The selective anticancer activity of the endogenous inhibitor of calcium-activated neutral proteinase. A histological, cytological and chemosensitivity study

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Summary. The cytotoxicity of an endogenous inhibitor of calcium-activated neutral proteinase (CANP-I) was evaluated using various mammalian tumor-derived cell lines and human cell cultures.

The inhibitor was selectively cytotoxic to human tumor cells from lung, bladder, melanoma and chronic myeloid leukemia tissues, in a dose-dependent manner, and was also cytotoxic to Walker rat tumor cells. The inhibitor was not cytotoxic to normal human, urothelial, fallopian tube, liver and resting white blood cells. Cytological examination of the treated malignant cells revealed cells with vacuolated cytoplasm, pyknotic, hyperchromatic nuclei and membranous, granular haematoxylinophilic extracellular matrix. The use of the inhibitor on urothelial tumor tissues caused great exfoliation of necrotic cells while not affecting normal urothelial tissues.

When the inhibitor was tested on mixed cell cultures, consisting of normal and malignant cell clones, a selective cytotoxicity to the malignant cells occurred allowing the normal cells to grow unaffected. Cyto-genetic and cytological examination of the remaining cells, after the inhibitor treatment, showed normal diploid karyotype and morphology.

The inhibitor was also tested *in vivo* on Wistar rats bearing Walker tumors. Treatment with 50 Units/100 g i.p. daily for 5 days caused 90% tumor regression and necrosis of metastatic foci in the liver and abdomen, without toxic side effects.

The protease inhibitors trypsin-chymotrypsin, aprotinin, leupeptin and E64 were also tested *in vitro* and showed no anticancer activity. In conclusion, the endogenous inhibitor of CANP selectively killed malignant cells of different chromosomal abnormalities, tissue and species origin; also nuclear vlimata and chemoresistant cells. These results are discussed in the context of a model for the action of the endogenous inhibitor of CANP, extracellular matrix and nuclear vlimata.

Key words: Proteinase inhibitors, Inhibitors of CANP, Selective malignant cell killing, Anticancer agent, Chemosensitivity assay, Extracellular matrix, Nuclear vlima

Introduction

Cancer chemotherapy has improved substantially within the past decade. However, toxic side effects and resistance following chemotherapy, has not yet been eliminated (Calvert, 1990).

The main weapon for fighting cancer is selective malignant cell killing by chemical or physical means. A selective antineoplastic agent may be defined as a substance which kills only malignant cells thereby inhibiting tumor development, and arresting the process by which malignant cells disseminate in the host.

Differences between malignant and normal cells at the cellular and subcellular level may serve as the basis for a universally effective selective antineoplastic agent.

One difference between malignant and normal cells is the quantity and the quality of proteinases. Proteinases are intrinsically involved in the process of metastasis (Khokha et al., 1992) and increased levels are often associated with malignancies (Enomoto et al., 1987).

In addition, glycosaminoglycan (GSG)-sac formation (Logothetou-Rella et al., 1992a), as well as nuclear vlimata (NV) production and function, by malignant cells (Logothetou-Rella, 1993a) *in vitro* and *in vivo*, have indicated involvement of intracellular proteinases.

One class of intracellular proteinase is the so called calcium-activated neutral proteinase (CANP). CANPs are present in the cytosol as inactive proenzymes that are converted to the active forms by a process that requires both Ca^{2+} and substrate. CANPs exist ubiquitously in various tissues and cells of vertebrates and are presumed to function in various fundamental cellular events mediated by Ca^{2+} such as: hydrolysis of epidermal and platelet growth factor receptors; cleavage of membrane

proteins before cell fusion; and activation of protein kinase C (Suzuki et al., 1987). Moreover, CANP levels in malignancies were increased when compared to normal tissues (Nishiura et al., 1979; Enomoto et al., 1987) and lymphocytic NVs were sensitive to the endogenous inhibitor of CANP (Logothetou-Rella, 1994a).

An inhibitor of CANP has been found in the cytosol of rabbit liver. This CANP inhibitor has been characterized as an endogenous inhibitor, being a protein of 240 KDa molecular weight containing 4 subunits, each of approximately 60 KDa (Melloni et al., 1984). CANP-I has also been purified from other sources (Suzuki et al., 1987). In this study, the selective malignant cell killing action of the endogenous inhibitor of CANP (CANP-I) from rabbit skeletal muscle is investigated *in vitro* and *in vivo*, for its possible use as a new effective antineoplastic agent.

