# Human and murine macrophages mediate activation of MEN 4901/T-0128: a new promising camptothecin analogue–polysaccharide conjugate

Monica Binaschi, Massimo Parlani, Daniela Bellarosa, Mario Bigioni, Carmela Salvatore, Carla Palma, Attilio Crea, Carlo Alberto Maggi, Stefano Manzini and Cristina Goso

**MEN 4901/T-0128 is a new cytotoxic prodrug constituted by the camptothecin analogue T-2513 bound to carboxymethyl dextran through a triglycine linker. MEN 4901/T-0128 was designed to target the active camptothecin at the tumour site. MEN 4901/T-0128 is weakly cytotoxic *in vitro* and thus T-2513 must be released from the conjugate to become active. Here, we demonstrated that human purified cathepsin B releases T-2513 from MEN 4901/T-0128 at pH values ranging from 3 to 5. pH dependency of this reaction suggests that cleavage of the linker should mainly occur in the lysosomes. As elevated cathepsin B activity has been described in macrophages, human tumour monocytic THP-1 cells differentiated into macrophage-like cells were used to study the cellular mechanisms responsible for MEN 4901/T-0128 antitumour activity. Here, we show that differentiated THP-1 internalizes MEN 4901/T-0128 efficiently in a time-dependent and concentration-dependent manner. After phagocytosis, THP-1 cells can cleave the prodrug and release T-2513 in the media. On the contrary, undifferentiated THP-1 cells or pancreatic ASPC-1 tumour cells, although expressing high levels of cathepsin B, are much less efficient in the release of cytotoxic moieties in the culture media. Moreover, normal murine macrophages, recovered from the peritoneal cavity or from the spleen, when activated (*in vitro* by 100 ng/ml phorbol 12-myristate-13-acetate and *in vivo* by 300 µl of 3% w/v thioglycollate solution), were able to release (after incubation with 10 µg/ml MEN 4901/T-0128) cytotoxic moieties in the culture supernatant, in an amount sufficient to kill human carcinoma A2780 cells. Thus, we suggest that tumour-associated macrophages may play a key role in the uptake of MEN 4901/T-0128, cleavage and local release of active moiety T-2513. This mechanism should support a tumour targeting of the cytotoxic moieties, allowing an improved antitumour efficacy/safety ratio for MEN 4901/T-0128. *Anti-Cancer Drugs* 17:1119–1126**


**Anti-Cancer Drugs** 2006, **17**:1119–1126

**Keywords: cathepsin B, macrophages, MEN 4901/T-0128, phagocytosis, tumour targeting**

Menarini Ricerche Pomezia, Italy.

Correspondence to Dr Monica Binaschi, PhD, Menarini Ricerche-Pharmacology Department, Via Tito Speri 10, 00040 Pomezia, Rome, Italy.
Tel: +390691184485; fax: +39069100220;
e-mail: mbinaschi@menarini-ricerche.it

**Received** 8 May 2006 **Revised form accepted** 19 July 2006

## Introduction

Camptothecin (CPT) is a plant alkaloid, isolated from the Chinese tree *Camptotheca acuminata*. Although CPT has good antitumour activity in preclinical models, its clinical development was discontinued due to toxicity [1,2]. Several alternative strategies are currently used to improve the therapeutic index of this class of compounds. A possible way is the use of the active moiety attached to water-soluble macromolecules. This strategy can lead to increase in solubility, reduction in systemic toxicity, alteration in biological distributions and possible improvement in therapeutic index. MEN 4901/T-0128 is a new CPT analogue–polysaccharide conjugate prodrug composed of a CPT analogue (T-2513) and carboxymethyl dextran (CM-dextran) linked through a triglycine spacer [3]. It is characterized by improved antitumour activity and tolerance in preclinical models [3]. *In-vitro* tests indicated that MEN 4901/T-0128 is inactive and the enzymatic release of its active moiety is necessary in order to display its potent cytotoxic activity.

Previous data suggested the ability of rat lysosomal cysteine-proteases (but not of other classes of proteinases) to release the active moiety from MEN 4901/T-0128 [4].

Several classes of cysteine-proteinases are known to be overexpressed by many tumour cells, including cathepsins [5]. In particular, cathepsin B has been investigated most extensively, owing to its critical role in tumour progression [6].

Thus, the aim of our work was the understanding of the exact mechanism responsible *in vitro* and *in vivo* for T-2513 release from MEN 4901/T-0128.

0959-4973 

## Materials and methods

### Materials

MEN 4901/T-0128, T-2513 and CM-dextran were obtained from Tanabe Seiyaku (Osaka, Japan). Stock solutions were dissolved in sterile distilled water and then appropriately diluted in complete culture medium before use. Phorbol 12-myristate-13-acetate (PMA), lipopolysaccharides (from *Escherichia coli* Sero-type 0111:B4) and nitroblue tetrazolium (NBT) were purchased from Sigma (St Louis, Missouri, USA). MTS [3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxy-phenyl)-2-(4-sulfophenyl)-2H-tetrazolium], inner salt was purchased from Promega (Madison, Wisconsin, USA). Stock solutions (3% w/v) of thioglycollate (Sigma-Aldrich) were prepared in NaCl 0.9% solution. Purified human liver cathepsin B, fluorogenic substrate Z-Arg-Arg-AMC and CA-074 Me {cathepsin B inhibitor IV; [L-3-*trans*-(polycarbamoyl)oxirane-2-carbonyl]-L-isoleucyl-L-proline methyl ester} were purchased from Calbiochem Novabiochem (Darmstadt, Germany). Anti-CD11b monoclonal antibody and isotypic control were purchased from Becton Dickinson (Mountain View, California, USA).

