STUDIES ON MECHANISMS OF BUSULPHAN CYTOTOXICITY AND PHARMACOKINETICS

WITH SPECIAL REFERENCE TO LIPOSOMAL BUSULPHAN

Zuzana Hassan

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DEPARTMENT OF MEDICINE, DIVISION OF HEMATOLOGY,
KAROLINSKA INSTITUTET AT HUDDINGE UNIVERSITY HOSPITAL

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AKADEMISK AVHANDLING

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Tisíc vynálezců udělalo krach
hvězdy nevyšinuly se z věčných drah
pohleďte jak tisíc lidí klidně žije
ne to není práce ani energie
to je dobrodružství jako na moři
uzamykati se v laboratoři
pohleďte jak tisíc lidí klidně žije
ne to není práce to je poezie

Vítězslav Nezval, 1927

Many inventors have amazed the world,
Still constant in their course the stars were hurled.
A thousand people live thus calmly, see!
No, this is not work, this is not energy,
This is a venture like the Golden Hind,
In a laboratory to seal your mind.
A thousand people live thus calmly, see!
No, this is not labour, this is poesy.

Translation Edith Pargeter

To my family

ABSTRACT

Busulphan is an alkylating agent currently used in conditioning regimen prior to stem cell transplantation (SCT). High dose therapy with busulphan has been shown to contribute to transplantation-related toxicities, such as veno-occlusive disease (VOD) and interstitial pneumonia. Previous pharmacodynamic studies have attempted to define a target systemic exposure to busulphan, and thresholds for different side effects. Pharmacokinetic studies have shown a wide inter-patient variation in the systemic exposure to busulphan after a fixed dose. The wide variation may be due to bioavailability, age, underlying disease, drug-drug interaction and circadian rhythm.

The aim of the first part of this thesis was to develop and evaluate an intravenous formulation of busulphan encapsulated in liposomes. Small, uncharged liposomes containing cholesterol suspended in 5% glucose were selected for the preparation of liposomal busulphan (LBu). The distribution of LBu was assessed with ^{14}C labeled busulphan in a rat model, and compared with a solution of free drug. The distribution of LBu was higher to bone marrow and spleen, and lower to brain, lung and heart, compared with free drug, while the distribution to liver was similar. The myelosuppressive effect of LBu was stable and reproducible in a mouse model. Pharmacokinetics of liposomal busulphan was studied in man after either low dose or high dose of LBu. A linear relation between the systemic exposure and the dose was found. The pharmacokinetic parameters were in agreement with those previously reported for orally administered drug. It was feasible to use liposomal busulphan for high dose therapy. The advantageous distribution and good myelosuppressive effect in animals, and the more predictable pharmacokinetics in man have motivated the launching of a phase I/II clinical trial.

The aim of the second part of the thesis was to investigate mechanisms of busulphan-induced cytotoxicity, and the role of glutathione (GSH) in this process. The myeloid P39 cell line was used in vitro as a model for induction of differentiation and/or apoptosis with the differentiating agent all-trans retinoic acid and the cytostatic agent etoposide. Both agents induced apoptosis that was mediated through caspase activation, but different pathways were involved. These pathways diverged in kinetics, preceding maturation, cleavage of Bcl-2 and actin, and rescue from apoptosis by granulocyte colony-stimulating factor (G-CSF). Busulphan- and etoposide-induced apoptosis shared common features, but differed in kinetics. The P39 cells were arrested in G2 phase of the cell cycle before apoptotic morphology developed. Proliferation and clonogenic capacity of the P39 cells showed an inverse linear relation to the exposure to busulphan expressed as AUC. Also in busulphan-treated human CD34+ hematopoietic progenitors, clonogenic capacity was inversely and linearly related to the exposure to busulphan expressed as AUC. Myeloid progenitors were more sensitive than erythroid progenitors (AUCs completely inhibiting colony formation were 69 ± 7.5 μg.hr/ml and 140 ± 36 μg.hr/ml for CFU-GM and erythroid colonies, respectively). Neither an increase (induced by N-acetylcysteine), nor a decrease (induced by buthionine sulfoximine) of the cellular content of GSH affected busulphan-induced cytotoxicity in human CD34+ hematopoietic progenitors in vitro, or in murine bone marrow cells in vivo.

In conclusion, liposomal busulphan is a suitable formulation for high dose treatment in conditioning regimen prior to stem cell transplantation. N-acetylcysteine does not decrease busulphan-induced cytotoxicity in hematopoietic progenitor cells, and may thus serve as a potential prophylactic agent against transplantation-related hepatotoxicity.

LIST OF PAPERS

This thesis is based on the following papers, which will be referred to by Roman numerals.

I. **Hassan M, Hassan Z, Nilsson C, Rehim MA, Kumlien S, Elfsson B, Kållberg N.** Pharmacokinetics and distribution of liposomal busulfan in the rat: a new formulation for intravenous administration. Cancer Chemother Pharmacol 1998;42(6):471-8.

II. **Hassan Z, Nilsson C, Hassan M**. Liposomal busulphan: bioavailability and effect on bone marrow in mice. Bone Marrow Transplant 1998;22(9):913-8.

III. **Hassan Z, Ljungman P, Ringdén O, Winiarski J, Nilsson C, Aschan J, Rosengren Whitley H.** Pharmacokinetics of liposomal busulphan in man. Bone Marrow Transplant 2000, In press.

IV. **Hassan Z, Fadeel B, Zhivotovsky B, Hellstrom-Lindberg E.** Two pathways of apoptosis induced with all-trans retinoic acid and etoposide in the myeloid cell line P39. Exp Hematol 1999;27(8):1322-9.

V. **Hassan Z, Hassan M, Hellström-Lindberg E.** The pharmacodynamic effect of busulfan in the P39 myeloid cell line in vitro. (Submitted)

VI. **Hassan Z, Hellström-Lindberg E, Alsadi S, Edgren MR, Hägglund H, Hassan M.** The role of glutathione in busulphan-induced cytotoxicity in hematopoietic cells in vitro and in vivo. (Manuscript)

CONTENTS

ABBREVIATIONS

ALL	acute lymphoblastic leukemia
AML	acute myeloid leukemia
ARC	area under the radioactivity-time curve
ATRA	all-trans retinoic acid
AUC	area under the concentration-time curve
Bcl-2	B cell lymphoma/leukemia 2
BFU-E	burst forming-unit erythroid
BM	bone marrow
BSO	L-buthionine [S,R]-sulfoximine
Bu/Ac	busulphan dissolved in aceton
Bu/CY	conditioning regimen containing busulphan and cyclophosphamide
Bu/DMSO	busulphan dissolved in DMSO
Bu/susp	busulphan suspended in water
Caspase	cysteinyl aspartate-specific proteinase
CFU-E	colony forming-unit erythroid
CFU-GM	colony forming-unit granucolyte-macrophage
CFU-S	colony forming-unit spleen
Cl	clearance
Cmax	maximum concentration
CML	chronic myeloid leukemia
CSF	cerebro-spinal fluid
Css	steady state concentration
CY/TBI	conditioning regimen containing TBI and cyclophosphamide
DMSO	dimethyl sulfoxide
F	bioavailability
Fas	FS-7 associated surface antigen
FBS	fetal bovine serum
G-CSF	granulocyte colony-stimulating factor
GSH	glutathione
GST	glutathione transferase
LBu	liposomal busulphan
LC	liposomal control, i.e. liposomal preparation without busulphan
LC_x	lethal concentration, i.e. concentration killing cells by x%
NAC	N-acetyl-L-cysteine
PB	peripheral blood
SCT	stem cell transplantation
t½	half-life
TBI	total body irradiation
Tmax	time to maximum concentration
Vdss	distribution volume at steady state
VOD	veno-occlusive disease of the liver
zVAD-fmk	a general caspase inhibitor

INTRODUCTION

Cytotoxic agents constitute an important part of cancer therapy and are essential in the treatment of hematological malignancies. Treatment schedules have often been developed empirically, based on “trial and error” experience, and dosages have been adjusted to the highest tolerable level with reasonable toxicity. Myelosuppression is the dose-limiting toxicity for the majority of cytostatic drugs, but high doses causing severe myelosuppression are sometimes needed for cure of more resistant cancer diseases (van der Wall *et al.* 1995). The lethal myelosuppressive effect of high dose chemotherapy may be overcome by rescue with autologous or allogeneic hematopoietic stem cells (Copelan *et al.* 1992, Ghalie *et al.* 1994). Stem cell transplantation (SCT) may also be used as a curative treatment for other primary or secondary defects in the hematopoietic system, such as aplastic anemia, thalassemia, osteopetrosis, lysosomal storage diseases, and immunodeficiencies (Vassal *et al.* 1993, Lucarelli *et al.* 1998, Poonkuzhali *et al.* 1999, Bolinger *et al.* 2000).

HISTORICAL BACKGROUND AND INDICATIONS FOR TREATMENT WITH BUSULPHAN

Busulphan is a cytotoxic agent that was synthesized in the early 1950’s by G. M. Timmis. It was shown to be cytotoxic to myeloid cells in rat and in man (Haddow *et al.* 1953). In 1953, Galton reported a clinical trail on busulphan in chronic myeloid leukemia, in which therapeutic doses of busulphan inhibited the leukemic proliferation and induced regression of splenomegaly (Galton 1953). Based on these results, busulphan became the drug of choice for treatment of chronic phase CML for almost three decades. Only recently, it has been substituted with less toxic agents, such as interferon-alfa, hydroxyurea or the tyrosine kinase inhibitor STI571. Busulphan in low or “standard” dose was also used in the treatment of polycythemia rubra vera, essential thrombocythemia, and chronic granulomatous disease (Thompson 1974, Brodsky 1982, Van de Pette *et al.* 1986).

The remarkable myelosuppressive properties of busulphan led to investigations on the use of busulphan as a preparative agent before bone marrow transplantation (Santos 1993, Santos 1995). In rat, busulphan was highly myeloablative, while having only a modest effect on lymphatic tissues (Santos *et al.* 1974). Thus, the combination of busulphan with an immunosuppressive agent was a prerequisite for a successful allogeneic transplantation (Tutschka *et al.* 1975). Based on these animal experiments, Santos et al conducted a phase I/II trial of the combination of busulphan and cyclophosphamide (BuCY) as conditioning regimen before bone marrow transplantation in acute non-lymphocytic leukemia (Santos *et al.* 1983). The BuCY regimen provided an effective alternative to cyclophosphamide and total body irradiation (CY/TBI) giving an equivalent rate of engraftment.

The ideal preparative regimen for SCT should meet the following criteria: 1.have marked antileukemic and myeloablative activity, 2.induce sufficient immunosuppression, and 3.not cause irreversible toxicity in non-target organs. None of the currently available conditioning regimens completely fulfills these criteria; however, the combination of BuCY shows several of these features (Santos 1993).

Currently, busulphan is mainly used as a part of the conditioning regimen prior to SCT for malignant and non-malignant diseases. Classical conditioning regimen contains busulphan in a dose of 16 mg/kg (divided in 4 × 1 mg/kg/day for four consecutive days) in combination with cyclophosphamide (60 mg/kg/day for 2 or 50 mg/kg/day for 4 days) (Santos *et al.* 1983). Due to higher systemic clearance of busulphan in young children, a dose of 600 mg/m² (Vassal *et al.* 1992, Shaw *et al.* 1994) or 640mg/m² (Yeager *et al.* 1992) is recommended as an equivalent to 16 mg/kg in adults. The time schedule has been modified to one single daily dose, two or three doses per day in several centers (Filipovich *et al.* 1992, Shaw *et al.* 1994, Hassan *et al.* 1996, Pawlowska *et al.* 1997). Intensified conditioning regimens consisting of various combinations containing thiotepa, etoposide, melphalan or TBI in addition to BuCY are suggested for more chemotherapy-resistant diseases, such as multiple myeloma, advanced myeloid leukemia and metastatic breast cancer (Crilley *et al.* 1995, Demirer *et al.* 1996, Demirer *et al.* 1996, Schiffman *et al.* 1996, Meloni *et al.* 2000).

ADVERSE EFFECTS OF BUSULPHAN

Treatment with low dose busulphan is associated with a low incidence of acute side effects. Adverse effects, such as Addison-like pigmentation of the skin and mucosa, cataract and pulmonary fibrosis, “busulphan lung”, have been observed only after long-standing therapy (Bishop *et al.* 1986). Busulphan has also been found to be teratogenic, mutagenic and cancerogenic, which is the reason for why its use in treatment of chronic myeloproliferative disorders has decreased (Bishop *et al.* 1986).

Regimen-related toxicity is a dose-limiting factor for the preparative regimen prior to SCT. Reports on the incidence of side effects differ considerably among studies (Shaw *et al.* 1986, Clift *et al.* 1994, Klingemann *et al.* 1994, Ljungman *et al.* 1997: Ringden, 1999 #155). This may reflect differences in preparative regimens, doses, or schedules of chemotherapy and/or TBI, age of the patients, underlying diagnosis, type of HLA-match, and source of stem cells (Table 1).

Several treatment components or their interactions may be responsible for the various transplantation-related toxicities. However, side effects such as neurotoxicity and VOD are likely to be related to treatment with high dose busulphan. A higher incidence of seizures has been observed after increased doses of busulphan in young children (Vassal *et al.* 1990). Prophylactic use of anticonvulsive drugs has minimized this complication. Veno-occlusive disease (VOD) of the liver is a life-threatening complication and reported incidences vary from 5% to 40% (Shulman *et al.* 1992, Bearman 1995). VOD may improve spontaneously, but the mortality of severe VOD is about 20-50%. Mechanisms underlying VOD are not fully understood, but low constitutive levels of glutathione (the most important intracellular antioxidant) in centrilobular hepatocytes, together with further glutathione exhaustion by cytotoxic drugs or radiation may contribute to the development of VOD (Bearman 2000, Carreras 2000). The relation of VOD to the exposure of busulphan is discussed under pharmacodynamics.

Table 1: Adverse effects of busulphan

Acute adverse effects of busulphan: • gastro-intestinal: mucositis, nausea, vomiting, diarrhea • hepatic: elevated bilirubin, veno-occlusive disease • neurological: general or localized seizures • pulmonary: interstitial pneumonitis • urogenital: hemorrhagic cystitis
Late adverse effects of busulphan: • endocrinous impairment • gonadal impairment and infertility • growth impairment • cataract • alopecia, hyperpigmentation of skin and mucosa

PHYSICOCHEMISTRY

Busulphan [1,4-bis-(methanesulfonoxy)butane] is a bifunctional alkylating agent. It is lipophilic and poorly soluble in water. The chemical structure is represented in Fig 1.

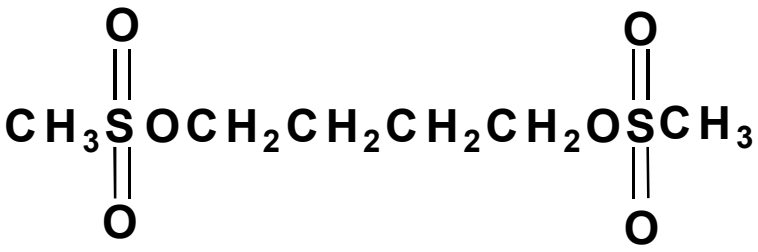

Fig 1: Busulphan [1,4-bis-(methanesulfonoxy)butane] (Myleran ®)

PHARMACOKINETICS

Pharmacokinetics of busulphan in animals and man was studied already in the 1950's, but until 1980's, there was no assay for measuring of drug concentrations available. Pharmacokinetic parameters were investigated using busulphan labeled with radioactive compounds (^{35}S, ^{14}C or ^{3}H) (Peng 1957, Nadkarni *et al.* 1959, Vodopick *et al.* 1963, Vodopick *et al.* 1969). These studies, however, could not differ between the parent compound and metabolites. When the analytic methods for plasma concentration of busulphan became available, pharmacokinetics of busulphan has been extensively studied. Busulphan pharmacokinetics is mostly described using a one-compartment model. A summary on reported pharmacokinetic parameters of busulphan is presented in Table 2.

