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Thimet oligopeptidase (EC 3.4.24.15) activates CPI-0004Na, an extracellularly tumour-activated prodrug of doxorubicin

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ABSTRACT

CPI-0004Na is a tetrapeptidic extracellularly tumour-activated prodrug of doxorubicin. The tetrapeptide structure ensures blood stability and selective cleavage by unidentified peptidase(s) released by tumour cells. The purpose of this work was to identify the enzyme responsible for the first rate-limiting step of CPI-0004Na activation, initially attributed to a 70 kDa acidic (pI = 5.2) metallopeptidase active at neutral pH that was subsequently purified from HeLa cell homogenates. Two electrophoretic bands were isolated and identified by matrix-assisted laser desorption ionisation-time of flight (MALDI-tof) and electrospray ionisation-quadrupole-time of flight (ESI-Q-tof) mass spectrometry as thimet oligopeptidase (TOP). The identity of the CPI-0004Na activating enzyme and TOP was further supported by the similar substrate specificity of the purified enzyme and recombinant TOP, by thiol stimulation of CPI-0004Na cleavage by cancer cell conditioned media (unique characteristic of TOP) and by the inhibition of CPI-0004Na activation by specific inhibitors or immunoprecipitation. Although other enzymes can be involved, TOP clearly appears to be a likely candidate for extracellular activation of the CPI-0004Na prodrug.

1. Introduction

CPI-0004Na[1] was developed as an extracellularly tumour-activated prodrug (ETAP) of doxorubicin (Dox)[2] with the objective of overcoming the two major factors that limit the efficacy of classical, cytotoxic, anticancer agents. These factors are the severe side-effects resulting from the action of anticancer drugs on normal cell types and tissues, and the inherent or acquired resistance of tumours to those same compounds.[3,4]

Abbreviations: AEBSF, aminoethylbenzenesulfonyl fluoride; Dox, doxorubicin; DTT, dithiothreitol; EDTA, ethylenediaminetetraacetic acid; ESI-Q-tof, electrospray ionisation-quadrupole-time of flight; ETAP, extracellularly tumour-activated prodrug; FCS, foetal calf serum; HATU, *O*-(7-azabenzotriazol-1-yl)-*N*,*N*,*N*′,*N*′-tetramethyluronium hexafluorophosphate; HEPES, hydroxyethylpiperazine; HPLC, high performance liquid chromatography; MALDI-tof, matrix-assisted laser desorption ionisation-time of flight; MMP, matrix metallopeptidase; MS, mass spectrometry; PSA, prostate-specific antigen; SDS, sodium dodecyl sulphate; TOP, thimet oligopeptidase; Tris, tris(hydroxymethyl) aminomethane; UPS, Universal protease substrate.

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The deal ETAP compound has: (i) to be pharmacologically inactive as a result of its inability to access the intracellular compartments of both normal and cancer cells; and (ii) to be converted into an active cytotoxic agent at the tumour site only. Prevention of intracellular uptake of the cytotoxic drug can be achieved through conjugation to a non-cell penetrating, blood-stable peptide. Conversion to the active agent is mediated by the action of extracellular peptidases found specifically in tumours.[2] Indeed, a number of hydrolases, and particularly peptidases, have been found to be overexpressed and oversecreted by tumour cells, tumour stromal cells or endothelial cells involved in tumour neoangiogenesis.[5–7]

The appropriate tetrapeptide, which constitutes the promoiety of CPI-0004Na, was selected in vitro among several peptidic conjugates of doxorubicin or daunorubicin, on the basis of its stability in whole human blood and for its selective reactivation by cancer cell conditioned media.[2] At the time of selection, the peptidase(s) responsible for prodrug activation was unidentified and referred to as "enzyme X". When CPI-0004Na showed a clear potential for further development, the characterisation and identification of the enzyme(s) involved in its tumour-selective activation became crucial. In vitro[2,8] and in vivo studies[1] had indicated that the extracellular activation of CPI-0004Na was a two-step process, the rate-limiting one being initial hydrolysis of *N*-(succinyl)-β-alanyl-L-leucyl-L-alanyl-L-leucyl-doxorubicin into *N*-L-alanyl-L-leucyl-doxorubicin. The second extracellular cleavage step yields *N*-L-leucyl-doxorubicin, a compound that is able to permeate passively inside cells where it is further cleaved into fully active doxorubicin by leucine aminopeptidases also overexpressed in tumour cells.[9,10]

Here, we report the results of the characterisation, purification and subsequent identification of thimet oligopeptidase (EC 3.4.24.15), a thiol-dependent metallo-oligopeptidase,[11] as an enzyme mediating the key initial step of extracellular tumour-selective activation of CPI-0004Na. Another enzyme could however also be involved in the activation of CPI-0004Na. Indeed, work conducted in parallel with this study, and recently published,[12] showed that CD10, a membrane-bound metallopeptidase also known as neprilysin, is able to cleave CPI-0004Na directly into *N*-L-leucyl-doxorubicin.

2. Materials and methods

2.1. Materials

Doxorubicin was supplied by Meiji Seika Kaisha Ltd. (Tokyo, Japan), synthesis solvents and *O*-(7-azabenzotriazol-1-yl)-*N*,*N*,*N*′,*N*′-tetramethyluronium hexafluorophosphate (HATU) by Aldrich (Milwaukee, WI). All resins and amino acids were either supplied by ABI (Foster City, CA), Novabiochem (San Diego, CA), Advanced ChemTech (Louisville, KY), Peptide International (Louisville, KY), or SynPep (Dublin, CA). Super-Leu-Dox (*N*-β-alanyl-L-leucyl-L-alanyl-L-leucyl-doxorubicin; SLD), *N*-L-alanyl-L-leucyl-doxorubicin, CPI-0004Na (*N*-succinyl-β-alanyl-L-leucyl-L-alanyl-L-leucyl-doxorubicin) and all other doxorubicin derivatives were prepared by conjugation of fluorenylmethoxycarbonyl-protected peptides using HATU as the activation agent and subsequent deprotection using a 10–100-fold excess of piperidine.[8] *N*-[1(*R*,*S*)-Carboxy-3-phenylpropyl]-Ala-Ala-Phe-*p*-aminobenzoate (Cpp-AAF-*p*AB),[13] prepared as described in Orlowski and colleagues,[14] was a gift from Marc Glucksman and James Roberts (Mount Sinai School of Medicine, New York, NY). Aminoethylbenzenesulfonyl fluoride (AEBSF) was obtained from Calbiochem (Darmstadt, Germany), and the other peptidase inhibitors from Sigma (Bornem, Belgium; ethylenediaminetetraacetic acid (EDTA), E64, 1,10-phenanthroline, fumagillin, Pro-Ile), Bachem (Bubendorf, Switzerland; CA074), or Roche Molecular Biochemicals (Mannheim, Germany; pepstatin, leupeptin, aprotinin).

