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Comparison of Different Busulfan Analogues for Depletion of Hematopoietic Stem Cells and Promotion of Donor-Type Chimerism in Murine Bone Marrow Transplant Recipients[1]

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ABSTRACT

Busulfan (1,4-butanediol dimethanesulfonate, BU) is relatively unique among other standard chemotherapy compounds in its ability to deplete noncycling primitive stem cells in the host and consequently to allow for high levels of long-term, donor-type engraftment after bone marrow transplantation (BMT). Such a property explains why this drug can be used as an alternative to total body irradiation in preparative regimes for BMT. However, as with radiation, BU conditioning is still troubled by severe toxicities that limit its applications to suboptimal drug doses. These problems stress the need for other BMT-conditioning drugs that are better tolerated and more selectively targeted toward normal and malignant hematopoietic stem cells. We have therefore compared the effects of various novel dimethanesulfonate compounds (related to BU) in terms of their toxicity to different stem cell subsets *in vivo* and *in vitro* and their ability to provide for long-term donor bone marrow engraftment using the congenic glucose-6-phosphate isomerase type 1 marker. Introduction of a benzene or cyclohexane ring in some of these drugs affords rigidity to the molecule and restricts the spatial positioning of the alkylating groups. Among 25 different compounds thus far tested at single doses, PL63 [*cis*-1,2-(2-hydroxyethyl) cyclohexane dimethanesulfonate] proved to be the most effective in providing for hematopoietic engraftment. The *trans*-isomer of the same compound gave significantly less engraftment and was comparable with the effects of dimethylbusulfan and Hepsulfam. The engraftment data correlated well with the depletion of different bone marrow stem cell subsets in the host as measured using the cobblestone area forming cell assay. The extent of stem cell depletion could not be explained on the basis of the distance and orientation of the two alkylating groups. Pharmacokinetic data, however, indicate that there is a correlation between biological activity and plasma levels reached.

The diverse cytotoxic effects shown by these novel analogues of BU have provided a basis for relating biological activity with pharmacokinetic properties rather than with structural properties such as distance and orientation of the two alkylating groups. The identification of highly active compounds such as PL63 offers an opportunity for further developing other closely related drugs for potential application in clinical BMT conditioning therapy.

INTRODUCTION

Recipient conditioning for BMT[4] is an important determinant in the overall clinical outcome in the treatment of many diseases of both a malignant (acute and chronic leukemias, myelodysplastic syndrome) as well as a nonmalignant nature (thalassemia major and inborn errors of the immune system). The two most frequently applied regimens are high-dose BU, followed by CY, or CY followed by TBI. In this respect, it is important to note that BU and TBI share the relatively unique property of depleting noncycling primitive stem cells and consequently provide for long-term donor stem cell engraftment (1–3). The efficacy of both preparative regimens in the treatment of leukemias are similar, although there seems to be a small advantage for CY/TBI in autologous transplantation for acute lymphoblastic leukemias in terms of leukemia-free survival and relapse incidence (4) and in patients with acute myeloid leukemia in first remission (5). In the case of allogeneic BMT for chronic myeloid leukemia, the BU/CY combination is slightly preferable over CY/TBI, both in terms of tolerance and in clinical outcome (6), possibly because of the higher antileukemic effect of BU/CY. Although better tolerated in some cases, the use of BU as well as radiation for recipient conditioning is still hampered by severe and in some cases lasting toxicities (Refs. 7 and 8; reviewed in Refs. 9 and 10). These toxicities include seizures (11), veno-occlusive disease (7), and pulmonary complications (12). The BU-induced hematological toxicities may not always be reversible; hematopoietic recoveries can be impaired for up to 4 years after BU treatment (13). Although damage to stromal cell populations are often assumed to occur in patients after BU administration, its contribution to impaired hematological reconstitution may be relatively minor as compared with the direct and chronic effects on stem cells (3, 14). Because of the poor solubility of BU, an oral formulation is needed, resulting in large variations in plasma concentrations between patients. The plasma levels reached relate to the extent of toxicity and graft rejection (11, 15–17) as well as to the probability of leukemic relapse (18).

These factors illustrate the demand for new drugs that are at least as effective and specific as BU and, preferably, show less toxicity. These novel agents should be targeted toward both normal and malignant primitive hematopoietic stem cells to allow for high levels of engraftment with minimal risk of relapse. In the past decades, a number of both aliphatic and aromatic DMS compounds have been synthesized. Of the aliphatic compounds with the general formula $CH_3{\cdot}SO_2O{\cdot}(CH_2)_n{\cdot}OSO_2{\cdot}CH_3$, all compounds (including BU, $n = 4$) have reported DNA interstrand cross-linking activity, except where

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[4] The abbreviations used are: BMT, bone marrow transplantation; CAFC, cobblestone area forming cell; Gpi-1, glucose-6-phosphate isomerase type 1; DMS, dimethanesulfonate; MTD, maximal tolerated dose; ALAT, alanine amino transferase; BUN, blood urea nitrogen; AUC, area under the plasma-concentration *versus* time curve; BU, busulfan, 1,4-butanediol dimethanesulfonate; CY, cyclophosphamide; DMB, dimethylbusulfan, 2,5-hexanediol dimethanesulfonate; Hepsulfam, 1,7-heptanediol disulfamate; Treosulfan, L-threitol-1,4-dimethanesulfonate; PL26, bis-1,2-(2-hydroxyethyl)benzene DMS; PL32, *cis*-1,2-cyclohexanedimethanol DMS; PL33, *trans*-1,2-cyclohexanedimethanol DMS; PL34, *cis*-1,4-cyclohexanedimethanol DMS; PL35, *trans*-1,4-cyclohexanedimethanol DMS; PL39, *cis*-1,3-cyclohexanedimethanol DMS; PL40, *trans*-1,3-cyclohexanedimethanol DMS; PL48, 1,8-octanediol DMS; PL49, 1,7-heptanediol DMS; PL50, 1,6-hexanediol DMS; PL51, 1,5-pentanediol DMS; PL63, *cis*-1,2-bis-(2-hydroxyethyl)cyclohexane DMS; PL64, *trans*-1,2-bis-(2-hydroxyethyl)cyclohexane DMS; PL84, 1-(3-hydroxypropyl)-2-(hydroxyethyl)benzene DMS; PL87, bis-1,2-(3-hydroxypropyl)benzene DMS; PL102, *trans*-2,3-bis-hydroxymethyloxirane DMS; PL103, *trans*-1,3-bis-(2-hydroxyethyl)cyclohexane DMS; PL104, *cis*-1,3-bis-(2-hydroxyethyl)cyclohexane DMS; PL106, *trans*-1,2-bis-(2-hydroxyethyl)cyclopentane DMS; PL107, *cis*-1,2-bis-(2-hydroxyethyl)cyclopentane DMS; PL108, *trans*-1,4-bis-(2-hydroxyethyl)cyclohexane DMS; PL109, 1,4-butanediol disulfamate; TBI, total body irradiation.