### Cell lines

Human monocytic leukaemia THP-1 and human ovarian carcinoma A2780 cell lines were purchased from the European Collection of Cell Culture (Salisbury, Wiltshire, UK). Human pancreatic tumour ASPC-1 cells were purchased from the American Type Culture Collection (Distribution by LGC Promochem, Milan, Italy). Cells were cultured in RPMI 1640 medium (Gibco, Glasgow, UK) containing 10% fetal bovine serum (FBS, Gibco) and glutamine 0.1% at 37°C in a humidified atmosphere of 5% $CO_2$ in air. Cell viability was checked by the ability of cells to exclude Trypan blue.

### Cathepsin B activity

All experiments were performed at 37°C. Human liver cathepsin B was prepared in 40 mmol/l acetate buffer (1 mmol/l ethylenediaminetetraacetic acid, 5 mmol/l reduced glutathione and 0.1% Triton X-100; pH 4.0–3.0) or phosphate buffer (1 mmol/l ethylenediaminetetraacetic acid, 5 mmol/l reduced glutathione and 0.1% Tween; pH 7.0–8.0). The enzymatic reaction (200 µl volume) was run in 96-well plates in the presence of 0.01 units/well cathepsin B and 20 µmol/l fluorogenic substrate Z-Arg-Arg-AMC. The release of fluorigenic substrate was measured using a Victor 1420 fluorescent plate reader with an excitation wavelength of 390 nm and an emission wavelength of 460 nm. The reaction was run for 60 min at 37°C with readings taken every 5 min. MEN 4901/T-0128 cleavage was assessed after incubation of 0.1 mg/ml MEN 4901/T-0128 with 5 units of cathepsin B for 24 h. The amounts of released T-2513 and Gly-T-2513 were determined using high-pressure liquid chromatography (HPLC) with LOQ = 200 ng/ml. To evaluate cathepsin B activity of cell lines, cells were resuspended at $3.2 \times 10^6$ cells/ml in PAB [Hank's balanced salt solution lacking sodium bicarbonate and containing 0.6 mmol/l $CaCl_2$, 0.6 mmol/l $MgCl_2$, 2 mmol/l L-cysteine and 25 mmol/l piperazine-1,4-bis(2-ethanesulphonic acid); pH 7.0]. The enzymatic reaction for cellular cathepsin B was run in 96-well black plates (320 000 cells/well) in the presence of 200 µmol/l Z-Arg-Arg-AMC for 60 min at 37°C with readings taken every 5 min. In all experiments, 0.1% Triton X-100 was added to liberate total cellular cathepsin B activity.

### Differentiation procedures of THP-1 cells

The monocytic human THP-1 cell line was maintained in culture medium (RPMI 1640 containing 10% fetal calf serum). To induce differentiation, $1.6 \times 10^6$ cells were seeded in 25-$cm^2$ flasks for 72 h at 37°C in the presence of 160 nmol/l PMA. Macrophage differentiation was monitored incubating cells for 30 min on ice with diluted monoclonal antibody anti-CD11–phycoerythrin or related isotypic controls. After washing, samples were acquired by flow cytometry (FACSORT; Becton Dickinson). The fluorescence intensity of 10 000 cells was collected on a logarithmic scale. The percentage of positive cells is indicated for each experimental condition.

### Nitroblue tetrazolium assay

Briefly, cells were seeded in Eppendorf tubes ($6 \times 10^4$ cells/100 µl RPMI) and NBT (0.12%) was added to each sample. After incubation at 37°C for 20 min, cells were pelleted and resuspended in 120 µl of KOH 2 mol/l. Cells were then transferred to clear flat-bottom 96-well plates and 140 µl dimethylsulfoxide was added. The absorbance at 450 nm was determined with a microplate reader (BioRad 3550; Bio-Rad Laboratories, Segrate, Milan, Italy). Moreover, to test the cell viability, 20 µl of MTS was added to each well of 96-well plates containing $6 \times 10^4$ cells. Cells were incubated at 37°C in a humidified atmosphere of 5% $CO_2$ in air. After 4 h, the absorbance at 490 nm was determined. Each assay was performed in triplicate; percentage of NBT reduction is calculated as a ratio between NBT and MTS values.

### MEN 4901/T-0128 uptake experiments in differentiated THP-1 cells

Cells were incubated with different concentrations of MEN 4901/T-0128 for different times (1, 4, 24, 48 h) at 37°C in a 5% $CO_2$ atmosphere.

Cells were rinsed with ice-cold phosphate-buffered saline (PBS pH 7.0) and then lysed by detergent solution (Triton X-100, 0.1% in $H_2O$). MEN 4901/T-0128 concentration was measured as polymer-bound T-2513, after chemical hydrolysis of the samples, using a sensitive (LOQ = 0.5 µg/ml of MEN 4901/T-0128 corresponding to 25 ng/ml of T-2513) validated HPLC spectrofluorimetric method.

Free T-2513 concentrations were directly measured using a sensitive (LOQ = 1 ng/ml) and validated spectrofluorimetric HPLC method.

