The novel melphalan prodrug J1 inhibits neuroblastoma growth *in vitro* and *in vivo*

Malin Wickström,[1] John Inge Johnsen,[2] Frida Ponthan,[2] Lova Segerström,[2] Baldur Sveinbjörnsson,[2] Magnus Lindskog,[1,2] Henrik Lövborg,[1,4] Kristina Viktorsson,[3] Rolf Lewensohn,[3] Per Kogner,[2] Rolf Larsson,[1] and Joachim Gullbo[1]

[1]Division of Clinical Pharmacology, Department of Medical Sciences, Uppsala University, Uppsala, Sweden; [2]Childhood Cancer Research Unit, Departments of Woman and Child Health, and [3]Oncology-Pathology, Karolinska Biomics Center, Karolinska Institutet, Stockholm, Sweden; and [4]Division of Clinical Pharmacology, Department of Medicine and Care, Faculty of Health Sciences, Linkoping University, Linkoping, Sweden

Abstract

Neuroblastoma is the most common extracranial solid tumor of childhood. The activity of J1 (L-melphalanyl-*p*-L-fluorophenylalanine ethyl ester), an enzymatically activated melphalan prodrug, was evaluated in neuroblastoma models *in vitro* and *in vivo*. Seven neuroblastoma cell lines with various levels of drug resistance were screened for cytotoxicity of J1 alone or in combination with standard cytotoxic drugs, using a fluorometric cytotoxicity assay. J1 displayed high cytotoxic activity *in vitro* against all neuroblastoma cell lines, with IC_{50} values in the submicromolar range, significantly more potent than melphalan. The cytotoxicity of J1, but not melphalan, could be significantly inhibited by the aminopeptidase inhibitor bestatin. J1 induced caspase-3 cleavage and apoptotic morphology, had additive effects in combination with doxorubicin, cyclophosphamide, carboplatin, and vincristine, and synergistically killed otherwise drug-resistant cells when combined with etoposide. Athymic rats and mice carrying neuroblastoma xenografts [SH-SY5Y, SK-N-BE(2)] were treated with equimolar doses of melphalan, J1, or no drug, and effects on tumor growth and tissue morphology were analyzed. Tumor growth *in vivo* was significantly inhibited by J1 compared with untreated controls. Compared with melphalan, J1 more effectively inhibited the growth of mice with SH-SY5Y xenografts, was associated with higher caspase-3 activation, fewer proliferating tumor cells, and significantly decreased mean vascular density. In conclusion, the melphalan prodrug J1 is highly active in models of neuroblastoma *in vitro* and *in vivo*, encouraging further clinical development in this patient group. [Mol Cancer Ther 2007;6(9):2409–17]

Received 3/6/07; revised 5/29/07; accepted 7/6/07.

Grant support: The Swedish Children's Cancer Foundation, the European Commission (contract no. QLK3-CT-2002-01956), and the Lions Cancer Research Fund.

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked *advertisement* in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

Requests for reprints: Malin Wickström, Division of Clinical Pharmacology, Department of Medical Sciences, Entrance 61, 4th Floor Uppsala University Hospital, 75185 Uppsala, Sweden. Phone: 46-18-6112931; Fax: 46-18-519237. E-mail: Malin.Wickstrom@medsci.uu.se

Copyright © 2007 American Association for Cancer Research.

doi:10.1158/1535-7163.MCT-07-0156

Introduction

Neuroblastoma is the most common and deadly tumor of childhood often associated with therapy-resistance. For children with metastatic disease at diagnosis or certain genetic tumor features such as MYCN amplification or 1p deletion, the survival remains poor despite intensive multimodal treatment protocols (1).

The alkylating drug melphalan is routinely used in high-dose protocols for children with advanced neuroblastoma. Some decades ago, a mixture of six oligopeptides containing the *meta*-isomer of melphalan (*m*-L-sarcolysin), designated Peptichemio, was developed by Italian researchers and initial clinical trials seemed promising with significant response rates in several common diagnoses; among them, childhood neuroblastoma (2–5).

As a result of a screening of peptides, based on pharmacologic data on Peptichemio and melphalan, a highly effective drug candidate, J1 (L-melphalanyl-*p*-L-fluorophenylalanine ethyl ester), exhibiting higher *in vitro* and *in vivo* cytotoxicity than melphalan, was synthesized (6–8). J1 is rapidly incorporated into the cytoplasm followed by intracellular hydrolysis, which results in the release of melphalan. The enzymes responsible for the activation have been identified to be aminopeptidases (9), which may provide an attractive cancer target because the activity of several aminopeptidases is elevated in plasma and effusions from patients with cancer (10–12). A phase I trial with J1 in adult patients with advanced cancers is currently ongoing in Sweden.

There is a need for novel therapies able to bypass drug resistance of high-risk neuroblastoma, and based on previous experience with melphalan and Peptichemio in this diagnosis, it seemed adequate to investigate if J1 is active as a single agent or in combination with standard drugs in experimental models of neuroblastoma *in vitro* and *in vivo*. This is the first study showing that J1 effectively inhibits neuroblastoma cell growth *in vitro* and *in vivo* by the induction of apoptosis, being significantly more effective than melphalan and with additive or synergistic effects in combination with cytotoxic drugs routinely used for children with advanced neuroblastoma. Furthermore, this is the first report of J1 efficacy using the xenograft model including immunohistochemical examination of the tumors.

Downloaded from http://aacrjournals.org/mct/article-pdf/6/9/2409/1848860/2409.pdf by guest on 13 June 2024

Materials and Methods

Chemicals and Reagents

The molecular structures of J1 and melphalan are shown in Fig. 1. J1 was synthesized as described previously (6) and dissolved in DMSO (Sigma-Aldrich) and further diluted with sterile water or PBS (Sigma-Aldrich). The DMSO concentration did not exceed 1% v/v in any experiment. For the *in vivo* studies, J1 was dissolved in *N,N*-dimethyl acetamide (Sigma-Aldrich) and further diluted in glucose solution (50 mg/mL; Apoteket AB). Fluorescein diacetate (Sigma-Aldrich) was dissolved in DMSO and kept frozen (−20°C) as a stock solution protected from light. Carboplatin, doxorubicin, etoposide, melphalan, and vincristine were obtained from the Swedish Pharmacy (Apoteket AB) and dissolved according to guidelines from the manufacturer and further diluted in sterile PBS (except in the xenograft studies, where melphalan was further diluted in 50 mg/mL glucose). 4-Hydroxycyclophosphamide (the active metabolite of cyclophosphamide) was a kind gift from S. Ludeman (Duke Comprehensive Cancer Center, Duke University Medical Center, Durham, NC). The aminopeptidase inhibitor bestatin (Sigma-Aldrich) was dissolved in 50% ethanol to a 4.0 mmol/L stock solution and further diluted in PBS. FAM-DEVD-FMK, supplied as part of the CaspaTag kit (Chemicon) and chloromethyl-X-rosamine (MitoTracker Red CMXRos; Molecular Probes) were dissolved in DMSO and further diluted in PBS to its final concentration. Hoechst 33342 (Sigma-Aldrich) was dissolved in water.

