Preclinical Studies of the Melphalan Prodrug J1 for Cancer Therapy

MALIN WICKSTRÖM
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Abstract

J1 (L-melphalanyl-L-p-fluorophenylalanoyl ethyl ester) is a dipeptide derivative of the alkylating agent melphalan with increased cytotoxicity. In this thesis the preclinical pharmacology of J1 has been characterized.

Our results show that J1 rapidly enters the cells, where melphalan is released by hydrolysis. The maximum concentration (C_{max}) of melphalan was detected 15 min after exposure to J1 in human cancer cell lines. In comparison, melphalan exposure resulted in a 10-fold lower C_{max} that was shifted to later time points. J1 induced more DNA damage and apoptosis than melphalan. The cytotoxic activity and release of melphalan from J1 were inhibited by preincubating cells with the aminopeptidase inhibitor bestatin. In accordance with these results, we showed that J1 is a substrate for aminopeptidase N (APN), which may result in increased tumor selectivity.

J1 effectively inhibited cell growth in a set of neuroblastoma cell lines. Athymic mice carrying neuroblastoma xenografts were treated either with equimolar doses of melphalan or J1. J1 inhibited the tumor growth more effectively than melphalan and the untreated control, and was associated with higher caspase-3 activation, fewer proliferating tumor cells and decreased mean vascular density.

J1 and melphalan showed similar activity profiles when tested in 176 primary tumor cell cultures from patients, but J1 exhibited 50- to 100-fold higher potency. The difference was greater in some diagnoses (e.g. breast cancer, NHL and AML), and was exceptionally large in some breast cancer samples with aggressive phenotypes. A combination screening of J1 and standard chemotherapeutics yielded mostly additive interactions, except for etoposide which induced synergy in all tested cell lines.

In conclusion, the melphalan prodrug J1 is effectively transported into the cells, where aminopeptidases (for example APN) catalyze the formation of melphalan. J1 shows promising preclinical potential in the diagnoses neuroblastoma and breast cancer.

Keywords: Pharmacology, Chemotherapy, Melphalan prodrug, Cancer

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Every beginning
is only a sequel, after all,
and the book of events
is always open halfway through.

_Wisława Szymborska_
This thesis is based on the following papers, which will be referred to in text by their Roman numbers:


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Additional papers:


- Åberg M, Wickström M and Siegbahn A. Simvastatin induces apoptosis in human breast cancer cells in a NFkB-dependent manner and abolishes the anti-apoptotic signaling of TF/FVIIa and TF/FVIIa/FXa. Thrombosis Research. 2007. (Accepted).


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## Abbreviations

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<th>Abbreviation</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>ALL</td>
<td>Acute Lymphocytic Leukemia</td>
</tr>
<tr>
<td>AML</td>
<td>Acute Myelocytic Leukemia</td>
</tr>
<tr>
<td>APN</td>
<td>Aminopeptidase N</td>
</tr>
<tr>
<td>APP</td>
<td>Appendix Cancer</td>
</tr>
<tr>
<td>BS-1</td>
<td>Bandeiraea Simplicifolia</td>
</tr>
<tr>
<td>CD13</td>
<td>Aminopeptidase N</td>
</tr>
<tr>
<td>CE</td>
<td>Carboxylesterases</td>
</tr>
<tr>
<td>CI</td>
<td>Combination Index</td>
</tr>
<tr>
<td>CLL</td>
<td>Chronic Lymphocytic Leukemia</td>
</tr>
<tr>
<td>CML</td>
<td>Chronic Myelocytic Leukemia</td>
</tr>
<tr>
<td>CNGRC</td>
<td>Cysteine-Asparagine-Glycine-Arginine-Cysteine</td>
</tr>
<tr>
<td>EC&lt;sub&gt;50&lt;/sub&gt;</td>
<td>Effective Concentration 50% (resulting in 50% effect)</td>
</tr>
<tr>
<td>FDA</td>
<td>Fluoroscein Diacetate</td>
</tr>
<tr>
<td>FMCA</td>
<td>Fluorometric Microculture Cytotoxicity Assay</td>
</tr>
<tr>
<td>HPLC</td>
<td>High Performance Liquid Chromatography</td>
</tr>
<tr>
<td>IC&lt;sub&gt;50&lt;/sub&gt;</td>
<td>Inhibitory Concentration 50% (resulting in 50% survival)</td>
</tr>
<tr>
<td>IC&lt;sub&gt;70&lt;/sub&gt;</td>
<td>Inhibitory Concentration 70% (resulting in 30% survival)</td>
</tr>
<tr>
<td>J1</td>
<td>L-melphalanyl-&lt;i&gt;p&lt;/i&gt;-L-fluorophenylalanine ethyl ester</td>
</tr>
<tr>
<td>LAP</td>
<td>Leucyl Aminopeptidase</td>
</tr>
<tr>
<td>LD&lt;sub&gt;50&lt;/sub&gt;</td>
<td>Lethal Dose 50% (resulting in 50% death)</td>
</tr>
<tr>
<td>Mel-pFPhe-OH</td>
<td>L-melphalanyl-&lt;i&gt;p&lt;/i&gt;-L-fluorophenylalanine</td>
</tr>
<tr>
<td>MMP</td>
<td>Mitochondrial Membrane Potential</td>
</tr>
<tr>
<td>NHL</td>
<td>Non-Hodgkin’s Lymphoma</td>
</tr>
<tr>
<td>NSCLC</td>
<td>Non-Small Cell Lung Cancer</td>
</tr>
<tr>
<td>P2</td>
<td>L-prolyl-&lt;i&gt;m&lt;/i&gt;-L-sarcolysin-&lt;i&gt;p&lt;/i&gt;-L-fluorophenylalanine ethyl ester</td>
</tr>
<tr>
<td>PBMC</td>
<td>Peripheral Blood Mononuclear Cell</td>
</tr>
<tr>
<td>PMP</td>
<td>Pseudomyxoma Peritonei</td>
</tr>
<tr>
<td>PSA</td>
<td>Puromycin Sensitive Aminopeptidase</td>
</tr>
<tr>
<td>PTC</td>
<td>Peptichemo</td>
</tr>
<tr>
<td>SEM</td>
<td>Standard Error of the Mean</td>
</tr>
<tr>
<td>SI</td>
<td>Survival Index</td>
</tr>
</tbody>
</table>
1 Introduction

Cancer is one of the major causes of death in the developed nations. In Europe and North America it represents approximately 20% of total mortality, which makes it the second most common cause of death. In Sweden, 51000 new cases were diagnosed in 2005 of which the most common types were prostate cancer and breast cancer [1]. There are three major approaches to treating malignancies: surgery, radiation and chemotherapy. The role of each of these treatments depends on the type of tumor and the stage of its development. Chemotherapy is the main method of treatment only in a few types of cancer, but it is increasingly used as an addition to surgery or radiation for many types of tumors.

The history of chemotherapy starts during the First World War, when sulfur mustard was observed to have effect on rapidly dividing cells. This raised the idea that there would be a possible treatment of diseases characterized by uncontrolled cell proliferation, like leukemia. The first clinical trial was presented in 1931, where sulfur mustard solution was applied topically onto, or injected directly into tumors in humans [2]. The procedure was later abandoned due to high toxicity in the patients, but the attempts to cure cancer with similar compounds continued, and in 1942, nitrogenmustards were successfully used in clinical trials [3].

Since then hundreds of chemotherapeutic compounds have been developed and used clinically. Most of them target cellular nucleic acids either by direct reaction (alkylating agents, platinum compounds), by synthesis inhibition (topoisomerase inhibitors, antimetabolites) or by interruption of the cellular machinery for separating the DNA-copies in mitosis (taxanes, vincaalkaloids). Therefore, most anticancer drugs display high activity against proliferating cells or tissues with rapidly dividing cells and thus they are likely to produce, to greater or lesser extent, some general toxic effects e.g. bone marrow toxicity, impaired wound healing, loss of hair, damage of gastrointestinal epithelium, depression of growth in children, sterility and teratogenicity [4].

Most of the anticancer treatments in widespread use today were developed prior to 1975, without knowledge about the genetic and biological mechanisms of cancer pathogenesis [5]. During the last decades, the understanding of signaling pathways in cancer cells has uncovered entirely new targets in cancer treatment, including growth factors, signaling molecules, cell-cycle proteins, modulators of apoptosis and molecules that promote
angiogenesis [6]. A number of drugs targeting these kinds of targets have been approved, for example the small molecule inhibitors imatinib (Gleevec) and gefinitib (Iressa), which act on tyrosine kinases and the antibody trastuzumab (Heceptin) against the Her2 receptor in breast cancer. The attempts to create more targeted anticancer drugs have been extensive; in 2003, a compilation of anticancer therapies in preclinical development or in clinical testing listed more than 1300 research and development projects that were being purchased by pharmaceutical or biotech companies [5]. Unfortunately, clinical trials with many of these agents have been discouraging. It was anticipated that these therapies would be so effective that they would replace older, non-specific cytotoxic agents and that their effects would be limited to cancer cells. With the exception of imatinib, none of these anticipations proved to be true when evaluated in clinical trials [7, 8]. Some of these “failures” can be explained by inadequate patient selection and poor understanding of the targets, which have highlighted the need for predictive tests and further evaluation of the targets [8].

So even though we have entered the “targeted era” in cancer research there still seems to be a need for cytotoxic drugs, especially if they can be further developed to have increased tumor specificity and thereby decreased toxicity. This thesis focuses on J1, a new formulation of the old drug melphalan. It is more than 50 years since melphalan was introduced but it still has a role in treating cancer; for example, melphalan still remains a component in the treatment of multiple myeloma even though new approaches like the proteasome inhibitor bortezomib have been introduced [9].

