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ORIGINAL ARTICLE

Serum FLT-3 ligand in a busulphan-induced model of chronic bone marrow hypoplasia in the female CD-1 mouse

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Summary

The concentration of the cytokine fms-like tyrosine kinase-3 ligand (FL) is elevated in the plasma of patients treated with chemotherapy or radiotherapy for malignant conditions. In addition, plasma FL is increased in patients with bone marrow failure resulting from stem-cell defects (e.g. aplastic anaemia). Our goal in the present study was to measure the concentration of serum FL in mice treated with the chemotherapeutic agent busulphan (BU) to induce bone marrow depression and relate changes in FL to effects on haemopoiesis. Female CD-1 mice were treated with BU (9.0 mg/kg) or vehicle by intraperitoneal injection on 10 occasions over 21 days. Animals were autopsied on days 1, 23, 72, 119 and 177 postdosing. A full blood count was performed, and serum prepared for FL analysis. Femoral marrow cell suspensions were prepared to assess the total femoral nucleated cell count (FNCC) and the number of committed haemopoietic progenitor cells (CFU-C). On days 1 and 23 postdosing, significant decreases were evident in many peripheral blood parameters; the FNCC and CFU-C were also reduced in BU-treated mice, in conjunction with increases in serum FL levels. On days 72, 119 and 177 postdosing, several peripheral blood and bone marrow parameters remained reduced and the concentration of serum FL continued to be significantly increased. Linear regression analysis demonstrated significant correlations between the concentration of serum FL in BU-treated mice and peripheral blood and bone marrow parameters; this suggests the possible use of serum FL as a potential biomarker for drug-induced bone marrow injury.

Keywords
aplasia, bone marrow, busulphan, fms-like tyrosine kinase-3 ligand, hypoplasia, mouse

The bifunctional alkylating agent busulphan (BU) is commonly used in the treatment of chronic granulocytic (myeloid) leukaemia, polycythemia vera and myelofibrosis. In addition, the myelo-ablative property of BU is regularly employed in conditioning regimens used prior to bone marrow transplantation in patients with malignant diseases and aplastic anaemia (AA) (Dollery 1999; Sweetman 2002; BNF 2004).

Busulphan is a small and highly lipophillic molecule that, once administered, undergoes extensive enzymatic metabolism; studies have found 12 metabolites of BU; however, many of these metabolites have not been fully characterized; indeed, <2% of the drug is excreted unchanged (Buggia *et al.* 1994). Busulphan interacts with nucleic acids to form DNA inter-strand cross-links resulting in significant DNA damage. However, cells that are resting in the G_0 phase of the cell cycle are more sensitive to the effects of BU (Dollery 1999).

In man, the major adverse effect of BU treatment is bone marrow hypocellularity resulting in anaemia, leucopaenia and thrombocytopaenia. In some cases, long-term exposure to BU, or incidences of overdose, result in significant bone marrow toxicity leading to complete bone marrow failure, the outcome of which may be pancytopaenia and AA (Buggia *et al.* 1994; Bright *et al.* 2001).

In laboratory animals, the extent to which the bone marrow is suppressed immediately following exposure to BU is directly proportional to the dose administered (Gibson *et al.* 2003). In the mouse, bone marrow depression occurring as a result of administering a single low dose of BU is readily reversible (Boggs & Boggs 1980). However, studies in both mice and rats have shown that single doses of BU at very high concentrations result in a more severe bone marrow injury. Haematological changes reported in animals treated with high doses of BU include a decrease in erythropoiesis and granulopoiesis, bone marrow hypocellularity, and a reduction in the number of committed progenitor cells (CFU-C) and spleen-colony forming units (Dunn & Elson 1970; Santos & Tutschka 1974; Boggs & Boggs 1980; Anderson *et al.* 1982).

However, the effects on the haemopoietic system of repeat dose regimens of BU are unusual compared to other cytotoxic agents. In the mouse, studies have shown that following treatment with cytotoxic agents such as 5-fluorouracil, methotrexate and vinblastine, the bone marrow is initially depressed before returning towards normal control values (Trainor *et al.* 1979). This is in contrast to studies in the mouse using BU which show that following an initial period of bone marrow depression and a return towards control values, normality may not be achieved and at 2-month postdosing, mice treated with BU demonstrate a secondary 'late-stage' or 'residual' phase of relatively stable bone marrow aplasia/hypoplasia. This phenomenon, first described by Morley and Blake (1974a) as chronic hypoplastic marrow failure (CHMF), shares many similarities with the human condition of AA. For example, BU-treated mice have significant reductions in peripheral blood cell counts, bone marrow cellularity and the number of CFU-Cs (Morley & Blake 1974a,b; Morley *et al.* 1975, 1978).

In the experiments of Morley and Blake (1974a) using the outbred female Swiss mouse and the inbred BALB/c mouse, BU was administered by intraperitoneal (i.p.) injection at fortnightly intervals on four occasions at 20, 20, 20 and 10 mg/kg, with CHMF occurring between 100 and 313 days after BU treatment. In order to induce 'late-stage' or 'residual' bone marrow aplasia in a more convenient time frame, Gibson *et al.* (2003) modified the dosing regimen of BU, treating mice with the drug at 10.50 mg/kg on eight occasions over 23 days, inducing a stabilized chronic bone marrow aplasia (CBMA) between 91 and 112 days postdosing.