#### In-vitro cytotoxicity assay

Human differentiated or not THP-1 cells or human pancreatic tumour ASPC-1 cells were incubated for 48 h with 0.5 mg/ml MEN 4901/T-0128 at 37°C in a 5% $CO_2$ atmosphere. A2780 cells were plated at 2000 cells/well on 96-well tissue culture plates in 100 µl of complete medium. After cell adhesion, different volumes (1, 4, 20 and 100 µl) of supernatants from THP-1 or from human pancreatic tumour APC-1 cells were added to A2780 cells, reaching a final volume of 200 µl/well. In control conditions, 20 µl of complete medium was added to the cells. After 24 h, fresh complete medium was added. The viability of A2780 cells was assessed after 96 h with Alamar Blue assay (Biosource International, Camarillo, California, USA).

Fluorescence was monitored with a multilabel counter Victor 1420 (Wallak, Turku, Finland) at 530 nm excitation wavelength and 590 nm emission wavelength.

#### Preparation and treatment of murine peritoneal macrophages/granulocytes and adherent splenocytes

Swiss mice were purchased from Harlan Italy [S. Pietro al Natisone (UD), Italy] and maintained under specific pathogen-free conditions according to the UK Co-ordinating Commitee on Cancer Research guidelines [7]. Mice were then killed and peritoneal lavage and/or spleen removal were carried out using aseptic techniques.

Splenocytes, obtained from mechanically disrupted spleens, following lysis of red blood cells, were plated in 24-well tissue culture plates in RPMI 1640 medium containing 10% heat-inactivated fetal bovine serum, 100 units/ml penicillin/streptomycin, 2 mmol/l glutamine and 10 mmol/l *N*-2-hydroxyl piperazine-*N*′-2-ethane sulfonic acid (complete medium). After 24 h of culture, nonadherent cells were removed after washing with PBS and the remaining adherent cells were used for the experiments.

Peritoneal exudate macrophages were recovered by peritoneal lavage with ice-cold PBS from mice treated intraperitoneally with 300 µl of thioglycollate (Sigma-Aldrich) solution (3%w/v) 3 days before. After washing, cells were plated in 24-well tissue culture plates in complete medium for 24 h. Nonadherent cells were removed by washing and adherent cells were used for the experiments. Cells stimulated or not with 100 ng/ml PMA were cultured in the presence or absence of MEN 4901/T-0128 (10 µg/ml) or CM-dextran (10.8 µg/ml) for 5 days. Cell culture supernatants were harvested, spun free from cells and debris, and assayed *in vitro* for cytotoxic activity on A2780 cells.

## Results

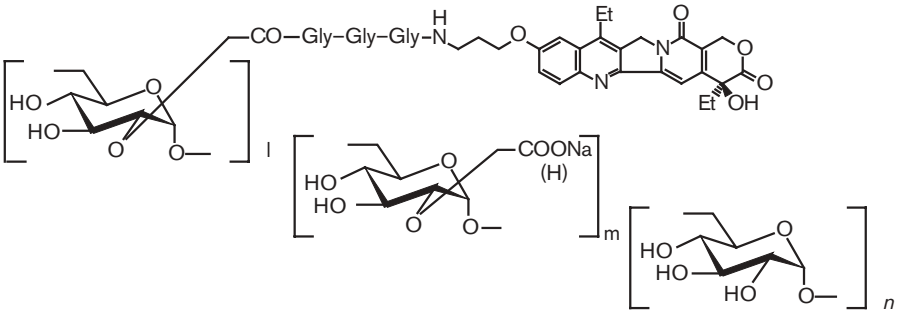
#### Human cathepsin B releases T-2513 from MEN 4901/T-0128

MEN 4901/T-0128 (Fig. 1) is a prodrug unable to effect *in vitro* the growth of A2780, differentiated THP-1 and ASPC-1 cells (data not shown). The cytotoxic moiety (T-2513) is released upon cleavage of the triglycine linker. Cathepsin B is the enzyme suggested to have this role.

The activity of human purified cathepsin B was evaluated on its specific substrate (Fig. 2a). The results show that the activity of the enzyme is pH dependent; indeed, the maximum activity is reached after 60 min at pH 6–7, whereas at pH 4 and at pH 8 cathepsin B is scarcely active.

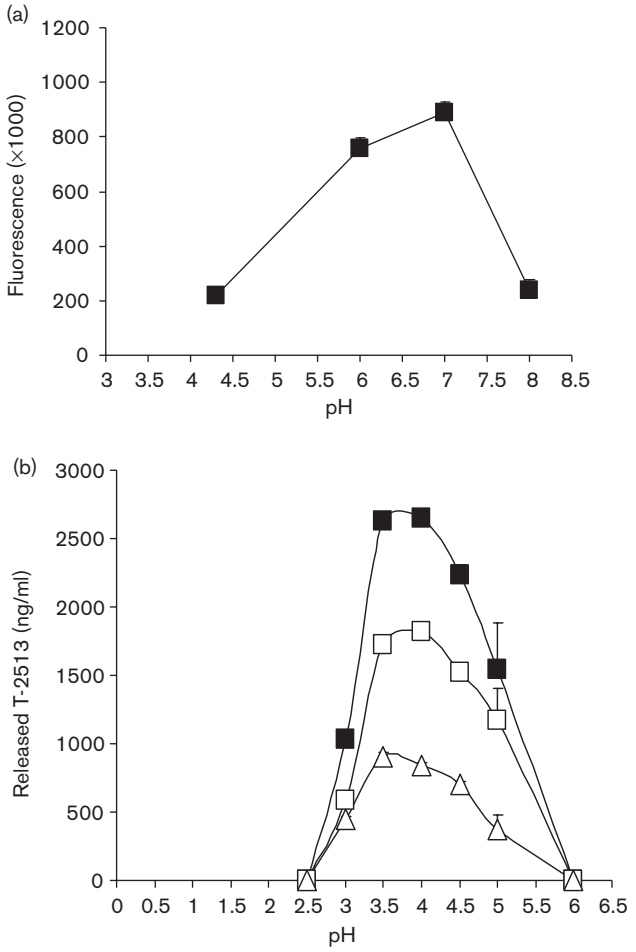
Results in Fig. 2(b) demonstrate that, after 24 h of incubation, human cathepsin B is able to cleave MEN 4901/T-0128 and to release not only T-2513, but also

**Fig. 1**




Chemical structure of MEN 4901/T-0128. MEN 4901/T-0128 is the camptothecin (CPT) T-2513 (7-ethyl-10 aminopropyloxy-CPT) bound to carboxymethyl dextran via a Gly-Gly-Gly linker. The average molecular weight is approximately 130 000.