Matrix metallopeptidases (MMPs) MMP-1, MMP-3, MMP-8, MMP-9, cathepsins B, D, G, H, L, human elastase, renin and prostate-specific antigen (PSA) were obtained from Calbiochem (Darmstadt, Germany), MMP-14, MMP-15 and MMP-16 from Chemicon International (Temecula, CA), and papain, chymotrypsin and porcine elastase were from Worthington Biochemical (Lakewood, NJ). Recombinant rat testis thimet oligopeptidase (rR-TOP) expressed and purified from *E. coli*,[15] as well as the rabbit affinity-purified polyclonal anti-TOP antibody were kindly provided by Marc Glucksman and James Roberts (Mount Sinai School of Medicine, New York, NY). The anti-TOP antibody was generated using a synthetic TOP peptide and did not cross-react with neurolysin, another intracellular metallopeptidase often copurified with TOP.[11]

2.2. Methods

2.2.1. Cell culture

The HeLa human cervical adenocarcinoma cell line was cultured at the Computer Cell Culture Center (Seneffe, Belgium) in MEM Eagle medium (Life Technologies, Inc., Rockville, MD) supplemented with 10% foetal calf serum v/v (FCS; Life Technologies, Inc.). The MCF-7/6 human breast cancer cell line was a gift of Professor M. M. Mareel (University of Ghent, Belgium) and was cultured in RPMI-1640 medium (Life Technologies, Inc.) supplemented with 10% FCS v/v. MCF-7/6 cell homogenate was prepared by ultrasonication of cells collected from subconfluent cultures in a 0.1 M, pH 7.2, triethanolamine–HCl buffer. Conditioned media were prepared by incubating subconfluent cultures for 24 h in a serum-free medium.[1,2] After removal of cells and debris by centrifugation (300*g* for 5 min at 4 °C), the conditioned medium was buffered to pH 7.2, using 50 mM tris(hydroxymethyl) aminomethane (Tris)-HCl and concentrated 20-fold by ultrafiltration with an Amicon 8200 stirred cell equipped with a YM10 membrane. The concentrate was used immediately.

2.2.2. CPI-0004Na cleavage by commercial enzymes

CPI-0004Na solutions (10 μM) were incubated (10 min at 37 °C) with different enzymes (MMP-1, MMP-3, MMP-8, MMP-9, MMP-14, MMP-15, MMP-16, cathepsin B, cathepsin D, cathepsin G, cathepsin H, cathepsin L, porcine elastase, human elastase, PSA, papain, chymotrypsin, renin) as recommended by the manufacturer (buffer and concentration). The reaction was stopped by the addition of acetonitrile and CPI-0004Na conversion was quantified by high performance liquid chromatography (HPLC) analysis. As activity control, enzymes were tested at experimental conditions using universal protease substrate (UPS; resofurin labeled casein; Roche Molecular Biochemicals, Mannheim, Germany).

2.2.3. *Inhibition studies*

MCF-7/6 cell homogenates or conditioned media, or HeLa cell fraction F1, were incubated with CPI-0004Na, *N*-L-alanyl-L-leucyl-doxorubicin or SLD, as indicated, at 17.2 μM at 37 °C, in the presence or absence of the different inhibitors tested at the maximal concentration recommended by the manufacturer. Substrate and product quantification were performed by HPLC as described previously.[2]

2.2.4. *Effect of metal ions*

MCF-7/6 cell homogenates or conditioned media were pre-treated with 1.3 mM EDTA for 2 h at 4 °C. EDTA was removed by desalting on PD-10 size exclusion columns (Amersham-Pharmacia Biotech, Roosendaal, The Netherlands), and the samples were then incubated for 2 h at 37 °C with SLD as the substrate and increasing concentrations (0.1 μM to 1 mM) of $CoCl_2$, $CuCl_2$, $MnCl_2$ or $ZnCl_2$. Substrate and product quantification were performed by HPLC as described previously.[2]

2.2.5. *Molecular weight and isoelectric point determination*

The molecular weight of enzyme X from MCF-7/6 cell homogenates was estimated by gel filtration chromatography (Superose S12, 10 × 300 mM column; Amersham-Pharmacia Biotech, Roosendaal, The Netherlands) with 0.1 M, pH 7.2, triethanolamine–HCl buffer as eluent.

Chromatofocusing (Mono P HR 5/5, 5 × 40 mM column; Amersham-Pharmacia Biotech, Roosendaal, The Netherlands) was used to determine enzyme X's isoelectric point according to manufacturer's instructions.