Table 2: Summary of pharmacokinetic parameters

Ref	No of pts	Age (Year)	Dose	t½ -E (hours)	C max (ng/ml)	Vd (l/kg)	Clearance (ml/min/kg)	Clearance (ml/min/m²)	AUC (ng.hr/ml)
Ehrsson *et al.* 1983	5	32-53	2 mg	2.57 ± 0.38	28 ± 5				125 ± 17
	5	32-53	4 mg	2.670 ± 1.03	65 ± 27				290 ± 66
	5	32-53	6 mg	2.48 ± 0.44	83 ± 31				366 ± 90
Hassan *et al.* 1989	5	32-47	1 mg/kg	3.38 ± 1.75	1730 ± 845				8170 ± 2170
Vassal *et al.* 1989	11	4-14	1 mg/kg	2.33 ± 0.51	803 ± 228			119 ± 54	3918 ± 1170
Grochow *et al.* 1990	14	0.17-3.6	1or 2 mg/kg	1.54 ± 0.90		1.42 ± 0.83	8.4 ± 4.3	197 ± 101	2931 ± 984
	25	≥16	1 mg/kg	2.33 ± 1.41	1047 ± 613	0.595 ± 0.421	2.5 ± 1.4	95 ± 54	8938 ± 4920
Hassan *et al.* 1991	4	<5	1mg/kg	2.05 ± 0.14				178 ± 8	2315 ± 149
	5	5-16	1mg/kg	2.74 ± 0.26				90 ± 10	5937 ± 891
	18	>16-50	1mg/kg	2.59 ± 0.09				105 ± 5	6135 ± 299
Vassal *et al.* 1992	27	1.9-13.8	37.5 mg/m²	2.94 ± 1.40	1258 ± 378	1.04 ± 0.38	4.5 ± 1.40		6404 ± 2378
Yeager *et al.* 1992	7	1.1-5.7	40 mg/m²						4531 ± 1918
Regazzi *et al.* 1993	16	0.5-19	1 mg/kg	2.4 ± 0.8	610 ± 225		8.9 ± 3.8		2154 ± 813
Vassal *et al.* 1993	33	0.2-2.8	0.9-2.6 mg/kg	2.83 ± 1.84		1.62 ± 1.29	6.8 ± 3.0		5748 ± 4897
Hassan *et al.* 1994	8	13-60	2 mg	2.68 ± 0.97	37 ± 14				161 ± 66
	8	1.5-6	2 mg	2.80 ± 1.14	88 ± 44				437 ± 254
Schuler *et al.* 1994	20	18-53	1 mg/kg	2.4	1512				
Shaw *et al.* 1994	9	1.3-13	4 mg/kg	2.3 ± 0.30	3448 ± 764	0.78 ± 0.19	3.96 ± .097		18719 ± 3940
	12	1.4-13.5	150 mg/m²	2.48 ± 0.52	5148 ± 1158	0.70 ± 0.10	3.44 ± 1.13		30541 ± 8651
Pawlowska *et al.* 1997	64	2.8-26	1 mg/kg	1.7 ± 0.9	764 ± 271	0.96 ± 0.43	7.1 ± 3.6		2870 ± 1599
Andersson *et al.* 2000	10	19-58	1 mg/kg	1.58 ± 0.66		0.56 ± 0.31	3.9 ± 1.94		4654 ± 1743

Absorption. Absorption kinetics was described to be of zero- or first-order**,** but was irregular in some patients (Ehrsson *et al.* 1983, Grochow *et al.* 1989, Vassal *et al.* 1989, Grochow *et al.* 1990, Hassan *et al.* 1994, Pawlowska *et al.* 1997). Zero-order dissolution of busulphan in the gastrointestinal tract was suggested as an explanation for zero-order absorption (Ehrsson *et al.* 1983).

Distribution. An administered drug (except drugs used for local application) has first to be distributed in the body and reach target organs before an effect may be attained. Drugs are usually distributed unselectively via blood and plasma to target and non-target organs. This may be a cause of unwanted side effects in non-target organs, as well as of treatment failure if the distribution to target organs is low. Thus, distribution is an important part of the characteristic of a drug.

The distribution of radiolabeled busulphan was studied in rat and a high distribution to liver, lungs, brain and kidney was observed within 15 minutes after the administration (Trams *et al.* 1957). Distribution of ^{11}C labeled busulphan was studied using a positron camera in cynomolgus monkey and in man (Hassan *et al.* 1992). The largest regional accumulation of radioactivity in the body was observed in liver, lungs, and brain. In man, ^{11}C busulphan rapidly entered the brain and reached maximum within 5 minutes. Grey matter to white matter ratio was 1.6, which indicated a flow-dependent delivery of the drug. Twenty percent of the total administered dose penetrated into the brain. Distribution of busulphan into brain was studied with ^{14}C labeled busulphan in rat. The ratio of concentration in plasma to concentration in brain was 0.74 at 24 hours. At the same time, the ratio of radioactivity in plasma and brain was 0.95. Elimination half-lives of busulphan and of the total radioactivity from brain did not differ from that of plasma (2.9 vs. 3.1 hours, and 9 vs. 8 hours, respectively) (Hassan *et al.* 1988).

Busulphan was detected in cerebrospinal fluid (CSF) in man and the CSF/plasma ratio was 1.3 in adults and 0.95 in children treated with doses of 16 mg/kg (Hassan *et al.* 1989, Vassal *et al.* 1989). When the dose administered to children was increased to 600 mg/m², the CSF/plasma ratio increased to 1.39 (Vassal *et al.* 1992). In an adult patient with Omaya shunt the concentrations of busulphan in CSF were comparable with those in plasma during conditioning regimen, and no accumulation was observed (Hassan *et al.* 1989). During high dose treatment, busulphan was detected in the saliva showing saliva to plasma ratio of 1.1 ± 0.1 (Hassan *et al.* 1989). Reversible protein binding was about 7.4% (range 2.7 – 14.7%) in high-dose treated patients (Ehrsson *et al.* 1984, Hassan *et al.* 1989).

Elimination. Elimination of busulphan is monoexponential (Ehrsson *et al.* 1983, Grochow *et al.* 1989, Vassal *et al.* 1989, Grochow *et al.* 1990, Vassal *et al.* 1992). The excretion of unchanged drug into urine was less than 2% within 24 hours and renal clearance was 1.1-7.1 ml/min in adults (Hassan *et al.* 1989). In children, about 5% of unchanged drug was found in urine over the four days treatment (Vassal *et al.* 1993).

Metabolism. Metabolism is an irreversible chemical change of drug to compounds that are usually easier to eliminate from the body. Metabolism of busulphan has been studied with ^{14}C labelled busulphan and metabolite γ-glutamyl-ß-(S-tetrahydrothiophenium)alanyl-glycine (sulfonium ion of GSH) has been detected in

bile from perfused rat liver and *in vivo* after intravenous administration (Roberts *et al.* 1959, Roberts *et al.* 1961, Hassan *et al.* 1987, Marchand *et al.* 1988). Several metabolites of busulphan have been detected in rat urine: 3-hydroxysulfolane, tetrahydrothiophene 1-oxide and sulfolane (Hassan *et al.* 1987). These metabolites have also been detected in the urine of patients treated with high-dose busulphan (Hassan *et al.* 1989). Tetrahydrothiophenium ion has been detected in plasma of patients treated with high dose busulphan (Gibbs *et al.* 1997). Glutathione transferases (GST) have been identified as the enzymes responsible for conjugation of busulphan with GSH. Ethacrynic acid (inhibitor of GST activity) was shown to inhibit the formation of sulfonium ion by in a perfused liver system (Hassan *et al.* 1987). GSTA1-1 is the major isoform of GST catalyzing busulphan-GSH conjugation in human liver, while other isoforms, GSTM1-1 and GSTP1-1 are less active in this process (Czerwinski *et al.* 1996, Gibbs *et al.* 1996).

Variability in pharmacokinetics of busulphan.

A linear relation between the dose and area under the plasma concentration-time curve (AUC) has been reported by several investigators (Vassal *et al.* 1993, Shaw *et al.* 1994, Hassan *et al.* 1996, Schuler *et al.* 1998). However, a wide inter- and intra-patient variability in pharmacokinetics of busulphan has been observed. This variation has been found to be due to age, bioavailability, underlying diseases, drug-drug interaction and circadian rhythm, as discussed bellow.

Absorption phase. Lag time (time from administration to start of absorption) varied between 0.05 and 2 hours and absorption half-life varied between 0.08 and 2 hours (Vassal *et al.* 1993, Hassan *et al.* 1996). The method of administration of busulphan (whole tablets vs. crushed tablets) was shown to affect the lag time in children, while pulverization of busulphan did not affect absorption time compared to tablets in another study (Shaw *et al.* 1994, Hassan *et al.* 1996). Simultaneous food intake decreased the systemic exposure to busulphan and increased Tmax (Schuler *et al.* 1994). Late Tmax was also found in some patients without any obvious cause (Schuler *et al.* 1994).

Bioavailability. As long as busulphan was available only in oral formulation, bioavailability was an important factor affecting the systemic exposure to busulphan. Bioavailability mirrors the first-pass effect of the drug. The bioavailability of low dose of busulphan was 68 ± 31% in children and 80 ± 19% in adults (Hassan *et al.* 1994). The development of intravenous formulations of busulphan have facilitated studies of bioavailability of high dose busulphan, which has been shown to be about 69 ± 28% in adult patients (Schuler *et al.* 1998, Andersson *et al.* 2000).

Busulphan disposition and age. The apparent clearance of busulphan and volume of distribution were higher in young children than in adults (Hassan *et al.* 1991, Regazzi *et al.* 1993, Tran *et al.* 2000), but busulphan clearance declined over the first decade of the life (Slattery *et al.* 1995). High apparent oral clearance in young children was explained by up-regulation of GST activity in enterocytes of young children (Gibbs *et al.* 1999). However, a wide inter-patient variation in elimination half-life has been reported within the same age groups (Grochow *et al.* 1989, Vassal *et al.* 1992, Regazzi *et al.* 1993, Hassan *et al.* 1996).

Underlying disease. Children with lysosomal storage disease showed longer elimination half-life (4.9 vs. 2.4 hours), a larger volume of distribution (3.4 vs. 1.2 l/kg) and higher clearance (8.7 vs. 6.3 ml/min/kg) compared to children with other non-malignant and malignant diseases (Vassal *et al.* 1993). In another study, systemic exposure to busulphan expressed as AUC corrected for dose (mg/kg) was higher in children transplanted for leukemia compared to children transplanted for inherited disorders (5527 and 2768 ng.hr/ml) and the elimination half-life was longer (Hassan *et al.* 1996).

Drug-drug interaction. Phenytoin decreased, and itraconazole, metoclopramide, and ketobemidone increased the systemic exposure to busulphan in patients undergoing SCT (Hassan *et al.* 1993, Schuler *et al.* 1994, Buggia *et al.* 1996, Hassan *et al.* 2000).

Chronopharmacokinetics. A circadian rhythm of AUC and trough levels was reported in adults and in children (Hassan *et al.* 1991, Grochow 1993, Vassal *et al.* 1993). Urinary excretion of unchanged busulphan varied according to physiological urine output (Vassal *et al.* 1993). Intra-patient variation throughout the conditioning regimen was also observed, but these changes are not consistent. The decrease of steady state levels and AUCs of busulphan were observed between doses 3 vs. 16 and doses 1 vs. 13, respectively (Yeager *et al.* 1992, Hassan *et al.* 1996). However, both studies used phenytoin as a prophylaxis of neurotoxicity, which may have influenced the results. In other studies, an increase of AUC between dose 1 and 13 was observed (Schuler *et al.* 1994, Li *et al.* 1999).

Thus, pharmacokinetic data show a wide inter-patient variability in exposure to a fixed dose of busulphan. There are two ways to attain an optimized exposure to busulphan. Firstly, drug concentrations may be assessed and the dose of busulphan adjusted according to the actual levels. Secondly, an intravenous administration of busulphan may decrease the variation in bioavailability and overcome the first-pass effect. However, before these strategies may be used, the target exposure and the thresholds for different adverse effects have to be defined.

PHARMACODYNAMICS

Pharmacodynamics of busulphan has been intensively studied with the aim to define the therapeutic window for myeloablative and antileukemic effects, and thresholds for major complications, such as VOD. Since SCT is a treatment comprising many components and several factors may interact, it is difficult to estimate the contribution of one single factor to the development of a particular complication.

Exposure to busulphan has been shown to affect graft failure. In one study in adults, the authors investigated the relation between the Css and engraftment failure. Four of four patients with Css <200 ng/ml, 4/11 patients with Css

200-600 ng/ml and only 1/23 with Css >600 ng/ml rejected their grafts (Slattery *et al.* 1995). Similar results in children were reported by the same authors (Bolinger *et al.* 2000). These results are highly significant and in agreement with data published by other groups, when Css is estimated from Cl/F and related to patients' outcome, as reviewed by Slattery (Slattery *et al.* 1995). A similar effect was also observed in rat experiments by Tutchka. Maintaining the same dose of cyclophosphamide, an increase in the dose of busulphan was shown to improve the engraftment of incompatible allogeneic donor cells (Tutschka *et al.* 1977).

A relation between the exposure and the antileukemic effect of busulphan was found in CML patients undergoing SCT. The cumulative incidence of relapse was significantly higher in patients with Css <917 ng/ml compared to patients with Css >917 ng/ml (Slattery *et al.* 1997).

The high cerebral uptake of busulphan is an important factor in busulphan-induced neurotoxicity and a relation to increased systemic exposure has been reported as discussed above. VOD has been related to increased systemic exposure to busulphan. The estimated threshold for VOD expressed as AUC was above 1500µM/min (6150 ng.hr/ml) (Grochow 1993, Dix *et al.* 1996). Another study found a threshold for VOD at much lower systemic exposure (Css > 900 ng/ml corresponding to AUC of 5400 ng.hr/ml) (Slattery *et al.* 1995).

THERAPEUTIC DRUG MONITORING AND DOSE ADJUSTMENT

Therapeutic drug monitoring with dose adjustment is an approach to optimize and individualize chemotherapy. There are five major criteria for a drug to be suitable for pharmacokinetic optimization, and busulphan fulfils all of them (Masson *et al.* 1997, McCune *et al.* 2000). 1. An assay for assessment of plasma concentrations of busulphan in a precise and reproducible manner is available. 2. Busulphan has a narrow therapeutic window, and over- or under-dosing may have fatal outcomes. 3. Busulphan pharmacokinetics shows a great degree of inter- and intra-patient variability. 4. Adverse effects are delayed thus adjusting the dose according to observed toxicity is not relevant. Moreover, as conditioning regimen is given as a single course, dose adjustment on "course to course" basis is irrelevant. 5. The relationship between the systemic exposure and VOD has been shown in several studies and has been discussed in the section about pharmacodynamics. The target AUC has been suggested to be about 1300 µM/min (5300 ng.hr/ml) with limits of 900 – 1400 µM/min (3700 – 5700 ng.hr/ml) (Grochow 1993, Chattergoon *et al.* 1997).

To make therapeutic drug monitoring more feasible, limited sampling models have been developed (Vassal *et al.* 1992, Schuler *et al.* 1994, Hassan *et al.* 1996, Chattergoon *et al.* 1997). Classical sampling for pharmacokinetic estimations uses between 10 to 12 samples after each dose, while limited sampling models decrease the number of samples to three or four. Nevertheless, the estimated AUCs obtained by limited sampling are of reasonable accuracy. The need for major dose adjustments (-33 % to +45 %) in a large proportion of patients has been reported both for children and for adults (Grochow 1993, Chattergoon *et al.* 1997).

INTRAVENOUS FORMULATIONS OF BUSULPHAN

An intravenous formulation of busulphan may circumvent an unpredictable intestinal absorption of busulphan and the hepatic first-pass effect. Several forms for intravenous administration have been reported recently. Two of them are based on dissolution of busulphan in organic solvents. In Busulfex®, busulphan is solved in a mixture of dimethylacetamide and polyethylene glycol 400 (Bhagwatwar *et al.* 1996, Andersson *et al.* 2000, Andersson *et al.* 2000). In the other one, busulphan is first dissolved in DMSO and than in water (Ehninger *et al.* 1995, Schuler *et al.* 1998, Deeg *et al.* 1999). Another type of intravenous formulation of busulphan is using Spartaject technology of microparticles (Olavarria *et al.* 2000). These are formed by encapsulation of a drug in a mixture of phospholipids.

Table 3: Pharmacokinetic parameters of intravenous formulation of busulphan

Reference	No of pts	Age (years)	Bu dose (mg/kg)	t½ (hours)	Clearance (ml/min/kg	AUC (ng.hr/ml)
Schuler *et al.* 1998	11	23-54	0.5-0.6		1.8 ± 0.7	4913 ± 1414
Andersson *et al.* 2000	10	19-58	0.08-0.8	4.1 ± 1.7	2.5 ± 0.78	4881 ± 928*
Olavarria *et al.* 2000	12	21-55	1	2.63 ± 0.75		6045 ± 1950a

Intravenous busulphan was administered as an infusion (0.5 – 2 hours). Data are expressed as a mean ±SD. * AUC obtained after dose 0.8 mg/kg.

LIPOSOMES

Liposomes are microscopic vesicles formed by phospholipids and other amphipathic lipids. In an aqueous environment, these lipids arrange themselves to concentric bilayers around an aqueous compartment with a structure similar to that found in cell membranes. Liposomes may carry hydrophilic substances in the aqueous compartment, and lipophilic substances within the lipid bilayers. The encapsulated drug does not exert pharmacological activity until it is released into an extracellular or intracellular space. Size, composition, and charge of the liposomes affect their distribution and make the targeting of specific sites of the body possible. Small uncharged liposomes containing cholesterol have been shown to be stable, circulate for a longer time in blood, and have a higher distribution to bone marrow than other types of liposomes (Gregoriadis 1989, Ostro *et al.* 1989, Fielding 1991). Several drugs in liposomal formulation, such as amphotericin B, cis-platinum, methotrexate and doxorubicin have already been introduced in clinical use (Steerenberg *et al.* 1988, Hay 1994, Mayer *et al.* 1995).

