

Dual targeting of Bruton's tyrosine kinase and Janus kinase 3 with rationally designed inhibitors prevents graft-versus-host disease (GVHD) in a murine allogeneic bone marrow transplantation model

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Summary

The purpose of the present study was to evaluate the effectiveness of targeting Bruton's tyrosine kinase (BTK) with a specific BTK inhibitor, α -cyano- β -hydroxy- β -methyl-*N*-(2,5-dibromophenyl)-propenamide (LFM-A13), for prevention of acute fatal graft-versus-host disease (GVHD) in a murine model of allogeneic bone marrow transplantation (BMT). Vehicle-treated control C57BL/6 mice receiving bone marrow/splenocyte grafts from allogeneic BALB/c donors developed severe multi-organ acute GVHD and died after a median survival time (MST) of 40 d. LFM-A13 treatment (25 mg/kg/d) significantly prolonged the MST of the BMT recipients to 47 d. The probability of survival at 2 months after BMT was $2 \pm 2\%$ for vehicle-treated control mice and $22 \pm 6\%$ for mice treated with LFM-A13 ($P = 0.0008$). Notably, the combination regimen of LFM-A13 plus the standard anti-GVHD drug methotrexate (MTX) (10 mg/m²/d) was more effective than LFM-A13 alone, while the combination regimen of LFM-A13 plus the novel anti-GVHD drug JANEX-1 (60 mg/kg/d), targeting Janus kinase 3, was more effective than LFM-A13, JANEX-1 or MTX alone. More than 70% of recipients receiving this most effective GVHD prophylaxis (LFM-A13 + JANEX-1) remained alive throughout the 80-d observation period with an MST of >80 d. Taken together, these results indicate that targeting BTK with the chemical inhibitor LFM-A13 may attenuate the severity of GVHD, especially when it is combined with other anti-GVHD drugs, such as MTX and JANEX-1.

Keywords: Bruton's tyrosine kinase inhibitor, LFM-A13, bone marrow transplantation, graft-versus-host disease, mouse.

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Graft-versus-host disease (GVHD) remains a significant cause of morbidity and mortality after allogeneic bone marrow transplantation (BMT) (Ferrara, 2003). GVHD occurs when alloreactive donor T cells become sensitized to the host, then expand and mediate host tissue destruction (Ferrara, 2003). Decades of experimental and clinical research have demonstrated that donor T cells are the principal mediators and effectors of GVHD. However, in addition to donor T cells, elements of the non-T-cell compartment have also been implicated in the pathogenesis of GVHD (Sprent *et al*, 1990,

1995; Mielcarek *et al*, 1997; Koh *et al*, 2000; Aranha *et al*, 2002; Rao *et al*, 2003; Tanaka *et al*, 1999).

Our earlier studies in a well-established murine model of allogeneic BMT (Cetkovic-Cvrlje *et al*, 2001, 2002) have revealed an important role for Janus kinase 3 (JAK3 kinase), which is abundantly expressed in T cells, in the pathogenesis of acute GVHD (Cetkovic-Cvrlje *et al*, 2002; Cetkovic-Cvrlje & Tibbles, 2004; Cetkovic-Cvrlje & Uckun, 2004). Targeting JAK3 with the specific tyrosine kinase inhibitor JANEX-1 was shown to prevent severe GVHD in mice undergoing allogeneic

BMT across the main major histocompatibility complex (MHC) barriers (Cetkovic-Cvrlje *et al*, 2001; Uckun *et al*, 2002a). The purpose of the present study was (a) to evaluate the effects of inhibiting Bruton's tyrosine kinase (BTK) within the non-T-cell compartment on development of severe GVHD after allogeneic BMT in mice and (b) to determine whether dual targeting of BTK and JAK3 kinases with rationally designed inhibitors would further improve the survival outcome of allogeneic BMT.

