present study, we examined the immunosuppressive effect of a new drug, FTY 720, on small bowel transplantation (SBT) in rats. Grafts from (LEW × BN) F1-to-LEW rats treated with FTY 720 at 0.5 mg/kg from day 0 to 14 post-SBT survived significantly longer than untreated grafts. In addition, the administration of FTY 720 combined with cyclosporin (CyA; 5 mg/kg per day) had a synergistic effect on allograft survival. The graft-versus-host reaction (GVHR) that occurred in the LEW-to-F1 rats was markedly reduced after the administration of FTY 720. FTY 720 combined with a low dose of CyA completely abrogated GVHR without any adverse reaction. FTY 720 treatment resulted in a significant decrease in the number of lymphocytes in the peripheral blood and the spleen, but the number of peripheral neutrophils was unchanged. Thus, FTY 720 would appear to be an ideal drug to combine with CyA in order to control the immune reaction after SBT.

Key words Immunosuppression, FTY 720, rat, small bowel transplantation · FTY 720, immunosuppression, rat, small bowel transplantation · Rat, small bowel transplantation, FTY 720 · Small bowel transplantation, rat, FTY 720

Introduction

It is well known that small bowel transplantation (SBT) may induce two types of immunological reactions in graft recipients: the host-versus-graft reaction (HVGR) and the graft-versus-host reaction (GVHR). The prevention of both types of reactions is a key requirement for successful SBT [6]. SBT has recently become feasible in humans using new immunosuppressive drugs including cyclosporin A (CyA) and FK 506 [3, 12]. Nevertheless, it is still more difficult to control the immune reaction after SBT than after transplantation of other organs, especially in the late stages of transplantation [13, 16].

FTY 720 is a chemical substance derived by modifying an immunosuppressive metabolite from *Isaria sinclairii,* and its chemical structure is completely different from conventional immunosuppressants [1]. Rat spleen cells incubated with FTY 720 have demonstrated characteristics of apoptosis, such as the absence of surface microvilli, chromatin condensation, and the formation of apoptotic bodies by electron microscopy, as well as genomic DNA fragmentation by agalose gel electrophoresis [8]. The induction of apoptosis was seen specifically in the lymphocyte population, but not in bone marrow-derived cells [2, 8]. Thymocytes from MRL-1pr/1pr mice with a mutant Fas gene were sensitive to FTY 720, indicating that the drug-induced apoptosis was not related to Fas antigens [10]. The intracellular ratio of bcl-2 to Bax proteins in the FTY 720-treated lymphocytes was decreased by the enhanced expression of Bax immediately after the drug treatment [9]. Thus, the drug displays bc1-2-associated apoptosis in lymphocytes. We have also shown that the drug has a potent immunosuppressive effect that prolongs graft survival in rat heart and liver recipients and in canine kidney recip-

ients [7, 8, 11]. It is worth noting that FTY 720 has a wide therapeutic window and induces no severe side effects in skin-allografted rats receiving oral doses ranging from 0.1 to 30 mg/kg as well as in heart-allografted rats at doses between 0.05 and 10 mg/kg [1, 7].

The purpose of the present study was to determine whether FTY 720 also presents HVGR and GVHR in rat SBT when used alone or in combination with CyA.

Materials and methods

Animals

Male LEW and (LEW × BN)F1 rats were purchased from Seiwa Experimental Animals (Fukuoka, Japan) and kept in a departmental animal room under specific pathogen-free conditions. Experiments were conducted using 12- to 18-week-old rats in accordance with the rules of the Animal Ethics Committee of National Children's Medical Research Center (Tokyo, Japan).

Surgical procedure

Heterotopic SBT was performed in the recipients under ether anesthesia using the technique with polyethylene cuff anastomosis, as described previously [5]. Briefly, the terminal ileum (10 cm long), together with the superior mesenteric artery and the aorta and the portal vein, was isolated from the donor and anastomosed to the left renal artery and vein of the recipient after removal of the kidney. Both ends of the grafted ileum were exteriorized as stomas in order determine graft viability, and the stomas were carefully cleansed of intestinal exudate daily to prevent occlusion. Complete necrosis of the stoma was regarded as the end point of rejection, as reported previously [14]. GVHD was diagnosed when there was evidence of weight loss redness, of skin, hair loss, and a hunched posture [5], and the day of recipient death was regarded as the end point of GVHD. Recipients showing necrosis in the stomas by postoperative day (POD) 5 were excluded from the present study as technical failures. The animals were autopsied in order to evaluate the etiology of their death.