1.1 Melphalan

1.1.1 History

In the early fifties, the British scientists Bergel and Stock began to work with cytoactive amino acids and their peptide derivatives in hope of finding compounds with antitumor activity and tumor selectivity [10]. The first publication in a series focused on derivatives of the amino acid phenylalanine and led to synthesis of several compounds. The derivative $L-p$-bis(2-chloroethyl) amino-$L$-phenylalanine was ultimately selected as the lead compound; it was later named “melphalan” (mustard-$L$-phenylalanine, molecular structure in Table 1) [11]. Some of the other derivatives were tested in vitro and in vivo but none of them reached the same clinical use as melphalan [12]. Among them is $m$-$L$-sarcolysin, an isomer with the bis(2-chloroethyl)amino group substituted in the meta position.
1.1.2 Pharmacodynamics

Nitrogen mustards i.e. bis(chloroethyl)amines are unstable molecules that spontaneously form aziridinium ions. These highly reactive intermediates can bind covalently to nucleophilic elements on DNA or other biological molecules (Figure 1). Bifunctional agents such as melphalan are more cytotoxic than monofunctional agents as they are able to crosslink a DNA strand within a double helix (intrastrand), between two strands (interstrand) or between DNA and proteins [13]. The reactivity of the nucleophilic sites of DNA relates to electronic and steric properties, as well as to the involvement in hydrogen bonds within the DNA double helix [14]. The most frequent site of alkylation of DNA is the 7 nitrogen (N7) in guanine, which is strongly nucleophilic. Adducts are also formed at the O6 and N1 of guanine, the N1, N3 and N7 of adenine and N3 of cytosine and O4 of thymine [13-15]. Crosslinking of DNA is probably the critical cause of cytotoxicity, inhibiting not only replication but also transcription, subsequently leading to cell death. Repair enzymes may correct the damage or further continue the damage process, which creates base deletions or misplacement DNA single- or double-strand breaks, apurinic sites or ring openings; all resulting in DNA that can no longer serve as a template [13].

Figure 1. Mechanism of action of bifunctional nitrogen mustards.

Melphalan uptake into cells is mediated by active, carrier-mediated processes; at low concentration it occurs through the ASC-like amino acid transport system that also transports alanine, serine and cysteine, whereas at high concentrations, the uptake is mostly mediated by the leucine-preferring amino acid transport system L [13]. Decreased melphalan uptake, increased sulfhydryl content and increased glutathione-S-transferase activity are associated with resistance in cells [13]. Changes in the capacity of enzymes to remove alkylated bases or repair other DNA damages are also related with melphalan sensitivity [15] and reparation of interstand crosslinks has been shown to be relevant for clinically observed melphalan resistance in chronic lymphocytic leukemia (CLL) and multiple myeloma [16].
1.1.3 Stability and pharmacokinetics

The half-life of melphalan in physiological buffer at 37°C is only about 1.5 h due to spontaneous formation of reactive intermediates [17]. The degradation in aqueous solutions is influenced by pH, temperature and chloride concentration of the solvents [17, 18]. By increasing the sodium chloride concentration to 3%, melphalan is stable for up to 6 h at room temperature, allowing longer infusion time [19].

The pharmacokinetics of melphalan in humans is well-studied, both when administered orally and intravenously. Following injection, drug plasma concentration declines rapidly in a biexponential manner with distribution and elimination phase half-lives of about 10 and 75 min respectively [20]. Similar circulating half-lives are obtained with oral and intravenous administration [13]. The plasma protein binding is high (60-90%) and albumin is the major binding protein. Melphalan is eliminated from plasma primarily by chemical hydrolysis to the mono- and bis-hydroxy derivatives [20]. After injection of radiolabeled melphalan, radioactivity is excreted in both urine and feces [17].

1.1.4 Clinical activity and toxicity

Like most alkylating agents, melphalan has showed significant activity in a broad spectrum of human malignancies. It is currently the primary choice in treatment of multiple myeloma and amyloidosis in high-dose treatments with stem cell support [15]. During recent years, there has been an increasing use of melphalan in high-dose regimens, including patients with, for example, lymphoma [21], neuroblastoma [22, 23], advanced breast [24] and ovarian cancers [25, 26]. Several clinical trials have showed interesting results for the combination of melphalan and bortezomib in multiple myeloma. In a study with 34 patients with relapsed or refractory multiple myeloma, 68% showed response, including two complete responses [27]. Similar results were received with additional treatment with thalidomide and prednisolone in another study in the same patient group (n=30); the response rate was then 67% [28]. Single agent activity of melphalan administered over a 24 h period with individual adapted dosing was evaluated in a Phase II study in 25 patients with advanced ovarian cancer. Partial responses were achieved in 16% and stable disease in 12%. The toxicity was acceptable and did not differ from 1 h infusion [29].

The dose-limiting toxicity of melphalan is suppression of hematopoiesis, including both leucopenia and thrombocytopenia, unless it is combined with bone marrow transplantation. High-dose treatment normally include side effects as mucositis, alopecia, diarrhea, nausea and vomiting [13, 15]. Treatment with chemotherapeutics, especially alkylating agents and topoisomerase inhibitors, is associated with an increased risk of secondary malig-
nancies following genetic damages. The most commonly occurring malignancy is acute myelocytic leukemia (AML), typically occurring after 5-7 years, often preceded by a preleukemic period of myelodysplastic syndrome and frequently associated with alterations on chromosomes 5 and 7 [30]. The total cumulative incidence varies between reports but is estimated to be about 10% among long-time survivors treated with alkylating agents [30, 31]. The following factors constitute a higher risk for developing a secondary leukemia: cumulative dose, long-term and maintenance therapy, additional radiation therapy and old age at the time of primary tumor treatment [30].

Table 1. Structures and chemical names of melphalan, m-L-sarcolysin, P2 and J1.

<table>
<thead>
<tr>
<th>Name</th>
<th>Structure</th>
<th>Chemical name</th>
</tr>
</thead>
<tbody>
<tr>
<td>Melphalan</td>
<td><img src="image" alt="Melphalan structure" /></td>
<td>1-(\text{p-bis}(2\text{-chloroethyl})\text{amino-L-phenylalanine} )</td>
</tr>
<tr>
<td>m-L-Sarcolysin</td>
<td><img src="image" alt="m-L-Sarcolysin structure" /></td>
<td>1-(\text{m-bis}(2\text{-chloroethyl})\text{amino-L-phenylalanine} )</td>
</tr>
<tr>
<td>P2</td>
<td><img src="image" alt="P2 structure" /></td>
<td>1-(\text{prolyl-m-L-sarcolysin-p-L-fluorophenylalanine ethyl ester} )</td>
</tr>
<tr>
<td>J1</td>
<td><img src="image" alt="J1 structure" /></td>
<td>1-(\text{melphalanyl-p-L-fluorophenylalanine ethyl ester} )</td>
</tr>
</tbody>
</table>
1.2  \textit{m-L-sarcolysin} and its conjugates

1.2.1 Peptichemio

In the nineteen sixties an Italian non commercial governmental organization (Istituto Sieroterapico Milanese) synthesized a few hundred small peptides based on \textit{m-L-sarcolysin} (molecular structure in Table 1) [32]. A cocktail of the six most interesting peptides was called Peptichemio (PTC) and after successful preclinical testing it entered clinical trials in the nineteen seventies. More than 100 scientific papers were published on PTC, of which at least 31 can be considered as clinical studies, before the production stopped in the mid-eighties.

1.2.1.1 Clinical studies

PTC has shown clinical activity in several human malignancies both as a single agent and as part of combination therapy. In one study, PTC was given to 32 children with various solid tumors. The efficacy was excellent in rhabdomyosarcoma and embryonal sarcoma and encouraging in neuroblastoma, Wilms tumor and histiocytosis X. The toxicity was minimal and further clinical investigations were performed in these diagnoses [33]. Similar results were reported from 25 children with various advanced and disseminated tumors, where PTC proved to be especially efficacious in advanced neuroblastoma [34]. This result was confirmed in two additional studies, in previously untreated children with advanced neuroblastoma; high response rates, 88 and 92\% (n=67 and 12), were reported including some complete responses (14\% and 17\%), although the duration seemed to be short [35, 36].

In children with neuroblastoma resistant to first line treatment or at relapse, 42 and 38\% (n=39 and 28) showed objective regression [37, 38]. Chronic use of PTC was limited by two major factors: long lasting thrombocytopenia and phlebosclerosis [35].

In a Phase II study in 56 patients, which were resistant to other alkylating agents, PTC induced an overall response rate of 32\%, including one complete response. Soft tissue and bone lesions were the primary sites of response. Major toxicities were myelosuppression (affecting mainly the platelets) and sclerosing phlebitis [39]. Melphalan and PTC were compared in a prospective randomized study in 56 previously treated breast cancer patients. There were no objective responses in the melphalan-group, but 14\% with stable disease. Among the PTC-treated patients, 25\% achieved a partial response, 3\% got less than partial response and 11\% stable disease. The major toxicity was myelosuppression. The authors concluded that PTC seemed to be an active agent in previously treated patients with metastatic breast cancer whereas melphalan was considered ineffective [40]. Similar results were
reported from 32 heavily treated, advanced breast cancer patients; the overall response rate was 18% [41].

Fifteen patients with plasma cell neoplasms were treated with PTC, 35% responded, including three complete responses, and the median duration of response was 8.5 months [42]. When PTC was given to previously treated patients with epithelial cancer of the ovary, objective responses were seen in 24% (n=42), including seven complete responses but most responders had a small tumor size (<2 cm) [43]. The most frequent side effects in both studies were myelosuppression and phlebosclerosis [42, 43].

Apart from these single agent studies, some combination studies were performed; several of these in multiple myeloma, were PTC was given in combination with prednisolone or prednisolone and vincristine [44-46]. PTC with vincristine and prednisolone was also given to patients with Non-Hodgkin’s lymphoma [47] and the same combination therapy, with additional melphalan and cyclophosphamide, was evaluated in CLL patients [48]. The combinations were generally considered well-tolerated and the results encouraging [44-48].