Human Tumor Cell Lines

Seven neuroblastoma cell lines, with different drug sensitivities, were grown in Eagle Minimal Essential Medium (SH-SY5Y; Sigma-Aldrich) or RPMI 1640 [SK-N-BE(2), SK-N-AS, SK-N-F1, SK-N-SH, SK-N-DZ, and IMR-32; Sigma-Aldrich] medium supplemented with 10% fetal bovine serum (Sigma-Aldrich) and 2 mmol/L of glutamine (Sigma-Aldrich), 100 μg/mL of streptomycin, and 100 units/mL of penicillin (Sigma-Aldrich) at 37°C humidified 5% CO_2 atmosphere.

Cytotoxicity Assay

The fluorometric microculture cytotoxicity assay was used (13, 14) to investigate the effect of J1 and the combination of J1 with other cytostatic drugs *in vitro*. Briefly, 96- or 384-well microtiter plates (NUNC Brand Products) were pre-prepared in duplicate with drug solutions at 10 times the desired final drug concentration. Cells were seeded into the drug-prepared microtiter plates at a cell density of 0.1×10^6 cells/mL and incubated for 72 h at 37°C in a humidified 5% CO_2 atmosphere. Fluorometric microculture cytotoxicity assay was done using an automated Optimized Robot for Chemical Analysis (Beckman Coulter) programmed through the software SAMI (Beckman Coulter). The plates were washed, fluorescein diacetate was added, and the fluorescence generated was measured at 485/520 nm using Fluoroscan Optima (BMG Labtech) after 50 min incubation. The resulting fluorescence is proportional to the number of intact cells in each well. A successful assay required a ratio of >5 between the signal in the control wells and the blank wells and a coefficient of variation of <30% in the control wells. Cell survival is presented as a survival index. For the combination studies, fixed concentration ratios of the drugs were used with 5-fold serial dilutions in five steps. All concentrations were tested in duplicate and the experiments were repeated thrice.

Bestatin was used to evaluate the effect of aminopeptidases on the cytotoxic effect of J1 in all seven neuroblastoma cell lines. In these analyses, cells were preincubated with 10 μmol/L of bestatin (nontoxic) for 60 min, seeded into drug-containing microtiter plates, incubated for another 30 min before the drug-containing medium was removed and fresh medium added. The activities were measured after 72 h using a fluorometric microculture cytotoxicity assay.

Apoptosis Analysis

A multiparametric high-content screening assay (15) was used for the measurement of apoptosis in the three neuroblastoma cell lines; SH-SY5Y, SK-N-AS, and SK-N-BE (2). Briefly, cells were seeded into 96-well plates (Perkin-Elmer, Inc.) and incubated with the indicated concentrations of drugs for 8 and 24 h. The FLICA probe FAM-DEVD-FMK (carboxyfluorescein-labeled fluoromethyl ketone peptide inhibitor of caspase-3; at a final concentration of 20 μmol/L) was added 1 h before the end of the drug exposure to stain activated caspase-3/7, and on the last 30 min chloromethyl-X-rosamine was included (at a final concentration of 100 nmol/L) to evaluate mitochondrial membrane potential. The plates were washed and stained with 10 μmol/L of Hoechst 33342 in 3.7% formaldehyde. Plates were analyzed using the ArrayScan high-content screening system (Cellomics®, Inc.). Images were acquired for each fluorescence channel, using suitable filters with a 20× objective, and in each well, at least 800 cells were analyzed. J1 and melphalan were tested in four different concentrations obtained by a 5-fold serial dilution. Each experiment was done thrice.

Treatment with J1 and Melphalan *In vivo*

Male nude rats (HsdHan: RNU-rnu; Harlan) at age 5 to 10 weeks with a weight of 150 to 275 g, and female nude mice (NMRI *nu/nu*; Taconic) at age 6 weeks with a weight of 18 to 25 g were used for the experiments. The animals were housed and maintained in laminar flow cabinets under specific pathogen–free conditions and given sterile

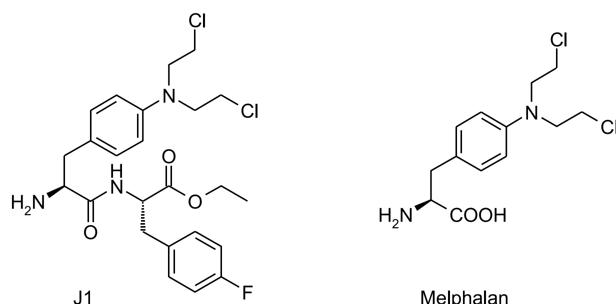

Figure 1. Molecular structures of J1 and melphalan.

Downloaded from http://aacrjournals.org/mct/article-pdf/6/9/2409/1848860/2409.pdf by guest on 13 June 2024

water and food *ad libitum*. 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). Establishment of neuroblastoma xenografts in nude rats was done as previously described (16). For the establishment of neuroblastoma xenografts in nude mice, animals were engrafted with 30×10^6 SH-SY5Y cells (in 0.1 mL of medium) s.c. in the flank of the right hind leg using a 23-gauge needle.

Three independent experiments were carried out. One using the multidrug-resistant, MYCN-amplified, p53-mutated cell line SK-N-BE(2) in nude rats and two experiments were done using the less drug-resistant SH-SY5Y cell line, one in rats and one in mice. Initial doses were selected based on previously determined LD_{50} values from other strains of rats/mice, which could not separate the toxic effects of melphalan and J1 at equimolecular doses.[5] In the first experiment, nude rats (n = 16) carrying SK-N-BE(2) xenograft tumors were randomly assigned to receive one dose of 10 μmol/kg J1 i.v. in the tail vein (n = 5), 10 μmol/kg of melphalan i.v. (n = 5) at day 0 or no treatment (n = 6), respectively. In the second experiment, nude rats (n = 16) carrying SH-SY5Y xenografts were randomized to receive 0.50 μmol/kg of J1 i.v. (n = 5), 0.50 μmol/kg of melphalan i.v. (n = 5) at day 0, or no treatment (n = 6), respectively. In the third experiment, nude mice (n = 25) engrafted with SH-SY5Y cells were randomly assigned to receive 0.50 μmol/kg of J1 i.v. (n = 9), 0.50 μmol/kg of melphalan i.v. (n = 8) at days 0 and 6, or no treatment (n = 9), respectively. Treatment continued for 12 days, starting when a tumor had reached a volume of 0.20 to 0.30 mL (mean tumor volume, 0.30 mL in experiments one and two, 0.26 mL in experiment three). Tumor volume was measured every other day as described previously (16). All animals were monitored for signs of toxicity including weight loss during the experiment.