**Fig. 2**



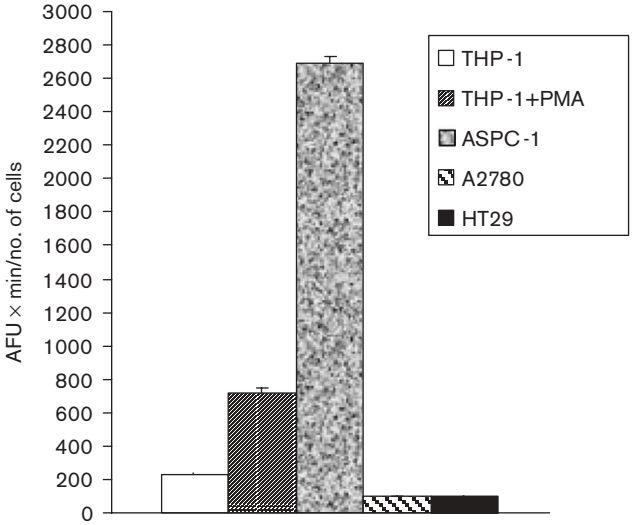

Cathepsin B activity on its specific peptidic substrate (a) and on MEN 4901/T-0128 (b). (a) Human liver-purified cathepsin B (0.01 units/sample) was incubated at 37°C with the specific fluorogenic substrate Z-Arg-Arg-AMC (20 μmol/l) at different pH values for 60 min. The released AMC was measured at excitation 390 nm and emission 460 nm. (b) Human liver purified cathepsin B (5 units/sample) was incubated at 37°C with MEN 4901/T-0128 (0.1 mg/ml) for 24 h at different pH values. T-2513 and Gly-T-2513 (open square, T-2513; open triangle, Gly-T-2513; full square, T-2513 + Gly-T-2513) were quantified using high-pressure liquid chromatography.

Gly-T-2513. The enzymatic process is pH dependent, being measurable in the pH range 3–5 and reaching the maximum at pH 4.

No drug activation is observed in the absence of cathepsin B.

As these data demonstrated that cathepsin B is involved in MEN 4901/T-0128 activation, we evaluated its activity in tumour cell lines, to verify whether the level of cathepsin B could have an impact on the release of T-2513.

**Fig. 3**




Cathepsin B fluorescence intensity in different tumour human cell lines: 320 000 differentiated or not THP-1 cells, ASPC-1 cells and A2780 cells were lysed in 0.1% Triton X-100 and 200 μmol/l of specific cathepsin B substrate (Z-arg-arg-AMC) was added for 60 min at 37°C. The released AMC was measured at excitation 390 nmol/l and emission 460 nmol/l. AFU, arbitrary fluorescence units.

Figure 3 shows that a human pancreatic tumour cell line, ASPC-1, has a high enzymatic activity. On the contrary, the level of cathepsin B in the monocytic leukemia cell line, THP-1, is definitely lower. Only after PMA activation, which induces an increase in CD11b positivity from 46 to 85% (Fig. 4), did THP-1 cells display an increase in cathepsin B activity.

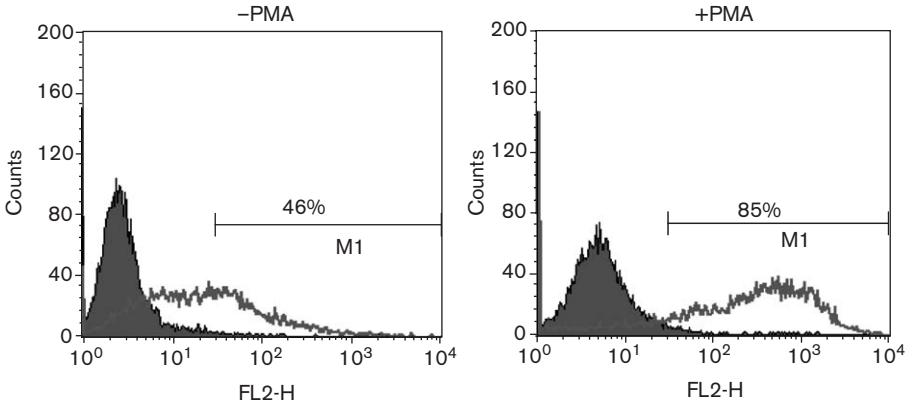
The specificity for the enzymatic assay was confirmed by the inhibition of the substrate cleavage operated by CA074Me (10 μmol/l), a selective cathepsin B inhibitor ($N = 2$).

**Differentiated THP-1 cells phagocyte MEN 4901/T-0128**

Differentiated THP-1 cells are a good human model to evaluate the involvement of macrophages in the activation of MEN 4901/T-0128 to generate CPT(s) and mediate the potent antitumoral effect of this molecule.