$n = 2$ (ethylene DMS), with a maximal activity with 1,6-hexanediol DMS ($n = 6$; Refs. 19 and 20). The cyclic DMSs synthesized previously include cyclohexane-1,3- and cyclohexane-1,4-dimethanesulfonate (21), but these compounds have not been tested on their ability to deplete bone marrow stem cells. Although DNA-DNA cross-linking is often considered to be the toxic lesion, DNA-protein binding has also been reported (20), mainly between DNA and the cysteine of histone H_3 (22). The *in vitro* or *in vivo* toxicity of the aliphatic and cyclic DMSs or sulfamates synthesized to date has been determined on either peripheral blood cells (23, 24), tumor cell lines (25–27), tumor xenografts (20, 28, 29), primary chronic myeloid leukemia cells (30), and spleen colony-forming units (31). In addition to BU, only three DMS and sulfamate compounds have actually been clinically evaluated: DMB (32), Treosulfan (33, 34), and Hepsulfam (35).

In the present study, we compared the efficacy of host pretreatment with 25 different aliphatic and cyclic analogues of BU, which have either been described before (20, 28, 29) or synthesized recently. These compounds were tested for induction of short- and long-term donor bone marrow engraftment *in vivo* using the Gpi-1 congenic marker and their ability to deplete various host stem cell subsets, as defined in the CAFC assay *in vitro*. In addition to syngeneic BMT, engraftment of allogeneic bone marrow was evaluated after conditioning regimens that included BU and another active compound, PL63, in combination with CY.

MATERIALS AND METHODS

Animals

Male C57BL/6JIco (B6-*Gpi-1*^{b}/*Gpi-1*^{b}) mice (IffaCredo, L'Arbresle, France), 12–20 weeks of age, 25–30 g, were used as recipients. C57BL/6J-*Gpi-1*^{a}/*Gpi-1*^{a} (B6-*Gpi-1*^{a}) congenic mice (males) were used as a source of donor bone marrow. BALB-B10LiLa male mice (Jackson Laboratory, Bar Harbor, ME), also carrying the *Gpi-1*^{a} gene, were used as allogeneic donors that were H-2-matched (*H-2*^{b}) but mismatched on a number of minor histocompatibility loci. For the pharmacokinetic studies, groups of five to six C57BL/6 mice from either congenic strain were used for each time point. Animals were housed in approved facilities free of known pathogenic organisms (Sendai, MHV, PVM, GD VII, REO III, EMC, LMC, MVM, K, and *Mycoplasma pulmonis*). Experiments were performed in accordance with the Netherlands Experiments on Animals Act (1977) and the European Convention for the protection of vertebrate animals used for experimental purposes (Strasbourg, 18.III.1986).

Drugs

BU and CY were purchased from Sigma Chemical Co. (St. Louis, MO) and Aldrich-Chemie (Steinheim, Germany), respectively. The sulfamate derivative Hepsulfam (NSC 329680) was obtained from the Drug Synthesis and Chemistry Branch of the National Cancer Institute (Bethesda, MD). Treosulfan was a gift from Dr. J. Baumgart (Medac Hamburg, Hamburg, Germany). All other DMS or sulfamate compounds were synthesized within our own facilities (Department of Experimental Chemistry, Paterson Institute for Cancer Research, Manchester, United Kingdom), partly as described previously: PL51, PL50, PL49, and PL48 (20), PL26 (28), and PL84 and PL87 (29). The chemical structure of all compounds used in this study are shown in Fig. 1 for aliphatic (*a*), aromatic (*b*), and alicyclic (*c*) compounds.

In Vitro Treatment

Murine bone marrow cells were isolated from femurs from untreated mice and incubated in Iscove's modified Dulbecco's medium (Life Technologies, Inc., Breda, the Netherlands) supplemented with penicillin (100 units/ml), streptomycin (100 μg/ml), 100 μM β-mercapto-ethanol, 10% FCS, and 5% horse serum. Nucleated cells were incubated at 37°C (10^6/ml) with the appropriate drug for 18 h at a concentration of 30 μM. The concentration of the solute DMSO never exceeded 1%.

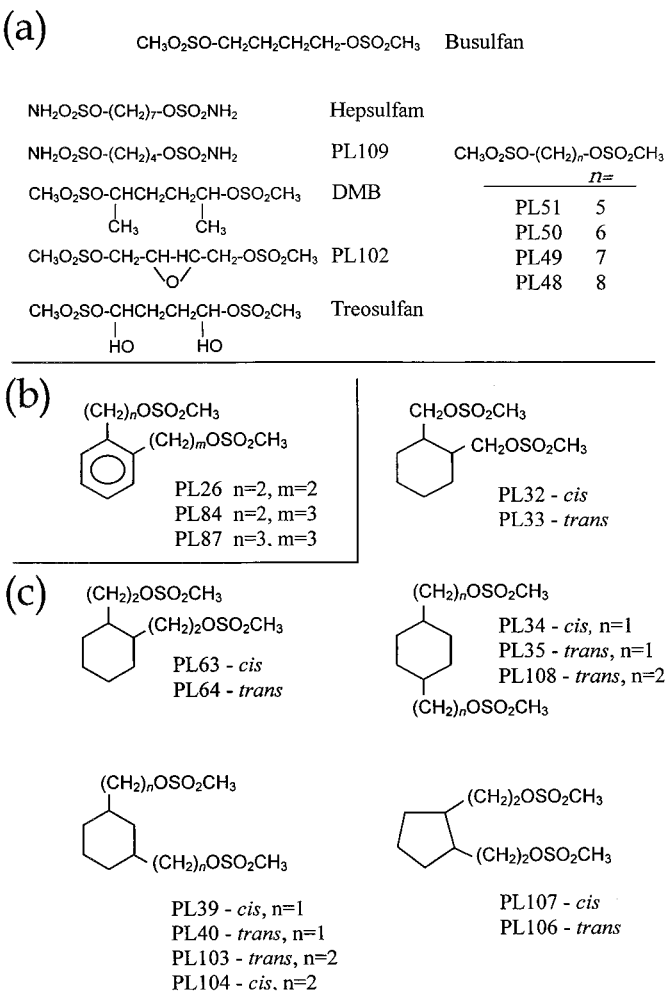

Fig. 1. Structural formulas of the aliphatic (*a*), aromatic (*b*), and alicyclic (*c*) analogues of BU that were investigated in the present study.