Immunohistochemistry

Apoptosis, proliferation, and angiogenesis *in vivo* were evaluated in deparaffinized tumor sections of neuroblastoma xenografts. For identification of apoptosis, sections were incubated overnight at 4°C with a rabbit monoclonal anti–active caspase-3 antibody (1:100; R&D Systems) and detected using anti-rabbit HRP-conjugated Superpicture Polymer Kit (Zymed Laboratories, Inc.). For assessment of proliferative activity, sections were incubated with a monoclonal rabbit anti–Ki-67 antibody (1:200; Neomarkers, Inc.) overnight at 4°C and detected using an anti-rabbit HRP SuperPicture Polymer Kit (Zymed). As a control for nonspecific background staining, sections were incubated with rabbit IgG isotype control (Zymed). Biotinylated *Bandeiraea simplicifolia* (BS-1, L3759; Sigma-Aldrich) lectin was used for highlighting endothelial cells as described previously (17). Apoptosis and proliferation were assessed by counting the number of positively stained nuclei and the total number of tumor cells in three representative regions in three tumors from each treatment group at ×400 magnification. The results are expressed as the proportion of positively stained cells. Sections were quantified for vessel density at ×200 magnification, and fields were chosen randomly with the inclusion criteria that it had to consist of viable tissue. The results are expressed as an average number of vessels per field.

Statistical Analysis

The cytotoxic IC_{50} values for drugs in the cell lines *in vitro* were determined from log concentration-effect (survival index %) curves in GraphPad Prism (GraphPad Software, Inc.) using nonlinear regression analysis. For the *in vitro* results, comparison of activity between two groups was made with a two-sided t test. To test combination effects *in vitro*, data were analyzed using the median effect method of Chou and Talalay (18) using the software CalcuSyn Version 2 (Biosoft). Each dose-response curve (individual agents as well as combinations) 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. Goodness-of-fit was assessed using the linear correlation coefficient, r, and $r > 0.85$ was required for a successful analysis. The extent of 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 concentrations of drugs 1 and 2 alone, required to produce a certain effect, and d_1 and d_2 are the concentrations 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 and a 70% effect was chosen for presentation. A CI lying on one indicates additivity; CI significantly lower was defined as synergy and CI significantly higher as antagonism. One-sample t tests were used to determine if the CIs differed from one (1.0). For the *in vivo* experiments, one-way ANOVA with Tukey multiple comparisons tests were used to compare the three treatment groups (GraphPad InStat). $P < 0.05$ was considered significant.

Results

The J1 Prodrug Is a Potent Inducer of Neuroblastoma Cell Death and Is Superior to Melphalan *In vitro*

The cytotoxic activity of J1, melphalan, and five cytotoxic drugs commonly used in the treatment of patients with neuroblastoma was investigated in seven neuroblastoma cell lines. In two of the neuroblastoma cell lines (SH-SY5Y and SK-N-SH), J1 was found to be the most effective drug with IC_{50} values of 0.0028 and 0.0051 μmol/L, respectively. In the remaining cell lines, only vincristine was more effective than J1 in inducing cell death (Fig. 2). J1 exhibited a significant increase (mean, 270-fold; range, 35- to 810-fold) in cytotoxicity compared with melphalan in all neuroblastoma cell lines investigated (t test, $P < 0.001$; Fig. 2).

[5] Unpublished data.

Downloaded from http://aacrjournals.org/mct/article-pdf/6/9/2409/1848860/2409.pdf by guest on 13 June 2024

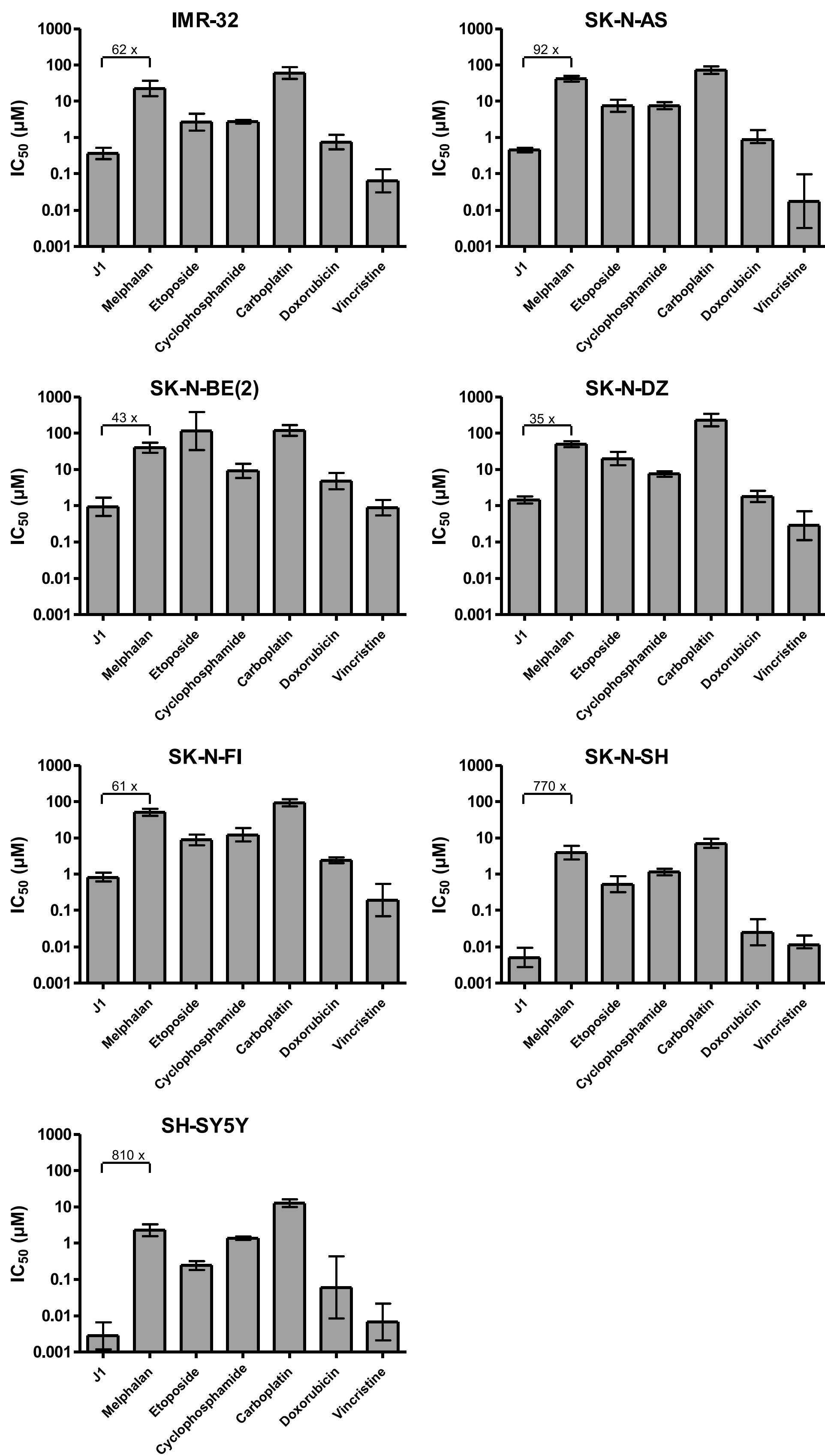

Figure 2. The IC_{50} (with 95% confidence interval) of J1, melphalan, and five cytotoxic drugs commonly used in the treatment of neuroblastoma in seven neuroblastoma cell lines. The increase in activity of J1 compared with melphalan (defined as IC_{50} melphalan/IC_{50} J1, indicated by numbers) was significant (*t* test, $P < 0.001$) for all cell lines.

