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

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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].

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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.

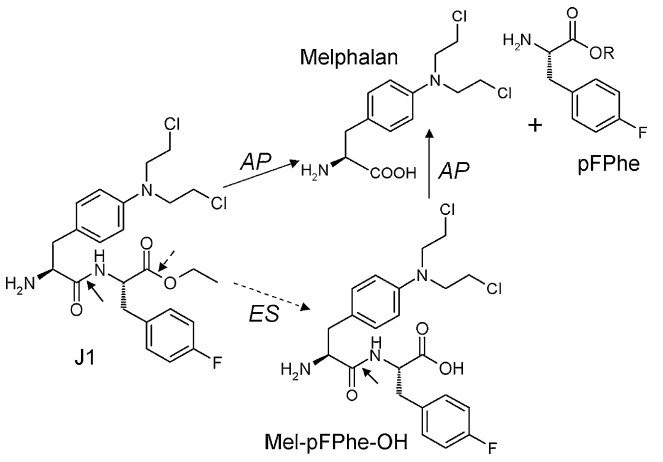

Fig. 1. Schematic picture of the cleavage of J1 (melphalanyl-L-*p*-fluorophenylalanyl ethyl ester hydrochloride), by aminopeptidases (AP) to melphalan or by esterases (ES) to L-melphalanyl-*p*-L-fluorophenylalanine (Mel-pFPhe-OH).

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