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TOXICOLOGICAL AND PHARMACOLOGICAL STUDIES OF BUSULPHAN, CYCLOPHOSPHAMIDE AND TREOSULFAN IN THE CONDITIONING REGIMEN PRIOR TO ALLOGENEIC STEM CELL TRANSPLANTATION

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Stockholm 2011

Published by Karolinska Institutet. Printed by Reproprint AB

ISBN 978-91-7457-394-7

ABSTRACT

Allogeneic stem cell transplantation (SCT) is a curative treatment for malignant and non-malignant diseases. However, transplantation related morbidity and mortality are major drawbacks affecting the survival and life quality of the patients. The major complications of SCT are infections, hemorrhagic cystitis, liver toxicity, interstitial pneumonia and GVHD. Busulphan (Bu), treosulfan (Tr) and cyclophosphamide (Cy) are alkylating agents. They are currently used in high doses as preparative regimen before SCT. Pharmacokinetics and pharmacodynamics of these drugs have been intensively studied with the aim of defining a therapeutic window to achieve a satisfactory myeloablation and immunosuppression with less treatment related toxicity.

Study I: We administered N-acetyl-L-cysteine (NAC) during conditioning to patients at risk of Sinusoidal obstructive Syndrome (SOS) due to pretransplant liver disorders or elevated liver enzymes. No side effects related to the NAC administration were observed and Bu-kinetics was not affected. All patients became pancytopenic and engrafted with100% donor cells. None of the patients developed SOS or liver failure. Increased liver enzymes during conditioning decreased or normalized in all patients. We suggested that NAC therapy is safe and does not impair the myeloablative effect of Bu during conditioning prior to SCT and hence NAC may be an effective prophylactic treatment against SOS and hepatic toxicity during conditioning.

Study II In a preclinical study, myeloablative as well as immunosuppressive properties of Tr were compared with those of Bu and Cy in a mouse model. The animals were treated with Tr, Cy, or Bu at sublethal doses that maintained survival without bone marrow support. The myeloablative effect was evaluated using colony-forming unit granulocyte macrophages (CFU-GM), while the immunological effect was performed using spleen cells. We found that Tr and Bu induced a high and persistent myeloablation compared to Cy. Moreover, Tr was more effective in depletion splenic B and T cells compared to Bu and Cy. T-cells isolated from the spleens of Tr- or Bu-treated mice were not responsive to allogeneic cells compared with those observed in Cy treated mice. Our findings suggested that Tr possesses both myeloablative and immunosuppressive properties and may be used as a single agent for conditioning prior to SCT.

Study III. Therapeutic drug monitoring (TDM) of Bu-iv was performed in 34 pediatric SCT patients. Bu-iv was administered twice daily according to recommended weight-based doses. Bu levels were measured and pharmacokinetic analysis was performed. The targeted Bu exposure was aimed to range between areas under the curve (AUC) of 9000–12000 ng/mLxh. In 23/34 patients (68%) Bu dose had to be adjusted at least once. In 16/23 patients the dose had to be increased in a range of 7-33%, while in 7/23 patients (30%) the dose had to be decreased by 7-20%. The need of dose adjustment was not related to weight, age or underlying disease. SOS was observed in 21% of the patients in spite of total AUC's within the target AUC. We concluded that TDM of iv Bu is essential to increase the efficacy and safety of Bu-based conditioning protocols in pediatric HSCT recipients.

Study IV. Limited sampling models for use in TDM of Bu in patients treated for hematologic malignancies. 23 patients were sampled according to standard protocol (8 samples). AUC calculated from three limited sampling models were compared with WinNonLin compartment modeling. Combining a curve fitting model and a compartment model, using the average AUC estimate, gave a concordance correlation coefficient of 0.85 with the described standard sampling protocol. Using Bland-Altman plots it was evident that most patients would have been treated the same regarding dose adjustment using the combined method as well as standard rich sampling. The results support the use of limited sampling in clinical therapeutic drug monitoring, provided adequate algorithms are used for evaluation. Both models included in the combined method utilized four concentrations points. The model is reliable, solid and user friendly providing the clinician with a graph and a numeric AUC estimate.

These four studies taken together may provide a step forward in treatment optimization and dose individualization to the benefit of SCT patients.

ISBN 978-91-7457-394-7

LIST OF PUBLICATIONS

I. F Sjöö, J Aschan, L Barkholt, Z Hassan, O Ringden and M. Hassan, N-acetyl-l-cysteine does not affect the pharmacokinetics or myelosuppressive effect of busulfan during conditioning prior to allogeneic stem cell transplantation. Bone Marrow Transplantation (2003) 32, 349–354

II. Fredrik Sjöö, Zuzana Hassan, Manuchehr Abedi-Valugerdi, Laimonas Griskevicius, Christina Nilsson, Mats Remberger, Johan Aschan, Hernan Concha, Una Gaughan, and Moustapha Hassan. Myeloablative and immunosuppressive properties of treosulfan in mice. Experimental Hematology 34 (2006) 115–121

III. R. Malär, F. Sjöö, K. Rentsch, M. Schmugge, M. Hassan, T. Güngör. Therapeutic drug monitoring is essential for intravenous busulfan therapy in pediatric hematopoietic stem cell recipients. (Accepted in Ped. Transp)

IV. Fredrik Sjöö and Moustapha Hassan. Combining methods for evaluating AUC from limited sampling protocols for use in TDM of Busulfan. (Manuscript)

To my family

LIST OF ABBREVIATIONS

AML	Acute myeloid leukemia
ANOVA	Analysis of variance
AUC	Area under the curve
BMT	Bone marrow transplantation
BSA	Body surface area
Bu	Busulphan
CCC	Concordance correlation coefficient
CFU-GM	Cluster forming unit granulocyte macrophage
CML	Chronic myeloic leukemia
CRAN	Comprehensive R archive network
Cy	Cyclophosphamide
DEB	Di-epoxy-butane
DNA	Deoxyribonucleic acid
GSH	Glutathione
GVHD	Graft versus host disease
HPLC-MS	High performance liquid chromatography – mass spectrometry
HLA	Human leukocyte antigen
HSCT	Hematopoietic stem cell transplantation
ICC	Intraclass correlation coefficient
IFN-γ	Interferon-gamma
IL-2	Interleucin-2
IP	Interstitial Pneumonia
LSM	Limited sampling model
MLR	Mixed lymphocyte reaction
MUD	Matched unrelated donor
NAC	N-acetyl-L-cysteine
PCV	Polycythemia vera
RICT	Reduced intensity conditioning transplantation
(H)SCT	(Hematopoietic) stem cell transplantation
SOS	Sinusoidal obstructive syndrome
TBI	Total body irradiation
TDM	Therapeutic drug monitoring
Th1	Type 1 T-helper cell
TNF-α	Tumor necrosis factor alpha
VOD	Veno occlusive disease

CONTENTS

1. INTRODUCTION

Drugs of varying kinds have been used since ancient times to fight disease. From old herbal remedies to modern bioengineered molecules, the means have changed but the goal remains the same: expedient cure and no adverse effects. Even if we may have come a long way since Hippocrates and Galen, a panacea for all human illness still seems far away.

The discoveries of modern biology and medicine have given us a dramatically widened perspective, but it has also unveiled the complexity of disease and in some cases produced more questions than answers. As doctors we can no longer hope for restitution of homeostasis in bodily fluids to be a cure for cancer or infection; instead, we have to combat microbes and malignant cells capable of adapting to and counteracting our moves in apparently deliberate ways.

Cancer is a disease as old as mankind; the Edwin Smith papyrus and the Ramayana describe malignant diseases and their treatments[1-3]. Most frequently therapy consisted of topical preparations, although removal of neoplasms had been practiced in old Egypt[3]. Mastectomy was described during the Roman period by Celsus and Leonides[4]. In the first century A.D., Dioscorides compiled a list of medicinal herbs and botanicals[5], including topical applications for treatment of tumors and carcinomas. By the beginning of the eleventh century, an Arabic physician, Avicenna "Ibn Sina", used the arsenic therapy systemically to treat leukemia; however, due to its high toxicity, it received little attention[6]. Preparations containing Arsenic known as Unguentum Aegypticum were used topically until late sixteenth century[6]. Potassium arsenite was reintroduced by Lissauer in 1865 as the first instance of effective chemotherapy to treat chronic myelogenous leukemia[7]. The use of Arsenic compounds (Fowler's solution) to treat leukemia continued until the 1930s[8]. Surprisingly, the use of arsenical therapy (arsenic trioxide) has become a part of modern therapy during the last decade and is now established as an effective treatment for acute promyelocytic leukemia (AML-M3) [9-10].

The cure for cancer has been sought for since the dawn of modern medicine, but for most patients with disseminated cancer it still remains elusive. There are, however, some malignancies for which the prognosis of disseminated disease has indeed improved considerably; treatment options for leukemia 75 years ago were generally limited to radiation therapy and arsenic with a prognosis of less than 10-15% long term survival[11]. Today we have an arsenal of treatments available that can cure or long-

term stabilize leukemia, ranging from tyrosine kinase inhibitors to blood stem cell transplantation. The prospect for these patients has changed dramatically. It is somewhat notable that in most cases this progress has come from new means of dealing with toxicity and adverse effects rather than from utilizing principally new drug targets or mechanisms. The now well established graft-versus-leukemia effect can be considered a novel treatment principle, but radiation and alkylating agents that constitute the basis for most conditioning regimens in allogeneic stem cell transplantation were discovered as early as a century ago.

