METABOLIC AND PHARMACOKINETIC STUDY TO ENHANCE BUSULPHAN THERAPEUTIC EFFICACY

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METABOLIC AND PHARMACOKINETIC STUDY TO ENHANCE BUSULPHAN THERAPEUTIC EFFICACY

THESIS FOR DOCTORAL DEGREE (Ph.D.)

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Say not, “I have found the truth,” but rather, “I have found a truth.”

Khalil Gibran (1883-1931)
Public Defense for Doctoral Thesis

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Lecture Hall Lissma

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ABSTRACT

Busulphan (Bu) is an alkylating agent used in high-doses as part of the conditioning regimen prior to hematopoietic stem cell transplantation (HSCT). HSCT is a curative treatment for hematological malignancies and other disorders. Bu can be administered by oral or intravenous route. The intravenous formulation contains high concentrations of dimethylacetamide (DMA), a potent solvent, but both hepatotoxic and neurotoxic. High-dose Bu treatment was shown to induce hepatotoxicity, which can develop to sinusoidal obstruction syndrome (SOS). Another side effect of high-dose Bu treatment is convulsions or generalized tonic-clonic seizures. The general aim of this thesis was to investigate the pharmacokinetics of Bu and DMA including their metabolites and understand the mechanisms underlying their toxicities.

In Study I. A liquid chromatography-mass spectrometry (LC-MS) based method was developed to quantify DMA and its primary metabolite methylacetamide (MMA) in human plasma. By following the FDA guidelines for bioanalytical method validation, we established a robust, selective, reproducible, and sensitive method for quantification of DMA and MMA. The calibration curves were linear between 1 and 4000 µM, and the limits of quantification were 1.8 and 8.6 µM for DMA and MMA, respectively. The developed method can be used for occupational exposure studies as well as monitoring both compounds in patients receiving drugs where DMA is present as an excipient.

In Study II. The pharmacokinetics of DMA and MMA was investigated in 18 pediatric patients receiving high-dose Bu treatment prior to HSCT. We used the LC-MS method developed in study I to quantify DMA and MMA in patients’ plasma. The results have shown accumulation of MMA throughout the treatment accompanied by an increase in the DMA clearance after the first dose administration of Bu. MMA had a slow elimination, and its half-life was calculated as 12.7h. In patients also, alanine transaminase (ALT) levels increased in more than 60% of the patients during conditioning with intravenous Bu. In vitro, cytotoxic tests on the hepatic cell line Huh 7 showed that the combination of Bu and MMA was more toxic than each compound separately. These findings imply that MMA might enhance Bu-induced hepatotoxicity.

In Study III. We investigated Bu-induced seizures, a neurotoxicity associated with high-dose Bu treatment. We quantified Bu and its four metabolites, tetrahydrothiophene (THT), THT 1-oxide, sulfolane, and 3-OH sulfolane in 18 patients receiving Bu prior to HSCT. The results showed that sulfolane, and to a less extend 3-OH sulfolane, were accumulated in patients throughout treatment with Bu. Sulfolane remained detectable up to 60h after last Bu administration. In mice, generalized seizures occurred in the group administered with sulfolane. Furthermore, pharmacokinetic studies in mice organs and plasma showed a high distribution of sulfolane into the brain where the ratio AUC brain/AUC plasma was the highest and calculated as 1.45. Neurotransmitters analysis in mouse brain showed that GABA and calbindin 28k levels were decreased in the group of mice injected with sulfolane. The levels of sulfolane in patients might have a role in potentiating Bu-induced seizures.

In Study IV. The role of N-acetylcysteine (NAC), a precursor of glutathione, on Bu-induced hepatotoxicity and the clinical outcome of HSCT was investigated. We evaluated the liver values, transplantation/conditioning complications and clinical outcome in two groups of patients receiving Bu (with and without NAC prophylaxis). The liver enzymes ALT, aspartate transaminase (AST) and alkaline phosphatase (ALP) were significantly decreased in the group of patients receiving Bu with NAC prophylaxis compared to the control group. Those levels were normalized even in patients who had high levels of liver enzymes before the start of Bu treatment. These observations suggest that NAC prophylaxis can potentially reduce Bu-induced hepatotoxicity without negatively affecting the clinical outcome of HSCT.
CONTENTS

I. Introduction ........................................................................................................... 9
   1. Cancer: definition and history ......................................................................... 9
   2. Theories of the cancer development .................................................................. 9
   3. Hematological malignancies ............................................................................. 10
   4. Treatment of hematological malignancies ....................................................... 11
   5. Hematopoietic stem cell transplantation ............................................................ 13
   6. Conditioning regimens, the good and the bad in HSCT ..................................... 13
   7. Busulphan, a key component in conditioning regimens for HSCT ...................... 14
   8. Dimethylacetamide, a solvent in the intravenous busulphan preparation .......... 17
   9. N-acetylcysteine, a potential hepato-protective agent for conditioning-induced hepatotoxicity in HSCT ............................................................. 18
  10. HSCT, an incomplete journey .......................................................................... 19

II. Aims ....................................................................................................................... 20

III. Methodology ....................................................................................................... 21
   1. Ethical implications ............................................................................................ 21
   2. High performance liquid chromatography-electrospray ionization mass spectrometry ............................................................................................................. 21
   3. Patients’ samples analysis .................................................................................. 22
   4. Patients’ clinical data analysis .......................................................................... 22
   5. Animal experiments and samples analysis ........................................................ 22
   6. Cell proliferation and cytotoxicity assay ............................................................ 23
   7. Statistical analysis .............................................................................................. 24

IV. Results and discussion ......................................................................................... 25
   1. Study I ............................................................................................................... 25
   2. Study II ............................................................................................................. 27
   3. Study III .......................................................................................................... 28
   4. Study IV ........................................................................................................... 30

V. Conclusions ......................................................................................................... 31

VI. Acknowledgements ............................................................................................ 32

VII. References ......................................................................................................... 33
LIST OF SCIENTIFIC PAPERS