### 1.2.1.2 The peptides of PTC

When studying the individual peptides of PTC in vitro (Table 2), it was shown that L-prolyl-\textit{m}-L-sarcolysin-p-L-fluorophenylalanine ethyl ester (P2, also called PSF, molecular structure in Table 1) possessed the highest activity among the peptides. It was, in fact, several times more toxic in human melanoma cells than \textit{m}-L-sarcolysin or melphalan in both non-clonogenic [49] and clonogenic [50] cytotoxicity assays. This could, at least partly, be explained by increased DNA crosslinkning after P2 exposure [50]. The superiority of P2 in vitro compared with the other peptides in PTC, melphalan or \textit{m}-L-sarcolysin was confirmed in cell lines and fresh human tumor samples. P2 was also shown to have less cross-resistance with standard drugs and be less affected by intracellular glutathione levels [51].

The pharmacokinetics of PTC was investigated in vivo. After infusion of PTC, \textit{m}-L-sarcolysin was rapidly released and disappeared with a half-life of 1.7 h. The stability of PTC’s individual peptides was investigated in vitro in blood and was concluded to be low, ranging from 1.1 (P2) – 21 (P4) min [52].

### Table 2. Individual peptides of Peptichemio, mSL = m-L-sarcolysin

<table>
<thead>
<tr>
<th>Peptide</th>
<th>Composition</th>
</tr>
</thead>
<tbody>
<tr>
<td>P1</td>
<td>l-Ser-l-pFPhe-l-mSL-OMe</td>
</tr>
<tr>
<td>P2</td>
<td>l-Pro-l-mSL-l-pFPhe-OMe</td>
</tr>
<tr>
<td>P3</td>
<td>l-pFPhe-l-mSL-l-Asn-OMe</td>
</tr>
<tr>
<td>P4</td>
<td>l-mSL-l-Arg(NO2)-l-Nval-OMe</td>
</tr>
<tr>
<td>P5</td>
<td>l-pFPhe-Gly-l-mSL-l-Nval-OMe</td>
</tr>
<tr>
<td>P6</td>
<td>l-mSL-l-Arg-l-Lys-l-mSL-l-His-OMe</td>
</tr>
</tbody>
</table>
1.2.2 Ambamustine (PTT.119) and MF13

A screening of over 300 \( m-L \)-sarcylsolin containing peptides resulted in a compound called PTT.119 (\( p-L \)-fluorophenylalanine-\( m-L \)-sarcylsyl-L-methionine ethyl ester, later called ambamustine) [53]. Ambamustine demonstrated good preclinical activity both \textit{in vitro} in cell lines and \textit{in vivo} in mouse models [53], and its uptake occurred at least by two natural pathways for amino acid transport [54]. It later entered clinical trials and appeared useful in some diagnoses for instance Non-Hodgkin’s lymphoma [55], but had no or modest activity in others such as small-cell lung cancer [56]. Ambamustine was however later abandoned for toxicity reasons [G. Bekesi, personal communication with J. Gullbo, 1999].

In a new screening, the tripeptide MF13 (\( L \)-prolyl-\( m-L \)-sarcylsyl-L-norvaline ethyl ester) was chosen as the new lead compound [57]. MF13 has shown to induce apoptosis \textit{in vitro} in several human cell lines and demonstrated higher antitumor activity than \( m-L \)-sarcylsolin \textit{in vivo} in xenograft models in mice [57, 58]. The activity and mechanism of MF13 in hepatocellular carcinoma were studied in a mouse model and MF13 was shown to inhibit the tumor growth by inducing apoptosis [59].

1.3 J1

Based on the knowledge that P2, the most active peptide in PTC, had a higher cytotoxic effect than both \( m-L \)-sarcylsolin and melphalan, a series of new alkylating oligopeptides were synthesized and tested \textit{in vitro}. Seventeen dipeptides were synthesized and analyzed for cytotoxicity in a cell line panel to determine which features of the dipeptide were most important for the cytotoxic effect. Factors such as amino acid composition, amino acid sequence, modifications of the \( C \)- and \( N \)-terminus, and to a minor extent the lipophilicity of the dipeptide derivatives, appeared to influence the \textit{in vitro} activity. The results indicated that the activity of the compounds not only relied on their chemical reactivity, but also on active biological interactions such as transport across membrane and/or enzymatic liberation of reactive entities [60].

\( L \)-melphalanyl-\( p-L \)-fluorophenyl-alanine ethyl ester, J1 (molecular structure, Table 1) was designed as an intermediate between melphalan and P2 and chosen as the lead compound. The activity of J1, P2 and melphalan was compared in different \textit{in vitro} assays; P2 was more effective than melphalan both in cell lines and patient tumor cells in a non-clonogenic assay. Interestingly, J1 was even more cytotoxic than P2 and produced more pronounced effects in a kinetic assay of macromolecular synthesis, metabolic activity and apoptosis [61]. An advantage with J1 is that it contains melphalan, which is a
well known chemotherapeutic agent that has been widely used as opposed to \textit{m-L-sarcolysin}.

J1 was evaluated for toxicity and antitumor activity in mice in parallel to melphalan. The highest investigated dose (25 μmol/kg), was considered close to maximal tolerated dose, with minor effects on body weight, but significant effect on the hematological parameters both for melphalan and J1. The drugs appeared to be equitoxic as no significant differences were detected. The hollow-fiber model with three different human tumor cell lines and two samples of primary human tumor cells was used for evaluating the antitumor effect. At the dose level tested (25 μmol/kg) there was a significantly decreased tumor cell growth and viability in response to J1 as compared with both melphalan and placebo in the cell lines CCRF-CEM and NCI-H69. In primary ovarian carcinoma cells only J1 treatment resulted in significant tumor regression [62].

1.4 Concept and examples of anticancer prodrugs
An increased expression of various hydrolytic enzymes like proteases, peptidases and esterases have been described in several types of human malignancies, especially those characterized as fast-growing and aggressive [63]. These enzymes include for example cathepsins, matrix metalloproteinases and plasmogen activators, which are all proteases known to be involved in tumor progression and the metastatic cascade [64]. Inhibitors of several of these proteases are already being evaluated clinically. However, some of these proteases, which are involved in tumor invasion and metastasis, could instead be used as target enzymes for design and synthesis of antitumor prodrugs [64].

1.4.1 Examples of enzymatically activated melphalan prodrugs
Prolidase is a cytosolic exopeptidase that cleaves imidodipeptides with C-terminal proline; an overexpression of the enzyme has been described in certain neoplastic tissues. Melphalan derivatives were synthesized with the intention of creating a targeted delivery through prolidase activation, and one derivative was found to be a good prolidase substrate [65]. \textit{In vitro} testing in a breast cancer cell line showed that the prodrug was more efficiently transported into the cells, evoked higher cytotoxicity and had similar effect on the DNA synthesis compared with melphalan itself [66].

Prolidase was also identified as an attractive target in melanoma from studies of gene expression databases and bioinformatics tools. A L-proline melphalan prodrug, prophalan-L, was synthesized and showed cytotoxicity similar to melphalan and the concept of targeting prolidase in melanoma cell lines was verified \textit{in vitro} [67]. Further \textit{in vitro} investigations showed that
the bioactivation and cytotoxicity of prophalan-L in cancer cell lines correlated well with their prolidase expression. There were also no differences in the uptake of melphalan, prophalan-L and its corresponding D-proline analog, which is resistant to prolidase activation. However, only melphalan and prophalan-L induced cytotoxic effects [68]. When prophalan-L, prophalan-D and melphalan were compared in a mouse melanoma model, a significant difference in tumor growth was demonstrated between the untreated control and melphalan or prophalan-L, but no significant difference between control and prophalan-D or between melphalan and prophalan-L. Prophalan-L was significantly less toxic than melphalan (measured as weight loss), whereas no difference was observed in toxicity between the prodrugs and untreated control. This suggests that prophalan-L has an enhanced therapeutic window compared with melphalan [69].

Collagenase, which degrades the extracellular matrix and leads to tumor cell metastasis, was the primary target for Timár and co-workers when synthesizing a melphalan containing hexapeptide. The product was shown to be a substrate for collagenase, resulting in cleaved products with higher potency than the prodrug in vitro [70].

1.4.2 Aminopeptidases as a target and examples

Aminopeptidases are metalloproteases that remove amino acids from unblocked N-terminal positions of oligopeptides. Recent studies have demonstrated that several aminopeptidases, including aminopeptidase N (APN, identical to CD13) [71] and dipeptidyl peptidase IV [72], are involved in tumor cell growth, differentiation and invasion in various malignancies through up- or downregulation of their expression, depending on the types of malignancy. The activity and/or the expression of APN is elevated in cancer of the breast [73], lung [74, 75], thyroid [76], pancreas [77] and ovaries [78, 79]. APN activity is also demonstrated to be elevated in plasma and effusions from cancer patients, corresponding to the tumor load [75, 80, 81]. In addition, an overexpression of leucyl aminopeptidase (LAP) is shown in breast cancer [82] and ovarian carcinomas (placental LAP) [83]. Primarily APN, but also aminopeptidase A, have been demonstrated to play a functional role in angiogenesis and thus be a target for its inhibition [84, 85]. Owing to overexpression of aminopeptidases in malignant cells, the enzymes may serve as an attractive target for cytotoxic agents; the APN inhibitor bestatin is, in fact, already in clinical trials in the diagnoses AML and chronic myelocytic leukemia (CML), lymphomas and Stage I squamous cell lung carcinoma and preliminary results suggest significant effects on survival [86].

A series of potential prodrugs of methotrexate was reported, in which the 2-amino group was coupled with amino acids with the intention that aminopeptidases should convert these prodrugs to generate free methotrexate. Of
the synthesized prodrugs, 2-L-pyroglutamyl-methotrexate was the best candidate, as it showed both stability in buffer solution and aminopeptidase activation [87].