Liposomes are distinctively different from the microparticles of Spartaject system in their biological and pharmacokinetic behavior (Olavarria *et al.* 2000).

BUSULPHAN-INDUCED CYTOTOXICITY

Busulphan is toxic to primary cells and cell lines of different origin as may be readily seen from the spectrum of adverse effects and studies on toxicity *in vivo* and *in vitro*. The most frequently investigated systems are hematopoietic cells and gonadal cells of testis.

Studies on busulphan-induced cytotoxicity *in vivo*

Animal studies

Mouse and rat models have been extensively used for studies of the myelotoxicity of busulphan. Major reasons are that the CFU-S assay allows detection of early hematopoietic progenitors, and that transplantation is feasible.

Early reports on the effect of busulphan on blood cells showed preferential sensitivity of granulocytic cells and almost no effect on erythrocytes or lymphocytes (Elson 1958). Studies on hematopoietic progenitors using the CFU-S and CFU-C assays showed a higher sensitivity of early progenitors compared to lineage committed cells. The effect was attributed to the G0/G1 phase of cell cycle of the sensitive cells (Dunn 1974). The probably most conclusive investigation is that of Down et al. In this study, the potential of busulphan to ablate different subsets of hematopoietic progenitor cells was assessed with cobblestone area-forming cell (CAFC) assay (Down *et al.* 1993, Down *et al.* 1994). This assay provides estimates of survival of hematopoietic progenitors according to their maturation status. CAFC analyzed on day 5 corresponded to CFU-GM, CAFC from day 10 to CFU-S and CAFC between day 30 to 40 to primitive stem cells with long repopulating ability (equivalent of pre-CFU-S in mice). Busulphan depleted both early and late CAFCs. Authors corroborated their data with transplantation studies of busulphan treated animals. Early CAFCs were necessary for early engraftment of transiently repopulating cells from the transplant, while late CAFCs were essential for achieving sufficient and lasting donor engraftment. The levels of chimerism were 70-80 % in blood, bone marrow, spleen, and thymus at 36 weeks after transplantation (Down *et al.* 1993, Down *et al.* 1994). Reports on differences in sensitivity of myeloid and erythroid colony-forming unit cells, respectively, vary between studies (Josvasen *et al.* 1973, Standen *et al.* 1980, Reynolds *et al.* 1986). However, in some studies these differences seem to reflect differences in maturation stage of the studied cells rather than a true difference between lineages (CFU-E or BFU-E vs. CFU-GM).

Morley and Blake developed a mice model of hypoplastic marrow failure based on repeated administration of non-myeloablative doses of busulphan with one or two week-intervals. Animals treated according to their protocols survived with normal or decreased cell count in peripheral blood. Persisting marrow impairment was always present months after the treatment and comprised either normocellular or hypocellular bone marrow with residual damage in clonogenic capacity. The severity depended on the total dose administered (Morley *et al.* 1974, Morley *et al.* 1974). Thus, as has been confirmed by several other authors, busulphan treatment induces residual damage of clonogenic potential of stem cells (Fitchen *et al.* 1980, McManus *et al.* 1984).

The ability of busulphan to cause damage of bone marrow microenvironment remains controversial. Impairment of stromal cells was found in long-term bone marrow cultures from busulphan treated mice (Hays *et al.* 1982), while other studies failed to confirm these results (Halka *et al.* 1987, Down *et al.* 1993). Involvement of other structures of the bone microenvironment was found in histological sections of bones after treatment with busulphan. In this investigation, a transient decrease in short-term adherent cell colonies (STACC) was observed. After 40 weeks, the STACC recovered up to 76%, while CFU-C remained less than 10% of control, over the study period. Morphological changes involving endosteum, bone, adipocytes and mast cells were observed (McManus *et al.* 1984).

Even more obscure is the residual decrease in clonogenic capacity of cells of donor origin in syngeneic transplantation in mice, despite a successful donor marrow engraftment and maintenance of blood and marrow cellularity. However, it cannot be explained by a defect of stromal cells, since stromal cells from treated animals support normal growth of CAFC from controls *in vitro* (Down *et al.* 1993).

Studies in CML patients after the treatment with busulphan

There are few studies on the effect of busulphan on clonogenic capacity of stem cells in human. Two investigations assessed the effect of busulphan on circulating CFU-GM. One study showed decreased CFU-GM growth within days after a single high dose of busulphan (100 mg), while another found an increase in circulating CFU-GM after low dose treatment of CML, compared with untreated patients or age-matched controls (Morstyn *et al.* 1981, Lopez-Karpovitch *et al.* 1995). Long-term cultures of bone marrow cells from CML patients treated with low dose busulphan showed, compared to untreated CML and normal control cultures, a decrease in clonogenic capacity after 15 days (Santucci *et al.* 1991). No difference between cluster and colony formation in PB and BM was found comparing treated and untreated CML patients (Preisler *et al.* 1983).

In vitro studies on busulphan-induced cytotoxicity

In vitro studies in primary hematopoietic cells

Human hematopoietic progenitors from bone marrow or peripheral blood from normal volunteers or patients with leukemia have been studied *in vitro* with regard to busulphan-induced toxicity (Table 4). These studies show that the decrease in colony formation is concentration- and time-dependent. A comparison of the data is difficult since incubation times differ between studies. One way to compare the results is to relate the cytotoxic effect to the exposure to busulphan. In an early study, Spiro et al calculated the exposure to busulphan as concentration × time of incubation. However, this study did not consider the degradation of busulphan (Spiro *et al.* 1981) thus overestimating the exposure.

Similar to what was observed *in vivo*, it was shown also *in vitro* that early progenitors were more sensitive than more mature progenitors. Clusters showed a

higher resistance to busulphan than colonies (Preisler *et al.* 1983). The sensitivity of BFU-E was found to be higher compared to CFU-GM in cells from healthy volunteers and CML patients (Kubota *et al.* 1983). Growth factors were shown to sensitize normal bone marrow cells to the effects of busulphan (IL-3 > GM-CSF > G-CSF) (Tohda *et al.* 1990). In a study on childhood acute leukemia, blasts from patients with AML were more resistant than blasts from the ALL cases. Moreover, different subgroups of AML showed different sensitivities to busulphan (order of resistance: M4 > M1/M2 > M5) (Zwaan *et al.* 2000).

Table 4: Summary of studies of busulphan-induced cytotoxicity in primary human hematopoietic cells *in vitro*

Reference	Cell source	Concentration (µg/ml)	Time	Results
Spiro *et al.* 1981	NPB CML-PB		continuous*	In CFU-C assay estimated LC_{90} for NPB (2.5 µg/ml) and CML (1.7 µg/ml)
Preisler *et al.* 1983	CML-BM	5, 10, 20	4 hours	Concentration-dependent decrease in CFU-C, wide inter-patient difference in sensitivity
Kubota *et al.* 1983	NBM NPB CML-BM CML-PB	5, 10, 20	4 hours	CFU-C more resistant than BFU-E; decrease in colonies concentration-dependent
Hincks *et al.* 1990	NBM NPB CML-BM CML-PB	1.2 to 246.3	2 to 18 hours	Inhibition of CFU-GM in concentration- and time-dependent manner
Tohda *et al.* 1990	NBM AML-PB	0.02 to 0.04	continuous*	Concentration-dependent decrease in blast-colony formation and CFU-GM
Berger *et al.* 1992	NBM	0.01 to 100	continuous*	Concentration-dependent inhibition of CFU-GM
Sanyal *et al.* 2000	NPB		7 days	LC_{50} (13.4 µg/ml) determined in MTT assay
Zwaan *et al.* 2000	BM or PB from ALL and AML	1.23 to 300	4 days	LC_{50} determined for AML (37.8 µg/ml) and for ALL (28.2 µg/ml) in MTT assay

BM = bone marrow; PB = peripheral blood; NBM = bone marrow from healthy volunteers; NPB peripheral blood from healthy volunteers; CML = chronic myeloid leukemia; ALL = acute lymphoblastic leukemia; AML = acute myeloid leukemia; LC_x = lethal concentration (concentration killing x % of the cells); *Continuous exposure to busulphan means the presence of busulphan in semi-solid medium in CFU assay

Clonogenic capacity was assessed with CAFC assay in murine bone marrow cells exposed to busulphan *in vitro*. The pattern of different sensitivities of early and late CAFC and the level of suppression were similar to what was obtained after treatment with busulphan *in vivo* (Westerhof *et al.* 2000). The sensitivity of CFU-E was higher compared with CFU-GM, when expressed as LC_{50} (0.25 vs. 5.59 µg/ml) (Sanyal *et al.* 2000)

In vitro studies of cell lines

As for primary cells, busulphan induces cytotoxicity in cell lines of hematopoietic and non-hematopoietic origin in a concentration- and time-dependent manner. Non-hematopoietic cell lines are in general more resistant than hematopoietic cell lines (Pacheco *et al.* 1990, Sanyal *et al.* 2000). However, it is difficult to compare those studies concerning busulphan-induced cytotoxicity since different methods, and different concentrations and times of incubation have been used for assessment of toxicity (Table 5).

In one study, differentiation of HL-60 cells was induced with busulphan (Michaeli *et al.* 1993), but this study does not indicate the solvent in which busulphan was dissolved. DMSO is frequently used as a solvent and it may act as a differentiating agent by itself.

Underlying mechanism of busulphan cytotoxicity

Despite the fact that busulphan has been in clinical use for 50 years, relatively little is known about its actual target molecules in cells. Busulphan is considered an alkylating agent, i.e. it acts by insertion of alkyl groups into nucleic acids and proteins. However, it possesses several unique properties, which distinguishes it from other alkylating agents, such as nitrogen mustard. Reactivity of busulphan was found to be low, compared to other alkylating agents, and the fraction of radiolabeled drug bound to DNA or RNA was less than 2% (Brookes *et al.* 1961). When busulphan reacted with DNA *in vitro*, formation of adducts on position N7 of guanine was observed.(Tong *et al.* 1980). Several investigations of crosslinking were published with no conclusive results. Interstrand crosslinking was found in two studies (Bedford *et al.* 1982, Hartley *et al.* 1991), while other studies failed to confirm this observation (Epstein *et al.* 1986, Pacheco *et al.* 1989, Pacheco *et al.* 1990, Knox *et al.* 1991). Busulphan was found to form DNA-protein crosslinks (Pacheco *et al.* 1989, Pacheco *et al.* 1990) or monoalkylates at guanine N-7 position (Ponti *et al.* 1991). In a study on DNA-crosslinking between histones and DNA, busulphan did not produce DNA-histone or histone-histone crosslinks, but monoadducts on histones were detected (Hartley *et al.* 1986). In contrast with the low reactivity of busulphan was the observation of a high mutagenic efficiency of busulphan, indicating that busulphan causes DNA damage (Sanderson *et al.* 1991, Sanderson *et al.* 1996). Thus, the mechanism underlying the cytotoxic effect of busulphan is not conclusively clarified.

Busulphan affects cycling cells through arresting them in G2 phase of the cell cycle. Busulphan-induced G2 phase arrest was detected in a mouse mammary and two human bladder tumor cell lines (Pauwels *et al.* 1995). Transient arrest in G2 phase was also detected in the chinese hamster V-79 cells, but normal cell cycling was restored within 3 days after the end of incubation (Millar *et al.* 1986).

Table 5: Summary of *in vitro* experiments in cell lines

Reference	Time (hrs)	LC_{50} (µg/ml)	Assay	Results
Sanyal *et al.* 2000	continuous	53.7	MTT	MT-4 T-lymphocytic
	continuous	41	MTT	H-9 T-cell lymphoma
	continuous	25.6	MTT	U-937 histiocytic
	continuous	47.3	MTT	PM-1 leukemic
	continuous	300.6	MTT	MCF-7 breast adenocarcinoma
	continuous	75.6	MTT	Hep-3B hepatocellular carcinoma
Pacheco *et al.* 1990	2 hrs	130*	CFU	K562 leukemic
	2 hrs	25*	CFU	HL-60 leukemic
	2 hrs	74*	CFU	BE human colon carcinoma
	2 hrs	86*	CFU	HT-29 human colon carcinoma
Epstein *et al.* 1986	1 hr	100	growth	HL-60 leukemic
Pacheco *et al.* 1989	2 hrs	74*	CFU	L1210 mouse leukemia
Knox *et al.* 1991	2 hrs	13.1 (LC_{90})	CFU	Walker 256 carcinoma
Hartley *et al.* 1986	NA	7.5		Yoshida wild-type solid rodent sarcoma
Sanderson *et al.* 1991	1 hr	113.3	Cloning efficiency	WIL2-NS B-cell lymphoblastoid
Millar *et al.* 1986	1 hr	49.3	CFU	V-79-753B Chinese hamster
Bedford *et al.* 1983	1 hr	7.6	growth	Yoshida lymphosarcoma
Teicher *et al.* 1986	0.5 hr	6.6	CFU	Raji human Burkitt lymphoma
	0.5 hr	15.5	colonies	SCC-25 human squamous cell carcinoma (tongue)

LC_x = lethal concentration (concentration killing x % of the cells)
* = LC_{50} estimated from published graph; NA = not available

CELL DEATH

There are two types of cell death, apoptosis and necrosis, which differ from each other by morphological and biochemical hallmarks (Majno *et al.* 1995). Apoptosis is encoded in cell genome and the mechanism is conserved through species from worms to mammals (Vaux *et al.* 1999). The process shows, irrespective of the organism, several common steps. Apoptotic morphology is characterized by cell shrinkage, membrane blebbing, condensation of cytoplasm and DNA and formation of apoptotic bodies. Biochemical changes comprise activation of caspases, cleavage of substrates participating in cellular homeostasis or metabolism, and finally activation of endonucleases responsible for fragmentation of DNA (Johnson 2000).

Apoptosis is a prerequisite for the homeostasis of a multicellular organism, and thus has to be in balance with cell proliferation and survival. Dysbalance in this regulation causes disease by deficient or excessive apoptosis (Kam *et al.* 2000). In multicellular organisms, apoptotic cells are dismissed through phagocytosis by neighboring cells without induction of an inflammatory reaction in the surrounding tissues.

Necrosis is characterized by cellular swelling, early loss of membrane integrity, release of lysosomal content and disintegration of nuclear chromatin without the formation of DNA ladder (Majno *et al.* 1995). Necrosis is caused by overwhelming noxious stimuli to the cells, and, in case of a multicellular organism, is always accompanied by an inflammatory reaction of the surrounding tissues.

The border between apoptosis and necrosis is not sharp. Type and strength of the stimulus together with the biological background (cell type, genotype and mutations in oncogenes regulating proliferation and cell death) and the actual status (phase of the cell cycle and differentiation stage) seem to regulate the type of cell death in many systems studied (Borner *et al.* 1999, Kitanaka *et al.* 1999, Kolenko *et al.* 1999, Blagosklonny 2000).

Apoptosis in both primary cells and cell lines may be triggered by a wide variety of causes (Table 6).

Table 6: Induction of apoptosis:

• activation of death receptors: Fas receptor, TNF receptor
• withdrawal of growth factors or hormones
• stimulation of downstream components of apoptotic pathways: phosphatases, kinase inhibitors
• differentiation agents: ATRA, DMSO
• DNA damage: ionizing radiation, anti-cancer drugs
• direct physical damage: radiation, alkylating agents

Every eukaryotic cell contains many proteins that participate in the balance between anti-apoptotic and pro-apoptotic signals (Jarpe *et al.* 1998, Utz *et al.* 2000). These proteins are divided in families and subfamilies based on their structural similarities. Members of the same protein family, however, may have

opposite effects on the apoptotic process. This has been well studied in the Bcl-2 family of proteins, which comprises anti-apoptotic proteins, such as Bcl-2, Bcl-X_L and Mcl-1, and pro-apoptotic proteins, such as Bax, Bak, Bcl-X_S, Bim, Bid and Bad (Tsujimoto *et al.* 2000). Moreover, an anti-apoptotic protein may be converted to pro-apoptotic by cleavage or phosphorylation, e.g. Bcl-2 (Cheng *et al.* 1997, Fujita *et al.* 1998, Grandgirard *et al.* 1998, Fadeel *et al.* 1999). Another possibility to switch form anti-apoptotic to pro-apoptotic settings in a cell is by up- or down-regulation of the pro- or anti-apoptotic proteins, respectively (Delia *et al.* 1992).

The caspase family of proteins plays a central role in apoptosis (Nicholson 1999). A recent definition encompasses the activation of the caspase cascade as a hallmark of apoptotic cell death (Samali *et al.* 1999). Caspases are cysteine proteases that require aspartic acid in the cleavage site to exhibit their proteolytic activity. Caspases cleave a substantial number of structural and signaling proteins, thus activating or deactivating their function (Earnshaw *et al.* 1999). The anti-apoptotic effect of Bcl-2 is converted to pro-apoptotic through cleavage by caspases, as mentioned above. Actin, a microfilamental cytoskeleton protein, is an example of the structural protein that may be cleaved during apoptosis (Mashima *et al.* 1995, Chen *et al.* 1996, Kayalar *et al.* 1996). Endogenous or exogenous inhibitors of caspases may block progress of biochemical changes underlying apoptosis (Earnshaw *et al.* 1999). However, blocking of the apoptotic cascade may not restore the vital functions of the cell.