Bruton's tyrosine kinase (Steller, 1995), a member of the BTK/Tec family of protein tyrosine kinases that includes TecI, TecII, Itk, Bmx/Etk, and DSrc28C (found in *Drosophila*; Mano, 1999), is a cytoplasmic protein tyrosine kinase of non-T lineage haematopoietic cells which plays a pivotal role in regulatory signal transduction pathways (Mukhopadhyay *et al*, 1999, 2002; Kurosaki, 2000; Horwood *et al*, 2003, Rawlings & Witte, 1994; Smith *et al*, 1994; Uckun, 1998). BTK is involved in signal transduction pathways that regulate growth, differentiation, and survival of B-lineage lymphoid cells (Rawlings & Witte, 1994; Uckun, 1998; Kurosaki, 2000). Recent studies have revealed that BTK is involved in induction of macrophage effector functions (Mukhopadhyay *et al*, 1999, 2002). Furthermore, BTK was found to be a key element of lipopolysaccharide-induced tumor necrosis factor production in monocytes (Horwood *et al*, 2003).

The structure-based design of specific inhibitors of BTK has been reported recently (Mahajan *et al*, 1999). Advanced docking procedures were used for the rational design of LFM analogues with a high likelihood to bind favourably to the catalytic site within the kinase domain of BTK. The lead compound α -cyano- β -hydroxy- β -methyl-N-(2,5-dibromophenyl)-propanamide (LFM-A13), inhibited recombinant BTK with an IC₅₀ value of 2.5 μ mol/l (Mahajan *et al*, 1999). Besides its remarkable potency in BTK kinase assays, LFM-A13 is also a highly specific inhibitor of BTK. Even at concentrations as high as 300 μ mol/l, LFM-A13 did not affect the enzymatic activity of other protein tyrosine kinases, including the Janus family tyrosine kinases JAK1 and JAK3, SYK, Src family tyrosine kinases HCK and LYN, or the receptor family tyrosine kinases EGF-Receptor Kinase (EGFR) and Insulin-Receptor Kinase (IRK) (Mahajan *et al*, 1999).

Here, we showed that a combination of the JAK3 inhibitor JANEX-1 and BTK inhibitor LFM-A13 confers a significant survival advantage for mice undergoing allogeneic BMT and is superior to GVHD prophylaxis using the standard anti-GVHD drug methotrexate (MTX). Notably, more than 70% of BMT recipients treated with JANEX-1 plus LFM-A13 remained alive throughout the 80-d observation period.

Material and methods

C57BL/6 and BALB/c mice

Eight to 10-week-old C57BL/6 (H-2^b) male mice and 6–8-week-old BALB/c (H-2^d) male mice were purchased from

Taconic, Germantown, NY, USA. Mice were housed in a controlled environment (12-h light/12-h dark photoperiod, 22 \pm 1°C, 60 \pm 10% relative humidity), which is fully accredited by the United States Department of Agriculture (USDA). All husbandry and experimental contact made with the mice maintained specific pathogen-free (SPF) conditions. All mice were kept in Micro-Isolator cages (Lab Products, Inc., Maywood, NY, USA) containing autoclaved food (Harlan Teklad LM-485), water and bedding. Animal studies were approved by the Parker Hughes Institute Animal Care and Use Committee, and all animal care procedures conformed to the Principles of Laboratory Animal Care (National Institutes of Health no. 85–23, revised 1985).

Pretransplant total body irradiation

For pretransplant conditioning, recipient C57BL/6 mice, positioned in a pie-shaped Lucite holder (Braintree Scientific Inc., Boston, MA, USA) underwent total body irradiation (TBI) (7.5 Gy) 1 d prior to BMT, which was delivered by a Cesium Instrument (JL Sheppard Labs, 47.08 rad/min). Recipients were given antibiotic-supplemented water (sulphamethoxazole/trimethoprim, Hi-Tech Pharmacal, Amityville, NY, USA) starting the day before transplantation.