In Vivo Treatment

All drugs were injected i.p. in volumes of 0.1 ml/10 g of body weight. DMB was dissolved in 5% DMSO in PBS, PL109 and Treosulfan in 50% DMSO in PBS, and CY in PBS. All other drugs were administered as a suspension in corn oil. TBI was applied using a ^{137}Cs gamma irradiation unit (IBL 637; CIS Biointernational, Gif-sur-Yvette, France) to a dose of 7 Gy (87 cGy/min). Each treatment group consisted of four mice allocated for the CAFC assay and/or five to six mice for bone marrow transplantation. When mice were treated with BU or PL63 combined with CY, BU or PL63 was given at day 2, CY at day 1, followed by BMT at day 0. Fractionated BU (4×25 or 4×12.5 mg/kg) was administered on four consecutive days (day 4 until day 1), followed by BMT at day 0.

Animals treated with TBI, CY, and PL63 received neomycin sulfate in their drinking water (3.5 g/l) to prevent treatment-related gut damage (2). Because of incisor damage starting 2–3 months after the administration of Hepsulfam, PL63, or CY, mice were provided moistened food from week 6 onward to prevent malnutrition.

Determination of Hematopoietic Stem Cell Subset Frequencies *in Vitro* (CAFC Assay)

At 24 h after drug treatment, bone marrow cells were harvested from either pooled groups of four mice or from conical tubes used for *in vitro* treatments and plated in limiting dilutions on confluent layers of the bone marrow stromal cell line FBMD-1 in 96-well plates, as described previously (36–38). These cultures provided growth of hematopoietic precursors under the stromal layer, giving the appearance of cobblestone areas in phase-contrast microscopy. The

frequency of CAFCs were assessed weekly from day 7 until day 35 after overlay. This *in vitro* system provided an estimate of the surviving fraction of CAFC day types that correspond to CFU-GM (CAFC day 5), transiently repopulating CFU-S day 12 (CAFC day 10), and the primitive stem cells with long-term repopulating ability (CAFC days 30–40; Refs. 36, 38, and 39).

BMT and Determination of Donor Engraftment *in Vivo* (Gpi-1 Chimerism Assay)

At 24 h after the drug treatments, allocated mice were transplanted i.v. with 10^6 nucleated bone marrow cells freshly harvested from the tibia and femur of B6-*Gpi-1*[a] or BALB.B10 donor mice. The level of erythroid chimerism in blood samples obtained at different intervals between 2 and 36 weeks after transplant was determined from glucose phosphate isomerase (*Gpi-1*) electrophoresis as described previously (40).

Pharmacokinetic Properties of BU Analogues

C57BL/6JIco or C57BL/6J mice were treated with BU (50 mg/kg), PL63 (50 or 300 mg/kg), PL26 (300 mg/kg), or PL108 (150 mg/kg). All drugs were i.p. administered. At the indicated time points, mice were anesthetized with isoflurane and bled, either by heart puncture or from the intraorbital cavity. Blood was collected in heparinized tubes and centrifuged, after which the plasma was collected and frozen at −80°C until analysis.

Determination of Serum Levels of BU Analogues

BU. Two hundred μl of the serum samples were diluted with PBS to a volume of 500 μl and were then spiked with deuterated BU as an internal standard and extracted as described below. The concentration of BU in the sample was then determined by comparing the ratio of BU and deuterated BU peaks.

BU Analogues. Two hundred μl of each sample were again diluted with PBS to give a volume of 500 μl and extracted as described below. In these cases, a standard curve of the analogue in question was prepared in mouse serum immediately prior to drug estimation and extracted simultaneously with the samples. This was used to determine the serum concentration of the analogue.

Sample Extraction. Sample extraction was by solid phase; briefly, a cyano-propyl SPE cartridge (Waters-Millipore, Watford, United Kingdom) was conditioned with 3 ml of methanol, followed by 3 ml of 50% methanol in water. The sample containing the compound in question was loaded onto the cartridge and allowed to equilibrate on the cartridge for 1 min. The cartridge was washed with 1 ml of water, and the analyte eluted with 1 ml of acetonitrile.

Chromatography. Extracted samples (40 μl) were analyzed by high-performance liquid chromatography using an Asahipak ODP50 analytical column (150 × 4.6 mm) using an isocratic mobile phase (70% methanol, 30% water, 0.1 M ammonium acetate). Detection was by thermospray mass spectrometry in selected ion recording mode.

Determination of the Internucleophile Distances of BU Analogues

These were calculated using a Silicon Graphics Iris 4d/310GTX Workstation using Quanta 4.0 software (including Charm 22.2) working under IRIS 4.0.5, as described previously (41).

Determination of the Partition Coefficients of BU Analogues

The experimental value of the partition coefficient of some of the compounds was determined using the colorimetric method monitoring the blue pigment formed by the reaction of alkane sulfonate with 4-nitrobenzylpyridine (NBP; Ref. 42). BU and other drugs were dissolved in a minimum volume of DMSO (<1%) and made up to 1 mg/ml in octanol. Serial dilutions were made in octanol. Five ml of each solution were mixed with 5 ml of deionized water and mixed for 30 min on a mechanical rotator. Aliquots (1 ml) of both aqueous and organic layer were removed, and 2 ml of 2% NBP in ethylene glycol were added, and the solution was boiled for 30 min. This was then cooled on ice, 2 ml triethylamine (50% w/v in acetone) were added, and the blue color was assayed at 565 nm. All concentrations were calculated using a standard curve. Experiments were performed in duplicate over a range of seven concentrations from 0.05 to 1 mg/ml. The theoretical partition coefficients were calculated from the structure of these compounds using the software package ClogP 4.0 (BioByte Corp., Claremont, CA).

RESULTS

Three assays were used to evaluate the biological activity of the various compounds: *in vivo* treatment of mice followed by either BMT or CAFC assay or *in vitro* treatment of freshly isolated murine bone marrow cells followed by the CAFC assay. Table 1 summarizes in which assay the various compounds were evaluated.

***In Vitro* Sensitivity of Murine Bone Marrow toward Different BU Analogues.** Fig. 2 shows the levels of CAFC survival after an 18-h incubation of marrow cells with 30 μM of each compound. A number of agents showed moderate activity that was comparable with that of BU. These include DMB, Hepsulfam, PL26, PL103, PL104, and PL106. The four compounds that appeared to exceed the activity of BU were Treosulfan, PL63, and PL64, whereas PL102, PL109, and PL107 had very little cell killing effect. These *in vitro* results after treatment at equimolar concentrations were compared with CAFC subset depletion and donor marrow engraftment after *in vivo* treatment so as to isolate possible differences in pharmacokinetic properties among the various compounds.