At least for now, optimizing therapy and the use of known drugs in new ways have been the basis of most of the progress made in improving the prognosis for patients suffering from malignant disease. Even if the advent of targeted drug development and recombinant gene technology may change that in the future, it is my belief that improving the use of known therapeutic principles and drugs will remain an important task and that pharmacokinetic and pharmacodynamic studies will continue to play a key role in such efforts.

The work for my thesis focuses on drug toxicity and optimization of treatment, specifically the improvement of myeloablative treatment as conditioning before allogeneic stem cell transplantation.

1.1 THE DEVELOPMENT OF CANCER TREATMENT

Alkylating agents were the first non-hormonal agents to show significant antitumor activity in humans [12-13]. The clinical trials of nitrogen mustards in patients with hematologic malignancies evolved from clinical observations of the victims of sulfur mustard gas used in World War I. This compound was found to produce more or less extreme leucopenia, which followed an initial leukocytosis. It is interesting to note that survival of mustard gas exposition was found to correlate inversely to post exposure leukocyte counts, just as is the case with accidental radiation exposure [14-15]. The time sequence of marrow depression revealed a maximal effect at two weeks after exposure when mortality was highest. Deaths were attributed to pneumonia associated with leucopenia as reported by Krumbhaar and Krumbhaar in 1919. The related, but less reactive, nitrogen mustards were found to be less toxic and to cause regression of lymphoid tumors in mice. The first clinical studies produced dramatic effects in lymphoma patients, and the antitumor effects in lymphoid malignancies were confirmed by an organized multi-institution study [12-13]. Investigation of the mechanism behind the observed biological effects revealed chromosome breakage due to cross-linking alkylation in cells treated with nitrogen mustards[16]. Alkylating agents still occupy a central position in cancer chemotherapy, both in conventional regimens and in high-dose protocols with allogeneic or autologous stem cell support. The first compound of the nitrogen mustard group examined for clinical use was mechlorethamine. After its introduction, several analogues in which the methyl group was replaced by other chemical groups were synthesized and tested for antitumor activity. Among them the well-known substances melphalan, chlorambucil, cyclophosphamide and ifosfamide were found to have a higher therapeutic index and a broader range of clinical activity, and they have now replaced mechlorethamine in clinical use.

HG&EB Krumbhaar, 1918

In the 1950s Timmis and coworkers introduced a new class of compounds. They found that compounds with one to eight methylene units placed between two sulfonate groups have alkylating and antitumor activity and that maximal activity is shown by the compound with four methylene units, busulphan [17-18].

Generally today, the antineoplastic agents are used for the purpose of killing cancer cells. Therefore the terms *cancer chemotherapeutic drugs* and *cytotoxic compounds* are interchangeable. Cancer drugs used today can generally be classified into classes such as *Alkylating agents, Antimetabolites, Antitumor antibiotics, Topoisomerase inhibitors, Alkaloids* and *Podophylotoxins.*

Among these drugs, the alkylating agents are the cornerstones of cancer treatment. Several other treatments like gene therapy, hormone treatment and cell therapy have been introduced in clinical use. Stem cell transplantation has introduced a curative treatment both for malignant and non- malignant diseases, as well as for different metabolic and genetic disorders.

1.2 HEMATOPOIETIC STEM CELL TRANSPLANTATION (HSCT)

HSCT is a procedure where hematopoietic stem cells are given to a recipient with the intention of replacing the recipient's own hematopoietic system in whole or in part. The stem cells can be derived from bone marrow, peripheral blood or cord blood. If the donor is the same person as the recipient it is called an autologous HSCT. If the donor is genetically identical to the recipient, as is the case for identical twins, we have a syngeneic HSCT. The syngeneic HSCT biology is immunologically equal to that of an autologous HSCT. HSCT with any other type of human donor, related or unrelated, is referred to as an allogeneic HSCT. An autologous transplantation is done in order to save the patient from the toxic effects of a high-dose treatment that would otherwise cause permanent or long-lasting aplasia of hematopoietic stem cells, a condition which is often lethal. The positive aspect of an autologous HSCT is that it does not cause graft versus host disease, and there is no risk for rejection. On the other hand, it does not offer any chance for immunologic graft versus tumor effect. Autologous HSCT is an important treatment modality, but it is not part of my research project. When I use the term HSCT without a qualifier in the rest of this text, I am referring to allogeneic HSCT.

Encouraged by the progress made in the first decades of the 20th century in treating pernicious anemia and iron deficiency anemia, medical researchers speculated that all kinds of blood disease could be cured by adding a "deficiency substance". Since this substance would have to be present in normally functioning bone marrow, it was presumed that providing healthy bone marrow extracts to the patient could cure aplastic anemia, granulocythopenia et cetera. Interestingly, oral administration provided results as beneficial as did sternal intraosseus administration[19-20]. The patients in these reports obviously had granulocythopenia caused by infection or other transient factors, and we now know that there is no "deficiency substance" that can cure aplastic anemia or leukemia patients. These unmatched bone marrow transplantation recipients would probably have died from graft versus host disease in the unlikely event of a successful bone marrow engraftment. Luckily for them, only patients with severe T-cell defect would run any risk for that from the procedures described, and it would take many years before graft versus host disease was reported in man.

During experiments with immunologically immature or deficient animals in the 1950s, it was found that an infusion of allogeneic leukocytes could react to the host and produce a lethal syndrome of wasting, hepatitis, dermatitis, aplastic anemia and diarrhea[21]. The probability of this reaction is correlated to the amount of allogeneic leukocytes given and the degree of immunodeficiency in the recipient. Blood transfusions had become an increasingly feasible clinical procedure during the first half of the 20th century, and with the dawning awareness that allogeneic leukocytes can cause this syndrome it was just a matter of time before an accidental case would be found in man. In 1965 the first proved cases were reported in two immunodeficient infants, who had received repeated blood transfusions due to smallpox vaccination complications. In both cases the syndrome was lethal[22]. By then, experiments in which patients were given intravenous bone marrow infusions had already been reported. In 1957 no adverse effects were seen from this procedure in a series of six patients. Even though recipients had received immunosuppressive treatments like radiation and busulphan, transiently circulating donor blood cells were found only in two patients, indicating the failure of permanent marrow engraftment. The author and later Nobel Prize laureate noted that no adverse effects had been seen. He further acknowledged that a graft versus host reaction may had been seen in animal experiments, but that the risk for occurrence in patients given sufficient immunosuppressive treatment for permanent engraftment was still "a matter of speculation"[23]. In 1963 the first successful bone marrow transplantation was reported in a patient with acute lymphoblastic leukemia who received a bone marrow mix from relatives. After what most reasonably was a moderate graft versus host disease, the cells of the donor with the best match in HLA-A took over and the rash and diarrhea resolved. Unfortunately, the patient later died from infectious complications[24]. From this, and from animal experiments, it was understood that bone marrow from a donor with a major histocompatibility locus identical to that of the recipient was more likely to engraft and less likely to produce a graft versus host reaction. Using HLA-matching, an infant with Wiskott Aldrich Syndrome was successfully transplanted with bone marrow from an HLA-A identical sibling after receiving immunosuppressive treatment with cyclophosphamide[25]. Successful treatment of aplastic anemia and leukemia with hematopoietic stem cell transplantation followed, but so did casualties from cytomegalovirus infections and other lethal adverse effects of the procedure[26-28]. Within a decade of the first successful hematopoietic stem cell transplantations, a team at Karolinska-Huddinge performed Sweden's first HSCT in 1975[29]. Today, HSCT is

a well-established curative procedure for several malignant and nonmalignant disorders in children as well as in adults[30]. The main problem with HSCT remains toxicity, limiting the use of this treatment to young patients free from serious co-morbidities. Even in this patient group, the procedure is dangerous and stressful. Cytomegalovirus killed the first successful transplantation patient reported in 1968 and was a serious threat during the early days of HSCT. Today, screened/filtered blood products, antiviral drugs and surveillance protocols have contributed to a low frequency of CMV related mortality[31]. However, even with the protocols refined through experience from the more than forty years that have passed since the first HSCT, patients still suffer from mucosal damage in the mouth and gastrointestinal tract. The damage of anatomical barriers renders the patient susceptible to various infectious agents, and during the weeks before the transplanted stem cells are able to produce an adequate amount of neutrophils, the patient is virtually defenseless against fungal and bacterial infections. Immunosuppressive drugs and slowly regenerating parts of the immune system cause a lasting increase in the risk of serious infections. In addition, graft versus host disease and malignant diagnose relapse adds to the mortality and morbidity[32].

The original combination of total body irradiation (TBI) and cyclophosphamide developed in Seattle provides effective immunosuppression and anti-tumor activity, but replacing TBI with busulphan is an effective alternative[33-36]. With this regimen one can avoid some problems with TBI, particularly growth retardation, cataracts and encephalopathy in children [37-38]. Moreover, it makes it possible to perform HSCT in centers without TBI facilities. Busulphan and TBI have similar myeloablative properties, but a higher engraftment level and a lower number of surviving CFUs in TBI treated mice compared to those treated with busulphan have been reported[39]. This may indicate a difference in their toxicity and mechanism of action. Despite being an effective myeloablative agent, busulphan does not possess sufficient immunosuppressive effects [40]. It is therefore of particular importance to add immunosuppressive drugs such as cyclophosphamide to busulphan based protocols in order to achieve an ideal pre transplant conditioning regimen.