Bone marrow transplantation

Donor bone marrow (BM) was collected into Roswell Park Memorial Institute (RPMI) 1640 medium supplemented with L-glutamine (Cellgro) (Mediatech, Herndon, VA, USA) by flushing the shafts of femurs and tibias and cell suspensions were prepared as previously described (Cetkovic-Cvrlje *et al*, 2001, 2002, Uckun *et al*, 2002a). In parallel, single cell suspensions of donor splenocytes (S) were prepared from minced spleens as a source of GVHD-causing T cells. The cells were washed and resuspended for i.v. injection via the caudal vein. The standard BM/S inoculum consisted of 25 \times 10⁶ BM cells and 25 \times 10⁶ splenocytes in 0.5 ml of RPMI 1640 medium.

Graft-versus-host disease monitoring

Bone marrow transplantation recipients were monitored daily for any clinical evidence of GVHD (weight loss, manifestations of skin erythema, alopecia, hunching, diarrhoea) and survival (Cetkovic-Cvrlje *et al*, 2001, 2002, Uckun *et al*, 2002a) during the 80-d observation period. Survival times were measured from the day of BMT (day 0).

Evaluation of engraftment status after BMT

The allo-engraftment was documented by flow cytometric (FACScan, Becton Dickinson, Mountain View, CA, USA) H-2D^d typing of peripheral blood nucleated cells using fluorescein isothiocyanate (FITC)-labelled anti-H-2D^d antibody

(clone 34-2-12; Pharmingen, San Diego, CA, USA), which marks BALB/c cells. Immunofluorescent staining of cells and flow cytometry were performed using standard procedures (Cetkovic-Cvrlje *et al*, 2001; Uckun *et al*, 2002a).

Drug treatments

For GVHD prophylaxis – injections of LFM-A13, JANEX-1, MTX (Immunex Corporation, Seattle, WA, USA) or vehicle control were administered to recipient mice. The BTK- and JAK3-inhibitory compounds LFM-A13 (25 mg/kg/d) and JANEX-1 (60 mg/kg/d), respectively, were administered daily starting on day 0 of BMT. These compounds were synthesized and characterized as previously described in detail (Mahajan *et al*, 1999; Sudbeck *et al*, 1999). The standard anti-GVHD drug MTX (10 mg/m²/d) was used for comparison. MTX was administered i.p. on days 1, 3, 6 and 11 post-BMT. All drugs were administered intraperitoneally (i.p.) in a volume of 200 µl.

Histopathologic examination of tissues

Recipient mice were electively sacrificed at specified time points and necropsied. Tissues were removed and fixed in 10% neutral-buffered formalin. Fixed tissues were embedded in paraffin, cut into 4 µm sections and stained with haematoxylin and eosin. All histopathology slides were blindly coded and graded by a veterinary pathologist. Livers were scored positive for GVHD when there was a periportal infiltrate, lungs were scored positive when there was evidence of vasculitis with a lymphocytic infiltrate, skin was scored positive when there was single cell necrosis, and colon was scored positive when there was single cell necrosis or crypt dropout. After initial scoring, all slides were reviewed for GVHD grading, as described previously by Bryson *et al* (1997). Our GVHD grading system was as follows: *Liver*: grade 0.5, focal portal lymphoid infiltrate; grade 1, widespread portal lymphoid infiltrate; grade 2, focal bile duct invasion or cellular injury; grade 3, multiple foci of bile duct injury and regeneration; grade 4, widespread injury and destruction of bile ducts; *Small and large intestine*: grade 0.5, occasional or rare necrotic cells in glands or crypts; grade 1, multiple foci of necrotic cells in glands or crypts; grade 2, necrosis involving several crypts or glands with focal abscess formation in crypts; grade 3, widespread crypt abscesses with focal glandular destruction; grade 4, loss of mucosa with granulation tissue response; *Skin-ear*: grade 0.5, occasional or rare single basal vacuolar necrosis; grade 1, several foci of single basal vacuolar necrosis; grade 2, contiguous single cell necrosis or multiple necrotic cells in proximity of lymphoid infiltrates; grade 3, confluent loss of cells with cleft formation or loss of skin appendages with extensive lymphoid infiltrates; grade 4, loss of epidermis or epithelium with or without granulation tissue response.