Attempts to target APN were also made by coupling the antitumor drug doxorubicin with a cyclic peptide containing the amino acid sequence Cysteine-Asparagine-Glycine-Arginine-Cysteine (CNGRC) which is preferentially bound to cells that exhibit high levels of APN. The aim was to improve doxorubicin delivery to tumor endothelium, i.e. vascular targeting. In preliminary xenograft studies in nude mice, it was shown that the efficacy of doxorubicin was enhanced when coupled to the motif [88] and it was later confirmed that phages expressing CNGFC bind to immunocaptured CD13 and to CD13 overexpressing tumor cells [85]. However, when the mechanism was further investigated, results showed that the cytotoxic effects were similar for doxorubicin-CNGRC in cell lines with different CD13 expression and that the cytotoxicity was the same as for the free doxorubicin both in vitro and in vivo. This indicated that the antiproliferative and antiangiogenic effects of doxorubicin-CNGRC were caused by the cytostatic effects of intracellularly released parent drug, independent of APN (CD13) expression/activity [89]. Similar efforts were also made with two prodrugs of 5-fluoro-2’-deoxyuridine coupled with CNGRC. Both prodrugs were of lower cytotoxicity compared to 5-FdUrd but showed more selective cytotoxicity toward APN/CD13 positive cells than negative cells [90].

1.5 Diagnosis of special interest: neuroblastoma

Neuroblastoma is one of the most common solid childhood cancers and originates from precursor cells of the sympathetic nervous system [91, 92]. In Europe the incidence is 11 cases per million children and the overall five-year survival is about 59% [93]. Approximately 20 new cases occur annually in Sweden [94]. Even though these tumors may regress spontaneously, particularly in infants, or mature into benign ganglioneuroma, the majority of older children have extensive or metastatic disease at the time of diagnosis, and their overall survival is poor. Metastasis to bone or bone marrow or finding specific genetic features, for example MYCN oncogene amplification, deletions of 1p or 11q, unbalanced gain of 17q in the tumor cells are major adverse prognostic factors [92, 95]. Long-term survival is seldom achieved for these patients despite intensive chemotherapeutic treatment. No cytotoxic agent has been universally successful in curing neuroblastoma and it is common that highly malignant neuroblastomas have multidrug resistance. There are 12 to 15 cytotoxic drugs frequently used in the treatment and cyclophosphamide, cisplatin/carboplatin, doxorubicin and teniposide/etoposide have become the backbone of many multidrug regimes [91]. Melphalan may be used as consolidation therapy for children with Stage IV diseases.
and for those with an amplified \textit{MYCN} gene copy number in tumor cells. High dose melphalan with autologous bone marrow support has been shown to improve the event-free and overall survival of children > 1 year of age with Stage IV neuroblastoma, who achieved complete or good partial response after OPEC (vincristine, cisplatin, teniposide, cyclophosphamide) induction and surgery [96].
2 Aims

J1 is a new alkylating dipeptide that has shown impressive activity \textit{in vitro}. The long clinical experience of melphalan and the clinical trials performed on PTC also supports the assumption that J1 is a promising candidate drug for treating cancer.

The specific aims of the project were:

- Perform detailed pharmacological profiling of J1 to find explanations for the higher cytotoxicity compared with melphalan; transportation into and activation inside the cells.
- Characterize the cell death patterns for J1, i.e., DNA-damage and apoptosis.
- Study the diagnosis directed activity of J1 by using human tumor samples \textit{ex vivo}.
- Investigate the potential of treatment with J1 in specific diagnosis of interest, such as neuroblastoma and myeloma.
- Study the interaction effects of J1 in combination with other standard chemotherapeutics.

All the studies were done in comparison with melphalan.
3 Methods used

The methods used in this thesis will be discussed here briefly, for further details see the individual papers.

3.1 Human tumor cell lines

Throughout the papers tumor cell lines were used to evaluate cytotoxic effects and for mechanistic studies. The cell lines used were mainly taken from two different sets of cell lines. The first one is a panel focusing on cellular drug resistance containing four parental cell lines from different tumor types with five sublines expressing different defined mechanisms of resistance and one primary resistant cell line. The panel has been described in detail before [97] (Paper I, III and IV). The other is a diagnosis specific panel with seven different neuroblastoma cell lines (Paper II and IV). Some additional cell lines were used to include more diagnoses (Paper III, IV). All cells were grown in complete cell medium at 37º C in a humidified atmosphere containing 5% carbon dioxide. They were split twice weekly and harvested in the log-phase for experimental use. Cell lines were kept for a maximum of 20 passages.

3.2 Patient tumor samples

In Paper III primary tumor cells from patients with twelve different cancer diagnoses were used to determine the diagnosis-specific activity of J1 and melphalan. The diagnoses were acute lymphocytic leukemia (ALL), AML, CLL, CML, non-Hodgkin’s lymphoma (NHL), myeloma, breast cancer, colorectal cancer, non-small cell lung cancer (NSCLC), ovarian cancer, renal cancer and appendix cancer/pseudomyxoma peritonei (app/PMP). The tumor samples were obtained by routine surgery, diagnostic biopsy or bone marrow/peripheral blood sampling. This sampling was approved by the ethical committee at the Uppsala University. Primary cultures of tumor cells from patients have, using the fluorometric microculture cytotoxicity assay, shown good correlation with tumor diagnosis-specific activity of standard and investigational new drugs. In contrast to established cell lines, patient cells retain their disease-specific phenotype with respect to drug sensitivity and
are better predictors for clinical activity [98]. For evaluation of cytotoxic activity in a normal phenotype, preparations of normal peripheral blood mononuclear cells (PBMC) from healthy donors were used.

### 3.3 The fluorometric microculture cytotoxicity assay

To measure cytotoxic effect of J1, melphalan and other test substances, the fluorometric microculture cytotoxicity assay (FMCA) was used. The method is a total cell kill assay based on measurement of fluorescence generated from hydrolysis of fluorescein diacetate to fluorescein by cells with intact plasma membranes, cultivated and exposed to drugs in 96- or 384-well microtiter plates. The fluorescence is proportional to the number of living cells in the well. Cell survival is presented as survival index (SI) and defined as the fluorescence in the experimental wells in percent of that in control wells, with blank values subtracted. The \( IC_{50} \)-value (inhibitory concentration 50%) was defined as the concentration giving a SI of 50%.

FMCA was originally developed as a method for semi-automated determination of cytotoxicity in human tumor cell lines in 96-well plates [99]; however, it was soon recognized for its clinical potential in predicting leukemia treatment outcome [100, 101] and later also on material from solid tumor [102, 103]. The sensitivity and specificity of FMCA and similar \textit{in vitro} methods used, in relation to clinical outcome, was 0.9 and 0.7 respectively [104]. Results from the FMCA have been shown to correlate with those of previously established \textit{in vitro} method such as MTT ([3-4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide) and DiSC (differential staining cytotoxicity) [100, 101]. Later the method has been developed for using the 384-well format both for testing of cell line and tumor samples from patients [105].

### 3.4 Chemical synthesis

The chemical synthesis in Paper I and IV followed standard procedures in solution phase peptide chemistry and were performed by Marcus Tullberg, Göteborg University. The dipeptides were synthesized using standard coupling reactions.

### 3.5 Intracellular concentrations

The intra- and extracellular concentrations and degradation of J1 were studied in Paper I and IV with two HPLC methods. In Paper I and partly IV, the chromatography method earlier developed for the PTC peptides [52], using
HPLC analytical separation and fluorometric detection of the autofluorescent melphalan and J1 respectively was used. In Paper IV, cell samples were analyzed by HPLC using a C₁₈ column with positive ion electrospray tandem-mass spectroscopic detection. This method was developed, validated and performed by Analyst Research Laboratories, Israel.

3.6 Human neuroblastoma xenografts model

Male nude rats (HsdHan: RNU-rnu) and female nude mice (NMRI nu/nu) were used for the xenograft experiments in Paper II. The animals were housed and maintained in laminar flow cabinets under specific pathogen-free conditions and given sterile water and food ad libitum. Animals were anaeasthetized and tumor cells were injected subcutaneously in the flank of the hind legs [106]. The procedure was carefully performed, to not pierce the muscle fascia, or lose cells by leakage from the injection site. When the tumors were evident by palpation and/or visible, the tumor length (along the tumor axis) and width (perpendicular to the axis) were measured with a caliper every second day and tumor volume was calculated by length x width² x 0.44. The true tumor weight was recorded at autopsy.

The animal experiments were approved by the regional ethics committee for animal research (N234-05 and N75-05) in accordance with the Animal Protection Law (SFS 1988:534), the Animal Protection Regulation (SFS 1988:539), and the Regulation for the Swedish National Board for Laboratory Animals (SFS 1988:541). The ability of the xenograft model to predict responses of patients has been questioned; disadvantages as an ectopic tumor site and the fact that the human tumor cells commonly used bear little resemblance to cells in real tumors have been expressed [5]. A great advantage with a subcutaneous xenograft tumor model is that subcutaneous tumors are easily evaluated for treatment response, without invasive methods, during the experiments. The xenograft model is an accepted method widely used in the pharmaceutical industry [5].

3.7 Immunohistochemistry

The in vivo apoptosis, proliferation and angiogenesis were evaluated in de-paraffinized tumor sections of neuroblastoma xenografts. For identification of caspase-3 activity and proliferation, sections were incubated with monoclonal anti-active caspase-3 antibody and anti-Ki-67 antibody respectively and detected using anti-rabbit-HRP-conjugated Super Picture Polymer Kit. Biotinylated Bandeiraea simplicifolia (BS-1) lectin was used for highlighting endothelial cells.
3.8 Multiparametric evaluation of apoptosis

To study cell death characteristics, a multiparametric single-cell assay was used (Paper II, IV and unpublished data) [107]. Cells were seeded and exposed to drugs in 96-well plates and probes were added to stain apoptotic markers: FAM-DEVD-FMK to stain activated caspase-3, MitoTracker Red to evaluate mitochondrial membrane potential (MMP) and Hoechst 33342 to stain the nucleus. Analysis was performed in the ArrayScan® high content screening system (Cellomics Inc.) which is a computerized automated fluorescence imaging microscope that automatically identifies stained cells and reports the intensity and distribution of fluorescence in individual cells. Automatic focusing, image acquisition and analysis were performed to collect data on a user-defined number of cells. Images and data regarding intensity and texture of the fluorescence within the individual cells, as well as the average fluorescence of the cell population within a well were stored in a database for easy retrieval and analysis.