Downloaded from http://aacrjournals.org/mct/article-pdf/6/9/2409/1848860/2409.pdf by guest on 13 June 2024

Table 1. Effect of J1 in combination with chemotherapeutic drugs in neuroblastoma cells *in vitro*

Combination	SK-N-AS		SK-N-BE(2)		SH-SY5Y	
	CI at IC_{70}	Effect	CI at IC_{70}	Effect	CI at IC_{70}	Effect
J1 + doxorubicin	0.86 (0.63–1.1)	Additive	0.88 (0.57–1.2)	Additive	0.78 (0.64–0.92)	Synergistic
J1 + carboplatin	0.69 (0.38–0.99)	Synergistic	1.1 (0.84–1.4)	Additive	0.96 (0.33–1.6)	Additive
J1 + cyclophosphamide	0.82 (0.37–1.3)	Additive	1.3 (0.58–2.0)	Additive	1.0 (0.69–1.3)	Additive
J1 + etoposide	0.47 (0.44–0.49)	Synergistic	0.75 (0.49–0.99)	Synergistic	0.63 (0.25–0.99)	Synergistic
J1 + vincristine	1.4 (0.34–2.5)	Additive	1.2 (0.46–1.9)	Additive	0.78 (0.30–1.3)	Additive

NOTE: Mean of CI at IC_{70} with 95% confidence interval. Mutual exclusivity is assumed. Synergism and antagonism are defined as a CI mean statistically significantly lower/higher than one with one-sample *t* test ($P < 0.05$).

To confirm that J1 acts as an enzymatic activated prodrug, as previously described (9), cells were pretreated with bestatin, a potent inhibitor of aminopeptidases (19). Pre-exposure to nontoxic concentration of bestatin resulted in a decreased activity of J1 in six of seven cell lines (*t* test, $P < 0.05$). In the IMR-32 cell line, the observed difference, however, did not reach statistical significance using this experimental setup. In general, bestatin pretreatment increased the IC_{50} by 5.5 times (paired *t* test, $P = 0.0006$). Bestatin did not affect neuroblastoma cell response to melphalan in any of the cell lines (*t* test, $P > 0.05$; paired *t* test $P = 0.30$).

J1 Potentiates Standard Neuroblastoma Chemotherapy *In vitro*

Combination chemotherapy, often supported with stem cell infusions, is considered as standard treatment in many clinical situations, and melphalan has been employed in several different protocols. Synergistic, additive, or antagonistic *in vitro* effects of J1 in combination with relevant chemotherapeutic drugs were evaluated in three neuroblastoma cell lines with different levels of drug resistance [SH-SY5Y, SK-N-AS, and SK-N-BE(2)]. As summarized in Table 1, which shows the CI at IC_{70}, the addition of J1 to standard drugs induced significant synergistic or additive cytotoxic effects in all tested cell lines. Synergism was observed when J1 was combined with etoposide (all cell lines), and for carboplatin (SK-N-AS), and doxorubicin (SH-SY5Y). The other combinations were found to be additive (Table 1).

J1 Triggers Apoptosis in Neuroblastoma

In order to examine if JI can trigger the apoptotic machinery, mitochondrial transmembrane potential and caspase-3 activity were measured, and nuclear DNA was assessed for apoptotic fragmentation in three neuroblastoma cell lines (Fig. 3). Mitochondrial depolarization was observed 8 hours after the addition of J1 (data not shown). At 24 h post–drug addition, J1 and melphalan were both associated with caspase-3 activation and nuclear DNA fragmentation. Taken together, these observations indicate that J1 induces the apoptosis of neuroblastoma cells by activation of the intrinsic apoptotic pathway. Notably, the proapoptotic effects of J1 were significantly more potent compared with melphalan (*t* test, $P < 0.001$; Fig. 3).

J1 Significantly Inhibits the Growth of Established Neuroblastoma Xenografts

To investigate the effect of J1 and melphalan on neuroblastoma growth *in vivo*, nude rats and mice carrying either SK-N-BE(2) or SH-SY5Y xenografts were used. In the first experiment, nude rats carrying xenografts from the drug-resistant neuroblastoma cell line SK-N-BE(2) were treated with a single dose of 10 μmol/kg of J1 or melphalan, a dose reported to be close to 50% of LD_{50}.[6] Significant inhibition of tumor growth was detected 2 days after J1 treatment (ANOVA, $P = 0.016$; Tukey, $P < 0.05$) and 4 days after melphalan treatment (ANOVA, $P = 0.0003$; Tukey, $P < 0.01$), respectively, compared with untreated tumors and throughout the observation period (ANOVA, $P > 0.001$; Fig. 4A). Using this dose scheduling, no significant difference in tumor growth was observed in rats treated with J1 compared with melphalan (Tukey, $P > 0.05$).

In the second experiment, nude rats carrying SH-SY5Y xenografts were treated with a single dose of 0.50 μmol/kg of J1 or melphalan, a dose corresponding to ~2.5% of LD_{50} (again estimated in Sprague-Dawley strain); thus, considerably lower than in the first experiment. Again, both treatments significantly inhibited tumor growth compared with untreated control tumors (ANOVA, $P < 0.001$; Fig. 4B). However, J1 was significantly more effective in tumor growth inhibition compared with melphalan using this lower concentration of drugs (Tukey, $P < 0.05$). Inhibitions were significant after 2 days for both J1 (ANOVA, $P = 0.0029$; Tukey, $P < 0.01$) and melphalan (Tukey, $P < 0.05$), compared with untreated tumors.

Previous *in vivo* studies with J1 were made with the hollow fiber method in mice only (8), showing the increased potency of J1 but a similar toxicity compared with equimolar doses of melphalan. This is the first presentation of J1's activity in a xenograft model, as well as its effect in a rat model for comparison, grafts were also studied in mice (having potentially different metabolisms and different maximum tolerated doses in previous studies). Tumor-bearing mice were treated i.v. with two doses of 0.50 μmol/kg, corresponding to ~0.6% of LD_{50}, of

[6] Unpublished data, estimation in Sprague-Dawley rats.