The relative merits of the two regimens are still debated, as studies comparing them report conflicting results concerning outcome and toxicities[41-43]. The important subgroup of unrelated donor transplantations has only recently been evaluated according to choice of TBI or busulphan; the results showed similar clinical outcomes for transplant recipients with myeloid malignancies indicating that other prognostic variables might have a larger influence on survival than the type of ablative preparative

regimen[44]. It must be remembered however, that there is presently no generally accepted standard strategy to handle the interpatient differencies in busulphan metabolism and bioavailabiliy. This becomes apparent in the wide variations in busulphan exposure seen when oral busulphan is used as replacement for TBI. Since several studies have demonstrated that adequate busulphan exposure levels are vital to minimize the risks for complications after HSCT[45-49], it is reasonable to hypothesize that this may at least in part explain the contradictory results.

Reduced Intensity Conditioning Transplantation (RICT) is a method for HSCT based on the philosophy of using a minimum of conditioning treatment, primarily immunosuppression, with the intent to utilize the immunologic graft versus leukemia alone as treatment against the malignant disease[50]. RICT has been shown to have less non relapse mortality and morbidity than myeloablative HSCT[51]. RICT is an important possibility for patients with co-morbidities excluding them from conventional HSCT, but the relapse risk especially in acute leukemia (the most common indication for HSCT) is unacceptably high for younger, fitter patients[52]. A substantial improvement is needed if RICT is to replace myeloablative treatment as the most important preparative strategy before HSCT. The success of HSCT is still limited due to several complications, including treatment related toxicity such as Sinusoidal obstructive Syndrome (SOS) formerly known as veno occlusive disease or VOD, Hemorrhagic cystitis (HC) and interstitial pneumonia (IP). Moreover, graft versus host disease and relapse are the most common immunological problems following HSCT.

1.2.1 Sinusoidal obstructive Syndrome (SOS)

Sinusoidal obstructive Syndrome (SOS) or 'Veno occlusive disease' is a serious liver complication caused by chemotherapy and/or radiation before HSCT. The reported incidence ranges from 3 to 50% and the mortality rate from 0 to 90 % (median 30 %)[53-56]. The symptoms usually appear 1-4 weeks after conditioning and are jaundice, enlarged liver, pain and tenderness in the area of the liver, rapid weight gain, swelling and accumulation of fluid. SOS is diagnosed on the basis of clinical signs - that is, the presence of hyperbilirubinemia (total serum bilirubin > 34umol/l) and the development of two of the following signs during the first 21 days post-transplant: weight gain >5% from preconditioning baseline weight, ascites and/or hepatomegaly. [57]

1.2.2 Graft versus host disease (GVHD)

Acute GVHD (aGVHD) remains the major complication of allogeneic SCT. Historically it was called the secondary disease, the disease that involved skin, gut and liver in allogeneically transplanted mice. It was renamed acute graft versus host disease in 1960. The incidence of GVHD ranges between 6-80% following allogeneic SCT, and it can be fatal in up to 50 % of cases.[58-60]. Elevated inflammatory Th1 cytokines appear to be responsible for the development of acute graft versus host disease (GVHD) following bone marrow transplantation (BMT) in a cascade that has been termed the 'cytokine storm'. [61] The development of GVHD has been suggested to occur in at least three phases: Phase 1 starts when the inflammatory cytokines (IL-1, IL-6, IL-8 and TNF-α) are released. Phase 2 starts with the activation of donor T-cells, reacting to up regulated host tissue antigens including human leukocyte antigen (HLA) and adhesion molecule expression. Phase 3 involves additional host tissue damage, activated T and NK cells, and the release of Th1 cytokines (IL-2,interferon-gamma and TNF-α)[61]. It has been shown that increased levels of TNF-α preceded BMT complications such as severe acute GVHD, interstitial pneumonitis, endothelial linkage syndrome and VOD.[62]. Usually prophylaxis treatment is used during and after SCT. Cyclosporine, prednisone and methotrexate are the most commonly used drugs.

Chronic GVHD (cGVHD) develops three or more months after SCT. The symptoms are a dry itchy rash, change in skin color and tautness or tightening of the skin. Liver abnormalities, dry or burning eyes, dry mouth, mouth sores, infections and stomach irritation are also common symptoms.

1.3 CYCLOPHOSPHAMIDE

As described earlier, cyclophosphamide (Fig 1) belongs to the nitrogen mustard group and is one of the most successful mechlorethamine analogues developed for clinical use against malignant disease. More than half a century after the introduction of cyclophosphamide it is still widely used both orally and intravenously, not only as an antineoplastic agent but also for immunosuppression. The pronounced immunosuppressive properties of cyclophosphamide have made it an important part of the therapeutic arsenal against autoimmune disease and a component of many pre transplant conditioning regimens[63].

Cyclophosphamide is a pro-drug activated by liver P450 enzymes including 2B6, 3A4, 2C19 and 2C9[64]. Metabolism of cyclophosphamide through the hepatic P450 enzymatic pathway produces active as well as toxic components, e.g. 4-hydroxycyclophosphamide(4-OH-Cy), chloroacetaldehyde and acrolein[64]. 4-OHCy forms the alkylating agent phosphoramide mustard which alkylates DNA. As substantial levels of glutathione are necessary to detoxify these metabolites it may be hypothesized that the administration of busulphan before cyclophosphamide may increase treatment related toxicity due to the decreased glutathione levels in the liver and tissues (after four days of busulphan), given that cyclophosphamide metabolites are the main components causing short term toxicity [65-66].

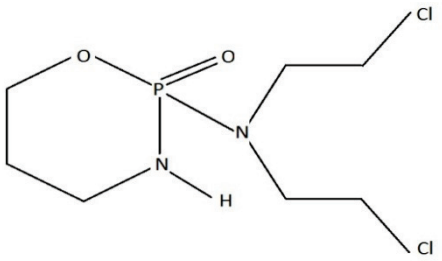


Figure 1: Cyclophosphamide (N,N-bis(2-chloroethyl)-1,3,2-oxazaphosphinan-2-amine 2-oxide)

1.4 TREOSULFAN

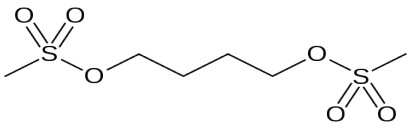
Busulphan (Fig 2) in combination with high-dose cyclophosphamide is one of the most common regimens of conditioning treatment prior to allogeneic HSCT. As described above, the combination has been in use for many years with only minor changes, despite the problems involved in high dose busulphan treatment. It is clear that the complicated and erratic pharmacokinetics of busulphan and the risks for, in particular, hepatotoxicity warrant the search for replacement drug candidates in the plethora of cytostatic agents available.

Treosulfan (Fig 2), synthesized first by Peter W Feit in 1961[67], is an alkylating agent structurally related to busulphan; it has traditionally been used primarily in treatment of late-stage ovarian carcinoma. It has many properties that make it interesting as a replacement for busulphan. In contrast to busulphan, treosulfan is water-soluble and can easily be administered by intravenous infusion.

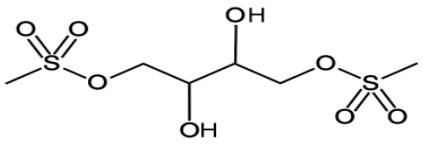
Treosulfan in doses exceeding 10 g/m^2 carries a high risk for permanent myeloablation, but no other frequently occurring toxicity is reported for doses up to 47 g/m^2. Beyond this dose, toxicities such as diarrhea, mucositis, stomatitis, skin toxicity, and metabolic acidosis have been observed. Very few cases of severe liver toxicity or VOD have been reported[68-70].

Figure 2: Busulphan and Treosulfan

Busulphan



Treosulfan



In spite of the obvious structural similarities between busulphan and treosulfan, the introduction of two hydroxyl moieties fundamentally changes the mechanism of action. Treosulfan is in principle a pro-drug that transforms to the active epoxide (2S, 3S)-1, 2; 3, 4-diepoxybutane (S, S-DEB) and Methanesulfonic acid non-enzymatically. The transformation depends mainly on pH and temperature; it is practically halted below pH 6.0. S, S-DEB is a bifunctional alkylating agent that has the ability to cross-link DNA[71]. The majority of cross-links are supposed to be of the 1, 3-interstrand type, causing cytotoxic effects. However, structural changes in DNA that fail to induce cell death are responsible for the well documented mutagenic effects of DEB [72-73].

Treosulfan bioavailability is close to 100% with low interpatient variability, and the pharmacokinetics can best be described by a two-compartment model with first-order kinetics of distribution, elimination and absorption. There is a linear relationship between C_{max} and AUC for doses from 20 up to 56g/m². To add to the favorable pharmacokinetic profile, available pediatric data indicate no important difference in pharmacokinetics between children and adults. About 30% of the total dose administered is excreted unchanged into the urine in a patient with normal renal function[74].

In conclusion, treosulfan possesses several attractive characteristics as a candidate for replacing busulphan in conditioning protocols before HSCT.

1.5 BUSULPHAN

$$CH_3SO_2O(CH_2)_4OSO_2CH_3$$

Figure 3: Busulphan (Myleran®)

Busulphan (Bu) 1,4-bis(methanesulfonoxy) butane is an alkylating agent. Busulphan (Fi 3) was first used in 1952 in low doses for the treatment of chronic myeloic leukemia (CML)[75] as well as for the treatment of polycythemia vera (PCV). Busulphan belongs to the alkyl alkane sulfonate group of alkylating agents.