Immunosuppression

FTY 720 was donated by Yoshitomi Pharmaceutical Industries (Osaka, Japan) as dry powder and dissolved in distilled water at a concentration of 0.1 mg/ml. CyA was supplied from Sandoz as dry powder and dissolved in olive oil at a concentration of 2 mg/ml. The drugs were administered orally via a gastric tube at a daily dose of 0.5 mg/kg (FTY 720) and/or 5 mg/kg (CyA) starting on the day of operation (day 0) and continuing for 14 days (day 14).

Cell count and flow cytometric analysis

Lymphocytes from the peripheral blood, spleen, and thymus of LEW rats treated with FTY 720 at a dose of 0.5 mg/kg for 15 days were taken 1 day after the final administration of the drug. Peripheral lymphocytes were purified by Ficoll-Conray gradient centrifugation and the spleen and thymus were minced and passed through 60-mesh stainless steel. The cells were then suspended in RPMI 1640 medium. The splenic cells were further purified by Ficoll-Conray gradient centrifugation. The splenic and thymic lymphocytes were calculated as the number per 400 g of body weight. The lymphocytes were then incubated with reagents for staining: R73 (mouse IgG1, MCA453, Serotec, UK) for T-cell receptors (TCR), OX33 (mouse IgG1, MCAP340, Serotec, UK) for CD45, FITC-conjugated W3/25 (mouse IgG1, MCA55F, Serotec, UK) for CD4, and biotinylated OX-8 (mouse IgG1, MCA48B, Serotec, UK) for CD8. FITC-conjugated anti-mouse rabbit IgG (STAR41, Serotec, UK) was used as the secondary antibody for R73 and OX33, and phycoerytherin-conjugated streptavidin (No. 9023, Becton Dickinson, USA) was used for biotinylated OX-8. Flow cytometric analysis was conducted using a FACScan (Becton Dickinson, USA)

Cellular study in parent-to-F1 combination

Lymphocyte numbers and subpopulations in normal and grafted F1 rats with or without 7-day administration of FTY 720 (0.5 mg/kg) were studied using flow cytometry. The splenic and peripheral blood lymphocytes were collected on POD 7 and analyzed with the same method mentioned above.

Statistics

All data were analyzed with a one-way ANOVA and either Gehan's generalized Wilcoxon test or Sheffe's test, using STATISTICA for Macintosh (StatSoft, USA). A difference was regarded as significant when the P-value was less than 5 %.

Results

Effect of FTY 720 on graft survival in the semiallogeneic combination

To evaluate the effect of FTY 720 on graft rejection while avoiding the influence of GVHR, the combination of (LEW × BN) F1 donor and LEW recipient was used. In this combination, a large number of donor lymphocytes existing in the ileum graft do not respond to recipient antigens since donor F1 rats share parental genetic traits. The results obtained are summarized in Table 1. None of the grafts in the syngeneic combination (group A) demonstrated any abnormal findings in the stomas after more than 100 days. These grafts did exhibit a slight atrophy of the villi, but there was no evidence of inflammation in the submucosal area upon histological examination. The untreated control grafts (group B) were completely rejected on day 8 (median). However, when the recipients were orally administered FTY 720 (group C), graft survival was significantly prolonged (median 20 days). The immunosuppressive effect of FTY 720 was more potent than that of low-dose CyA; the median survival time in the CyA-alone group (group D). was 14.5 days. The treatment consisting of FTY 720 in combination with CyA (group E) was the most effective in terms of graft survival (median 32 days). None of the recipient rats in the abovementioned

Table 1 The survival of small bowel grafts in LEW recipients after the administration of FTY720 and/or cyclosporin