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<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
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<tbody>
<tr>
<td>ALL</td>
<td>Acute lymphocytic leukemia</td>
</tr>
<tr>
<td>ALP</td>
<td>Alkaline phosphatase</td>
</tr>
<tr>
<td>ALT</td>
<td>Alanine transaminase</td>
</tr>
<tr>
<td>AML</td>
<td>Acute myeloid leukemia</td>
</tr>
<tr>
<td>AST</td>
<td>Aspartate transaminase</td>
</tr>
<tr>
<td>AUC</td>
<td>Area under the curve</td>
</tr>
<tr>
<td>BBB</td>
<td>Blood-brain barrier</td>
</tr>
<tr>
<td>Bu</td>
<td>Busulphan</td>
</tr>
<tr>
<td>CML</td>
<td>Chronic myeloid leukemia</td>
</tr>
<tr>
<td>CNS</td>
<td>Central nervous system</td>
</tr>
<tr>
<td>CTH</td>
<td>Cystathionine gamma lyase</td>
</tr>
<tr>
<td>Cy</td>
<td>Cyclophosphamide</td>
</tr>
<tr>
<td>CYP</td>
<td>Cytochrome P 450</td>
</tr>
<tr>
<td>DDI</td>
<td>Drug-drug interaction</td>
</tr>
<tr>
<td>DMA</td>
<td>Dimethylacetamide</td>
</tr>
<tr>
<td>ESI</td>
<td>Electrospray ionization</td>
</tr>
<tr>
<td>GABA</td>
<td>Gamma-aminobutyric acid</td>
</tr>
<tr>
<td>GC</td>
<td>Gas chromatography</td>
</tr>
<tr>
<td>GC-MS</td>
<td>Gas chromatography mass spectrometry</td>
</tr>
<tr>
<td>GSH</td>
<td>Glutathione</td>
</tr>
<tr>
<td>GST</td>
<td>Glutathione S-transferase</td>
</tr>
<tr>
<td>GVHD</td>
<td>Graft versus host disease</td>
</tr>
<tr>
<td>GVT</td>
<td>Graft versus tumor</td>
</tr>
<tr>
<td>HPLC</td>
<td>High performance liquid chromatography mass spectrometry</td>
</tr>
<tr>
<td>HSCT</td>
<td>Hematopoietic stem cell transplantation</td>
</tr>
<tr>
<td>JMML</td>
<td>Juvenile myelomonocytic leukemia</td>
</tr>
<tr>
<td>LC-MS</td>
<td>Liquid chromatography mass spectrometry</td>
</tr>
<tr>
<td>MAC</td>
<td>Myeloablative conditioning</td>
</tr>
<tr>
<td>Acronym</td>
<td>Definition</td>
</tr>
<tr>
<td>---------</td>
<td>------------------------------------</td>
</tr>
<tr>
<td>MDR</td>
<td>Multidrug resistance</td>
</tr>
<tr>
<td>MDS</td>
<td>Myelodysplastic syndrome</td>
</tr>
<tr>
<td>MMA</td>
<td>Methylacetamide</td>
</tr>
<tr>
<td>MTD</td>
<td>Maximum tolerated dose</td>
</tr>
<tr>
<td>NAC</td>
<td>N-acetylcysteine</td>
</tr>
<tr>
<td>NMAC</td>
<td>Non myeloablative conditioning</td>
</tr>
<tr>
<td>RIC</td>
<td>Reduced intensity conditioning</td>
</tr>
<tr>
<td>SOS</td>
<td>Sinusoidal obstruction syndrome</td>
</tr>
<tr>
<td>TBI</td>
<td>Total body irradiation</td>
</tr>
<tr>
<td>TDM</td>
<td>Therapeutic drug monitoring</td>
</tr>
<tr>
<td>THT</td>
<td>Tetrahydrothiophene</td>
</tr>
<tr>
<td>TRM</td>
<td>Transplant-related mortality</td>
</tr>
<tr>
<td>VOD</td>
<td>Veno-occlusive disease</td>
</tr>
</tbody>
</table>
I. INTRODUCTION

1. Cancer: definition and history

According to the National Institute of Cancer (NCI), cancer is a group of diseases in which cells are dividing uncontrollably (excessive proliferation), invading the adjacent tissues, and spreading to other parts of the body (metastasis) via blood circulation and the lymphatic system [1, 2]. The word “cancer” is originated from the Greek word for crab, “Karkinos” and was coined by the Greek physician Hippocrates (460-370 BC). The term was adopted by the Romans (cancer in Latin means crab) to explain malignant tumors [3, 4].

Historically, cancer is an ancient disease that has afflicted both humans and animals around the world. In fact, there is paleopathological evidence that exhibits the existence of cancer in prehistoric man and animals such as dinosaurs [3]. The earliest description of cancer in humans was discovered in an Egyptian papyrus that was sold to the antiquities dealer and collector Edwin Smith in 1862 [5]. This ancient surgical document was placed in a tomb in Egypt over 3000 years ago. Written around 1600 BC, the papyrus described 48 surgical cases of which 8 cases were breast tumors or ulcers; the document was based on an earlier reference dated 3000 BC. Other evidences of metastatic carcinoma were also found in the skeletons of mummies of ancient populations [6-10].

According to The International Agency for Research on Cancer (IARC), which is a part of the World Health Organization, cancer is the second leading cause of death worldwide; i.e. in every six deaths, one is caused by cancer [11].

2. Theories of the cancer development

Hippocrates established the first theory about the development of cancer as the humoral theory [4]. He believed that the human body consists of four types of fluids as humors (blood, phlegm, yellow bile and black bile) and that the correct balance between these humors is fundamental for maintaining the human health. Based on this theory, he described the development of cancer as a result of excess of dark bile in a particular body organ. The humoral theory was accepted for 1300 years and was dismissed during the middle ages [4]. Despite all, Hippocrates was the first to classify cancer to sarcomas and carcinomas. These terms are still used until today.

After dismissing the humoral theory and until the 1920s, several other theories such as lymph theory, parasite theory, chronic irritation theory and trauma theory were proposed to explain the development of cancer [4]. Upon discovery of DNA helical structure by the Nobel laureates Watson and Crick in the middle of the 20th century [12], scientists began to gradually unravel the complexity of cancer biology. Subsequently, the discovery of the two important gene families during the 1970s, oncogenes (involved in promoting cancer development), and tumor suppressor genes (controlling the cell proliferation), have increased our understanding about gene functions, transformations and mutations that can lead to different abnormalities, among them cancer [13-15].
3. **Hematological malignancies**

Hematological malignancies, known also as blood cancer, represent a heterogeneous group of cancers that affect the blood, the bone marrow, and the lymphatic system. Nearly all the hematological malignancies originate from the bone marrow. As shown in Figure 1 and 2, depending on the affected cell types, they are classified as myeloid neoplasms (myeloid lineage, Figure 1), or lymphoid neoplasms (lymphoid lineage, Figure 2). Although a variety of classifications have been described in the literature, in 2001, the World Health Organization (WHO) established a classification protocol that categorized and defined the different hematological malignancies according to their genetic abnormalities, morphological characteristics, and clinical features; this classification is updated every 8 years [16-19].