3.9 Alkaline single cell gel electrophoresis

To evaluate the DNA damage, alkaline single-cell gel electrophoresis (comet assay) [108, 109] was performed in Paper IV. Cells were exposed to drugs and then mixed with 0.8% low-melting point agarose and layered on top of an ordinary microscope slide pre-coated with 0.8% agarose. After the cells were lysed electrophoresis was performed, slides were neutralized and images analyzed using a fluorescence microscope attached to a black and white charge-coupled device video camera connected to a computer-based image analysis. The tail moment was used as an indicator of DNA damage (calculated as the tail length multiplied by the fraction of DNA in the comet tail); median values of 50 randomly captured comets were used as a summary measure on each slide and four slides were prepared per treatment.

3.10 Statistics

The IC_{50}-values for drugs in cell lines and primary tumor cells in vitro were determined from log concentration-effect (survival index %) curves in GraphPad Prism using non-linear regression analysis. All calculations on IC_{50}-values were performed on logarithmic values. In general results are presented as mean values ± standard error of the mean (S.E.M.) of three experiments, unless otherwise indicated. Comparison of activity between two groups was made with Student’s t-test and ANOVA with Tukeys post-tests were used to compare three or more treatment groups. All statistical tests were two-sided and p<0.05 was considered significant.
The combination effects of J1 and standard chemotherapeutics were analyzed using the median-effect method of Chou and Talalay [110] using the software CalcuSyn Version 2. All dose-response curves were fit to a linear model using the median effect equation, allowing calculation of a median effect value D (corresponding to the IC_{50}) and slope. The extent of drug interaction between the drugs was expressed using the combination index (CI) for mutually exclusive drugs: CI = d_1/D_1 + d_2/D_2 where D_1 and D_2 represent the concentration of drug 1 and 2 alone, required to produce a certain effect and d_1 and d_2 are the concentration of drugs 1 and 2 in combination required to produce the same effect. Different CI values are obtained when solving the equation for different effect levels. A CI equal to one (1.0) indicates an additive interaction; a significantly lower CI value was defined as synergy and significantly higher as antagonism. One-sample t-tests were used to determine if the CIs differed from one ($p<0.05$).
4 Results and discussion

4.1 J1 is a prodrug dependent on peptide hydrolysis (Paper I)

In Paper I the details of the increased activity of J1 compared with melphalan was investigated aiming to explore the hypothesis that J1 works as an enzymatically activated prodrug with possible active transport into the cells by membrane transporters. Evidence of an overexpression of such proteins in malignant phenotypes is described in scientific literature, for example various hydrolytic enzymes such as peptidases, esterases and proteases [63], and membrane transporters as the dipeptide transporter pepT1 [111, 112].

Attempts to competitively inhibit the active transport of J1, using the pepT1 substrate glycyl-sarcosine (Gly-Sar) or the dipeptide phenylalanyl-phenylalanine (Phe-Phe), were unsuccessful; addition of the transporter substrates had no effect of the cytotoxic activity of J1 (Figure 2). This suggests that passive diffusion is the predominantly transport mechanism for J1 into the cells.

![Figure 2](image-url)

Figure 2. Dose-response curves for J1 in the human tumor cell lines ACHN, CCRF-CEM and U937GTB (mean of two experiments in FMCA). No competitive inhibition from pre-exposure to the PepT1 substrate, Gly-Sar or the dipeptide Phe-Phe was shown (t-test, $p>0.05$).

Despite the lack of evidence for active transport, the results showed that the transport of J1 into the cells was rapid; limiting the exposure time in cell lines proved to be less unfavorable for J1 than for melphalan and six other standard chemotherapeutic agents, indicating a trapping mechanism. This was confirmed when measuring the intracellular melphalan concentration after J1 exposure. A high concentration was reached as soon as after 15 min.
Dipeptide derivatives designed to resist the action of peptidases were less active than the corresponding normal dipeptide in the same experimental setup. This demonstrated that the intact peptide clearly was less effective in the alkylation of nucleophilic sites as DNA, probably due to steric hindrance. The dependency of hydrolysis was verified when inhibition of peptidase activity resulted in a decreased activity of J1. The aminopeptidase inhibitor bestatin [113] caused a more pronounced shift in cytotoxic activity compared with a more general protease-inhibiting cocktail, indicating that aminopeptidases are an interesting group of enzymes.

In summary, Paper I demonstrated that the activity of J1 was highly dependent on intracellular hydrolysis, which created a rapid and efficient intracellular delivery of melphalan. In Paper IV, the enzymatic activation was further studied.

4.2 J1 inhibits neuroblastoma cell growth \textit{in vitro} and \textit{in vivo} (Paper II)

Paper II focuses on the potential of J1 in the treatment of a specific diagnosis, neuroblastoma. Neuroblastoma was chosen as the diagnosis of interest for several reasons: there is a need for novel therapies in high-risk neuroblastoma, clinical trials with PTC had shown promising results [33-37] and the fact that high-dose melphalan still has a role in treating advanced neuroblastoma [96].

4.2.1 The activity of J1 in neuroblastoma cell lines

J1 showed high activity in seven human neuroblastoma cell lines \textit{in vitro}, being 35-810 times more active than melphalan when J1, melphalan and five chemotherapeutic drugs commonly used clinically in neuroblastoma, were tested using the FMCA (Figure 3). The difference was considerable in especially two of the cell lines, SK-N-SH and SH-SY5Y, which indicates that those cell lines may express drug targets specific for J1 to a higher extent. These drug targets may be enzymes capable to activate J1.
4.2.2 J1 in vivo in neuroblastoma xenografts

To see if the impressive activity of J1 in neuroblastoma was translatable into the in vivo situation, three xenograft studies in rodents were performed. Two cell lines were used, the drug resistant SK-N-BE(2) and the, in general, more sensitive SH-SY5Y. J1 and melphalan were administered intravenously in the tail vein as one (at day 0) or two doses (at day 0 and 6). Both J1 and melphalan significantly inhibited tumor growth in rats with SK-N-BE(2) xenografts after one dose of 10 μmol/kg (approximately 50% of LD50 in rats) compared with the untreated control rats, but there was no difference between the two drugs. When rats carrying SH-SY5Y xenografts were treated with one lower dose J1 or melphalan, 0.50 μmol/kg (approximately 2.5% of LD50 in rats), both treatments still inhibited tumor growth significantly compared to the untreated controls. However, with this dose J1 was also significantly more effective than melphalan over the whole observation period. In the third setting, mice with SH-SY5Y-tumors were treated with two doses of 0.50 μmol/kg (approximately 0.6% of LD50 in mice) J1 or melphalan. Tumor growth was significantly inhibited by the treatment with J1 both compared with the untreated control and the melphalan treated mice. In Figure 4 the tumor weights at sacrifice are shown. Animal weight and other signs of toxicity were recorded. The high dose of melphalan and J1 (10 μmol/kg) caused a transient reduction in weight gain compared to the untreated control rats. In rats treated with the lower dose (0.50 μmol/kg) only melphalan, not J1, had a temporary inhibitory effect on the weight gain. No impact on weight gain was observed in mice treated with two doses of 0.50 μmol/kg J1 or melphalan.
Even though J1 proved to be more effective in inhibiting the tumor growth than melphalan in the two *in vivo* studies with lower doses, the differences were considerably less pronounced than in the *in vitro* setting. One possible explanation for this moderate difference may be related to the enzymatic differences between rodents and humans which will affect the activation of J1 to melphalan. J1 has two hydrolysis-susceptible bonds, one peptide- and one ester bond and cleaving of the peptide bond by peptidases leads to release of melphalan, an action which is crucial for the cytotoxic effect of J1. Reports of extended esterase activity in rodents compared to humans have been published previously [114], and it has been shown to be relevant for the efficacy of other prodrugs like CPT-11 (irinotecan) in *in vivo* experiments in mice [115]. Unpublished data have confirmed this hypothesis; J1 showed markedly shorter half-life in blood from mice and rats (9 and 16 seconds) and the de-esterified form of J1 was formed to a much higher extent in rodent blood compared with human, where the half-life was about 5 min and no de-esterified form could be detected [unpublished data, Nygren H and Ehrsson H, 2007]. With the rapid de-esterification of J1 some of the advantages compared with melphalan may be lost; the passive transportation into the cells may decrease since the de-esterified J1 is less lipophilic than J1. The de-esterified J1 indeed possesses reduced cytotoxic activity *in vitro*, see section 4.8.2.

![Figure 4](image.png)

*Figure 4.* Tumor weight on day 12 (at sacrifice) for rats and mice treated i.v. with J1 or melphalan, expressed as percent of the mean tumor weight for the untreated animals. Mice were treated with 0.5 μmol/kg day 0 and day 6 (SH-SY5Y-tumors, n=25) and rats were treated with either 0.5 μmol/kg (SH-SY5Y-tumors, n=16) or 10 μmol/kg [SK-N-BE(2)-tumors, n=16] on day 0. The tumor weights for animals treated with J1 were significantly lower than the untreated controls in all experiments, while melphalan-treated tumors only weighed significantly less in the high dose regime (*p*<0.05). Two mice in the melphalan-treated group had to be put to death before day 12 (at day 8 and 9) because they exceeded the maximal approved tumor size in the ethical approval.
4.2.3 Treatment with J1 induces apoptosis, reduces proliferation and inhibits angiogenesis in vivo

To evaluate the quality of the treatments, immunohistochemistry was performed on the tumors where a difference was observed in tumor growth between J1 and melphalan. In the SH-SY5Y-tumors from rats, J1 induced significantly more apoptosis (caspase-3 positive cells) than melphalan and untreated controls while difference in proliferation (Ki-67 positive cells) was only significantly less compared with untreated cells. Tumors from mice (SH-SY5Y-cells) treated with J1 showed not only significantly more apoptosis, but also less proliferation compared with the untreated controls and melphalan. Tumors were also stained with the endothelial cell marker BS-1 because inhibition of angiogenesis can contribute to a reduced cell proliferation. Interestingly, J1-treated tumors had a significantly decreased number of blood vessels compared with melphalan and untreated controls.

