Biochemical Pharmacology 79 (2010) 1281–1290

Contents lists available at ScienceDirect

Biochemical Pharmacology

journal homepage: www.elsevier.com/locate/biochempharm

The alkylating prodrug J1 can be activated by aminopeptidase N, leading to a possible target directed release of melphalan

Malin Wickström [a,1,*], Kristina Viktorsson [b,1], Lovisa Lundholm [b], Reidun Aesoy [b], Helen Nygren [c], Linda Sooman [d], Mårten Fryknäs [a], Lotte Katrine Vogel [e], Rolf Lewensohn [b], Rolf Larsson [a], Joachim Gullbo [a]

[a] Department of Medical Sciences, Division of Clinical Pharmacology, Uppsala University Hospital, S-75185 Uppsala, Sweden
[b] Karolinska Biomics Center, Department of Oncology/Pathology, Karolinska Institutet and Karolinska University Hospital, Stockholm, Sweden
[c] Karolinska Pharmacy, Karolinska University Hospital, Stockholm, Sweden
[d] Department of Oncology, Radiology and Clinical Immunology, Section of Oncology, Uppsala University Hospital, Uppsala, Sweden
[e] Department of Cellular and Molecular Medicine, Faculty of Health Science, University of Copenhagen, Denmark

ARTICLE INFO

Article history:
Received 8 October 2009
Accepted 29 December 2009

Keywords:
J1
Aminopeptidase N
Esterases
Prodrug
Cancer therapeutics
Alkylating agents

ABSTRACT

The alkylating prodrug of melphalan, J1 (melphalanyl-L-*p*-fluorophenylalanyl ethyl ester) is currently in early clinical trials. Preclinical studies have shown that J1-mediated cytotoxicity is dependent on hydrolytic activity of tumor cells. In this report we have analyzed potential peptidases and esterases of importance for release of free melphalan from J1. Exposure of tumor cell lines to J1 resulted in a significant increased level of free intracellular melphalan, at least tenfold at C_{max}, compared to exposure to melphalan at the same molar concentration. This efficient intracellular delivery could be inhibited in both magnitude and in time by bestatin, a broad spectrum inhibitor of the aminopeptidases, including the metalloproteinase aminopeptidase N (APN, EC 3.4.11.2.), and ebelactone A, an esterase inhibitor. These effects resulted, as expected, in decreased cytotoxic effects of J1. A specific role of APN in hydrolyzing J1 releasing free melphalan was demonstrated in vitro with pure APN enzyme. By using plasmid-based overexpression of APN or down regulation of endogenous APN with siRNA in different tumor cell lines we here confirm the involvement of APN in J1-mediated cytotoxic and apoptotic signaling. In conclusion, this study demonstrates a role of APN in the activation of the melphalan prodrug J1 and subsequently, its cytotoxicity. Given that APN is shown to be overexpressed in several solid tumors our data suggest that J1 may be activated in a tumor selective manner.

1. Introduction

During the last decade the development of novel cancer therapies with a rational molecular target has been intense, mostly aiming to affect a specific genetic difference between tumor- and normal cells, e.g. oncogenic activation of a tyrosine kinase. This approach has indeed generated drugs that revolutionized cancer therapy, herceptin and imatinib targeting Her-2 and Bcr-Abl being two examples. Yet another avenue to selectively target tumor cells is to take advantage of phenotypic rather than monogenetic differences between normal and tumor cells. These phenotypic differences can include elevated protease activity or overexpressed transporters, which can be used to selectively activate or accumulate a chemotherapeutic prodrug within tumor cells or in their close vicinity, thereby achieving an increased therapeutic ratio.

J1 (melphalanyl-L-*p*-fluorophenylalanyl ethyl ester hydrochloride) is a prodrug of melphalan and we have earlier in a number of publications described that it exhibits significantly higher in vitro and in vivo cytotoxicity than melphalan itself despite identical alkylating capacity [1–6]. These results indicate that J1 upon addition to cells becomes activated generating free melphalan. Thus in tumor cells a limited exposure time which simulate short half-life in vivo, proved more favorable for J1 than for melphalan, indicating a "trapping" mechanism [4]. In addition, inhibition of peptidase activity resulted in a decreased activity of J1, and derivatives designed to resist the action of peptidases were less active than the corresponding normal dipeptide [4].

* Corresponding author. Tel.: +46 18 6112931; fax: +46 18 519237.
E-mail addresses: malin.wickstrom@medsci.uu.se (M. Wickström), kristina.viktorsson@ki.se (K. Viktorsson), lovisa.lundholm@ki.se (L. Lundholm), Reidun.Aesoey@med.monash.edu.au (R. Aesoy), helen.nygren@apoteket.se (H. Nygren), linda.sooman@onkologi.uu.se (L. Sooman), marten.fryknas@medsci.uu.se (M. Fryknäs), vogel@sund.ku.dk (L.K. Vogel), rolf.lewensohn@ki.se (R. Lewensohn), rolf.larsson@medsci.uu.se (R. Larsson), joachim.gullbo@medsci.uu.se (J. Gullbo).
[1] These authors contributed equally to this work.

0006-2952/$ – see front matter © 2010 Elsevier Inc. All rights reserved.
doi:10.1016/j.bcp.2009.12.022

Aminopeptidases are metalloproteinases that remove amino acids from unblocked *N*-terminal positions of oligopeptides. These proteinases have broad substrate specificity and are widely distributed [7–9]. One of the most studied aminopeptidases is the membrane-bound aminopeptidase N (APN, EC 3.4.11.2.), also known as CD13, which is reported to be involved in different cellular processes that constitute the hallmarks of a tumor i.e. cell growth, invasion, metastasis [8,10]. APN is also shown to have a functional role in tumor angiogenesis [11,12] and there is a strong correlation between the expression of APN and the invasive capacity of a numerous tumor cell types [8]. Moreover, APN is reported to be overexpressed or have an altered enzymatic activity in many tumor types such as breast-, lung-, ovarial-, and thyroid cancer [8,13–15]. A fully enzymatically active soluble form of APN has been recognized to show an increased expression at tumor sites [8,16–19]. Also other aminopeptidases, e.g. leucyl aminopeptidase (LAP), has been associated with tumor development and is reported to be overexpressed in several tumor types [20,21].

Thus it seems reasonable to suggest aminopeptidases in general, and maybe APN in particular, as suitable targets for both antitumor- as well as antiangiogenic therapy towards tumors [9,22,23]. Along this line is the development of APN inhibitors that have shown promising results in tumor bearing animals and in early clinical trials against different tumor types either alone or in combination with conventional chemotherapy [24–26]. Interestingly, attempts to direct conventional chemotherapeutic agents towards tumors by adding an APN-homing sequence, Cys-Asn-Gly-Arg-Cys (CNGRC) to doxorubicin, 5-fluoro-2′-deoxyuridine, cisplatin or TNF-α have recently been described [11,27–30].

In this report we have investigated the function of the metalloproteinase APN and esterases in the hydrolytic activation of J1 generating a potential activation mechanism of J1 summarized in Fig. 1. Our results confirm that the hydrolysis results in an increased cytotoxic activity of J1, over melphalan. Using the purified enzyme we show that J1 in fact is a substrate for APN, and that melphalan is the product. Importantly, manipulation of APN protein expression in human tumor cell lines altered J1-mediated pro-apoptotic signaling and cytotoxicity. Taken together, the results provide evidence that APN is involved in the activation of J1, a mechanism associated with the tumor cells *per se* but which may also influence the vascular compartment.