Toxicity. A number of different dose levels for each new compound was applied in pilot studies to establish a dose that approximated to the MTD. The dose levels and animal survival for each different compound is listed in Table 2. Large differences in dose exist between some of the compounds, *e.g.*, 12 mg/kg for DMB and 3000 mg/kg for Treosulfan. However, for the majority of the cyclic compounds, the MTD was reached at doses of ~300 mg/kg. The toxicities that were encountered included: (*a*) supra-acute toxicity within the first day (lethargy, hypothermia), which was the dose-limiting toxicity for most drugs except DMB and Hepsulfam; (*b*) gut damage (diarrhea between 1 and 3 weeks) was seen for PL63 and Hepsulfam; and (*c*) incisor damage started 6 weeks after CY, PL63, or Hepsulfam administration or the combinations PL63/CY or BU/CY. The extent of this latter damage was such that the animals were provided moistened food to avoid malnutrition.

Liver and kidney toxicity were determined by measuring ALAT

Table 1 *Assays used for each compound*

	In vitro	*In vivo*	
Compound	CAFC assay	CAFC assay	BMT
Busulphan	+	+	+
DMB	+	+	+
PL109	+	+	+
Hepsulfam	+	+	+
Treosulfan	+	+	+
PL102	+	+	+
PL48		+	
PL49		+	
PL50		+	
PL51		+	
PL26	+	+	+
PL84			+
PL87		+	+
PL32	+	+	+
PL33		+	+
PL34	+	+	+
PL35		+	+
PL39		+	+
PL40		+	+
PL63	+	+	+
PL64	+	+	+
PL103	+		
PL104	+	+	
PL106	+		
PL107	+		
PL108	+	+	

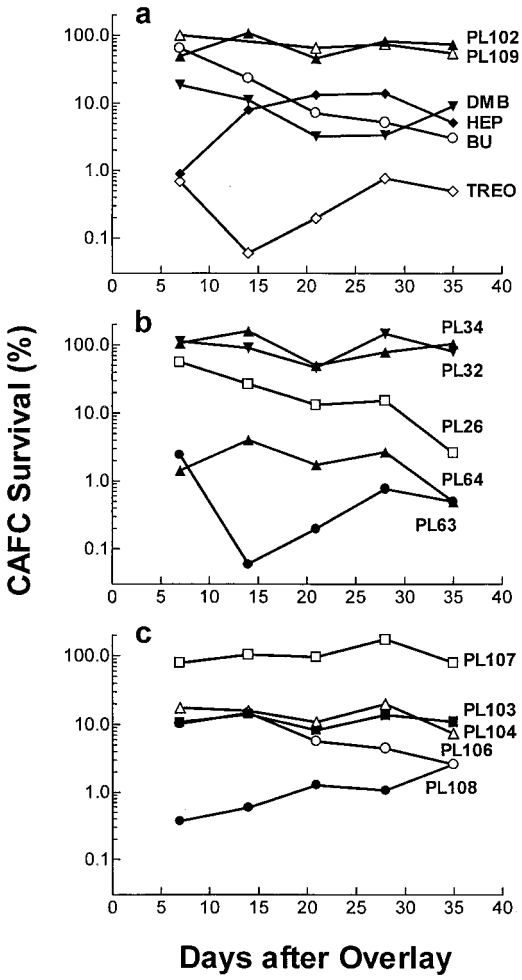

Fig. 2. CAFC frequencies (percentage of control) of murine bone marrow cells treated *in vitro* with the indicated drugs (18 h, 30 μM).

and BUN, respectively, in plasma of control and treated animals. Control levels of ALAT ranged from 14.5 to 18.6 IU/l within a range between 2 days and 36 weeks after transplant. Within that time frame, BUN varied between 8.57 and 10.12 mmol/l. Neither ALAT nor BUN values changed after treatment with either DMB, Hepsulfam, PL26, PL63, PL64, PL87, or TBI (7 Gy).

Host CAFC Depletion and Syngeneic Donor Marrow Engraftment. The extent of depletion of the various stem cell subsets in the host as measured in the CAFC assay differed widely among the 25 compounds tested. Figs. 2 and 3 contain data of a number of compounds (aliphatic and cyclic, respectively) that were tested in both the CAFC and syngeneic bone marrow transplantation. The depletion of primitive hematopoietic cells (CAFC day 35; Fig. 3*a* and 4*a*) appeared to be reflected in the subsequent engraftment of donor bone marrow cells (Figs. 3*b* and 4*b*). PL102, the epoxide analogue of BU, showed little *in vivo* activity, whereas PL109, the sulfamate analogue of BU, showed comparable engraftment levels to BU, although the CAFC data indicate a lower activity in the depletion of stem cells. The other aliphatic BU analogues with carbon chain lengths of 5–8 (PL51, PL50, PL49, and PL48, respectively) were also tested in both assays but appeared to be inactive (Table 2).

Only one compound exceeded BU in its ability to deplete CAFC day 35, the *cis*-cyclohexane analogue PL63. This compound also proved to be as equally effective as 7 Gy irradiation in depleting early- and late-developing CAFC subsets and in providing for long-term donor marrow engraftment. The *trans*-isomer PL64 gave moderate stem cell toxicity and engraftment levels that were comparable with the effects of DMB and Hepsulfam. The other cyclic analogues PL26 (Fig. 3), PL32, PL33, PL34, PL35, PL39, PL40, PL84, and PL87 (Table 2) were less effective in depleting stem cell subsets in the CAFC assay and showed minimal long-term syngeneic bone marrow engraftment.

When BU was administered in four daily doses of 12.5 mg/kg each, the overall profile of depleting the various hematopoietic subsets as measured with the CAFC assay was comparable with a single dose of 1×50 mg/kg (Fig. 5*a*), indicating an additive effect of fractionation. However, fractionation enabled at least a doubling in the tolerated dose and a further decline in the survival of primitive hematopoietic stem cells to below detection limits ($<0.05\%$). This difference is also reflected in the level of engraftment of syngeneic bone marrow cells: 85% *versus* 50% for fractionated and single dose, respectively (Fig. 5*b*).

Fig. 6*a* shows how the level of CAFC day 35 survival in the host inversely relates to the magnitude of long-term donor blood chimerism for each pretreatment group, whereas the large scatter in data points in Fig. 6*b* shows a poor correlation with the level of CAFC survival after *in vitro* treatment.