![Figure 1: World Health Organization Classification (WHO) of Myeloid Neoplasms (Adapted and modified from [18]).](image-url)
4. Treatment of hematological malignancies

A physics-based approach for the treatment of all hematological malignancies started in the beginning of the 20th century with the discovery of X-rays. The X-rays were used for palliative therapy in patients with Hodgkin disease [20]. On the other hand, the first chemical treatment for cancer was the systemic arsenic therapy performed by the Arab physician, Ibn Sina (known in the West as Avicenna) in the 11th century [21]. The modern era of cancer chemotherapy began in the 1940s as a result of observing the side effects of mustard gas on white blood cells (WBCs) of soldiers exposed to this chemical during World War-I and further the adverse effects of the same compound seen on the lymph nodes and bone marrow during World War-II [22]. Goodman and colleagues were able to temporarily induce tumor regression in patients with non-Hodgkin lymphoma using the more stable nitrogen mustard compounds such as beta-chloroethyl amines [23]. By the end of the 1940’s, Sidney Farber introduced the second group of anti-cancer drugs, folate analogues (known as antimetabolites) for the treatment of acute lymphatic leukemia in pediatric patients.

Today, more than 50 different chemotherapeutic agents categorized into 14 different classes are used for the treatment of hematological malignancies (Table 1) [24]. These agents are often used in different combinations depending on the diagnosis and prognosis of the malignancy as well as the age and health status of the patient. Based on these multidrug treatments, more than 70% of children with ALL and about 80% of children and adults with Hodgkin lymphoma are cured [24].
<table>
<thead>
<tr>
<th>Treatment</th>
<th>Mechanism of action</th>
<th>Usage</th>
<th>Example of Drugs</th>
</tr>
</thead>
<tbody>
<tr>
<td>Alkylating agents</td>
<td>Alkylate nucleic acids and cause apoptosis</td>
<td>Lymphoma, myeloma, leukemia</td>
<td>Busulphan (myleran®, Busulfex®), Cyclophosphamide (Cytoxan®, Neosar®)</td>
</tr>
<tr>
<td>Antitumor antibiotics</td>
<td>Interact with DNA and reduce cell survival</td>
<td>All blood cancers</td>
<td>Doxorubicin (Adriamycin®, Rubex®, Bleomycin (Blenoxan®)</td>
</tr>
<tr>
<td>Antimetabolites</td>
<td>Inhibit DNA /RNA synthesis and induce cell death</td>
<td>Most blood cancers</td>
<td>Fludarabine (Fludara®), Methotrexate</td>
</tr>
<tr>
<td>Antimitotic drugs</td>
<td>Inhibit the mitosis and induce apoptosis</td>
<td>Lymphoma</td>
<td>Vinblastine (Velban®), Paclitaxel (Taxol®)</td>
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<tr>
<td>Bisphosphonates</td>
<td>Prevent the bone resorption in myeloma</td>
<td>Myeloma</td>
<td>Pamidronate (Aredia®), Zoledronic acid (Zometa®)</td>
</tr>
<tr>
<td>Synthetic hormones</td>
<td>At high doses, kill malignant lymphocytes</td>
<td>Lymphoma</td>
<td>Dexamethasone (Decadron®, Prednisone (Deltasone®)</td>
</tr>
<tr>
<td>DNA-repair enzyme inhibitors</td>
<td>Target DNA repair enzymes and cause apoptosis</td>
<td>Lymphoma, leukemia</td>
<td>Etoposide (VePesid®, Etopophos®, Toposar®, Topotecan (Hycamtin®)</td>
</tr>
<tr>
<td>Histone deacetylase inhibitors</td>
<td>Inhibit histone deacetylase, induce histone hyperacetylation and in cause cell death</td>
<td>Leukemia</td>
<td>Vorinostat (Zolinza®)</td>
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<tr>
<td>Monoclonal antibodies</td>
<td>Target specific molecules on the malignant cells and cause apoptosis</td>
<td>Lymphoma, leukemia</td>
<td>Rituximab (Rituxan®), Gemtuzumab (Mylotarg®)</td>
</tr>
<tr>
<td>Bio modifiers</td>
<td>Exert immune or antiangiogenesis effects</td>
<td>Some blood cancers</td>
<td>Interferon-α (Roferon®A, Intron®A), Thalidomide</td>
</tr>
<tr>
<td>Cell-maturing agents</td>
<td>Cause maturation in leukemia cells</td>
<td>Leukemia</td>
<td>Tretinion (Vesanoid®), Arsenic trioxide (Trisenox®)</td>
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<tr>
<td>Phototherapy drugs</td>
<td>Stop malignant cell transformation by blocking mutant proteins</td>
<td>Skin lymphoma</td>
<td>Psoralen</td>
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<tr>
<td>Tyrosine kinase inhibitors</td>
<td>Tyrosine kinase inhibitors</td>
<td>Leukemia</td>
<td>Imatinib (Gleevec®), Dasatinib (Sprycel™)</td>
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<tr>
<td>Proteasome inhibitors</td>
<td>Inhibit the proteolytic function of proteasomes</td>
<td>Myeloma</td>
<td>Bortezomib (Velcade®)</td>
</tr>
</tbody>
</table>

**Table 1:** Classes of drug-based modalities used in the treatment of hematological malignancies (Adapted and modified from [24]).
5. **Hematopoietic stem cell transplantation**

Although the employment of chemotherapy in hematological malignancies has considerably improved the cancer treatment and increased the survival rate, the development of multidrug resistance or MDR (primary or acquired) [25, 26] has limited the success of curative potency of the chemotherapeutic agents. To improve treatment efficacy for hematological diseases and avoid the development of MDR, hematopoietic stem cell transplantation (HSCT) was introduced as a curative therapy for several hematological malignancies including those that are refractory to the conventional therapy [27-29]. Moreover, this treatment strategy is used for many acquired and congenital disorders of the hematopoietic system, as well as several immune and metabolic disorders [27-29]. The principle of HSCT lays in the replacement of the malignant/unhealthy hematopoietic stem cells in the patients either with their own healthy (isolated after induction treatment –autologous cells), or with other healthy donor stem cells (allogeneic or syngeneic). The first successful stem cell transplantation was performed by E.D. Thomas and his co-workers in 1959 [30]. The patient had an advanced stage of leukemia and received a preparative treatment with total body irradiation followed by infusion of bone marrow from her healthy identical twin (syngeneic HSCT). The transplant was successful and had a three-month remission [30]. Upon identification of the major histocompatibility complex molecules and typing of human leucocytes antigen (HLA)[31], several allogeneic bone marrow transplantations were successfully performed in the early 1960s [28, 32]. Currently, more than 40 000 allogenic HSCTs are performed in Europe annually [33]. The process of HSCT involves three main phases including:

1. Conditioning of the patient depending on the diagnosis and prognosis, the patient receives a specific conditioning treatment for the purposes of abolishing the bone marrow (myeloablation) and in some cases eliminating the malignant cells. The patient conditioning can also have a purpose of suppressing the recipient’s immune system (immunosuppression) [34, 35]. While myeloablation creates space for the new marrow, immunosuppression allows the engraftment and prevents the rejection of the transplanted marrow [34].