Downloaded from http://aacrjournals.org/mct/article-pdf/6/9/2409/1848860/2409.pdf by guest on 13 June 2024

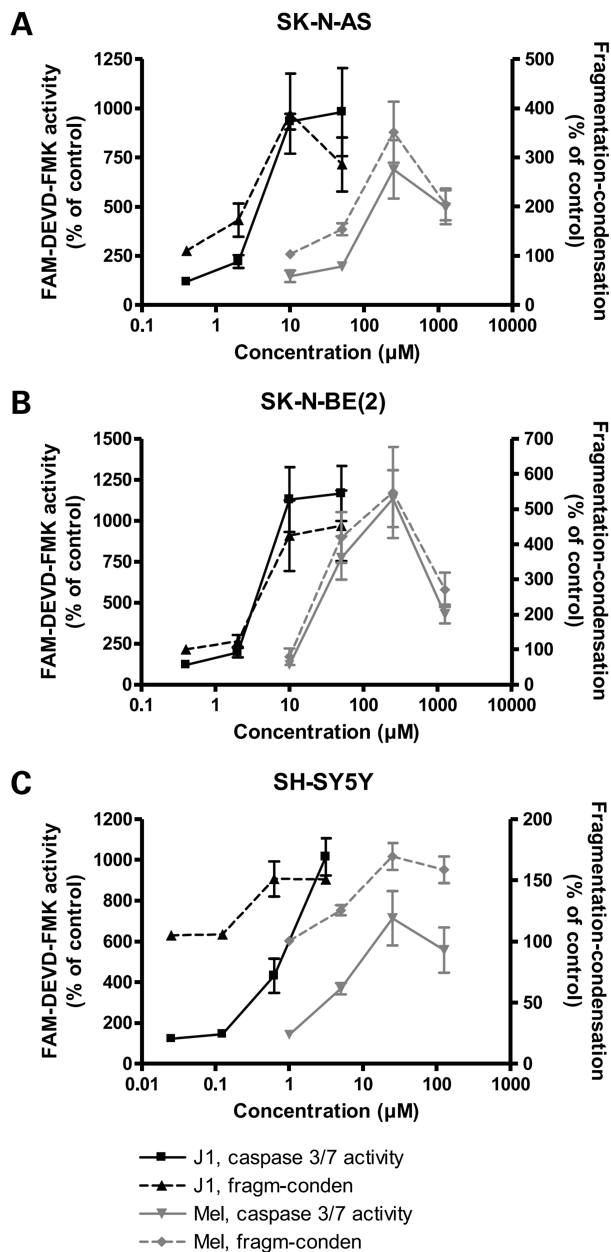

Figure 3. Cell death characteristics for J1 and melphalan after 24-h exposure in the three neuroblastoma cell lines SK-N-AS (**A**), SK-N-BE(2) (**B**), and SH-SY5Y (**C**). The nuclear fragmentation/condensation and caspase-3/7 activity (measured using FAM-DEVD-FMK) are both expressed as a percentage of the untreated control and averaged for at least 800 cells per well. *Points,* means of three independent experiments; *bars,* SE.

either J1[7] or melphalan. The drugs were given when the tumor volume had reached >0.20 mL (mean, 0.26 mL) and at 6 days after tumor establishment (Fig. 5A). Tumor growth was significantly inhibited 2 days after J1 injection compared with both melphalan and the untreated control (ANOVA, P = 0.0073; Tukey, P < 0.05) and throughout the observation period (ANOVA, P = 0.0002; Tukey, P > 0.01 J1 versus melphalan; P > 0.001 J1 versus control; Fig. 5A). No significant reduction in tumor volume was observed in animals treated with melphalan (Tukey, P > 0.05). Animal weight and other signs of toxicity were recorded in all the *in vivo* experiments. In rats treated with a single dose of 10 µmol/kg of J1 or melphalan, a transient reduction in weight gain was observed on the first 4 days after treatment compared with the untreated control animals (ANOVA, P = 0.011; Tukey, P < 0.05; Fig. 4B). No significant differences in weight gain were observed at the end of the experiments (ANOVA, P = 0.25; Fig. 4B). In rats treated with 0.50 µmol/kg of J1 or melphalan, a temporary reduction in weight gain was observed in rats treated with melphalan on the first 4 days after treatment (ANOVA, P = 0.014; Tukey, P < 0.05; Fig. 4D). No weight loss was observed in rats treated with 0.50 µmol/kg of J1 compared with untreated control rats throughout the experiment (Fig. 4D). In mice treated with two doses of 0.50 µmol/kg of J1 or melphalan at days 1 and 6, no weight loss was observed in the treated mice compared with untreated controls (Fig. 5B). No other signs of toxicity were observed in any of the treatments (data not shown).

Treatment with J1 Significantly Reduces Proliferation, Inhibits Angiogenesis, and Induces Apoptosis in Neuroblastoma *In vivo*

Having established that J1 seems more effective than melphalan in the treatment of established neuroblastoma xenografts, we investigated the effect of the drugs on the induction of apoptosis, tumor cell proliferation, and angiogenesis in SH-SY5Y xenografts.

A significantly increased level of cleaved caspase-3–positive cells was found in tumors from rats treated with J1 compared with untreated tumors (ANOVA, P = 0.0021; Tukey, P < 0.01; Fig. 4C). Furthermore, caspase-3 activity was significantly higher in J1-treated tumors than in xenografts from rats treated with melphalan (Tukey, P < 0.01; Fig. 4C). Cell proliferation as measured by Ki-67 was significantly inhibited after J1 treatment, but not after melphalan treatment compared with untreated tumors (ANOVA, P = 0.042; Tukey, P < 0.05 J1 versus control; P > 0.05 melphalan versus control; Fig. 4D).

A significantly elevated expression of cleaved caspase-3 was detected in SH-SY5Y xenografts from nude mice treated with J1 compared with melphalan-treated tumors (ANOVA, P = 0.017; Tukey, P < 0.05; Fig. 5B). Moreover, a significant reduction of tumor cell proliferation was also shown in J1-treated tumors compared with tumors treated with melphalan (ANOVA, P < 0.001; Tukey, P < 0.01; Fig. 5C). Because inhibition of angiogenesis could contribute to reduced cell proliferation and increased apoptosis, we also determined the effect of J1 and melphalan on mean vessel density. Treatment with J1 or melphalan induced a 49% (95% confidence interval, 27–72%; ANOVA, P < 0.001; Tukey, P < 0.001) or 20% (−0.35% to 41%; Tukey, P > 0.05)

[7] Unpublished data, estimation in specific pathogen–free Swiss mice.

Downloaded from http://aacrjournals.org/mct/article-pdf/6/9/2409/1848860/2409.pdf by guest on 13 June 2024

reduction in mean vessel density compared with untreated tumors. Mean vessel density was also significantly lower (36% reduction, 7.9–65%) in tumors treated with J1 compared with melphalan (Tukey, $P < 0.05$; Fig. 5D).

Discussion

Melphalan could prolong the survival of children with advanced neuroblastoma and is currently used in many high-dose protocols for this patient group (20). The present study shows for the first time that J1, a prodrug of melphalan, is highly active against human neuroblastoma cell lines *in vitro* and *in vivo*. The cytotoxic activity of J1 *in vitro* was found to be superior compared with melphalan, and statistical comparisons also favored J1 in terms of antiproliferative, proapoptotic, and antiangiogenic activity *in vivo*. These findings are particularly interesting in view of the demonstrated clinical activity of Peptichemio in neuroblastoma (4, 5).