Differentiated THP-1 cells were incubated with MEN 4901/T-0128 (0.1–2.5 mg/ml) for different times at 37°C. In Table 1(a), the intracellular levels of MEN 4901/T-0128 (measured as polymer-bound T-2513) and free T-2513 achieved after 1, 4, 24 or 48 h exposure to 2.5 mg/ml MEN 4901/T-0128 are reported. The intracellular T-2513 appeared after the uptake of MEN 4901/T-0128, suggesting intracellular cleavage of the peptidyl linker. Cells were also incubated with different concentrations of

**Fig. 4**




Phorbol 12-myristate-13-acetate (PMA) differentiated THP-1 cells upregulate CD11b differentiation antigen. Undifferentiated and differentiated cells with PMA were incubated with nonspecific RPE-labelled mouse antibody or with RPE-labelled mouse anti-human CD11b antibody. The percentage of positive cells is indicated for each experimental condition.

**Table 1 MEN 4901/T-0128 cellular uptake and T-2513 release in differentiated THP-1 cells**

(a) Time course: Human differentiated THP-1 cells were incubated with MEN 4901/T-0128 (2.5 mg/ml) at 37°C for different times

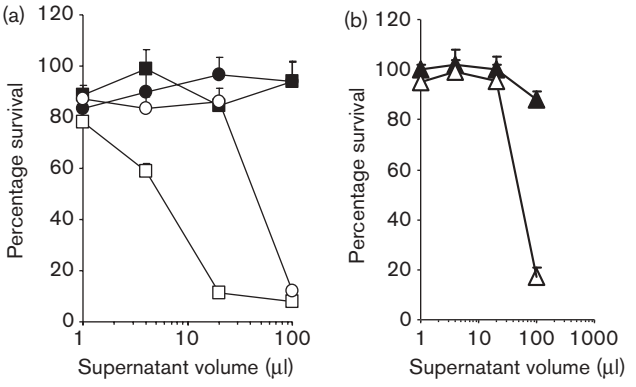
| Incubation time (h) | Polymer-bound T-2513: intracellular concentration (ng/$10^6$ cell) | Free T-2513: intracellular concentration (ng/$10^6$ cell) |
|---|---|---|
| 1 | 106 ± 42 | 0.2 ± 0.04 |
| 4 | 327 ± 100 | 1.8 ± 0.39 |
| 24 | 542 ± 12 | 8.4 ± 0.34 |
| 48 | 684 ± 146 | 15.7 ± 5.4 |

(b) Concentration dependence: Human differentiated THP-1 cells were incubated with different concentrations of MEN 4901/T-0128 for 48 h at 37°C

| MEN 4901/T-0128 (mg/ml) | Polymer-bound T-2513: intracellular concentration (ng/$10^6$ cell) | Intracellular released free T-2513 (ng/$10^6$ cell) |
|---|---|---|
| 0.1 | 29 ± 3 | – |
| 0.5 | 147 ± 20 | 5.75 ± 0.57 |
| 1 | 271 ± 42 | 7.94 ± 0.16 |
| 2.5 | 538 ± 68 | 10.3 ± 1.4 |

MEN 4901/T-0128 (measured as polymer-bound T-2513) and T-2513 uptake was evaluated using high-pressure liquid chromatography. Values are shown as mean ± SE from $n = 3$ experiments, two replicates each.

**Fig. 5**




Phorbol 12-myristate-13-acetate (PMA)-differentiated THP-1 cell supernatant kill A2780 human ovarian carcinoma cells. (a) Differentiated (empty squares) or not (empty circles) THP-1 cells were incubated for 48 h with 0.5 mg/ml MEN 4901/T-0128. As a control, both differentiated (full squares) or not (full circles) THP-1 cells were incubated with the vehicle. Collected supernatants were added in different volumes (1, 5, 20 and 100 µl) to A2780 cells for 24 h. The viability of A2780 was assessed after 96 h with the Alamar blue assay. (b) Human pancreatic tumour ASPC-1 cells were incubated for 48 h with 0.5 mg/ml MEN 4901/T-0128 (empty triangles) or with the vehicle (full triangles). Collected supernatants were added in different volumes to A2780 cells for 24 h. The viability of A2780 was assessed after 96 h with the Alamar blue assay.

MEN 4901/T-0128 for 48 h at 37°C. Table 1(b) shows that the uptake of MEN 4901/T-0128 was concentration dependent.

Human carcinoma A2780 cells were used to evaluate the ability of differentiated or not THP-1 cell supernatants to kill tumour cells. Supernatants, obtained from differentiated or not THP-1 cells previously exposed to MEN 4901/T-0128 (0.5 mg/ml; 48 h), were added in different amounts to A2780 cells (Fig. 5a).

Results reported in Fig. 5(a) demonstrate that only supernatants obtained from differentiated cells, incubated with MEN 4901/T-0128, exerted a powerful cytotoxic effect on A2780 cells. On the contrary, supernatants from undifferentiated cells were inactive (up to 20 µl of supernatant) in killing A2780 cells, suggesting that these cells were unable to phagocyte MEN 4901/T-0128 and release T-2513 in the culture medium. Moreover, ASPC-1 cells that displayed a high degree of cathepsin B activity were not able to release the cytotoxic

moiety from the prodrug (up to 100 µl of supernatant); indeed, Fig. 5(b) shows that supernatants obtained from ASPC-1 cells incubated with 0.5 mg/ml MEN 4901/T-0128 for 24 h are much less cytotoxic than supernatants obtained from differentiated THP-1 cells: 20 µl supernatants from differentiated THP-1 cells are able to kill 90% A2780 cells, whereas no cytotoxic effect is produced by the same amount of supernatants obtained from ASPC-1 cells.