Allogeneic BMT. BU and PL63, the analogue with the highest activity in syngeneic BMT, were also evaluated for their ability to provide for chimerism in an H-2-matched allogeneic BMT model (BALB.B10 transplanted into C57BL/6J). A fractionated course of BU (4×25 mg/kg) had a transient benefit over a single dose but did not allow for permanent donor marrow engraftment (Fig. 7*a*). Similar results were obtained after a single dose treatment with PL63 (300 mg/kg). Fractionation with concomitant increase of the dose was not possible for PL63 because this appeared to be too toxic. Transplantation of allogeneic bone marrow after conditioning with BU or PL63 combined with CY (200 mg/kg) were also performed. In this case, single-dose BU plus CY was not sufficient to overcome the immunological barrier resulting in immediate allograft rejection, whereas fractionated BU or single-dose PL63 combined with CY allowed for lasting and high levels of donor marrow engraftment after allogeneic BMT (100 and 80% after 20 weeks, respectively).

Chemical Properties of BU Analogues. Internucleophile distances were determined to establish whether there was a relationship biological activity and molecular structure. In this case, comparisons with the *in vitro* activity seems the most appropriate so as to exclude pharmacokinetic processes that could influence biodistribution to the target tissues. Table 3 shows the distance between the two alkylating carbons varied between 3.03 Å (PL32) and 7.63 Å (PL108). Compounds with comparable or higher activity than BU, *e.g.*, PL26, PL63, PL64, PL106, and PL108, have a wide range of internucleophile distances whereas compounds lacking any activity, *e.g.*, PL32 and PL33, have internucleophile distances that are almost identical to BU. The distances between the two oxygens in the neighboring mesylate groups were also estimated because these atoms are likely to be occupied by a nitrogen or oxygen of a nucleophile (*e.g.*, guanine in DNA). Again, the distances between active (PL63 and PL64, 3.27 and 3.24 Å, respectively) and inactive compounds (PL32 and PL34, 3.12 and 3.52 Å, respectively) do not differ significantly. Thus, it seems that the distances between the two leaving groups of the compounds tested do not correlate with the biological activity.

Partition Coefficients. The values for the partition coefficients were determined using either an experimental or a computer calculation method. Experimental values were determined using a range of concentrations, and the coefficients were taken from the linear part of the curves. The values are depicted in Table 3. In the cases in which we were unable to determine the experimental values because of lack of material, a theoretical calculation was made for the partition coefficients. Although the calculated values were mostly higher than the

Table 2 *Summary of data (d35 CAFC survival and percent engraftment) in mice treated with different compounds*

Compound	Carbon chain length	Dose (mg/kg)	Carrier	Survival	Survival time	d35 CAFC survival (%)	% donor engraftment (20 wk)
Busulfan	[C-4]	50	Oil	4/5	<1 day	0.67	49.2 ± 3.4
		50	Oil	2/4	<1 day	0.69	
		4 × 25	Oil	5/5		<0.05	87.5 ± 4.1
DMB	[C-4]	12	5% DMSO in PBS	5/5		2.9[a]	39.7 ± 4.7
		12	Oil	5/5		3.2[a]	38.5 ± 5.3
		12	5% DMSO in PBS	5/5		2.1	30.1 ± 6.6
PL109	[C-4]	100	5% DMSO in PBS	3/3			
		300	Oil	3/3			
		600	Oil	3/3			
		600	50% DMSO in PBS	5/5		4.66	52 ± 2.7
Hepsulfam	[C-7]	100	Oil	5/5			
		150	Oil	3/3			
		200	Oil	5/5		4.9	31.9 ± 4.8
		200	Oil	5/5			
Treosulfan	[C-4]	3000	50% DMSO in PBS	4/4			
		3000		5/5		21.7	6.54 ± 2.2
		5000		0/3	<1 day		
PL102	[C-4]	600	Oil	4/4		101.8	0
PL48	[C-8]	100	Oil	4/4		19.1	17.9[a]
		200	Oil	0/3	6–8 days		
PL49	[C-7]	100	Oil	4/4		19.4	17.8[a]
		200	Oil	1/3	2 h + 8 wk		
PL50	[C-6]	100	Oil	3/3			
		100	Oil	4/4		10.0	25.5[a]
		200	Oil	0/3	<2 h		
PL51	[C-5]	50	Oil	3/3			
		50	Oil	4/4		9.1	26.5[a]
		100	Oil	2/3	3 h		
PL26	[C-6]	300	Oil	5/5		49.6	5.2 ± 1.4
		300	Oil	5/5			
		400	Oil	0/3	<1 day		
PL84	[C-7]	300	Oil	5/5			
		400	Oil	2/3	<1 day	68.0[a]	3.1 ± 0.3
PL87	[C-8]	400	Oil	3/3			
		600	Oil	4/5	<1 day	41.5	8.8 ± 1.1
PL32	[C-6]	300	Oil	3/3			
		300	Oil	8/10	<1 h	110.1	0
PL33	[C-6]	300	Oil	3/3			
		300	Oil	9/10	<2 h	75.4	0
PL34	[C-8]	300	Oil	3/3			
		300	Oil	9/9		123.9	0
PL35	[C-8]	300	Oil	4/4			
		300	Oil	9/9		78.3	4.2 ± 2.5
PL39	[C-7]	300	Oil	4/4		34.6	11.1[a]
PL40	[C-7]	300	Oil	3/3			
		300	Oil	9/9		80.5	0
PL63	[C-6]	200	Oil	5/5		0.4[a]	63.6 ± 5.9
		300	Oil	10/11	10 days	0.57	70.2 ± 5.6
		400	Oil	2/3	8 days		
		400	Oil	4/5	6 days	0.03[a]	90.7 ± 8.0
		400	Oil	6/7	8 days	0.04[a]	88.4 ± 7.1
PL64	[C-6]	200	Oil	5/5		14.2[a]	21.5 ± 6.3
		300	Oil	5/5		3.8	34.9 ± 6.0
		400	Oil	3/3			
		400	Oil	4/4		2.7[a]	40.8 ± 2.4
PL104	[C-7]	300	Oil	2/4	<1 day	13.1	22.3[a]
PL108	[C-8]	150	Oil	3/3		28	13.5[a]
		200	Oil	1/3	<1 day		
		300	Oil	1/3	<1 day		
TBI		4 Gy		5/5			30.4
		5 Gy		5/5			
		7 Gy		5/5		0.43	67.6

[a] Values were calculated according to the curve in Fig. 5 (formula: $y = -26.67x + 52.13$).

experimentally determined values, both approaches do correlate (Spearman's ranking, correlation coefficient, 0.84; $P = 0.009$; not shown).