Group	Donor	Immunosuppression[a]		(*n*)	Graft survival[b] (Days)	Median survival time (Days)	Statistics[c] *P* value
		FTY720	Cyclosporin				
A	LEW	(–)	(–)	10	$>100\times10$	>100	
B	F1	(–)	(–)	10	$7\times4, 8\times5, 9$	8	
C	F1	(+)	(–)	10	$14, 17\times2, 20\times3, 21\times2, 22\times2$	20	< 0.01 vs B < 0.01 vs D
D	F1	(–)	(+)	6	$11, 13, 14, 15\times2, 19$	14.5	< 0.01 vs B
E	F1	(+)	(+)	6	28, 29, 31, 33, 34, 42	32	< 0.01 vs B < 0.01 vs C
F	F1	(–)	(+)	6	$7^d, 7^d, 8^d, 9^d, >100\times2$	8	

[a] FTY720 and/or cyclosporin were orally administered at a dose of 0.5 mg/kg and 5 mg/kg, respectively for 15 days from the day of operation. In group F, recipients received 15 mg/kg of CyA alone
[b] The total necrosis of the stoma was regarded as the end point of the rejection
[c] *P*-values were determined by Gehan's generalized Wilcoxon test after a one-way ANOVA
[d] Represents the day of animal death due to hemorrhagic pneumonia

Table 2 Recipient (LEW × BN) F1 rat survival after parental LEW rat small bowel transplantation after the administration of FTY720 and/or cyclosporin

Group	Donor	Immunosuppression[a]		(*n*)	Graft survival[b] (Days)	Median survival time (Days)	Statistics[c] *P* value
		FTY720	Cyclosporin				
G	LEW	(–)	(–)	10	$14, 15, 16\times2, 18, 19, 22\times2, 31, 32$	>100	
H	LEW	(+)	(–)	12	$41, 42\times2, >100\times9^d$	>100	< 0.01 vs G < 0.01 vs I
I	LEW	(–)	(–)	6	$22, 23, 25, 26\times2$	24	
J	LEW	(+)	(+)	6	$>100\times6^e$	>100	< 0.01 vs G < 0.01 vs H < 0.01 vs I

[a] FTY720 and/or cyclosporin were orally administered at a dose of 0.5 mg/kg and 5 mg/kg, respectively, for 15 days from the day of operation
[b] GVHD was diagnosed by daily observation of body weight loss, skin redness, hair loss, and hunched postures. The date of death was regarded as the end point of GVHD
[c] *P*-values were determined by Gehan's generalized Wilcoxon test after a one-way ANOVA
[d] Seven out of nine recipients showed transient GVHD before day 40
[e] Two out of six recipients showed slight skin redness at around day 14

groups died during the observation period; however, daily treatment with 15 mg/kg of CyA (group F) caused death in four of six recipients in the early post-transplant period. Autopsy revealed hemorrhagic pneumonia in all four rats.

Effect of FTY720 on GVHR induced by small bowel transplantation

The parent-to-F1 combination was used to evaluate the effect of FTY720 on GVHD. As shown in Table 2, the recipients (group G) showed severe weight loss accompanied by other signs of GVHD, resulting in death by POD 32. Treatment with FTY720 (group H) markedly improved recipient survival: 3 out of 12 recipients died on PODs 41 and 42, while survived indefinitely. Seven of these survivors showed a transient weight loss and redness of the skin. Starting on POD 40, the body weight of the surviving recipients increased at the same rate as that of recipients undergoing syngeneic SBT, suggesting that the recipients recovered completely from GVHD. Figure 1 shows the time course of body weight after SBT in groups G and I in comparison with the syngeneic group (group A). A low dose of CyA alone did not improve recipient survival (group I). The combined administration of FTY720 and CyA (group J) resulted in no animal deaths. However two recipients transiently showed a mild redness of the skin 2 weeks posttransplantation.