Enzyme X activity in fractions collected from gel filtration and chromatofocussing was determined after dilution in 0.1 M, pH 7.2, triethanolamine–HCl buffer and using SLD as the substrate and the HPLC method described previously.[2]

2.2.6. *Determination of pH optimum*

The pH optimum of enzyme X activity from MCF-7/6 conditioned medium was determined after buffering samples with 50 mM borate (pH 8–11), 50 mM 3,3-dimethylglutaric acid–NaOH (pH 5–7.4) or 50 mM triethanolamine–HCl (pH 7–9). SLD was used as the substrate and incubation time was 1 h at 37 °C. Substrate and product quantification were performed by HPLC as described previously.[2]

2.2.7. *Purification of initial cleavage enzyme from HeLa cells*

HeLa cells (50×10^9) were homogenised with a sonicator and Dounce homogeniser in a total of 180 ml of lysis solution consisting of 0.02% Triton X-100 w/v, 0.04% sodium azide w/v, and 4 Complete™ EDTA-free proteinase inhibitor cocktail tablets (Roche Molecular Biochemicals, Mannheim, Germany). The homogenate was centrifuged (30 min at 5000*g* and 4 °C) and the pellet was re-homogenised and centrifuged in the same conditions. The supernatants were combined and centrifuged (90 min at 145,000*g* and 4 °C). The homogenate was portioned into 6 samples corresponding to ~350 mg of protein (Protein Assay, BioRad, Nazareth Eke, Belgium) that were successively applied to a 2.6 × 9.4 cm Source™ 15Q (Amersham Pharmacia Biotech, Roosendaal, The Netherlands) equilibrated with a 20 mM, pH 7.2, triethanolamine–HCl buffer containing 0.01% Triton X-100 w/v, 0.02% sodium azide w/v and 50 μM $CoCl_2$ (equilibration buffer). After washing, proteins were eluted with a linear 0–0.5 M NaCl gradient in equilibration buffer. Fractions were collected and assayed for enzyme activity using SLD as the substrate. Activity-containing samples were pooled (fraction 1; F1) and supplemented with complete™ EDTA-free proteinase inhibitor cocktail tablets (Roche Molecular Biochemicals, Mannheim, Germany).

Twenty milligrams of protein from F1 (pre-treated with 45 mM EDTA) were loaded onto a 12 × 150 mM Chelating-Sepharose (Amersham Pharmacia Biotech, Roosendaal, The Netherlands) column saturated with cobalt. After washing, the proteins were eluted with a 600 ml of 0–0.2 M imidazole gradient in a 20 mM phosphate, 0.01% Triton X-100 w/v, 0.02% sodium azide w/v, 0.5 M NaCl, pH 7.2 buffer. The active fractions were pooled, concentrated by ultrafiltration (molecular weight cut-off: 10,000) and diluted (1:1) with electrophoresis sample buffer (0.12 M Tris–HCl, 5% glycerol w/v, 0.01% bromophenol blue w/v, pH 6.8). This sample was then fractionated by preparative native polyacrylamide gel electrophoresis. A Model 491 PrepCell (BioRad, Nazareth Eke, Belgium) was used with a 37 × 120 mM, 7% T, 2.6% C resolving gel buffered with 0.37 M Tris–HCl, pH 8.8, and a 37 × 5 mM, 4% T, 2.6% C concentrating gel buffered with 0.12 M Tris–HCl, pH 6.8. The electrode buffer was 25 mM Tris, 192 mM glycine, pH 8.3, and the elution buffer 100 mM triethanolamine, 0.01% Triton X-100 w/v, 50 μM $CoCl_2$, pH 7.2. After 30 min at 30 mA, separation was performed for 24 h at 40 mA. Fractions were collected at a flow rate of 0.4 ml/min. The active fractions (~150 mg of protein) were pooled, concentrated by ultrafiltration (molecular weight cut-off: 10,000) and were applied to a gel filtration HPLC column (TosoHaas TSK G3000SWXL, 7.8 × 600 mM) eluted at 0.3 ml/min with a pH 7.0, 50 mM phosphate buffer containing 0.2 M K_2SO_4. Fractions were collected and the active fractions were stored at −80 °C.

2.2.8. *Sample preparation for mass spectrometry*

Purified HeLa initial cleavage enzyme recovered from gel filtration HPLC was lyophilised overnight, dissolved in 30 μl electrophoresis sample buffer, heated at 90 °C for 1 min, centrifuged to remove any insoluble material and loaded on a sodium dodecyl sulphate (SDS) polyacrylamide gel. Running conditions were 30 mA, 300 V for 45 min. After staining with Coomassie blue R250, two bands (A and B) were detected. A 2 × 2 mM piece of each band was transferred into small tubes and washed in 15 min steps under agitation with each of the following solutions: 25 mM ammonium bicarbonate, 50% acetonitrile v/v in water, 25 mM ammonium bicarbonate. The samples were then dried in a centrifugal concentrator (Speedvac) before incubation 3 h at 37 °C with 10 μl of a 25 mM ammonium bicarbonate solution containing 0.5 mg trypsin. The trypsin digest was extracted with acetonitrile, dried in a centrifugal vacuum concentrator (Speedvac) and purified with a ZipTip™ C18 microextraction device.

2.2.9. *Matrix-assisted laser desorbtion ionisation-time of flight (MALDI-tof) and nanospray-mass spectrometry (MS)*

Mass spectra of the tryptic digests were acquired on a Biflex (Bruker-Daltonik, Bremen, Germany) MALDI-tof mass spectrometer equipped with delayed extraction operated in the reflector mode. The peptide mass fingerprint obtained for

each digest was matched to predicted digest patterns from known protein sequences using MS-Fit (http://prospector.ucsf.edu). Electrospray ionisation (ESI) quadrupole-time of flight (Q-tof) tandem mass spectrometry was performed using a Q-tof instrument (Micromass Ltd., Manchester, United Kingdom) with a Z-spray ion source working in the nanospray mode. MS/MS spectra were transformed using MaxEnt3 (MassLynx, Micromass Ltd.) and amino acid sequences were determined using PepSeq (Biolynx, Micromass Ltd.).

2.2.10. *Immunoprecipitation*

Five microliters of test samples was incubated 1 h at 4 °C with 10 μl 1:250 diluted anti-TOP or irrelevant affinity purified rabbit IgG in pH 7.2, 10 mM hydroxyethylpiperazine (HEPES), 150 mM NaCl solution. This mixture was added to 15 μl Protein A Sepharose (Amersham Pharmacia Biotech, Roosendaal, The Netherlands) equilibrated in the same buffer and incubated at 4 °C for 1 h. After centrifugation, residual enzyme activity was determined in the supernatant by incubation at 37 °C for 2 h with 17.24 μM CPI-0004Na as the substrate in pH 7.2, 20 mM HEPES buffer with 100 mM $MnCl_2$. The reaction was stopped by adding three volumes of acetonitrile. Precipitated protein was removed by centrifugation and the supernatant was diluted into three volumes of water and analysed by HPLC as described above for cleavage by commercial enzymes.