Busulphan alkylates through an S_N2 mechanism (Fig. 4), the rate of which is dependent on the concentration of both the alkylating agent and the target nucleophile. The compound reacts more extensively with thiol groups of amino acids and proteins than do the nitrogen mustards, and these findings have prompted the suggestion that the alkyl alkane sulfonates, unlike the nitrogen mustards, may exert their cytotoxic activities mostly through such reactions rather than through interactions with DNA[76-77]. In contrast to the nitrogen mustards, busulphan displays a more marked effect on myeloid cells than on lymphoid cells, and it was noted early that simultaneous administration of busulphan and a nitrogen mustard can imitate the hematological effects of radiation[78]. Busulphan is cytotoxic to hematopoietic stem cells. The effect is seen clinically in the prolonged aplasia that may be seen after busulphan administration and can be shown experimentally in stem cell cloning systems [79-80]. Posing problems when busulphan is used as low dose treatment for chronic myelogenous leukemia, the characteristics are exploited in high dose conditioning protocols for ablative hematopoietic stem cell transplantation [33, 35]. Alkylators are in general mutagenic, but there is a considerable difference between the compounds in their tendency to induce mutations relative to their cytotoxic effects. Busulphan compares unfavorably to most other alkylators in this respect, which means it is not suitable for long term therapy due to the risk for secondary malignancies [81-82]. Over time other less mutagenic treatment options have become available. Busulphan has been replaced by tyrosine kinase inhibitors, interferon and hydroxyurea in non-myeloablative treatment of chronic myelogenous leukemia and other myeloproliferative diseases, and high dose conditioning protocols is now the single most important clinical application of busulphan [83-84].

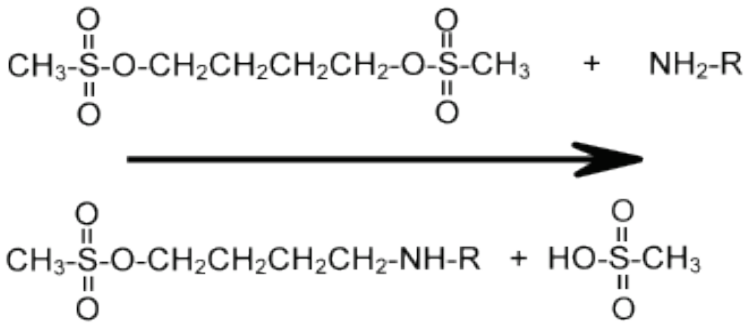


Figure 4: Busulphan alkylating mechanism

1.5.1 Pharmacokinetics of busulphan

The unpredictability of busulphan pharmacokinetics is notorious, and when used in doses close to maximum tolerated (as in HSCT) over- or under-dosing may have severe consequences. Busulphan is a hydrophobic drug; potentially toxic additives and costly liposomal packaging are needed to achieve a formulation suitable for intravenous administration. Accordingly busulphan is in most cases administered orally, adding bioavailability to the factors that affect drug exposure. Bioavailability is known to vary extensively, in pediatric patients as much as 2- to 5-fold[85]. Studies have found that approximately one third of the total amount of busulphan administered is irreversibly bound to plasma proteins and half is irreversibly bound to red blood cells[86].

The metabolism and elimination is quite complex (Fig 5), but the parent drug alone possesses alkylating activity and the metabolites are believed to be pharmacologically inactive.

Busulphan is deactivated by two mechanisms:

- Busulphan may spontaneously form tetrahydrofuran. This occurs at a low rate and is *in vivo* quantitatively of minor importance.
- Busulphan is enzymatically conjugated to glutathione. This is the major deactivating mechanism, its rate limited by hepatic enzyme function and glutathione availability.

When glutathione is conjugated to busulphan the sulfonium ion of glutathione is formed and some of the conjugate is excreted in the bile. The conjugate undergoes enzymatic cleavage in the intestine by forming tetrahydrothiophene which may be reabsorbed and oxidized in the liver to tetrahydrothiopheneoxide, sulfolane and eventually 3-hydroxy-sulfolane. Tetrahydrothiopheneoxide and 3-hydroxy-sulfolane

are polar and readily excreted in the urine. Some of the conjugate is metabolized in the mercapturic acid pathway to form the sulfonium ion of N-Acetyl-L-Cysteine and then excreted in the urine [87-88].

1.5.2 Busulphan metabolism

As may be inferred from the above description, the metabolism and elimination of busulphan (Fig 5) is highly dependent on glutathione. The intracellular concentration may be affected by nutritional status, hepatic function et cetera[89]. This may be the reason that intra-patient variation in busulphan exposure is even more pronounced than the variation in bioavailability, meaning that the latter can only partly be responsible for the former[90].

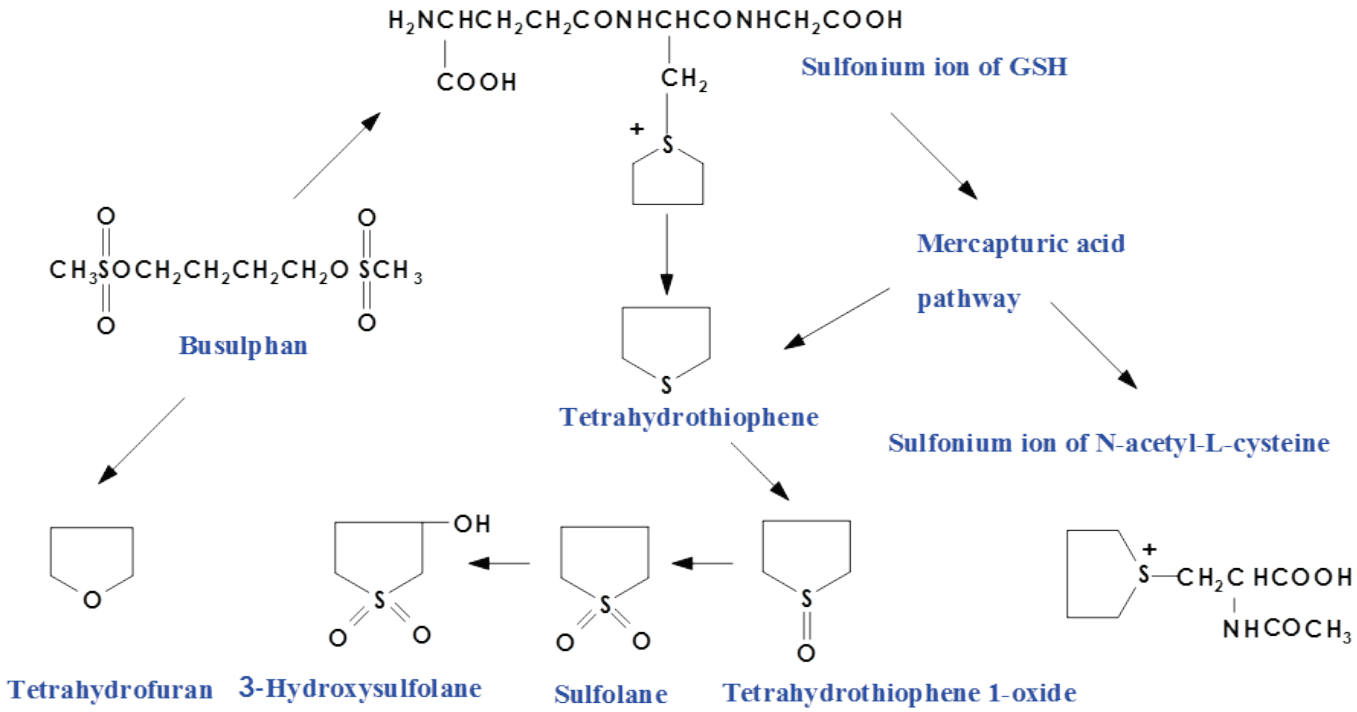
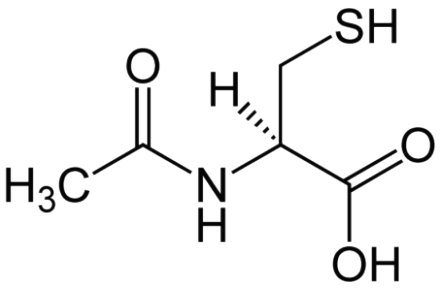


Figure 5: Busulphan metabolic Pathway

1.6 N-ACETYL-L-CYSTEINE (NAC)

Apart from acute graft versus host disease and infections, Sinusoidal Obstruction Syndrome (SOS), formerly known as Veno Occlusive Disease (VOD) of the liver, is one of the more common early complications with a potentially fatal outcome following HSCT [42, 90]. SOS is a clinical syndrome consisting of combinations of jaundice, ascites, unexplained weight gain, hepatomegaly and upper quadrant abdominal pain, as defined by McDonald *et al* [91] and Jones *et al*[92]. Low constitute levels of glutathione (GSH), the most important intracellular antioxidant, in centrilobular hepatocytes together with further GSH exhaustion by the conditioning chemotherapy given before HSCT is likely to contribute to the development of SOS[93]. In particular, busulphan is known to deplete GSH by 50-60% in murine hepatocytes. An increase of cellular levels of GSH has been demonstrated to prevent murine busulphan hepatocyte toxicity [94]. This can readily be accomplished by administering the GSH precursor N-acetyl-L-cysteine (NAC; Fig. 6)[95]. NAC is clinically used as a well-established and nontoxic treatment used to prevent hepatotoxicity due to acetaminophen poisoning. It is hence widely available[96] and there have been case reports of SOS patients successfully treated with NAC[97].