Statistical analysis

Group comparisons of continuous variables were done using Student's *t*-tests. The survival data were analysed by life-table methods. *P*-values of less than 0.05 (log-rank test) were considered significant.

Results

Targeting BTK with the chemical inhibitor LFM-A13 attenuates fatal acute GVHD across the major histocompatibility barrier in mice

Severe GVHD, associated with overt diarrhoea, hunching, weight loss and ruffled fur, was induced in lethally irradiated (7.5 Gy TBI) C57BL/6 mice (H-2^b) across the MHC barrier by injection of BM/S grafts from BALB/c mice (H-2^d). In an attempt aimed at preventing the development of fatal GVHD, recipient mice were treated with LFM-A13 (25 mg/kg/d) every day from the day of BMT until the end of the 80-d observation period. Control mice were treated with vehicle alone. All of the TBI-conditioned, vehicle-treated control C57BL/6 mice (*n* = 43) receiving BM/S grafts from BALB/c mice developed severe multi-organ GVHD, as clinically signaled by development of overt diarrhoea, hunching, weight loss and ruffled fur within 2–3 weeks and died with a median survival time (MST) of 40 d (Table I, Fig 1). Histopathological examination of multiple organs from seven control mice that either died or were terminated in moribund condition between the weeks 4 and 6 post-BMT confirmed the diagnosis of multi-organ GVHD (Table II). The average GVHD scores in these mice were 3.0 ± 0.1 for the liver, 1.9 ± 0.1 for the skin, 1.1 ± 0.1 for the small intestine, and 1.4 ± 0.2 for the large intestine (Table II).

LFM-A13 treatment significantly improved the survival of BMT recipients (*n* = 50, *P* = 0.0008 compared with vehicle-treated control recipients) and prolonged the MST to 47 d (Table I). The probability of survival at 2 months post-BMT was 2 ± 2% for vehicle-treated control mice and 22 ± 6% for mice treated with LFM-A13 (Table I, Fig 1). However, only 2 ± 2% of LFM-A13-treated mice survived the experimental period of 80 d (Table I, Fig 1).

The standard anti-GVHD drug MTX (10 mg/m²/d) and the recently described experimental anti-GVHD compound JANEX-1 targeting JAK3 (Cetkovic-Cvrlje *et al*, 2001; Uckun *et al*, 2002a) were used for comparison. Both drugs were found to have comparable efficacy in attenuating severe acute GVHD after allogeneic BMT in a murine models of GVHD (Cetkovic-Cvrlje *et al*, 2001; Table I). JANEX-1 and MTX significantly improved the survival of BMT recipients (*P* < 0.0001 and *P* < 0.0001 respectively), and prolonged the MST to 57 and 63 d respectively (Table I). The probability of survival at 80 d after BMT was 31 ± 12% for MTX-treated and 31 ± 9% for recipients treated with JANEX-1, compared with 0 ± 0% for

Treatment protocol	n	MST (d)	Cumulative proportion surviving (% \pm ,SEM)			P-value (log rank)	
			40 d	60 d	80 d	vs. (A)	vs. (B)
(A) Vehicle	43	40	47 \pm 8	2 \pm 2	0 \pm 0	–	0-0008
(B) LFM-A13	50	47	74 \pm 6	22 \pm 6	2 \pm 2	0-0008	–
(C) JANEX-1	26	57	69 \pm 9	46 \pm 10	31 \pm 9	<0-0001	0-0006
(D) MTX	16	63	88 \pm 8	56 \pm 12	31 \pm 12	<0-0001	0-0004
(E) LFM-A13 + JANEX-1*,**	11	>80	100 \pm 0	82 \pm 12	73 \pm 13	<0-0001	<0-0001
(F) LFM-A13 + MTX	19	>80	84 \pm 8	68 \pm 11	58 \pm 11	<0-0001	<0-0001