2.2.11. *Hydrolysis of different doxorubicin-linked substrates*

To compare hydrolysis rates of the different doxorubicin-linked substrates, test solutions were also incubated at 37 °C for up to 2 h with 17.24 μM substrate in pH 7.2, 20 mM HEPES buffer with 100 mM $MnCl_2$. The post-incubation samples were analysed as described above for immunoprecipitation supernatants.

2.2.12. *Thiol activation*

MCF-7/6 conditioned medium was pre-incubated for 30 min at room temperature with dithiothreitol (DTT). CPI-0004Na or *N*-L-alanyl-L-leucyl-doxorubicin (17.24 μM) was added before incubation at 37 °C. Hydrolysis products were extracted and analysed by HPLC as described previously.[1]

3. Results

3.1. *Initial characterisation of enzyme X activity*

Hydrolysis of CPI-0004Na was initially assessed with 18 commercial peptidases – many of them potentially cancer-related (see Section 2.2 for list) – in conditions (buffer, enzyme and substrate concentrations) appropriate for hydrolysis of the standard substrate used by the manufacturer to determine enzyme activity. However, no CPI-0004Na cleavage was observed with any of these enzymes although only 3 (cathepsin B, cathepsin L, and PSA) out of 15 proved inactive when similarly tested with the universal protease substrate as a control.

In order to determine the peptidase subclass of which enzyme X is a member, inhibition experiments were performed with MCF-7/6 breast cancer cell homogenates. Cleavage of CPI-0004Na into *N*-L-leucyl-doxorubicin (enzyme X activity) was almost 90% inhibited by 1 mM EDTA or 2 mM 1,10-phenanthroline treatment, but all other inhibitors tested (50 μM AEBSF, 4 μg/ml aprotinin, 20 μM E-64, 1.5 μM pepstatin, 1 μM CA074, 1 μM fumagillin, or 15 μM leupeptin) had no effect. On the other hand, EDTA and 1,10-phenantroline similarly inhibited CPI-0004Na cleavage by MCF-7/6 cell conditioned media but were totally unable to inhibit cleavage of *N*-L-alanyl-L-leucyl-doxorubicin into *N*-L-leucyl-doxorubicin by the same media. Based on these results, the enzyme mediating the initial step of extracellular CPI-0004Na activation was speculated to be a metallopeptidase and reactivation by divalent cations was studied. Following EDTA treatment of conditioned media or cell homogenates, reactivation of CPI-0004Na cleavage activity could be achieved by the addition of Co^{2+} or Mn^{2+} (50 μM). Zn^{2+} or Cu^{2+} at the same concentration did not allow to recover activity (Fig. 1). Co^{2+} concentrations higher than 100 μM led only to partial reactivation.

Enzyme X activity from MCF-7/6 cell homogenates was found to have an apparent molecular weight of about 70 kDa and an isoelectric point of 5.2. Its pH of optimum activity was found to be close to neutrality (7.2–7.7).

3.2. *Structural characterisation of initial cleavage peptidase*

Since no known enzyme capable of activating CPI-0004Na could be found, it was decided to purify and identify the main prodrug-activating peptidase (i.e. the one mediating initial cleavage into *N*-L-alanyl-L-leucyl-doxorubicin) from human cancer cells. A starting material likely to be obtained easily in large amounts was needed and HeLa cells were selected following confirmation of the presence in lysates of CPI-0004Na-activating peptidase similar in its major characteristics to that of MCF-7/6 cells (inhibition by EDTA and 1,10-phenanthroline, reactivation by Co^{2+} and Mn^{2+}, neutral pH optimum). The CPI-0004Na cleavage activity was purified from HeLa cell homogenates as described in Section 2. Analysis of fractions from the first, ion-exchange, chromatography step definitely confirmed a two-step process, involving two different enzymes, for CPI-0004Na activation into *N*-L-leucyl-doxorubicin. Indeed, fraction analysis using CPI-0004Na as the substrate revealed a single chromatographic peak corresponding to a peptidase yielding *N*-L-alanyl-L-leucyl-doxorubicin as the sole product. Subsequent re-analysis of all fractions using *N*-L-alanyl-L-leucyl-doxorubicin as the substrate revealed a separate peak eluting later and corresponding to a peptidase generating *N*-L-leucyl-doxorubicin. The first peak, corresponding to the activity of interest (rate-limiting step in CPI-0004Na activation), was called fraction 1 or F1 and further purified using Co^{2+}-chelation affinity chromatography, preparative polyacrylamide gel electrophoresis and finally gel filtration HPLC.

Progressive enrichment in initial cleavage activity (Fig. 2) led to a sample containing two major protein bands with apparent molecular weights of 74 and 63 kDa, respectively (Fig. 2, Lane 5). Both bands were excised from a SDS gel, digested with trypsin and fragments were analysed by MALDI-tof mass spectrometry. The peptide mass fingerprint obtained with the 74 kDa band digest was compared to predicted trypsin digests results of known protein sequences. The best