The immunohistochemistry evaluation showed that J1 was superior from a qualitative point of view compared to melphalan. In addition to its direct antitumoral effects, J1 was also able to significantly reduce the number of blood vessels. The metalloproteinase APN is highly expressed in vascular endothelial cells and has been shown to play multiple roles in angiogenesis [116]. J1 was shown to be a substrate for APN which may be of importance for the antiangiogenic effect of J1, see section 4.8.3.

4.3 Species differences in activity of J1 and melphalan in lymphocytes (unpublished data)

The activity of J1 and melphalan was studied in primary lymphocytes from human and mouse to explore possible differences between species [117]. As illustrated in Figure 5, lymphocytes from mice were less sensitive to J1 compared with human cells, the difference was 76-fold, and for melphalan the corresponding difference was 4.6-fold. Lymphocytes from rats and dogs have also been preliminary tested. Results indicated that IC$_{50}$ for J1 in rats was 7.3 μM and in dogs 0.16 μM and for melphalan 140 μM and 200 μM for rats and dogs, respectively.
Consequently, the difference in cytotoxicity between melphalan and J1 was much greater in humans compared to mice (220 and 13 times, respectively). This result is in concordance with the stability data of J1 in blood, previously discussed in regard to the \textit{in vivo} results in rodents (section 4.2.2). Carboxylesterases (CE) belong to a family of enzymes that are thought to be involved in detoxification of xenobiotics. They are known to hydrolyze many esterified drugs, such as lidocain and cocaine, to inactive products [118]. CE are located predominantly in the endoplasmatic reticulum but can be secreted from cells which result in a localized high extracellular concentration [119]. The activity of CE is described to be higher in serum in rodents compared with humans [120]. Hence, the species differences observed for J1 in lymphocytes may be due to higher extracellular levels of esterases such as CE in mice compared to humans. As hypothesized before, a more efficient de-esterification of J1 extracellularly may decrease the transport into the cells considerably.

4.4 Diagnosis directed activity of J1 \textit{ex vivo} (Paper III)

In Paper III the disease-specific activity \textit{ex vivo} for J1 and melphalan was studied in 176 primary cultures of human tumor cells using the FMCA. J1 demonstrated a significantly lower mean IC$_{50}$ than melphalan in all twelve diagnoses represented (Table 3). Low IC$_{50}$-values for J1 were observed especially in the hematological malignancies as well as breast cancer and ovarian cancer (Table 3). The correlation coefficient on all the samples was 0.78, indicating a common mechanism of action, in accordance with earlier results in cell lines [61]. When comparing the ratios of the IC$_{50}$-values of melphalan over J1, the highest ratios were observed in NHL, AML and breast cancer.
A striking observation was the great variation in the ratios among the individual breast cancer samples. A subgroup analysis (where early stage was defined as surgically resectable and advanced stage as non-resectable: Stage IV and locally advanced) revealed IC\textsubscript{50}-values of 0.84 µM and 600 µM, for J1 and melphalan, respectively, (ratio 713) in early stage breast cancer samples and in advanced stage samples of 0.50 µM and 37 µM, respectively (ratio 74) (Figure 6). This difference was mostly due to decreased melphalan sensitivity in the advanced stages compared to the early stage samples, while the J1 sensitivities were quite the same. This may be explained by alkylator resistance; all the advanced stage samples had previously been treated with cyclophosphamide, which is a first line drug used in breast cancer. PTC was compared with melphalan in a randomized Phase II trial in advanced breast cancer (previously discussed in section 1.2.1.1). PTC showed partial remission in 25% of the patients while no objective responses were observed in the melphalan-treated group, indicating that melphalan was ineffective in previously treated breast cancer patients, possibly because of cross-resistance with cyclophosphamide, whereas PTC did not show the same resistance problems [40]. Another diagnosis showing an interesting difference in sensitivity for J1 and melphalan was AML, where the ratio between the IC\textsubscript{50}-values was 107 and the Pearson correlation coefficient was as low as 0.37, indicating differences in the inherent sensitivity and prodrug activation. Unfortunately, clinical information on the AML patients was not available for subgroup analysis.

Drug effect in cells from CLL and PBMC were compared to roughly estimate tumor cell specificity. This showed that both J1 and melphalan were slightly more active against the normal phenotype, but the differences were not significant. A previous study has shown the same negative relationship for CLL/PBMC activity in 17 out of 25 tested standard chemotherapeutic drugs [121]. This was also confirmed in Paper III, where only two of the eight tested chemotherapeutics were more active in CLL than in PBMC.
Table 3. Median (range) of individual IC<sub>50</sub>-values for J1 and melphalan in 176 primary cultures of human tumor cells in twelve diagnoses and seven preparations of healthy lymphocytes. R was derived from Pearson correlation coefficient.

<table>
<thead>
<tr>
<th>Diagnosis</th>
<th>J1 (μM)</th>
<th>Melphalan (μM)</th>
<th>Ratio (melphalan/J1)</th>
<th>R (melphalan and J1)</th>
<th>n</th>
</tr>
</thead>
<tbody>
<tr>
<td>ALL</td>
<td>0.084 (0.01-20)</td>
<td>4.38 (0.97-84)</td>
<td>47</td>
<td>0.65</td>
<td>21</td>
</tr>
<tr>
<td>AML</td>
<td>0.057 (0.01-1.6)</td>
<td>7.27 (0.99-75)</td>
<td>107</td>
<td>0.37</td>
<td>26</td>
</tr>
<tr>
<td>CLL</td>
<td>0.23 (0.01-0.69)</td>
<td>15 (4.3-30)</td>
<td>68</td>
<td>0.41</td>
<td>18</td>
</tr>
<tr>
<td>CML</td>
<td>0.037 (0.01-0.39)</td>
<td>4.6 (0.10-54)</td>
<td>72</td>
<td>0.46</td>
<td>8</td>
</tr>
<tr>
<td>PBMC</td>
<td>0.12 (0.020-0.50)</td>
<td>10 (5.3-13)</td>
<td>85</td>
<td>0.41</td>
<td>7</td>
</tr>
<tr>
<td>Myeloma</td>
<td>0.29 (0.046-0.32)</td>
<td>11 (4.1-26)</td>
<td>54</td>
<td>0.75</td>
<td>3</td>
</tr>
<tr>
<td>NHL</td>
<td>0.042 (0.01-3.2)</td>
<td>7.0 (0.73-90)</td>
<td>160</td>
<td>0.88</td>
<td>14</td>
</tr>
<tr>
<td>Ovarian ca</td>
<td>0.57 (0.035-100)</td>
<td>32 (8.1-1000)</td>
<td>60</td>
<td>0.72</td>
<td>21</td>
</tr>
<tr>
<td>Breast ca</td>
<td>0.39 (0.01-21)</td>
<td>56 (9.9-1000)</td>
<td>150</td>
<td>0.46</td>
<td>20</td>
</tr>
<tr>
<td>Colorectal ca</td>
<td>24 (0.26-100)</td>
<td>1000 (24-1000)</td>
<td>27</td>
<td>0.75</td>
<td>11</td>
</tr>
<tr>
<td>Renal ca</td>
<td>95 (1.76-100)</td>
<td>1000 (400-1000)</td>
<td>11</td>
<td>0.70</td>
<td>7</td>
</tr>
<tr>
<td>NSCLC</td>
<td>0.41 (0.095-10)</td>
<td>28 (11-120)</td>
<td>84</td>
<td>0.68</td>
<td>5</td>
</tr>
<tr>
<td>App ca/PMP</td>
<td>1.2 (0.14-83)</td>
<td>51 (13-1000)</td>
<td>74</td>
<td>0.56</td>
<td>22</td>
</tr>
</tbody>
</table>

Figure 6. Breast cancer samples treated with J1 or melphalan (one dose-response curve per patient), divided into two groups: early stage breast cancer (solid line, ■: surgically resectable, n=13) and advanced stage breast cancer (dashed line, □: non-resectable: stage IV and locally advanced, n=7).

Since J1 is a prodrug depending on hydrolytic activation to deliver melphalan the different activity profile in various cell types may in part be due to different expression of hydrolytic enzymes such as aminopeptidases. Several studies have demonstrated an increased activity and/or expression of different aminopeptidases in malignant tissue compared to normal tissues, for example in breast [73, 82, 122], lung [74], ovary [79, 83] and AML [122]. In other diagnoses, a decreased activity and/or expression have been described such as in renal cell carcinoma [123, 124] and colorectal cancer [125, 126]. These data are in concordance with the differences in activity between J1 and melphalan. The highest ratios were found in NHL, AML, breast cancer and lung cancer and the two diagnoses with the lowest ratios were renal cancer and colorectal cancer.
4.5 Activity of J1 in human myeloma cell lines (unpublished data)

When the diagnosis-directed activity of J1 was investigated very few human multiple myeloma samples were included due to difficulties in achieving cell preparations of adequate purity. Therefore, J1, melphalan and some standard chemotherapeutics used in myeloma therapy were used in a panel of eight myeloma cell lines to further investigate the activity of J1. The results are presented as IC_{50}-values (μM) in Table 4. J1 showed significantly lower IC_{50} than melphalan in all the tested cell lines (t-test, \( p < 0.05 \)) and the mean ratio in potency between J1 and melphalan was 37 (ranging 20 to 71).

Table 4. Activity as IC_{50} (μM) from FMCA of J1, melphalan and six standard drugs in human multiple myeloma cell lines.