C57BL/6 (H-2^b) recipients were lethally irradiated (TBI = 7.5 Gy) and transplanted with BM/FS grafts from MHC-disparate BALB/c (H-2^d) mice and subjected to the treatment regimens presented above (the details of these treatment regimens are given in Materials and Methods); * P < 0.05 compared with group D, and ** P = 0.0008 compared with group C; statistically significant differences obtained by life table analysis (logrank test).

vehicle-treated mice (Table I). Table I, as well as Fig 1A and B emphasize that both JANEX-1 (P = 0.0006) and MTX (P = 0.0004) were more effective than LFM-A13 in improving the survival outcome postallogeneic BMT.

Efficacy of combined LFM-A13 plus MTX treatment in prevention of fatal acute GVHD across the major histocompatibility barrier in mice

We next sought to identify an effective GVHD prevention regimen that employs the BTK inhibitor LFM-A13 in combination with a standard immunosuppressive agent. As shown previously (Cetkovic-Cvrlje *et al*, 2001; Uckun *et al*, 2002a), MTX is a potent anti-GVHD drug. MTX was more potent than LFM-A13 in the attenuation of acute GVHD (P = 0.0004, Table I and Fig 1A). The combination regimen LFM-A13 plus MTX was more effective than LFM-A13 alone (P < 0.0001, Table I and Fig 1A). However, the combination regimen LFM-A13 plus MTX was not statistically more effective than MTX alone, despite the prolongation of MST to >80 d compared with 63 d in the MTX only group (Table I). More than half of the C57BL/6 recipients receiving this (LFM-A13 + MTX) GVHD prophylaxis remained alive and healthy throughout the 80-d observation period with a cumulative survival probability of 58 \pm 11% compared with 31 \pm 12% in MTX-treated group (Table I, Fig 1A).

The histopathologic examination of organs of five representative long-term survivors of the LFM-A13 + MTX-treated group revealed that average liver, skin and large intestine scores in these mice were significantly lower than the GVHD scores of vehicle-treated control mice (Table II). According to the scoring system, the histologic GVHD grades were 2.2 \pm 0.3 for the liver, 0.9 \pm 0.3 for the small intestine, 0.5 \pm 0.0 for the large intestine, and 0.6 \pm 0.3 for the skin (Table II).

The long-term survival of LFM-A13 + MTX-treated mice was not due to poor engraftment of donor cells. Notably, 98.5 \pm 1.1% H-2D^d-positive donor cell engraftment was observed in LFM-A13 + MTX-treated mice, indicating that,

Table I. Attenuation of lethal GVHD in murine allogeneic BMT recipients by targeting BTK with LFM-A13.

under these experimental conditions, donor cell engraftment was not prevented and the attenuation of GVHD in LFM-A13 + MTX-treated recipient mice was not due to lack of donor cell engraftment with concomitant autologous recovery.

GVHD Prophylaxis with LFM-A13 plus JANEX-1 markedly improves the survival outcome of recipient mice postallogeneic BMT