Pharmacokinetics. Table 4 shows some pharmacokinetic properties of BU, PL63, PL26, and PL108. The average AUC after a single dose (50 mg/kg) of BU was 133 μg·h/ml. The AUC after an equal dose of PL63 was much lower (50.5 μg·h/ml), whereas it was comparable with that of BU at a higher dose approximating the MTD (300 mg/kg). The AUC after 300 mg/kg PL26 was again much lower than after an equal dose of PL63. PL108 gave the lowest AUC of the four compounds listed in this table; the AUC at 150 mg/kg is 3-fold lower than BU at one-third of the dose (50 mg/kg). From the AUCs, the relative bioavailabilities (F_r) were calculated. The F_r of PL63 at 300 mg/kg was five times lower as compared with BU. The F_r of PL26 at 300 mg/kg and PL108 at 150 mg/kg were ~10-fold lower as compared with BU.

DISCUSSION

Previous studies on different chemotherapeutic agents and radiation modalities have demonstrated that depletion of primitive stem cells in the recipient is an important prerequisite to achieving lasting engraftment of both transiently and long-term repopulating stem cells from

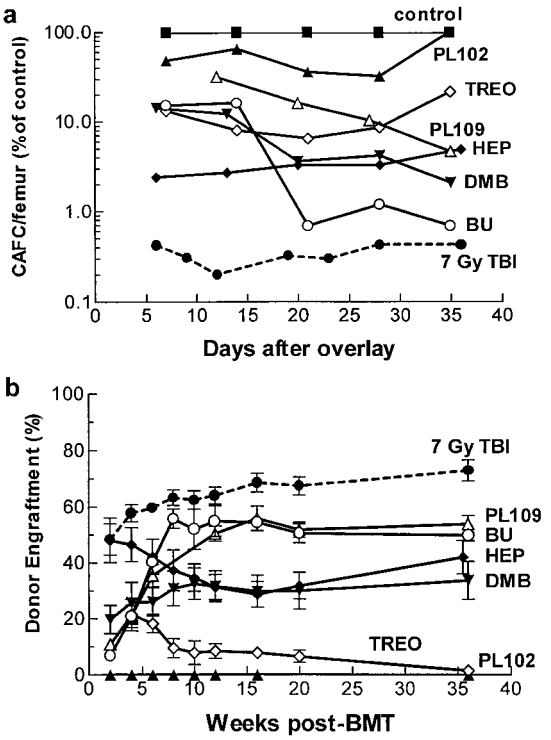

Fig. 3. *a*, CAFC frequencies (percentage of control) of mice treated with BU, aliphatic analogues of BU (Treosulfan, PL109, Hepsulfam, and DMB), and TBI. For determination of CAFC, bone marrow was harvested from treated and control mice 24 h after treatment, pooled, and seeded onto confluent layers of murine stromal cells. Doses were as follows: Treosulfan, 3000 mg/kg; PL109, 600 mg/kg; Hepsulfam, 200 mg/kg; DMB, 12 mg/kg; BU, 50 mg/kg; and TBI, 7 Gy. *b*, development of bone marrow chimerism after syngeneic BMT (10^6 B6-*Gpi-1*^{a} bone marrow cells) in mice treated as in *a*. Values are the means of four to six mice; *bars*, ±1 SE.

the donor (2, 3, 43). Among the standard chemotherapeutic drugs, BU was found to be particularly efficient in this respect, and it is therefore of interest in the present study to establish whether a relationship exists between the molecular or physical properties of new BU analogues and their biological activity in the context of BMT conditioning therapy. Our design of new compounds was first based on earlier findings that the antitumor activity of BU analogues changed according to the distance and orientation of the two methane-sulfonate groups (28, 29, 44). Many of these compounds contained either a cyclohexane or benzene ring, thus rendering rigidity to the molecule and restricting the positioning of the alkylating groups.

The present study shows that there is indeed a large variation in activity among the group of closely related BU analogues, although there does not seem to be a correlation between the spacing or orientation of the two methane-sulfonate or -sulfamate groups and their biological activity. We decided to use these drugs at doses that were equitoxic to the animals rather then using the same dose for every drug because almost all drugs tested *in vivo* did not show any activity at the dose normally used for BU (50 mg/kg). Figs. 2*a* and 3*a* show that of the compounds tested, only PL63 exceeded the activity of BU in depleting primitive stem cells. Indeed, PL63 had a similar effect to 7 Gy TBI in its ability to diminish the number of transiently repopulating stem cells (CAFC days 7–14) as well as the primitive stem cell population (CAFC day 35). Albeit lower in activity than BU, Hepsulfam, DMB, and PL109 showed a 1–2-log depletion of late-developing CAFCs. PL109 was designed to be a more soluble drug as compared with BU through replacement of the methane-sulfonate moieties by sulfamate groups. However, it required a 12-fold increase in dose compared with BU to achieve a similar level of stem cell depletion. Other members of the aliphatic series with varying length of the carbon chain were at least 10-fold less active than BU. This is in accordance with a previous report in which the biological activity of a series of dimethane sulfonate compounds (n = 1–10) was measured from the assessment of marrow CFU-S content in rats (31).

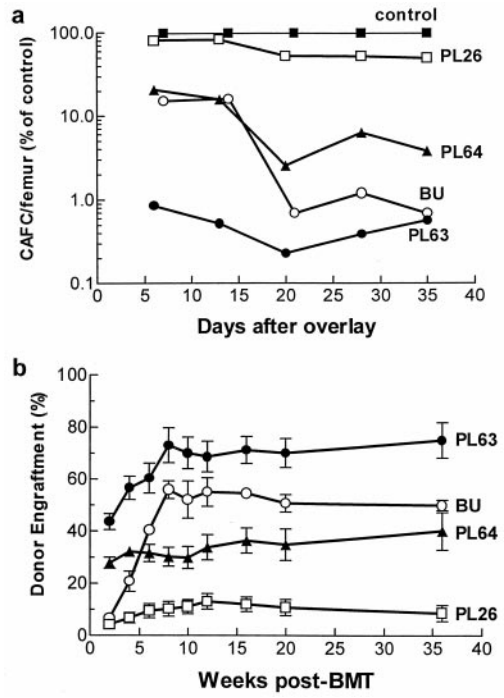

Fig. 4. CAFC frequencies (percentage of control; *a*) and development of bone marrow chimerism after syngeneic BMT (*b*) of mice treated with cyclic analogues of BU. Doses of all three drugs were 300 mg/kg. Values in *b* are the means of four to six mice; *bars*, ±1 SE.