2. Intravenous infusion of HSCs which can be harvested from the bone marrow, peripheral blood or cord blood [34].

3. Monitoring the engraftment of the new marrow and determining the outcomes of HSCT [34].

Regarding the last stage, it is well known that although HSCT can be a curative treatment strategy, its efficacy is far from being satisfactory. For instance, several acute and/or chronic complications may emerge post HSCT [34, 35]. In fact, the occurrence and development of these complications (as detailed bellow) can hamper the success of HSCT [34-36].

6. **Conditioning regimens, the good and the bad in HSCT**

Conditioning regimen is a key element in both allogeneic and autologous HSCT. Treatment combination, protocol, length, and dosage of the selected drug/radiation depend on the transplantation center, diagnosis, age, remission and health status of the patient.
This preparative treatment can consist of various combinations of different myeloablative and immunosuppressive chemotherapeutic agents, with or without total body irradiation (TBI).

The aim of conditioning regimens with high intensity is to eliminate, or strongly reduce the number of malignant cells in order for the donor marrow to regenerate hematopoiesis in the cancer patients. [28]. However, as it became evident that donor immune cells play a substantial role against tumor cells in the host (graft-versus-tumor (GVT) effect), many investigators reduced the dose of radiation and chemotherapeutic agents used in the conditioning regimens in order to enhance the GVT effect [37]. Today conditioning regimens can be divided into three main categories: myeloablative (MAC), reduced-intensity (RIC), and non-myeloablative (NMAC):

1. **Myeloablative Conditioning (MAC)** generally involves a combination of cytotoxic drugs that are given at maximally tolerated doses in order to eliminate the recipient’s hematopoietic cells in the bone marrow. This type of regimen is associated with high myelotoxicity, and mainly includes combination of high doses of busulphan (Bu), cyclophosphamide (Cy), melphalan, thiotepa (TT), and etoposide with or without TBI. Some years ago, fludarabine was introduced to substitute cyclophosphamide in the combination (Bu/Cy) in order to minimize the Cy-related toxicities. Today, busulphan in combination with fludarabine is being used frequently [38, 39]. Another conditioning regimen designed to provide anti-lymphomal activity is the BEAM, which is a combination of the four alkylating agents: carmustine (BiCNu®), etoposide, cytarabine (Ara-C), and melphalan [38].

2. **Reduced Intensity Conditioning (RIC)** was introduced mainly to condition old patients who cannot tolerate the toxic effects of high dose treatment. Patients with diseases that are associated with high rates of non-relapse mortality (e.g., Hodgkin’s disease and myeloma) also receive RIC [40, 41]. Today RIC is also used for several metabolic and genetic disorders in pediatric patients [42].

3. **Non-Myeloablative Conditioning (NMAC)** induces less organ toxicity as it might reduce the risk of acute graft versus host disease (GVHD) by limiting the release of inflammatory cytokines and minimizing cytopenia [38].

Despite being a key component of HSCT, conditioning regimens are considered as the main risk factor for the development of many of acute and chronic HSCT-associated complications (Table 2) [36, 43]. In fact, these complications, in particular GVHD, relapse in malignancy, infections and drug-induced toxicities are the main causes for an increased mortality and morbidity following HSCT [35, 44].

**7. Busulphan, a key component in conditioning regimens for HSCT**

Busulphan (1, 4-butanediol dimethanosulfonate) is a bifunctional alkylating agent originally used at low doses for the treatment of some hematological malignancies such as chronic myeloid leukemia (CML) and polycythemia Vera (PCV) [45-47]. However, at high doses, busulphan has been a potent myeloablative agent (comparable to TBI) in conditioning regimens prior to HSCT [41, 48]. As part of various conditioning protocols, busulphan is either used orally or intravenously in doses of 0.8-1 mg/kg four times daily for...
two to four days [49]. Busulphan exerts its cytotoxic effects by inter or intra-strand DNA crosslinking mainly at guanine N7 position causing a disturbance in DNA replication and leading to cell death [50, 51].

Busulphan is mainly metabolized in the liver via conjugation with a key tripeptide antioxidant, glutathione (GSH) [52]. This conjugation is catalyzed by the glutathione S-transferase isoforms GSTA1, GSTM1, GSTP1, and GSTT1 (Figure 3) [53]. The formed conjugate, Bu-sulfonium ion of GSH, is unstable and rapidly broken down (non-enzymatic reaction) to form tetrahydrothiophene (THT). The conversion of Bu-sulfonium ion of GSH to THT can also be catalyzed by cystathionine gamma lyase (CTH) (Figure 3) [54]. The Bu-sulfonium ion of GSH conjugate can also form THT by the alternative mercaptopurate pathway forming N-acetylated-cysteine conjugate, a reaction that is catalyzed by the enzymes, dipeptidase/cysteinylglycinase (DPEP), gamma glutamyl transfrase (GGT) and N-acetyl transfrase (NAT) (Figure 3)[55]. An intermediate product of mercaptopurate pathway, THT-A (cysteine-S-conjugate [β-(S-tetrahydrothiophene L-alanine)], can undergo enzymatic conversion to form THT facilitated by CTH [56]. Furthermore, an in vitro study has shown that THT-A can slowly undergo a non-enzymatic beta-elimination reaction at pH 7.4 and 37°C to yield THT [56]. Finally, the formed THT is rather a lipophilic compound, and subsequently undergoes an oxidation to produce tetrahydrothiofene 1-oxide (THT 1-Oxide) (Figure 3). Cytochrome P 450 (CYP2C9) and Flavin-containing monooxygynase 3 (FMO3) were found to be involved in THT metabolism [57, 58]. The THT 1-oxide itself undergoes further oxidation resulting in the formation of sulfolane, a product that is further oxidized to form 3-hydroxysulfolane (Figure 3)[59-61] [55, 58]. Although busulphan is a lipophilic compound, some of it can be hydrolyzed in aqueous environment and converted to tetrahydofurane releasing methane sulfonic acid (Figure 3) [62].
Several pharmacokinetic studies have revealed a wide inter- and intra-individual variation of bioavailability in patients receiving busulphan orally [63]. The variability observed in busulphan kinetics was more pronounced in children compared to adult patients [59, 64-66]. Moreover, these studies have shown a great variability in drug exposure in those patients. Other studies have shown that low drug exposure can lead to engraftment failure and relapse while high exposure can be associated with the development of hepatic sinusoidal obstruction syndrome (SOS), formerly known as hepatic veno-occlusive disease (VOD) (Figure 4) [63-66]. In another investigation, a positive correlation was found between high plasma levels of busulphan and the increase of transplant-related mortalities (TRM) [67]. The optimal therapeutic window of busulphan was identified for a target concentration range of 600-900 ng/mL at a steady state (Figure 4) [55]. Thus, in patients with myeloid malignancies, therapeutic drug monitoring (TDM) and dose adjustment are recommended in order to reach the targeted drug exposure, which might result in better transplantation clinical outcome [65, 68]. With respect to busulphan toxicity, the major general and life-threatening complication associated with this drug is the development of SOS [49, 69]. Moreover, since busulphan is a lipophilic drug that can easily cross the blood-brain barrier (BBB) [70], neurotoxicity is also considered as an adverse effect of this drug [69, 71]. Indeed, one of the indications of busulphan-induced CNS toxicity is the development tonic-clonic seizures (busulphan-induced seizures) that occurs during the third and fourth days of conditioning in patients undergoing HSCT [72]. In order to avoid these CNS-related side effects, the conditioning regimen is accompanied with
anticonvulsant prophylaxis such as phenytoin, lorazepam, levetiracetam, or others [71-73]. However, studies have shown that the use of phenytoin increased busulphan clearance [71, 74, 75]. Moreover, it has been reported that seizures occurred in 1.3% of patients despite treating concomitantly with anticonvulsant prophylaxis [76]. Thus, further studies are required to elucidate the mechanisms underlying the development of busulphan-induced neurotoxicity and to develop better preventive strategies for this HSCT-associated complication.