Figure 6: N-acetyl-L-cysteine



Because the desired myeloablative effect of busulphan consists of cytotoxicity towards hematopoietic cells, there has been concern that NAC treatment to avoid hepatocyte toxicity would impair myeloablation. Busulphan, however, is metabolized in the liver through conjugation with GSH catalyzed most effectively by glutation-transferase isoenzyme GST-A1 (an isoenzyme not present in hematopoietic cells) [98-99]. Accordingly a selective liver protection effect by NAC treatment is probable. In fact, in vitro as well as animal in vivo studies have shown that modulation of GSH content in hematopoietic cells does not counteract the hematotoxic effect of busulphan[100].

1.7 THERAPEUTIC DRUG MONITORING AND DRUG DOSING

In many cases a single fixed dose can be prescribed to all adults. Certainly that does not imply that every adult needs the same dose for adequate effect or that every adult could tolerate the same amount of drug before experiencing toxic effects. Fixed dosing is justified if there is a sufficiently wide difference in the amount of drug that produces the desirable effect and in that which have toxic effects, i.e. a sufficiently wide therapeutic window, to compensate for individual variation in population physiology, pharmacokinetics and pharmacodynamics.

An obvious and relatively simple method of individualizing the dose is to compensate for body mass. In a subpopulation with a very wide variation of body mass, such as children, this is done for most drugs. In adults, recommendations for a higher or lower dose than standard are commonly provided for patients with a body mass that differs substantially from the average.

Several factors complicate the correlation between patient body mass and adequate dosage. Most drugs are not evenly distributed in the body. Thus, if a patient has 20 kg of extra fat, this may not affect the target organ concentrations of a lipophobic drug.

Several other factors such as renal, intestinal or hepatic dysfunction and genetic differences can affect the needed and tolerated dose more than the patient's body mass.

In the late 19th century it was discovered that small animals utilized proportionately more oxygen and produced proportionately more heat per kg body mass, than did larger animals. Instead, there is a correlation between metabolism and body surface area (BSA). Later it was also discovered that BSA was correlated to blood volume and circulating plasma proteins.

BSA was initially recommended in dosage calculations for intravenous fluids, electrolytes and blood replacement in children, an approach that was proven to produce better outcome than traditional dosing according to body mass alone. In 1958, Donald Pinkel proposed that the use of dosing by BSA should replace dosing by weight, which had been the traditional method until then [101]. He argued that the determined adequate dose of the previously mentioned substances mechlorethamine and methotrexate was much better correlated to BSA than to weight in different species such as man, rat and mouse. The article had a tremendous impact, and today BSA dosing is the most commonly used approach in cancer chemotherapy.

Prof Eugene F duBois

It seemed reasonable to adopt BSA dosing for anti-cancer drugs at the time. However, the evidence presented does not meet modern scientific standards. Inter-species differences in BSA are much larger than differences in BSA between human patients, and the evaluation of animal toxicity used as a reference did not attempt to relate therapeutic doses in various species. There are also several methodological problems with estimating BSA. The customary practice in the clinic is to use the height-weight formula of cousins Du Bois and Du Bois[102]:

$$\text{Area } (\text{cm}^2) = \text{BW } (\text{kg})^{0.425} \times \text{height } (\text{cm})^{0.725} \times 71.84$$

The formula was constructed by iteration and graphical interpolation of data from nine subjects, who were measured by covering the whole body with soft paper in 1916. Even if later studies, comparing the formula to laser scanning measurements of BSA, have found a reasonable agreement[103], it still is surprising that hundreds of thousands of patients world-wide during almost a hundred years have had their cytostatic drug-dose determined by results from a single study on a small number of patients using questionable statistic methods. The rationale for BSA dosing strategies has not been supported by pharmacokinetic studies and pharmacological investigations[104]. It is, however, important to note that for a few cytotoxic drugs such as oral busulphan, BSA dosing has not gained widespread use. For oral busulphan, the initial dose is calculated according to body mass in most protocols.

In conclusion, calculations based on BSA or body mass are unlikely to produce acceptable estimates of the accurate dose for drugs with a narrow therapeutic window. For cytotoxic drugs the therapeutic window is frequently more or less narrow; complicating the matter further, the therapeutic and toxic effects are often delayed. Although toxicity is commonly regarded as the most important effect to control, the risk of under dosing and reduced efficacy must also be considered.

Obviously more robust dosing strategies have to be developed to improve therapy. Such strategies need to be based on predictions of clinical effect. Pharmacokinetic parameters are surrogate markers that are often used since they are clinically measurable. Therapeutic drug monitoring means that serum or plasma concentrations are measured during therapy and used for correcting consecutive doses. In this manner

it is possible to ensure that the treated patient is subjected to the intended degree of drug exposure. Exposure is in this case defined as the area under the plasma concentration curve, AUC. High dose Busulphan regimen is given orally for four days with a total dose of 16mg/kg divided into smaller doses. Pharmacokinetic studies of oral busulphan have found that bioavailability (AUC oral/AUC iv) varies 2- to 5-fold in pediatric patients.[85] Numerous studies have correlated busulphan AUC with regimen related toxicity, engraftment and relapse in patients receiving the busulphan and cyclophosphamide preparative regimen. A busulphan AUC of 3600-5400ngh/ml in partially matched or unrelated bone marrow transplant recipients and 1200-5400ngh/ml in allogeneically matched sibling bone marrow transplant patients is desired to minimize toxicity and prevent rejection.[46, 105-106] Based on these facts, TDM is widely considered standard procedure and a validated method for improving the results of HSCT with high dose oral busulphan as part of the conditioning protocol. Intravenous formulations of busulphan have recently become available and studies have confirmed reduced toxicity problems, apparently due to elimination of the unpredictable absorption kinetics of oral busulphan. [107-109] However, it is likewise clear that several sources of interpatient variation in pharmacokinetics remain, such as differences in e g metabolism.[105, 110]

1.8 LIMITED SAMPLING MODELS (LSM) IN THERAPEUTIC DRUG MONITORING

Traditional TDM is demanding for the lab, the clinical staff and the patient. Determination of AUC involves multiple blood samples which must frequently be collected from an already anemic patient, sometimes from a pediatric patient. Since dose adjustments need to be made as quickly as possible, both the collecting and the analyzing of blood samples often have to take place during off-hours when staff is reduced. It should be remembered that analysis of pharmacokinetic parameters has in general been developed with the preclinical evaluation of new drugs in mind, and not for the assessment of doses to individual patients during treatment. None of the above complications apply with a healthy study subject in evaluating the pharmacokinetic profile of a new drug. On the other hand, as long as the correct decision regarding dose adjustment can be reached, precision is not as critical in TDM as when assessing a new drug. The needs and complications in TDM are quite different from those in preclinical drug evaluation studies and that has prompted new approaches to assessing drug exposure in an individual patient as measured by AUC. One important objective is to reduce the number of samples and instead use mathematical models for compensating the missing data. Several reports on Limited Sampling Models (LSM) have been published, but there is a plethora of models and no consensus on which one to use.

A rough division of models can be made as follows.

Models based on Bayesian inference: Bayesian statistics are based on the concept of aggregating previous knowledge, an informative prior, with current observations for making conclusions regarding the likelihood for a certain case. For example, an informative prior can be constructed from patient medication, liver enzyme levels and what is known in general about busulphan pharmacokinetics. The previously known data is allowed to affect the interpretation of the measured plasma concentrations [111-112].

Models based on linear regression: In this case previous results from earlier conventional rich sampling TDM is used to construct an equation; the plasma concentrations are used as determining factors and AUC as the dependent variable. The factors with most impact on the dependent variable are selected for the equation. A small initial patient cohort is used and the equation is then verified on a larger cohort [113-114].

Models based on compartments: These kinds of models are constructed from the theoretical principles of pharmacology where the body is visualized as a number of

compartments and the drug is absorbed into, transferred between and finally eliminated from these compartments at certain rates. Since it is difficult to construct compartment models with noisy, sparse sampling data, these models are sometimes combined with a Bayesian approach as described earlier.[112]

Models where a mathematical curve is approximated to the measured dataset: These can range from simple trapezoidal rule approximations to elaborate spline constructions with correction factors.[115]

The plasma concentration curve after busulphan treatment is notoriously unpredictable and involves not only wide inter- and intraindividual variations in uptake and elimination, but also double-peak patterns often seen in drugs with low solubility[116]. Reducing the number of samples (and thus the amount of data) on which to base estimates of drug exposure will inevitably downgrade precision. Considering the benefits of limited sampling, the relevant question is whether this will significantly affect the clinical usefulness of TDM. In other words, can the method identify patients with busulphan exposure outside the desired interval with enough sensitivity and specificity?

Presently there is no consensus on the optimal method for limited sampling calculations or on whether limited sampling is at all acceptable in TDM.

2. GENERAL AIMS

The overall aim of this thesis has been to improve high dose chemotherapy; in particular, busulphan based conditioning regimens used prior to HSCT. My hope is that these findings may benefit patients in need of this treatment and possibly also patients treated with chemotherapy for other diseases.

2.1 SPECIFIC AIMS

- To investigate the myeloablative and immunosuppressive properties of treosulfan and to evaluate treosulfan as a candidate drug for use in HSCT.
- To investigate the effect of N-acetyl-L-cysteine in conditioning chemotherapy on busulphan kinetics, and hence its effect on immunosuppressive or myeloablative properties of the conditioning regimen.
- To evaluate the necessity of TDM and dose adjustment of intravenous busulphan, particularly in pediatric patients.
- To improve limited sampling strategies for TDM of busulphan and to evaluate the efficacy of these strategies in estimating drug exposure compared to conventional methods.