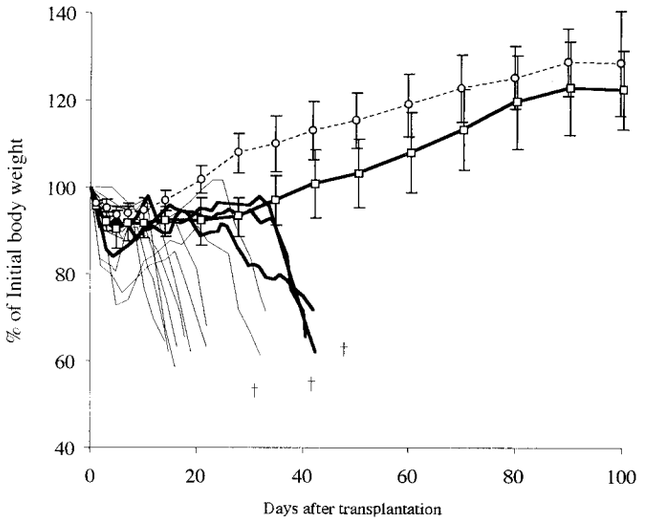


Fig. 1 Time frame of body weight in recipients with syngeneic or parental LEW small bowel. *Dotted line with open circles* represents the mean ± SD ($n = 10$) in the syngeneic controls. *Fine solid lines* denote the changes in body weight of individual recipients suffering from lethal GVHD after parental small bowel grafting. *Bold solid line with open squares* represents the mean ± SD ($n = 9$) body weight of FTY720-treated recipients that survived GVHD. *Three bold solid lines* denote the weight changes of recipients that died of GVHD despite FTY720 treatment

Changes in lymphocyte number and subpopulation after administration of FTY720

The flow cytometric analysis was performed to examine the effect of oral administration of FTY720 on the immune system in LEW rats. One day after the final administration of FTY720, peripheral lymphocytes significantly decreased in number of approximately 22% of the control (Fig. 2A, $P = 0.0002$), whereas polymorphonuclear cells did not decrease ($1.90 \pm 0.84 \times 10^6$ and $2.49 \pm 0.42 \times 10^6$/ml in the control and the treated groups, respectively). In addition, the number of splenic lymphocytes decreased to 47% of the control (Fig. 2B), while that of thymocytes did not change (Fig. 2C).

The percentages of TCR^+ and $CD45^+$ cells in the peripheral lymphocytes were reduced to a great extent by the administration of FTY720; TCR^+ cells decreased to 7.21% (vs 54.86% in untreated rats) and $CD45^+$ cells to 5.33% (vs 25.1% in untreated rats; Fig. 3A). Although an absolute number of peripheral lymphocytes decreased significantly (Fig. 2), the proportion of $CD8^+$ cells showed a slight but significant increase, while that of $CD4^+$ cells remained unchanged (Fig. 3A). In the spleen, TCR^+ cells decreased to 28.91% (vs 56.49% in the controls) after FTY720 administration, but other cell populations did not (Fig. 3B). The percentages of CD4 and CD8 double-positive cells in the thymus also decreased, whereas those of CD4 single-positive cells exhibited an increase (Fig. 3C). Nevertheless, histological examination of the thymus showed no significant findings, including atrophic changes in the cortical area.

Changes in lymphocyte number and subpopulation under the GVHD condition with or without FTY720

Redness of the ears and splenomegaly were observed in F1 recipients that were not treated with FTY720 on POD7. The number of splenic lymphocytes markedly increased in these rats (Fig. 4). When the rats were treat-