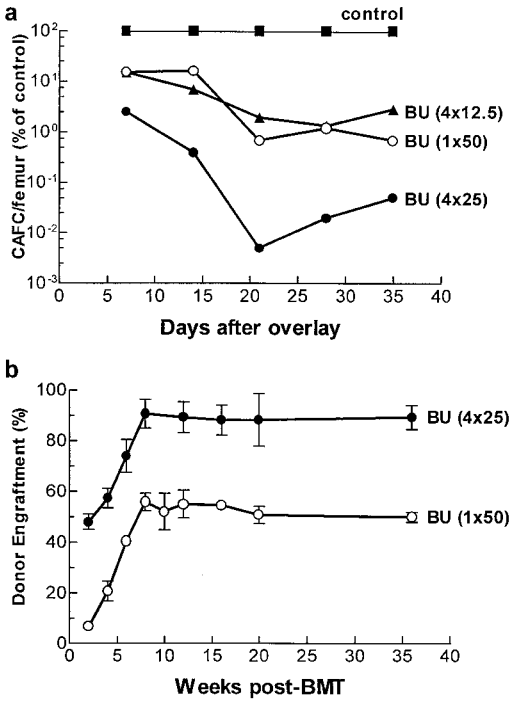

Fig. 5. CAFC frequencies (percentage of control; *a*) and development of bone marrow chimerism after syngeneic BMT (*b*) of mice treated with BU, either a single dose (1 × 50 mg/kg) or fractionated (4 × 12.5 or 4 × 25 mg/kg). Values in *b* are the means of four to six mice; *bars*, ±1 SE.

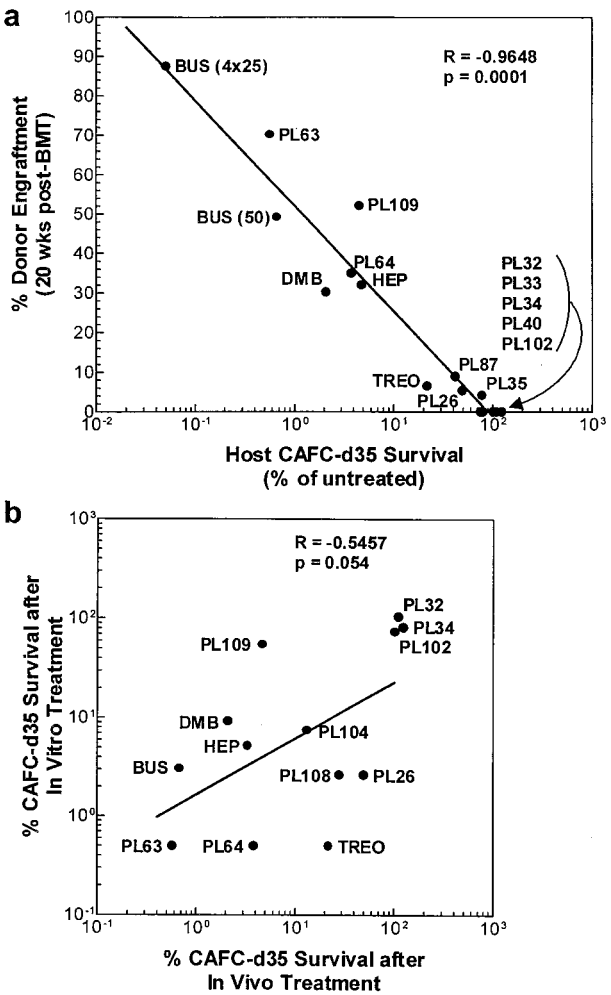

Fig. 6. *a*, correlation between percentage of host CAFC day 35 survival in murine bone marrow and donor engraftment 20 weeks after syngeneic BMT in mice treated with various BU analogues. The formula of the straight line is: $y = -26.67x + 52.13$, and has been used to calculate some theoretical values for Table 2. *b*, relationship between CAFC day 35 survival after *in vitro versus in vivo* treatment for each compound.

The epoxide analogue studied in this paper (PL102) did not show any activity in our assay systems. Various epoxide analogues have been synthesized previously, but neither of them is more active than BU in depleting the neutrophils, lymphocytes, or platelets (45). Treosulfan is a registered prodrug indicated for recurrent or progressive ovarian cancer (34). This compound is spontaneously converted into corresponding mono- and diepoxides responsible for alkylation (46, 47). It is as yet unclear why the metabolite of Treosulfan seems to be more active in stem cell depletion as compared with the monoepoxide PL102.

Our data show that the spacing between the two alkylating groups does not bear a relationship with cytotoxicity. This is exemplified by the fact that sulfamate analogues of BU and PL49, *i.e.*, PL109 and Hepsulfam, respectively, show a decreased activity while the carbon-chain length is the same. The cyclic analogues also show large variations in activity with relatively minor structural differences between compounds. There is, for instance, a large difference in activity between PL63 (*cis*-1,2-(2-hydroxyethyl) cyclohexane DMS and its *trans*- counterpart (PL64). Also, replacing the cyclohexane ring of PL63 by a benzene ring (PL26) almost fully diminishes the *in vivo* activity of the BU analogue. In both compounds, the spacing is virtually the same, as is the orientation of both side groups. The cyclic compounds that are particularly inactive are PL32, 33, 34, 35, 39, 40, 84, and 87, although the internucleophile distances of the more rigid molecules PL32 and PL33 are the same as in BU, and the internucleophile distance of PL34 is similar to that of PL63. It thus appears that factors other than spacing and orientation of the two alkylating groups are of major importance.

In addition to single-dose BU, we also treated mice with a more clinically relevant scheme: four smaller doses administered over four

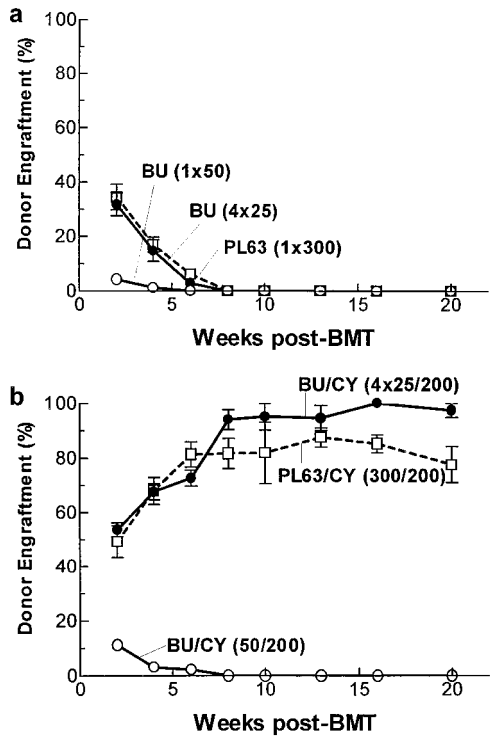

Fig. 7. Development of donor-type chimerism after allogeneic BMT (10^6 BALB.B bone marrow cells) in B6 mice pretreated with BU and PL63 without (*a*) or with (*b*) CY. Values are the means of four to six mice; *bars*, ±1 SE.