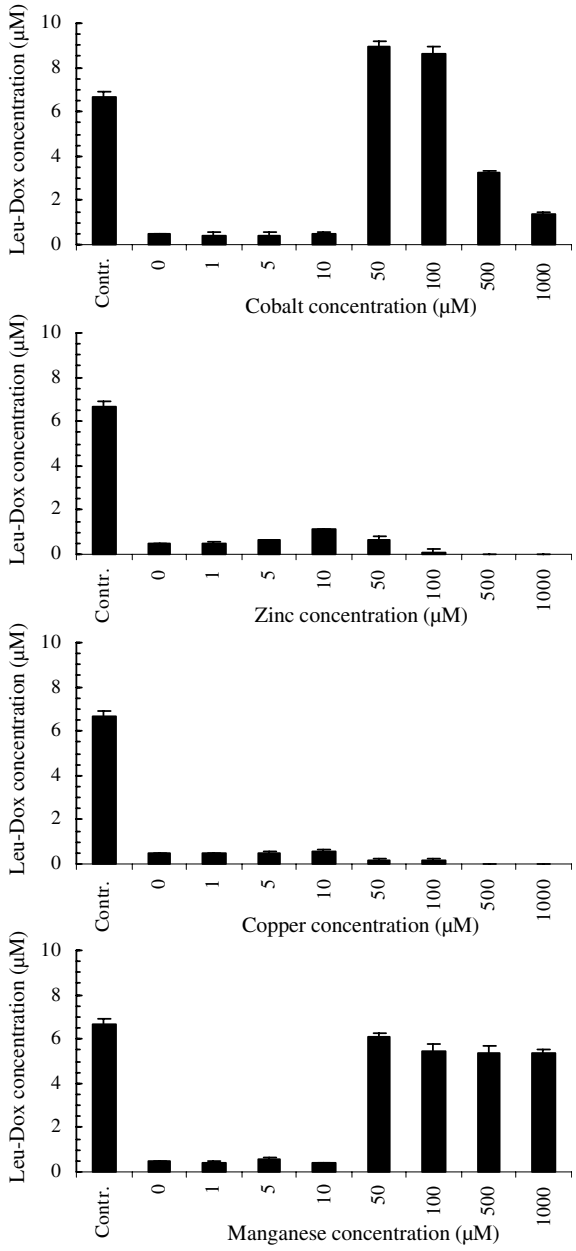

Fig. 1 – Reactivation of ethylenediaminetetraacetic acid (EDTA)-inactivated enzyme X from MCF-7/6 breast cancer cell homogenates. MCF-7/6 cell homogenates were pre-treated with 1.3 mM EDTA for 2 h at 4 °C. EDTA was then removed by gel filtration chromatography and the samples were incubated 2 h at 37 °C with *N*-β-alanyl-L-leucyl-L-alanyl-L-leucyl-doxorubicin as the substrate and increasing concentrations of Co^{2+}, Zn^{2+}, Cu^{2+} or Mn^{2+}(chloride salts). Residual substrate and product quantifications were performed using high performance liquid chromatography as detailed in Section 2. Contr., corresponding homogenate activity prior to EDTA treatment; Leu-Dox, *N*-L-leucyl-doxorubicin.

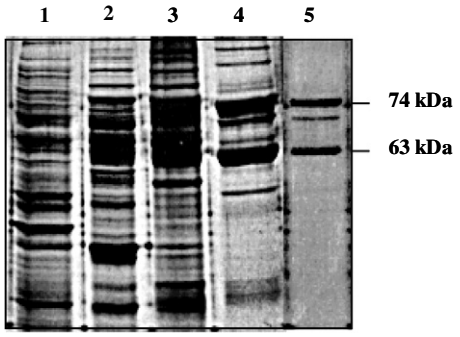

Fig. 2 – Sodium dodecyl sulphate-polyacrylamide gel electrophoresis analysis (silver staining) of the different steps of the purification of HeLa cell initial cleavage enzyme. The HeLa cell enzyme responsible for cleavage of CPI-0004Na into *N*-L-alanyl-L-leucyl-doxorubicin was purified from cell homogenates (lane 1, ultracentrifugation supernatant). Activity-containing fractions were identified, pooled and used for the next step as described in Section 2. Pooled fractions from the successive steps are presented: lane 2, ion-exchange chromatography; lane 3, cobalt-chelating affinity chromatography; lane 4, preparative electrophoresis; lane 5, high performance gel filtration chromatography. The pool collected from gel filtration chromatography was used for matrix-assisted laser desorption ionisation-time of flight mass spectrometry analysis of tryptic fragments following in-gel digestion.

score was obtained for thimet oligopeptidase (TOP, EC 3.4.24.15) with 20 fragments corresponding to predicted mass, which accounts for 33% of the known human TOP sequence.[16]

MALDI-tof analysis of the 63 kDa band trypsin digest indicated that it shares the same sequence but with the absence of a small portion of the carboxy-terminus.

To further confirm this result, two of the HeLa cell 74 kDa band tryptic fragments were sequenced by electrospray ionisation-quadrupole-time of flight (ESI-Q-tof) tandem mass spectrometry. Both fragments analysed (A[I/L]ADVEVTYTVQR, *m/z* 1464.65 and WDLSAQQIEER, *m/z* 1374.43) completely matched (100% identity) the known sequence of human TOP (residues 66–78 and 25–35, respectively).[16] Homology to rat and pig TOP's corresponding sequences ranges from 73% to 100%.

Western immunoblots performed with an anti-TOP antibody stained both the 74 and 63 kDa bands in HeLa cell homogenates, and only a 74 kDa band in homogenates of MCF-7/6 or MDA-MB-231 breast cancer cells (not shown). Immunoprecipitation of partially purified HeLa cell homogenate (F1) with the same antibody completely removed CPI-0004Na-cleavage activity from the solution, as in the case of rR-TOP. In HeLa cell total homogenates, enzyme X activity was 71% reduced by immunoprecipitation with the anti-TOP antibody. Immunoprecipitation of F1, rR-TOP or HeLa cell homogenates in the same conditions but with an irrelevant antibody had no effect on enzyme X activity.

3.3. *Functional evidence of TOP involvement in CPI-0004Na activation*

Substrate specificity of partially purified HeLa cell homogenate (F1) was compared to that of rR-TOP. Eight peptidyl

conjugates were prepared and conjugated to doxorubicin before being used as substrates in comparison with CPI-0004Na. As shown in Table 1, all eight conjugates and CPI-0004Na were found to have essentially the same rate of hydrolysis with HeLa cell fraction F1 and rR-TOP. In all cases, the product was X–Y–doxorubicin.