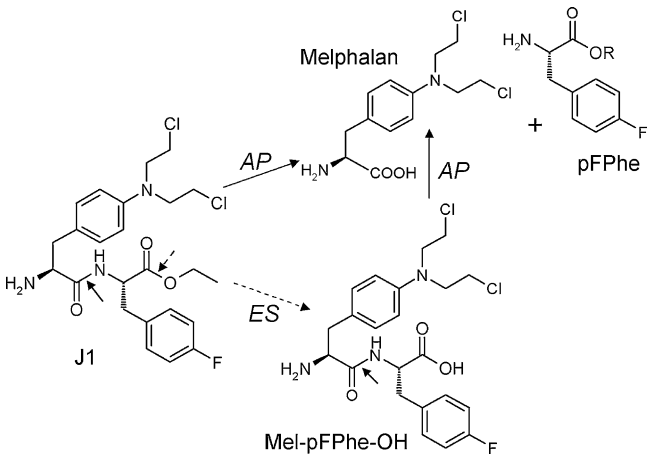

Fig. 1. Schematic picture of the cleavage of J1 (melphalanyl-L-*p*-fluorophenylalanyl ethyl ester hydrochloride), by aminopeptidases (AP) to melphalan or by esterases (ES) to L-melphalanyl-*p*-L-fluorophenylalanine (Mel-pFPhe-OH).

2. Materials and methods

2.1. Human tumor cell lines

Tumor cell lines used were the non-small cell lung carcinomas NCI-H23 (ATCC, LGC Standards, Borås, Sweden) and U1810 [31], the cervical adenocarcinoma HeLa (ATCC), the breast cancer line MCF-7 (ATCC), the neuroblastoma SH-SY5Y (kind gift from Professor Per Kogner, Karolinska Institutet, Sweden), and histiocytic lymphoma U937 [32]. Cells were grown in RPMI 1640 medium (H23, U1810, U937) or Minimal Essential Medium Eagle (SH-SY5Y, MCF-7, HeLa) supplemented with 10% heat-inactivated fetal calf serum, 2 mM glutamine, 100 μg/ml streptomycin, and 100 U/ml penicillin (plus 100 mM sodium pyruvate for MCF-7, HeLa) at 37 °C in a humidified 5% CO_2 atmosphere. Medium and supplements were purchased at Sigma–Aldrich, Stockholm, Sweden.

2.2. Drugs

J1 and its de-esterified derivative L-melphalanyl-*p*-L-fluorophenylalanine triflouroacetate salt (Mel-pFPhe-OH, obtained by LiOH-hydrolysis of J1) (gifts from Oncopeptides AB, Stockholm, Sweden) were dissolved in DMSO or in 99.5% ethanol with 2% HCl. Melphalan was obtained as Alkeran® from the Swedish Pharmacy (Apoteket AB, Uppsala, Sweden) and was dissolved according to the manufacturer. All substances were further diluted in sterile water, phosphate-buffered saline (PBS; Sigma–Aldrich) or cell culture media to appropriate concentration prior to start of experiments to avoid mustard hydrolysis. Molecular structures of the three studied compounds are shown in Fig. 1.

2.3. Measurement of intracellular concentrations of J1 and melphalan

Cells were suspended (2.5×10^6/ml) in warm (37 °C) complete medium and 10 μM J1 or melphalan was added. At 0, 5, 15, 30, 60 and 120 min a sample of 5×10^6 cells were withdrawn, immediately put into 8 ml ice-cold PBS and centrifuged ($200 \times g$, 5 min). A sample (150 μl) of the supernatant was taken and frozen, the PBS was carefully removed and the cells were washed again in 10 ml of ice-cold PBS. After centrifugation ($200 \times g$, 5 min) the cell pellet was solubilized in 200 μl ethanol/acetonitrile (1:1, v/v), thereafter the precipitate was removed by centrifugation ($7800 \times g$, 3 min) and 150 μl of the supernatant was saved. The samples were immediately frozen at −70 °C until analysis. Samples were analyzed as previously been described (modified from [4,33]) or by HPLC using C_{18} column with positive ion electrospray tandem-mass spectroscopic detection by Analyst Research Laboratories, Israel or by OncoTargeting AB, Sweden.

2.4. Fluorometric microculture cytotoxicity assay

Cell viability after drug exposure was analyzed with fluorometric microculture cytotoxicity assay (FMCA) as described [34,35]. The FMCA is a total cell kill assay, based on measurement of fluorescence generated from hydrolysis of fluorescein diacetate (FDA; Sigma–Aldrich) to fluorescein (measured at 485/520 nm).

2.5. Enzyme inhibition experiments

The enzyme inhibitors bestatin (inhibiting a number of aminopeptidases including APN, LAP, aminopeptidase B), actinonin (inhibitor of LAP but also APN), chymostatin (inhibitor of

chymotrypsin, papain and cathepsins A, B and C), puromycin (inhibitor of puromycin-sensitive aminopeptidase (PSA)) and ebelactone A (inhibitor of esterases) (all purchased from Sigma–Aldrich) were used to evaluate the impact of the different enzymes on the cytotoxic effect of J1, melphalan and Mel-pFPhe-OH, respectively. In these analyses, cells were pre-incubated with non-toxic concentrations (determined in preparatory experiments, data not shown) of bestatin (10 μM), actinonin (200 μM), chymostatin (100 μM), puromycin (100 nM), and ebelactone A (100 μM) for 60 min, before drug exposure.

2.6. Enzyme kinetics

To establish if J1 and Mel-pFPhe-OH are substrates for APN-mediated hydrolysis, J1 or Mel-pFPhe-OH (10 μM) was added to a buffer solution (0.05 M Hepes, 0.15 M NaCl, pH 7.0) with the pure enzyme (final concentration of 0.125 U/ml aminopeptidase N, EC 3.4.11.2; Sigma–Aldrich) and samples of 50 μl were withdrawn at 1, 5, 10, 15 and 30 min. Adding acetonitrile and subsequent incubation on ice interrupted the reaction. To assess spontaneous hydrolysis under these conditions, samples of 10 μM of melphalan or J1 incubated in buffer solution alone were analyzed at 0 and 30 min, respectively [4,33].

2.7. Transfection of aminopeptidase N

H23 or HeLa cells were seeded in 6-well plates and the next day transiently transfected with the cDNA coding for human APN, full length in the pTej4 or pTej8 vector (H23 and HeLa) or APN lacking the membrane attaching sequence (representing the soluble form of APN) in the pTej4 vector (HeLa) [36] using Lipofectamine 2000 (Invitrogen, Stockholm, Sweden) or Fugene HD transfection reagent (Roche Diagnostics Scandinavia AB, Bromma, Sweden) according to the standard protocols. H23 and HeLa cells were harvested 24 and 72 h after transfection, respectively. Specific increase in APN expression and enzyme activity was confirmed by Western blot and colorimetric analysis, respectively.

2.8. Small interfering RNA (siRNA) analysis

U1810 cells were transfected with ON-TARGETplus SMARTpool Human APN (NM_001150) using the DharmaFECT1 reagent as recommended by the manufacturer (Dharmacon, Inc., Lafayette CO, USA). In preparatory experiments, a siControl Non-targeting pool (D001810) (Dharmacon) was used (data not shown). Briefly, 2.0×10^5 cells/well were seeded into 6-well plates and the next day transfected with 50 nM siRNA mixed with 2 μl DharmaFECT1. Treatment with J1 was performed 48 h after transfection. siRNA suppression of APN expression and enzymatic activity in U1810 cells was confirmed by Western blot and colorimetric analysis, respectively.

2.9. Western blot analysis

Total protein content was extracted from cells using RIPA buffer (50 mM Tris–HCl (pH 7.4), 150 mM NaCl, 0.5% Igepal, 5 mM EDTA (pH 8.0), 0.1% SDS) supplemented with 1× PhosStop and Mini Protease inhibitor cocktail tablets (Roche Diagnostics, Basel, Switzerland). Thirty μg protein from each sample was resolved on SDS polyacrylamide gels and transferred to nitrocellulose membrane (Hybond ECL, Pharmacia Biotech, GE Healthcare, Uppsala, Sweden), blocked in 5% BSA and probed with primary antibody. For APN detection, 3–8% Tris–acetate gels in NuPage Tris–acetate running buffer (EA0378BOX, Invitrogen) and for Jun *N*-terminal kinase (JNK) and c-Jun detection, 10% Bis–Tris gels in NuPage MOPS running buffer (NP0322BOX, Invitrogen) were used. The membranes were probed with the following commercial and validated primary antibodies: phospho-JNK (Thr183/Tyr185) (#9251) [37], phospho c-Jun (Ser63) (#9261) [38] (both from Cell Signaling Technology, Danvers, MA), JNK (sc-571) [39] and APN/CD13 (sc-13536) [40] (both from Santa Cruz Biotechnology Inc., Santa Cruz, CA, USA) and GAPDH (#2275 PC-1) from Trevigen (Gaithersburg, MD, USA). The membranes were immunoblotted with primary antibodies overnight at 4 °C followed by probing with either mouse or rabbit HRP-conjugated secondary antibody (GE Healthcare) for 45 min at room temperature before ECL-reagent was added (Pierce, Rockford, IL, USA). Images were scanned and quantified by Quantity One software (Bio-Rad, Hercules, CA, USA).