We showed previously that the JAK3 kinase inhibitor JANEX-1 is a potent anti-GVHD agent (Cetkovic-Cvrlje *et al*, 2001; Uckun *et al*, 2002a). Here we set out to determine if the survival outcome of allotransplanted bone marrow recipients could be further improved by using a combination of JANEX-1 (60 mg/kg/d) plus LFM-A13 (25 mg/kg/d) for GVHD prophylaxis. As shown in Table I and Fig 1B, the combination treatment of LFM-A13 plus JANEX-1 was highly effective in the attenuation of acute GVHD, and significantly better than the treatment with either LFM-A13 alone (P < 0.0001) or JANEX-1 alone (P = 0.0006). While the MST of recipients treated with LFM-A13 alone and JANEX-1 alone increased to 47 and 57 d, respectively, compared with the MST of 40 d in vehicle controls, the combination treatment (LFM-A13 + JANEX-1) increased the MST to >80 d. Notably, 73 \pm 13% of recipients treated with this combination treatment survived beyond the 80-d observation period, compared with only 2 \pm 2% and 31 \pm 12% recipients of LFM-A13- and JANEX-1-treated groups respectively (Table I). Histopathologic examination of the organs of LFM-A13 + JANEX-1-treated long-term survivors (n = 7) showed that average liver, skin, small and large intestine GVHD scores in these mice were significantly lower than those of vehicle-treated control mice (Table II). Average GVHD scores of long-term survived LFM-A13 + JANEX-1-treated BMT recipients were 0.9 \pm 0.2 for the liver, 0.3 \pm 0.1 for the skin, 0.6 \pm 0.1 for the small intestine and 0.4 \pm 0.1 for the large intestine. Flow cytometric H-2D^d-typing of splenocytes obtained from seven long-term H-2D^b-positive survivors treated with this effective two-drug combi-

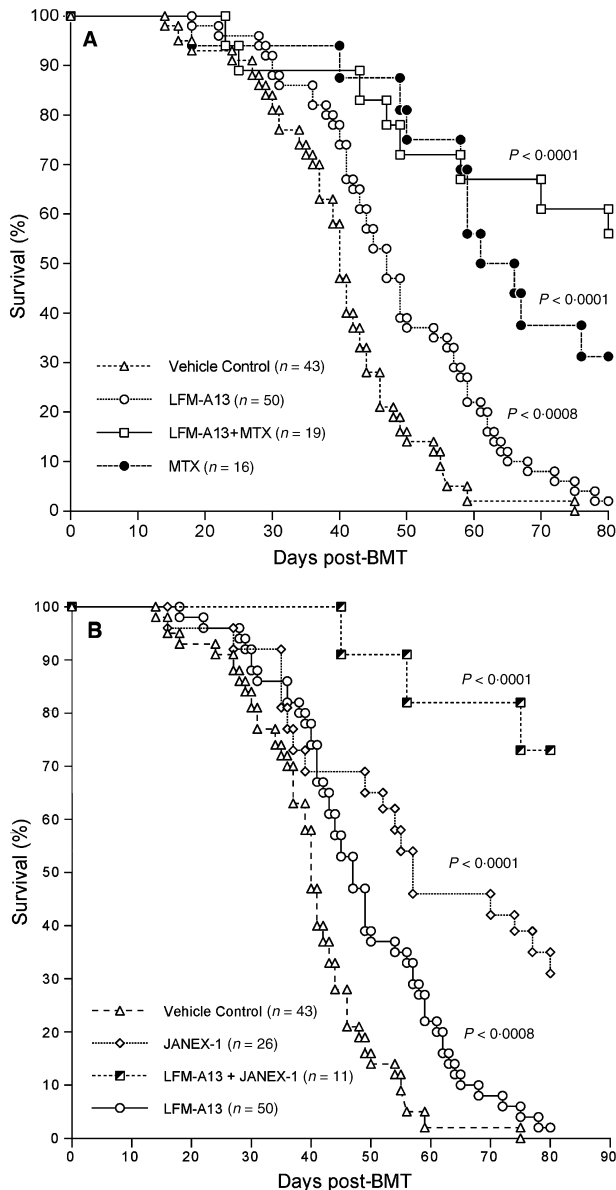


Fig 1. Effects of the BTK inhibitor LFM-A13 in combination with methotrexate (MTX) (A) and JANEX-1 (B) on the post-BMT survival outcome in a murine model of acute GVHD. Irradiated (7.5 Gy) C57BL/6 (H-2^b) recipients were given BM and splenocytes (25×10^6 of each) from BALB/c (H-2^d) mice. LFM-A13 was administered i.p. at 25 mg/kg/d from day 0 to day 80. Methotrexate (MTX) was used at a dose level of 10 mg/m²/d and administered i.p. on days 1, 3, 6 and 11 post-BMT. JANEX-1 was administered i.p. at 60 mg/kg/d from day 0 to day 80. P-values obtained in comparison with control group by the life table analysis (log-rank test). See Table I for details of the life table analysis.

nation showed $95.6 \pm 1.8\%$ H-2D^d donor cell engraftment (Table III).