The model of CBMA developed by Gibson *et al.* (2003) shared many similarities with the human condition of AA. However, mouse mortality in these studies was high, with 49.3% of animals treated with BU being killed '*in extremis*' or being 'found dead' (Turton *et al.* 2006). To reduce the level of mouse mortality associated with BU administration, further studies on the development of a BU-induced animal model of CBMA were conducted (Turton *et al.* 2006). Busulphan was administered to BALB/c mice at 8.25, 9.0 and 9.75 mg/kg by i.p. injection on 10 occasions over a 21-day dosing period. Immediately postdosing (days 1–23 postdosing) animals treated with BU developed significant bone marrow depression with many peripheral blood values being significantly reduced. A period of recovery then followed and from approximately days 50–120 postdosing animals displayed the characteristics of CBMA which was stabilized and long-lasting (as previously reported by Gibson *et al.* (2003)). The severity of the 'late-stage' or 'residual' (i.e. postday 50) CBMA induced by BU treatment, and the level of mortality recorded, appeared to be related to the dose of BU administered. In conclusion, this study (Turton *et al.* 2006) demonstrated that BU administered at 9.0 mg/kg induced a stabilized CBMA without significant mortality.

In the present experiment, BU was administered to CD-1 mice at 9.0 mg/kg on 10 occasions by i.p. injection over a 21-day dosing period. This experiment was devised to use the BU-treated mouse model to examine the changes in the concentration of the serum cytokine fms-like tyrosine kinase-3 (FLT-3) ligand (FL) in relation to other haematological parameters. Although studies in laboratory animals on FL in radiation-induced bone marrow injury have been carried out (Gratwohl *et al.* 1998; Bertho *et al.* 2001; Prat *et al.* 2005), detailed investigations on changes in the levels of FL in mice following the administration of BU, have not been reported previously although initial studies with

mitomycin in the mouse and chlorambucil in the rat have been described (Molyneux *et al.* 2004, 2005). The CD-1 mouse was selected for use in the present experiment as this strain (stock) of mouse is larger than the BALB/c mouse, and therefore yields greater volumes of peripheral blood at autopsy for blood analysis and serum preparation. The CD-1 mouse is derived from the Swiss mouse (Charles River 2005) that was used in the initial studies of Morley and Blake (1974a) to study the development of CHMF. Therefore, it was considered likely that the CD-1 mouse would respond to BU treatment in a similar way as the BALB/c mouse. An additional objective of the present investigation was to assess further the potential usefulness of the BU-treated mouse model of CBMA in a study having some relevance to the possible elucidation of the basic mechanisms of AA development, and the pathogenesis and pathophysiology of this condition in man.

Materials and methods

Mice

Female CD-1 mice ($n = 96$; mean body weight 17.0 g; Charles River UK Ltd, Margate, Kent, UK) were caged in groups of 5–10 with access to diet (Rat and Mouse No. 1; SDS Ltd, Witham, Essex, UK) and drinking water *ad libitum*. A temperature of 19–22 °C was maintained, with relative humidity of 45–65% and a light:dark cycle of 12:12 h (lights on at 07.00 a.m.). Before the initiation of the experimental procedures animals were allowed at least 7 days to acclimatize. During the BU dosing period, and in the post-dosing period, animals were observed daily for signs of declining health and body weights were determined weekly throughout the course of the experiment. All animal procedures were conducted under local Ethical Committee guidelines and approval for Home Office Project and Personal Licences, and followed the UK Home Office 'Code of Practice for the Housing and Care of Animals used in Scientific Procedures' (Home Office 1989).

BU administration

Busulphan (Sigma Chemical Co., Poole, Dorset, UK) was dissolved in acetone at a concentration of 10 mg/ml and immediately before administration, deionized water was added to the BU-acetone solution at a volume of 2 (water):1 (acetone). Busulphan was administered at a dose volume of approximately 0.1–0.2 ml per mouse by i.p. injection; control animals were treated with water:acetone (vehicle) at the same dose volume.

Experimental design

Mice were divided into two groups, BU-treated ($n = 45$; mean body weight 18.4 g) and control (vehicle-treated; $n = 51$; mean body weight 15.8 g) and were treated with 10 i.p. injections of BU (9.0 mg/kg) or vehicle over a 21-day period (days 1, 3, 5, 7, 9, 11, 14, 16, 18 and 21). On days 1, 23, 72, 119 and 177 after the final dose of BU, animals ($n = 4$–9 per group) were killed by an i.p. injection of pentobarbitone sodium (Sagatal; Rhône Mérieux Ltd, Harlow, UK) for blood and bone marrow investigations. The spleen was removed and weighed.

Analysis of blood and marrow suspensions

At autopsy, a thoracotomy incision was performed, and blood removed from the right ventricle. A 0.5 ml aliquot of blood was anti-coagulated with 1.5 mg/ml dipotassium EDTA (Teklab; Sacriston, Durham, UK); the remaining blood was collected into serum separator tubes (Microtainer; Becton Dickinson and Co., Franklin Lakes, NJ, USA). The contents of the left femur were aspirated into 5 ml Iscove's modified Dulbecco's medium (IMDM; Life Technologies, Paisley, UK) supplemented with 10% foetal calf serum (FCS; PAA Laboratories GmbH, Linz, Austria) to give a marrow cell suspension. Blood samples, and bone marrow suspensions in IMDM, were analysed with the ADVIA 120 haematology analyser (Bayer Diagnostics UK Ltd, Newbury, UK) with mouse specific software. The femoral marrow cell suspension in IMDM was used to obtain the total bone marrow nucleated cell count (femoral nucleated cell count; FNCC) using the basophil channel of the ADVIA 120.