The compound *N*-[1-(*R*,*S*)-carboxypropyl]-Ala-Ala-Phe-*p*-aminobenzoate is a selective inhibitor of TOP and of the closely related metallopeptidase neurolysin.[17] At the concentration of 3 μM, this compound completely inhibited CPI-0004Na hydrolysis by HeLa cell fraction F1. On the other hand, the dipeptide Pro-Ile, a selective inhibitor of neurolysin,[18] had no effect at the recommended concentration of 5 mM.

For a metallopeptidase, a unique characteristic of TOP is the fact that it is activated by low levels of thiol reducing agents such as 50 μM DTT or 1 mM 2-mercaptoethanol, but inhibited by higher concentrations of the same agents such as 5 mM DTT.[19,20] MCF-7/6 cell conditioned media were therefore pre-treated or not with increasing concentrations of DTT. These experiments indicated a maximal 10-fold activation of CPI-0004Na hydrolysis at the concentration of 1 mM, higher concentrations were ineffective (Fig. 3). It should be noted that the product observed is *N*-L-leucyl-doxorubicin rather than *N*-L-alanyl-L-leucyl-doxorubicin as in all experiments performed with crude material that also contains exopeptidases able to remove the terminal alanine residue of TOP's cleavage product. However, no effect of DTT is observed on the hydrolysis of *N*-L-alanyl-L-leucyl-doxorubicin by the same conditioned media.

Table 1 – Digestion rate (relative to CPI-0004Na) of different peptidyl conjugates of doxorubicin by HeLa cell fraction F1 and recombinant rat thimet oligopeptidase (rR-TOP)

Substrate[a]	HeLa cell fraction F1	rR-TOP
Succ-β-Ala-Leu-Ala-Leu-Dox[b]	1.0	1.0
Succ-β-Ala-Ile-Ala-Leu-Dox	0.025	<0.03
Succ-Leu-Ala-Leu-Dox	0.64	0.66
Succ-Met-Ala-Leu-Dox	0.41	0.97
Succ-Leu-Tyr-Leu-Dox	0	0
Succ-Ile-Ala-Leu-Dox	0	0
Succ-Leu-Ala-Gly-Dox	0.40	0.43
Succ-Leu-NmAla-Leu-Dox	0	0
Succ-Ile-NmAla-Leu-Dox	0	0

Test solutions were incubated up to 2 h with 17.24 μM substrate in a 20 mM hydroxyethylpiperazine, 100 mM $MnCl_2$ buffer pH 7.2. The reaction was stopped by the addition of acetonitrile and the supernatant was analysed by high performance liquid chromatography as detailed in Section 2.
a All optically active amino acids are from the L series: Succ, succinyl; Dox, doxorubicin; Nm, *N*-methyl.
b CPI-0004Na.

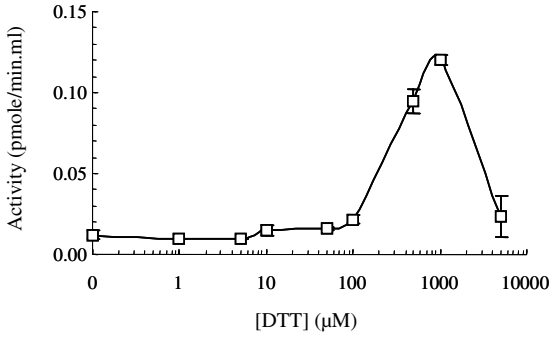

Fig. 3 – Effect of dithiothreitol (DTT) on the activation of CPI-0004Na by MCF-7/6 breast cancer cell conditioned media. MCF-7/6 cell conditioned medium was pre-incubated 30 min at room temperature with increasing concentrations of DTT. In both cases, CPI-0004Na was added (17.2 μM) before incubation at 37 °C. The samples were then analysed by high performance liquid chromatography as described in Section 2. □, *N*-L-leucyl-doxorubicin.

4. Discussion

CPI-0004Na, an extracellularly tumour-activated prodrug of doxorubicin, is the result of studies that began with in vitro screening of several oligopeptidic derivatives of doxorubicin (known to be unable to permeate cells) for their stability in whole human blood as well as for their capacity to be cleaved into *N*-L-leucyl-doxorubicin by MCF-7/6 breast cancer cell conditioned media.[1,2,8] The β-alanyl-L-leucyl-L-alanyl-L-leucine tetrapeptide allowing stability of the corresponding doxorubicin conjugate in blood and its extracellular activation by cancer cells was therefore not selected on the basis of the substrate specificity of any known tumour-associated peptidase. Ignoring the identity of the activating peptidase(s) we progressively gathered in vitro[2] and then in vivo[1] evidence that the activation of CPI-0004Na could be a two-step process, the limiting one being initial cleavage into *N*-L-alanyl-L-leucyl-doxorubicin. In view of the characterisation and identification of the enzyme responsible for this initial activation step, certain commercially available peptidases, including most of those known to be associated to the malignant phenotype (such as matrix metallopeptidases or cathepsins[21,22]), were tested for their ability to cleave CPI-0004Na. Since the results were totally negative in spite of positive controls, the CPI-0004Na activating peptidase was thought to be either unknown or a peptidase not known to be related to cancer. The enzyme has all the characteristics of a metallopeptidase, being inhibited by EDTA and 1,10-phenanthroline and reactivated by Co^{2+} or Mn^{2+} ions.[23] Preliminary studies of the MCF-7/6 cell extract hydrolytic activity indicated an enzyme with molecular weight close to 70 kDa, an isoelectric point of about 5.2, and maximum activity at neutral pH.