2.10. Colorimetric analysis of APN

Enzymatic activity of APN was measured with the APN substrate L-alanine-4-nitro-anilide (Sigma–Aldrich) as previously described [41]. Briefly, intact cells were incubated with L-alanine-4-nitro-anilide (2 mM) for 30 or 60 min and free nitro-aniline was detected by absorbance measurement at 405 nM.

2.11. Caspase-3 activation analysis

Caspase-3 activity was analyzed using FITC conjugated antibody against caspase-3 in flow cytometry. Briefly, cells were harvested and fixed in 4% phosphate-buffered formaldehyde for 5 min at RT. After rinsing in PBS, cells were incubated in 100 μl permeabilizing buffer (10 mM HEPES, 0.5% Triton-X-100, 3% BSA) containing antibody against the active form of Caspase-3 (1:20) (BD#559341, FITC-conjugated) (BD Biosciences, Stockholm, Sweden) on a rotating wheel at 4 °C o/n. Flow cytometry analysis was done in the FL-1 channel of a FACS calibur (BD Biosciences).

2.12. Statistics

The cytotoxic IC_{50}-vaules (inhibitory concentration 50%) of the drugs were determined from log concentration–effect curves in Graph Pad Prism (GraphPad software Inc., La Jolla, CA, USA) using non-linear regression analysis. Comparison between two groups was made with unpaired *t*-test, $P < 0.05$ was considered significant.

3. Results

3.1. Treatment of human tumor cell lines with J1 results in higher intracellular concentration of melphalan than after melphalan treatment

The intracellular kinetics of J1 and melphalan following exposure to equal molar concentrations was analyzed in the tumor cell lines U1810, H23, HeLa, SH-SY5Y, and MCF-7. Results for U1810 are displayed in Fig. 2A, the curves for H23, HeLa, MCF-7, and SH-SY5Y showed a similar pattern (supplementary Fig. S1). Exposure of the tumor cells to J1 rapidly resulted in a high intracellular concentration of liberated melphalan, with a peak concentration reached after approximately 15 min. In comparison, after melphalan exposure the C_{max} of intracellular melphalan was detected after 60 min, and more importantly, the obtained maximum intracellular concentration of melphalan was only one twentieth to one tenth of the concentration achieved after J1 exposure (Fig. 2A and B). The same relationship was found for the total melphalan exposure after J1 and melphalan addition respectively as estimated by the $AUC_{0-120\ min}$ (Fig. 2B). The de-esterified metabolite Mel-pFPhe-OH was detected as a relatively

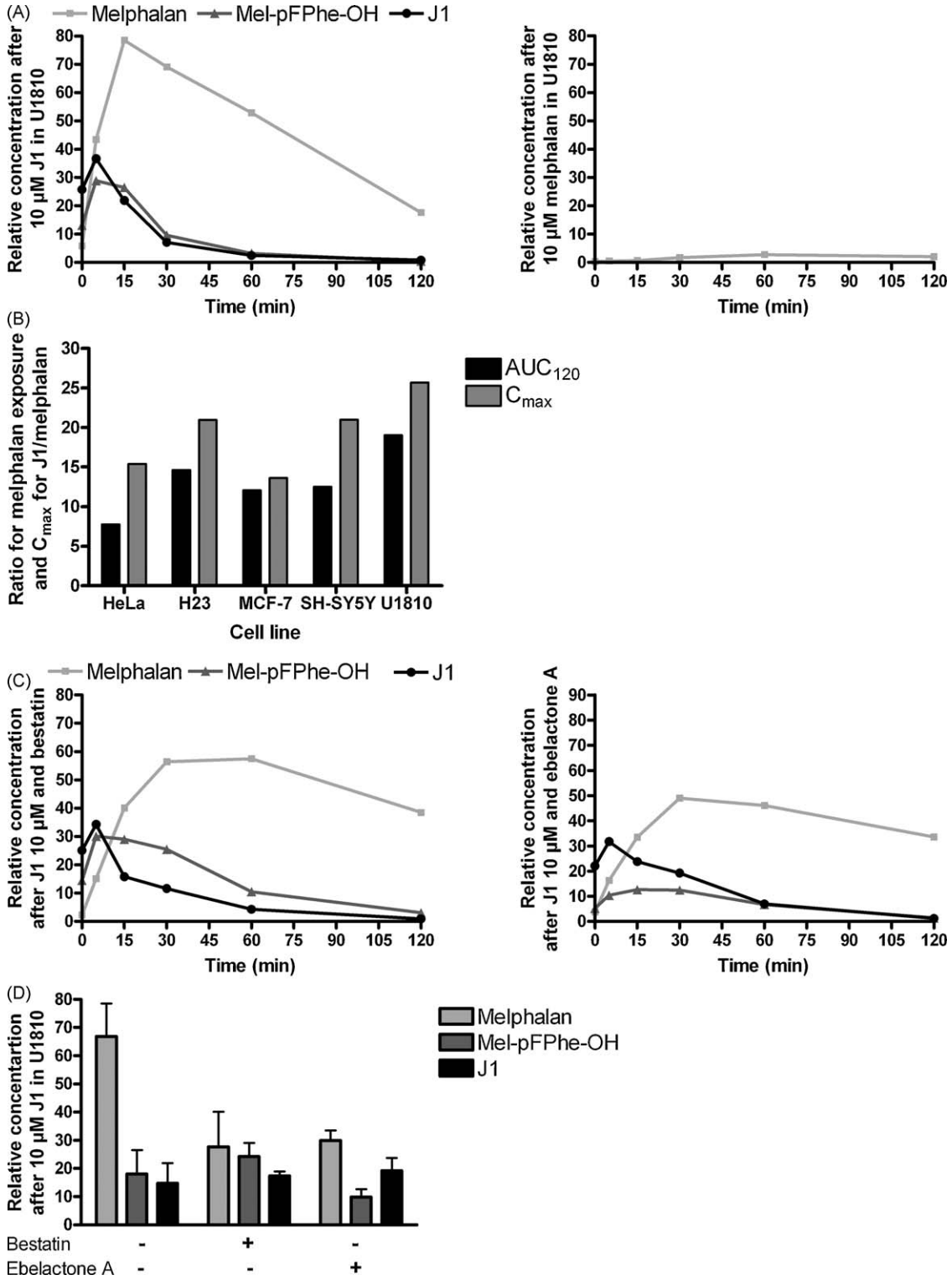

Fig. 2. J1 treatment of human tumor cells results in intracellular accumulation of melphalan and is decreased and delayed by inhibitors of aminopeptidases and esterases. The intracellular concentrations of J1, melphalan and the de-esterified form Mel-pFPhe-OH were analyzed in cell pellets of the tumor cell line U1810 after exposure to 10 μM of J1 (A, left panel) or melphalan (A, right panel) at 0–120 min. One representative experiment of two is shown. The relationship of C_{max} and total exposure of melphalan (measured as $AUC_{0-120\ min}$) after J1 and melphalan addition respectively is displayed in five tumor cell lines (B). Intracellular concentrations were also measured after J1 exposure (10 μM) in the presence or absence of pretreatment of bestatin (10 μM, 60 min; C, left panel) or ebelactone A (100 μM, 60 min; C, right panel), at 0–120 min or at 15 min (D, mean of three experiments ±S.E.M.).

short-lived intermediate in the activation of J1, with C_{max} within a few minutes after start of exposure and with varied quantity within the different cell types examined, presumably as a consequence of differences in esterase activities (Fig. 2A and supplementary Fig. S1).