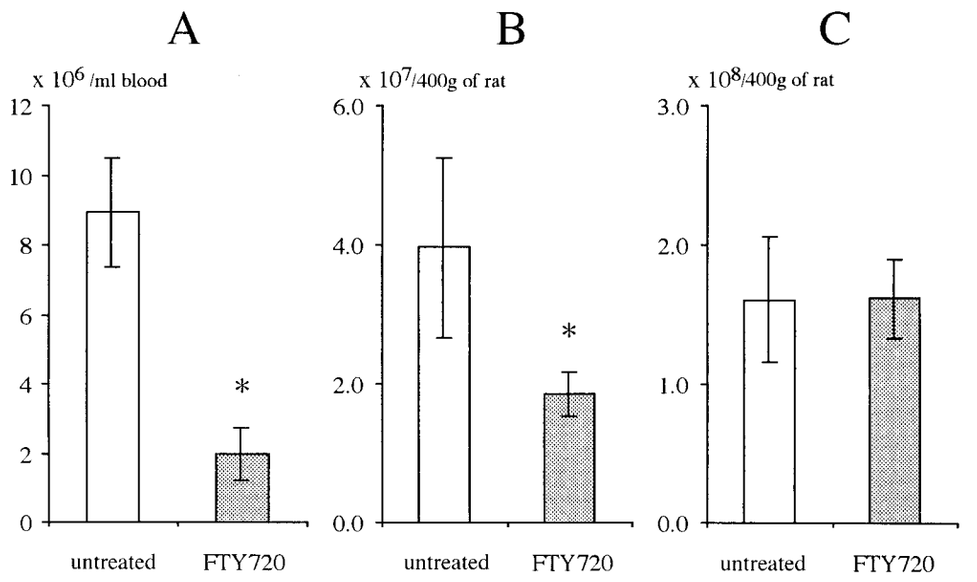
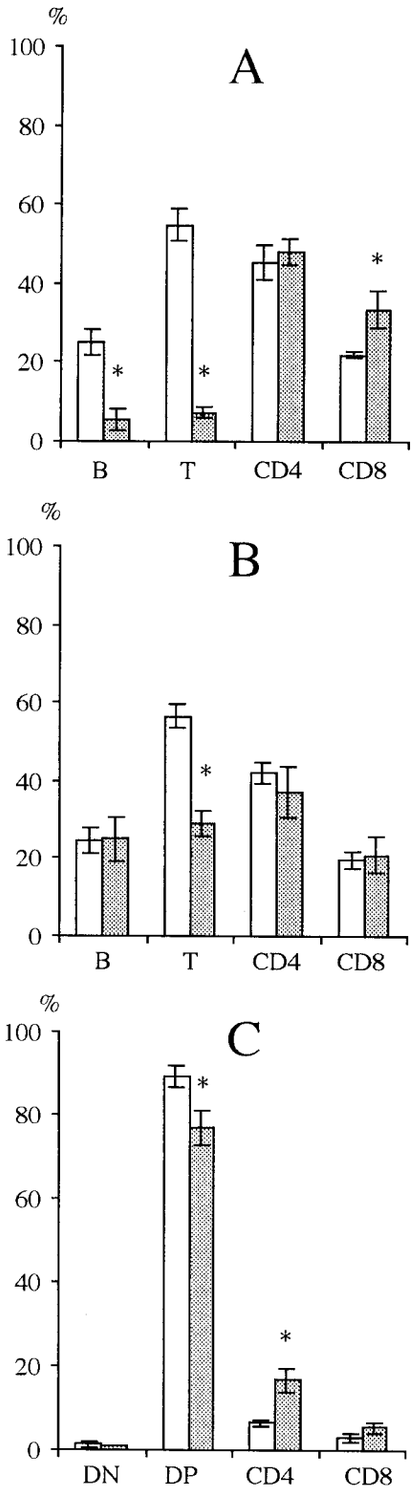


Fig. 2 Lymphocyte numbers in **A** peripheral blood, **B** spleen, and **C** thymus after treatment with FTY720 (0.5 mg/kg) for 15 days in LEW rats ($n = 3$). Lymphocytes were counted 24 h after the last administration of the drug. Each *bar* denotes SD and *asterisk* indicates statistical significance ($P < 0.05$) by one-way ANOVA and Sheffe's test



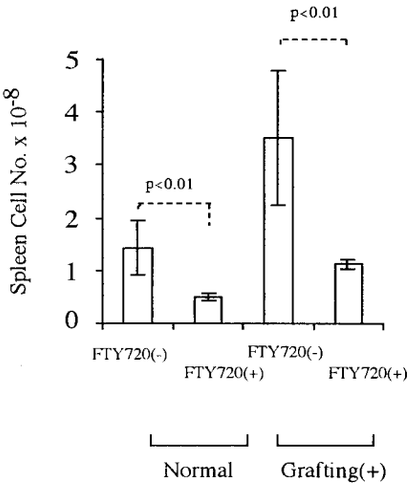


Fig. 4 The number of splenic lymphocytes in normal and F1 recipients. FTY720 was orally administered at a daily dose of 0.5 mg/kg and cell samples were obtained on POD 7

ed with FTY720, the spleen cells significantly decreased to a near-normal level (Fig. 4). There was no significant difference in subpopulation of the splenic lymphocytes between the rats treated with FTY720 and those not treated with FTY720. In the peripheral blood, the ratios of TCR^+ and $CD45^+$ cells decreased markedly in the rats treated with FTY720, although these cell ratios did not decrease in the spleen (Table 3). However, absolute numbers of TCR^+ and $CD45^+$ cells decreased remarkably because of the reduced number of total splenocytes.