Purification proved impossible from MCF-7/6 cell conditioned media or homogenates, the availability of starting material being too restricted. Therefore, homogenates of HeLa cells grown in suspension were selected because they contained a CPI-0004Na cleavage activity similar in its major features (inhibition, reactivation, pH optimum, etc.) to that of MCF-7/6 cells. The mediation of the two steps of CPI-0004Na activation by two different enzymes was confirmed when they were separated by ion-exchange chromatography. Further purification of the initial cleavage enzyme yielded a final sample characterised by two major bands of 74 and 63 kDa in SDS–polyacrylamide gel electrophoresis. Enough material was produced for identification by mass spectrome-

try following in-gel trypsin digestion. Comparison of the peptide mass fingerprint of the 74 kDa band with predicted patterns of known proteins using MS-Fit indicated homology of 20 fragments to human thimet oligopeptidase (TOP), covering 33% of its entire sequence and distributed over its full length.[16] MALDI-tof analysis of the 63 kDa band following trypsin digestion showed that it shares the same sequence with the exception of a missing part of the carboxy-terminus. A 63 kDa form of TOP has not been previously reported and it may be a proteolytic product formed during purification. The identification of the two purified bands with TOP was confirmed by ESI-Q-tof mass spectrometry sequencing of two tryptic fragments that perfectly match the published human sequence. Furthermore, bands of the same molecular weight (74 and 63 kDa) were observed in western immunoblots of HeLa cell fraction 1 when stained with anti-TOP antibodies.

TOP is a thiol-dependent metalloendopeptidase. It is a single chain, monomeric, non-glycosylated protein comprised of 687 amino acids.[24] The size (74 kDa) of the purified protein was well within the range of reported molecular weight for human,[16] rabbit,[25,26] rat[27] and chicken[19] TOP. It was also in good agreement with the initial estimation of 70 kDa made for enzyme X. Indeed, this value was determined by gel filtration chromatography and the difference can easily be explained by the inherent error of such molecular weight determinations. Similarly, the isoelectric point of enzyme X was initially estimated by chromatofocusing to be 5.2, a value which is similar to the reported isoelectric point of TOP (5.0 ± 0.2[27]). The same is also true for the pH optimum.[28]

More experiments were performed in order to support the identity of TOP and the initial CPI-0004Na cleavage enzyme. Substrate specificity of HeLa cell fraction 1 was found to be similar to that of recombinant rat TOP by testing nine different peptidyl conjugates of doxorubicin, including CPI-0004Na and non-substrates. The CPI-0004Na cleaving activity of HeLa cell fraction 1 could also be inhibited by an inhibitor of both TOP and neurolysin, but not by a specific inhibitor of the latter. Immunoprecipitation of HeLa cell fraction 1 or of HeLa cell total homogenates using a specific anti-TOP antibody resulted in removal of CPI-0004Na cleaving activity from solution. This removal was total in the case of HeLa cell fraction 1 but only 71% complete in the case of total cell homogenates indicating that other enzymes could be involved in CPI-0004Na activation. The role of TOP in CPI-0004Na activation was further supported by the observation that thiol treatment of MCF-7/6 cell conditioned media greatly enhanced the prodrug's cleavage rate, and thiol activation is a unique feature of TOP.[20]

The only apparent discrepancy between our characterisation of enzyme X and TOP characteristics is the fact that although it is also possible to reactivate EDTA-deactivated rat[29] or human[30] TOP with Zn^{2+}, such reactivation was not seen with the EDTA-treated MCF-7/6 cell enzyme. The specific methods used for EDTA treatment and removal could have affected the results, and the fact that concentrations of Zn^{2+} as low as 100 μM are inhibitory to TOP[29] could also be a factor.

Our results show that thimet oligopeptidase (TOP; EC 3.4.24.15), a thiol-dependent metallopeptidase, is a very likely candidate for the extracellular activation of the prodrug CPI-0004Na. TOP is found in tumour cell conditioned media, and our unpublished results indicate that this is not likely to be the result of leakage from dead cells because there is no correlation between the released levels and those of lactate dehydrogenase, a typical cytosolic enzyme. Although TOP has also been described as a mainly cytosolic enzyme, our results suggest that this peptidase is secreted by tumour cells, at least in vitro. TOP secretion was previously described in the case of glioma cells[31] or of corticotrope AtT-20 cells.[32] It was also found to be associated with the extracellular surface of these same AtT-20 cells.[33] Finally, and interestingly, it should also be noted that TOP could be particularly well suited for the in vivo activation of a prodrug such as CPI-0004Na since its activity is inhibited in oxygenated media such as blood and enhanced in mildly reducing and anoxic environments that are very often characteristic of solid tumours. Of course, the role actually played by TOP – as well as by CD10[12] – in the in vivo activation of CPI-0004Na remains to be formally demonstrated. CD10 cleavage activity (yielding directly N-L-leucyl-doxorubicin from CPI-0004Na; 12) was not detected as part of this work, likely because of the fact that CD10 is mainly a membrane-bound enzyme and that membranes were eliminated from the cell preparations used here. The relative importance of both peptidases (TOP and CD10) with regard to CPI-0004Na's activity will receive particular attention in future human studies.

Conflict of interest statement

None declared.

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REFERENCES

1. Dubois V, Dasnois L, Lebtahi K, et al. CPI-0004Na, a new extracellularly tumour-activated prodrug of doxorubicin: in vivo toxicity, activity, and tissue distribution confirm tumor cell selectivity. *Cancer Res* 2002;**62**(8):2327–31.
2. Trouet A, Passioukov A, Van derpoorten K, et al. Extracellularly tumor-activated prodrugs for the selective chemotherapy of cancer: application to doxorubicin and preliminary in vitro and in vivo studies. *Cancer Res* 2001;**61**:2843–6.
3. Kaufman DC, Chabner BA. Clinical strategies for cancer treatment: the role of drugs. In: Chabner BA, Longo DL, editors. *Cancer chemotherapy and biotherapy: principles and pratice*. Philadelphia (PA): Lippincott Williams and Wilkins; 2001. p. 1–16.
4. Robert J. Multidrug resistance and its reversal. General review of fundamental aspects. *Ann Biol Clin* 1996;**54**(1):3–8.
5. Koblinski JE, Ahram M, Sloane BF. Unraveling the role of proteases in cancer. *Clin Chim Acta* 2000;**291**:113–35.
6. Del Rosso M, Fibbi G, Pucci M, et al. Multiple pathways of cell invasion are regulated by families of serine proteases. *Clin Exp Metastasis* 2002;**19**(3):193–207.