3.2. Impaired cytotoxic activity of the de-esterified form of J1: Mel-pFPhe-OH

Mel-pFPhe-OH, demonstrated a significantly lower cytotoxic activity compared to J1 in all tested cell lines ($P < 0.001$, Table 1).

Table 1
Cytotoxic activity as IC_{50} (μM) of J1, melphalan and L-melphalanyl-*p*-L fluorophenylalanine (Mel-pFPhe-OH) after 30 min drug exposure and fold change for cells pretreated with the aminopeptidase inhibitor bestatin (10 μM, 60 min) or the esterases inhibitor ebelactone A (100 μM, 60 min) compared to the non pretreated cells. The activity was analyzed in FMCA (tested in duplicated, two–three individual experiments). Statistics from *t*-tests of pretreated cells vs untreated controls on log IC_{50} values, significance levels: $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$ and NS non-significant. *Nd* = not determined.

Cell line	Drug	IC_{50} (μM)	+Bestatin		+Ebelactone A	
			Fold change	Significance	Fold change	Significance
MCF-7	J1	3.41	2.0	**	2.5	**
	Melphalan	503	0.9	NS	1.5	NS
	Mel-pFPhe-OH	222	1.5	*	1.4	NS
HeLa	J1	9.06	2.5	***	2.3	***
	Melphalan	879	1.0	NS	1.1	NS
	Mel-pFPhe-OH	562	1.9	*	1.1	NS
U1810	J1	0.437	2.5	**	1.1	NS
	Melphalan	162	1.0	NS	1.1	NS
	Mel-pFPhe-OH	61.1	2.1	*	1.1	NS
H23	J1	5.32	3.0	***	1.7	**
	Melphalan	792	1.1	NS	1.1	NS
	Mel-pFPhe-OH	401	2.2	*	1.5	NS
U937	J1	0.327	2.4	**	1.7	*
	Melphalan	15.6	1.2	NS	0.8	NS
	Mel-pFPhe-OH	45.5	*Nd*	–	*Nd*	–
SH-SY5Y	J1	0.227	2.9	**	1.7	*
	Melphalan	36.9	0.8	NS	1.1	NS
	Mel-pFPhe-OH	56.5	*Nd*	–	*Nd*	–

The activity was approximately in the same order of magnitude as melphalan.

3.3. J1-induced cytotoxicity is reduced by inhibitors of aminopeptidases and esterases

Several inhibitors were screened for their effect on J1-mediated cytotoxicity in different human tumor cell lines (Table 1). When the tumor cells were pretreated with the aminopeptidase inhibitor bestatin, the cytotoxic activity of J1, but not melphalan, was significantly decreased in all the tested cell lines ($P < 0.01$, Table 1) and a mean fold change of 2.5 times in IC_{50} relative to cells treated with J1 alone was observed. Pretreatment with the esterase inhibitor ebelactone A significantly reduced the cytotoxic activity of J1 in five of six cell lines tested ($P < 0.05$, Table 1), the mean fold change in IC_{50} was 1.9 times. The cytotoxic activity of Mel-pFPhe-OH was significantly inhibited by bestatin pretreatment in all of the four tested cell lines ($P < 0.05$, Table 1) while ebelactone A did not impair this activity in any of the tested cell lines (Table 1). Examining the hydrolysis of the substrates L-alanine-4-nitroanilide and FDA respectively further substantiated the inhibited activity of APN by bestatin and esterase by ebelactone A. The APN activity was 10–55% in the bestatin-treated cells compared to the untreated cells and the esterase activity was 25–43% in the ebelactone A treated cells compared to the untreated cells (data not shown).

In contrast, the other tested enzyme inhibitors: puromycin (primarily inhibitor of PSA but also APN), actinonin (inhibitor of LAP and APN) and chymostatin (inhibitor of chymotrypsin, papain, cathepsin A, B, C) did not have any significant effect on the J1-induced cytotoxicity in any of the cell lines examined (U937 and SH-SY5Y, data not shown).

3.4. Conversion of J1 to melphalan is decreased and delayed by inhibitors of aminopeptidases and esterases

The effects of bestatin and ebelactone A on the intracellular accumulation of melphalan, J1 and Mel-pFPhe-OH after J1 exposure were studied in U1810 (Fig. 2C and D), H23, HeLa, MCF-7 and SH-SY5Y (supplementary Fig. S2). Both bestatin and ebelactone A were found to reduce intracellular accumulation of melphalan from J1 shown as decrease and shift in time of melphalan C_{max} (U1810, Fig. 2C). In bestatin-treated U1810 cells Mel-pFPhe-OH remained in the cells for a longer time compared to the cells not treated with inhibitor (Fig. 2A, C, D), whereas in ebelactone A-treated U1810 cells the formation of Mel-pFPhe-OH was inhibited resulting in less melphalan (Fig. 2A, C, D). These observations were confirmed in HeLa, H23, MCF-7 and SH-SY5Y (supplementary Fig. S2).

3.5. J1 is a substrate for APN and melphalan is the product

To confirm the involvement of APN in conversion of J1 to free melphalan experiments were performed using purified APN in a buffer system (Fig. 3). J1 disappeared within this buffer with a half-life of about 2.5 min and melphalan was formed at the same rate

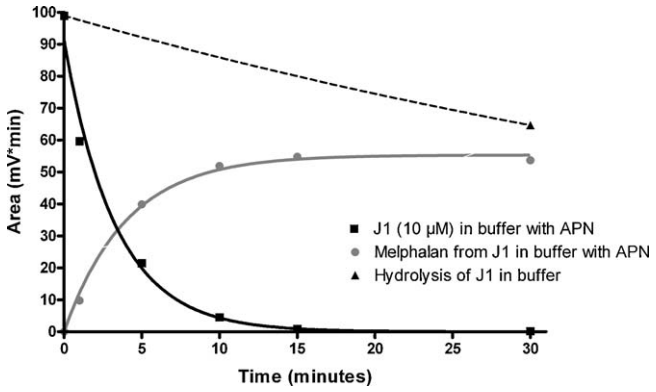

Fig. 3. J1 is a substrate for aminopeptidase N and melphalan is the product. J1 (10 μM) was added to a buffer solution containing APN (0.125 U/ml) and the degradation of J1 and liberation of free melphalan were followed over time using HPLC. For comparison, spontaneous hydrolysis of J1 (the bis (2-chloroethyl) amines part) in the same buffer solution is depicted. After the non-enzymatic hydrolysis of J1 no melphalan was detected. Melphalan 10 μM yielded a peak signal at 81 mV min. One representative experiment of three is shown.