<table>
<thead>
<tr>
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</tr>
</thead>
<tbody>
<tr>
<td>Karpas 707</td>
<td>0.51</td>
<td>12</td>
<td>0.018</td>
<td>55</td>
<td>15</td>
<td>710</td>
<td>8.7</td>
<td>&gt;100</td>
</tr>
<tr>
<td>U-1957</td>
<td>0.12</td>
<td>3.9</td>
<td>0.021</td>
<td>30</td>
<td>0.62</td>
<td>430</td>
<td>19</td>
<td>&gt;100</td>
</tr>
<tr>
<td>U-1958</td>
<td>0.23</td>
<td>14</td>
<td>0.019</td>
<td>500</td>
<td>3.4</td>
<td>&gt;2000</td>
<td>&gt;100</td>
<td>&gt;100</td>
</tr>
<tr>
<td>LP-1</td>
<td>1.7</td>
<td>34</td>
<td>0.019</td>
<td>360</td>
<td>43</td>
<td>240</td>
<td>2.9</td>
<td>45</td>
</tr>
<tr>
<td>U-266-1970</td>
<td>0.41</td>
<td>29</td>
<td>0.017</td>
<td>3.7</td>
<td>3.2</td>
<td>940</td>
<td>&gt;100</td>
<td>&gt;100</td>
</tr>
<tr>
<td>U-266-1984</td>
<td>0.80</td>
<td>27</td>
<td>0.022</td>
<td>700</td>
<td>9.0</td>
<td>&gt;2000</td>
<td>&gt;100</td>
<td>&gt;100</td>
</tr>
<tr>
<td>RPMI 8226/S</td>
<td>1.0</td>
<td>27</td>
<td>0.085</td>
<td>280</td>
<td>1.7</td>
<td>750</td>
<td>87</td>
<td>&gt;100</td>
</tr>
<tr>
<td>U-1996</td>
<td>1.3</td>
<td>40</td>
<td>0.091</td>
<td>100</td>
<td>110</td>
<td>1700</td>
<td>410</td>
<td>&gt;100</td>
</tr>
</tbody>
</table>


4.6 J1 shows synergistic effects in combination with standard chemotherapeutic agents (Paper II and III)

Combination chemotherapy is considered to be standard treatment in many clinical situations, and melphalan has been used in several different regimes. Combination effects of J1 and standard chemotherapeutics have been investigated both in Paper II and III. An effect level at 70%, IC_{70}, was chosen for statistical analysis. The most striking result was that J1 and the topoisomerase II inhibitor etoposide demonstrated synergistic effects in all twelve cell lines tested (Figure 7, CI at IC_{70}). Synergy between melphalan and topoisomerase inhibitors has been reported before [128, 129]. TopoIIa is required for normal processing of melphalan-induced DNA damages, which may explain the positive interaction. In its absence, DNA damages are repaired through recombinational repair, which is associated with increased cytotoxicity [128]. Combination with doxorubicin, the other investigated topoisom-
erase II inhibitor resulted in five synergistic and seven additive effects in the cell lines (Figure 7, CI at IC\textsubscript{70}). The combination of melphalan and etoposide has been evaluated clinically in different high-dose regimes. In previously treated patients with advanced Hodgkin’s disease, the complete response rate was 75% (n=50) and the combination was considered well-tolerated [130]. Also in children with Stage IV neuroblastoma, the combination was concluded as well-tolerated, with gastrointestinal toxicity as the main dose-limiting effect [22].

The combination of melphalan and bortezomib has been found to be interesting, since several clinical studies have shown good activity, see section 1.1.4 [27, 28]. A mechanistic explanation has also been suggested. A DNA damage-activated signaling pathway which regulates cellular resistance to DNA crosslinking agents requires an intact proteasome function to be activated [131]. In our analysis, the effect of the combination of J1 and bortezomib was additive in all cell lines but one, in which it was synergistic (Figure 7, CI at IC\textsubscript{70}).

Except for some sporadic antagonisms and a few other synergies, two thirds of the tested combinations were evaluated as additive at IC\textsubscript{70}. For comparison, CIs at IC\textsubscript{50} are also shown in Figure 7, with similar interaction patterns as at IC\textsubscript{70}. When considering drug interactions in cancer therapy, it should be noted that combinations that do not show true synergism can still be valuable clinically, as long as the combination produces a better effect than each drug alone. This is frequently termed “therapeutic synergy”, and can be important if the drugs produce different types of toxicity [132].

![Figure 7. Combination index at 50% and 70% effect level. Mean CI of three experiments are shown in each cell line. Synergism and antagonism are defined as a CI statistically lower/higher than 1.0.](image-url)
4.7 J1 induces apoptosis in human tumor cell lines
(Paper II, IV and unpublished data)

The cell death characteristics caused by J1 and melphalan were evaluated in a multiparametric assay addressing three fundamental aspects of apoptosis: nuclear morphological changes, caspase activation and changes in MMP. Both J1 and melphalan induced caspase-3 activity, a modest reduction in MMP and nuclear DNA fragmentation after 24 h in U937GTB cells (Figure 8). There were not yet any pronounced effects on nuclear morphology and caspase activity 8 h after drug application but the reduction in MMP was similar to that at 24 h. At 4 h no changes in any of the parameters were recorded (data not shown). The ability of J1 and melphalan to induce apoptosis was confirmed in two other cell lines, HeLa (cervical carcinoma, data not shown) and CCRF-CEM (T-cell leukemia, data presented in Paper IV), where the cell death kinetics were verified. J1 was significantly more potent in inducing apoptosis than melphalan in all three cell lines (t-test, \( p < 0.001 \); Table 5). Also, in Paper II the apoptotic properties of J1 and melphalan were investigated in three neuroblastoma cell lines [SH-SY5Y, SK-N-AS and SK-N-BE(2)] and the same conclusions concerning time and potency were drawn. In short, J1 induces apoptosis in a more effective manner than melphalan, probably through the intrinsic apoptotic pathway since a reduction in MMP was recorded.

![Figure 8](image_url)

**Figure 8.** Dose response curves of caspase 3/7 activity (left axis), nuclear fragmentation/condensation (right axis) and MMP (right axis) as percent of the untreated control. U937GTB cells were exposed to J1 or melphalan for 24 h. Data points represent the mean of three experiments ± S.E.M.

<table>
<thead>
<tr>
<th>Cell line</th>
<th>J1 (μM)</th>
<th>Melphalan (μM)</th>
<th>( p )</th>
</tr>
</thead>
<tbody>
<tr>
<td>U937GTB</td>
<td>0.73 (0.23-2.3)</td>
<td>28 (9.6-83)</td>
<td>0.0066</td>
</tr>
<tr>
<td>CCRF-CEM</td>
<td>1.4 (1.0-1.9)</td>
<td>32 (17-58)</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>HeLa</td>
<td>4.6 (2.5-8.4)</td>
<td>65 (33-130)</td>
<td>0.0022</td>
</tr>
</tbody>
</table>

Table 5. EC\(_{50}\) (with 95% confidence interval) for caspase 3/7 activity after 24 h drug exposure. Calculations were made using non-linear regression analysis and a fixed maximum value was set on the maximum obtained value in each cell line.
4.8 Cellular loading and activation of the prodrug J1 (Paper IV, unpublished data)

In Paper IV, the details of the cellular pharmacology of J1, including the kinetics of the cell death, DNA damage and melphalan release from J1 and the nature of the enzymatic activation, were studied.

4.8.1 J1 more efficiently produces intracellular melphalan and induces more DNA damage and apoptosis than melphalan

The relative intra- and extracellular (in the surrounding cell medium) concentrations of J1 and melphalan following exposure to J1 or melphalan were analyzed with a validated HPLC/MS method in three cell lines (CCRF-CEM, MCF-7 [breast cancer] and SH-SY5Y). The results showed that J1 rapidly entered the cells and released melphalan with a peak concentration already after approximately 15 min. This confirms the results from Paper I performed with a detection system of much lower sensitivity. After melphalan exposure the concentration peaked at about 60 min and was only about one tenth as high as after J1 addition. The release of melphalan was inhibited when the cells were pre-exposed to the aminopeptidase inhibitor bestatin.

The capacity of J1 and melphalan to induce DNA damage and subsequent apoptosis was measured in CCRF-CEM with alkaline single-cell gel electrophoresis and a multiparametric assay. When cells were exposed to J1 or melphalan for 8 h, both drugs induced DNA damage; for J1 all the tested concentrations (0.01 μM, 0.1 μM and 1.0 μM) showed significantly more DNA damage than the untreated control, whereas only melphalan in the concentrations 1.0 μM and 10 μM did the same. Although roughly estimated with tail moment as the only quantitative measure and with no qualitative measure, it appears that J1 was 10 times more potent than melphalan in this respect, a difference that coincides with the levels of intracellular concentrations. As previously described (see section 4.7) J1 triggered apoptosis more potently than melphalan, with nuclear fragmentation and caspase-3 activity (Table 5).

The fact that the difference in melphalan concentration after exposure to J1 versus melphalan was about the same as the difference in apoptosis and DNA damage may indicate that the increase in potency is mostly due to cellular loading and trapping through the enzymatic activation.

4.8.2 Activity of the de-esterified form of J1, Mel-pFPhe-OH

J1 has two hydrolysis-susceptible bonds in its molecule formula, the peptide bond and the ester bond. When the peptide hydrolysis was inhibited by bestatin and consequently also the conversion of J1 to melphalan, another me-
tabolite was formed to a greater extent. It was later identified as the de-esterified form of J1, L-melphalanyl-\textit{\textit{p}}-L-fluorophenylalanine (Mel-pFPhe-OH) (Figure 9).

The activity of Mel-pFPhe-OH was studied in a cell line panel using the FMCA and showed approximately the same cytotoxic activity as melphalan, which was significantly lower than J1. Previous experiments suggested passive diffusion as the predominantly transport mechanism for J1 and since the ethyl ester increases the lipophilicity, the transport inside the cells of the more hydrophilic Mel-pFPhe-OH is probably decreased which may be responsible for its lower cytotoxicity.