A supra-threshold stimulus may activate one or several pathways that constitute a network of molecular events (Solary *et al.* 2000). Some of these events are unique for a single agent, while others are common for several different agents. Initiation of multiple pathways seems to be common in treatment with anticancer drugs and differentiating agents, as they often have several targets within the cell (Calabresse *et al.* 1995).

Etoposide is a toposisomerase II inhibitor, which is used in the treatment of hematological malignancies. It is also well known inducer of apoptosis in several cell types (Fujita *et al.* 1998). All-trans retinoic acid (ATRA) is a potent differentiating agent that is used as the first choice in the treatment of acute promyelocytic leukemia. It also induces differentiation of cells of myeloid origin *in vitro* (Nagy *et al.* 1998).

There are plenty of models for investigation of apoptosis *in vitro* and many methods to assess different changes throughout the course of apoptosis. However, not all methods are suitable for all cell types and a selection of methods has to be done for each investigation. P39 is a myeloid cell line established from a patient suffering from acute leukemia following a myelodysplastic syndrome of the chronic myelomonocytic leukemia subtype (Nagai *et al.* 1984). P39 cells are easily induced to differentiation and/or apoptosis with different agents (Anzai *et al.* 1994). Apoptotic cells show typical morphological changes and form DNA ladder, which makes this cell line a suitable model for our purpose.

Busulphan-induced apoptosis

Busulphan-induced cell death has been studied in several cell types *in vivo* and *in vitro*. Cells in crypts of small intestine of mice were shown to undergo apoptosis as a response to busulphan treatment *in vivo* (Ijiri *et al.* 1983). However, these cells were not stem cells, but cells that were already partly differentiated. On the other hand, no increase in apoptosis was detected in bone marrow smears or trephine biopsies from patients treated with busulphan for CML (Thiele *et al.* 1996, Thiele *et al.* 1997). A recent study showed that busulphan induced apoptosis in malignant pre-B cell or B cell lines *in vitro*. Apoptosis was decreased when B-cells were co-cultured with follicular dendritic cells. The mechanism by which follicular dendritic cells inhibited apoptosis remains unknown (Schwarz *et al.* 1999).

The role of glutathione in busulphan-induced cytotoxicity

Glutathione is a thiol-containing tripeptide and is found in all animal and plant cells. It acts as a scavenger in the antioxidant defense system. Another important function of GSH is detoxicification of xenobiotics and thus protection of cells against exogenous toxins (Tew 1994, Tew *et al.* 1996). Under physiological circumstances, the cellular level of GSH is stable and synthesis is in balance with degradation. GSH plays an important role in the metabolism of alkylating agents and alteration of the cellular level of GSH may affect the toxicity of these drugs. The cellular content of GSH may be modulated *in vitro* and *in vivo* by different treatments (Meister 1988). Some of these agents may be used clinically with the aim to improve the effect or decrease the side effects of alkylating agents. Precursors of GSH, such as methionine or N-acetylcysteine, increase the cellular content of GSH and may decrease the toxicity of alkylating agents. On the other hand, depletion of GSH increases the toxicity of alkylating agents in most cell systems studied. GSH may be depleted *in vivo* by treatment with buthionine sulfoximine, and *in vitro* by incubation in medium without sulphur amino acids, or by treatment with buthionine sulfoximine or ethacrynic acid.

Busulphan is metabolized in the liver by conjugation with glutathione catalyzed by GST, as discussed in the paragraph about metabolism. The final products of this metabolic pathway are water soluble, harmless and easily excreted from the body via urine. Intermediate products have reactive potential, but there is no direct evidence that they participate in busulphan-induced toxicity. However, an increased toxicity of busulphan in animals pretreated with cysteine was observed in one study (Addison *et al.* 1971).

There are only a few published reports on the role of GSH in busulphan cytotoxicity and the results are not conclusive. In xenografts of human glioblastoma multiforme, no differences in GSH and GST levels were found between sensitive and procarbazine-resistant (cross-resistant to busulphan) cell lines (Friedman *et al.* 1997). However, another xenograft treated with busulphan resulted in busulphan-resistant cells and these cells contained about 50% less of intracytoplasmic GSH than the parent cells (Hare *et al.* 1997). Depletion of GSH in sensitive cells did no decrease their sensitivity to busulphan. GSH and enzymes involved in the GSH metabolism were assessed in a pre-cataract lens model in rat (Cherian *et al.* 1991).

All enzymes, glutathione reductase, γ-glutamyl cysteine synthetase, γ-glutamyl transpeptidase, GST, and adenosine triphosphatase were increased in the pre-cataract lens, but GSH was increased with only 8% compared to the control. Cataractous lenses, on the other hand, showed a decrease in all these parameters. Busulphan may thus increase the GSH turnover and potentate its own metabolism in rat lens.

Murine hepatocytes were depleted by 50 % in GSH after treatment with busulphan *in vivo* and *in vitro* (DeLeve *et al.* 2000). Modulation of the cellular level of GSH altered busulphan cytotoxicity to hepatocytes, in that cells depleted of GSH were more sensitive and cells with increased GSH less sensitive to the effect. Moreover, GST inhibitors protected hepatocytes from busulphan toxicity *in vitro*.

The effect of GSH modulation on busulphan-induced cytotoxicity has not been studied in hematopoietic cells. Hematopoietic stem cells do not express GSTA1, which might explain their sensitivity to busulphan. However, hematopoietic cells express other GST isoforms. GSTP1 predominates in most hematopoietic cells, while GSTT1 and low levels of GSTA1 are expressed in erythroid cells and GSTM1 in lymphoid cells (Wang *et al.* 2000). GSTM and GSTP isoforms both show lower activity in the conjugation of busulphan with GSH compared to GSTA (Czerwinski *et al.* 1996). The relative activity compared to GSTA1 is 46% for GSTM1 and 18% for GSTP1. The activity of these GST isoforms has anyhow to be considered, since busulphan might induce GST expression in hematopoietic cells in similarity with what was found in the lens model.

RESISTANCE TO BUSULPHAN

Despite high dose chemotherapy and graft-versus-leukemia effect, malignant diseases may recur after SCT. Thus, increased antileukemic and antitumor effect of busulphan may further improve the outcome of transplanted patients. There are several mechanisms of resistance to alkylating agents (Table 7).

Table 7: Mechanisms of resistance to alkylating agents

- Low primary sensitivity of cells: type of the cell, expression of anti-apoptotic proteins
- Decreased drug uptake
- Drug efflux: MDR
- Drug detoxification: GSH
- Enhanced DNA repair
- Biochemical changes in target molecules

Some of these mechanisms are relevant for busulphan, but very few thorough investigations have been done (Hall *et al.* 1992). The wide difference in the exposure to busulphan needed for induction of a cytotoxic effect reflects different sensitivities of the cells. A decreased uptake of busulphan by resistant cells and a

different expression of mismatch repair proteins were found in resistant cells of xenografts of human glioblastoma multiforme (Friedman *et al.* 1997, Hare *et al.* 1997).

It is clear from the present level of knowledge that studies on basic mechanisms underlying busulphan-induced cytotoxicity are important. Such knowledge may help to find effective strategies to increase the effect and decrease adverse effects of busulphan in the conditioning regimen prior to stem cell transplantation.

AIMS OF THE THESIS

The objectives of my thesis were to improve busulphan-based conditioning regimens before stem cell transplantation by developing a new formulation of busulphan for an intravenous administration, and to increase the understanding of the mechanisms underlying busulphan-induced cytotoxicity in hematopoietic cells.

SPECIFIC AIMS OF THE STUDY

Development and evaluation of an intravenous formulation of busulphan

I + II To develop an intravenous formulation of busulphan encapsulated in liposomes and to investigate its distribution and pharmacokinetics, as well as its myelosuppressive effect in animal models.

III To investigate the pharmacokinetic parameters of liposomal busulphan in man, and the feasibility of using it in high dose in conditioning regimen before stem cell transplantation.

Investigation of mechanisms underlying busulphan-induced cytotoxicity

IV To develop and evaluate a functional *in vitro* model of maturation and/or apoptosis in myeloid cells, and to select suitable methods for the following studies.

V To study *in vitro* pharmacodynamics of busulphan in the myeloid P39 cell line, and mechanisms behind busulphan-induced cell death.

VI To assess *in vitro* pharmacodynamics of busulphan in hematopoietic progenitor cells. To investigate the effects of modulation of glutathione on busulphan-induced cytotoxicity in hematopoietic progenitor cells *in vitro,* or *in vivo* in an animal model.

"OF MICE AND MAN" AND METHODS

MAN

Patients (Study III)

The study followed the guidelines of the Research Board of Sweden and the Ethics Committee of Karolinska Institutet, and the Swedish Drug Agency approved the LBu for intravenous administration. Seventeen consecutive patients undergoing busulphan-based conditioning regimen for stem cell transplantation at Huddinge University Hospital were enrolled. Six adults and six children were given a low dose of LBu and five patients (four adults and one child) were given two high doses. Informed consent was obtained from the adult patients and from the parents of the children, and assent from the children 8 years or older. The patients' characteristics are described in paper III (Table 1 and Table 2).

Volunteers (Study VI)

The study design was approved by Local Ethics Committee of Huddinge Hospital at Karolinska Institutet. Ten healthy volunteers were found among the medical staff and students without age or sex selection. The health condition was examined by questioning and bone marrow was harvested from spina illiaca anterior superior in local anesthesia.

ANIMALS

Rat (Study I)

Male Sprague-Dawley rats (200-230g) were purchased from B&K Universal Limited, Sollentuna, Sweden. Local animal ethical committee approved animals housing conditions and experiment design. Animals were fed with standard pelleted food and water *ad libitum*.

Mice (Study II and VI)

BALB/C mice were purchased from B&K Universal Limited, Sollentuna, Sweden. Male and female mice were 6 to 14 weeks old with a weight ranging from 16 to 30g. Local animal ethical committee approved animals housing conditions and experiment design. Each experiment was performed using animals within the same age and weight group. Animals were fed with standard pelleted food and water *ad libitum*.

PREPARATION OF LIPOSOMAL BUSULPHAN (STUDY I-III AND VI)

Lipids (L-α-phosphatidylcholine, 1,2-dioleolyl-sn-glycero-3-phosphate and cholesterol) were dissolved in chloroform, and busulphan or ^{14}C-busulphan (study I only) was added. The mixture of lipids and busulphan was dried by

evaporation to a thin film in a round vessel and traces of solvent were removed under a gentle stream of nitrogen. After hydration with glucose (50mg/ml, pH 4.0), multilamellar vesicles were formed by vortexing. The suspension was extruded five times under nitrogen pressure through two stacked polycarbonate filters with a pore size of 100 nm. Volumes within 50 ml of LBu were prepared using the extruder LiposoFast 50 (Avestin, Ottawa, Canada) and volumes larger than 50 ml were prepared using a holder with a 2 liter capacity barrel (Aka Filter, Göttingen, Germany). The LBu was prepared for each experiment and each patient under aseptic conditions, sterile filtered and then stored at +4 °C for no more than 24 hours pending use.

BUSULPHAN DETERMINATION (STUDY I-III AND V)

Busulphan concentrations in plasma and in medium were assessed using gas chromatography with electron capture detection. Busulphan and the internal standard (1,5-bis(methanesulfonoxypentane)) were converted to 1,4-diiodobutane and 1,5-diiodopentane, respectively, and assayed (Hassan *et al.* 1983).

PHARMACOKINETICS AND STATISTICS

Pharmacokinetics in vivo. The concentration-time curves were adjusted to data sets via non-linear iterative least square regression analysis. The pharmacokinetic parameters were calculated using WINNONLIN (Pharsight Corporation, Mountain View, CA, USA).

Pharmacokinetics in vitro. The half-life of busulphan in RPMI at 37 °C was determined to 16 hours and thus k=0.043. The concentration of busulphan at the end of incubation was calculated as $C=C_0.e^{-kt}$ and AUC was calculated according to the trapezoidal rule.

Statistics. GraphPad Instat (GraphPad Software Inc., San Diego, CA, USA) was used in statistical analysis. Differences between two groups were assessed using the Mann-Whitney *U* test, Kruskal-Wallis test or Wilcoxon paired *t*-test, whenever appropriate. In *in vitro* studies, differences between two or more time points were assessed using the Student's *t*-test and one-way Anova, whenever appropriate.

METHODS USED IN EVALUATION OF LIPOSOMAL BUSULPHAN (STUDY I-III)

Characteristics of LB

The characteristics of the final liposomal preparation (after the fifth filtration) stored at 4 °C was studied over a period of 20 days. The distribution of size of liposomal vesicles was assessed using dynamic light scattering (Malvern Autosizer) and laser difraction (Malvern Mastersizer). Formation of free crystals of busulphan in LBu was assessed using electron microscopy.

Preparation of busulphan formulation other than LBu

Busulphan for oral administration was either suspended in water (Bu/susp) or diluted in acetone as a stock solution and then was mixed with water before administration (Bu/Ac). Busulphan or ^{14}C-busulphan (study I only) for intravenous administration was dissolved in DMSO: ethanol: propyleneglycol mixed in ratio 0.35:0.25: 0.40 (Bu/DMSO). The control group for study II consists of untreated animals and animals treated with preparations containing solvent without busulphan: *i.e*. liposomal solution (LC) or organic solvent solution (DMSO).

Treatment schedules

Treatment of rats. LBu and Bu/DMSO were administered in a single dose through the tail vein, under anesthesia with ether.

Treatment of mice. LBu and Bu/DMSO were administered through the tail vein, and the oral forms were administered through a gastric tube. Two treatment schedules were used: single high dose, and a conditioning regimen-like schedule with busulphan administered twice a day for four consecutive days.

Treatment of patients. Two dosing schedules were used in the trial. Low dose of LBu was 2, 4, 6 or 8 mg in a single dose, and the dose of each patient was selected randomly (except the youngest child). LBu was administered as a short intravenous infusion (20 minutes) one day before starting the conditioning regimen. High dose of LBu was included in the conditioning regimen consisting of sixteen doses of busulphan (1 mg/kg every 6 hours), followed by cyclophosphamide. LBu replaced the first and the last doses (1 and 16) of the regimen, while doses 2 to 15 were given orally. LBu was increased from 0.4 mg/kg to 0.9 mg/kg and administered as an intravenous infusion for 1.5 hours. Drugs known to interact with Bu were not used during the Bu treatment.

Sampling schedules

Blood sampling of rats. Blood samples were collected at the time intervals of 10 and 30 minutes, and at 1, 2, 3, 4, 6, 8, 10, 12, 18 and 24 hours after the administration of busulphan. The blood was sampled by heart puncture under general anesthesia with pentobarbital. The blood was immediately centrifuged at 2000g and plasma was frozen at - 20 ^{o}C until assay.

Blood sampling of mice. Blood samples were collected at time intervals 5, 10, 15 and 30 minutes and at 1, 2, 3, 4, 6, 8 and 10 hours after the administration of busulphan. The blood was sampled by heart puncture under general anesthesia with ether. The blood was immediately centrifuged at 2000g and plasma was frozen at - 20 ^{o}C until assay.

Blood sampling of patients. Samples were collected from permanently placed central venous catheters. Two sampling schedules were used. In the low dose group, blood samples were collected before infusion, 5 and 15 minutes after starting the infusion, at the end of the infusion, and at 2.5, 5, 15 and 30 minutes and 1, 2, 3, 4, 6, 8 and 12 hours after the infusion. In the high dose group, blood samples were collected before infusion, 15, 30 and 45 minutes after starting the infusion, at 2.5, 5,

15 and 30 minutes and 1, 2, 3, 4 and 6 hours after stopping the infusion. The blood was centrifuged at 2000g, the plasma was separated and frozen at -20 ^{o}C until analysis.

Clonogeneic assay

Mice were sacrificed under general anesthesia with ether and both femurs were removed. Bone marrow was flushed out under sterile conditions. After lysing red cells with ammonium chloride solution, nucleated cells were washed twice in PBS and cell number and viability were determined by trypan blue exclusion. Nucleated cells ($1x10^5$) were plated in MethoCult M3530 in 35 mm Petri dishes in triplicates and incubated at 37 ^{o}C in a humidified 5% CO_2 atmosphere. Colonies more than 50 cells were counted on the day seven using an inverted microscope.

Distribution of ^{14}C busulphan in rat organs

Rats were sacrificed under general anesthesia with pentobarbital. Lungs, heart, liver, spleen, kidney, testis and brain were removed and washed of blood. Bone marrow was flushed from femurs and its weight was estimated as a difference in weight of femurs before and after flushing. About 400 mg of each organ was homogenized and solubilized (Soluene-350/isopropanol 1:1). Samples were decolorized with hydrogen peroxide and scintilation was determined in Hionic Fluor with beta-counter (WALLAC, EG&G Comp, Turku, Finland).