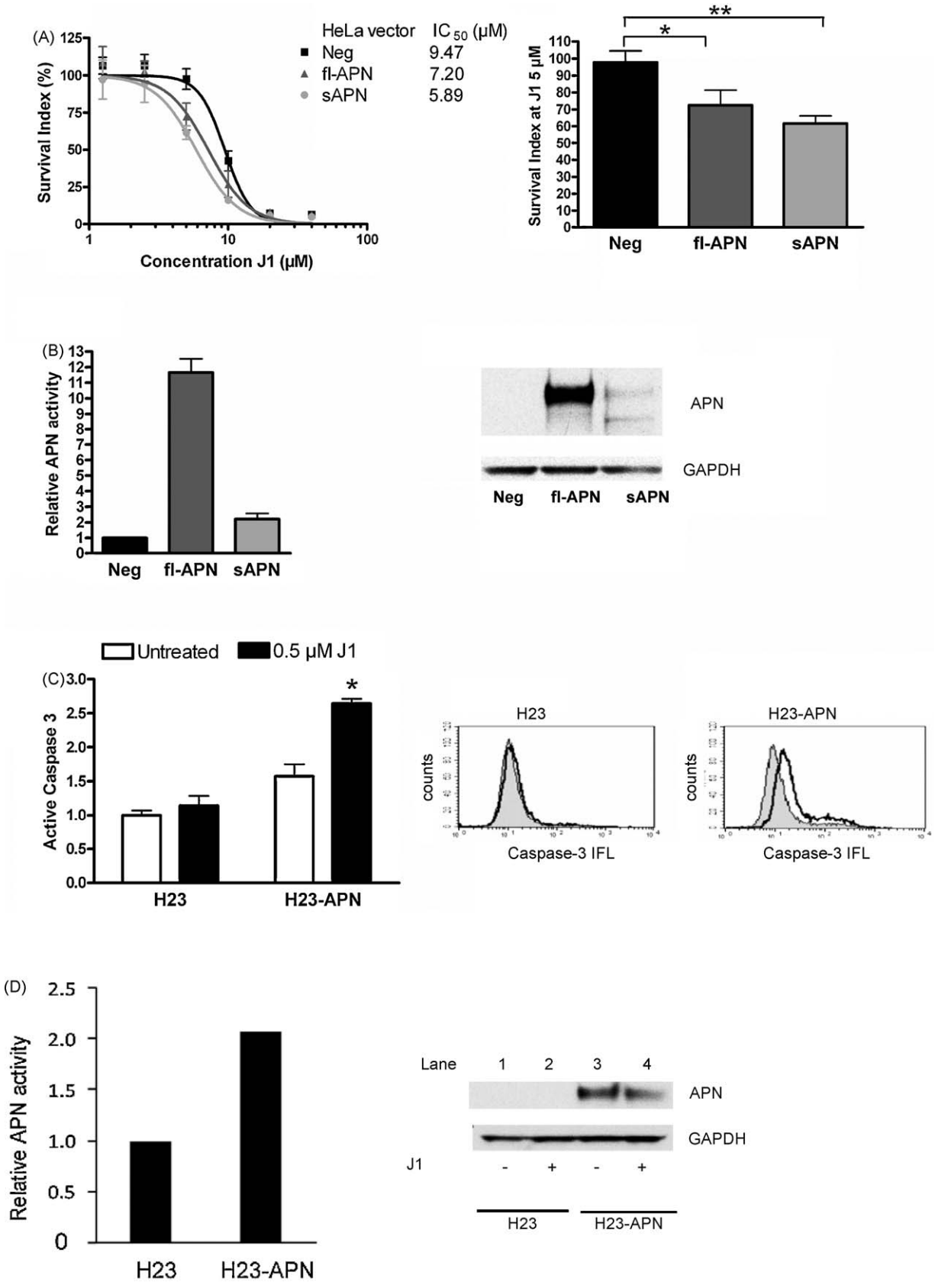

Fig. 4. Overexpression of aminopeptidase N increases J1-induced cell cytotoxicity and activation of caspase-3. Two different cell types, cervical cancer cells HeLa (A, B) or non-small cell lung carcinoma cells H23 (C, D) were transiently transfected with a vector encoding full-length APN (fl-APN) or soluble APN (sAPN). At 72 h post-transfection, HeLa cells were treated with J1 or melphalan for 15 min and cell viability assessed using FMCA at 72 h (A). A change on log IC_{50} in cells transfected with sAPN was demonstrated compared to the negative control vector ($P < 0.05$; A, left upper panel). The survival index at 5 μM of J1 in the different transfectants were found to be statistically increased between negative control and fl-APN (*t*-test, $^{*}P < 0.05$) and sAPN (*t*-test, $^{**}P < 0.01$) respectively (A, right panel). Mean ± S.E.M. of three experiments is shown. The APN activity and expression in the HeLa cells were measured using colorimetric assay (L-alanine-4-nitroanilide) and Western blot respectively (B, left and right panels respectively). H23 cells

(Fig. 3). In the buffer, non-enzymatic hydrolysis of J1 occurred with a half-life of approximately 41 min, similar to what would be expected for bis (2-chloroethyl) amines in aqueous solutions [42]. No melphalan was released in the absence of APN. Thus we can, for the first time, show that J1 indeed is a substrate for APN and that free melphalan is the cleavage product. In a similar manner Mel-pFPhe-OH was investigated as a substrate for APN and indeed it was yielding free melphalan, but cleavage of the peptide bond appeared approximately 50 times slower (corrected for spontaneous hydrolysis of chloroethyl groups, data not shown).

3.6. Overexpression of APN increases J1-induced tumor cell cytotoxicity and activation of caspase-3

Effects of APN overexpression on J1-induced cytotoxicity and pro-apoptotic signaling were investigated (Fig. 4). HeLa cells were transiently transfected with either full-length APN or APN lacking the membrane attaching sequence (soluble APN). Increases in sensitivity towards J1 were observed in both transfectants, the IC_{50} was decreased to 76% (full-length APN) and 62% (soluble APN) of the IC_{50} for the parental HeLa cells (Fig. 4A). As expected, the sensitivity towards melphalan did not change with APN overexpression (data not shown). Increased APN activity and expression were confirmed in the transfected HeLa cells (Fig. 4B).

To further confirm the increased sensitivity of J1 in cells overexpressing APN, H23 cells with low endogenous APN expression were transiently transfected with full-length APN. Activation of caspase-3, a marker of apoptotic signaling, was examined in parental H23 and cells transfected with full-length APN after exposure to J1 (0.5 μM, 24 h) (Fig. 4C, left and right panels). Overexpression of APN (confirmed by colorimetric assay and Western blot, Fig. 4D, left and right panels) was indeed found to increase J1-induced caspase-3 activity in J1-exposed cells, as shown by 1.7-fold increase in caspase-3 activity while no caspase-3 activation was observed in parental H23 cells (*t*-test, $P < 0.05$, Fig. 4C).

3.7. siRNA mediated silencing of APN partly reduces J1-induced pro-apoptotic signaling

In order to verify the importance of APN in J1-mediated cell death, we analyzed J1-induced signaling effects after knockdown of APN by siRNA in U1810, a cell line with high expression level of endogenous APN. Almost complete suppression of APN expression level was obtained (Fig. 5A, left panel) leading to decreased APN activity (Fig. 5A, right panel). Activation of JNK is a central step in signaling from initial DNA damage to activation of apoptotic signaling. The effects of J1 on pro-apoptotic signaling in the presence or absence of APN were analyzed by quantification of phosphorylated and total forms of JNK1 and JNK2 and phospho c-Jun respectively at 4 h post-J1 treatment (1 μM, Fig. 5B and C). In untransfected control cells, levels of phosphorylated JNK 1 and 2 were increased following J1 exposure (non-significant tendency, $P > 0.05$). This tendency could not be detected for siRNA transfected cells (Fig. 5B), suggesting that silencing of APN expression reduce J1-induced pro-apoptotic signaling (i.e. phosphorylation of JNK). Analysis of a JNK-target protein, i.e. the Ser63-phosphorylation of c-Jun yielded similar results. A dose-dependent increase in phospho c-Jun (1.5- and 2-fold at 1 and 2 μM J1, respectively, $P < 0.05$ vs control) is observed in control (i.e. non-transfected) cells, but not in APN-silenced cells (Fig. 5C). Finally, effects further downstream the apoptotic signaling cascade, and at a later time-point (24 h), were analyzed by the activation of caspase-3. In the control U1810 cells, J1 caused a 4-fold increase in caspase-3 activity, which was partly reduced in APN siRNA cells (duplicate experiments, Fig. 5D). In conclusion, this series of measurements of early pro-apoptotic and apoptotic signaling, suggest that APN is one of several peptidases that are required for J1-induced apoptotic effects.