**Figure 4:** The therapeutic window of busulphan.

### 8. Dimethylacetamide, a solvent in the intravenous busulphan formulation

Since busuphan is a water insoluble drug, its intravenous preparation requires a potent solvent [77]. Currently, the available intravenous buslphan (Busulfex™) is dissolved in 33% wt/wt dimethylacetamide (DMA) and 67% wt/wt polyethylene glycol 400. This formulation was developed by B.S. Andersson and co-workers to minimize the inter-individual variations observed by the treatment with oral busulphan, particularly in pediatric patients [63, 78]. Although the administration of the i.v. formulation has decreased the inter- and intra-individual variability of busulphan pharmacokinetics, the variability is still considerable [78, 79], [80]. Thus, intensive monitoring and dose adjustment is required for patients receiving busulphan, orally or intravenously, to improve the outcome of HSCT.

DMA (CH₃CN (CH₃)₂) is an organic colorless solvent that is used to dissolve high molecular-weight polymers and a wide range of organic and inorganic compounds. Because of its excellent solvent properties, DMA is widely used in chemical and pharmaceutical industries for the production of films, acrylic and elastane fibers as well as in the manufacturing process of some antibiotics such as cephalosporin.
In the 1960s, DMA was studied as a potential anti-tumor drug; however, severe liver toxicities (shown by elevated transaminase) along with neurotoxicity (manifested as hallucinations, confusion, and lethargy) were revealed [81]. These adverse reactions were observed at a dose around 400 mg/kg/day [81]. Most of the patients included in phase I studies experienced other side effects like nausea and vomiting. Additionally, studies have shown that DMA induced hepatic injury in workers exposed to DMA during spandex fibers manufacturing process [82].

In patients receiving intravenous busulphan, DMA can reach very high plasma levels, around 166 mg/kg/day, which represent 42% of the maximum tolerated dose (MTD). Whether or not these high plasma levels exert hepatotoxicity remains to be further investigated. Moreover, there is a lack of knowledge about the metabolism of DMA although it has been reported that DMA is metabolized in the liver by Cytochrome P450 2E1 (CYP2E1) to N-methylacetamide and acetamide [83].

9. N-acetylcysteine, a potential hepato-protective agent for conditioning-induced hepatotoxicity in HSCT

N-acetylcysteine (NAC) is an old and affordable nutritional supplement/medication [84, 85]. NAC is a thiol-containing compound that promotes glutathione biosynthesis and acts as a free radicals scavenger [86]. NAC is a stable, membrane permeable delivery system of cysteine (Figure 5). Upon administration, NAC undergoes deacetylation by deacetylases and provides L-cystein, which is one of the three essential amino acids for GSH synthesis (Figure 5) [87, 88]. NAC was first introduced as a mucolytic agent for treatment of lung diseases such as chronic bronchitis and cystic fibrosis [89, 90]. It is also used as a supporting treatment in combination with clomiphene citrate (CC) in CC-resistant polycystic ovary syndrome (PCOS) [91]. One of the most common uses of NAC is in treatment of acetaminophen-induced hepatotoxicity [92, 93]. Oxidative stress plays a pivotal role in neurodegenerative diseases; accordingly and with reference to different studies, NAC is also used to treat several neurological diseases [94, 95]. In HSCT, NAC is used as a prophylactic drug given concomitantly with cytostatic drugs such as busulphan. It has been shown that NAC might exert a protective effect for post transplantation complications such as sinusoidal obstruction syndrome (SOS) [96-98]. The main characteristics of conditioning-induced SOS include hepatomegaly, hyperbilirubinemia, ascites and weight gain that emerge 21-30 days after HSCT [99, 100]. SOS is a potentially lethal complication, and its incidence ranges from 5% to over 50% [101]. Thus, it is of high importance to establish preventive and therapeutic strategies to decrease the incidence of SOS, and thereby improve overall survival of patients undergoing HSCT. Busulphan metabolism consumes large amounts of GSH [102] causing the depletion of the latter can contribute to the development of SOS [103]. In line with that, it has been shown that GSH infusion to rats with SOS prevented the development of the disease [97].
In the 60 years since the first report on HSCT, the use of this treatment procedure for hematological malignancies and other blood disorders has increased dramatically. Indeed, the data from the Worldwide Network for Blood and Marrow Transplantation (WBMT) has shown that until 2012, more than 1,000,000 HSCT were performed at 1516 transplant centers in 75 countries [104]. Moreover, the results of HSCT are improving constantly; for instance, the overall mortality and the risk for developing transplant-related complications (such as GVHD, infections, and organ toxicity) have been significantly reduced [105]. Unfortunately, despite all improvements, significant morbidity and mortality still occur in many patients after HSCT. This clearly suggests that there are still some unresolved issues related to patients’ response to different treatment strategies which results in alteration of treatment efficacy and lack of complete remission of patients. The findings presented in this thesis focus on unraveling busulphan, DMA and metabolites-related toxicities, including toxicity due to its metabolites and to excipients. Unravelling and understanding the toxicities connected to the conditioning agents, will hopefully benefit patients undergoing HSCT.

**Figure 5:** NAC as a cysteine delivery system for GSH biosynthesis.
II. AIMS

**General Aim**

The overall aim of the study is to investigate the metabolism and the pharmacokinetics around busulphan treatment as part of the conditioning regimen prior to hematopoietic stem cell transplantation (HSCT), and elucidate the mechanisms underlying some of busulphan-related side effects.