Discussion

Modulating the immunological responses in clinical recipients of small bowel transplants is currently one of the most difficult challenges in the solid organ transplantation. We previously demonstrated the effectiveness of a novel immunosuppressant, FTY720, on heart, liver, and kidney allograft survival in experimental studies using rats and dogs [8, 11]. The present study investigated the effect of FTY720 on rat SBT using unidirectional, semiallogenic combinations. In rats in which HVGR was induced, FTY720 treatment significantly

◀ **Fig. 3** Lymphocyte subpopulations in **A** peripheral blood, **B** spleen, and **C** thymus after treatment with FTY720 (0.5 mg/kg) for 15 days in LEW rats ($n = 3$). Lymphocytes were isolated 24 h after the final administration of the drug, stained with surface markers, and analyzed by FACScan. Each *bar* denotes SD and *asterisk* indicates statistical significance ($P < 0.05$) by one-way ANOVA and Sheffe's test

Table 3 Changes in lymphocyte subpopulation under GVHD condition in (LEW × BN) F1 rats treated with (+) or without (–) FTY720

Group[a]	TCR^+ cells %	$CD45^+$ cell %	$CD4^+$ %	$CD8^+$ %
Spleen				
Intact F1 (Control)	38.23 ± 3.73	37.91 ± 0.21	39.26 ± 1.18	12.62 ± 1.34
Grafting +, FTY720 –	40.44 ± 0.68	40.38 ± 1.14	30.15 ± 2.30	15.17 ± 0.54
Grafting +, FTY720 +	33.76 ± 2.13	35.95 ± 1.77	22.83 ± 0.05	20.14 ± 0.22
Peripheral blood				
Intact F1 (Control)	46.48 ± 1.65	33.98 ± 0.60	46.95 ± 1.20	11.39 ± 0.35
Grafting +, FTY720 –	42.96 ± 4.43	27.89 ± 11.88	47.24 ± 2.60	7.46 ± 1.59
Grafting +, FTY720 +	3.64 ± 0.04	15.99 ± 6.02	41.82 ± 0.76	14.44 ± 4.71

[a] FTY720 was orally administered at a daily dose of 0.5 mg/kg from the day of operation and cell samples were obtained on POD 7

prolonged graft survival, and to a greater extent than with low-dose CyA alone. Worth noting is that FTY720 combined with CyA synergistically prolonged graft survival without any adverse reaction.

It has been reported that daily administration of CyA at 15 mg/kg induces indefinite graft survival in the same donor-recipient combination as used in the present study [4]. However, in this study, daily treatment with 15 mg/kg of CyA resulted in the death of four out of six rats, all of which showed hemorrhagic pneumonia on autopsy. As the administration of FTY720 does not affect the blood concentration level of CyA [8], the synergistic mechanism in the combination therapy has been emphasized. In the parent-to-F1 combination, FTY720 remarkably inhibited GVHR and prevented recipient death in 75 % of the animals, even when they were given a low dose (0.5 mg/kg) of FTY720 alone. The preventive effect of FTY720 on GVHR was also revealed by the flow cytometric analysis of splenic and peripheral lymphocytes (Fig. 4, Table 3). In addition, the combination of FTY720 and CyA almost completely abrogated the lethal GVHR in all recipients, indicating a more excellent outcome than in a previous study using high-dose CyA [4]. Long-term recipient survival in rats exhibiting GVHR has been thought to result from complete suppression of alloreactive cells in the grafted organ, without any adverse reactions, such as infection due to overimmunosuppression [12, 15]. From this point of view, FTY720 may be considered a useful drug for SBT recipients suffering from complications that include infectious episodes.

To study the cellular mechanism of action of FTY720, lymphocyte subpopulations in peripheral blood and lymphoid organs in the drug-treated rats were analyzed by flow cytometry. It was shown that the number of lymphocytes in peripheral blood and in the spleen was significantly decreased in rats treated with FTY720. Both TCR^+ and $CD45^+$ cells in the peripheral blood were dramatically reduced in number, while polymorphonuclear cells and monocytes were not affected, suggesting that FTY720-treated animals were relatively resistant to infectious complications. Indeed, none of the rats died of infection or of other complications in our present or previous studies [7, 8, 11].

In conclusion, the present results support the feasibility of including FTY720 in the immunosuppressive regimen to control the immune reaction and to protect transplant recipients from the undesirable complications after clinical SBT.

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