4. Discussion

A prodrug approach where the prodrug is selectively activated in tumors (either directly by the cancer cells or by stromal cells), by overexpressed prodrug-activating enzyme(s) could create important therapeutic advantages. In this respect APN seems as an adequate enzymatic target for antitumor- as well as antiangiogenic therapy towards tumors [22,23]. In the current report, the novel alkylating prodrug J1 was investigated with respect to its trapping and activation mechanism by esterases and aminopeptidases in general, and APN in particular. In a clinical setting, J1 may through such activation mechanisms cause increased accumulation of free melphalan within or in very close vicinity of tumor cells to induce alkylation of tumor cell DNA and cell death. J1 is currently in phase I-IIa clinical trial in advanced solid tumor malignancies. It is, from these studies, suggested that that the advantage of J1 vs melphalan will be most evident in tumors expressing higher levels of APN, which may be those with an aggressive phenotype including high angiogenic potential.

Previous preclinical studies with J1 have revealed high in vitro activity and a beneficial therapeutic index in mice and rats compared to melphalan [1–6]. Structure–activity relationship studies suggested influence of enzymatic activation since peptide composition (both selection of amino acid moieties and order) had dramatic effects on the observed cytotoxicity, but also minor dependency on lipophilicity suggesting passive transport into the tumor cells [3]. Peptide analogues designed to resist enzymatic cleavage of the peptide bond lost all advantage over melphalan [4].

In this report it is demonstrated that APN plays a specific role in the hydrolytic activation of J1, we show that the purified enzyme indeed acts on the prodrug to release melphalan. In line with our previous findings [4], intracellular measurements revealed that J1 was quickly transported into tumor cells, resulting in rapid accumulation of released melphalan. Bestatin, an inhibitor of aminopeptidases including APN, PSA, and LAP [43], significantly could decrease both intracellular melphalan concentrations and the cytotoxic effects of J1. The different aminopeptidases have closely related enzymatic activities with sometimes broad and overlapping specificities, i.e. many substrates may be hydrolyzed by more than one enzyme. Human APN, preferably hydrolyzes substrates with an *N*-terminal alanine residue, but other neutral amino acids may also be removed, the order of favored substrates is: Ala > Phe > Tyr > Leu > Arg etc [8,44]. As a consequence, most aminopeptidase inhibitors also lack tight specificity [25]. The *N*-terminal phenylalanine analogue of J1 makes it a reasonable substrate for APN, and overexpression of APN in tumor cell lines

were transiently transfected with fl-APN (H23-APN), at 48 h post-transfection and 24 h after 0.5 μM J1 treatment, the effect on J1-mediated caspase-3 activation was analyzed using flow cytometry (C). C, left panel: the activity of caspase-3 is given as fold increase in caspase-3 associated immunofluorescence signal and data presented are the mean ± S.E.M. of three experiments. H23 transfected with fl-APN (H23-APN) showed significantly more active caspase-3 compared to parental H23 after J1 treatment (*t*-test, $^*P < 0.05$). C, right panel: histogram showing caspase-3 associated fluorescence in parental H23 cells and APN-transfected H23 cells (H23-APN). Filled grey: untreated cells; black line: 0.5 μM J1, 24 h. The APN activity and expression in H23 cells and H23-APN were measured using colorimetric assay (as above) and Western blot respectively (D, left and right panels, respectively). The data shown in the Western blot correspond to the data shown in C, right panel and the loading are as follows: (1) non-transfected H23, untreated; (2) non-transfected H23, 0.5 μM J1, 24 h; (3) H23 cells transfected with fl-APN (H23-APN), untreated; (4) H23 cells transfected with fl-APN (H23-APN), 0.5 μM J1, 24 h.

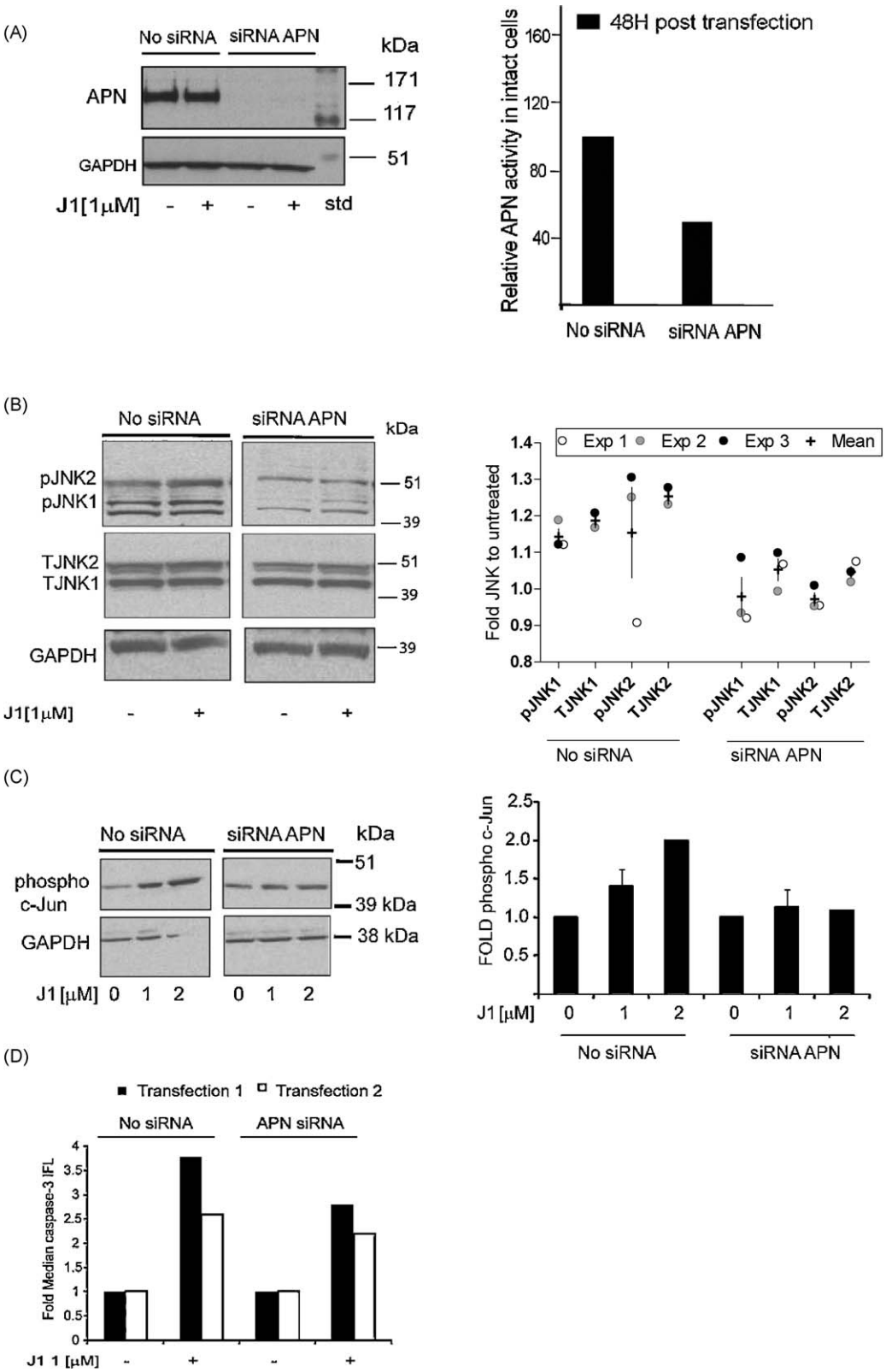

Fig. 5. Inhibition of aminopeptidase N expression and activity reduces J1-induced pro-apoptotic signaling. U1810 cells were transiently transfected with siRNA against APN. At 48 h post-transfection, parental and siRNA transfected U1810 cells were treated with 1 or 2 μM J1 for 4 h. The expression (A, left; Western blot) and the activity of APN (A, right; colorimetric assay) were reduced in U1810 cells transfected with siRNA against APN compared to non-siRNA transfected cells. For space constraints cropped images are presented but full pictures are found in supplementary Fig. S3A. Western blot analysis was used to examine levels of phosphorylated JNK1 and JNK2 and total JNK1 and JNK2 (B) and phospho c-Jun (C) in U1810 controls and cells transfected with APN siRNA (siRNA APN). (B) Levels of phospho- and total JNK1 and JNK2 4 h after exposure to J1 1 μM were quantified from 3 independent experiments and corrected for loading differences using GAPDH. Outliers were detected with Grubb's test (one for TJNK1 and one for TJNK2). (C) Phospho c-Jun (ser 63) was quantified from 3 independent experiments for 1 μM J1 and from 2 independent experiments for 2 μM J1 after correction for loading differences and is given as fold to control with the mean values ±SD after 1 μM J1 and mean values for 2 μM J1. For space constraints cropped images are presented but full pictures are found in supplementary Fig. S3B (JNK) and S3C (phospho c-Jun). (D) FACS analysis of caspase-3 activity was performed at 72 h after transfection start and 24 h after J1 addition. Data show two independent APN siRNA transfections where the caspase-3 response to J1 treatment, in comparison to treatment of non-siRNA transfected cells, was decreased by 28 and 12%, respectively.

