Anticancer Activity of Melflufen

Preclinical Studies of a Novel Peptidase-Potentiated Alkylator

SARA STRESE
Melflufen (melphalan flufenamide, chemical name L-melphalanyl-p-L-fluorophenylalanine ethyl ester hydrochloride, previously called J1) is a derivative of the classical alkylating agent melphalan. Melflufen is potentiated by hydrolytic cleavage by aminopeptidase N (APN), leading to high intracellular concentrations of alkylating moieties and subsequent cell death. Increased APN expression is associated with the malignant phenotype of several human cancers, including acute myeloid leukemia, lymphoma and ovarian cancer, and plays a functional role in tumor angiogenesis. Therefore investigations of melflufen activity in these malignancies as well as detailed studies of inhibition of angiogenesis are interesting. The aim of this project was to investigate the cytotoxic and antiangiogenic effect, in vitro and in vivo, of melflufen, compared to melphalan and other cytotoxic drugs used in the clinic.

We showed that melflufen was more effective than its parental drug melphalan in lymphoma, AML and ovarian cancer in cell lines as well as in primary patient samples. An improved in vitro therapeutic index was demonstrated by an increased cytotoxic activity in the patient samples compared to normal peripheral blood mononuclear cells (PBMCs). Furthermore, melflufen in combination with cytarabine was synergistic in an AML cell line in a sequence-dependent manner. Melflufen was shown effective in several animal models using lymphoma, AML and ovarian cell xenografts (single drug or in combination), including an intraperitoneal ovarian xenograft. Finally, we demonstrated that melflufen had antiangiogenic properties in several different models.

Keywords: cancer therapy, preclinical studies, melflufen, aminopeptidase-potentiated, cancer, angiogenesis
Do. Or do not. There is no try

-Yoda
List of Papers

This thesis is based on the following papers, which are referred to in the text by their Roman numerals.


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Abbreviations

AML  Acute Myeloid Leukemia
APN  Aminopeptidase N
BCE  Bovine Capillary Endothelial
BLI  Bioluminiscence Intensity
BRCA Breast Cancer
CAM  Chorioallantoic Membrane
DIVAA Directed In Vivo Angiogenesis Assay
DMSO Dimethyl Sulphoxide
EMA  European Medicines Agency
FACS Fluorescence-Activated Cell Sorting
FDA  Fluorescin Diacetate
FDA  Food and Drug Administration
FGF  Fibroblast Growth Factor
FMCA Fluorometric Microculture Cytotoxicity Assay
FMCA-GM FMCA-Granulocyte Macrophage
GFP  Green Fluorescent Protein
HPLC High Performance Liquid Chromatography
HUVEC Human Vein Umbilical Endothelial Cells
IC$_{50}$ Half maximal Inhibitory Concentration
IP  Intraperitoneal
J1  Melflufen (L-melphalan)-p-L-fluorophenylalanine ethyl ester
MS/MS Tandem Mass Spectrometry
MTT  3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide
NHL  Non Hodgkin’s Lymphoma
OC  Ovarian Cancer
PARP Poly (ADP-Ribose) Polymerase
PDX  Patient Derived Xenograft
PBMC Peripheral Blood Mononuclear Cell
PBS  Phosphate Buffered Saline
PTC  Peptichemio
RWD Relative Wound Density
SD  Standard Deviation
SEM  Standard Error of the mean
SI  Survival Index
SP  Subperitoneal
sPCI  simplified Peritoneal Carinomatosis Index
VEGF Vascular Endothelial Growth Factor
Introduction

Every year about 58 000 people in Sweden is diagnosed with cancer, where-of 48 000 for the first time [1]. Cancer is the collective name of about 200 different diseases, which is characterized by abnormal cell growth and the ability to spread metastases throughout the body. The term cancer is a common name for both hematological malignancies (such as leukemia) as well as different solid tumors, the latter consisting of proliferating cells (parenchyma), and the stroma (connective tissue and blood vessels).

Surgery and radiation are the oldest and most used approaches for cancer therapy, often in combination with chemotherapeutic drugs. Treatment is specific for each location and tumor type, to give the greatest cytoreductive effect and to minimize the normal cell toxicity. To maximize tumor-killing effect, and limit drug-specific toxicity, combination regimens with two or more compounds is considered as standard treatment in most cases.

In the early 1930s the first clinical trial for cancer treatment was conducted on a sulfur mustard solution [2], thus initiating the future development of chemotherapy. Although the earliest clinical trials was abandoned because of toxicity, research continued, and in 1942 a successful clinical study on nitrogen mustards was presented [3]. Since then, many chemotherapeutic drugs, with various mechanisms of action, have been developed for clinical use. The most common classical approach is targeting cellular nucleic acids of proliferating cells. Cancer cells do indeed proliferate more rapidly than most normal cells, and therefore chemotherapeutic drugs displays preferential activity towards the malignant cells. Unfortunately high general toxicity of other renewing normal cells such as bone marrow, hair follicles, gastrointestinal mucosa and germ cells is also common, contributing to the narrow therapeutic index of most chemotherapeutics. Because the systemic effect of presently used chemotherapy is associated with high toxicity, it is important to find new chemotherapeutic drugs with selectivity towards malignant cells and with a broader therapeutic index.

Melphalan

In the early fifties work was begun to find cytoactive amino acids and their peptide derivatives that might selectively target tumor cells by interfering with the nucleic acid or protein metabolism of malignant cells [4]. A series
of publications presented synthesis of several amino acid phenylalanine derivatives, and later the highly active L-form of \( p\)-\(bis\)(2-chloroethyl) amino-L-phenylalanine, or melphalan, was chosen as lead compound [5].

Since the start of the development 60 years ago, melphalan has been used as an important part of treatment of a variety of cancers and as part of several combinations regimens, today the clinical use is mostly limited to multiple myeloma and some high dose myeloablative regimens prior to stem cell transplantation. Melphalan is a nitrogen mustard that spontaneously forms reactive aziridium ions of both chloroethyl groups. These ions binds covalently to intracellular nucleophilic guanine bases on DNA, leaving the DNA unavailable for replication and transcription and thereby killing the cell [6]. Melphalan is a bifunctional agent able to crosslink a DNA strand within a double helix (intrastrand), between two strands (interstrand) or between DNA and proteins [7]. Despite the similar mechanism of action of all alkylating agents, potency, bio-distribution and normal tissue toxicity differs greatly, probably due to the differences in non-alkylating portions of these drugs [7]. The short half-life of melphalan, around 1.5 hours in physiological buffer at 37°C, might be explained by the formation of reactive intermediates [8]. There is a large variety in the absorption of melphalan when administered orally, with a bioavailability ranging between 56 and 85% and \(C_{\text{max}}\) reached within 0.5 to 2.0 hours [9].

**Melflufen**

A cocktail of six interesting sarcloysine (a stereoisomer of melphalan) containing peptides, called Peptichemio (PTC), was synthesized [10] and studied in several preclinical and clinical trials in the end of the seventies, before it was abandoned. When studying the peptides of PTC individually, it was discovered that the tripeptide L-prolyl-\(m\)-L-sarcolysin-\(p\)-L-fluorophenylalanine ethyl ester (P2) was the most effective of the six and even more effective than melphalan [11-13].

In the light of the successful PTC-peptide P2, a series of new alkylating nitrogen mustard dipeptides was synthesized and analyzed for cytotoxic activity. It was found that the different compounds did not differ in activity only because of their chemical reactivity, but also because of biological interactions like membrane transport and enzymatic liberation of reactive units [14]. The dipeptide melflufen (L-melphilanyl-\(p\)-L-fluorophenylalanine ethyl ester, previously called J1) was synthesized as an intermediate of melphalan and P2 [15], and was soon chosen as the lead compound. Chemically, melflufen can be described as the ethyl ester of a dipeptide consisting of melphalan and \(para\)-fluoro-L-phenylalanine. Melflufen was shown to be more cytotoxic than both melphalan and P2 in cell line and patient sample assays, as well as having a more rapid onset of action [15]. When melflufen
is hydrolyzed, it leads to high intracellular concentrations of alkylating moieties, i.e. melphalan, which interact with DNA inside the tumor cells. Exposure of melflufen to various malignant cells in vitro resulted in at least a 10-20 fold superior intracellular concentration of melphalan in comparison with direct treatment with equimolar doses of melphalan, indicating a “trapping” mechanism. The intracellular entry was rapid as the hydrolysis occurred only a few minutes after melflufen entered the cell. The intracellular melphalan (liberated from melflufen) $C_{\text{max}}$ was reached after 15 minutes and the half life was approximately 1 hour [16]. Antitumor activity and acute toxicity of melflufen was investigated in a hollow fiber in vivo assay. Melflufen expressed significant antitumor activity superior to melphalan in several cell line implantations, with minor effects of toxicity (no difference towards melphalan) [17]. When melflufen was tested on patient samples from different diagnoses it was shown that melflufen was more active than melphalan in all cases, especially in breast cancer, non-Hodgkin’s lymphoma and acute myeloid leukemia (AML). Synergistic interactions in vitro in several cell lines of different origin was also revealed when melflufen was analyzed in combination with a panel of drugs with different mechanisms of action [18]. Furthermore, melflufen showed significant activity in neuroblastoma [19] and multiple myeloma [20] in vitro and in vivo, encouraging further clinical development in both patient groups to increase therapeutic response, reduce side effects and improve patient outcome.