**Specific Aims**

- To develop a robust and sensitive bioanalytical method for quantification of dimethylacetamide (DMA) and methylacetamide (MMA) in biological matrices.

- To investigate the pharmacokinetics and the toxicity of DMA and its metabolite MMA in patients receiving intravenous busulphan.

- To explore the mechanisms of neurotoxicity caused by busulphan and/or its metabolites.

- To evaluate the beneficial effects of N-acetylcysteine (NAC) on the hepatotoxicity and the clinical outcome of HSCT in patients receiving busulphan as part of conditioning regimen.
III. METHODOLOGY

1. Ethical implications

The content of this thesis describes findings from patients with hematological disorders. The samples collection from those patients was primarily for dose adjustment purposes of busulphan prior to HSCT at Drottning Silvias Barn och Ungdomssjukhus in Gothenburg, Sweden (study I and II) and the Center for Allogeneic Stem Cell Transplantation (CAST), Karolinska University Hospital in Huddinge (study III and IV). The further use/analysis of the samples, extra-samples and clinical data in study I, II, III, and IV was approved by the Regional Ethical Committee in Stockholm, and informed consents were obtained from the patients, parents, or legal guardians (Ethical Permits #: 616/03, 65/03).

The design of the animal experiments in paper III followed the principles of the 3Rs: replacing, reducing and refining. The animal experiments were performed after receiving the ethical approval from the Board of Agriculture in Stockholm (Ethical Permit #: ID 619).

2. High performance liquid chromatography-electrospray ionization mass spectrometry

Mass spectrometry is an analytical tool that can provide qualitative and quantitative information about the compound analyzed. The analyte can acquire positive/negative charges in the ionization chamber of the mass spectrometer; this step is called ionization. In the electrospray ionization mass spectrometer (ESI-MS), the ions are transformed from the solution to gaseous phase using electrical energy. Later on, the ionic species travel through the mass analyzer arriving to the detector where different signals are generated according to their mass/charge ratio (m/z). Those signals are recorded and graphically displayed on a computer system as a spectrum. The mass spectrum represents the relative abundance of the ions according to their m/z [104].

In study I, we validated a bioanalytical method using a Finnegan TSQ Quantum Ultra triple quadrupole mass spectrometer (MS) with electrospray ionization (ESI) coupled with high performance liquid chromatography (HPLC). Detecting and quantifying DMA and MMA, was achieved using a YMC Pack ODS-AQ/S-5 µm for separation and two mobile phases, acetonitrile (Phase A) and water (Phase B) with 0.1% formic acid. In order to acquire sensitivity, we optimized and tuned the MS parameters by flow injection analysis (Table 2).

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mode</td>
<td>Positive (+)</td>
</tr>
<tr>
<td>Ion spray voltage</td>
<td>5000 V</td>
</tr>
<tr>
<td>Sheath gas</td>
<td>20 arbitrary units</td>
</tr>
<tr>
<td>Auxiliary gas</td>
<td>5 arbitrary units</td>
</tr>
<tr>
<td>Capillary temperature</td>
<td>237 °C</td>
</tr>
</tbody>
</table>

Table 2: Optimization of different MS parameters for detection and quantification of DMA and MMA.
3. Patients’ samples analysis

In study I and II we used the validated HPLC-MS (ESI) based method to elucidate the pharmacokinetics of DMA and MMA. Therefore, we analyzed plasma samples from patients with different blood malignancies and other disorders. Those patients received high-dose intravenous busulphan (Busulfex) for a total period of four days followed by HSCT. In study III, samples (plasma and urine) were collected from patients who received oral busulphan prior to HSCT. We measured the concentrations of four busulphan metabolites, THT, THT 1-oxide, sulfolane, and 3-OH sulfolane, using gas chromatography mass spectrometry (GC-MS), a method that was developed by El Serafi et al [61]. The combination of GC and MS is ideal for generating quantitative and qualitative data for volatile and semi-volatile organic compounds. The method allowed us to follow the kinetics of the four metabolites in plasma and urine samples from 18 patients during the four days treatment with busulphan and 24h 48h and 72h after the last dose administration.

4. Patients’ clinical data analysis

Large volumes of important clinical data are generated daily on clinical care sites. The secondary use of this clinical information can improve and help understanding and answering the questions around many diseases and treatments. In this thesis, we performed retrospective analysis and compared a set of clinical data in patients treated with busulphan prior to HSCT. In study II we performed a comparison of different liver enzymes levels, such as ALT, AST and bilirubin, before and after treatment with intravenous busulphan. We also made the correlation between those levels and the clinical data from the patients, i.e., the outcome of transplantation and the occurrence of transplant/conditioning-related complications. Study IV included clinical data from 108 patients. In this study, the levels of the liver enzymes ALT, AST, ALP, and bilirubin were routinely measured and compared before and after busulphan conditioning in two groups, with and without prophylactic treatment with NAC. The clinical outcome of HSCT (i.e. relapse, graft failure, GVHD, SOS, and survival rate) was also compared in both patients groups.

5. Animal experiments and samples analysis

The use of animals for medical research is a longstanding practice. The physiological similarities between animals and humans have allowed investigating and understanding a large number of diseases, mechanisms, and novel therapies [107]. Mouse as an experimental animal model, has been mostly used by researchers not only to provide insight into elucidating different mechanisms underlying different diseases, but also to study the toxicology and pharmacology of potential candidate drugs before the transition to human testing [108].
The appropriate selection of the animal and strain is very important for different types of studies [105]. In order to understand the neurological side effects of busulphan and its metabolites, in study III, we used the mouse strain C57BL/6N because it is commonly used for behavioral studies [106]. The animals (kept in standard housing conditions) received busulphan or one of the four metabolites (THT, THT1-oxide, sulfolane, and 3-OH sulfolane) intraperitoneally, and were closely monitored and video recorded for different behavioral changes including the occurrence of seizures.

In the same study (study III), we used the same mouse strain to investigate and follow the kinetics of busulphan and the four metabolites in plasma and different organs. Measuring the levels of these compounds at different time point using a GC-MS based method developed by El-Serafi et al [61], would help identify the compounds responsible for the behavioral changes and occurrence of seizures. Furthermore, we analyzed mice brains for different neurotransmitters such as, serotonin, dopamine, glutamate, GABA, and calbindin D28K. The measurements of the neurotransmitters were performed using commercially available enzyme-linked immunosorbent assay (ELISA) according to the manufacturer’s instructions.