METHODS USED IN STUDIES OF MECHANISM OF BUSULPHAN (STUDY IV-VI)

CD34+ cells separation

Mononuclear cells were separated by density gradient centrifugation on sodium diatrizoate (Lymphoprep). CD34+ cells were separated by positive selection using CD34+ Separation Kit (Miltenyi Biotec GmbH, Bergisch Gladbach, Germany). After separation, CD34+ cells were incubated in medium RPMI 1640 supplemented with 10% heat inactivated fetal bovine serum at 37°C in a humidified 5% CO_2 atmosphere.

Cell line cultures

The P39 cell line was kindly provided by Prof. Y. Yoshida, Center for South East Asian Studies, Kyoto University, Kyoto, Japan. Cells were grown in RPMI 1640 medium supplemented with 10% heat inactivated fetal bovine serum (FBS), penicillin (25 IU/mL) and streptomycin (25 µg/mL) at 37°C in a humidified 5% CO_2 atmosphere. All experiments were run with exponentially growing cells in medium supplemented with 10% FBS without antibiotics.

All-trans retinoic acid and etoposide cultures in vitro

Cells were incubated with ATRA (0.1, 1 or 10 µM) for 96 or 120 hours and with etoposide (1 or 6 µg/ml) for 48 hours. G-CSF concentrations of 50 or 100 ng/ml were added to the cultures simultaneously with ATRA and etoposide. Concentrations of CD95 antibodies were 1 µg/ml of CH-11 and 1 µg/ml of ZB4 and concentration of the general caspase-inhibitor zVAD-fmk was 10 µM.

Busulphan cultures in vitro

Busulphan for *in vitro* experiments was diluted in sterile DMSO and before the addition to cell suspension it was further diluted in medium. CD34+ cells were incubated in busulphan at final concentrations of 1 to 60 µg/ml for 2, 4 or 8 hours for pharmacodynamic experiments and 4.5 or 10 µg/ml for 4 hours for GSH-modulation experiments. P39 cells were incubated with busulphan at final concentrations of 10 to 100 µg/ml for 2, 4 or 8 hours. After incubation, P39 cells were washed and re-cultured in busulphan-free medium until 72 hours.

Modulation of apoptosis and GSH cellular content

Modulators of apoptosis or GSH cellular content were used as follows: G-CSF 100 ng/ml, ZVAD-fmk 50 µM, N-acetyl-L-cysteine 1 mM or L-butionine-[S,R]-sulfoximine 50 µM. The final concentration of DMSO was 0.05% for CD34+ cells and equal or less than 0.5% for P39 cells.

Treatment of mice

Animals were treated once daily for four consecutive days with single drug or in combinations. Treatment combinations sustained from N-acetyl-L-cysteine orally or L-butionine-[S,R]-sulfoximine intraperitoneally followed by liposomal busulphan intraperitoneally.

Clonogeneic assay

CD34+ cells. Two thousands CD34+ cells were washed in medium and plated in MethoCult™ GF H4434 in 35 mm Petri dishes in triplicates. Colonies (CFU-GM ≥ 20 cells; erythroid colony ≥ 2 clusters, each containing minimum of 8 cells) were scored on day 14 using an inverted microscope.

Mice. Mice were sacrificed under general anesthesia and both femurs were removed. Bone marrow was flushed out under sterile conditions. Nucleated cells ($0.5x10^5$) were plated in MethoCult™ GF M3534 in 35 mm Petri dishes in triplicates. Colonies containing 50 or more cells were counted on the day 7 using an inverted microscope.

P39 cell line. Five hundred cells were plated in 0.9% methylcellulose in 35 mm Petri dishes in triplicates. Colonies ≥ 50 cells were counted on day 7 using an inverted microscope.

Cell number and viability

Cell numbers were determined in Turk solution (3% glacial acetic acid and 0.01% aqueous gentian violet) and viability was determined with trypan blue exclusion.

Proliferation

Aliquots of 0.2 ml of P39 cell suspension were incubated in triplicates on 96-well microplates with 1 µCi (20 µl of 50 µCi/ml) ^{3}H-labelled thymidine for 4 hours at 37 °C. Incubation was initiated before incubation with busulphan, after incubation with busulphan and after 24, 48 and 72 hours in busulphan-free cultures. The cells were harvested on filter with a Cell Harvester (Skatron Instruments Ltd, Suffolk, UK).

The activity was measured in scintillation fluid (OptiScint Hisafe) using a liquid scintillation counter (WALLAC, EG&G Comp, Turku, Finland).

Differentiation

Myeloid differentiation was determined using nitroblue-tetrazolium test. Cytospins preparations were air dried, fixed in methanol and counter-stained with May-Grünwald-Giemsa. The percentage of cells containing dark blue formazan deposits was counted in a total of 400 cells.

Assays for apoptosis

Apoptotic cells were assessed in May-Grünwald-Giemsa staining on cytospined slides. Cells with morphological features such as fragmented nuclei and condensed chromatin were defined as apoptotic and apoptosis was expressed as a percentage of a minimum of 400 counted cells per slide. DNA fragmentation assay was used to confirm the apoptotic origin of these morphological changes.

Analysis of surface markers and cell cycle with flow cytometry (FACS)

Fresh P39 cells were stained for CD95 (Fas) surface antigen. Cell cycle analysis of P39 cells fixed in ethanol was performed using hypotonic PI solution containing DNase-free RNase. Cell cycle was analysed using the “halving-peak” method. The acquirement and the analysis were preformed on a FACScan flow cytometer using CELL Quest software (Becton-Dickinson, San Jose, CA, USA).

Protein quantification

Total cellular protein was quantified with a colorimetric assay (Bio-Rad protein assay) based on Bradford dye-binding or with colorimetric method according Lowry (Lowry *et al.* 1951).

SDS-PAGE and Western blotting

Cell lysate in Laemmli buffer was electrophoresed by SDS-PAGE and then transferred to a PVDF membrane. The membrane was blocked in 5% non-fatty dry milk and than incubated with antibodies against Bcl-2 (1:100, DAKO, Glostrup, Denmark), actin (1:1000 Sigma, St. Louis, MO, USA), PARP (1:100, Oncogene Research Products, Boston, MA, USA) or caspase-3 (1:1000, PharMingen International, San Diego, CA, USA). After washing, the membrane was incubated with a peroxidase-conjugated secondary antibody (1:2500 Amersham Pharmacia Biotech AB, Uppsala Sweden). The enhanced chemiluminiscence (ECL+Plus, Amersham Pharmacia Biotech AB, Uppsala Sweden) was used for visualization.

GSH quantification

Intracellular concentrations of glutathione was determined using the enzymatic assay according to Tietze (Tietze 1969).

RESULTS

DEVELOPMENT AND EVALUATION OF A LIPOSOMAL BUSULPHAN

Development and evaluation of liposomal busulphan in animal models (Study I and II)

An intravenous formulation of busulphan was developed using a technique of encapsulation of busulphan into liposomes. The optimal composition of mixture of lipids, busulphan and solvent, and several factors influencing its stability were studied. The incorporation of busulphan into liposomes was dependent on the solvent used and was more efficient in 0.9% NaCl solution compared with 5% glucose. The start concentrations of Bu inversely affected incorporation of Bu into liposomes in both solvents (Fig 2). The major lose of busulphan concentration was observed after the first filtration, however, no further decrease in concentration was observed from filtration two to five.

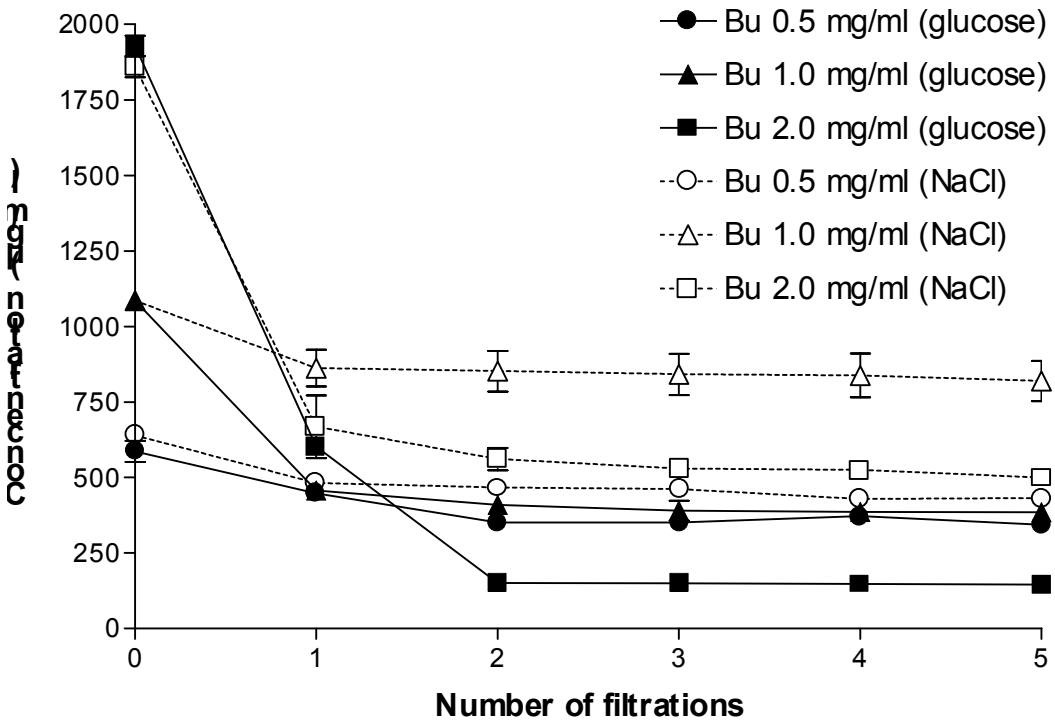

Fig 2: The effect of solvent, starting concentration of busulphan and number of filtrations on busulphan concentration in liposomal busulphan formulation.

Stability of LBu was assessed in terms of half-life of Bu, aggregation of liposomes and formation of free crystals of busulphan. Size of liposomal vesicles was assessed after the fifth filtration and expressed as the diameter of vesicles. The results are presented in Table 8.

Table 8: Characteristics of liposomal busulphan

	0.9% NaCl	5% Glucose
Incorporation of Bu into liposomes with starting concentration of Bu 1 mg/ml (mg/ml)	0.83 ± 0.08	0.31 ± 0.03
Half-life of Bu in liposomal formulation at 4 °C (days)	8.7 ± 2.7	
Stability of liposomes at 4 °C (days)	5	20
Formation of free crystals of Bu (days)	x	20
Diameter of liposomes (nm)		220 ± 14

Results are presented as a mean ± SD of 10 separately prepared LBu

The following parameters for preparation of LBu were selected: 5% glucose as a solvent, the starting concentration of Bu 0.5 mg/ml and five filtrations. The study progressed to an investigation of LBu for intravenous administration using animal models.

Pharmacokinetics and distribution of liposomal busulphan were studied in rat using ^{14}C labeled busulphan and compared with Bu/DMSO. Plasma concentrations of busulphan were fitted to the classical one- or two-compartment models. LBu was eliminated from plasma according to biexponential decay, while Bu/DMSO was eliminated according to a monoexponential model. Bu pharmacokinetics were linear within the range of dose studied (0.5 – 3.5 mg/kg) for both LBu and Bu/DMSO. Pharmacokinetic parameters obtained after the Bu dose of 3.5 mg/kg are presented in Table 9.

Table 9: Pharmacokinetic parameters in rat

Parameter	Cmax (µg/ml)	t½ (hr)	Vdss (l/kg)	Cl (ml/min)	Auc (µg.hr/ml)
Bu/DMSO					
Blood	2151 ± 729	1.75 ± 0.37	0.68 ± 0.06	0.072 ± 0.004	11.82 ± 2.22
BM		1.53 ±0.01		0.066 ± 0.01	9.85 ± 0.67
LBu					
Blood	1817 ± 557	2.52 ± 0.09	1.39 ± 0.22	0.099 ± 0.02	9.93 ± 0.15
BM		3.08 ± 1.06		0.055 ± 0.03	15.82 ± 2.25

Data are presented as a mean ± SD from three separate experiments

The bioavailability of LBu was 0.85 compared to Bu/DMSO. However, the exposure of bone marrow to LBu was 1.6 fold higher ($p<0.05$) compared to Bu/DMSO, when exposure was expressed as AUC in bone marrow. Higher exposure of bone marrow to LBu compared to Bu/DMSO was also achieved when exposure was expressed as a ratio between AUC_{BM} / AUC_{blood} (1.59 and 0.83, respectively).

The distribution volume of LBu was higher ($p<0.01$) and the elimination half-life of LBu in blood was longer ($p<0.05$) compared to Bu/DMSO. No difference in clearance was found when LBu was compared to Bu/DMSO.

Distribution of Bu/DMSO and liposomal busulphan to organs was assessed as the area under the radioactivity-time curve (ARC) from 0 to 18 hours after the treatment adjusted for 1 g of wet tissue (Fig 3).

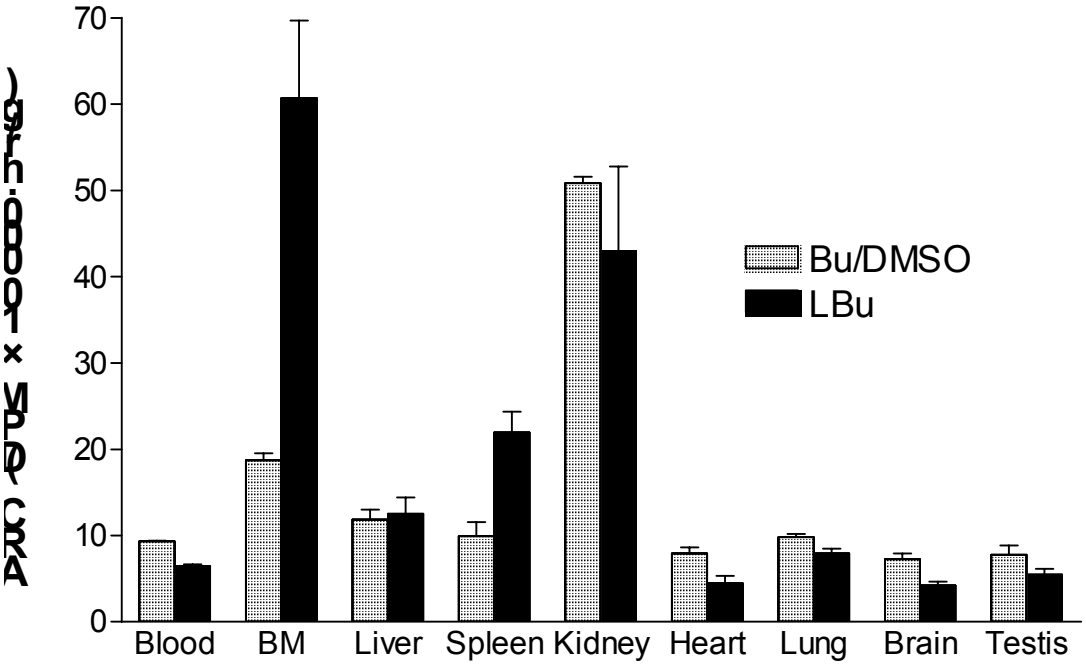

Fig 3: Distribution of the total radioactivity in rat organs. Data are presented as a mean ± SD of three separate experiments.

A significant accumulation ($p<0.01$) of radioactivity in bone marrow and spleen was observed compared to Bu/DMSO. A significant decrease ($p<0.05$) in the distribution of LBu and/or its metabolites to heart, lungs, brain, and blood compared to Bu/DMSO was found. The distribution of the radioactivity after the administration of LBu to brain was 58% of that of Bu/DMSO ($p<0.01$). No significant differences in the distribution of radioactivity into liver, kidney, and testis were observed.

Pharmacokinetics and myelosuppressive effect were investigated in mice. Pharmacokinetics was fitted to the one compartment open model with first order absorption. Pharmacokinetic parameters are listed in Table 10.

The bioavailability of LBu was 0.86 (range 0.84 to 0.88) and biovailability of oral preparations was 0.74 (range 0.67 to 0.86) for Bu/Ac and 0.40 (range 0.38 to 0.47) for Bu/susp compared to Bu/DMSO.

Table 10: Pharmacokinetic parameters in mice

Parameter	Cmax (µg/ml)	t½ (hr)	Vss (l/kg)	Cl (ml/min)	AUC (µg.hr/ml)
Bu/DMSO	26.6 ± 3.4	1.60 ± 0.30	0.012 ± 0.001	0.0054 ± 0.0009	55.94 ± 1.72
LBu	13.0 ± 2.7	1.30 ± 0.21	0.021 ± 0.006	0.0117 ± 0.004	48.01 ± 1.47
Bu/Ac	15.6 ± 3.4	2.36± 0.74	0.0226 ± 0.006		42.27 ± 5.20
Bu/susp	10.2 ± 0.9	1.35 ± 0.18	0.0262 ± 0.001		23.24 ± 2.63

Data are presented as a mean ± SD from three experiments; AUC is corrected for dose of 16 mg/kg

Myelosuppressive effect after the conditioning-like regimen with LBu, or with two oral formulations of Bu was studied over a period of 9 days after the last dose of Bu (Fig 4).