It was shown that inhibition of peptidase activity resulted in a decreased activity of melflufen and dipeptide derivatives designed to resist the action of peptidases, were less active than the corresponding normal (melflufen like) dipeptide [16]. It was later confirmed that hydrolysis by the metalloproteinase aminopeptidase N (APN) and esterases potentiated the activity of melflufen, releasing its alkylating moieties (i.e. melphalan; Figure 1), and resulted in an increased cytotoxic activity of melflufen over melphalan. Manipulation of APN expression in human tumor cell lines altered melflufen-mediated pro-apoptotic signaling and cytotoxicity, providing evidence that APN-mediated cleavage of the dipeptide is an important mediator of its activity [21].
Figure 1. The chemical structure of melflufen (J1) and the cleavage by aminopeptidases to melphalan and p-Fluorophenylalanin (pFPhe), or by esterases to des-ethylmelflufen (Mel-pFPhe-OH). Figure from Wickström et al. [21] with permission.

Aminopeptidase N

Several types of human malignancies, especially those with a fast-growing and aggressive phenotype, display an increased expression of various hydrolytic enzymes such as peptidases, esterases and proteases [22]. Aminopeptidases are metalloproteinases that remove amino acids from unblocked N-terminal positions of oligopeptides. These proteinases have a broad substrate specificity and are extensively spread [23, 24]. One of the most noted enzymes is the metalloprotease aminopeptidase N (APN; EC 3.4.11.2, also known as CD13), a membrane-bound cell surface protein over-expressed in several different hematopoietic and solid malignancies, which is involved in cellular processes such as uncontrolled cell growth, invasion and metastasis [23-28]. APN is also associated with angiogenesis and is up regulated in response to hypoxia and angiogenic growth factors, but not in normal blood vessels and tissue [29-32]. Since APN cleaves melflufen to a more potent alkylating moiety inside the tumor cell or in environment with high concentration of APN [21], it is indicated that melflufen is clinically useful as a chemotherapeutic and anti-angiogenic drug for cancer therapy.
Aminopeptidase inhibitors in cancer therapy

APN is evidently dysregulated in many human malignancies, and therefore it has been suggested as a suitable target for anti-cancer therapy and development of novel anticancer drugs. Currently, there are no approved aminopeptidase inhibitors for anti-cancer therapy by the European Medicines Agency (EMA) or the American Food and Drug Administration (FDA), although the aminopeptidase inhibitor bestatin (Ubenimex) has been used in Japan as an antitumor drug for lung cancer and AML [33]. There are also many APN-targeted therapeutic drugs under clinical and late pre-clinical development [27]. Two main approaches for aminopeptidase targeting have been suggested; by direct inhibition or via prodrugs, which are enzymatically metabolized into pharmacologically active acid products [27]. Bestatin is the most studied direct aminopeptidase inhibitor, which in clinical trials has shown to be generally well tolerated with mild adverse events in diseases like lymphomas [25, 28, 34], AML [25, 28] and ovarian cancer [35-38].

Diagnoses of interest

Three cancer diagnoses were of special interest in this thesis, namely lymphoma, acute myeloid leukemia (AML) and ovarian cancer (OC).

Lymphoma

Lymphoma originates from malignant cells in the lymphatic system, and is classified into many different entities. First line chemotherapy, usually including alkylators, are combination treatments such as R-CHOP for Diffuse Large B-cell Lymphoma (DLBCL) and ABVD for Hodgkin’s lymphoma. Generally chemotherapy may be combined with radiotherapy and early relapse patients at young age are often considered for high dose chemotherapy and stem cell transplantation. Despite the relative high survival rate in lymphoma patients, many patients still relapse in the disease, posing a great need for new drugs with improved results and reduced toxicity [39, 40].

Increased APN expression is associated with anaplastic large cell lymphoma [41, 42], and it has been shown that the aminopeptidase inhibitor bestatin inhibits the lymphoma proliferation in vitro [25, 34] and in vivo [25]. This promoted further investigations of the APN-potentiaged drug melflufen in lymphoma.

Acute myeloid leukemia

Acute myeloid leukemia (AML) is a rapidly growing hematologic malignancy divided into several different subgroups. AML originates from hemato-
poietic blast cells in the bone marrow, leading to drastic inhibition of normal hematopoiesis [43]. Initial treatment of AML consists of the anti-metabolite cytarabine in combination with an anthracycline, usually daunorubicin.

Extensive expression of APN has been described in all AML subtypes [25, 44-47], where it has been found on the cell surface, in the cytoplasm [48] and in microvesicles [49]. The aminopeptidase inhibitor bestatin has been clinically approved for AML in Japan [33], and has shown therapeutic efficacy and survival benefits, with mild adverse events in clinical trials [25, 28].

Ovarian cancer

Ovarian cancer (OC) is the collective name of malignancies located around the ovaries. Ovarian cancer is often diagnosed in a late stage, due to mild clinical symptoms [50]. Surgery, carboplatin/cisplatin and/or paclitaxel-based chemotherapy are the primary treatments for ovarian cancer [51], but historically melphalan has been an important therapeutic alternative [52]. The high mortality rate of ovarian cancer patients [40, 53] illustrates the need for new chemotherapeutic options. Given that ovarian cancer mostly remains confined in the peritoneal cavity, locoregional administration of intraperitoneal (IP) chemotherapy has been investigated, allowing higher peritoneal fluid concentrations leading to less systemic toxicity [51, 54, 55].

Increased expression of APN has been observed in tumor-associated blood vessels in ovarian serous or mucinous carcinoma [35, 37, 38]. APN is expressed to a higher extent prior to chemotherapy, with less APN expression post treatment [36], suggesting APN as a target for chemotherapy in ovarian carcinoma patients.

Ovarian cancer usually remains confined to the peritoneal cavity. Catheter-delivered intraperitoneal (IP) chemotherapy [56-59], or intraoperative chemoperfusion [60-62] in combination with cytoreductive surgery and intravenous chemotherapy has demonstrated a significant survival benefit. Intraoperative chemoperfusions also permits higher chemotherapy concentrations, offering beneficial treatment compared to intravenous chemotherapy, because of less systemic toxicity [51, 54]. As melphalan is a promising agent for aggressive and recurrent peritoneal surface malignancies [63], it is postulated that the superior tissue penetration and lipophilicity of melflufen and susceptibility to enzymatic hydrolysis would make melflufen an ideal intraperitoneal agent.

Angiogenesis

The inability to provide adequate oxygen concentration to the tumor cells is caused by variable blood flow and obstructions in the tumor vessels. Initially the vessels do not expand as a function of tumor growth and therefore the
exchange of oxygen, nutrients and waste products is reduced. Later, pre-existing host vessels are incorporated, through migration and proliferation of endothelial cells and re-arrangements of extra-cellular matrix, into the tumor tissue but are to some extent compressed or obstructed by the growing tumor [64]. This neovascularization, or angiogenesis, is required to keep the growing tumor oxygenated and differs from normal physiological angiogenesis [65]. Increased pathological angiogenesis is correlated with tumor metastasis and decreased patient survival [66]. The new tumor micro-vessels are hastily formed and can therefore show rigorous structural malformations. The blood-flow in these vessels becomes irregular due to blind ends, leakage or lack of smooth muscle, and the vessels can be dilated or have incomplete membranes [64, 67]. Angiogenesis is normally minimal in non-malignant tissue in adults, but may be activated by controlled processes, e.g. wound healing.

Several angiogenesis factors, such as vascular endothelial growth factor (VEGF), act to mediate signals of vascular formation in endothelial cells as well as to increase the vascular permeability to enhance the availability of oxygen and nutrients from capillaries [68, 69]. The delicate balance between activators (such as VEGF) and inhibitors of angiogenesis regulate an “angiogenic switch” in growing tumor nodules [70]. The aminopeptidase APN is up-regulated in response to hypoxia, angiogenic growth factors and tube formation signals [29, 30], and regulates proliferating endothelial cells and angiogenic tube formation but does not effect normal cells and tumor cell lines [32]. This suggests APN as a promising target for anti-angiogenic therapy in cancer treatment.
The aims of this project was to investigate:

- The cytotoxic effect, *in vitro* and *in vivo*, of melflufen compared to melphalan and other standard chemotherapeutic drugs in lymphoma, AML and ovarian cancer.
- The effect of melflufen in combination with clinically relevant cytotoxic drugs for these diagnoses.
- The possibility of using melflufen in localized intraperitoneal treatment.
- The antiangiogenic effect of melflufen, useful in the treatment of cancer.
Methods

This section is a summary of the methods used in this thesis, for further details see individual papers.