![Figure 9. Schematic picture of the cleavage of J1: by aminopeptidase to melphalan or by esterases to Mel-pFPhe-OH.](image)

4.8.3 Does aminopeptidase N activate J1 enzymatically?

Paper I showed that the enzymatic activation of J1 was essential for its activity and with the aim of identifying the activating enzyme(s) of J1, numerous enzyme inhibitors were tested. The only inhibitors succeeding to significantly shift the activity of J1 in a majority of the investigated cell lines were the multi-aminopeptidase inhibitor bestatin (t-test, $p<0.05$, Figure 10) and the esterase inhibitor ebelactone A (in CCRF-CEM, SH-SY5Y and U937GTB cells). Bestatin did not affected the activity of melphalan in any of the tested cell lines (t-test, $p>0.05$, investigated in all cell lines shown in Figure 10, data not shown). Besides the peptidase inhibitors described in
Paper IV (actinonin, chymostatin and puromycin) cathepsin inhibitors were also tested. U937GTB and CCRF-CEM cells were preincubated with nontoxic concentrations of CA-074 (cathepsin B inhibitor), Z-Phe-Phe-fluoromethyl ketone (cathepsin B and L inhibitor) or pepstatin D (cathepsin D inhibitor) and were tested with the FMCA. None of the inhibitors had any effects on the cytotoxic activity of J1 or melphalan (t-test, \( p > 0.05 \), data not shown).

Figure 10. IC\(_{50}\) (μM) for J1 (30 min) in cells pre-exposed to the aminopeptidase inhibitor bestatin (10 μM for 60 min) compared to untreated cells. Mean values with 95% confidence interval are shown. All the cell lines have been previously described except from the fibrosarcoma cell line HT1080.

Melphalan was formed in approximately the same rate as J1 was disappearing when the degradation of J1 was investigated in a buffer solution containing the pure APN enzyme. This concluded that J1 is indeed a substrate for APN but it still remains to investigate the relevance of APN for the activity of J1 in cells in vitro and in vivo.
5 Summary and conclusions

In this thesis the details of the pharmacology of J1 have been further characterized: the activity in vitro and in vivo in neuroblastoma and ex vivo in primary tumor cell cultures from patients with a variety of diagnoses, along with mechanistic studies to explain the higher cytotoxicity. The results may be summarized as:

- J1 was shown to be highly dependent on intracellular hydrolysis since its activity was inhibited by peptidase inhibitors and dipeptide derivatives designed to resist the action of peptidases were less active than the corresponding normal dipeptide. Results also indicated a rapid intracellular loading of J1; limiting the cells exposure time to drugs did not reduce the activity for J1 as much as for melphalan and some other standard chemotherapeutic drugs.
- J1 effectively inhibited neuroblastoma cell growth in vitro in a set of cell lines. When nude rats and mice carrying neuroblastoma xenografts were treated with equimolar doses of melphalan, J1, or no drug, the tumor growth was significantly inhibited by J1 compared with untreated controls in all the conducted in vivo studies. Compared with melphalan, J1 inhibited the tumor growth more effectively in mice, where J1 treatment also was associated with higher caspase-3 activation, fewer proliferating tumor cells and significantly decreased mean vascular density.
- J1 showed a similar activity profile as melphalan in 176 primary tumor cell cultures from patients (representing twelve diagnoses), but expressed 50- to 100-fold higher potency compared with melphalan. The difference was greater in some diagnoses (e.g. breast cancer, NHL and AML), and exceptionally high in some breast cancer samples with aggressive phenotypes.
- J1 acted synergistically when combined with the topoisomerase II inhibitor etoposide in all the tested cell lines in an in vitro combination screening. Most of the effects of the combinations with the other seven standard chemotherapeutics were additive.
- The fast intracellular loading of melphalan after J1 addition was confirmed when measuring relative intra- and extracellular concentrations in cells exposed to melphalan or J1. The top melphalan concentration occurred later and was about one tenth as high as after exposure to melphalan compared with J1. Also, the release of melphalan from J1 was inhibi-
ited when cells were preincubated with an aminopeptidase inhibitor. The tenfold difference between J1 and melphalan was approximately the same when measuring DNA damage induced after J1 or melphalan treatment. Hydrolysis of both the ester- and peptide bond was shown to be of significance for the cytotoxic activity of J1, but not for melphalan. Furthermore, J1 showed to be a substrate for aminopeptidase N.

In conclusion, J1 is a melphalan prodrug which rapidly enters the cells, where it releases melphalan. The enzymatic activation is performed by aminopeptidases, for example aminopeptidase N, an enzyme described to be overexpressed in certain malignancies, which may lead to tumor selectivity. J1 showed promising preclinical activity in the diagnoses neuroblastoma, breast cancer, AML and NHL.
Future outlooks

During the years I have worked with the project, J1 has entered clinical trials. After a successful Phase I dose-finding study, J1 is now entering Phase IIa in patients that have diagnoses considered to be interesting for J1, in hope of observing objective responses. Even though J1 is already in the clinic there still remains some interesting preclinical work.

The experiments presented in the thesis indicate that APN may be an important enzyme in the cytotoxic activity of the prodrug J1 as it can release melphalan from J1 and the cytotoxicity of J1 decreases when the APN activity is inhibited. The scientific literature of the described APN overexpression in different diagnoses is in accordance with the activity of J1 in human tumor samples from these diagnoses. Still, the target of APN needs to be validated with transfection experiments, either by selectively inhibiting APN through siRNA or overexpressing the target. A predictive test to determine the levels of aminopeptidase in patients also needs to be considered for clinical use. This is crucial to find the “right” patients as it is the patients with high amounts of aminopeptidases that would benefit from treatment with J1 instead of another alkylating agent.

Another interesting finding in this thesis was the antiangiogenic effects of J1 compared with melphalan in the neuroblastoma xenograft study, especially in relation with aminopeptidases. Primarily APN, but also aminopeptidase A, have been demonstrated to play a functional role in angiogenesis and thus be a target for the inhibition thereof [84, 85]. Pasqualini et al. showed that APN expression is up-regulated in endothelial cells within tumors, but apart from those and other blood vessels undergoing angiogenesis, APN was not detected in blood vessels of various other normal tissues [85]. The selective expression was later confirmed and APN was suggested to have an important role in the endothelial morphogenesis during angiogenesis [116]. It would be of great interest to evaluate the antiangiogenic effect of J1 further, maybe by using metronomic therapy schedules.

In the ex vivo screening in Paper III, J1 was investigated in human tumor samples from twelve different diagnoses. It would seem reasonable to continue and complete ex vivo profiling studies with diagnoses and situations where melphalan is routinely used in the clinic, for example multiple myeloma. Some of the tested diagnoses were identified as being of extra interest such as breast cancer and AML, and these findings need to be validated in vivo in proof of concepts studies in a relevant animal model.
7 Svensk populärvetenskaplig sammanfattning


Det första och det sista arbetet handlade om att hitta förklaringen till varför J1 är mer potent än melfalan. En rad olika försök visade att J1 tar sig in i cellen mycket snabbt och där omvandlas till melfalan genom att en peptidbindning bryts. Detta innebär att J1 fungerar som en prodrug, dvs. ett läkemedel som ges i en inaktiv form och sedan görs om till den aktiva formen i kroppen. När cellerna exponerades för J1 ökade koncentrationen av melfalan inne i cellerna både mer och snabbare än när de fick melfalan direkt. J1 orsakade även mer DNA-skador och därmed också mer kontrollerad celldöd, apoptos. Både den celldödande effekten och frisättningen av melfalan inne i cellen från J1 kunde hämmas med en substans som hämmede aminopeptidaser, ett enzym som annars bryter peptidbindningar. Vissa aminopeptidaser tros finnas i överskott i olika cancerceller, vilket skulle kunna leda till att J1 aktiveras i större utsträckning i tumörer än i andra celler och därmed ger färre biverkningar. När man tillsatte J1 i en buffertlösning med enzymet aminopeptidas frisattes melfalan i ungefär samma takt som J1 bröts ned.

För att utvärdera J1 i behandling av neuroblastom studerades den prekliniska aktiviteten. J1 hämmede tillväxten av neuroblastomcellerna väldigt effektivt i försök med cellinjer. Aktiviteten undersöktes också genom att möss med nedsatt immunförsvar injicerades med humana neuroblastomceller
så att de utvecklade tumörer. Sedan behandlades de med antingen J1 eller melfalan, obehandlade möss användes som kontroll. Tillväxten av tumörerna hämmades mer effektivt i mössen som fått J1 än i kontrollgruppen eller gruppen som behandlats med melfalan. Dessutom hade tumörerna från mössen som behandlats med J1 mer apoptos, färre aktivt delande celler och färre blodkärl än tumörerna från kontrollmössen eller de möss som fått melfalan.


Ett läkemedel mot cancer ges sällan ensamt, utan i kombinationer för att ge bättre effekt. Därför testade vi J1 tillsammans med olika cytostatika som används kliniskt idag, till det använde vi cellinjer. De flesta av kombinationerna gav resultat som var likvärdiga med summan av läkemedlens enskilda effekter, men J1 tillsammans med läkemedlet etoposid gav en bättre effekt än summan av de två läkemedlen var för sig. Detta är fördelaktigt eftersom det skulle kunna leda till sänkta läkemedelsdoser och därmed minskade bipverkningar.

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Fina lilla krumelur, jag vill inte bliva stur

Pippi Långstrump
A doctoral dissertation from the Faculty of Medicine, Uppsala University, is usually a summary of a number of papers. A few copies of the complete dissertation are kept at major Swedish research libraries, while the summary alone is distributed internationally through the series Digital Comprehensive Summaries of Uppsala Dissertations from the Faculty of Medicine. (Prior to January, 2005, the series was published under the title “Comprehensive Summaries of Uppsala Dissertations from the Faculty of Medicine”.)