also resulted in increased J1-induced cytotoxicity and apoptotic signaling. These effects were constricted to J1 and were not seen for melphalan, and thus suggest a targeting mechanism by APN-associated activation in situ (in the cytoplasm or at the cellular membrane). However, experiments to demonstrate a direct correlation to increased melphalan concentrations in these cells were unsuccessful (not shown), this is most likely related to the rather simple and numb method for measurements of intracellular concentrations including two steps with 5 min washing while also the parental cells presented with a very rapid formation of J1 to melphalan, already after 5 min the majority of the added J1 had been converted to melphalan. Specific inhibition of APN with siRNA demonstrated partial reduction of J1-induced JNK or c-Jun phosphorylation and showed decreased J1-induced caspase-3 activation. Although being non-significant from a statistical point of view, these results are consequent and consistent with the other experiments performed.

Although APN is mainly described as a membrane-bound protein, human plasma contains a significant amount of an active soluble form of APN [16,45], which suggests that certain cells may secrete soluble APN. In the transfection experiments soluble APN induced a more pronounced increase in J1 sensitivity, compared to the full-length APN. As indicated above, the full-length APN is described as a membrane-bound protein with the catalytic activity on the outside of the cell surface [8]. In vivo such localization would probably result in a high local concentration of melphalan around APN-expressing tumor cells. However, in these in vitro cell experiments, with cell suspended in cell growth medium (analogous to an enormous amount of extracellular fluid), the released melphalan can diffuse away from the cells and hence degrade by spontaneous aqueous hydrolysis since melphalan clearly does not permeate into the cells as readily as J1.

Theoretically J1 can be enzymatically hydrolyzed in two places, the peptide bond and the ester bond (Fig. 1). Beside the APN-dependence we found that also ebelactone A, an esterase inhibitor, significantly decreased intracellular melphalan concentrations as well as the cytotoxic effects of J1. With a fast passive transport into the cells, inhibition of the esterase activity may lead to a decreased trapping effect, allowing J1 to diffuse out of the cells again. Correlation between activity difference ($\Delta \log IC_{50}$) for J1 and melphalan and esterase activity yielded a higher correlation coefficient than the correlation to peptidase activity in a prior publication [4]. The de-esterified metabolite Mel-pFPhe-OH was detected as a relatively short-lived intermediate (peaking in concentration prior to melphalan) in the activation process of J1 in the intracellular compartment, but when added in the medium its cytotoxic activity was comparable to melphalan, significantly lower than J1. While APN was able to hydrolyze both J1 and Mel-pFPhe-OH, cleavage of the former appeared much more efficient. Our interpretation of the data is that the de-esterified form is less lipophilic than J1 and therefore reduced passive transport into the cells is expected. Taken together these data may indicate that the hydrolysis of the ester bond happens prior or in parallel to the hydrolysis of the peptide bond, but that hydrolysis of the peptide bond is a prerequisite for efficient nucleic acid interaction as we previously showed [4].

As APN has been indicated to be involved in the metastatic process, a higher expression of APN has been suggested in metastasis [8,13,15,18]. This implies that J1 may work in the metastatic setting and we have indeed reported on a more favorable ratio between J1 and melphalan in advanced breast cancer samples compared to early breast cancer samples in a cytotoxic activity screening in human tumor samples [5].

The importance of APN in J1 metabolism, shown in the present paper, and the reported high expression of APN in endothelial cells e.g. in vascular endothelium [11,12], raises the hypothesis that J1 may have, beside a cytotoxic effect on the tumor cells *per se*, an antiangiogenic effect on the tumor associated vessel formation. Although further studies are required to prove such an hypothesis, we previously found that J1 not only inhibited tumor growth more efficiently than melphalan, J1 also caused a significantly more pronounced reduction of micro-vessel density than melphalan in a neuroblastoma xenograft model in mice [6].

In summary, these studies demonstrate that the alkylating prodrug J1 is a substrate for APN and that melphalan is the product from such hydrolysis. Influence of esterase trapping may also be of importance, and together these two systems render high intracellular melphalan concentrations, at least ten times higher than after exposure to melphalan directly. Moreover using chemical inhibitors, overexpression and siRNA we show that also in cell systems, APN is critically involved in J1 metabolism and cytotoxicity. Given that APN is found to be overexpressed in several solid malignancies and found in both tumor cells *per se* as well as in the proangiogenic endothelial cells, the identification of APN as a player in J1-induced cell death may imply a tumor selective mechanism for this prodrug.

Conflict of interest

L. Lundholm and K. Viktorsson: in part funding of employment, Oncopeptides AB. R. Lewensohn, R. Larsson, K. Viktorsson and J. Gullbo: ownership interest, Oncopeptides AB. The other authors disclosed no potential conflict of interest. Oncopeptides AB has also supplied research support.

Acknowledgements

The work of Caroline Haglund and Birgitta Mörk is gratefully acknowledged as is the valuable comments on this study by Dr. Jack Spira. This work was supported by Swedish Cancer Society (R.La., J.G. and R.Le.), the Stockholm Cancer Society (K.V. and R. Le.), the European Commission to R.Le. (6th FP CHEMORES-project, 7th FP ApoSYS-project), and Oncopeptides AB (defined in conflict of interest).

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bcp.2009.12.022.

References

[1] Gullbo J, Dhar S, Luthman K, Ehrsson H, Lewensohn R, Nygren P, 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.
[2] Gullbo J, Lindhagen E, Bashir-Hassan S, Tullberg M, Ehrsson H, Lewensohn R, 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.
[3] Gullbo J, Tullberg M, Vabeno J, Ehrsson H, Lewensohn R, Nygren P, et al. Structure–activity relationship for alkylating dipeptide nitrogen mustard derivatives. Oncol Res 2003;14:113–32.
[4] Gullbo J, Wickstrom M, Tullberg M, Ehrsson H, Lewensohn R, Nygren P, 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.
[5] Wickstrom M, Haglund C, Lindman H, Nygren P, Larsson R, Gullbo J. The novel alkylating prodrug J1: diagnosis directed activity profile ex vivo and combination analyses in vitro. Invest New Drugs 2008;26:195–204.
[6] Wickstrom M, Johnsen JI, Ponthan F, Segerstrom L, Sveinbjornsson B, Lindskog M, et al. The novel melphalan prodrug J1 inhibits neuroblastoma growth in vitro and in vivo. Mol Cancer Ther 2007;6:2409–17.
[7] Sanderink GJ, Artur Y, Siest G. Human aminopeptidases: a review of the literature. J Clin Chem Clin Biochem 1988;26:795–807.
[8] Mina-Osorio P. The moonlighting enzyme CD13: old and new functions to target. Trends Mol Med 2008;14:361–71.