Drugs
Melflufen was a kind gift from Oncopeptides AB (Stockholm, Sweden). Melphalan was purchased from the local pharmacy as Alkeran and dissolved according to the manufacturer’s instruction, or as a pure chemical dissolved in DMSO. Because of the rapid mustard hydrolysis of melflufen ($t_{1/2} = 1.6$ hours) and melphalan ($t_{1/2} = 2.1$ hours) all water dilutions were instantly frozen (-70 °C) or prepared immediately prior to use [71]. All additional drugs and chemicals used in the studies were bought as pharmaceutical preparations from the local pharmacy or from commercial distributors or manufacturers.

Cell lines and primary cells
A number of cell lines with lymphoma (DB, DOHH-2, HDLM-2, KM-H2, L-428, Ly-3, RC-K8, SU-DHL-6, SU-DHL-10, U-2932, U-2940, WSU-NHL), AML (MV4-11, HL60, Kasumi, KG1-A) and ovarian cancer (A2780, A2780cis, ES-2, SKOV-3, SKOV-3 Luc IP1) origin were used in the thesis for cytotoxicity assessment (papers I-III), cell cycle distribution analysis (paper I) and xenograft studies (paper I and III). The effect of single melflufen and melphalan in combinations with a selection of PARP-inhibitors was assessed in the BRCA-mutated ovarian cancer cell line UWB1.289-BRCA1 and the corresponding non-mutated cell line UWB1.289+BRCA1; with restored wt-BRCA1. A panel of various cell lines with different origin; A2780, ACHN, H69, HCT116, MCF-7, PC3 and U-937, was used in the investigation of hypoxia and hypoglycemia. Ovarian cell lines A2780 and SKOV-3 were used in the attempt of making spheroids for three-dimensional (3D) studies. All cells lines were cultured in recommended complete medium in 37 °C, in a humidified environment with 5% carbon dioxide and split twice weekly.
Human CD34+ umbilical cord blood cells (commercially available) were stimulated towards granulocytopyoietic differentiation [72] to investigate the cytotoxicity at different degrees of cell differentiation using the FMCA-GM7 and FMCA-GM14 assays in paper II.

Human umbilical vein endothelial cells (HUVEC) (commercially available cells accounted to expand for approximately 15 populations) and bovine capillary endothelial (BCE) cells (harvested from calf adrenal gland cortex) were used in the assessment of antiangiogenic effect of melflufen in paper IV.

Patient lymphoma (paper I), AML (paper II) and ovarian cancer (paper III) cells were also analyzed for in vitro sensitivity. Patient samples can be a more accurate predictor of clinical activity, since primary tumor cells retain their disease-specific phenotype to a larger extent than established cell lines [73]. Peripheral blood mononuclear cells (PBMC; paper II) from healthy donors were used to assess the cytotoxic phenotype in normal cells. Patient samples and PBMCs were obtained by surgery, needle biopsies, bone marrow or peripheral blood approved by the Ethics Committee of Uppsala University. Cancer cells were purified by density gradient centrifugation (solid samples first dispersed in collagenase), viability was determined by trypan or toluidine blue exclusion and a cytopathologist estimated the proportion of tumor cells by inspection of stained cytospin preparations [74].

Cytotoxicity assays
The fluorometric microculture cytotoxicity assay (FMCA) and the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) assay were used to evaluate the cytotoxicity of melflufen and standard drugs in several cell line and primary cell models.

Fluorometric microculture cytotoxicity assay
The fluorometric microculture cytotoxicity assay (FMCA) is a non-clonogenic assay that measures the cell viability after exposure to cytotoxic compounds [75, 76]. FMCA is a sensitive assay that can be adapted to most conditions and has previously been used for cell lines and patient sample cancer cells [18, 73], malignant [73] and non-malignant cells [72], to investigate the cellular effects of single drugs as well as the combination of drugs [18]. Cells was counted and diluted to an appropriate concentration depending on cell type. The cells were seeded as monolayers (tumor cells, HUVEC and BCE), or in suspension (leukemic cells and PBMCs) in multiwell microtiter plates before or after addition of drug and incubated in 37°C, with 5% carbon dioxide. After 72 hours, the cells were washed with PBS, and fluoresceindiacetate (FDA) was added to the cells. FDA is hydrolyzed to fluo-
rescent fluorescein by esterases in cells with intact cell membranes, a progress that generates a fluorescence signal directly proportional to the number of viable cells (Figure 2). Wells with untreated cells served as control and wells with medium only served as blank. Cell survival was expressed as Survival Index in percent (SI%), compared to control wells, and was calculated as (drug signal- blank)/(control signal- blank) for each concentration tested. From the mean SI% dose-response curves, the half maximal inhibitory concentration (IC_{50}) was determined by non-linear regression analysis in the GraphPad Prism Software. Quality criteria for a successful assay included >75% viable cells on day 0 (judged by trypan blue exclusion), a control signal more than ten times the blank, and a coefficient of variation in control and blank wells of <30%.

Figure 2. Flow chart of the FMCA procedure

MTT assay
In the colorimetric 3-(4,5-dimethythiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) assay, viable cells with active metabolism convert MTT into a purple-colored formazan product. The MTT assay measures cell metabolism rather than cell proliferation [77] and is widely used because of its simplicity to use and low cost [78]. In paper III, an ovarian cell line (SKOV-3 IP1) was seeded as a monolayer in a microculture plate at a suitable density and left to equilibrate for 24 hours. The cells were treated with drugs and incubated for 72 hours (37°C, 10% CO_{2}). MTT was then administered to each well and absorbance of viable (metabolizing) cells was measured with a spectrophotometer. IC_{50}-values were calculated using non-linear regression analysis (dose-response inhibition) in the Graphpad Prism software.

Melflufen combinations
Combinations of melflufen and clinically used drugs were investigated in AML (paper II) and ovarian cancer (paper III) cell lines. In the AML cell line HL-60, 7x9 drug concentration matrices of melflufen+cytarabine and melflufen+daunorubicin was analyzed by FMCA. The effect of the combinations was determined by the multiplicative Bliss model, allowing measurement of antagonistic, additive and synergistic drug combinations [79]. The effect of the combinations was determined by subtracting the experimental
values from theoretical additive values in the MacSynergy II program. A three-dimensional surface of the whole 7x9 matrix was also plotted and the combination was considered synergistic if the volume under this plane (volume of synergy) was larger than the theoretical additive plane [80].

Ovarian cancer combination analysis was performed in four different ovarian cancer cell lines (A2780, A2780cis, ES-2 and SKOV-3) with melflufen and standard drugs at concentrations yielding a viability of 60-80% in single drug analysis. A fixed concentration of melflufen and a concentration range for the standard drug was added to the cells and analyzed by FMCA. The effect of the combination was defined as synergistic if it was higher than the sum of both drugs administered alone, i.e. the expected additive effect.

Progenitor cell assay (FMCA-GM)

Human CD34+ umbilical cord blood cells were stimulated towards the granulocyte-macrophage (GM) lineage to study the effect of drug treatment starting at different degrees of cell differentiation [72]. The more stem cell like cells, developed in the 7-day model (FMCA-GM7) was analyzed after seven days of cell differentiation and drug exposure. In the 14-day model (FMCA-GM14), the cells were cultured in the absence of drugs for seven days and thereafter exposed to drugs until day 14 of differentiation. Readout for both models was the FMCA.

Cell cycle distribution

In paper I, cell cycle distribution was analyzed by flow cytometry (FACS) in selected lymphoma cell lines treated with melflufen to illustrate the distribution of the cell cycle phases G0/G1, S and G2/M. Cells were treated with melflufen before propidium iodide DNA staining and analyzed with flow cytometry.

In vivo studies

A number of different in vivo studies were performed to investigate the antitumoral effect of melflufen in mice. In addition, the Direct Angiogenesis In Vivo Assay (DIVAA) was performed in paper IV, presented in section Angiogenic assays. All in vivo experiments were performed according to ethical guidelines for animal experiments, and all animals were terminated at the end of the experiment or at humane endpoint.
Subcutaneous xenografts

Subcutaneous xenografts, with lymphoma cell line DOHH-2 or ovarian cancer cell line A2780, in mice were performed by Pipeline Biotech A/S (paper I) or at the MTC Animal Facility at Karolinska Institutet (paper III). The animals were inoculated subcutaneously with applied cell lines and randomized into treatment groups. The resulted tumors were treated with melflufen or comparing drug (or combination) and antitumor efficacy was estimated by tumor volume, overall survival (paper I) or tumor weight (paper III). Toxicity was monitored by body weight change of the animals.