Bone marrow clonogenic assay

At autopsy, the right femur was removed with surrounding muscle and placed in 5 ml sterile IMDM with 10% FCS; under sterile conditions the muscle and epiphyses were removed from the femur and the marrow flushed into 5 ml sterile IMDM supplemented with 10% FCS. Using trypan blue exclusion the nucleated femoral bone marrow cells were counted and cultured at 10^5 cells in 1 ml IMDM supplemented with 30% FCS, 1% bovine serum albumin (Sigma Chemical Co.), 10^{-4}M β-mercaptoethanol (Sigma Chemical Co.), 0.05% $NaHCO_3$, 2.1 mM L-glutamine (Sigma Chemical Co.) and 0.9% methylcellulose (Stem Cell Technologies Inc., London, UK). Cultures were set up in duplicate in 35 mm dishes (Nunclon; Loughborough, UK) with the following growth factors added to each dish: 4 IU human erythropoietin (hEPO; Janssen-Cilag Ltd,

High Wycombe, Bucks, UK), 50 ng murine interleukin-3 (mIL-3; R&D Systems Europe Ltd, Abingdon, UK), 50 ng murine stem cell factor (mSCF; R&D Systems), 50 ng human interleukin-6 (Novartis Pharmaceuticals Ltd, Langley, UK) and 50 ng human granulocyte colony stimulating factor (Amgen UK Ltd, Cambridge, UK). The cultures were incubated at 37 °C in 5% CO_2 in air for 14 days. On day 14, granulocyte-macrophage colony forming units (CFU-GM), erythroid burst forming units and colonies containing both granulocyte-macrophage and erythroid elements were counted. The number of CFU-GM and erythroid colonies were added together to give a total number of CFU-C per femur.

Serum FLT-3 ligand analysis

Blood samples in separator tubes were allowed to stand (75–90 min), centrifuged (400 ***g***, 5 min, room temperature), the serum harvested and stored at −80 °C. The presence of the cytokine FL was measured in serum samples using an ELISA (R&D Systems) with a sensitivity of typically <5 pg/ml according to the manufacturer's instructions.

Statistical analysis

Busulphan-treated and control (vehicle-treated) groups were compared using a one tailed Student's *t*-test (Microsoft EXCEL; Microsoft Ltd, Microsoft UK, Reading, UK). To identify possible relationships between the concentration of FL in the serum, and peripheral blood and bone marrow parameters, scattergrams were drawn. To produce linear relationships a $\log_{10}$-transformation was performed on serum FL data. The relationship between FL and both peripheral blood and bone marrow counts were analysed using linear regression with Runs post-test performed to verify linearity (GRAPHPAD PRISM version 4.00 for Windows, GraphPad Software, San Diego, CA, USA).

Results

Body weight changes

On day 1 of BU dosing, the mean body weight of vehicle-treated (control) animals was 15.8 g increasing to 19.1 g over the 21-day dosing period (20.9% increase). The mean body weight of mice treated with BU was 18.4 g on day 1 of dosing. Over the period of BU treatment the mean body weight of BU-treated mice decreased by 6.5% to 17.2 g. After the period of BU dosing, the mean body weight of BU-treated mice rapidly increased to values comparable to the controls.

Haematology results

On day 1 postdosing, a significant peripheral blood pancytopaenia was evident in BU-treated mice (Table 1). The red blood cell (RBC), haemoglobin (Hb), haematocrit (HCT) and the reticulocyte mean counts were all significantly reduced to 62.1%, 65.0%, 61.5% and 19.2% of the control mean values, respectively ($P < 0.001$ for all parameters). In addition, the mean cell haemoglobin (MCH) and mean cell haemoglobin concentration (MCHC) were significantly increased ($P < 0.001$) but the mean cell volume (MCV) was normal. The mean platelet count on day 1 was also significantly reduced ($P < 0.001$), to 3.3% of the control mean. The profound effects of BU administration were also seen in the leucocyte parameters, the white blood cell (WBC), neutrophil, lymphocyte, monocyte and eosinophil counts were all reduced significantly, to 21.0%, 5.1%, 27.0%, 8.3% and 0.0% of the control mean values respectively.

On day 23 postdosing, significant reductions continued to be seen in the mean erythrocyte parameters (RBC, Hb, HCT), leucocyte counts (WBC, neutrophils, lymphocytes and monocytes) and in the platelet counts of BU-treated mice (Table 1). In addition to these reductions, there was a significant increase in MCV and MCH at this time point ($P < 0.001$ for both values). Although many parameters continued to be significantly reduced in BU-treated animals on day 23 postdosing there was some evidence of a recovery, with the mean reticulocyte count being comparable to the controls at this time.

On day 72 postdosing, the return of peripheral blood counts in BU-treated mice to values comparable to controls was not completed in all blood parameters, with significant reductions still continuing in the RBC, Hb and HCT values in addition to the platelet, lymphocyte and monocyte counts (93.9%, $P < 0.001$; 95.6%, $P < 0.001$; 94.5%, $P < 0.001$; 68.0%, $P < 0.001$; 68.2%, $P < 0.05$; 50.0%, $P < 0.01$, of mean control values respectively).

Many peripheral blood parameters of BU-treated mice were comparable to control values on day 119 postdosing; however, significant reductions continued to be seen in the RBC, HCT and platelets (Table 1). Furthermore, in BU-dosed animals on day 177 postdosing, erythrocyte parameters including, RBC, Hb and HCT continued to be significantly reduced, and the MCV significantly increased. White blood cell, lymphocytes and eosinophils also continued significantly reduced at day 177, to 67.0%, 55.4% and 63.6% of the control means respectively.

Table 1 Haematological results from female CD-1 mice treated with 10 doses of busulphan (BU) over a period of 21 days and sampled at 1–177 days after the final dose