Discussion

Acute graft-versus-host disease is a major cause of morbidity and mortality in patients undergoing allogeneic BMT (O'Reilly

& Papadopoulos, 1997). To date, most therapeutic approaches designed to reduce acute GVHD, including *ex vivo* T cell depletion of marrow grafts (Martin *et al*, 1988; Poynton, 1988; Marmont *et al*, 1991), use of positively selected CD34⁺ haematopoietic precursor cells (Berenson *et al*, 1996), and systemic immunosuppression (O'Reilly, 1983) are associated with an increased rate of graft rejection, more severe immunosuppression, and higher relapse rate of the original malignancies. Therefore, novel anti-GVHD agents with potent anti-leukaemic activity are urgently needed for effective prevention of GVHD after BMT without facilitating the recurrence of leukaemia.

Several decades of the studies in area of BMT have demonstrated that donor T cells are the principal mediators of GVHD. However, recent data obtained from clinical and experimental transplantation indicated the role of non-T-cell compartment in development of GVHD (Sprent *et al*, 1990, 1995; Mielcarek *et al*, 1997; Koh *et al*, 2000; Aranha *et al*, 2002; Rao *et al*, 2003; Tanaka *et al*, 1999). Here, we show that targeting BTK within the non-T-cell compartment using the rationally designed BTK inhibitor LFM-A13 attenuated the severity of GVHD postallogeneic BMT in an acute murine GVHD model. However, LFM-A13 was less effective than the standard anti-GVHD drug MTX or the recently described anti-GVHD agent JANEX-1. Therefore we investigated whether the inhibition of BTK in combination with the inhibition of JAK3 could have beneficial effects on GVHD development. Indeed, combined prophylactic treatment with LFM-A13 and JANEX-1 exhibited potent *in vivo* biologic activity. Notably, the combination regimen LFM-A13 + JANEX-1 was more effective than LFM-A13 alone or JANEX-1 alone (73% recipients treated with LFM-A13 + JANEX-1 survived throughout 80-d observation period, with a MST of >80 d, compared with 2% and MST of 47 d in LFM-A13-treated, and 25% and MST of 56 d in JANEX-1-treated recipients). The combination regimen LFM-A13 + MTX was as effective as LFM-A13 + JANEX-1 (58% recipients survived on day 80 post-BMT with a MST of >80 d). However, LFM-A13 + MTX treatment was not significantly more effective than treatment with MTX alone. In contrast, LFM-A13 + JANEX-1 treatment was more effective than each of the drug treatments alone (either LFM-A13, JANEX-1, or MTX alone). The H-2D typing of nucleated peripheral blood cells from LFM-A13 + JANEX-1- and LFM-A13 + MTX-treated recipients confirmed that >90% of the circulating cells were of donor origin. Thus, the attenuation of GVHD in recipient mice treated with both dual treatments LFM-A13 + JANEX-1- and LFM-A13 + MTX was not due to lack of donor cell engraftment with concomitant autologous recovery.

LFM-A13 was described as the first BTK-specific tyrosine kinase inhibitor and the first anti-leukaemic agent targeting BTK (Uckun & Zheng, 2000, 2001). Notably, treatment of leukaemic cells with LFM-A13 resulted in abrogation of BTK activity at a concentration of 10 μ mol/l (Mahajan *et al*, 1999). LFM-A13 disrupted BTK-Fas association and rendered resistant leukaemic

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