Patient derived xenograft

In paper II, an AML disseminated patient derived xenograft (PDX) study was performed by Accelera Srl. The PDX model was used because of the improved estimates of clinical value and is indicated to reflect the clinical situation to a higher extent [81]. Mice were injected with AML cells intravenously, randomized in experimental groups and treated with melflufen or comparator drug. The efficacy of melflufen was measured by mice mortality and percent leukemia free animals. The toxicity was evaluated in body weight change. Furthermore, circulating leukemia cells were determined by FACS on day 30.

Intraperitoneal models

The effect of melflufen was analyzed in two localized ovarian cancer models at Ghent University (paper III). An ovarian cancer cell line (SKOV-3 LucIP1) was engrafted intraperitoneally (IP) or in the subperitoneal (SP) space into mice and treated IP with melflufen or comparing drug cisplatin [82, 83]. The antitumor efficacy of melflufen was evaluated by bioluminiscence (BLI) over treatment time, tumor weight, tumor volume, peritoneal metastases score (Peritoneal Carinomatosis Index; sPCI) and ascites score.

In addition, the pharmacokinetics was assessed in peritoneal fluid and blood at different timepoints after addition of melflufen and the amounts of melflufen, des-ethylmelflufen and melphalan were analyzed using HPLC-MS/MS.
Angiogenic assays

Evaluation of potential anti-angiogenic effect of melflufen was done by a series of experiments. In addition, investigation of intracellular melflufen/melphalan concentrations and APN activity in endothelial cells was conducted.

Cytotoxic activity in primary endothelial cells

FMCA was used to evaluate the cytotoxic effect of melflufen and melphalan in primary endothelial cells. Bovine capillary endothelial (BCE) cells have previously been used in cell models to study the in vitro anti-angiogenic effect of various substances, measuring both cytotoxicity [84, 85] and migration/DNA synthesis [86, 87]. Human umbilical vein endothelial cells (HUVEC) have been successfully cultured since 1973 [88] and are frequently used to study angiogenesis in vitro.

Intracellular melflufen/melphalan concentrations

To investigate the metabolism of melflufen in endothelial cells, intracellular concentrations of melflufen, des-ethylmelflufen and melphalan were measured as described previously [21]. Melflufen (with or without pre-exposure of aminopeptidase inhibitor bestatin) or melphalan was added to HUVECs suspended in warm medium, and after 15 and 60 min aliquotes were withdrawn. The cells were washed, re-dissolved in ethanol/acetonitrile and the supernatant was analyzed by HPLC with mass spectroscopic detection (performed by OncoTargeting AB, Sweden).

Aminopeptidase activity in HUVEC

Aminopeptidase enzymatic activity in HUVECs was assessed by measuring the free nitro-aniline generated by APN-mediated hydrolysis from the APN substrate L-alanine-4-nitro-anilide, as previously described [89]. The intention was to investigate if the antiangiogenic effect of melflufen was attributed to APN inhibition. Briefly, HUVECs were pre-treated with melflufen or melphalan before addition of L-alanine-4-nitro-anilide for 30 min. Absorbance was measured in a spectrophotometer.

Chick embryo chorioallantoic membrane (CAM) assay

The chick embryo chorioallantoic membrane (CAM) is an embryonic membrane in the chicken egg, with similar gas exchange properties as the lung. Because of the immense vascularization in the membrane, the CAM-assay has been used as an in vivo or in ovo model to study tumor growth [90] and
test angiogenic stimulators and inhibitors [91]. A window in the shell of the egg was opened and a gel-filled construct, containing fibroblast growth factor-2 (FGF-2) and the tested drug, was placed directly on the CAM (Figure 3). After 5 days of incubation the gel with ingrown vessels was removed from the eggs, the erythrocytes were stained for analysis [92, 93] and visually scored by two independent observers.

The CAM assay can be conducted in several ways; with (in ovo) or without (ex ovo) the shell; tissue or cell line tumor xenografts; administration of test substance via some kind of construct, direct or by injection. The CAM assay is cheap and simple, and thereby it is suitable for large-scale screening, although it is hard to distinguish between actual new vessel growth due to damage from irritants (like shell dust) or contraction due to high osmolarity or low pH. The big advantage of a vertical construct is that new vessel growth is easier to identify and distinguish from the normal horizontal vessel growth [93]. A commonly used alternative to the CAM model is the cornea angiogenesis system, and although it enables the quantification of vascularization, it is expensive, complicated and the tissue is very different from tumor tissue [94].

Figure 3. CAM construct containing collagen, FGF and melflufen or melphalan.

Co-culture angiogenesis assay

The AngioKit™ and GFP-AngioKit™ are two in vitro angiogenesis assay systems, commercially available kits with pre-prepared plates containing HUVEC in co-culture with normal human fibroblast matrix [95]. The endothelial cells proliferate to form threadlike tubule structures, resembling capillaries, and the test compound and conditioned media can be added to the cultures within individual wells. In the AngioKit™-assay the tubules are stained with CD31 (PECAM-1) antibodies for image analysis with AngioSys Software at endpoint (day 11). This assay reflects the angiogenic process in its entirety, studying endothelial cell proliferation, endothelial cell migration
and endothelial cells association into tubules when in contact with matrix proteins.

The GFP-AngioKit™ is very similar to the AngioKit™, but can be used to recapitulate the fluorescent imaging of angiogenesis over time. In this kit, the HUVECs are infected with Green Fluorescent Protein GFP-lentivirus in co-culture with normal human dermal fibroblasts (NHDF). During the course of the study, HUVEC tube formation is visualized through the IncuCyte FLR software [96]. The GFP-label allows fluorescent imaging of the HUVEC tube-formation, not visualized in a regular phase image IncyCyte reader. Similar dynamic information during the experiment is not available in any end-point assay.

HUVEC Scratch Wound (Migration) assay

The Scratch Wound assay was mentioned but not shown in paper IV. The assay was conducted according to the protocol provided by Essen BioScience [97]. The HUVECs were seeded in 96-well ImageLock plates and grown to 90% confluency. On the day of the experiment, a scratch wound was made in each well. After wounding, the wells were washed with PBS to remove debris and avoid reattachment of dislodged cells and fresh media containing test compounds were then added to the wells. Melflufen and melphalan (in eight different concentrations) were tested. The actin polymerization inhibitor and known blocker of HUVEC migration cytochalasin D was included as a positive control. The plate was placed in an IncuCyte™-FLR phase-contrast microscope and images of the cells/wounds were taken. Analysis algorithms identified the wound boundaries and data was expressed as percentage relative wound density (% RWD; measurement of the spatial cell density in the wound area relative to the spatial cell density outside of the wound area at each time point). The area under the curve (AUC) for the % RWD from the entire time-course of HUVEC migration was calculated and the % inhibition was determined relative to the vehicle (0.1% DMSO) control.

Directed In Vivo Angiogenesis Assay (DIVAA™)

DIVAA™ is a quantitative in vivo method for the investigation of angiogenesis in mice. Silicone cylinders filled with Matrigel and angiogenic factors, angioreactors, were implanted subcutaneously in the dorsal flanks of nude mice. Vascular endothelial cells migrated into the gel, stimulated by growth factors, for neovascularization and at the selected time point the extracted angioreactor gel was digested and the number of endothelial cells measured (Figure 4) [98]. Although the need for quantitative methods for angiogenic responses in vivo is high, it has been difficult to develop an adequate reproducible method for this purpose [99]. There are several other in vivo angio-
genesis assays that study the formation of new, functional blood vessels but they often require considerable amounts of test compound, are technically challenging and expensive, and are also mostly based on morphometric analysis, like vessel counts, for quantitation of neovascularization [94]. DIVAA uses less material and several angioreactors can be implanted in the mouse, thereby making it more cost- and time-effective than for example CAM assay, corneal angiogenesis assay or Matrigel plug assay [98].

Figure 4. Angioreactor for DIVAA, filled with Matrigel and angiogenic factors

Hypoxia and hypoglycemia

The effect of melflufen and melphalan in a panel of cell lines with different origin was assessed when deprived of oxygen or glucose. For the hypoxia study, the cells (ovarian cancer A2780, renal adenocarcinoma ACHN, small cell lung cancer H69, breast cancer MCF7 and leukemic monocyte lymphoma U-937) were seeded in microtiter plates and placed in a hypoxic incubator in extreme oxygen deprivation (anoxia (0.1% O$_2$) or hypoxia (1.0% O$_2$)) [100]. For the hypoglycemia study, the cells (A2780, ACHN, MCF7, U937, colorectal carcinoma HCT116 and prostate carcinoma PC3) were seeded in either regular cell culture medium (hyperglycemic; 11-25 mM glucose) or hypoglycemic (no added glucose) cell medium (Sigma Aldrich) in microtiter plates. Melflufen or melphalan was added to the cells, and after incubation FMCA was used to evaluate the viability of the cells.