	Day of sampling									
	1		23		72		119		177	
	Control	BU	Control	BU	Control	BU	Control	BU	Control	BU
RBC	9.83 (0.36)	6.10 (1.12)***	10.15 (0.14)	7.88 (1.68)***	10.02 (0.29)	9.41 (0.27)***	9.99 (0.19)	9.70 (0.33)*	10.31 (0.22)	9.51 (0.42)***
Hb	16.0 (0.6)	10.4 (1.9)***	16.4 (0.4)	14.6 (2.6)*	16.0 (0.4)	15.3 (0.3)***	15.0 (0.7)	14.7 (0.5)	15.0 (0.5)	14.1 (0.5)**
HCT	48.1 (1.7)	29.6 (5.6)***	48.6 (1.3)	42.6 (7.6)*	47.6 (1.3)	45.0 (0.4)***	47.1 (1.1)	45.7 (1.7)*	48.1 (1.3)	45.5 (1.3)**
MCV	49.0 (1.6)	48.6 (0.5)	47.9 (1.7)	54.6 (3.0)***	47.5 (0.5)	47.9 (1.2)	47.1 (0.2)	47.1 (0.4)	46.7 (0.6)	47.9 (1.0)**
MCH	16.3 (0.5)	17.1 (0.1)***	16.2 (0.5)	18.6 (1.0)***	15.9 (0.1)	16.2 (0.3)**	15.0 (0.5)	15.1 (0.2)	14.5 (0.2)	14.8 (0.5)
MCHC	33.0 (0.4)	35.2 (0.3)***	33.7 (0.7)	34.1 (0.4)	33.6 (0.4)	33.9 (0.5)	31.8 (1.0)	32.2 (0.6)	31.1 (0.4)	30.9 (0.6)
Retic	339 (114)	65 (21)***	340 (42)	365 (125)	343 (72)	301 (39)	407 (57)	393 (39)	318 (74)	301 (80)
Plt	908 (101)	30 (10)***	1040 (80)	335 (245)***	1030 (86)	700 (111)***	1010 (105)	708 (49)***	1084 (116)	900 (103)**
WBC	1.67 (0.55)	0.35 (0.26) ***	2.44 (0.94)	0.87 (0.30)***	2.20 (0.79)	1.67 (0.39)	2.62 (1.48)	1.93 (0.49)	2.30 (0.58)	1.54 (0.96)*
Neut	0.39 (0.12)	0.02 (0.01)***	0.43 (0.18)	0.18 (0.13)**	0.51 (0.20)	0.44 (0.12)	0.45 (0.16)	0.52 (0.12)	0.45 (0.14)	0.48 (0.30)
Lymph	1.22 (0.42)	0.33 (0.25)***	1.93 (0.78)	0.65 (0.24)***	1.57 (0.59)	1.07 (0.41)*	1.98 (1.26)	1.32 (0.44)	1.68 (0.54)	0.93 (0.64)*
Mono	0.02 (0.01)	0.00 (0.00)***	0.04 (0.02)	0.01 (0.01)**	0.04 (0.03)	0.02 (0.01)**	0.04 (0.02)	0.02 (0.02)	0.07 (0.02)	0.06 (0.03)
Eo	0.02 (0.01)	0.00 (0.00)**	0.03 (0.05)	0.01 (0.03)	0.07 (0.06)	0.13 (0.01)	0.12 (0.11)	0.06 (0.07)	0.11 (0.03)	0.07 (0.02)**
Baso	0.00 (0.01)	0.00 (0.00)	0.00 (0.00)	0.00 (0.00)	0.00 (0.00)	0.00 (0.00)	0.00 (0.00)	0.00 (0.00)	0.01 (0.01)	0.00 (0.01)
FNCC	2.17 (0.30)	0.44 (0.20)***	1.72 (0.35)	0.88 (0.48) ***	2.68 (0.56)	1.88 (0.58)**	2.50 (0.82)	1.93 (0.46)	4.64 (0.45)	3.35 (1.75)
Spleen	5437 (541)	3853 (403)***	5319 (589)	5807 (1412)	5583 (691)	5337 (477)	5275 (509)	4666 (455)*	4784 (493)	4425 (328)

Values are mean, SD in parenthesis; $n = 8$ for control and $n = 6$ for BU groups at all time points, except on day 72 and 119 where $n = 7$ (control), and day 177 where $n = 9$ (controls) and $n = 4$ (BU).

RBC, red blood cells ($\times 10^6/\mu l$); Hb, haemoglobin (g/dl); HCT, haematocrit (%); MCV, mean cell volume (fl); MCH, mean cell haemoglobin (pg); MCHC, mean cell haemoglobin concentration (g/dl); Retic, absolute reticulocyte count ($\times 10^3/\mu l$); Plt, platelets ($\times 10^3/\mu l$); WBC, white blood cells ($\times 10^3/\mu l$); Neut, neutrophils ($\times 10^3/\mu l$); Lymph, lymphocytes ($\times 10^3/\mu l$); Mono, monocytes ($\times 10^3/\mu l$); Eo, eosinophils ($\times 10^3/\mu l$); Baso, basophils ($\times 10^3/\mu l$); FNCC, femoral bone marrow nucleated cell count ($\times 10^7$); Spleen, relative spleen weight (mg/kg body weight).

Significantly different from control values, $^*P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$.

Table 2 Results for committed progenitor cell (CFU-C) content of the femoral bone marrow and serum FL values in female CD-1 mice treated with busulphan (BU; 9.0 mg/kg) on 10 occasions over a period of 21 days and sampled on days 1, 23, 72, 119 and 177 postdosing

	Day of sampling				
	1	**23**	**72**	**119**	**177**
CFU-C					
Control	10,220 (3151)	15,816 (3256)	12,202 (2087)	16,365 (4560)	12,904 (3447)
BU	186 (250)***	3096 (3359)***	7996 (2059)***	6389 (4082)***	14,481 (9959)
BU (as % of control)	1.82	19.58	65.53	39.04	112.22
Serum FL					
Control	321.7 (8.3)	326.4 (10.1)	329.3 (9.2)	284.5 (13.8)	303.0 (18.1)
BU	2370.8 (528.2)***	1337.6 (454.5)***	645.4 (213.8)***	567.5 (155.5)***	558.0 (199.6)**
BU (as % of control)	737.05	409.75	196.02	199.50	184.18

Values are mean, SD in parenthesis. The mean results for BU-treated animals is also presented as a percentage of the mean concurrent control value. For CFU-C, cultures were set up in duplicate and the mean CFU-C number per femur was calculated per mouse; for serum FL mean values are in pg/ml.
Numbers of animals per group, as Table 1; significantly different from concurrent controls, $^{**}P < 0.01$; $^{***}P < 0.001$.