6. Cell proliferation and cytotoxicity assay

Cell culture or in vitro studies are the one key to the replacement and refinement of animal experimentation. It has been a rapid way of toxicological testing and screening of many candidate drugs for different diseases [107]. In study II, we performed cytotoxicity tests on two cell lines. As DMA was reported to have hepatotoxic effects [81], we decided to perform cell proliferation assay using the hepatic cell line Huh 7 after incubation with DMA, MMA and busulphan. We also investigated the cytotoxicity of those compounds in the promyelocytic leukemia cell line, HL-60. For monitoring the cells proliferation and viability in response to DMA, MMA, and busulphan at different concentrations and combinations, we used the tetrazolium based assay, WST-1. WST-1 is based on measuring
the activity of mitochondrial dehydrogenases that convert tetrazolium salt to formazan. The increase of formazan represents the correlation to the metabolically active cells [108].

7. Statistical analysis

In study I, the values are expressed as means ± standard deviation (STDV) for each step of the validation, the STDV was also converted to coefficient of variation (CV %) for each parameter (i.e. accuracy, reproducibility, stability, recover). In study II and IV we used a non-parametric t-test, or Mann Whitney to compare liver enzymes levels in two groups using GraphPad Prism 5. The same t-test was used further in study II for comparing AUC and clearance in two groups, after 1st dose and after 8th dose administration of busulphan. The different compounds concentrations measured patients specimens, in study II and III, were plotted using GraphPad Prism 5 and expressed as mean ± SEM. Animal experiments data analysis were expressed as mean ± SEM. The cytotoxicity in study II was estimated by calculating the half maximal inhibitory concentration (IC50), using nonlinear regression analysis (fit curve) on GraphPad Prism 5. All the pharmacokinetic parameters in study II and III were estimated using the PK/PD software WinNonLin.
IV. RESULTS AND DISCUSSION

1. Study I

DMA is a powerful solvent widely used in chemical and pharmaceutical industries to dissolve compounds with limited water solubility. In pharmaceuticals, DMA is used in the manufacturing process of certain antibiotics (i.e. cephalosporin)[109], and as an excipient in some drug preparations such as i.v busulphan (Busulfex or Busilvex), teniposide (Vumon), and Amsacrine (Amsidine)[110-112]. DMA has shown antitumor effects in vitro and in vivo [113, 114]. In patients, a phase I study of DMA has revealed that its administration exerted antitumor effects where two patients have shown response to the treatment [81]. Moreover, a recent study has shown that DMA acts as a bromodomain ligand, and therefore induced anti-osteoporotic and anti-inflammatory effects [115]. During the phase I clinical trials, DMA revealed several toxicities (i.e. hepatotoxicity, neurotoxicity), and some of those toxicities were seen in spandex fibers workers [82]. Accordingly, following the pharmacokinetics of this compound in biological matrices is important due to its impact on safety and human health. Therefore, several methods have been developed to quantify DMA in plasma using GC-MS and LC-MS [116, 117]. MMA, the primary metabolite to DMA, was also quantified previously in urine from workers occupationally exposed [118]. We developed and validated an LC-MS based method according to the FDA guidelines for bioanalytical method development [119]. We validated this method in human plasma, and the results obtained from the accuracy, reproducibility, stability, and recovery tests, have met the criteria described in the guidelines (Table 3). Furthermore, the sample preparation is fast and simple, and allows a relatively quick quantification of DMA and MMA in the same sample simultaneously using the same instrument parameters and internal standard, DMA-d9. The limits of quantification (LOQ) for DMA and MMA were 1.8 µM and 8.6 µM, respectively. The calibration curves were linear for both compounds and cover a concentration range between 1 and 4000 µM (12 points/concentrations); the correlation coefficient was $r^2=0.9966$ for DMA, and $r^2=0.9911$ for MMA. These values represent the sensitivity of our method and the ability to measure not only low plasma concentrations of these two compounds, but also relatively high concentrations which can also be used for environmental analysis. In conclusion, the new LC-MS method is a reproducible, sensitive, selective and robust tool that can be used in further investigations about DMA and MMA. It can also be used in occupational exposure studies in workers of different DMA-related products manufacturing. In a clinical setting, patients receiving high-dose Bu treatment are exposed to high concentrations of DMA; our method can help monitor the exposure in this group of patients and therefore avoid undesirable side effects.
### Table 3: Summary results for the bioanalytical method validation parameters

CV=Coefficient of variation (Lowest-Highest).

<table>
<thead>
<tr>
<th>Validation Parameter</th>
<th>DMA CV (%)</th>
<th>MMA CV (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Accuracy</td>
<td>0.74-2.92%</td>
<td>6.67-8.16%</td>
</tr>
<tr>
<td>Reproducibility</td>
<td>1.51-5.42%</td>
<td>2.14-6.90%</td>
</tr>
<tr>
<td>Stability 4°C</td>
<td>1.24-3.52%</td>
<td>2.01-5.24%</td>
</tr>
<tr>
<td>Stability -20°C</td>
<td>1.94-6.75%</td>
<td>3.43-4.50%</td>
</tr>
<tr>
<td>Post Preparation</td>
<td>0.89-3.00%</td>
<td>4.30-14.03%</td>
</tr>
<tr>
<td>Recovery</td>
<td>97-100%</td>
<td>76-100%</td>
</tr>
</tbody>
</table>
2. Study II

High-dose busulphan treatment is an essential part and one of the most common conditioning regimens administered to patients prior to HSCT. Patients receiving oral busulphan have shown a large intra-individual variation; therefore, the i.v formulation was introduced to assure high bioavailability and minimize the variability between individuals [78, 110, 120]. DMA is present at high concentrations in the i.v formulation of busulphan, and the total dose for 4 days treatment is approximately 1 g/kg (for 16 mg/kg total dose of busulphan). It has previously been reported that DMA exerts several toxic effects such as gastrointestinal toxicity (nausea, vomiting, and anorexia) [81]. Several patients displayed hepatotoxicity during phase I studies of DMA when administered with 400 mg/kg/day for 3 days [81]. The same dose of DMA induced neurotoxic effects manifested in depression, lethargy, confusion and hallucinations [81]. During conditioning treatment, patients receive between 3.2-4.8 mg/kg/day of busulphan over 4 days depending on the body weight; this dosage translates to 166-248 mg/kg/day for four days of DMA which represents 42-62% of the maximum tolerated dose [121-123]. It is possible that there is a relative contribution of DMA and/or its metabolite MMA to the hepatotoxicity and neurotoxicity-related to busulphan conditioning. Accordingly, the results in our study have revealed that MMA, the primary metabolite of DMA, was accumulated in patients receiving i.v busulphan and still detectable even 40h after the last dose administration. In contrast, DMA was cleared from the blood within 12h post-administration, and its clearance increased throughout the treatment period. The results of cytotoxicity analysis in the hematological and hepatic cell lines (HL-60 and Huh 7) showed that DMA and MMA exert cytotoxic effects in both cells lines. The IC_{50} values in HL-60 and Huh 7 respectively were 20 mM and 52 mM for DMA and 44 mM and 70 mM for MMA. Furthermore, in vitro experiments showed that busulphan dissolved in DMA and combined with MMA exerted the most cytotoxic effects on Huh 7. We therefore compared the liver enzymes before and after busulphan conditioning and found the following: a significant increase in ALT, which is an enzyme indicating liver toxicity (Figure 6). Considering the relatively long half-life of MMA (≈ 12.7h), the presence of the 3 compounds (DMA, MMA and busulphan) at the same time in patients raises the question of possible combined toxicities. Additionally, the presence of MMA in patients’ blood for 40h after the end of busulphan conditioning should be investigated further for possible drug-drug interactions (DDI) with the next treatment.