An attempt was made to make spheroids with ovarian cells to study the effect of melflufen in an oxygen- and nutrient-deprived 3D cell model [101, 102]. Ovarian cell lines A2780 and SKOV-3 were seeded in several different concentrations in a round-bottomed ultra low attachment plate, with or without Matrigel matrix. The cells formed loose aggregates (without Matrigel) but did not form spheroids under any of the tested conditions, so therefore the experiment was abandoned.
Results and discussion

Superior cytotoxicity of melflufen in lymphoma, AML and ovarian cancer cell lines

Cytotoxic activity of melflufen and melphalan in well-established cell lines was investigated using the FMCA, showing a general activity in all cell lines (Table 1). Melflufen was active in both Hodgkin’s and non-Hodgkin’s lymphoma, yielding IC\(_{50}\)-values as low as 11 nM, but with a high grade of variation between the different cell lines. Melflufen also exhibited potent cytotoxic activity in the AML cell lines with IC\(_{50}\)-values ranging from 18 to 170 nM. The tumor cell lines with ovarian cancer origin had cytotoxic IC\(_{50}\)-values for melflufen in the range of 0.32 to 3.1 µM, suggesting that these cell lines were less sensitive.

We also observed an overall supreme activity of melflufen compared to melphalan in all tested cell lines, with ratios ranging from 5 (OC cell line A2780) to 106 (lymphoma cell line HDLM-2). Melflufen was more cytotoxic than melphalan in the lymphoma cell lines with an average IC\(_{50}\)-ratio of 55 (range 13 to 106). The lowest melflufen IC\(_{50}\)-value and the highest melflufen/melphalan ratio in the AML cell lines was observed in the FLT3 mutated MV4-11 cell line. Mutations in the receptor tyrosine kinase FLT3 are common in pluripotent hematopoietic AML cells and have been associated with a reduced overall patient survival [103, 104], so melflufen activity against cells with this mutation is promising. In the ovarian cancer cell lines there was a 5 to 38 times potency difference between melflufen and melphalan, with lower mean ratio (22-fold) than the other two diagnoses.

Among the ovarian cancer cell lines, the largest difference between melflufen and melphalan was observed in the BRCA-mutated cell line UWB1.289-BRCA1 (not published). BRCA is a common germ-line and somatic mutation in ovarian cancer patients [105, 106]. The major cause of hereditary breast and ovarian cancer is mutations in the BRCA1 (breast cancer 1) and the BRCA2 (breast cancer 2) genes. Targeting the poly (ADP-Ribose) polymerase (PARP) is a promising targeted therapeutic strategy to treat BRCA mutated ovarian and breast cancer. PARP enzymes are involved in DNA repair and other multiple cellular processes and contribute to cell survival after DNA damage, and if this pathway is inhibited the cell will die after a collapse in the DNA replication forks [107].
Melphalan and melflufen, and the melphalan/melflufen ratio, in lymphoma (paper I), AML (paper II), ovarian cancer (paper III) and BRCA-mutated (unpublished results) cell lines. All differences between melphalan and melflufen were statistically significant (two-tailed t-test).

<table>
<thead>
<tr>
<th>Cell line</th>
<th>Origin</th>
<th>IC$_{50}$ (µM) melphalan</th>
<th>IC$_{50}$ (µM) melflufen</th>
<th>Ratio</th>
</tr>
</thead>
<tbody>
<tr>
<td>DB</td>
<td>Lymphoma</td>
<td>44</td>
<td>0.92</td>
<td>48</td>
</tr>
<tr>
<td>DOHH-2</td>
<td>Lymphoma</td>
<td>17</td>
<td>0.39</td>
<td>44</td>
</tr>
<tr>
<td>HDLM-2</td>
<td>Lymphoma</td>
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<td>0.088</td>
<td>106</td>
</tr>
<tr>
<td>KM-H2</td>
<td>Lymphoma</td>
<td>8.6</td>
<td>0.22</td>
<td>39</td>
</tr>
<tr>
<td>L-428</td>
<td>Lymphoma</td>
<td>9.4</td>
<td>0.73</td>
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</tr>
<tr>
<td>Ly-3</td>
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<td>0.011</td>
<td>47</td>
</tr>
<tr>
<td>RC-K8</td>
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<td>0.45</td>
<td>27</td>
</tr>
<tr>
<td>SU-DHL-6</td>
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<td>38</td>
<td>0.42</td>
<td>90</td>
</tr>
<tr>
<td>SU-DHL-10</td>
<td>Lymphoma</td>
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<td>0.71</td>
<td>41</td>
</tr>
<tr>
<td>U-2932</td>
<td>Lymphoma</td>
<td>19</td>
<td>0.52</td>
<td>37</td>
</tr>
<tr>
<td>U-2940</td>
<td>Lymphoma</td>
<td>9.8</td>
<td>0.12</td>
<td>82</td>
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<td>WSU-NHL</td>
<td>Lymphoma</td>
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<td>0.077</td>
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<td>MV4-11</td>
<td>AML</td>
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<td>0.018</td>
<td>94</td>
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<td>HL60</td>
<td>AML</td>
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<td>0.13</td>
<td>29</td>
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<td>Kasumi</td>
<td>AML</td>
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<tr>
<td>KG1-A</td>
<td>AML</td>
<td>8.6</td>
<td>0.17</td>
<td>51</td>
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<tr>
<td>A2780</td>
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<td>0.26</td>
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<td>A2780cis</td>
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<td>15</td>
</tr>
<tr>
<td>ES-2</td>
<td>OC</td>
<td>2.0</td>
<td>0.29</td>
<td>7</td>
</tr>
<tr>
<td>SKOV-3</td>
<td>OC</td>
<td>&gt;100</td>
<td>3.1</td>
<td>32</td>
</tr>
<tr>
<td>SKOV-3 Luc IP1</td>
<td>OC</td>
<td>-</td>
<td>0.89</td>
<td>-</td>
</tr>
<tr>
<td>UWB1.289-BRCA1</td>
<td>OC (BRCA-mutated)</td>
<td>42</td>
<td>1.1</td>
<td>38</td>
</tr>
<tr>
<td>UWB1.280+BRCA1</td>
<td>OC (restored BRCA)</td>
<td>75</td>
<td>2.2</td>
<td>34</td>
</tr>
</tbody>
</table>

**Melflufen display cytotoxic activity in primary cultures of lymphoma, AML and ovarian cancer**

The cytotoxicity of melflufen in primary patient samples was investigated using the FMCA (Figure 5). We demonstrated that melflufen was active against all primary patient samples tested, with IC$_{50}$-values of 0.078 µM (lymphoma), 0.067 µM (AML) and 0.35 µM (ovarian cancer). Melflufen also exhibited an overall superior mean cytotoxicity compared to melphalan, 114-fold in lymphoma, 82-fold in AML and 43-fold in ovarian cancer (Table 2). The results from primary cultures correlated with the results obtained in the cell lines for each diagnosis.
Figure 5. Concentration-effect curves for melflufen in lymphoma (paper I), AML (paper II) and ovarian cancer (paper III) patient samples (error bars denote SEM).

Table 2. Mean IC$_{50}$ (µM) for melphalan and melflufen, and the melphalan/melflufen ratio, in primary lymphoma (paper I), AML (paper II), ovarian cancer (paper III) and PBMCs (paper II). All differences between melphalan and melflufen were statistically significant (p<0.0001), in a two-tailed t-test.

<table>
<thead>
<tr>
<th>Diagnosis</th>
<th>IC$_{50}$ (µM) melphalan</th>
<th>IC$_{50}$ (µM) melflufen</th>
<th>Ratio</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lymphoma (n=16)</td>
<td>10</td>
<td>0.088</td>
<td>114</td>
</tr>
<tr>
<td>AML (n=82)</td>
<td>5.5</td>
<td>0.067</td>
<td>82</td>
</tr>
<tr>
<td>Ovarian cancer (n=84)</td>
<td>15</td>
<td>0.35</td>
<td>43</td>
</tr>
<tr>
<td>PBMC</td>
<td>12</td>
<td>0.38</td>
<td>32</td>
</tr>
</tbody>
</table>

High activity of melflufen in lymphoma and AML primary cells compared to normal blood cells

The activity of melflufen in patient samples was also compared to normal peripheral blood mononuclear cell (PBMC) cultures. The mean IC$_{50}$ difference between PBMC and lymphoma or AML patient samples was approximately 5-fold, whereas no difference was observed between the PBMC and solid ovarian cancer samples (Table 2). When sensitivity of melphalan was compared, the difference was only 1.5-2-fold (in lymphoma and AML vs PBMC), indicating a higher therapeutic index for melflufen. Primary cancer cultures show as little proliferation as normal PBMCs during the assay conditions, leaving out the proclamation that melflufen is more active in the cancer samples because of higher proliferation rate. Increased APN expression in lymphoma and AML cells could be a reasonable explanation to the high therapeutic index observed for melflufen. Studies have demonstrated
that melflufen is susceptible to hydrolysis by APN, resulting in intracellular enrichment of alkylating moieties and subsequent cell death [20, 21]. Therefore it is plausible to consider the melflufen hydrolysis by APN is preferentially occurring in APN overexpressing lymphoma or AML cells.