Femoral nucleated cell count

On day 1 after the final BU dose the mean FNCC of BU-treated mice was significantly reduced to 20.3% ($P < 0.001$) of the control mean (Table 1). On days 23 and 72 postdosing, the FNCC was returning towards the control value, however, the count continued to be significantly reduced to 51.2% ($P < 0.001$) and 70.1% ($P < 0.01$) of the control mean respectively. On days 119 and 177 postdosing, the decreased marrow cellularity of BU-treated mice was further sustained (7.2% and 77.2% of the controls, respectively), but these reductions were not statistically significant.

Bone marrow clonogenic assay

The number of CFU-C per femur, within the bone marrow of BU-treated mice is shown in Table 2, expressed as a percentage of the control mean at each concurrent time point. On day 1 postdosing, the number of committed progenitors was significantly reduced, in mice treated with BU, to 2.0% of the control mean ($P < 0.001$). On day 23 and 72 postdosing, the number of colonies per femur in BU-dosed animals continued to be significantly reduced, to 19.6% and 65.5% of the control mean, respectively ($P < 0.001$ for both time points). However, there was evidence of recovery and a return towards normal control values at these times. The return of the number of progenitor cells towards control values was not completed on day 119 postdosing, and the mean number of colonies per femur of BU-treated mice was reduced to 39.0% of the control mean ($P < 0.001$). However, on day 177 postdosing, the number of committed progenitor cells within the bone marrow of BU-treated mice was comparable to the control mean (Table 2).

Serum FLT-3 ligand analysis

At the scheduled autopsy points (days 1, 23, 72, 119 and 177 postdosing), the concentration of the serum cytokine FL

Table 3 Linear regression analysis and correlation coefficients of $\log_{10}$ FL and haematological parameters in busulphan-treated mice autopsied on day 1, 23, 72, 119 and 177 postdosing

Parameter	Equation	r
Plt	$y = -1046.70x + 3607.80$	0.90***
RBC	$y = -5.05x + 23.40$	0.86***
Neut	$y = -0.71x + 2.41$	0.84***
FNCC	$y = -3.15x + 10.90$	0.77***
WBC	$y = -1.99x + 7.14$	0.76***
CFU-C/femur	$y = -13{,}729x + 46{,}058$	0.73***
MCHC	$y = 3.76x + 22.30$	0.71***
Hb	$y = -5.60x + 30.35$	0.70***
MCH	$y = 3.43x + 6.34$	0.66***
Mono	$y = -0.05x + 0.16$	0.66***
Lymph	$y = -1.10x + 4.11$	0.62***
Retic	$y = -292.78x + 1150.60$	0.61***
Eo	$y = -0.11x + 0.38$	0.46***
MCV	$y = 4.63x + 35.55$	0.42*

Abbreviations and units as Table 1. Relationships between serum FL values and the basophil count and the HCT were not linear and therefore linear regression analysis was not performed on these parameters. FL results were converted logarithmically to linearize data for linear regression analysis.
Significant correlation, $^{*}P < 0.05$; $^{***}P < 0.001$.

Table 4 Haematological results from 2 groups of 4 female CD-1 mice treated with 10 doses of busulphan (BU; 9.0 mg/kg) over a period of 21 days and killed *in extremis* on day 43 and 55 after the final dose; 4 control mice were also sampled at each time point

	Treatment			
	Day 43 mean (SD)		Day 55 mean (SD)	
	Control	BU	Control	BU
RBC	10.42 (0.54)	4.47 (1.24)***	10.64 (0.25)	2.69 (1.87)***
Hb	16.5 (0.7)	8.6 (2.6)*	16.9 (0.4)	4.7 (3.2)***
HCT	48.6 (2.5)	23.9 (6.2)***	48.7 (2.2)	14.0 (9.8)***
MCV	46.6 (0.9)	53.8 (1.9)***	45.7 (1.3)	52.4 (3.8)**
MCH	15.9 (0.2)	19.3 (2.3)**	15.8 (0.2)	17.8 (0.7)***
MCHC	34.0 (0.5)	35.9 (4.2)	34.7 (0.8)	34.0 (1.4)
Retic	282 (25)	43 (11)†,***	332 (21)	64 (69)***
Plt	904 (116)	38 (18)***	1007 (88)	38 (12) ***
WBC	1.18 (0.46)	0.28 (0.08)**	3.25 (1.24)	0.38 (0.23)**
Neut	0.29 (0.08)	0.05 (0.02)***	0.49 (0.09)	0.03 (0.02)***
Lymph	0.77 (0.49)	0.22 (0.07)*	2.63 (1.19)	0.33 (0.21)**
Mono	0.03 (0.01)	0.00 (0.00)***	0.06 (0.01)	0.00 (0.00)***
Eo	0.00 (0.01)	0.00 (0.00)	0.00 (0.00)	0.00 (0.00)
Baso	0.07 (0.07)	0.00 (0.00)*	0.05 (0.08)	0.00 (0.00)
FNCC	1.98 (0.09)	0.19 (0.06)***	1.79 (0.21)	0.11 (0.02)**
Spleen	5615 (1708)	4218 (973)	4851 (238)	2945 (1067)**

Values are mean, SD in parenthesis. Abbreviations and units: as Table 1.
Significantly different from controls, $*P < 0.05$; $** P < 0.01$; $*** P < 0.001$.
†Mean (SD) of $n = 3$ mice (insufficient sample volume for analysis from the fourth animal in the group).

was measured in control (vehicle-treated) and BU-treated mice (Table 2). A significant increase in serum FL was evident in BU-treated mice immediately postdosing with the mean value for FL being 2370.8 pg/ml compared to 321.7 pg/ml in the control animals ($P < 0.001$). The concentration of FL in the serum of BU-treated mice continued to be elevated above the mean control level on day 23 postdosing to approximately four times the concentration in vehicle-treated mice (control mean 326.4; BU mean 1337.6 pg/ml; $P < 0.001$). However, there was evidence of a return towards normal control values at this time. On days 72, 119 and 177 the concentration of FL in the serum of BU-treated animals was maintained at a plateau, remaining significantly elevated above the control mean value (Table 2).