[9] Zhang X, Xu W. Aminopeptidase N (APN/CD13) as a target for anti-cancer agent design. Curr Med Chem 2008;15:2850–65.
[10] Saiki I, Fujii H, Yoneda J, Abe F, Nakajima M, Tsuruo T, et al. Role of aminopeptidase N (CD13) in tumor-cell invasion and extracellular matrix degradation. Int J Cancer 1993;54:137–43.
[11] Pasqualini R, Koivunen E, Kain R, Lahdenranta J, Sakamoto M, Stryhn A, et al. Aminopeptidase N is a receptor for tumor-homing peptides and a target for inhibiting angiogenesis. Cancer Res 2000;60:722–7.
[12] Fukasawa K, Fujii H, Saitoh Y, Koizumi K, Aozuka Y, Sekine K, 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.
[13] Kehlen A, Lendeckel U, Dralle H, Langner J, Hoang-Vu C. Biological significance of aminopeptidase N/CD13 in thyroid carcinomas. Cancer Res 2003;63:8500–6.
[14] Martinez JM, Prieto I, Ramirez MJ, Cueva C, Alba F, Ramirez M. Aminopeptidase activities in breast cancer tissue. Clin Chem 1999;45:1797–802.
[15] Tokuhara T, Hattori N, Ishida H, Hirai T, Higashiyama M, Kodama K, et al. Clinical significance of aminopeptidase N in non-small cell lung cancer. Clin Cancer Res 2006;12:3971–8.
[16] van Hensbergen Y, Broxterman HJ, Hanemaaijer R, Jorna AS, van Lent NA, Verheul HM, et al. Soluble aminopeptidase N/CD13 in malignant and nonmalignant effusions and intratumoral fluid. Clin Cancer Res 2002;8:3747–54.
[17] Severini G, Gentilini L, Tirelli C. Diagnostic evaluation of alanine aminopeptidase as serum marker for detecting cancer. Cancer Biochem Biophys 1991;12:199–204.
[18] Murakami H, Yokoyama A, Kondo K, Nakanishi S, Kohno N, Miyake M. Circulating aminopeptidase N/CD13 is an independent prognostic factor in patients with non-small cell lung cancer. Clin Cancer Res 2005;11:8674–9.
[19] Favaloro EJ, Bradstock KF, Kabral A, Grimsley P, Zowtyj H, Zola H. Further characterization of human myeloid antigens (gp160, 95; gp150; gp67): investigation of epitopic heterogeneity and non-haemopoietic distribution using panels of monoclonal antibodies belonging to CD-11b, CD-13 and CD-33. Br J Haematol 1988;69:163–71.
[20] Pulido-Cejudo G, Miranda H, El Abdaimi K, Wang C, Kar B, Medina Acevedo J, et al. A monoclonal antibody driven biodiagnostic system for the quantitative screening of breast cancer. Biotechnol Lett 2004;26:1335–9.
[21] Carl-McGrath S, Lendeckel U, Ebert M, Rocken C. Ectopeptidases in tumour biology: a review. Histol Histopathol 2006;21:1339–53.
[22] Huang PS, Oliff A. Drug-targeting strategies in cancer therapy. Curr Opin Genet Dev 2001;11:104–10.
[23] Sato M, Arap W, Pasqualini R. Molecular targets on blood vessels for cancer therapies in clinical trials. Oncology (Williston Park) 2007;21:1346–52 (discussion 54-5, 67, 70 passim).
[24] van Herpen C, Eskens F, de Jonge M, Desar I, Hooftman L, Bone E, et al. A phase Ib dose escalation study to evaluate safety and tolerability of the combination of the 344 aminopeptidase inhibitor CHR-2797 and paclitaxel in patients with advanced or treatment refractory tumors. Mol Targets Cancer Ther (Geneva) 2008.
[25] Bauvois B, Dauzonne D. Aminopeptidase-N/CD13 (EC 3.4.11.2) inhibitors: chemistry, biological evaluations, and therapeutic prospects. Med Res Rev 2006;26:88–130.
[26] Krige D, Needham LA, Bawden LJ, Flores N, Farmer H, Miles LE, et al. CHR-2797: an antiproliferative aminopeptidase inhibitor that leads to amino acid deprivation in human leukemic cells. Cancer Res 2008;68:6669–79.
[27] van Hensbergen Y, Broxterman HJ, Elderkamp YW, Lankelma J, Beers JC, Heijn M, et al. A doxorubicin-CNGRC-peptide conjugate with prodrug properties. Biochem Pharmacol 2002;63:897–908.
[28] Zhang Z, Hatta H, Tanabe K, Nishimoto S. A new class of 5-fluoro-2′-deoxyuridine prodrugs conjugated with a tumor-homing cyclic peptide CNGRC by ester linkers: synthesis, reactivity, and tumor-cell-selective cytotoxicity. Pharm Res 2005;22:381–9.
[29] Mukhopadhyay S, Barnes CM, Haskel A, Short SM, Barnes KR, Lippard SJ. Conjugated platinum(IV)-peptide complexes for targeting angiogenic tumor vasculature. Bioconjug Chem 2008;19:39–49.
[30] Sacchi A, Gasparri A, Gallo-Stampino C, Toma S, Curnis F, Corti A. Synergistic antitumor activity of cisplatin, paclitaxel, and gemcitabine with tumor vasculature-targeted tumor necrosis factor-alpha. Clin Cancer Res 2006;12:175–82.
[31] Bergh J, Nilsson K, Ekman R, Giovanella B. Establishment and characterization of cell lines from human small cell and large cell carcinomas of the lung. Acta Pathol Microbiol Immunol Scand A 1985;93:133–47.
[32] Sundstrom C, Nilsson K. Establishment and characterization of a human histiocytic lymphoma cell line (U-937). Int J Cancer 1976;17:565–77.
[33] Ehrsson H, Lewensohn R, Wallin I, Hellstrom M, Merlini G, Johansson B. Pharmacokinetics of peptichemio in myeloma patients: release of m-L-sarcolysin in vivo and in vitro. Cancer Chemother Pharmacol 1993;31:265–8.
[34] 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.
[35] Lindhagen E, Nygren P, Larsson R. The fluorometric microculture cytotoxicity assay. Nat Protoc 2008;3:1364–9.
[36] Vogel LK, Noren O, Sjostrom H. The apical sorting signal on human aminopeptidase N is not located in the stalk but in the catalytic head group. FEBS Lett 1992;308:14–7.
[37] Xiao GG, Wang M, Li N, Loo JA, Nel AE. Use of proteomics to demonstrate a hierarchical oxidative stress response to diesel exhaust particle chemicals in a macrophage cell line. J Biol Chem 2003;278:50781–90.
[38] Lee SA, Dritschilo A, Jung M. Role of ATM in oxidative stress-mediated c-Jun phosphorylation in response to ionizing radiation and CdCl2. J Biol Chem 2001;276:11783–90.
[39] Lahti A, Sareila O, Kankaanranta H, Moilanen E. Inhibition of p38 mitogen-activated protein kinase enhances c-Jun N-terminal kinase activity: implication in inducible nitric oxide synthase expression. BMC Pharmacol 2006;6:5.
[40] Fontijn D, Duyndam MC, van Berkel MP, Yuana Y, Shapiro LH, Pinedo HM, et al. CD13/Aminopeptidase N overexpression by basic fibroblast growth factor mediates enhanced invasiveness of 1F6 human melanoma cells. Br J Cancer 2006;94:1627–36.
[41] Yeager CL, Ashmun RA, Williams RK, Cardellichio CB, Shapiro LH, Look AT, et al. Human aminopeptidase N is a receptor for human coronavirus 229E. Nature 1992;357:420–2.
[42] Jones RB. Clinical pharmacology of melphalan and its implications for clinical resistance to anticancer agents. Cancer Treat Res 2002;112:305–22.
[43] Scornik OA, Botbol V. Bestatin as an experimental tool in mammals. Curr Drug Metab 2001;2:67–85.
[44] Huang K, Takahara S, Kinouchi T, Takeyama M, Ishida T, Ueyama H, et al. Alanyl aminopeptidase from human seminal plasma: purification, characterization, and immunohistochemical localization in the male genital tract. J Biochem (Tokyo) 1997;122:779–87.
[45] Favaloro EJ, Browning T, Nandurkar H, Sartor M, Bradstock KF, Koutts J. Aminopeptidase-N (CD13; gp 150): contrasting patterns of enzymatic activity in blood from patients with myeloid or lymphoid leukemia. Leuk Res 1995;19:659–66.