Sequence-dependent synergy of melflufen and cytarabine combinations in AML

Melflufen was tested in combination with cytarabine or daunorubicin, standard treatment for AML, in the HL-60 cell line (paper II). When both drugs were added simultaneously synergy was observed for melflufen in combination with cytarabine whereas only sub-additive interactions were seen for the combination of melflufen and daunorubicin. The volume of synergy for the complete melflufen+cytarabine combination matrix (9x7) was 111 (Figure 6), which means that the synergy is strong and likely to be important in vivo [108]. The volume of synergy for the melflufen+daunorubicin combination matrix was in comparison merely 3, i.e. an additive combination. Interestingly the synergy between melflufen and cytarabine seemed to be sequence dependent, with strong synergy when melflufen was administrated before cytarabine (236), whereas the opposite sequence showed only moderate synergy (58). This sequence-dependent synergistic interaction could be the outcome of the induced cell cycle arrest caused by cytarabine, as previously reported for other combinational therapeutics [109].

![Figure 6](image)

**Figure 6.** The complete combination matrix of melflufen and cytarabine in the AML cell line HL-60.
Synergistic and additive effects of melflufen in combination with gemcitabine and doxorubicin in ovarian cancer

The combination of melflufen and several standard and experimental drugs was investigated in four human ovarian cancer cell lines (A2780, A2780cis, ES-2 and SKOV-3). Mainly additive and antagonistic effects were observed but no specific pattern could be detected. The synergistic interactions observed were mainly seen in the cisplatin-resistant cell line A2780cis between melflufen and gemcitabine (Figure 7). It should also be noted that the combination of melflufen and doxorubicin was often additive (not shown) motivating further studies for melflufen in combination with doxorubicin and gemcitabine in vivo.

In the BRCA mutated cell line UWB1.289-BRCA1 no synergistic effects of melflufen or melphalan combined with any of the tested PARP-inhibitors (olaparib, Merck chemicals DPQ (PARP III inhibitor) and IQD (PARP IV inhibitor) were observed. Furthermore, none of the PARP inhibitors had a dose dependent effect alone, and there was no difference in sensitivity between the BRCA mutated and non-mutated cell line (not published).

![Figure 7](image-url)

**Figure 7.** Synergistic effects of melflufen (4.0, 2.0 and 1.0 µM) and gemcitabine 2.0 µM, compared to the expected additive effect in the cisplatin-resistant ovarian cancer cell line A2780cis.

Superior activity of melflufen over melphalan in stem cell like progenitor cells

In paper II, melflufen and melphalan were tested on CD34⁺ progenitor cells with treatment starting at different degrees of differentiation (FMCA-GM7
and FMCA-GM14; Table 3). A very similar sensitivity in the stem cell like FMCA-GM7 cells and the more differentiated FMCA-GM14 cells was observed for melflufen with IC$_{50}$-values of 0.028 and 0.029 µM, respectively. Notably, melphalan was less active in the stem cell like cells (1.9 µM) than in the differentiated cells (0.68 µM), with almost a three-fold difference. The superior activity of melflufen over melphalan in the stem cell like cells (x68) could suggest a potential use for melflufen in myeloablative conditioning regimens prior to stem cell transplantation [110-112].

Table 3. Mean IC$_{50}$ (µM) for melphalan and melflufen, and the melphalan/melflufen ratio, in CD34$^+$ progenitor FMCA-GM7 and FMCA-GM14 cell models. Differences between melphalan and melflufen were statistically significant (p= 0.0004 for GM7 and p= 0.0003 for GM14), in a two-tailed t-test.

<table>
<thead>
<tr>
<th></th>
<th>IC$_{50}$ (µM) melphalan</th>
<th>IC$_{50}$ (µM) melflufen</th>
<th>Ratio</th>
</tr>
</thead>
<tbody>
<tr>
<td>FMCA-GM7</td>
<td>1.9</td>
<td>0.028</td>
<td>68</td>
</tr>
<tr>
<td>FMCA-GM14</td>
<td>0.68</td>
<td>0.029</td>
<td>23</td>
</tr>
<tr>
<td>Ratio</td>
<td>3</td>
<td>1</td>
<td>1</td>
</tr>
</tbody>
</table>

Melflufen treated lymphoma cells accumulates in the G2/M phase

Flow cytometry was performed in lymphoma cell lines to determine cell cycle phase distribution after treatment with melflufen (paper I). A clear accumulation of cells in the G2/M phase was seen after 24 hours in the SU-DHL-10 cell line and after 48 hours G2/M phase arrest was seen in both the KM-H2 (not shown) and SU-DHL-10 cell lines (Figure 8). This increase in G2/M arrest correlates with DNA damage caused by melflufen, preventing the cells from exiting mitosis.
Melphalan is active in *in vivo* models of cancer

After the positive results of melphalan cytotoxicity *in vitro, in vivo* studies in mice were conducted with cell line xenografts inoculated subcutaneously (lymphoma and ovarian cancer), patient derived xenografts (AML) and intraperitoneally inoculated cell line xenografts (ovarian cancer).

Moderate activity in lymphoma, and high activity in ovarian cancer subcutaneous xenografts

Melphalan was moderately to very active in subcutaneously inoculated lymphoma or ovarian cancer cell line xenografts (*Figure 9*). Xenografted DOHH-2 lymphoma tumors were modestly but significantly inhibited by melphalan 3 mg/kg compared to the vehicle treated control (paper I). Overall health and weight changes were not influenced by the melphalan treatment and all animals survived until termination. Sufficient tumor control and low toxicity of the melphalan regimen opens up for an increase in dose or intensity of the treatment, which would also increase the inhibition of tumor growth.

*Figure 8*. Cell cycle distribution (G1, S or G2/M phases) in lymphoma SU-DHL-10 cell line after melphalen (0.1, 0.2 and 0.4 μM) treatment for 24 and 48 hours.
Figure 9. Tumor reduction for melflufen and used standard drug compared to control in the in vivo models performed with lymphoma (tumor volume), ovarian cancer (tumor volume) or AML (percent AML cells in blood) xenografts.

In the ovarian A2780 xenograft, melflufen 8 mg/kg significantly inhibited tumor growth and displayed superior activity compared to melphalan 4 mg/kg and 8 mg/kg (paper III). Median survival after single melflufen treatment was longer than for melphalan treatment; untreated 6 days, melphalan 2mg/kg 7.5 days, melphalan 4mg/kg 12.5 days, melflufen 4mg/kg 18 days and melflufen 8mg/kg 18 days (not published; Figure 10). Combination therapy of melflufen 4 mg/kg, with liposomal doxorubicin (Doxil) 2 mg/kg or gemcitabine 5 mg/kg was performed in the same ovarian carcinoma xenografts, where an increased inhibition of tumor growth was seen in both combinational treatments compared to the control. None of the mice was prematurely sacrificed because of weight reduction or any other clinical signs of toxicity.
High activity and low toxicity in AML patient derived xenografts

Melflufen displayed significant activity in the AML PDX in comparison to control, in Figure 9, presented as the amount of circulating leukemia cells in the blood from melflufen treated mice (paper II). Although the cell count difference on day 30 was not significantly different from cytarabine 75 mg/kg, the toxicity was considerately lower for melflufen 8 mg/kg, as all melflufen-treated animals were alive and mostly considered tumor free at the end of the study.

Moderate activity and low hematologic toxicity in ovarian cancer intraperitoneal xenografts

The effect of melflufen in the ovarian cancer intraperitoneal (IP) xenograft model appeared modest, although a (non significant) difference could be observed in the BLI signal, ascites score and modified sPCI in comparison to NaCl control (paper III). An additional study is currently underway.

In the subperitoneal (SP) xenograft model there were significant differences between the treatment groups. Tumor volume was reduced when treated with melflufen and tumor weight was significantly lower when comparing the melflufen-treated group to the control group. A significant reduction in sPCI score was observed for the melflufen (and cisplatin) treated groups compared to the control group, although no statistical differences were detected between the melflufen and control animals.

Figure 10. Survival for ovarian cancer xenografted mice treated with melflufen (4 mg/kg or 8 mg/kg) and melphalan (2 mg/kg or 4 mg/kg). Additional data from experiments presented in Figure 2A in paper III.
regarding ascites score and BLI signal. There was no distinguished difference between melflufen-treated and cisplatin-treated animals with regards to sPCI score, tumor volume, tumor weight, ascites score and BLI signal. However, melflufen treated mice seemed to be in a better physical state reflected by a significant weight increase. All mice were eutanized at the end of the experiment.