Despite different levels of absolute sensitivity, the seven human neuroblastoma cell lines displayed a similar pattern of sensitivity against the tested drugs (Pearson correlation of log IC_{50}, 0.85–0.99), reflecting the importance of a common cytopathologic origin. The *in vitro* activity of J1 was, on average, 270-fold that of melphalan, with a range from 35- to 810-fold. There is a clear tendency toward a greater difference in the generally more sensitive cell lines (i.e., SH-SY5Y and its parental cell line SK-N-SH) compared with the more resistant cell lines (Fig. 2). Thus, one or more drug targets specific for J1 may be more abundantly expressed in SH-SY5Y/SK-N-SH and the observation warrants further mechanistic studies. No obvious differences in IC_{50} of J1 between MYCN and non-MYCN–amplified cell lines could be observed. Both melphalan and J1 activated the intrinsic apoptotic pathway in neuroblastoma cells. However, a reduction in the levels of activated caspase-3/7 was observed for high concentrations of melphalan, which suggests that melphalan may induce neuroblastoma cell death by other mechanisms at higher concentrations (Fig. 3).

The major use of melphalan in neuroblastoma therapy is in high-dose regimens with stem cell support. For this purpose, melphalan has been evaluated in combination

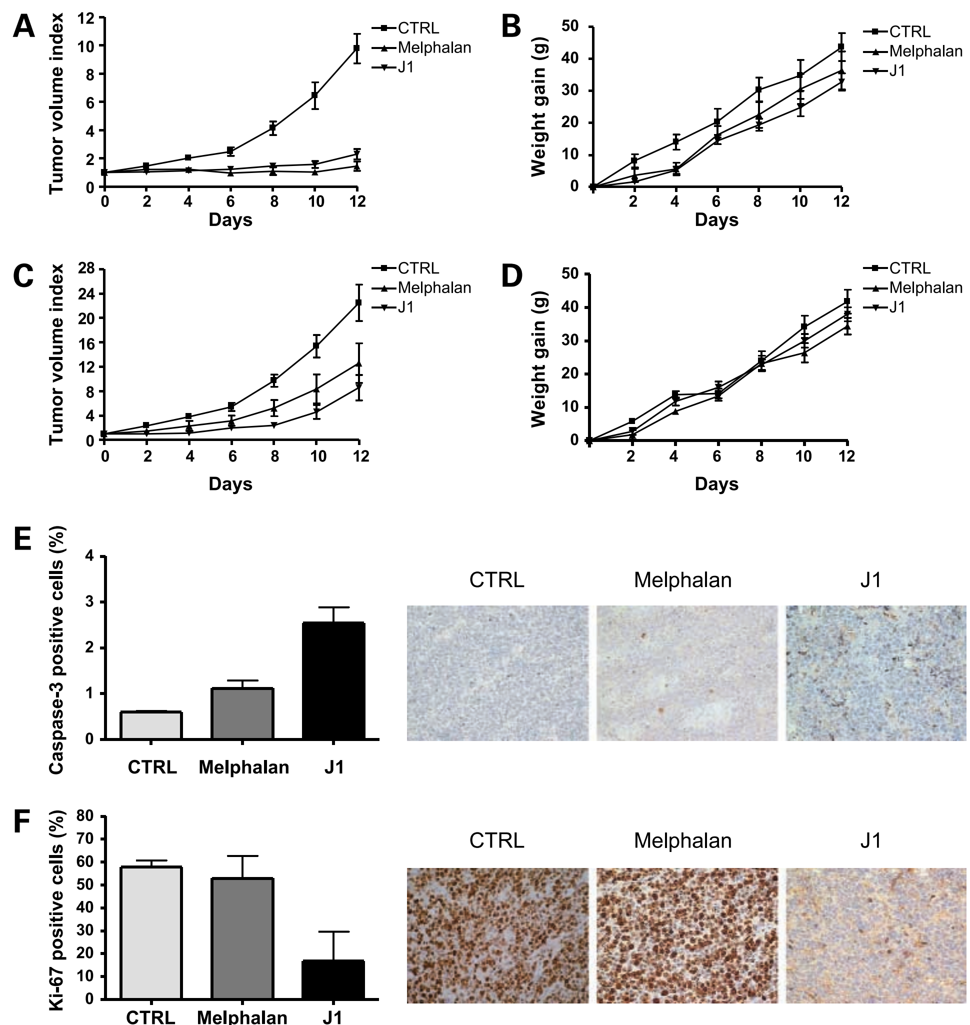

Figure 4. Treatment with J1 inhibits neuroblastoma tumor growth in nude rats. **A** and **C,** tumor volume is illustrated as tumor volume index which is defined as the tumor volume at each day compared with the tumor volume at day 0. **A** and **B,** nude rats with SK-N-BE(2) xenografts treated i.v. with 10 μmol/kg of J1, melphalan, or no drug on day 0: *points,* mean tumor volume index; *bars,* SE (**A**); *points,* mean weight gain; *bars,* SE (**B**). Both drugs inhibited tumor growth compared with the untreated controls ($P < 0.001$). **C** and **D,** nude rats with SH-SY5Y xenografts treated i.v. with 0.50 μmol/kg of J1, melphalan, or no drug on day 0: *points,* mean tumor volume index; *bars,* SE (**C**); *points,* mean weight gain; *bars,* SE (**D**). J1 was significantly more effective in tumor growth inhibition compared with melphalan ($P < 0.05$). **E** and **F,** immunohistochemistry of percentage of caspase-3 (**E**) and Ki-67 (**F**) positively stained cells in tumors treated with J1 and melphalan, and untreated controls from SH-SY5Y xenografts. Three slides from three different tumors were analyzed (magnification, ×400). *Columns,* mean; *bars,* SE. J1 significantly increased caspase-3–positive cells compared with melphalan ($P < 0.01$) and cell proliferation was significantly inhibited after J1 treatment, but not after melphalan treatment, compared with untreated tumors ($P < 0.05$).

Downloaded from http://aacrjournals.org/mct/article-pdf/6/9/2409/1848860/2409.pdf by guest on 13 June 2024