3. MATERIALS AND METHODS

3.1 ANIMALS

For the treosulfan study, an animal model was used. Female BALB/c mice, 10 to 12 weeks old and weighing approximately 20g were purchased from B&K Universal Limited (Sweden). The local ethics committee approved the experimental protocol, conditions and design. Animals were fed with standard pelleted food and water ad libitum.

3.2 PATIENTS

The patients for the NAC study were all recruited from Huddinge University Hospital (presently Karolinska, Huddinge) where they underwent allogeneic SCT between October 2000 and May 2002. Six patients were transplanted with stem cells from matched unrelated donors, three with stem cells from HLA identical sibling donors and one with cord blood. The criterion for inclusion was a high risk for liver toxicity from the chemotherapy. Most patients had elevated liver enzymes, but some were included due to very high busulphan concentrations in plasma or preexisting hemochromatosis.

The study on TDM of intravenous busulphan was made in cooperation with the Children's University Hospital in Zurich, Switzerland. Between August 2006 and March 2009, thirty-four patients were transplanted in Zurich using a busulphan based conditioning regimen in which the busulphan was administered intravenously. The majority received allogeneic grafts, but three patients received autologous stem cell grafts. According to the underlying disease and current treatment protocols, busulphan was used with ATG, fludarabine, cyclophosphamide, melphalan, etoposide or combinations thereof. There were 9 patients with malignant disease and 25 patients with nonmalignant disease.

For the limited sampling study, patients were recruited from the center for allogeneic stem cell transplantation at Karolinska, Huddinge. All patients had been diagnosed with malignant hematological disease and were treated with busulphan as part of the conditioning therapy before allogeneic stem cell transplantation. According to local guidelines, oral busulphan was administered in two daily doses of 2 mg/kg for four days, preceding cyclophosphamide.

3.3 ANALYTICAL METHODS

3.3.1 Cytokines

We assessed the immunosuppressive effect of treosulfan by examining T-cell expression of interleukin-2 (IL-2), tumor necrosis factor-α (TNF-α) and interferon-γ (IFN-γ) after stimulation with PMA/Ionomycin. We also performed a Mixed Lymphocyte Reaction (MLR) study. A clonogeniec assay was used to test the myeloablative properties.

3.3.2 Clonogeneic assay

Both femurs were removed and placed in a sterile Petri dish. Bone marrow was flushed from both femurs with Iscove's modified Dulbecco's medium. Repeated flushing using 14-gauge needle formed a single cell suspension. A volume containing 2.5 x 10^6 cells was transferred to sterile tube. Iscove's modified Dulbecco's medium was added. The mixture was vortexed and transferred to Methocult GF M3534. This media specifically encourages the growth of CFU-GM. 0.5 x10^5 cells were plated in triplicate in 35mm Petri dishes and incubated at 37°C, 5% CO_2 and 100% humidity. A colony count was performed on day seven.

3.3.3 Busulphan determination

In the Zurich protocol, busulphan plasma concentrations were analyzed using a HPLC-MS/MS instrument after liquid-liquid extraction into dichloromethane. Separation was performed on an Uptisphere 5µ ODB column (125 x 2 mm; Montluçon, France) and busulphan was detected as ammonium adducts after electrospray ionization. Spiked plasma samples were used to calculate the calibration curve. The calibration curve was linear within the range 100-2500 ng/ml. Busulphan concentrations were calculated from the calibration curve.

At Karolinska University Hospital, the Bu concentration was measured using gas chromatography. An aliquot of 50µl of internal standard [1,5-bis(methanesulfonoxy)pentane] at a concentration of 10µg/ml dissolved in acetone was added 0.5ml of the plasma. 400µl of n-heptane and 1ml of 8 M sodium iodine were added. The reaction between Bu and the internal standard and NaI was carried out at 70°C for 45 min under magnetic stirring. 200µl of n-heptane was added, and the organic phase was removed and analyzed using gas chromatography equipped with electron capture detector. The injection temperature was 250^{o}C, the column was

operated isothermically at 135°C and there was a detector temperature of 300°C. The calibration curve was linear within the range 10-2600ng/ml.

3.3.4 Pharmacokinetics and model development

Conventional calculation of AUC was made utilizing WinNonLin compartment modeling. Models for limited sampling were implemented using Microsoft Visual Studio 2010 Professional with the C# programming language and the NMath mathematical library from CenterSpace Software Inc. for the Microsoft .NET platform. The LSM program was constructed on and compiled for a computer running 32 bit Windows 7.

3.4 STATISTICS

Microsoft Excel was used for simple general statistic calculations of average and standard deviations et cetera. For LSM method development and assessment, R version 2.12.1 from the R Foundation for Statistical Computing and CRAN packages were used. In particular R implementations of algorithms for ICC calculus, Bland-Altman plots, normality test and a method for finding the most predictive design points in a model were utilized.

3.5 EVALUATION OF METHODS

In clinical medicine there is frequently a need for quantitative measurements upon which to base decisions regarding treatment. TDM is a particularly complicated example of this, where the results from plasma concentration analyses must be processed mathematically to obtain an estimate of drug exposure, which can be used for decisions regarding dose adjustments. Not only must the plasma concentration analysis be valid, but also the mathematical method used for calculating AUC. When introducing a new method, some lack of agreement with the old methods is inevitable. We need to know by how much the new method is likely to differ from the old, so that if this difference is not big enough to cause problems in clinical interpretation we can replace the old method with the new. How far apart measurements can be without leading to problems is a question of clinical judgment. Statistical methods cannot answer such a question.

Historically, accuracy has been used to measure systematic bias while precision has been used to measure random error. Agreement measures the “closeness” between readings. The term contains both accuracy and precision.

The Correlation Coefficients is one of the most popular indices in statistical literature for assessing agreement. Many kinds of correlation coefficients have been proposed. There are several correlation coefficients available; the best choice depends on the nature of the data to be analyzed. The most commonly used correlation coefficient is the concordance correlation coefficient (CCC). The CCC was developed for comparing two series of observations, generating continuous data on each subject[117]. The CCC is suitable when the subjects and the observers are randomly chosen but not when the observers are replaced by fixed AUC calculation algorithms (methods). A more appropriate parametric method for this situation is the Intraclass Correlation Coefficient which can be used for calculating agreement as well as consistency (precision)[118]. The ICC is based on analysis of variance (ANOVA) calculations, and different models are used depending on the data. The following model applies for assessing agreement for fixed observations on random targets. This type of the ICC is based on a two-way mixed model ANOVA[119]. An ANOVA separates the variance of the observations into components derived from interpatient variance and intrapatient variance. The intrapatient variance, in two-way analysis, can be further divided into variance from interaction between patient and method and residual variance. The equation for the agreement parameter ρ is seen in (5). The parameter can be estimated from an analysis of variance table and the sum of squares as described in (6).

$$\rho = \frac{\sigma_P^2}{\sigma_P^2+\sigma_M^2+\sigma_I^2+\sigma_E^2} \quad (5)$$

ρ=correlation

σ_P^2=variance of patients

σ_M^2= variance of methods

σ_I^2= variance of patient-method interaction

σ_E^2= variance of the residual error

$$ICC = \frac{BMS - EMS}{BMS + (k-1)EMS + \frac{k}{n}(MMS - EMS)} \quad (6)$$

ICC=Intraclass Correlation Coefficient

BMS=Between Patients Mean Square

EMS=Residual Error Mean Square

MMS=Methods Mean Square

k= number of methods compared (in this case 2 each comparison)

n=number of patients (23)

Bland-Altman plots give graphical representations of precision and accuracy in relation to exposure range[120]. A Bland-Altman plot is a plot showing the difference of the two methods against the mean result. The plots provide important information on how well the limited sampling models perform in specific areas of interest. Patients with busulphan exposure in the vicinity of the cut-off point for dose adjustments can be identified, and the agreement of the LSM to the reference for these patients can be studied. The main disadvantage with a Bland-Altman plot is the difficulty in making a quantitative objective comparison between the different LSM performances. The choice of scale is also vital for an informative plot.

4. RESULTS

Treosulfan and busulphan induce a high and persistent degree of myeloablation in comparison with cyclophosphamide. Treosulfan was more effective in depletion of splenic B and T cells as compared to busulphan and cyclophosphamide, and T cells isolated from the spleens of treosulfan- or busulphan-treated mice were not responsive to allogeneic cells.