The pharmacokinetic analysis after IP administration of melflufen showed that cellular uptake was complete and rapid as no melflufen could be detected in the intraperitoneal cavity after only 5 minutes. However des-ethylmelflufen and melphalan were detected in the intraperitoneal fluid at all time points in increasing and decreasing concentration (Table 4). Melphalan and des-ethylmelflufen were detected in plasma after 30 minutes, a time point estimated close to melphalan C\textsubscript{max}, at a considerably lower concentration than of C\textsubscript{max} of melphalan administered at the LD\textsubscript{10}, implying IP melflufen to be safe and likely to cause low systemic/hematologic toxicity.

Table 4. Concentrations of melflufen, des-ethylmelflufen and melphalan in intraperitoneal fluid after 5, 15 and 30 minutes post IP-administration of melfufen.

<table>
<thead>
<tr>
<th>Time (minutes)</th>
<th>Melflufen (µM)</th>
<th>Des-ethylmelflufen (µM)</th>
<th>Melphalan (µM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>5</td>
<td>-</td>
<td>8.8</td>
<td>9.7</td>
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<tr>
<td>30</td>
<td>-</td>
<td>10</td>
<td>7.5</td>
</tr>
</tbody>
</table>

Melflufen inhibits angiogenesis

In paper IV, several assays were used to investigate the antiangiogenic activity of melflufen.

Primary endothelial cells are more sensitive to melflufen than to melphalan

The cytotoxicity of melflufen in comparison to melphalan was assessed in two endothelial cell types, BCE cells and HUVECs (Figure 11). There was a large difference between the two drugs in the BCE cells, i.e. 247-fold, and in HUVECs the difference was estimated as >30-fold (since IC\textsubscript{50} for melphalan was >10 µM which was the highest concentration used). To compare it to results retrieved in this thesis, the differences in human tumor cell lines was at the most 106-fold (lymphoma cell line HDLM-2), and in human tumor specimens at the most 114-fold (lymphoma).
APN inhibition reduced the intracellular concentrations of alkylating moieties in HUVEC

Intracellular concentrations of melflufen, melphalan and des-ethylmelflufen were measured in HUVECs after exposure to melflufen, melflufen+bestatin or melphalan. The concentration of melphalan in melflufen-treated cells was about 40 times higher than for the melphalan treated cells after 60 minutes. When the cells were pre-incubated with the aminopeptidase inhibitor bestatin before treatment with melflufen, the intracellular concentration of melphalan was decreased with an accompanied increase in des-ethylmelflufen. This clearly indicated a forced switch from APN cleavage of the peptide bond, to the de-esterification by esterases (see Figure 1). Also, the cytotoxic activity of melflufen pre-incubated with bestatin was reduced by half compared to melflufen alone.

No inhibition of APN activity in HUVEC

Aminopeptidase activity was assessed in HUVECs by an aminopeptidase probe measuring aminopeptidase activity. Neither melflufen nor melphalan inhibited APN activity, however, at a very high concentration of melflufen (17 µM, i.e. 50 x IC₅₀) a decrease in APN activity was observed, compared to control and the melphalan groups (p< 0.05 in a one-way ANOVA; Figure 12). It was concluded that this effect could not contribute to the anti-angiogenic properties of melflufen.
Modest antiangiogenic effect in the chick embryo chorioallantoic membrane assay

In the traditional CAM assay the angiogenic activity of melflufen and melphalan, was moderately effective, showing less vessel ingrowth compared to FGF-treated positive control and similar response as untreated negative control with no FGF. There was no difference between melflufen and melphalan. The modest antiangiogenic effect in this assay may be annotated to the short half-life, i.e. 1.5 hours in aqueous solution [71], of melflufen in the gel construct during the experiment.

Long-term exposure inhibited HUVEC tubule growth, but did not effect normal human dermal fibroblast, in two co-culture angiogenesis assays

Inhibition of blood vessel growth by melflufen and melphalan was assessed in two different AngioKit-assays (AngioKit™ and GFP-AngioKit™) as tubule length and number of tubule junctions. While the melflufen single drug exposure in the CAM assay presented moderate inhibition, these long-term drug exposure assays revealed a strong angiogenic inhibition. Melflufen caused a dose-dependent inhibition on tubule length in both assays, as well as an inhibition on vessel junction measured by AngioKit™. There was a significant difference between melflufen and melphalan in both assays, where melphalan showed almost no effect at all during the entire

Figure 12. APN-activity in HUVECs when treated with melflufen or melphalan.
experiment in the GFP-AngioKit™ assay. The anti-angiogenic effect of melflufen was also time-dependent and the majority of cell migration occurs during the first few days after the co-culture is created. Melflufen produced little or no effect on tube formation until after day 4. Only the HUVECs, but not the co-cultured normal human dermal fibroblasts, were affected by any of the administrated drugs, suggesting a selective anti-angiogenic effect even at high concentrations (Figure 13).

![Figure 13. Vessel growth in the AngioKit™ assay, when treated with 1 μM melflufen and VEGF (A) or VEGF alone (B).](image)

No inhibition of HUVEC migration during a 24-hour scratch wound assay

In addition, HUVEC cell migration was studied using a 24 h scratch wound assay, where neither melflufen (0.01–1 μM) nor melphalan (0.08–10 μM) inhibited migration of HUVEC cells (highest concentrations shown in Figure 14; not published). In the scratch wound assay, HUVEC migrated rapidly reaching 90% relative wound density (RWD) within 18 hours. Consistent wounds were obtained with a mean wound width of 656 ± 53 μm (mean ± SD, 89 replicates). The actin polymerization inhibitor cytochalasin D (3 μM) abolished the migration of the HUVEC (88 ± 2% inhibition; mean ± SEM, 8 replicates). Melflufen (1 μM) produced little or no inhibition of the migration of HUVEC (-1 ± 2% inhibition; mean ± SEM, 5 replicates). Similarly, melphalan (10 μM) failed to attenuate HUVEC migration (-1 ± 5% inhibition; mean ± SEM, 3 replicates). These findings were consistent with the effects observed in the co-culture angiogenesis assays. In the AngioKit™ model the majority of cell migration occurs during the first few days after the co-culture and inhibition was not seen until around day 4.
Anti-angiogenic effect of melflufen in the Directed In Vivo Angiogenesis Assay

The DIVAA™ angioreactor assay was used to assess the anti-angiogenic effect of melflufen in vivo. The total amount of endothelial cells retrieved from the angioreactor (measured with fluorescein fluorescence and confluence) was significantly lower for melflufen and the positive control bevacizumab than for the negative control. This in vivo study confirmed that melflufen could indeed be used as an anti-angiogenic drug utilized in cancer therapy.

Melflufen activity in hypoxia and hypoglycemia

The blood flow in a tumor varies considerably and is often lower than in normal tissue, leading to oxygen- and nutrient-deficiency inside the tumor [113, 114]. In the regular monolayer cell culture (two-dimensional FMCA) used throughout this thesis, there is a lack of insight to how the oxygen- and glucose-deficient tumor cells respond to the administered drugs. Therefore, we induced hypoxia/anoxia and hypoglycemia in several cell lines from different origins, to compare the response to normoxic and hyperglycemic responses. The effect of melflufen and melphalan in a panel of cell lines with

Figure 14. Relative wound density (RDW) for control (0.1% DMSO), melflufen 1 µM, melphalan 10 µM and cytochalasin D 3 µM.
different origin was assessed when deprived of oxygen or glucose (not published).

Melflufen was slightly more effective in oxygen-deprived cells

Melflufen and melphalan were tested in several cell lines cultured in a hypoxic/anoxic environment and compared to results obtained in cell lines cultured in a normoxic environment [100]. The results were presented as hypoxic or anoxic IC_{50}/normoxic IC_{50} ratios, where a result under 0.8 indicated a higher activity in the hypoxic cells, and over 1.2 indicated a higher activity in normoxic cells. In 50% of the ratios it was revealed that melflufen was more effective in oxygen-deprived cells (30% was less effective and 20% was equally effective), and 50% of the ratios displayed that melphalan was less effective in oxygen-deprived cells (37.5% was more effective and 12.5% was equally effective) (Table 5). The renal adenocarcinoma ACHN was less sensitive, and the lung cancer H69 was more sensitive to the effects of melflufen in anoxic and hypoxic conditions, as consistent with results from several other drugs tested [100]. Here it can also be noted that melflufen was slightly less active in oxygen-deprived cells in the ovarian cancer cell line A2780. Remarkably, melflufen seemed to be marginally more active in hypoxia/anoxia in the lymphoma cell line U-937, even tough this cell line was most sensitive to oxygen deprivation (reduced proliferation) and should render lower sensitivity to cytotoxic drugs [100].