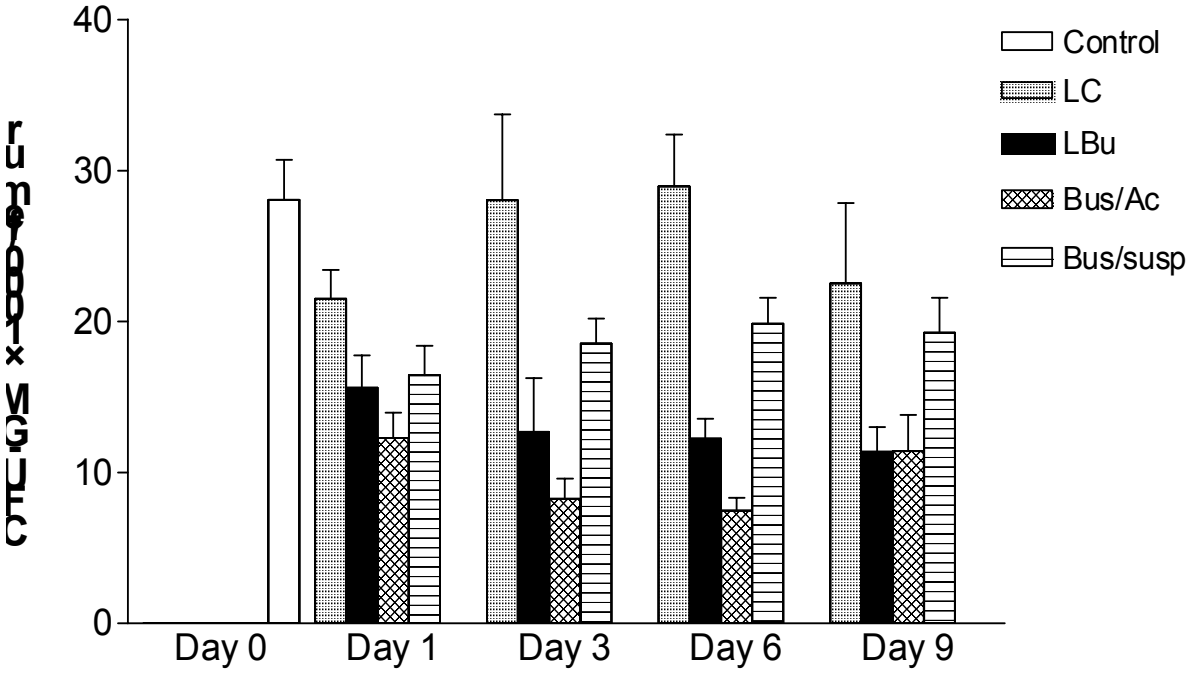

Fig 4: Kinetics of myelosuppressive effect of LBu and orally administered busulphan after conditioning regimen. Data are presented as a mean ± SD of three experiments.

LBu and Bu/Ac showed similar effect. The clonogenic capacity of bone marrow was decreased significantly ($p<0.05$) at day 1 to 40-50 % compared with untreated animals. This decrease was stable and continued trough day nine. Myelosuppression induced with Bu/susp was about 65 % of clonogenic capacity of untreated animals. A significant difference ($p<0.001$) was found when all treatment groups were compared from day 1 to day 9. LBu and Bu/Ac had a significantly higher effect on bone marrow compared to Bu/susp, while no difference was observed comparing LBu with Bu/Ac. Neither a decrease in colony-formation capacity of bone marrow, nor any adverse effects were observed after the administration of blank liposomes to the mice.

Pharmacokinetics of LBu in man (Study III)

Twelve patients undergoing SCT were given low dose of liposomal busulphan one day before they start their conditioning regimen. Another five patients received two high doses of liposomal busulphan replacing the first and the last dose of the conditioning regimen. The data fitted a two-compartment model. Pharmacokinetic parameters are presented in Table 11.

Table 11: Pharmacokinetic parameters in man

Patient group		t½ (hr)	Cl (ml/min/kg)	Vss (l/kg)	AUC_{corr} (ng.hr/ml)
Low dose Children	Mean	2.53	3.12	0.61	5491
	SD	0.72	0.59	0.13	912
Low dose Adults	Mean	2.73	2.86	0.58	5955
	SD	0.66	0.30	0.21	627
High dose Dose 1	Mean	2.90	2.81	0.58	6167
	SD	0.54	0.61	0.14	1385
High dose Dose 16	Mean	2.84	2.45	0.52	6933
	SD	0.79	0.24	0.17	656

A linear relation was found between the dose and area under the plasma-concentration-time curve. No correlation was found between patients' age and the apparent volume of distribution. A single episode of mild reaction (flushing of the face, ears, neck and upper chest) was observed during the first high dose of LBu in patient Nr 3. Symptoms did not occur when the second high-dose of LBu was administered and no other sign of toxicity related to liposomal formulation by itself was observed.

STUDIES ON THE MECHANISM OF BUSULPHAN INDUCED CYTOTOXICITY

Differentiation and apoptosis in a myeloid cell line (Study IV)

Maturation and apoptosis were induced by ATRA and etoposide in the myeloid P39 cell line. ATRA induced maturation and apoptosis over a period of days (Fig 5), while etoposide induced apoptosis over a period of hours without preceding maturation.

Apoptosis induced with both agents was mediated through caspase activation, but showed different pattern of intracellular events. Actin was cleaved in ATRA-induced apoptosis while Bcl-2 remained intact. The opposite pattern was found in etoposide-induced apoptosis, in which Bcl-2 was cleaved and actin remained intact. Both, a general caspase inhibitor zVAD-fmk and G-CSF partially blocked ATRA-induced apoptotic morphology in P39 cells and a mild additive effect

of both agents was observed in simultaneous cultures. However, neither zVAD-fmk nor G-CSF blocked cleavage of actin induced with ATRA. The agonistic or antagonistic Fas antibodies did not affect either ATRA- or etoposide-induced apoptosis. Suitable methods for *in vitro* investigations of differentiation and/or apoptosis were selected for the following study.

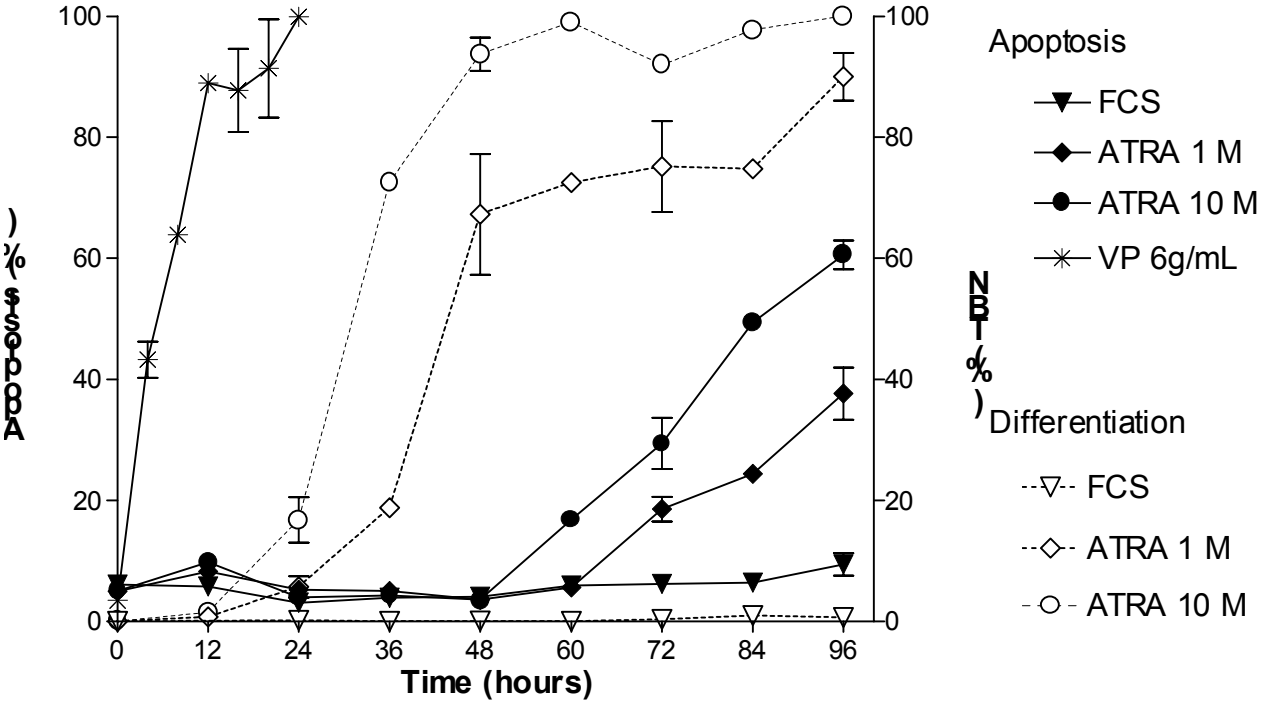

Fig 5: Differentiation and/or apoptosis induced with ATRA and etoposide in P39 cell line. Data are presented as a mean ± SD of three to seven separate experiments.

Pharmacodynamics of busulphan *in vitro* (Study V and VI)

The P39 myeloid cell line

The effect of busulphan on proliferation, clonogenic capacity and apoptosis was studied in the myeloid P39 cell line (Fig 6). Proliferation was AUC-dependent and decreased with increasing AUC. The effect was more expressed when the cells were re-cultured in busulphan-free medium for 72 hours. Colony-forming capacity of P39 cells was also AUC-dependent and the most pronounced effect was observed immediately after incubation with busulphan. Cells incubated in busulphan of AUC less than 150 µg.hr/ml and re-cultured in busulphan-free medium fully or partially recovered.

Apoptosis induced with busulphan was concentration- and time-dependent, but apoptotic morphology developed within days in busulphan-free cultures. A linear relation between AUC and fraction of apoptotic cells was observed at 48 and 72 hours after the incubation with busulphan. Cells were arrested in G2

phase of cell cycle before appearance of apoptotic morphology. In lower concentrations and shorter time of incubation, cells were able to recover their normal distribution of cell cycle. Apoptosis was mediated through caspase activation as detected by the active subunit p17 of caspase 3. Bcl-2 and PARP were cleaved while no actin cleavage was observed.

The general caspase inhibitor zVAD-fmk decreased apoptotic morphology by about 50 %, however, no corresponding improve in thymidine incorporation was observed. G-CSF could not rescue the cells from toxicity as detected either with proliferation assay or apoptotic morphology.

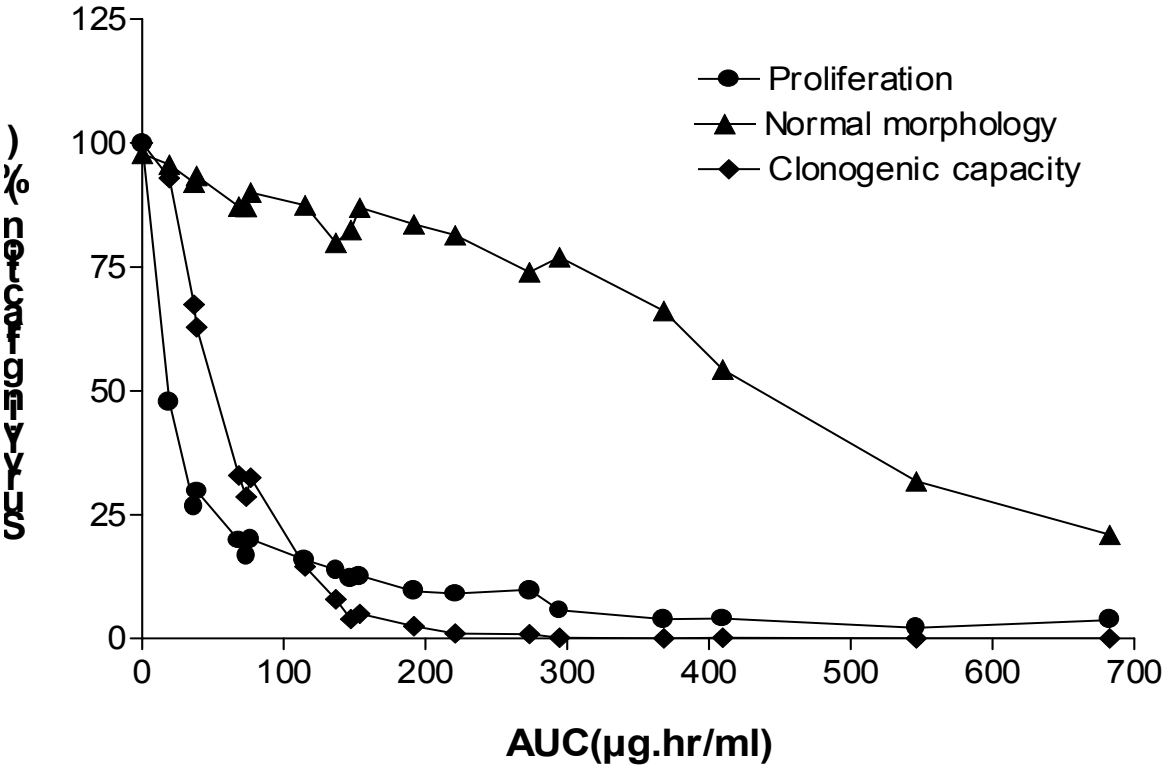

Fig 6: Surviving fraction of P39 cells treated with busulphan and incubated in busulphan-free cultures for 48 hours. Data are presented as a mean ± SD of three separate experiments.

Normal bone marrow

Busulphan inhibition of colony-formation of CD34+ cells from healthy volunteers was AUC-dependent and linear for both CFU-GM and erythroid colonies (Fig 7).

The sensitivity of CFU-GM was twice as high as the sensitivity of erythroid colonies, when results are expressed as AUC inhibiting colony formation by 50% (10.61 ± 2.89 and 26.29 ± 5.29 µg.hr/ml, respectively) or AUC inhibiting colony formation by 100% (68.66 ± 7.48 and 140.26 ± 35.65 µg.hr/ml, respectively).

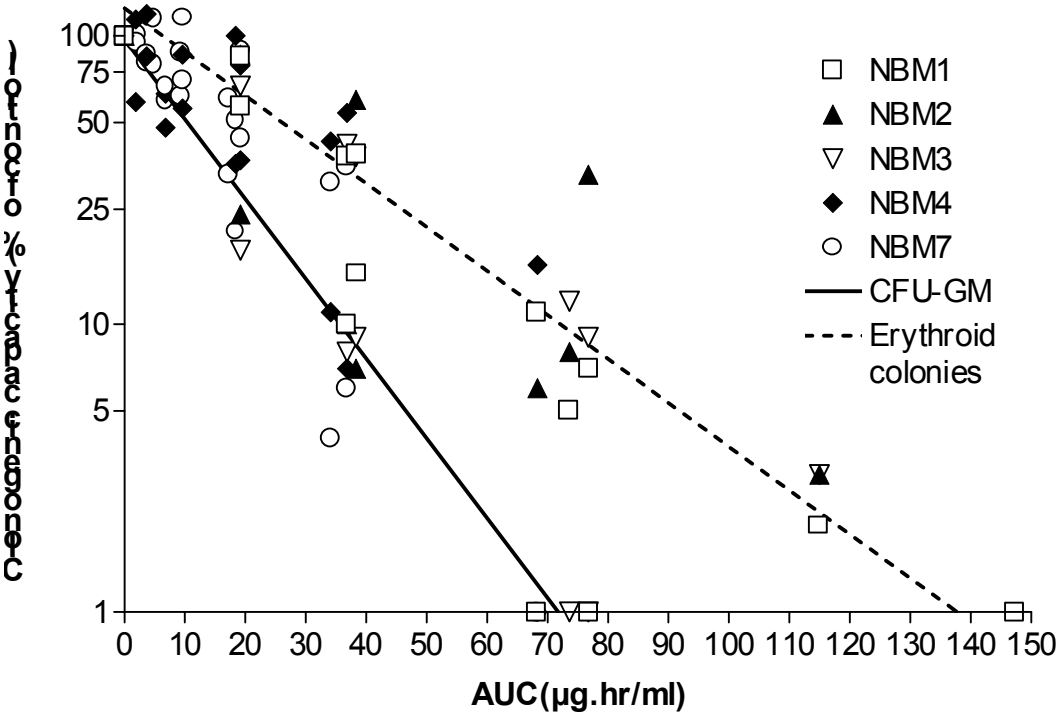

Fig 7: Clonogenic capacity of CD34+ progenitors from 5 healthy volunteers related to AUC. Compact line represents linear regression on all CFU-GM and dot line represent linear regression on erythroid colonies.

The role of GSH in busulphan-induced cytotoxicity in hematopoietic cells (Study VI)

The effect of modulation of the intracellular level of GSH on busulphan-induced cytotoxicity was studied in three parallel systems, normal human bone marrow and a human leukemic cell line *in vitro*, and in a mice model *in vivo*.