7. De La Haba RJ, Macho A, Calzado MA, et al. Soluble dipeptidyl peptidase IV (CD26) in serum of patients with colorectal carcinoma. *Neoplasma* 2002;**49**(5):307–11.
8. Fernandez AM, Van derpoorten K, Dasnois L, et al. *N*-Succinyl-(beta-alanyl-L-leucyl-L-alanyl-L-leucyl)doxorubicin: an extracellularly tumour-activated prodrug devoid of intravenous acute toxicity. *J Med Chem* 2001;**44**(22): 3750–3.
9. Canal P, Robert J, Ramon M, et al. Human pharmacokinetics of *N*-L-leucyl-doxorubicin, a new anthracyclin derivative, and its correlation with clinical toxicities. *Clin Pharmacol Ther* 1992;**51**(3):249–59.
10. Breistøl K, Hendriks HR, Fodstad Ø. Superior therapeutic efficacy of *N*-leucyl-doxorubicin versus Doxorubicin in human melanoma xenografts correlates with higher tumour concentrations of free drug. *Eur J Cancer* 1999;**35**(7):1143–9.
11. Barrett AJ, Chen JM. Thimet oligopeptidase. In: Barrett AJ, Rawlings ND, Wooessner JF, editors. *Handbook of proteolytic enzymes*. San Diego (CA): Academic Press; 1998. p. 1108–12.
12. Pan C, Cardarelli PM, Nieder MH, et al. CD10 is a key enzyme involved in the activation of tumor-activated peptide prodrug CPI-0004Na and novel analogues: implications for the design of novel peptide prodrugs for the therapy of $CD10^+$ tumors. *Cancer Res* 2003;**63**:5526–31.
13. Knight CG, Barrett AJ. Structure/function relationships in the inhibition of thimet oligopeptidase by carboxyphenylpropyl-peptides. *FEBS Lett* 1991;**294**(3):183–6.
14. Orlowski M, Michaud C, Molineaux CJ. Substrate-related potent inhibitors of brain metalloendopeptidase. *Biochemistry* 1988;**27**(2):597–602.
15. Mc Kie N, Dando PM, Brown MA, Barrett AJ. Rat thimet oligopeptidase: large scale expression in *Escherichia coli* and characterization of recombinant enzyme. *Biochem J* 1995;**309**:203–7.
16. Thompson A, Huber G, Malherbe P. Cloning and functional expression of a metalloendopeptidase from human brain with the ability to cleave a beta-APP substrate peptide. *Biochem Biophys Res Commun* 1995;**213**(1):66–73.
17. Serizawa A, Dando PM, Barrett AJ. Characterization of a mitochondrial metallopeptidase reveals neurolysin as a homologue of thimet oligopeptidase. *J Biol Chem* 1995;**270**(5):2092–8.
18. Dauch P, Vincent JP, Checler F. Specific inhibition of endopeptidase 24.16 by dipeptides. *Eur J Biochem* 1991;**202**:269–76.
19. Barrett AJ, Brown MA. Chicken liver Pz-peptidase, a thiol-dependent metallo-endopeptidase. *Biochem J* 1990;**271**:701–6.
20. Shrimpson CN, Glucksman MJ, Lew RA, et al. Thiol activation of endopeptidase EC 3.4.24.15. *J Biol Chem* 1997;**272**(28):17395–9.
21. Nelson AR, Fingleton B, Rothenberg ML, Matrisian LM. Matrix-metalloproteinases: biologic activity and clinical implications. *J Clin Oncol* 2000;**18**(5):1135–49.
22. Rochefort H, Garcia M, Glondu M, et al. Cathepsin D in breast cancer: mechanisms and clinical applications, a 1999 overview. *Clin Chim Acta* 2000;**291**:157–70.
23. Auld DS. Removal and replacement of metal ions in metallopeptidases. In: Barrett AJ, editor. *Methods in enzymology*, Vol. 248. San Diego (CA): Academic Press; 1995. p. 228–42.
24. Mc Kie N, Dando PM, Rawlings ND, Barrett AJ. Thimet oligopeptidase: similarity to 'soluble angiotensin II-binding protein' and some corrections to the published amino-acid sequence of the rat testis enzyme. *Biochem J* 1993;**295**:57–60.
25. Kawabata S, Nakagawa K, Muta T, Iwanaga S, Davie EW. Rabbit liver microsomal endopeptidase with specificity for processing proproteins is structurally related to rat testes metalloendopeptidase 24.15. *J Biol Chem* 1993;**268**(17):12498–503.
26. Hayashi MA, Gomes MD, Reboucas NA, Fernandes BL, Ferro AC, Camargo AC. Species specificities of thimet oligopeptidase. *Biol Chem Hoppe Seyler* 1996;**377**(5):283–91.
27. Pierroti A, Dony KW, Glucksman MJ, Orlowski M, Roberts JL. Molecular cloning and primary structure of rat testes metalloendopetidase EC 3.2.24.15. *Biochemistry* 1990;**29**:10323–9.
28. Barrett AJ, Molly AB, Dando PM, et al. Thimet oligopeptidase and oligopeptidase M or Neurolysin. In: Barrett AJ, editor. *Methods in enzymology*, vol. 248. San Diego (CA): Academic Press; 1995. p. 529–56.
29. Tisljar U, Barrett AJ. A distinct thimet peptidase from rat liver mitochondria. *FEBS Lett* 1990;**4**(1):84–6.
30. Dando PM, Brown MA, Barrett AJ. Human thimet oligopeptidase. *Biochem J* 1993;**294**(Pt2):451–7.
31. Ferro ES, Tambourgi DV, Gobersztejn F, et al. Secretion of a neuropeptide-metabolizing enzyme similar to endopeptidase 22.19 by glioma C6 cells. *Biochem Biophys Res Commun* 1993;**191**(1):275–81.
32. Ferro ES, Tullai JW, Glucksman MJ, Roberts JL. Secretion of metalloendopeptidase 24.15 (EC 3.2.24.15). *DNA Cell Biol* 1999;**18**(10):781–9.
33. Crack PJ, Wu TJ, Cummins PM, et al. The association of metalloendopeptidase EC 3.4.24.15 at the extracellular surface of the AtT-20 cell plasma membrane. *Brain Res* 1999;**835**:113–24.