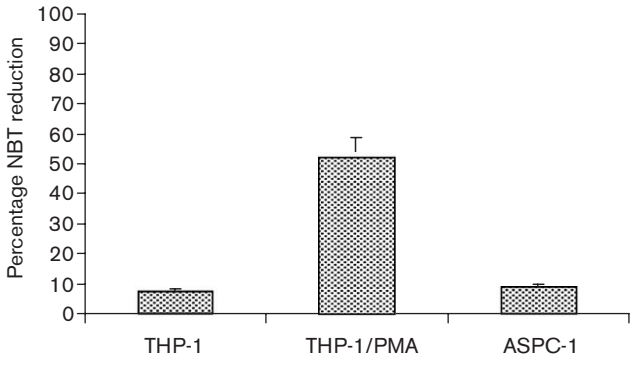
In agreement with these results, Fig. 6 shows that differentiated THP-1 cells have phagocytic activity assessed with the NBT assay, whereas ASPC-1 cells are not able to reduce NBT.

Thus, our results demonstrate that the human tumour monocytic THP-1 cells, once differentiated in macrophages, internalize MEN 4901/T-0128 very efficiently and liberate T-2513 in the medium in an amount sufficient to kill A2780 human ovarian carcinoma cells. This ability seems to be linked not only to the presence of the cathepsin B, but also to the phagocytic property of THP-1 cells, ASPC-1 cells being (expressing cathepsin B but without phagocytic properties) unable to release the active moiety.

### Role of murine immune cells in MEN 4901/T-0128 activation

The role of normal macrophage populations in the uptake and cleavage of MEN 4901/T-0128 was assessed by incubating murine macrophages with the prodrug and evaluating the ability of the conditioned culture media to kill ovarian carcinoma cell line A2780.

**Fig. 6**




Phagocytic activity of different human cell lines. Cells were exposed to MTS to measure cell viability for 4 h or to NBT to measure cell phagocytic activity for 20 min. Cell culture plates were then read with a fluorimeter. Percentage of NBT reduction is calculated as a ratio between NBT values and MTS values. PMA, phorbol 12-myristate-13-acetate.

Adherent splenocytes and peritoneal exudate macrophages were recovered from thioglycollate-injected mice. These cells, activated or not with PMA, were exposed to MEN 4901/T-0128. Figure 7 shows that both macrophage populations, recovered from the spleen (Fig. 7a) and from the peritoneal cavity (Fig. 7b), are able to generate cytotoxic culture media after exposure to MEN 4901/T-0128. In peritoneal exudate macrophages recovered from thioglycollate-injected mice, and therefore already activated, no increase in A2780 cytotoxicity was observed following a further *in-vitro* stimulation with PMA (Fig. 7b). Moreover, the CM-dextran moiety of MEN 4901/T-0128 did not contribute to the observed cytotoxic effects, as supernatants of murine macrophages and splenocytes obtained from thioglycollate-treated mice and cultured with equimolar concentration of CM-dextran (10.8 µg/ml) were ineffective in inhibiting A2780 cellular growth (Fig. 7).

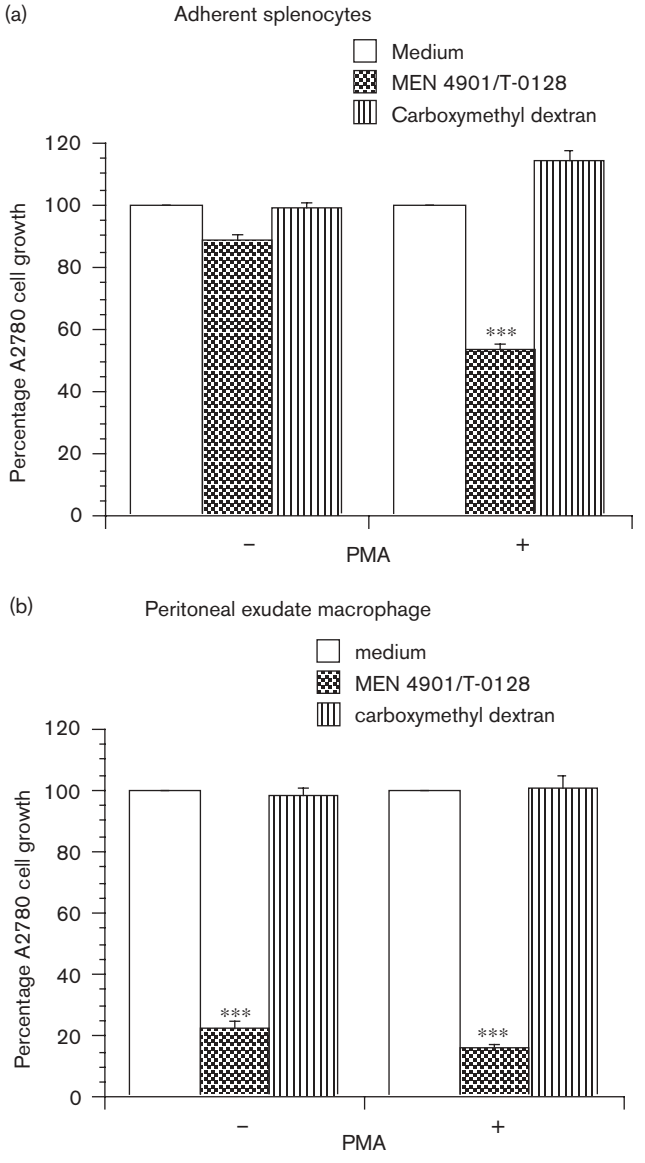
Thus, from these results, we suggested that normal activated macrophages are able to phagocyte MEN 4901/T-0128 and release cytotoxic moieties in the culture media.