Preincubation of CD34+ hematopoietic progenitors with NAC did not affect busulphan-induced inhibition of colony formation of CFU-GM or erythroid colonies. Neither did preincubation of CD34+ cells with BSO change busulphan-induced inhibition of clonogenic growth (Fig 8a and 8b). NAC or BSO alone did not influence the colony formation of CFU-GM or erythroid colonies in CD34+ cells.

Similarly, NAC did not influence the effect of busulphan on clonogeneic capacity in the myeloid P39 cells line. BSO increased the toxicity of busulphan when cells were studied immediately after the incubation with drugs, while no increased toxicity was observed after 48 hours in drug-free cultures. Moreover, BSO alone decreased the clonogenic capacity of P39 cells by 50% immediately at the end of incubation, but the cells fully recovered after 24 hours in BSO-free media. Modulation of GSH did not influence the proliferation of busulphan treated cells, assessed with ^{3}H-thymidine incorporation. Pretreatment of P39 cells with NAC or BSO did not change levels of apoptosis induced by busulphan.

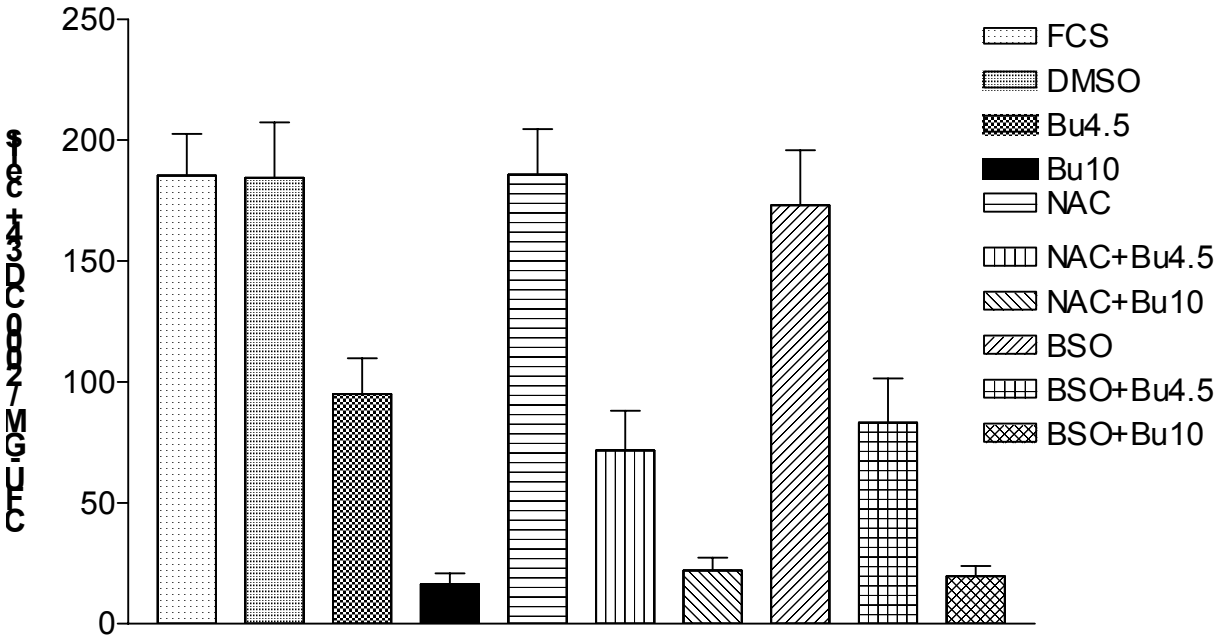

Fig 8a: The effect of NAC and BSO on busulphan-induced cytotoxicity in CD34+ hematopoietic progenitors. Concentration of busulphan is in µg/ml. Bars represent mean ± SD of 5 healthy volunteers.

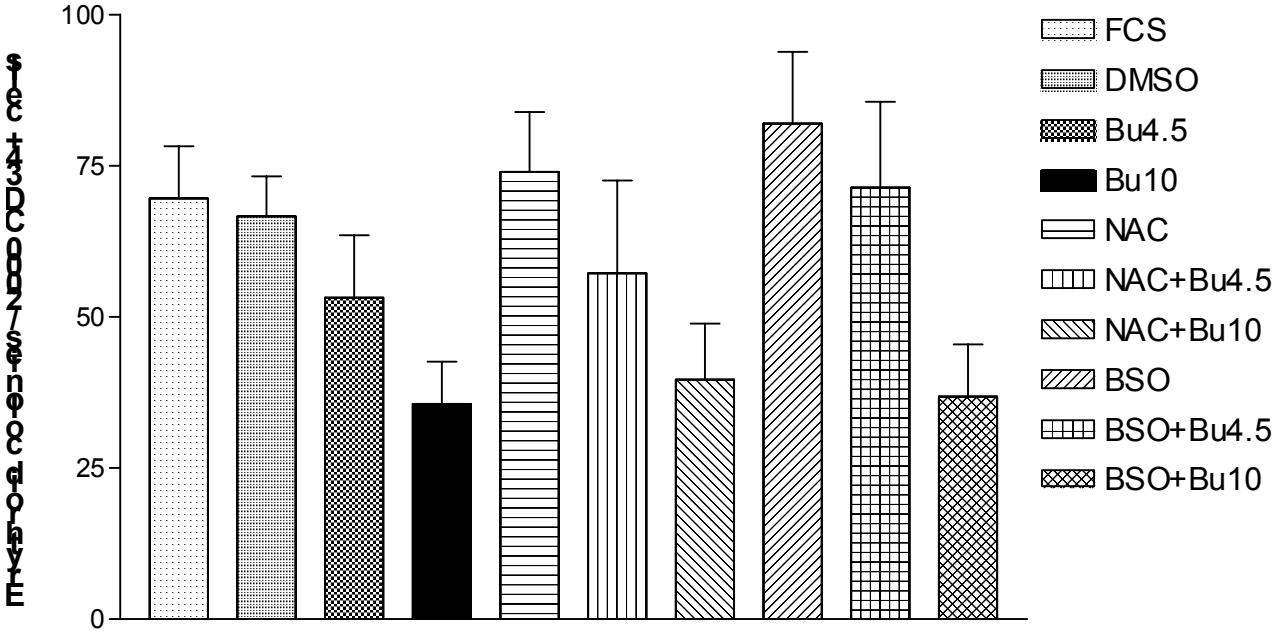

Fig 8b: The effect of NAC and BSO on busulfanbusulphan-induced cytotoxicity in CD34+ hematopoietic progenitors. Concentration of busulfanbusulphan is in µg/ml. Bars represent mean ± SD of 5 healthy volunteers.

Busulphan induced stable myelosuppression on days 3 through day 9 in the mice model *in vivo* assessed with CFU-GM colony-formation assay. Combination of NAC with busulphan did not significantly influence the myelosuppressive effect of busulphan. Treatment of mice with BSO and busulphan did not change the level of myelosuppression compared to animals treated with busulphan only. However, BSO alone caused significant myelosuppression on day 9 compared to the control group, while NAC alone did not influence the CFU-GM formation compared to control animals (Fig 9).

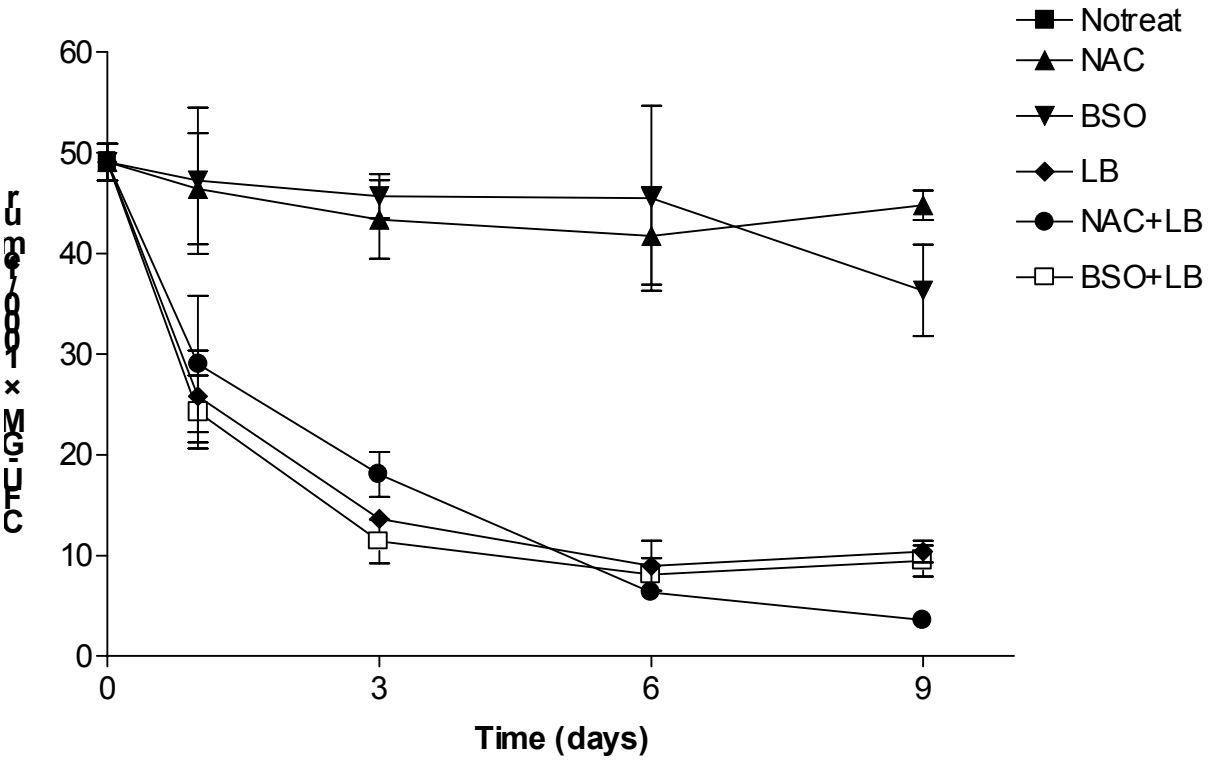

Fig 9: Effect of pretreatment with NAC or BSO on busulphan-induced myelosuppression in mice. Data are presented as a mean ± SD of three separate experiment sets.

DISCUSSION

The studies on the novel intravenous formulation of busulphan were stimulated by our previous investigation on the bioavailability of busulphan (Hassan *et al.* 1994). The considerable inter-patient variation, particularly in young children, constitutes a distinct clinical problem. A formulation of busulphan for intravenous administration might decrease the variation in systemic exposure, thus making treatment more safe and predictable. It would also overcome the first-pass effect, which would decrease the toxic load to the liver.

Busulphan is poorly soluble in water. An intravenous formulation may be based on either organic solvents or on lipid-transporter systems, such as liposomes or lipid microparticles. Organic solvents have their own toxicities and may increase the risk for adverse effects of the conditioning regimen (Yellowlees *et al.* 1980, Kennedy *et al.* 1986, Kinney *et al.* 1993). Lipid-transporter systems are prepared from lipids closely related to those naturally occurring in the body (Ostro *et al.* 1989). Moreover, a liposomal vehicle offers the possibility of selective targeting based on size, composition, and surface charge of the liposomes (Gregoriadis 1989). This theoretical background motivated the design of liposomal busulphan based on small, uncharged liposomes containing cholesterol.

In a rat model, the distribution of LBu was assessed with ^{14}C labeled busulphan. A higher distribution of LBu to bone marrow and spleen, and a lower distribution of LBu to brain, lungs, heart and blood compared to Bu/DMSO was observed. No differences in the distribution to liver, kidney and testis were found between LBu and Bu/DMSO. Since acute neurotoxicity has been related to the exposure to busulphan, the decreased distribution of LBu to brain is of great importance. Free busulphan easily crosses over the blood brain barrier and the concentrations in cerebrospinal liquor are comparable with those in plasma. Although seizures may be prevented with anticonvulsant therapy, a reduced exposure, especially in children, of the brain to any toxic agent is always preferable with regard to eventual late adverse effects. Higher exposure of bone marrow to LBu compared with Bu/DMSO was detected with two independent methods, assessment of free drug concentrations, and assessment of radioactivity. Exposure to free drug expressed as AUC was 1.6 fold higher for LBu than for Bu/DMSO. Accumulation of busulphan and its metabolites, expressed as ARC, was 3.2 fold higher for LBu than for Bu/DMSO. The latter observation shows that busulphan has been released from liposomal vesicles, taken up by bone marrow cells and metabolized/reacted. It is an important observation, since the assay for free drug cannot differ between encapsulated and already released drug. However, from a pharmacological point of view, the encapsulated drug is “outside the body” in the sense that it cannot act until it is released from the liposomes (Fielding 1991).

We then studied the effect of liposomal busulphan in mice. In a conditioning-like regimen, the myelosuppressive effect of LBu was similar to that obtained after administration of Bus/Ac, and significantly higher than after Bus/susp. This is most likely due to differences in bioavailability, which was 0.86 for LBu, 0.74 for Bu/Ac and 0.40 for Bu/susp. The difference in bioavailability between the two orally administered forms is probably due to enhancement of the absorption of busulphan by acetone. Acetone in small amount does not have toxic effect on the

mice or the bone marrow, as described previously (Trainor *et al.* 1980, Dietz *et al.* 1991). Administration of liposomes without busulphan did not show any effect on CFU-GM. The effect of a single high-dose busulphan on bone marrow cells was similar to that obtained after conditioning-like regimen. These results are in agreement with clinical experience that different schedules of busulphan treatment in the conditioning regimen (4 mg/kg divided in 4 doses daily for four days versus 4 mg/kg once daily for four days) give similar engraftment rates (Santos *et al.* 1983, Shaw *et al.* 1994, Hassan *et al.* 1996). The myelosuppressive effect of LBu was stable and reproducible over the studied period. However, based on the results from previous study of distribution of LBu in rat, a more pronounced myelosuppresion with LBu in mice than we actually observed could be expected. This may be due to differences in physiology and metabolism between mice and rats.

The two animal studies were followed by an investigation of the pharmacokinetics of liposomal busulphan in man. LBu was administered in low dose prior to treatment, or in two high doses replacing the first and the last dose in a conventional BuCY regimen. The data fitted to a two-compartment model. Two other intravenous formulations of busulphan dissolved in a mixture of organic solvents were also described with a two-compartment model (Hassan *et al.* 1994, Andersson *et al.* 2000). However, it differs from studies on orally administered busulphan, in which data are usually described using a one-compartment model (Ehrsson *et al.* 1983, Grochow *et al.* 1989, Hassan *et al.* 1989, Vassal *et al.* 1989).

In our studies, we observed a linear relation between the dose of LBu and the systemic exposure to busulphan. A linear relation between doses within the 0.02 – 4 mg/kg range, and AUCs has also been reported by others (Vassal *et al.* 1993, Shaw *et al.* 1994, Hassan *et al.* 1996, Schuler *et al.* 1998). Pharmacokinetic parameters after low doses of LBu were similar to those obtained after low doses of other intravenous formulation used for assessment of bioavailability (Hassan *et al.* 1994). Corrected AUCs did not differ between LBu and busulphan dissolved in organic solvents, regardless if correction was done for a dose of 1 mg/kg or a total dose of 2 mg. Thus, the bioavailability of LBu compared to busulphan dissolved in organic solvent is almost 100%. Clearance of LBu did not differ from data in previously published studies. No significant differences in clearance between children and adults and no correlation between the age and clearance were observed. These results differ from the bioavailability study, and from reports of other teams (Hassan *et al.* 1991, Regazzi *et al.* 1993, Hassan *et al.* 1994, Slattery *et al.* 1995, Tran *et al.* 2000). It may be due to the higher age of the children in the present study, which was not designed to investigate age-dependent variations in pharmacokinetics. Reported clearance values for busulphan are based on orally administered drug and most of these studies do not consider busulphan bioavailability. None of the recent investigations on intravenous formulations of busulphan have studied very young children. It will be of great interest if the intravenous formulations show decrease in clearance in young children, since higher clearance in young children is ascribed to the first pass effect in gut and/or in liver (Hassan *et al.* 1994). Similar concerns as for clearance are valid for volume of distribution, which was similar in children and adults. The values we found in children differed from those in other studies, which may be due to the above-mentioned difference in age, differences in underlying diseases and/or a difference in distribution of different formulations (Table 2). Elimination half-lives were similar in children and adults and did not differ from those in previously published studies (Table 2).

Pharmacokinetic parameters were assessed also after high doses of LBu. Since we did not want to change the conditioning schedule for patients participating in the study, the follow-up time was 6 hours. This is a relatively short time interval for a pharmacokinetic investigation. However, we have considered it to be long enough to estimate the parameters with appropriate accuracy, since the infusion time was about 1.5 hour and the half-life of Bu is 2.5 hours. Clearance, the apparent volume of distribution, and half-life did not differ from those obtained with a low dose of LBu. The clearance of LBu was similar to clearance reported for Busulfex®, but higher than the clearance reported for Bu/DMSO in man (Schuler *et al.* 1998, Andersson *et al.* 2000).