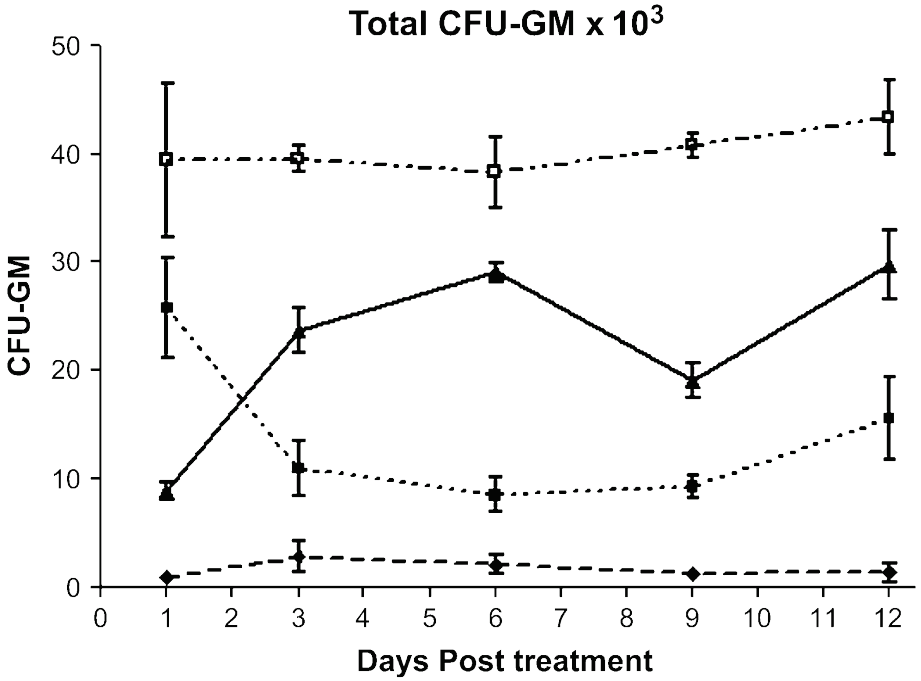


Figure 7: CFU-GM colony-forming unit granulocyte macrophages

Treosulfan induces durable myeloablation in mice (Fig 7). Mice were treated with treosulfan (solid diamond and dashed lines), busulphan (solid squares with dotted lines), and cyclophosphamide (solid triangle symbols and solid lines). Control animals were left untreated (empty squares).

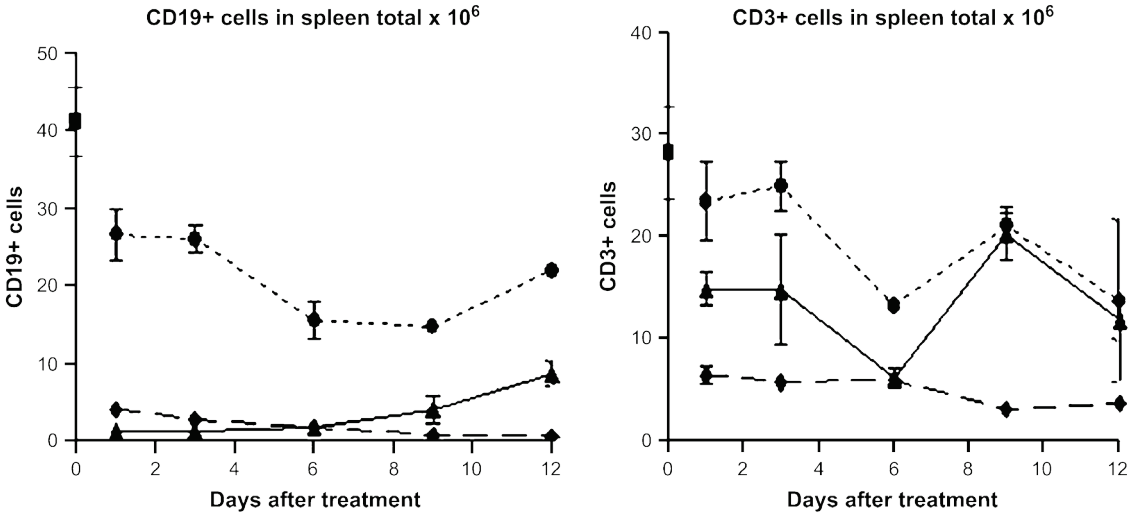


Figure 8: Splenic B- and T-cell depletion respectively

Treosulfan induces splenic B- and T-cell depletion in mice (Fig 8). Mice were treated with treosulfan (solid diamond and dashed lines), busulphan (solid squares with dotted lines), and cyclophosphamide (solid triangle symbols and solid lines). Control animals were left untreated (solid square symbols on the Y axes).

After the administration of NAC during conditioning to patients with increased risk for hepatotoxicity and SOS due to pre-transplant liver disorders or elevated liver enzymes, no side effects related to the NAC infusions were observed and busulphan concentrations were not affected. All patients became pancytopenic and engrafted with 100% donor cells (Fig 9).

Patient no.	*First day of SCT, WBCs* $\leq 0.5 \times 10^9/l$	*Engraftment day, WBC* $\geq 0.5 \times 10^9/l$	*Chimerism at 1 month post-SCT*	*Recurrence of the malignancy*	*Acute GVHD – grade and onset*	*Patient outcome*
1	2	16	100% donor	15 months after transplantation	I – skin, day + 16	
2	−2	12	100% donor	No	III – liver and intestine, day 22	Died day + 55 due to intestinal GVHD
3	1	13	100% donor	No	No	
4	−1	12	NA	No	No	
5	−3	15	NA	No	III – liver and skin, day + 15	
6	0	24	NA	No	III – liver, day + 18	
7	4	13	100% donor	No	I – skin, day + 13	
8	5	10	100% donor	No	II – skin, day + 10	
9	−1	14	NA	No	No	
10	6	12	NA	No	No	

Day of SCT = day 0, WBC = white blood cell count, SCT = stem cell transplantation, GVHD = graft-versus-host disease grades I–IV according to Seattle,[33] NA = not available.

Figure 9: Course of transplantation for NAC treated patients

In patients treated with intravenous busulphan, the busulphan dose had to be adjusted at least once for 23/34 patients (68%) studied. The need for dose adjustment could not be predicted by age or weight as shown in figure 10.

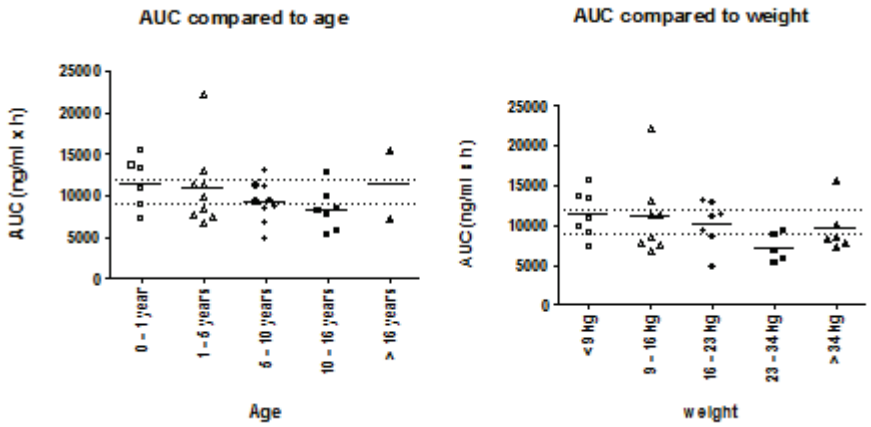


Figure 10: AUC compared to age and weight (respectively)

A comparison of three different principles for constructing a limited sampling model with the industry standard/rich sampling/WinNonLin compartment model as reference proved that there is a substantial variation in quality and reliability of AUC estimate depending on the choice of algorithm and model. Bland Altman plots for each model (Fig 11-12) showed that using limited sampling with a one compartment model or a modified Purves curve fitting model, one can arguably obtain a clinically useful estimate. Using an average of both methods tends to improve agreement to the reference method further. Comparing the average to the reference method (Fig 13) we found an intraclass correlation coefficient (ICC) of 0.86. In contrast, the ICC for the regression model was 0.62. Bland-Altman plots found that using this method would frequently lead to other decisions regarding dose reduction than if the decisions were based on the reference rich sampling model. Further analysis of data found that only one of the 23 examined cases had an AUC estimate from the combined AUC that differed from the reference method in a manner that would affect decisions regarding dosing of busulphan.

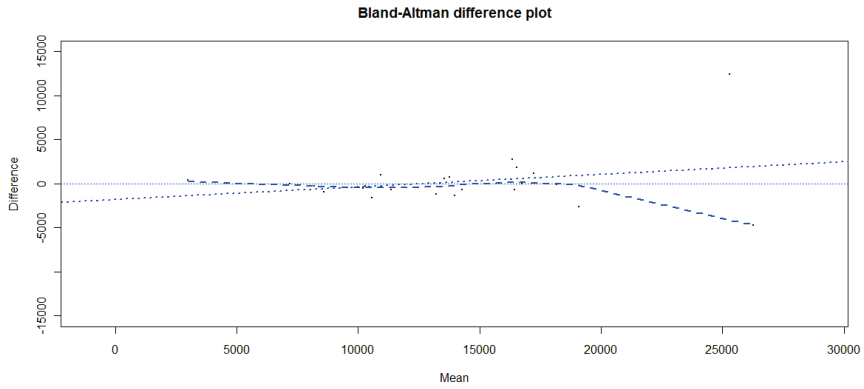


Figure 11: Agreement of estimates from combining the compartment and the curve fitting model

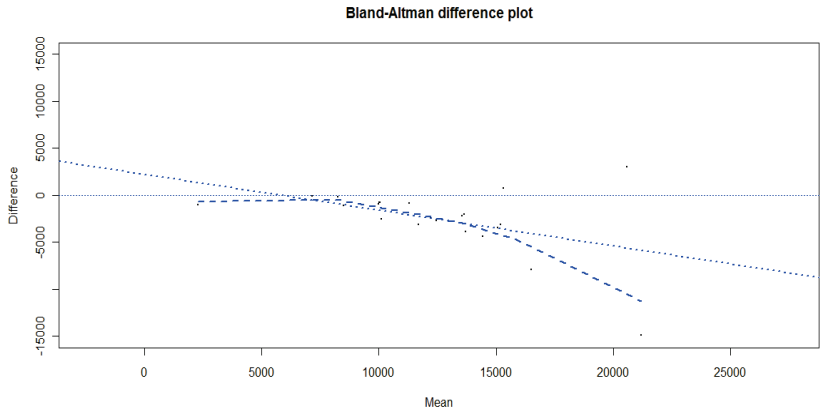


Figure 12: Agreement of the three sample linear regression model

Limited sampling model	Estimated ICC	95% CI lower limit for ICC	95% CI upper limit for ICC
Non-Compartment curve-fitting model	0.82	0.61	0.92
One-Compartment model	0.77	0.53	0.90
Linear Regression model	0.62	0.16	0.84
Using Average AUC from Compartment + Non-Compartment curve fitting model	0.86	0.69	0.94