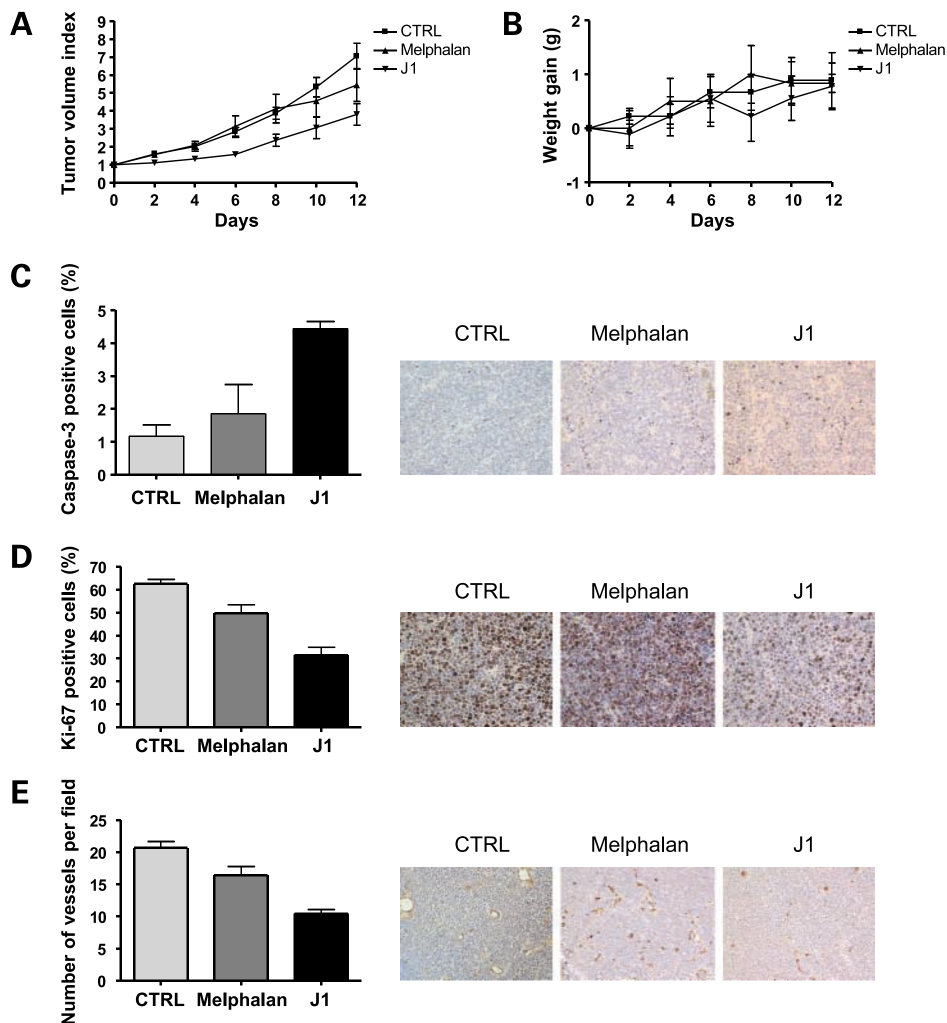

Figure 5. Treatment with J1 inhibits neuroblastoma tumor growth in nude mice. **A,** tumor volume index (*points,* mean; *bars,* SE) from nude mice with SH-SY5Y xenografts treated i.v. with 0.50 μmol/kg of J1, melphalan, or no drug on days 0 and 6. Tumor growth was significantly inhibited by J1 compared with melphalan ($P > 0.01$). Two mice in the melphalan-treated group had to be killed before day 12 (at day 8 and 9) due to exceeding the maximal approved tumor size in the ethical approval, their volume indexes for the remaining days were set at the same level as that when they were killed. **B,** *points,* mean weight gain; *bars,* SE. **C – E,** immunohistochemistry of the percentage of caspase-3 (**C**) and Ki-67 (**D**) positively stained cells and microvessel density (**E**) in tumors treated with J1 or melphalan, and untreated controls. Three slides from three different tumors were analyzed (magnification, ×400 for Ki-67 and caspase-3; ×200 for vessels). *Columns,* mean; *bars,* SE. Treatment with J1 was associated with higher caspase-3 activation ($P < 0.05$), fewer proliferating tumor cells ($P < 0.01$), and significantly decreased mean vascular density ($P < 0.05$) compared with both the untreated controls and melphalan.

with, for example, cyclophosphamide (21), etoposide or carboplatin (22), and etoposide (23). In this work, we chose to study combinations with common neuroblastoma drugs representing different structural classes or mechanisms, and several interesting observations were made; most notably, J1 and the topoisomerase II inhibitor etoposide induced neuroblastoma cell death in a synergistic manner, the mean CI_{70} being significantly lower than one in all three cell lines. Notably, synergistic interactions between melphalan and topoisomerase inhibitors *in vitro* have been reported (24), and high-dose regimens of melphalan and etoposide have been tested clinically in patients with stage IV neuroblastoma and were found to be well-tolerated (23).

Previous *in vivo* studies were done in mice and included dose-finding toxicity and antitumor efficacy in the hollow fiber model. Monitored variables included behavior, weight change, blood cell analysis, autopsy, and histopathologic examinations of sternal bone marrow and the intestines. Early weight loss comparisons favored J1 over melphalan, but differences in all other variables were not significant (significant effects on blood cell count verses control for both drugs; ref. 8). Despite being simple, elegant, and robust, the hollow fiber method suffers from some disadvantages. For example, an almost complete lack of tumor-matrix interaction and no simple histopathologic or immunohistochemical evaluation of treatment results. The *in vivo* findings presented in this article showed that J1 is highly active, both against drug-sensitive (SH-SY5Y) and multidrug–resistant [SK-N-BE(2)] xenografts, at doses giving no observed toxicity except for a transient reduced weight gain using high doses of J1 in rats. Compared with melphalan, J1 seems to induce a more potent proapoptotic as well as the antiangiogenic activity in neuroblastoma *in vivo*. That J1, in addition to its direct antitumoral effects, is able to significantly reduce the number of blood vessels in neuroblastoma, is a novel finding. The metalloproteinase aminopeptidase N is highly expressed in vascular endothelial cells and has been shown to play multiple roles in angiogenesis (25). Preliminary findings in our lab show that aminopeptidase N is a target of J1,[8] which may

[8] Wickström et al., unpublished observation.

Downloaded from http://aacrjournals.org/mct/article-pdf/6/9/2409/1848860/2409.pdf by guest on 13 June 2024

be relevant in view of the antiangiogenic effect of J1 presented here. In their recent work, Pastorino et al. showed that doxorubicin-containing liposomes coupled to aminopeptidase N–directed peptides successfully targets the vasculature of neuroblastoma xenografts (26). Our finding that the aminopeptidase inhibitor bestatin partly inhibits the direct antitumoral effects of J1 against neuroblastoma cells further supports a role for this enzyme as an important target of J1. Studies to further unravel the involvement of aminopeptidase N as a target of J1 are now ongoing.

One possible explanation for the somewhat moderate activity differences between J1 and melphalan compared with the *in vitro* data in the neuroblastoma xenograft studies in rodents may be related to the enzymatic differences between rodents and humans. The prodrug has two hydrolysis susceptible bonds, one peptide and one ester bond. Cleaving of the peptide bond (i.e., by peptidases) leads to melphalan, and in a previous publication, we presented evidence that this cleavage results in the increased activity of J1 compared with melphalan (8). Reports of extended esterase activity in rodents compared with humans have been published previously (27). This has been shown to be important for other prodrugs like CPT-11 (irinotecan) efficacy and toxicity in rodent experiments *in vivo* (28). With the rapid de-esterification of J1, some of the advantages compared with melphalan may be lost, e.g., because the de-esterified J1 is less lipophilic than J1, passive transportation into the cells may decrease. Indeed, de-esterified J1 also possesses reduced cytotoxic activity *in vitro*.[9]

In summary, these studies show significant activity of J1, a novel alkylating dipeptide and prodrug of melphalan, in neuroblastoma *in vitro* and *in vivo*, involving multiple parallel effects on tumor growth. Furthermore, additive or synergistic *in vitro* interactions with standard chemotherapeutics, particularly etoposide, are shown. These novel findings should encourage further preclinical studies to support clinical studies in children with neuroblastoma refractory to standard treatment.