A clinically relevant question is whether the volumes and amounts of lipids in liposomal busulphan are feasible for use in the preparative regimen before SCT in man. The mean concentration of Bu in the liposomal preparation was about 0.2 mg/ml and the lipid content was 16 mg/ml. For an adult patient of 70 kg treated with 4 mg/kg/day it means a volume of 1400 ml, and 22.4 g of lipids (320 mg/kg/day), which is acceptable. The amount of lipids administered in liposomal formulations of other drugs range from 1.5 to 104 mg/kg/day and the upper limit for the amount of lipids administered during parenteral nutrition is 1500 mg/kg/day (Janoff 1999). The volume of lipid-based microsuspension of busulphan was 500 ml in one-hour infusion four times daily during a phase I/II clinical trial, in which no acute complications related to either a volume or a lipid formulation were observed (Olavarria *et al.* 2000). Alternatively, incorporation of busulphan into liposomes may be increased by the use of 0.9% NaCl as a solvent. However, the stability of the liposomes in 0.9% NaCl is shorter than the stability of the liposomes in 5% glucose, which is currently used in liposomal busulphan. Lyophilisation may resolve stability problems and prolong the expiration time.

In summary, busulphan is an important component of the conditioning regimen prior to stem cell transplantation and affects the overall outcome of the patients. The efficacy and adverse effects of busulphan are related to the systemic exposure to busulphan, which shows a high inter-patient variation after a fixed oral dose. Our novel intravenous formulation offers an alternative to overcome the first-pass effect and decrease the variation in systemic exposure. The liposomal formulation is in itself non-toxic and may, due to its physico-chemical properties, more selectively target the bone marrow than solutions of free busulphan. In the animal experiments, we showed a beneficial distribution of liposomal busulphan and a good and stable myelosuppressive effect. The pharmacokinetic study in man showed a linear relation between dose and exposure, along with pharmacokinetic parameters consistent with the data reported for other formulations of busulphan. We believe that this approach, busulphan encapsulated in liposomes, may increase the efficacy and decrease the toxicity of busulphan-based conditioning regimens for stem cell transplantation and have therefore recently launched a phase I/II clinical trial.

In the second part of this thesis we investigated the mechanisms underlying busulphan-induced cytotoxicity on hematopoietic cells, and the role of glutathione in this process. Cell lines are easily available models for *in vitro* studies, but as these cells are transformed, they may have specific properties that are not found in primary cells derived from the same tissue. The advantage of cell lines is

that they provide good possibilities for method development before experiments on primary cells are performed. *In vitro* studies in primary cells are important in the investigation of molecular mechanisms, while animal models are needed for a more complex investigations of interacting factors in the multicellular organism.

We selected the P39 cell line for our *in vitro* studies for two reasons. Firstly, it is a myeloid cell line, and secondly, apoptotic P39 cells show typical morphological changes easily detected in Giemsa staining (Anzai *et al.* 1994). A model of differentiation and/or apoptosis was developed, and two different pathways of apoptosis induced with ATRA and etoposide were found. Both were mediated through caspase activation, but differed from each other in terms of kinetics of apoptotic changes, presence or absence of maturation, the effect of granulocyte colony-stimulating factor rescue, and cleavage of Bcl-2 and actin.

In recent studies of several cell types, Bcl-2 was found to be cleaved by caspases after Fas ligation, interleukin-3 withdrawal, alpha-virus infection or etoposide-treatment (Cheng *et al.* 1997, Fujita *et al.* 1998, Grandgirard *et al.* 1998). In a previous study, we found that Bcl-2 cleavage in P39 cells occurred early in the course of apoptosis and selectively involved cleavage of Bcl-2 in the mitochondrial compartment (Fadeel *et al.* 1999). This cleavage was blocked by the general caspase inhibitor zVAD-fmk.

In ATRA-induced apoptosis, which was preceded by differentiation, Bcl-2 was not cleaved, but downregulated as shown also in other reports (Calabresse *et al.* 1995). Downregulation of Bcl-2 was recently reported in HL-60 cells induced to maturation with ATRA or DMSO (Watson *et al.* 1997).In our study, the cleavage of actin was observed in ATRA-induced apoptosis, but not in the etoposide-treated cells. Actin cleavage has been detected in several cell types *in vitro* and *in vivo* (Mashima *et al.* 1995, Chen *et al.* 1996, Kayalar *et al.* 1996), while other authors have failed to confirm this observation (Song *et al.* 1997). Nevertheless, our data indicate that actin cleavage may serve as a useful marker of differentiation-associated apoptosis.

G-CSF decreased ATRA-induced apoptosis by 50%, but it did not affect spontaneous or etoposide-induced apoptosis. G-CSF itself did not induce maturation in P39 cells. These findings support G-CSF as a potent survival factor in myeloid cells, but indicates that it mainly acts on the more mature precursors (Begley *et al.* 1988, Moore 1990). ATRA-induced apoptosis was blocked with zVAD-fmk, and the combination of zVAD-fmk and G-CSF additively blocked the appearance of apoptotic morphology. However, neither zVAD-fmk nor G-CSF blocked actin cleavage. This may imply that caspases are not responsible for actin cleavage in these cells and that other proteases, such as calpain, have to be considered.

P39 cells were weakly positive for the Fas (CD95) antigen, and ATRA or G-CSF did not influence this expression. Agonistic Fas antibody induced limited apoptosis, which corresponds to the usually low Fas sensitivity of cells of myeloid origin (Dirks *et al.* 1997). The Fas system did not seem to be involved in either pathway and neither agonistic nor antagonistic CD95 antibodies affected etoposide- or ATRA-induced apoptosis. Notwithstanding, it was proposed in recent reports that various anticancer drugs cause both upregulation of FasL and its interaction with Fas at the cell surface (Friesen *et al.* 1996, Fulda *et al.* 1997). In another study, HL-60

cells induced to differentiation with ATRA were more sensitive to agonistic anti-Fas antibody than undifferentiated cells, while Fas expression remained at the same level (Watson *et al.* 1997).

Busulphan-induced cell death shared several features with apoptosis induced by etoposide, but had different kinetics. Busulphan induced apoptosis with a typical morphology and DNA ladder formation in the P39 cell line, but in contrast to etoposide, apoptotic morphology developed slowly over a period of days. Blagosklonny recently defined slow cell death as an alternative to apoptosis (Blagosklonny 2000). The main difference between slow death and apoptosis is that the latter is associated with activation of the caspase cascade. Slow death shows condensation and cleavage of DNA, since endonucleases may be activated via another pathway. In P39 cells we detected the active subunit of caspase-3, thus assuming that cells died through apoptosis, in spite of the slow kinetics of apoptotic changes. Proteolytic events in busulphan-induced apoptosis showed a similar pattern to etoposide-induced apoptosis: Bcl-2 and PARP were cleaved, while actin remained intact. A general caspase inhibitor, zVAD-fmk, blocked the development of apoptotic morphology, inhibited Bcl-2 cleavage, but failed to block the formation of the p17 subunit of caspase-3 or restore proliferation. G-CSF did not rescue P39 cells from busulphan-induced apoptosis and no signs of preceding maturation were found either in morphology or NBT test. This opposes previous findings in HL-60 cells, where the exposure to busulphan was accompanied by NBT positivity (Michaeli *et al.* 1993).

As frequently observed with cytotoxic drugs, busulphan-treated cells were arrested in the G2 phase of cell cycle. However, some cells escaped busulphan-induced toxicity at low AUCs, and cell cycle was restored in busulphan-free cultures. Cell cycle arrest in G2 phase was also observed in several human cancer cell lines cultured with busulphan for 72 hours (Pauwels *et al.* 1995). In the Chinese hamster cells incubated with busulphan, cell cycle was transiently arrested in G2 phase but the cells subsequently returned to normal cell cycle distribution (Millar *et al.* 1986). A comparison of kinetics of the changes showed that G2 arrest preceded development of apoptotic morphology. It is possible that busulphan does not induce apoptosis by a direct trigger, and apoptosis may be a secondary event following the block of important metabolic functions. This phenomenon has been observed also in other forms of chemotherapy-induced apoptosis (Vaux *et al.* 1999).

To enable a comparison of *in vitro* pharmacodynamics of busulphan with *in vivo* studies, the exposure to busulphan was expressed as AUC. A linear relation between the AUC and the effect of busulphan was observed in most of the assays studied. Proliferation showed an inverse linear relation to AUC within the studied range, and the effect was more pronounced with time in busulphan-free medium. Busulphan has been shown to decrease proliferation in several cell lines, but as these systems differ from each other they are difficult to compare (Table 5). Even results obtained in the same cell line may be contradictory. The concentration of busulphan inhibiting growth by 50% was about 100 µg/ml in HL-60 cells treated for 1 hour (Epstein *et al.* 1986). On the contrary, no effect of busulphan in concentrations ranging from 8.6 to 85.5 µg/ml was found in a radiometric assay in the same cell line exposed continuously for 6 days (Marshall *et al.* 1993).

The clonogenic capacity of the P39 cells decreased immediately after the exposure to busulphan and the effect was AUC dependent. Fifty percent inhibition,

and a complete abolition of colony growth were obtained with AUCs of 35.5 µg.hr/ml and more than 150 µg.hr/ml, respectively. These results are in agreement with other reports showing suppression of clonogeneic formation in K562 and HL-60 cell lines after busulphan treatment *in vitro* (Pacheco *et al.* 1990). P39 cells incubated at low AUC partially recovered in busulphan-free cultures as some cells probably escaped busulphan-induced toxicity and continued to divide. This recovery was also qualitative, i.e. the size of colonies at the day of assessment was comparable with the control cultures.

As in the P39 cell line, an inverse linear relation between the AUC and clonogenic growth was observed in CD34+ cells isolated from bone marrow of healthy volunteers. The AUCs that completely inhibited colony formation were 69 ± 7.5 µg.hr/ml and 140 ± 36 µg.hr/ml for CFU- GM and erythroid colonies, respectively. This is in an agreement with cumulative AUC ($AUC_{single\ dose}$ × number of dose) of busulphan during conditioning regimen before SCT, calculated from the data in Table 2 (mean 82 µg.hr/ml, median 75 µg.hr/ml, range 34 to 143 µg.hr/ml). Similar attempts to define a relation between the exposure and the effect of busulphan were reported by Spiro et al. They studied *in vitro* inhibition of colony formation of progenitor cells from normal and CML peripheral blood using CFU-C assay, and calculated an exposure (concentration × time). The exposure that inhibited 90 % of CFU-C formation in normal and CML progenitors was higher compared to that what we found in the present study. However, the authors did not consider degradation of busulphan in their calculations of exposure. The decrease in survival of CFU-GM is in good agreement with other reports (Kubota *et al.* 1983, Hincks *et al.* 1990). However, all previously reported studies were done with light density cells, and not with CD34+ cells, which we believe is a more accurate system.

In our study, CFU-GM were more sensitive to busulphan than erythroid colonies. This contradicts the report of Kubota et al (Kubota *et al.* 1983). They observed a higher sensitivity to busulphan in BFU-E compared to CFU-GM grown from light density cells from peripheral blood, and bone marrow from both healthy volunteers and CML patients. This difference might be explained by the differences in growth factors used in semisolid medium. In our experiments, a combination of SCF, IL-3, GM-CSF and erythropoietin was used, while in their study only erythropoietin was added to agar medium. Differences between cell lineages or cells of the same lineage but at different levels of differentiation have been shown also in *in vivo* studies. In mice, immature erythroid progenitors were more sensitive than more mature cells (Udupa *et al.* 1979, Reynolds *et al.* 1986). Another study in rats found a higher sensitivity to busulphan in the granulocytic compared to the erythroid repopulating ability (Standen *et al.* 1980). Our study was not designed to investigate the pharmacodynamic effect of busulphan on the very primitive hematopoietic progenitors but these are, as reported by other investigators, more sensitive to busulphan than committed progenitors.

Although it is difficult to make clinical interpretations from *in vitro* data, pharmacodynamic studies *in vivo* are cumbersome and suffer from certain problems of interpretation. While concentrations of a drug are usually studied in the plasma compartment, the distribution of a drug to tissues and cells may be different and reflect the chemical property (lipophility), the blood supply to target organs, and the body composition of the patient. Thus, the exposure of target cells to a drug may significantly differ from an exposure estimated from the concentrations in plasma.

Ethical and practical reasons limit the number of samples that can be used for AUC estimation *in vivo*, which adds to the variation in exposure estimates. On the other hand, pharmacodynamic studies *in vitro* are limited to drugs, which do not need to be activated in the body. In addition, the effects of potential yet unknown metabolites are disregarded *in vitro*, as well as the network of agonistic and antagonistic factors. Being aware of the above-mentioned problems, we believe that *in vitro* studies may give considerable insight into the pharmacodynamics of a drug as well as increase the knowledge of cellular events underlying drug effects.

Altogether, we found that the pharmacodynamics of busulphan in vitro is linear to exposure and that the total exposure to busulphan probably is the main factor both for its myeloablative effect and unwanted toxicity. It is in agreement with clinical experience, that different dosing schedules give the same effects and that conditioning-related complications are AUC related.

Two of the most serious conditioning-related complications are veno-occlusive disease of the liver and interstitial pneumonia. A prophylactic agent that does not interfere with myelo- and immunosuppressive effects of the conditioning regimen may improve the outcome of the patients. N-acetylcysteine is a candidate drug for VOD prophylaxis (Ringden *et al.* 2000). In order to study the effect of modulation of cellular content of GSH on busulphan cytotoxicity, we designed a study comprising three parallel systems: a normal human bone marrow and a human leukemic cell line *in vitro*, and a mice model *in vivo*. Increased cellular levels of GSH did not protect CD 34+ cells from busulphan-induced toxicity in vitro. This differs from a study, in which N-acetylcysteine was shown to protect CFU-GM from normal bone marrow to irradiation (Selig *et al.* 1993). Neither could we demonstrate that NAC decreased busulphan-induced myelotoxicity in mice. Lack of protection of murine bone marrow cells *in vivo* by NAC was reported also for other drugs, such as cyclophosphamide, doxorubicin or cis-dichlorodiammineplatinum(II) (Massa *et al.* 1985, Lerza *et al.* 1986). The observed additive effect of NAC to busulphan-induced cytotoxicity at day 9 after the treatment is not significant and needs further investigation.

To corroborate that GSH is not involved in busulphan-induced myelotoxicity, we investigated the effect of GSH depletion by BSO on busulphan-induced cytototoxicity. GSH was depleted with 50% of control in CD34+ cells. This level of GSH should not induce endogenous oxidative stress to cells, and only an additive effect to busulphan toxicity might be expected. However, no effect of BSO on busulphan-induced toxicity was observed in the CD34+ cells. Neither did treatment of mice with BSO before busulphan influence the myelosuppressive effect. GSH depletion was shown to increase busulphan toxicity in hepatocytes in vitro (DeLeve *et al.* 2000). However, it did not have an additive effect to busulphan-induced growth delay and tumor regression in human glioblastoma multiforme xenograft (Hare *et al.* 1997).

Similar to our findings in CD34+ cells, and in mice, we could not demonstrate that an increased cellular content of GSH affected busulphan-induced cytotoxicity in the P39 cell line. This indicates that an increase in intracellular GSH does not interfere with the anti-leukemic effect of busulphan. However, this particular cell line shows a similar sensitivity to busulphan as primary hematopoietic progenitor cells, and studies of more resistant leukemic cells are warranted.

In conclusion, modulation of the cellular content of GSH did not affect busulphan-induced cytotoxicity in hematopoietic stem cells, in a leukemic cell line or in murine bone marrow. Thus, N-acetylcysteine might serve as a prophylactic agent against hepatotoxicity induced by busulphan.

CONCLUSIONS

In this thesis we have developed and evaluated a new formulation of busulphan for intravenous administration, and studied mechanisms behind busulphan-induced cytotoxicity.

I + II The novel liposomal formulation of busulphan is suitable for intravenous administration. In rat, distribution of liposomal busulphan was higher to bone marrow and spleen, and lower to brain, lung and heart compared to solution of free drug. The distribution to liver was similar in both formulations. The myelosuppressive effect of LBu was stable and reproducible in mice.

III Pharmacokinetic parameters of liposomal busulphan in man were in agreement with those previously reported for orally administered drug. It is feasible to use liposomal busulphan for high dose therapy in conditioning regimen prior to stem cell transplantation, and a phase I/II clinical trial is thus motivated.

IV ATRA- and etoposide-induced apoptosis was mediated via caspase activation, but involved different pathways. These diverged in kinetics, preceding maturation, cleavage of Bcl-2 and actin, and rescue from apoptosis by G-CSF.

V Busulphan showed linear in vitro pharmacodynamics in the myeloid P39 cell line. Busulphan- and etoposide-induced apoptosis shared common features, but differed in kinetics.

VI The in vitro pharmacodynamics of busulphan was linear also in human hematopoietic progenitor cells. An increase in the cellular content of glutathione did not affect busulphan-induced cytotoxicity in human CD34+ cells in vitro, or in murine bone marrow *in vivo*.

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