Individual animal serum FL values measured in BU-treated mice at all five time points (i.e. days 1, 23, 72, 119 and 177 postdosing) were analysed in relation to individual peripheral blood and bone marrow parameters. To linearize the data, the concentration of serum FL was converted logarithmically ($\log_{10}$). Significant correlations were identified between the majority of blood and bone marrow parameters tested (Table 3). Results show that when a significant change in peripheral blood and bone marrow values is evident, serum FL is elevated, with the strongest relationships identified between $\log_{10}$-FL and the platelets, RBC, neutrophils and the FNCC ($r = 0.90$, $r = 0.86$, $r = 0.84$ and $r = 0.77$, respectively; $P < 0.001$ for all four parameters).

Clinical observations and BU-induced myelotoxicity

During the course of the experiment, several animals exhibited clinical evidence of BU-induced toxicity. These mice tended to show a loss of condition, with the fur becoming dull and dry, with piloerection. Also there was a decrease in activity and responses, and in some individuals an abnormal gait and a hunched posture developed. The ears, paws and tails of these animals often became pale or white, losing the normal pink colour. The condition of these mice deteriorated quickly, some were killed *in extremis* and a small number of animals were 'found dead'. Such mice are categorized as 'inter-current death' (ICD) animals. To investigate the basis of the toxicity and in an attempt to identify a cause of death, efforts were made to identify animals that were becoming ill. At two time points, days 43 and 55 postdosing, BU-treated animals ($n = 4$ on each day) showing significant evidence of toxicity were autopsied. In addition, a group of control mice ($n = 4$) were autopsied at each time point for comparison and statistical analysis. A full blood count was performed and a femoral marrow flush prepared to assess marrow cellularity. The peripheral blood counts

Table 5 Serum FL values (pg/ml), femoral bone marrow clonogenic assay (CFU-C) results, femoral bone marrow nucleated cell counts (FNCC) and peripheral blood haematological findings in five individual female BALB/c mice treated with busulphan (BU; 9.0 mg/kg) on 10 occasions over a period of 21 days and sampled on days 1, 50, 126 and 154 postdosing; data from concurrent control animals are also presented

	Day of sampling								
	1			50		126		154	
	1	2	Control	3	Control	4	Control	5	Control
FL	1023.8	1496.0	376.0 (127.5)	3760.0	274.4 (72.3)	986.8	265.4 (45.6)	1333.8	260.2 (15.4)
CFU-C	2646	743	14,546 (4680)	NS	NS	431	11,908 (2965)	2485	13,978 (4041)
FNCC	2.14	1.65	4.1 (0.62)	2.28	3.86 (0.68)	2.71	4.22 (0.17)	0.88	3.60 (0.84)
RBC	8.60	9.10	9.49 (0.18)	9.04	9.83 (0.30)	8.99	9.69 (0.15)	7.50	9.89 (0.23)
Hb	14.0	15.0	15.1 (0.2)	14.5	15.2 (0.5)	13.7	14.7 (0.3)	12.6	14.9 (0.4)
HCT	44.0	46.7	48.7 (1.1)	46.4	48.3 (1.8)	43.2	46.5 (1.8)	38.6	48.2 (1.6)
MCV	51.2	51.2	51.3 (0.8)	51.4	49.3 (0.4)	49.3	48.0 (1.1)	53.2	48.7 (1.2)
MCH	16.3	16.4	15.9 (0.2)	16.0	15.4 (0.1)	15.2	15.2 (0.1)	16.8	15.1 (0.2)
MCHC	31.9	32.1	30.9 (0.6)	31.2	31.3 (1.5)	31.0	31.7 (0.6)	31.5	31.0 (0.8)
Retic	500	247	368 (59)	300	322 (52)	305	315 (27)	307	325 (28)
Plt	195	116	953 (111)	541	955 (37)	486	916 (84)	206	1110 (122)
WBC	1.17	0.54	1.46 (0.30)	1.81	3.41 (1.17)	2.61	3.37 (0.9)	0.56	2.72 (1.08)
Neut	0.16	0.09	0.25 (0.06)	0.23	0.62 (0.18)	0.65	0.73 (0.17)	0.21	0.65 (0.34)
Lymph	0.97	0.44	1.11 (0.27)	1.51	2.58 (0.95)	1.87	2.41 (0.72)	0.34	1.86 (0.79)
Mono	0.00	0.00	0.02 (0.01)	0.02	0.06 (0.03)	0.02	0.09 (0.02)	0.00	0.06 (0.03)
Eo	0.02	0.01	0.06 (0.03)	0.04	0.13 (0.04)	0.05	0.12 (0.05)	0.01	0.14 (0.07)
Baso	0.00	0.00	0.00 (0.00)	0.00	0.01 (0.01)	0.01	0.00 (0.01)	0.00	0.00 (0.00)

For concurrent control animals, values are means, SD in parenthesis. There were $n = 6$ control animals (days 1 and 50), $n = 5$ (day 126) and $n = 8$ (day 154). For CFU-C, cultures were set up in duplicate and the number of CFU-C per femur calculated per mouse. NS, no sample available for analysis. Other information as Tables 1 and 2.

and marrow cellularity of the BU-treated mice and the controls are shown in Table 4. It is seen that at day 43 and 55 postdosing, BU-treated mice had significant bone marrow hypocellularity (reduced FNCC values) and significant reductions in peripheral blood values for RBC, neutrophils and platelets; the MCV was increased ($P < 0.01$ or $P < 0.001$ for all five parameters). It is therefore considered likely that the BU-treated animals were losing condition and becoming ill as a result of bone marrow suppression.