## Discussion

T-2513 is a new camptothecin analogue characterized by a broad cytotoxicity against a range of human tumour cell lines. MEN 4901/T-0128, the macromolecule constituted by T-2513 bound to CM-dextran, is instead inactive *in-vitro*. Nevertheless, in-vivo MEN 4901/T-0128 treatment was able to significantly reduce or abolish the growth of many different human tumour models [3], even after a single administration. These data suggest that MEN 4901/T-0128 antitumour activity *in vivo* requires host cells or factors able to release the cytotoxically active compound T-2513 from the prodrug. Thus, the aim of our work was to better define the determinants for MEN 4901/T-0128 cytotoxic activity, clarifying (i) which enzymes are able to cleave the T-2513 moiety from the CM-dextran backbone, and (ii) which cells are able to phagocyte the macromolecule, cleave MEN 4901/T-0128 and then release T-2513 in the culture medium.

Previous data suggested an involvement of lysosomal cysteine-proteases (cathepsin B, in particular) in the release of the active moiety from MEN 4901/T-0128 [4]. Cathepsin B can catalyze the cleavage of peptide bonds by two different mechanisms: endoproteolytic attack and attack starting from the C-terminus by both peptidyl-dipeptidase and carboxypeptidase activity [8]. These two catalytic mechanisms have two different optimal pH. At an acidic pH of 4.5–5.5, which corresponds to the pH in lysosomes and other acidic compartments, cathepsin B has mainly peptidyl-dipeptidase and carboxypeptidase activity. Conversely, the endopeptidase activity has optimum pH around 7.4 [8]. Many tumour cell lines

**Fig. 7**




A2780 cell growth inhibition by culture supernatants obtained from murine macrophage populations recovered from thioglycollate-treated mice and incubated with MEN 4901/T-0128. Splenocytes (a) and peritoneal exudate macrophages (b) were obtained from Swiss mice injected with 300 µl of 3% w/v thioglycollate solution 3 days before. Adherent cells (700 000/well/ml), stimulated (+) or not (–) with 100 ng/ml phorbol 12-myristate-13-acetate, were cultured for 5 days in the presence of 10 µg/ml MEN 4901/T-0128 (black bars), 10.8 µg/ml carboxymethyl dextran (stripped bars) or culture medium (white bars). At the end of incubation, culture supernatants, diluted 1 : 2, were assayed for cytotoxic activity on A2780 cells. A2780 cell growth was measured as chemical reduction of Alamar blue. Data are presented as percentage of A2780 cell growth in the absence of murine cell culture supernatants and are the mean ± SE from $N = 2$ experiments, four replicates each. ***$P < 0.001$ (with respect to A2780 cell growth in the absence of murine culture supernatants, one-way analysis of variance; Tukey's test).

have a large quantity of pericellular (cell surface and secreted) cathepsin B [9,10]. Cathepsin B expression is also enhanced in malignant tumours of various origins, suggesting that the enzyme may be involved in tumour invasiveness and metastasis [9–15]. For this reason, inhibitors of cathepsin B are sought as potential anticancer and antimetastatic agents [16]. In this study, we show that human purified cathepsin B releases T-2513 at pH values ranging from 3 to 5. pH dependency of this reaction suggests that the cleavage of the linker should mainly occur in the lysosomes, and that both peptidyldipeptidase and carboxypeptidase activities of the enzyme should be involved.

Thus, we believe that MEN 4901/T-0128 activation requires the presence of the cathepsin B enzyme to release T-2513 inside the tumour and that phagocytic cells must be involved in this process. Indeed, it has been published [17] that in the presence of macrophage-like cells (mouse macrophage-like tumour J.774.1) the growth of B16 melanoma cells is more inhibited than in the absence of macrophage-like cells.

To further address this issue, normal murine and human phagocytic immune cells were studied.

We demonstrated here that murine macrophage populations recovered from the peritoneal cavity as well as from the spleen and exposed to MEN 4901/T-0128 were able to activate MEN 4901/T-0128, releasing cytotoxic moieties in their culture supernatants. Although we have not directly assayed T-2513 in these media, the involvement of this CPT is supported by the demonstration that supernatants obtained from macrophages incubated with equimolar concentrations of CM-dextran moiety were inactive in killing A2780 cells. It cannot, however, be ruled out that T-2513 metabolites or gly-T-2513 derivatives can also contribute to the cytotoxic effects of supernatants. Interestingly, macrophage activation (*in vitro* with PMA or *in vivo* with thioglycollate) was necessary to obtain cytotoxic culture media.

Furthermore, using a human cell model system we demonstrated that MEN 4901/T-0128 can enter the macrophage cell very efficiently and that T-2513 can be released in an amount sufficient to kill A2780 human ovarian carcinoma cells. These data were obtained using THP-1 cells, a monocytic cell line that can be differentiated in macrophage-like cells after *in-vitro* stimulation. Interestingly, these cells, once differentiated, upregulated the expression of cathepsin B. The increase in enzymatic activity showed a good correlation with the ability of the cells to release T-2513, thus supporting the key role of cathepsin B in the release process.