Acknowledgments

The work of Ninib Baryawno, Lotta Elfman, Helena Gleissman, and Agnes Rasmusson are gratefully acknowledged.

References

1. Brodeur GM. Neuroblastoma: biological insights into a clinical enigma. Nat Rev Cancer 2003;3:203 – 16.

2. Buzdar AU, Legha SS, Blumenschein GR, et al. Peptichemio versus melphalan (L-PAM) in advanced breast cancer. Cancer 1982;49:1767 – 70.

3. Paccagnella A, Tredese F, Salvagno L, et al. Peptichemio in pretreated patients with ovarian cancer. Cancer Treat Rep 1985;69:17 – 20.

4. De Bernardi B, Nicolas B, Boni L, et al. Disseminated neuroblastoma in children older than one year at diagnosis: comparable results with three consecutive high-dose protocols adopted by the Italian Co-Operative Group for Neuroblastoma. J Clin Oncol 2003;21:1592 – 601.

5. Pastore G, De Bernardi B, Carli M, et al. Peptichemio in neuroblastoma at relapse. Med Pediatr Oncol 1984;12:162 – 5.

6. Gullbo J, Dhar S, Luthman K, et al. Antitumor activity of the alkylating oligopeptides J1 (L-melphalanyl-*p*-L-fluorophenylalanine ethyl ester) and P2 (L-prolyl-m-L-sarcolysyl-*p*-L-fluorophenylalanine ethyl ester): comparison with melphalan. Anticancer Drugs 2003;14:617 – 24.

7. Gullbo J, Tullberg M, Vabeno J, et al. Structure-activity relationship for alkylating dipeptide nitrogen mustard derivatives. Oncol Res 2003;14:113 – 32.

8. Gullbo J, Lindhagen E, Bashir-Hassan S, et al. Antitumor efficacy and acute toxicity of the novel dipeptide melphalanyl-p-L-fluorophenylalanine ethyl ester (J1) *in vivo*. Invest New Drugs 2004;22:411 – 20.

9. Gullbo J, Wickstrom M, Tullberg M, et al. Activity of hydrolytic enzymes in tumour cells is a determinant for anti-tumour efficacy of the melphalan containing prodrug J1. J Drug Target 2003;11:355 – 63.

10. van Hensbergen Y, Broxterman HJ, Hanemaaijer R, et al. Soluble aminopeptidase N/CD13 in malignant and nonmalignant effusions and intratumoral fluid. Clin Cancer Res 2002;8:3747 – 54.

11. Suganuma T, Ino K, Shibata K, et al. Regulation of aminopeptidase A expression in cervical carcinoma: role of tumor-stromal interaction and vascular endothelial growth factor. Lab Invest 2004;84:639 – 48.

12. Martinez JM, Prieto I, Ramirez MJ, Cueva C, Alba F, Ramirez M. Aminopeptidase activities in breast cancer tissue. Clin Chem 1999;45: 1797 – 802.

13. Larsson R, Kristensen J, Sandberg C, Nygren P. Laboratory determination of chemotherapeutic drug resistance in tumor cells from patients with leukemia, using a fluorometric microculture cytotoxicity assay (FMCA). Int J Cancer 1992;50:177 – 85.

14. Rickardson L, Fryknas M, Haglund C, et al. Screening of an annotated compound library for drug activity in a resistant myeloma cell line. Cancer Chemother Pharmacol 2006;58:749 – 58.

15. Lovborg H, Nygren P, Larsson R. Multiparametric evaluation of apoptosis: effects of standard cytotoxic agents and the cyanoguanidine CHS 828. Mol Cancer Ther 2004;3:521 – 6.

16. Ponthan F, Johnsen JI, Klevenvall L, Castro J, Kogner P. The synthetic retinoid RO 13 – 6307 induces neuroblastoma differentiation *in vitro* and inhibits neuroblastoma tumour growth *in vivo*. Int J Cancer 2003;104:418 – 24.

17. Ponthan F, Wickstrom M, Gleissman H, et al. Celecoxib prevents neuroblastoma tumor development and potentiates the effect of chemotherapeutic drugs *in vitro* and *in vivo*. Clin Cancer Res 2007;13:1036 – 44.

18. Chou TC, Talalay P. Quantitative analysis of dose-effect relationships: the combined effects of multiple drugs or enzyme inhibitors. Adv Enzyme Regul 1984;22:27 – 55.

19. Scornik OA, Botbol V. Bestatin as an experimental tool in mammals. Curr Drug Metab 2001;2:67 – 85.

20. Pritchard J, Cotterill SJ, Germond SM, Imeson J, de Kraker J, Jones DR. High dose melphalan in the treatment of advanced neuroblastoma: results of a randomised trial (ENSG-1) by the European Neuroblastoma Study Group. Pediatr Blood Cancer 2005;44:348 – 57.

21. Hartmann O, Valteau-Couanet D, Benhamou E, et al. Stage IV neuroblastoma in patients over 1 year of age at diagnosis: consolidation of poor responders with combined busulfan, cyclophosphamide and melphalan followed by *in vitro* mafosfamide-purged autologous bone marrow transplantation. Eur J Cancer 1997;33:2126 – 9.

22. Yanik GA, Levine JE, Matthay KK, et al. Pilot study of iodine-131-metaiodobenzylguanidine in combination with myeloablative chemotherapy and autologous stem-cell support for the treatment of neuroblastoma. J Clin Oncol 2002;20:2142 – 9.

23. Valteau-Couanet D, Vassal G, Pondarre C, et al. Phase I study of high-dose continuous intravenous infusion of VP-16 in combination with high-dose melphalan followed by autologous bone marrow transplantation in children with stage IV neuroblastoma. Bone Marrow Transplant 1996;17:485 – 9.

24. Kaufmann SH, Peereboom D, Buckwalter CA, et al. Cytotoxic effects of topotecan combined with various anticancer agents in human cancer cell lines. J Natl Cancer Inst 1996;88:734 – 41.

25. Fukasawa K, Fujii H, Saitoh Y, et al. Aminopeptidase N (APN/CD13) is selectively expressed in vascular endothelial cells and plays multiple roles in angiogenesis. Cancer Lett 2006;243:135 – 43.

26. Pastorino F, Brignole C, Di Paolo D, et al. Targeting liposomal chemotherapy via both tumor cell-specific and tumor vasculature-specific ligands potentiates therapeutic efficacy. Cancer Res 2006;66:10073 – 82.

27. Quon CY, Stampfli HF. Biochemical properties of blood esmolol esterase. Drug Metab Dispos 1985;13:420 – 4.

28. Morton CL, Iacono L, Hyatt JL, et al. Activation and antitumor activity of CPT-11 in plasma esterase-deficient mice. Cancer Chemother Pharmacol 2005;56:629 – 36.

[9] Unpublished data.

Downloaded from http://aacrjournals.org/mct/article-pdf/6/9/2409/1848860/2409.pdf by guest on 13 June 2024