Discussion

In the present experiment the outbred CD-1 mouse replaced the previously-used inbred BALB/c mouse (Gibson *et al.* 2003; Turton *et al.* 2006). The CD-1 mouse (Charles River 2005) is derived from the 'Swiss' mouse used by Morley & Blake (1974a,b) and Morley *et al.* (1975). The results from the current study suggest that the CD-1 mouse is susceptible to BU-induced bone marrow depression. Also, the CD-1 has the advantage, in comparison to the BALB/c, that this strain (stock) is widely used in the pharmaceutical industry for drug safety evaluation studies, and is also popular in the field of experimental toxicology. However, the CD-1 also has the major advantage over the BALB/c of larger size (i.e. greater body weight) and thus can provide larger volumes of blood at autopsy for haematological analysis and the preparation of serum. Nevertheless, a total of 7 CD-1 mice out of 45 (i.e. 15.6%) treated with BU were categorized as ICD animals in the current experiment, suggesting that the dose level of BU employed (9.0 mg/kg), should be slightly reduced in future studies to avoid such mortalities.

In a previous report (Turton *et al.* 2006), the use of the term 'CBMA', to describe the late-stage (residual) changes induced by repeated BU administration in the mouse, was discussed; it was pointed out that the late stage bone marrow effects could be more appropriately described as 'chronic bone marrow hypoplasia' (CBMH) rather than CBMA. The view on the use of the term hypoplasia *vs.* aplasia for the late-stage lesion has been influenced by the fact that Morley & Blake (1974a,b) and Morley *et al.* (1975, 1978) described the BU-induced condition in their mice as CHMF (Pugsley *et al.* 1978). The present experiment

appears to lend support to the use of the term CBMH to describe the late stage (residual) BU-induced effects, rather that the previously-used (Gibson *et al.* 2003) CBMA.

The FLT-3 receptor is expressed on haemopoietic stem cells (HSCs) and early progenitor cells, particularly those of the B-lymphoid lineage (Rosnet *et al.* 1996). The FLT-3 ligand, in the soluble and membrane bound forms of the ligand, may therefore be involved in the control of haemopoiesis.

Studies in the mouse (Brasel *et al.* 1996) have shown that daily administration of FL at 10 μg/mouse for 15 days by i.p. injection promotes lymphopoiesis with peripheral blood and spleen WBC counts increasing significantly. The experiments of Brasel *et al.* (1996) also showed that the administration of FL had a positive effect on the number of HSCs. Indeed, HSCs characterized as lin^-, $Sca\text{-}1^+$ and $c\text{-}Kit^+$, were found in higher numbers in the bone marrow, spleen and peripheral blood of FL-treated mice.

The FL administration (1 μg/mouse) to neonatal mice within 24 h of birth, and then daily for 6 days thereafter, has also been shown to enhance immune function (Vollstedt *et al.* 2003). The FL-treated mice had improved immune responses when challenged with bacteria and viruses. This improvement in immune response occurred in addition to an increase in dendritic cell number in the spleen, liver and peritoneal fluid.

Further evidence for the role of FL in lymphopoiesis and the immune response has been reported using mice that have been genetically manipulated and do not express the FLT-3 receptor (McKenna *et al.* 2000). Such FLT-3 knock out mice were shown to have a significant reduction in lymphopoiesis, with WBC counts significantly reduced in the peripheral blood and bone marrow. Additionally, immune cells were affected with a significant reduction in the number of dendritic cells in the spleen, thymus and lymph nodes as well as evidence of a reduction in the number and function of natural killer cells.

Administration of FL by subcutaneous injection has been shown to protect against bone marrow failure in rabbits exposed to total body irradiation (TBI) (Gratwohl *et al.* 1998). When given at 500 μg/kg for 14 days starting 2 days before TBI, FL-treated rabbits had a delayed and a less pronounced reduction in peripheral blood cells compared to FL-untreated rabbits. The FL-treated rabbits also had an increased survival rate, with the best outcome achieved when administering FL in conjunction with granulocyte colony stimulating factor (Gratwohl *et al.* 1998).

In the present study, CD-1 mice treated with the chemotherapeutic drug BU, an agent reported to destroy HSCs (Jopling & Rosendaal 2001), initially (i.e. day 1 postdosing) demonstrated bone marrow hypoplasia (i.e. a reduced FNCC) and peripheral blood pancytopaenia (Table 1). There was also a very significant reduction in the number of haemopoietic progenitor cells (CFU-C; Table 2). During this initial phase of bone marrow hypoplasia, the concentration of FL in the serum of BU-treated mice was increased to a mean of 2370.8 pg/ml compared to 321.7 pg/ml in the vehicle-treated control mice (Table 2). The increase in FL levels continued to be evident throughout the postdosing period with FL concentrations being increased significantly at all time points (days 23, 72, 119 and 177 postdosing).

Studies have shown that patients receiving chemotherapy for the treatment of malignant diseases, or as part of a conditioning regimen to prepare for bone marrow transplant, also have significantly elevated plasma FL levels (Chklovskaia *et al.* 1999). The increase in plasma FL corresponds to the period of bone marrow aplasia. Indeed, studies show the concentration of FL in the plasma to be increased immediately following chemotherapy, and returning to pretreatment levels following successful haematological recovery (Chklovskaia *et al.* 1999; Blumenthal *et al.* 2000; Huchet *et al.* 2003).

An increase in plasma FL has also been identified in patients with diseases characterized by bone marrow failure such as AA and Fanconi's anaemia (Wodnar-Filipowicz *et al.* 1996; Lyman *et al.* 2005). Serum levels of FL have been reported to be as high as 2653 pg/ml in AA patients upon presentation and when the bone marrow is markedly aplastic. This is in comparison to a serum FL concentration of 14 pg/ml in normal subjects. The increased concentration of FL in the serum of AA patients then reduces following successful bone marrow reconstitution, for example, as a result of anti-lymphocyte globulin treatment or bone marrow transplant (Wodnar-Filipowicz *et al.* 1996). However, if patients relapse, FL levels have been reported to increase in the serum once again, to the concentrations seen prior to treatment (Wodnar-Filipowicz *et al.* 1996).