A high level of cathepsin B is, however, not a sufficient requirement for MEN 4901/T-0128 activity. Actually, the enzymatic activity must be present in a cell characterized

also by a high phagocytic activity, like that shown by differentiated THP-1 cells.

Recently, phagocytosis has also been observed in human tumours [18]; thus, it is possible to suggest that besides macrophage population, some specific tumour cell types could also be able to internalize and activate MEN 4901/T-0128. Here, we evaluated ASPC-1, a pancreatic tumour cell line, expressing an elevated level of cathepsin B, but without any phagocytic activity. These cells were less efficient in the release of T-2513 in the culture media, thus supporting the hypothesis that the presence of the enzyme should be coupled with high phagocytic activity in order to obtain T-2513 release.

In conclusion, our studies support the hypothesis that macrophage populations could be the cells deputed to activate the prodrug MEN 4901/T-0128 inducing the intratumoral release of the active moiety. As tumour-associated macrophages are a prominent component of the mononuclear leucocyte population of the solid tumours [19], we speculate that after treatment with MEN 4901/T-0128, they should play a major role in the local generation of active CPT (T-2513), thus mediating the potent antitumoral effect of this drug.

## Acknowledgements

We acknowledge S. Mauro for analytical assays and C. Gicca for her helpful assistance in typing the manuscript.

## References

1 Slichenmyer WJ, Rowinsky EK, Donehower RC, Kaufmann SH. The current status of camptothecin analogues as antitumor agents. *J Natl Cancer Inst* 1993; **85**:271–291.
2 Chabot GG. Clinical pharmacokinetics of irinotecan. *Clin Pharmacokinet* 1997; **33**:245–259.
3 Okuno S, Harada M, Yano T, Yano S, Kiuchi S, Tsuda N, *et al.* Complete regression of xenografted human carcinomas by camptothecin analogue–carboxymethyl dextran conjugates (T-0128). *Cancer Res* 2000; **60**:2988–2995.
4 Harada M, Sakakibara H, Yano T, Suzuki T, Okuno S. Determinants for the drug release from T-0128, camptothecin analogue–carboxymethyl dextran conjugate. *J Control Release* 2000; **69**:399–412.
5 Otto HH, Schirmeister T. Cysteine proteases and their inhibitors. *Chem Rev* 1997; **97**:133–172.
6 Qian F, Chan SJ, Gong Q, Bajkowski AS, Steiner DF, Frankfater A. The expression of cathepsin B and other lysosomal proteinases in normal tissues and in tumors. *Biomed Biochim Acta* 1991; **50**:531–540.
7 United Kingdom Co-ordinating Commitee on Cancer Research (UKCCCR). Guidelines for the welfare of animals in experimental neoplasia (Second Edition). *Br J Cancer* 1998; **77**:1–10.
8 Linebaugh BE, Sameni M, Day NA, Sloane BF, Keppler D. Exocytosis of active cathepsin B. Enzyme activity at pH 7.0, inhibition and molecular mass. *Eur J Biochem* 1999; **264**:100–109.
9 Rozhin J, Sameni M, Ziegler G, Sloane BF. Pericellular pH effects distribution and secretion of cathepsin B in malignant cells. *Cancer Res* 1994; **54**:6517–6525.
10 Hulkower KI, Butler CC, Linebaugh BE, Klaus JL, Keppler D, Giranda VL, *et al.* Fluorescent microplate assay for cancer cell-associated cathepsin B. *Eur J Biochem* 2000; **267**:4165–4170.
11 Sloane BF, Dunn JR, Honn KV. Lysosomal cathepsin B: correlation with metastatic potential. *Science* 1981; **212**:1151–1153.
12 Konduri S, Lakka SS, Tasiou A, Yanamandra N, Gondi CS, Dinh DH, *et al.* Elevated levels of cathepsin B in human glioblastoma cell lines. *Int J Oncol* 2001; **19**:519–524.
13 Tzanakakis GN, Margioris AN, Tsatsakis AM, Vezeridis MP. The metastatic potential of human pancreatic cell lines in the liver of nude mice correlates well with cathepsin B activity. *Int J Gastrointest Cancer* 2003; **34**:27–38.
14 Staack A, Tolic D, Kristiansen G, Schnorr D, Loening SA, Jung K. Expression of cathepsin B, H, and L and their inhibitors as markers of transitional cell carcinoma of the bladder. *Urology* 2004; **63**:1089–1094.
15 Troy AM, Sheahan K, Mulcahy HE, Duffy MJ, Hyland JM, O'Donoghue DP. Expression of cathepsin B and L antigen and activity is associated with early colorectal cancer progression. *Eur J Cancer* 2004; **40**:1610–1616.
16 Redwood SM, Liu BC, Weiss RE, Hodge DE, Droller MJ. Abrogation of the invasion of human bladder tumor cells by using protease inhibitor(s). *Cancer* 1992; **69**:1212–1219.
17 Harada M, Imai J, Okuno S, Suzuki T. Macrophage-mediated activation of camptothecin analogue T-2513–carboxymethyl dextran conjugate (T-0128) possible cellular mechanism for antitumor activity. *J Control Release* 2000; **69**:389–397.
18 Lugini L, Lozupone F, Matarrese P, Funaro C, Luciani F, Malorni W, *et al.* Potent phagocytic activity discriminates metastatic and primary human malignant melanomas: a key role of ezrin. *Lab Invest* 2003; **83**:1555–1567.
19 Bingle L, Brown NJ, Lewis CE. The role of tumour-associated macrophages in tumour progression: implications for new anticancer therapies. *J Pathol* 2002; **196**:254–265.