![Figure 6](image-url)  
**Figure 6:** Comparison of ALT levels before and after high-dose i.v busulphan in 18 patients.
3. Study III

Convulsions or busulphan-induced seizures, are one of the side effects associated with high-dose busulphan conditioning [72]. Since approximately 10% of patients receiving high-dose busulphan experienced generalized seizures, prophylactic treatment was introduced concomitantly with the conditioning [71, 72, 124, 125]. The underlying mechanism of busulphan-induced seizures is not fully understood. In this study, we investigated the pharmacokinetics of busulphan and its metabolites, THT, THT 1-oxide, sulfolane, and 3OH-sulfolane in 18 patients receiving high-dose treatment with busulphan. Although busulphan kinetics have been intensively studied, there is only little information available about the pharmacokinetics of its metabolites. The method developed by El-Serafi et al [61] has allowed us to follow the kinetics of these compounds in patients and mice. The results from this investigation have revealed accumulation of the two busulphan metabolites, sulfolane and 3-OH sulfolane, in plasma from patients. These two metabolites, particularly sulfolane, showed the highest plasma concentrations in patients; these levels remained relatively high and quantifiable even 40h after the last busulphan dose administration. It has been reported that sulfolane presented some neurotoxicities, such as hypothermia, in rodents [126], and we confirmed those findings in the group of mice intraperitoneally injected with sulfolane. In addition to hypothermia, generalized seizures also occurred in mice administered with sulfolane. The pharmacokinetics of busulphan and its metabolites in mice organs showed that the highest ratio AUC brain/AUC plasma belonged to busulphan and sulfolane, indicating the high distribution of both compounds to the brain. The findings regarding the high levels of sulfolane measured in patients warrant for further investigations on the role of sulfolane in busulphan-induced neurotoxicities. Furthermore, these levels could raise questions about possible DDI between sulfolane and concomitant medications and/or the next treatment. The neurotransmitters measurements results have shown that Calbindin 28K levels were low in brains from sulfolane and 4 day busulphan groups, while GABA was reduced in the sulfolane group only. In fact, it has been reported previously that calcium-binding protein levels were reduced in kindled rats [127]. Additionally, seizures are also associated with a decrease in the levels of inhibitory neurotransmitter GABA [128, 129]. In the counterpart, and given the anticonvulsant effect of dopamine, its high levels in the brain in the sulfolane and 4 day busulphan mice groups could have been for reversing the seizures [130-132].
Figure 7: Plasma concentrations of busulphan metabolites in 18 patients throughout conditioning with busulphan and up to 72h after last dose administration.
4. Study IV

Busulphan is metabolized through GST-catalyzed conjugation with GSH; hence, the latter is depleted from hepatocytes by 60% in vivo and 50% in vitro[102]. In patients, it has been suggested that the depletion of GSH in the hepatocytes may augment the cyclophosphamide-induced liver injury [133]. NAC is a precursor for GSH synthesis, and therefore it increases the cellular content of GSH [86]. NAC is widely used as a treatment to reverse acetaminophen toxicity [134], and it was suggested to be used as a treatment for SOS [135]. The main purpose of using NAC concomitantly with the busulphan conditioning is to enhance the GSH synthesis and prevent its depletion that might afterwards contribute to conditioning-related toxicities, particularly hepatotoxicity. Our study was retrospective; we followed the clinical outcome, conditioning/transplantation-related complications, and liver enzymes (AST, ALT, ALP, and Bilirubin) in 108 patients. In this study, we compared a group of patients (n=54) that received conditioning using oral busulphan together with prophylactic treatment with NAC, to the second group that received oral busulphan without prophylactic treatment (control group). The clinical outcome of the transplantation was not affected by absence/presence of prophylaxis with NAC. However, the liver enzymes (ALT, AST, and ALP) were significantly decreased (normalized) after the end of busulphan conditioning in the NAC treated group, indicating less hepatotoxicity, compared to the untreated group (Figure 8). From these observations, we can conclude that NAC is a potential prophylactic treatment for hepatotoxicity during conditioning with busulphan.

**Figure 8:** The mean aspartate transaminase (AST), alanine transaminase (ALT) and alkaline phosphatase (ALP) values before and after busulphan conditioning for patients treated with N-acetyl-l-cysteine (NAC) versus the control group.
V. CONCLUSIONS

Study I

- We developed and validated, according to the FDA guidelines for bioanalytical method validation, a sensitive and robust LC-MS based method for simultaneous quantification of DMA and MMA in plasma.
- The Method can be used for occupational exposure studies as well as monitoring DMA and MMA in patients receiving DMA-containing medications.

Study II

- DMA clearance was increased throughout treatment with intravenous busulphan as part of the conditioning treatment prior to HSCT.
- MMA was accumulated in patients throughout the treatment period with busulphan and was still quantifiable 40h after the last dose administration of busulphan.
- The liver enzyme ALT levels were increased after the end of conditioning with intravenous busulphan.
- Hepatic-derived cells Huh 7 were more sensitive to the combination busulphan-MMA than busulphan or MMA alone.
- Conditioning with intravenous busulphan, MMA might increase the busulphan-induced hepatotoxicity.

Study III

- Sulfolane, and to a lesser extent 3-OH sulfolane, exhibited accumulation in patients receiving busulphan prior to HSCT.
- Sulfolane induced generalized seizures in mice.
- The inhibitory neurotransmitter GABA and the calcium binding protein (Calbindin 28K) were low in the group of mice injected with sulfolane.
- The busulphan-induced seizures might occur as a result of sulfolane accumulation in patients.

Study IV

- NAC prophylaxis normalized the liver enzymes levels, ALT, AST and ALP in patients during treatment with busulphan prior to HSCT.
- The clinical outcome of the transplantation was NAC-independent.
- Prophylaxis with NAC might prevent busulphan-induced hepatotoxicity in patients undergoing HSCT.
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