In the present mouse study, changes were measured in the concentration of serum FL and levels were compared with changes in other haematological parameters such as peripheral blood counts, bone marrow cellularity (FNCC) and the number of CFU-C. Linear regression analysis showed significant correlations between the concentration of FL in the serum of BU-treated mice and many of the haematological parameters measured (Table 3). However, the relationship between FL levels and HCT and the basophil count were not linear, and linear regression analysis could not therefore be conducted; the reasons for these anomalous results are not clear, particularly in the case of the HCT values.

Studies describing changes in the concentrations of serum FL in mice following treatment with BU have not previously been reported, although we have described initial findings on bone marrow depression and FL levels in the mitomycin-treated CD-1 mouse (Molyneux *et al.* 2005) and in the chlorambucil-treated Wistar-Hanover rat (Molyneux *et al.* 2004) which find a direct parallel with the present results in the BU-treated CD-1 mouse. However, in addition, a recent report by Prat *et al.* (2005) has demonstrated that levels of FL are elevated in the plasma of BALB/c mice and non-obese diabetic/severe combined immunodeficient mice following exposure to radiation. An increase in plasma FL has also been shown in nonhuman primates exposed to varying levels of TBI (Bertho *et al.* 2001). This last study also showed that a significant correlation exists between the neutrophil count at its lowest point and the concentration of FL in plasma. In addition, the level of plasma FL was found to correlate with the intensity of radiation exposure.

In man, inverse correlations have been described between the concentration of plasma FL and both the neutrophil count and the number of CFU-C within the bone marrow of AA patients (Wodnar-Filipowicz *et al.* 1996). Similarly, a study by Pfister *et al.* (2001) reported that membrane bound FL is increased on $CD4^+$ and $CD8^+$ lymphocytes of severe AA patients. In the study of Pfister *et al.* (2001) the level of membrane bound FL was inversely correlated with the number of $CD34^+$ cells of the bone marrow.

The results presented in Table 4 demonstrate that a proportion of individual animals treated with BU appear to be particularly sensitive to the drug, and the haematological parameters of these animals become more significantly affected than the changes seen in the rest of the BU-treated cohort. This type of response has been noted in earlier studies with BU (Gibson *et al.* 2003; Turton *et al.* 2006). The effect, on a minority of animals, was also reported by Morley and Blake (1974a,b) and Morley *et al.* (1975) in studies on BU-induced CHMF in the Swiss or BALB/c mouse. Following a BU treatment regimen, Morley *et al.* described their mice as being in a 'latent' phase, showing only minor haematological abnormalities. However, at times after day 60 postdosing, individual 'latent' animals began to show a developing bone marrow aplasia, and these animals were referred to as 'aplastic' mice. We consider that the animals presented in Table 4 may compare with the 'aplastic' mice of Morley *et al.*, and it is suggested that the haematological changes evident in Table 4 animals show parallels with the haematological changes seen in AA in man. Unfortunately, it was not possible to prepare serum samples for FL evaluation from the animals presented in Table 4, as in mice becoming unwell in this way it is often difficult to obtain a suitably large volume of blood for both haematological analysis and serum preparation. In the case of the animals described in Table 4, the small volumes of blood obtained at autopsy were used for peripheral blood assays, rather than serum preparation, in an attempt to identify the cause of illness/death at these relatively chronic time points following BU administration (i.e. day 43/55). In retrospect, this action may have been inopportune, as it would have been of considerable interest to assay for serum FL in these individual animals becoming seriously unwell. Nevertheless, some limited results, examining FL in conjunction with CFU-C values, FNCC counts and significantly changed blood parameters, in individual BU-treated animals are available. In a recent study involving female BALB/c mice treated with BU (9.0 mg/kg on 10 occasions over a 21-day period), individual animal data from five animals was obtained (Table 5); the study involved autopsies at days 1, 50, 126 and 154 post-treatment. It is seen (Table 5) that although the effects of BU in these five mice were not as severe as in the animals of Table 4, the overall pattern of changes (in comparison with the concurrent controls) show greatly increased FL levels, in conjunction with reduced CFU-C counts, lowered FNCC values, and a reduced peripheral blood RBC and platelet counts, and generally decreased WBC/neutrophil/lymphocyte values.

In conclusion, in the present study in the BU-treated female CD-1 mouse, the concentration of FL in the serum (Table 2) correlated with bone marrow injury as measured by bone marrow cellularity (FNCC; Table 1), the number of CFU-C (Table 2) and peripheral blood counts (Table 1). The return of FL towards normal (control) levels in BU-treated mice (Table 2) correlated with the time of partial haematological recovery. However, the concentration of serum FL continued to be significantly increased in BU-treated mice until day 177 postdosing during which time some peripheral blood parameters remained significantly affected in comparison to vehicle-treated control mice. These findings indicate the possible usefulness of serum FL levels as a potential marker of drug-induced bone marrow injury. Also, the data generated appear to confirm the possible value of the BU-induced CD-1 mouse model of bone marrow depression in investigations to explicate mechanisms of AA pathogenesis in man. However, the role played by FL in bone marrow injury is unclear, and more studies need to be completed to further elucidate the mechanistic basis for elevated serum FL concentrations during bone marrow damage. It is possible that after treatment with chemotherapy or radiotherapy, and in conditions such AA, increases in FL occur in an attempt to stimulate an increase in the number of HSCs, and hence the number of mature cells. Cell cycle analysis of HSCs

following BU-treatment in the mouse may provide evidence for such processes.

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