Table 3 *Summary of chemical properties of DMS compounds*

Compound	Partition coefficient (log *P*)		Internucleophile distance (Å)	
	Calculated	Experimental (SD)	C–C	O–O
Busulphan	−0.59	−0.585 (0.007)	3.09	2.93
DMB	0.03		3.93	4.68
PL109	−2.25		3.91	6.13
Hepsulfam			5.32	4.66
Treosulfan	−2.20		3.91	6.15
PL102	−0.45		3.89	5.38
PL48	1.52		8.91	11.09
PL49	0.995	0.413 (0.061)	7.64	9.88
PL50	0.466	0.146 (0.082)	6.42	8.66
PL51	−0.063	−0.109 (0.009)	5.08	7.37
PL26	0.746	0.535 (0.077)	4.04	3.85
PL84	1.28		5.36	6.71
PL87	1.804	1.352 (0.009)	6.62	8.85
PL32		0.83	3.03	2.83
PL33		0.83	3.07	3.12
PL34	0.99	0.470 (0.056)	4.88	3.52
PL35		0.66	5.84	6.78
PL39		0.66	5.03	5.02
PL40		0.66	4.56	4.77
PL63	2.048	0.757 (0.213)	4.66	3.27
PL64	2.048	0.925 (0.009)	4.65	3.24
PL103	2.05		6.29	8.40
PL104	2.05		5.26	6.03
PL106	1.49		4.51	6.46
PL107	1.49		4.69	6.17
PL108	2.05		7.63	8.23

Table 4 *Summary of pharmacokinetic properties of some DMS compounds*

Drug	Dose (mg/kg)	AUC (μg·h/ml)	Mean AUC	Bioavailability (relative to BU)
Busulfan	50	97.24		
	50	174.91	133.11	
	50	127.19		1.00
PL63	50	50.48		0.38
	300	149.91	157.38	
	300	164.85		0.20
PL26	300	87.45		0.11
PL108	150	35.63		0.09

consecutive days. The effect of dividing the dose over 4 days (4×12.5 mg/kg) produced an additive effect; the depletion of the different hematopoietic subsets was very similar to those seen after a single dose of 50 mg/kg. However, fractionation of the dose did allow for a doubling of the dose (4×25 mg/kg) without compromising its toxicity. This dose escalation had a profound effect on stem cell killing; CAFC frequencies fell below detectable levels (>0.05%) after 2 weeks in culture. This decrease is again reflected in the ability to allow for high levels of syngeneic donor marrow engraftment.

In the allogeneic setting, both single-dose PL63 and fractionated BU were also very active in allowing for high and permanent levels of engraftment but only when used in combination with CY. The benefit of adding CY to either TBI or BU prior to allogeneic BMT in terms of promoting donor-type chimerism is well recognized in animal models (48–50) and is most likely attributed to the immune suppressive properties of CY in preventing allograft rejection. Although it is difficult to discern whether fractionated BU or PL63 also contributed to immune suppression, the lack of allogeneic chimerism in mice given single-dose BU with CY may reflect insufficient stem cell ablation that is required for donor cell engraftment across the immunological barrier used here.

Thus far, we have evaluated the activity of these compounds using two biological end points: depletion of CAFC subtypes and blood chimerism after BMT in treated animals. Femoral CAFC (day 35) depletion directly after treatment with a variety of DMS compounds shows a highly significant correlation with development of donor marrow engraftment in the months thereafter (Fig. 6*a*). The data strongly suggest that the extent of depletion of primitive stem cells (CAFC day 35) fully determines the establishment of primitive donor stem cells with long-term repopulating ability. Therefore, the *in vitro* CAFC assay system alone may be sufficient for the initial identification of new compounds that have the ability of promoting long-term chimerism in pretreated recipients.

We tested a number of drugs for their activity *in vitro* in which fresh bone marrow cells were treated with equimolar concentrations of drug as opposed to equitoxic doses used *in vivo*. These *in vitro* treatments of murine bone marrow cells may answer the question as to whether *in vivo* distribution is an important determinant in the efficacy of these compounds. Indeed, we found that a number of compounds exhibited relatively high *in vitro* activity but with poor activity *in vivo*. These include Treosulfan, PL26, and PL108. With respect to molecular structure, it is important to note that of the cyclic compounds that show the highest activity (>90% depletion of CAFC day 35) are all analogues with two hydroxyethyl moieties ($n = 2$) attached to the ring. It seems that the distance between the active groups and the cyclohexane ring to which they are attached is more important than the distance between the active groups themselves; PL108, PL64, and PL63 show higher activity as compared with their methane-sulfonate counterparts PL35, PL33, and PL32. The differences between the *in vitro* and *in vivo* activities can be reconciled, at least partially, by the pharmacokinetic profiles of four tested compounds. From the drug levels measured in the blood, it appeared that the AUC of PL63 was 2.5-fold lower as compared with BU after the same dose. AUCs were comparable when the dose of PL63 was 6-fold higher than BU, and this is in keeping with the higher stem cell activity based on the *in vitro* treatment experiment. PL26 given at a dose of 300 mg/kg results in an AUC that is 2-fold lower than that of PL63 at the same dose. From the AUCs, relative bioavailabilities (F_r) were calculated. At an equal dose of 50 mg/kg, the F_r of PL63 was 2.5-fold lower than BU, and at a higher dose of 300 mg/kg, the F_r further decreases to 20%. The F_r of PL26 at 300 mg/kg and PL108 at 150 mg/kg were even lower, ~10% of the value for BU. The comparison between bioavailability among these four compounds is therefore consistent with the lower activity *in vivo* as compared with *in vitro*.

In conclusion, a wide range of stem cell toxicities for different BU analogues was seen that correlated with their ability to induce donor-type chimerism after BMT. The compounds BU, PL63, PL26, PL108, and Treosulfan showed a comparable activity *in vitro*. However, significant activity *in vivo* was only seen for BU and PL63, which correlated with the bioavailability at the MTD. There seems to be no clear correlation between biological activity and either internucleophile distance or partition coefficient. The high levels of engraftment reached with the use of PL63 in syngeneic or allogeneic BMT and the activity of some of these analogues *in vitro* demonstrate the potential for improving the selectivity of this class of compound in BMT conditioning therapy. Such an approach is becoming increasingly pertinent to the current clinical efforts of establishing less toxic, pretransplant protocols that favor the growth of donor stem cells and consequently establish lymphohematopoietic chimerism.

ACKNOWLEDGMENTS

This report is dedicated to the memory of Prof. Brian Fox, whose ideas inspired this work. We are very grateful for the technical assistance of Rita Setroikromo in performing the chimerism assays. Nicola Carrol, Anila Khan, Robert Hargreaves, Séverine Altwiesm and Davina Oanpher are acknowledged for the synthesis of some of the DMS compounds and for the determination of their partition coefficients and internucleophile distances.

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