Table 5. Hypoxic IC_{50}/normoxic IC_{50} and anoxic IC_{50}/normoxic IC_{50} ratios for melflufen and melphalan in five different cell lines. Ratio value for a drug: 0.8-1.2 = equally effective in anoxia/hypoxia and normoxia, <0.8 = more effective in anoxia/hypoxia, >1.2 = more effective in normoxia, NA = not applicable.

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<th></th>
<th>Melflufen</th>
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<tr>
<td></td>
<td>Anoxia</td>
<td>Hypoxia</td>
</tr>
<tr>
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<tr>
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<td>10</td>
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<tr>
<td>H69</td>
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<tr>
<td>U-937</td>
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<td>0.48</td>
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No detectable trends for melflufen in hypoglycemic cells

Melflufen and melphalan were also tested in several cell lines (with different origin) cultured in a hypoglycemic environment and compared to results obtained in the cell lines cultured in a hyperglycemic environment. The results were presented as hypoglycemic IC_{50}/hyperglycemic IC_{50} ratios, where a result under 0.8 indicated a higher activity in the hypoglycemic cells, and over 1.2 indicated a higher activity in hyperglycemic cells. There were no detectable trends for either of the drugs (Table 6), although the cells were
seeded in a high concentration, leading to quick cell confluence that affects cell proliferation and consequently drug sensitivity. Further studies, with altered cell concentrations, needs to be conducted to be able to reach a conclusion about the sensitivity of melflufen in a hypoglycemic environment.

**Table 6.** Hypoglycotic IC$_{50}$/hyperglycotic IC$_{50}$ ratios for melflufen and melphalan in six cell lines. Ratio value for a drug: 0.8-1.2 = equally effective in hypoglycemia and hyperglycemia, <0.8 = more effective in hypoglycemia, >1.2 = more effective in hyperglycemia.

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<tr>
<td>ACHN</td>
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<td>PC3</td>
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<td>U-937</td>
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Conclusion and future outlook

In conclusion, melflufen is an aminopeptidase-potentiated alkylator that rapidly enters the cell, where it is hydrolyzed, for example by APN, to smaller alkylating moieties. We have found that melflufen showed promising preclinical activity in the diagnoses lymphoma, AML and ovarian cancer, and had anti-angiogenic effects. The results may be summarized as:

• Melflufen was more effective than its precursor melphalan in all tested lymphoma, AML and ovarian cancer cell lines.
• Melflufen was highly active in human patient lymphoma, AML and ovarian cancer samples, motivating further preclinical studies in these particular diagnoses.
• Melflufen was more cytotoxic in lymphoma and AML patient samples than in normal peripheral blood mononuclear cells (PBMCs), indicating a large therapeutic index with low systemic toxicity.
• Melflufen in combination with cytarabine was synergistic in an AML cell line in a sequence dependent manor.
• Melflufen was equally effective in stem cell like progenitor cells as in more differentiated cells, suggesting a potential role in stem cell transplantation.
• Melflufen was highly effective in in vivo lymphoma, AML and ovarian cell xenograft models.
• Melflufen show strong in vivo activity in subcutaneous ovarian cancer xenografts, alone and in combination with gemcitabine or liposomal doxorubicin.
• Melflufen was moderately active with low systemic toxicity in ovarian cancer intraperitoneal xenografts.
• Melflufen inhibited angiogenesis in several different models, suggesting an anti-angiogenic effect useful in the treatment of cancer.

Melflufen was recently designated orphan drug status by the EMA and FDA, for the treatment of multiple myeloma [115]. Promising results from a phase II clinical trial for multiple myeloma was reported with 67% of patients showing objective responses (refractory to immunomodulatory drugs and proteasome inhibitors) evaluable for efficacy, and clinical responses was even observed in melphalan resistant patients [116]. In the light of the strong clinical activity in the ongoing study, it seems warranted to identify other possible diagnoses for the clinical development of melflufen.
I would like to thank everyone who in any way has contributed to this thesis. In particular I would like to express my sincere gratitude to:

My outstanding supervisor Joachim Gullbo for your excellent scientific guidance, for always having the patience to share your knowledge and experience with me, but also for being generous, sociable and fun. Without you I would still be confused.

Rolf Larsson, my co-supervisor, for giving me the opportunity to work in your group, and for sharing your extensive expertise in science.

Mårten Fryknäs, my co-supervisor, for sharing your experience and knowledge, for your positive disposition and for being a good roommate.

Caroline Haglund for life-saving scientific discussions, but foremost for being a fabulous friend, and sharing all the adventures of being a parent.

Linda Rickardson for sharing your experiences in the world of science, for your generosity and your wonderful friendship.

Anna Eriksson for good scientific talks, being an awesome friend and roommate with a wonderful spirit.

Malin Wickström Näsman for all the amazing and fun help with lab work and collaborations writing articles.

Fantastic companions Malin Jarvius, Hanna Göransson Kultima, Sofie Schwan and Åsa Fransson for great talks about anything, everything, life and science.

Grand laboratory guidance and assistance from Lena Lenhammar, Nasrin Najafi and Christina Leek.

Anna-Karin Lannergård, Kristin Blom and Annika Jonsson for great help digging through the massive collection of patient samples.
All my excellent co-authors and associates, especially **Peder Fredlund Fuchs**, **Pär Gerwins**, Tim Dale, Johan Lennartsson, Lisa Rebello, Mar- yam Delforoush, Gunilla Enblad, Sadia Hassan, Martin Höglund, Charlotte Carlier, Kristina Viktorsson, Peter Nygren, Maria Uustalu and **Therese Juntti** for interesting collaborations.

Everybody at OncoPeptides, mainly **Jack Spira** and **Jakob Lindberg** for letting me do exciting investigations and write my thesis about melflufen.

**Jenny Felth** for guiding me through my master thesis and introducing me to the lab.

**Anders Backlund** at the Division of Pharmacognosy for letting me be a part of the fantastic Global Pharmacy-course, and **Professor Chang** for taking such good care of us at the Kaohsiung Medical University, Taiwan.

**Shima Momeni** for sharing the joy and frustration of the dissertation process. We made it!

Tremendous room mates **Sharmineh Mansoori**, **Wojciech Senkowski** and **Henning Karlsson**, and great master students **Emelie Larsson** and **Isabelle Morin**.

And all other past and present work friends; **Håkan**, Gunilla, Lena F, Nad- ja, Malin B, Hugo, Vendela, Frida, Ebba, Mats, Christofer, Obaid, Kashif, Mahdia, Jenny, Claes, Kim, Payam, Shibu, David, Kristin, Pär, Anna-Karin L, Eva, Elisabeth, Ulrica, Sofie, Jessica, Anna S, Mia, Mao, Anna-Karin H, Gabriella, Anders, Maria, Anna H, Malin O, Markus, Martin, Sebastian for contributing to the warm atmosphere at the department.

I would also like to acknowledge the importance of my family and friends outside the lab, especially:

**Ida Eklund**, Jenny Fundell, Pia Kollberg, Nermina Kosovac, Katrin Nilsson and **Antionette Teir** with families. ”True friendship isn’t about being inseparable, it’s about being separated and nothing changes”. #minat-jeper94 forever!

Uppsala friends, with partners, from Master of Science Program in Pharmacy; **Emelie Bengtsson**, Sandra Creutz, Emelia Holm, Andreas Jendeberg, Ghazaleh Karimi, Camilla Ledin, Erika Olsson and Caroline Thordenberg. 10 awesome years of laughter, hard work, fika, comfort, parties and travels. Lets have a 100 more!
Albert Elmsjö and Ellinor Nilsson for trusting us to be godparents to your wonderful daughter Myra.

The rest of the book club Kajsa Björner, Elin Matson, Maria Swartling and Frida Wilske for great discussions about books and other good things in life.

Relatives from the Markström-clan for Christmas celebrations, family parties, endless board games and many laughs.

The Laitinens, especially mummu Kirsti, for relaxing times and saunas in Olhava. And for the Finnish Sisu mentality.

My Strese-family; Else-Marie, Berndt, Malin and Rasmus for opening your arms, your home and your hearts to me.

My Laitinen-family; Marianne, Matti, Anna, Getu and Ronja for your unconditional love and life-long support. You are my safe haven!

Åke, the love of my life, for being the greatest husband anyone can have. And Rut, my sunshine and my hurricane, for brightening my life. Every day I am thankful for having the privilege to be a part of our amazing family!
References

9. Melphalan Summary of Product Characteristics, SmPC [https://lakemedelsverket.se/LMF/Lakemedelsinformation/?nplid=19650121000014]


116. Heavily Pretreated, Relapsed and Relapsed Refractory Multiple Myeloma Patients Showed Significant Clinical Benefit, as Measured by Overall Response Rate and Progression Free Survival, when Treated with Melflufen and Dexamethasone [http://www.oncopeptides.se/wp-content/uploads/2015/06/Oncopeptides-Presents-at-EHA-150612-F4-ND.pdf]

You may now call me Dr Strese.
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