Figure 13: Intraclass correlation coefficient for the various kinds of lsm with rich sampling as reference

5. DISCUSSION

The thesis deals with different ways to reduce toxicity and adverse effects associated with chemotherapy as conditioning before HSCT, particularly in busulphan chemotherapy. The least radical measure would be to improve dosing strategy and assure that every patient is given the optimal dose. Due to the unpredictable bioavailability and metabolism of busulphan this is, however, a formidable task. I have found that only part of the interpatient variations in exposure can be overcome with intravenous administration. It can be speculated that this may be the result of unpredictable phase II metabolism and glutathione conjugation capacity. An unexpected high risk for SOS also raises concern about adverse effects of additives needed for intravenous formulations of busulphan. Regardless of the possible future replacement of oral busulphan with intravenous formulations, the development of improved strategies for therapeutic drug monitoring is important. Since the number of samples is one of the most important factors in determining resource demand as well as patient discomfort, it is highly desirable to limit the number of blood samples collected during therapeutic drug monitoring. The catch is that reducing the number of samples increases the size of plasma concentration curve segments necessary to simulate in between measured concentrations and causes a single measurement error to have more impact on the final parameter estimate. The question is: How few samples are enough for a reasonably safe and accurate pharmacokinetic evaluation? The answer to the question must be somewhat subjective and perhaps should be different for different patient populations. Therapeutic drug monitoring guided by rich sampling, i.e. 8-12 samples, has been demonstrated to benefit clinical outcome. Ideally, any change in this procedure should be verified by studies to provide the same benefits. In reality, testing any possible procedure in this manner would raise several difficult ethical questions. Instead, it makes sense to search for a method that shows as much agreement as possible with the verified procedure in use, and especially to assess how frequently decisions based on the new model could differ from decisions based on the verified model. I investigated limited sampling model calculations based on three different principles and their respective agreement with rich sampling for actual patients. The best possible algorithm should be used in future research and direct studies on the clinical benefits of TDM based on limited sampling models. I found that there is reason to believe that only very few patients would not get the same dose adjustments with

estimates from the proposed LSM strategy as they would with estimates from rich sampling.

Adding drugs to reduce toxicity from chemotherapy is also a possibility, but this may impair the desired therapeutic effect. In HSCT that may be just as fatal as any toxicity. Detoxification of busulphan is almost entirely dependent on glutathione conjugation in the liver. As stated earlier, the unpredictable metabolism of the drug is probably caused primarily by this fact as well as by variations in nutritional status and hepatic function between patients. Glutathione hepatocellular content can be replenished by administration of NAC, and this is done routinely in some intoxications, most notably paracetamol intoxication. The time from intoxication to NAC treatment is vital. According to current treatment guidelines for paracetamol intoxication, treatment should *not* be delayed until signs of hepatic damage occur, since this renders NAC treatment virtually ineffective. In a pilot study of ten patients we showed that NAC concomitant with conditioning chemotherapy did not reduce the myeloablative properties of treatment warranting further studies with concomitant NAC to high risk patients before signs of liver damage. It is my firm belief that this possibility has to date not been sufficiently explored.

The most radical approach to handling busulphan toxicity would be to simply replace the drug with another drug capable of myeloablation but without the variations in bioavailability and metabolism, and with less hepatotoxicity. Ideally this drug would also be soluble and easy to administer intravenously. In fact, there is such a candidate drug available: treosulfan. An animal model allows a much more invasive and complete assessment of myeloablative and immunosuppressive properties *in vivo* than would be ethically possible in human subjects. The study on treosulfan was an important step towards in-clinic studies of actual patients. It confirmed the expectations of treosulfan properties on hematopoietic cells and immune system, indicating that it is suitable as a drug for use in HSCT conditioning regimens. The drug has lately gained widespread use in the clinic in various regimens for HSCT conditioning for patients with a high risk of hepatotoxicity.

6. CONCLUSIONS

- Treosulfan possesses both myeloablative and immunosuppressive properties, and our findings affirmed treosulfan as a candidate drug for conditioning prior to bone marrow transplantation. Lately treosulfan has come in clinical use for patients at high risk (especially for liver toxicity), and it is increasingly accepted as an interesting new alternative to busulphan.
- NAC therapy is safe and does not impair the myeloablative effect of busulphan during conditioning prior to SCT. None of the patients in the study developed VOD or liver failure. Increased liver enzymes during conditioning decreased or normalized in all patients. In spite of these interesting findings, the number of patients was too small to provide evidence of the beneficial effects of NAC during conditioning.
- TDM is also essential when busulphan is administered intravenously. It is clear that TDM increases the efficacy and safety of intravenous busulphan-based conditioning protocols in pediatric HSCT recipients. The unexpectedly high risk for SOS warrants further studies to determine whether additives in intravenous formulations could be the cause.
- Decisions regarding dose adjustments based on the proposed approach of using an average of a compartment model and a curve fitting model will seldom differ from decisions based on rich sampling. The conclusion is that the clinical use of this implementation of a limited sampling algorithm with four samples for busulphan therapeutic drug monitoring is justifiable, considering the practical aspects of using rich sampling. In contrast, it was found that using the regression model based on three samples would quite frequently impact decisions regarding (particularly) dose reductions. This means that the clinical usefulness of TDM based on the latter model cannot be verified.

7. FUTURE PERSPECTIVES

Developing models for therapeutic drug monitoring in order to individualize dose and treatment will certainly benefit patients treated with cytostatic drugs that have a narrow therapeutic window.

Even if monoclonal antibodies, tyrosine kinases and other designed drugs will be increasingly important for the treatment of malignant disease in the future, it is likely than cytostatic agents will remain necessary for most patients. In addition, cytostatic drugs are not the only drugs to have a narrow therapeutic window.

Individually optimized therapy is an important concept that must be used much more than it is today, and it there is a potential for extending this concept to new areas as well. I believe that an important step in facilitating this is to make TDM more expedient and less costly. Developing limited sampling models for use in TDM is the most obvious way to achieve this.

Validating the benefits of TDM in new areas of cytostatic treatment requires clinical studies and accepted standardized techniques that can realistically be used in daily clinical practice. One future perspective is to use a LSM in this kind of study, providing direct evidence of the benefits from LSM TDM for certain patients and treatments.

7.1 ON THE USE OF COMPUTERS IN MEDICAL RESEARCH

The calculations necessary for the evaluation of plasma concentrations cannot practically be done without the use of computers. With an approachable modern high level language such as C# there is, however, a new possibility for researchers to construct Windows based applications and to achieve individually tailored solutions.

I have chosen to supply the final version of the Windows application used for AUC calculus freely for download in the hope that this will facilitate the use and implementation of my findings. In this I was inspired by the practice in the scientific field of statistics, where scientists have long provided implementations of their work as R scripts. R is an open-source statistical programming language.

8. ACKNOWLEDGEMENTS

I owe my deepest gratitude to all those who have contributed to the realization of this thesis and supported me during all these years, especially to:

Moustapha Hassan: my supervisor, tutor and friend who introduced me to the world of research and pharmacokinetics. He is the kind of true scientist of which the world needs many more. He convinced me that I could finish this work, although I left the university hospital for Capio St Görans hospital at an early stage. Without his guidance and persistent help, the completion of this dissertation would not have been possible.

Johan Aschan, my co-supervisor: for his advice and help, particularly on the clinical aspects of my thesis.

Zuzana Hassan: for her advice and help, including some very important comments that led to last minute changes in the design of my half time seminar presentation.

Olle Ringdén, former head of Center for Allogeneic Stem cell Transplantation at Huddinge: for the inspiring atmosphere at CAST where my research project once started.

My other co-*authors Lisbeth Barkholt, Reta Malär, Katharina Rentsch, Tayfun Güngör, Manuchehr Abedi-Valugerdi, Laimonas Griskevicius, Christina Nilsson, Mats Remberger, Hernan Concha* and *Una Gaughan*.

Jennifer Usterud, secretary to Moustapha Hassan: for correcting my English.

Jörgen Sörstad, former head of the internal medicine section at the department of medicine, Capio St Görans hospital: for allowing me to continue my research and to attend graduate courses even though St Göran is not a university hospital. It required a lot of creativity in planning my work schedule.

Lennart Wennerström, head of the department of medicine at Capio St Görans Hospital: for his talent and genuine interest in leadership and management. For providing probably the best terms for professional development of any department of medicine in Stockholm, making it possible to attend international congresses and courses to an extent clinicians at a university hospital I know well can only dream of.

My colleagues at the section of internal medicine at St Görans Hospital: for your patience as I finish this work during my first months as the new head of the internal medicine section.

Marika, my beloved wife: for bringing color, beauty and happiness to my life, for her loyal support, patience and simply for her enchanting presence during our 15 years of marriage, for being a perfect mother, compensating my being a quite often too busy father.

My daughters *Carin-Linnea* and *Miranda*: for being wonderful.

I would like to express my gratitude to the Swedish Cancer Foundation (Cancer Fonden) and the Swedish Children Cancer Society (BarnCancerFonden) for their support during these studies.

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