Materials and methods

Cell cultures

Cell cultures were established from human tissue specimens by enzymatic digestion (Logothetou-Rella et al., 1988a) and designated by the initials of the patients. A total of ten specimens from various human tumors were included in this study (Table 1). There were five malignant urothelial lines (Pa, Br, S, IG, R), three malignant lung cell lines (M, P, B), one melanoma (Ha) and one bone marrow aspirate from a patient with chronic myeloid leukemia (BM). In addition, Walker rat tumor cells were tested (W-cells).

The human normal liver cell line (Li-cells), the normal urothelial cell line (N-cells), resting white blood cells from five healthy persons and normal fallopian-tube cells (F-cells), were used as control cells.

Most of these cell lines have recently been characterized cytologically and identified as malignant or normal by cytogenetic analysis (Logothetou-Rella et al., 1992b; Logothetou-Rella, 1993a).

All cell cultures were used at passage 10-15, grown in complete RPMI-1640 medium (Gibco) supplemented with 10% heat-inactivated foetal bovine serum (FBS, Seromed), penicillin (100 U/ml, Gibco), streptomycin (100 µg/ml, Gibco) and incubated at 37 °C in a humidified 5% CO_2-incubator.

Protease inhibitors

Three CANP inhibitors were tested: the endogeneous CANP inhibitor (Sigma P-0787), leupeptin (Sigma L2884) and E64 (Sigma E-3132). Trypsin-chymotrypsin inhibitor (Sigma T-9777) and aprotinin (Sigma A-4529) were also tested.

All inhibitors were dissolved in RPMI-1640 with 25 mM HEPES (Seromed), filtered through 0.22 µm Sartorious filters, dispersed in aliquots and frozen at -20 °C. Fresh or thawed inhibitor solutions were used.

Chemosensitivity assay

Two techniques were used to determine the inhibitor cytotoxicity on malignant and normal cells.

a) Cytological changes of cell cultures in continuous presence of the inhibitors.

Seven kinds of complete medium RPMI-1640 were prepared. One was supplemented with 1 U/ml CANP-I; the second with 1 mg/ml leupeptin; the third with 1 mg/ml E 64; the fourth with 2 mg/ml trypsin-chymotrypsin inhibitor; the fifth with 1 mg/ml aprotinin; the sixth with all five inhibitors; and the seventh complete RPMI-1640 as a control medium.

Fourteen glass Petri dishes (5 cm diameter) were each seeded with 1 x 10^6 M-cells and another fourteen dishes, each with 1 x 10^6 P-cells. Duplicate cell cultures received each kind of complete medium containing the inhibitors and control medium. The cell cultures were incubated at 37 °C in a humidified 5% CO_2-incubator for 120 hours. The culture medium was changed with a fresh one of the same kind in each case, 24 and 72 hours after culture initiation. Half of the cell cultures were fixed in 50% ethanol 72 hours, and the other half 120 hours after culture initiation. All cell cultures were stained by the Papanicolaou method.

Postconfluent cell cultures of M-cells and P-cells received fresh complete medium RPMI-1640 supplemented with 1 U/ml CANP-I. They were then incubated at 37 °C for 3 days, fixed in 50% ethanol and stained by the Papanicolaou method.

b) Liquid medium short-term culture method

The cells were detached with trypsin-EDTA (Seromed) resuspended in complete RPMI-1640 and cell counts were made using a hemocytometer. Viable counts were assessed using the 0.4% trypan blue exclusion method. The cells were then washed once with complete RPMI-1640, centrifuged at 200 g for 8 min, resuspended in complete RPMI-1640 and used for chemosensitivity testing (at 50,000-200,000 cells per tube) in accordace with the method of Chang et al. (1989).

Duplicate samples of cells were incubated for one hour for each concentration of the inhibitors in a shaking water bath at 37 °C for one hour. The cells were then washed twice with complete RPMI-1640 by centrifugation at 200 g for 8 min. Each rinsed cell pellet was resuspended in 1 ml complete RPMI-1640. The cells were rendered single by gentle pipetting and were then seeded in 24-well microplates (Costar, Cambridge Mass.) for a 4-day period of short term culture at 37 °C under a humidified atmosphere of 5% CO_2. The cytotoxicity assessment was made using the dye exclusion method of 0.4% trypan blue. The degree of cytotoxicity was measured according to the following formula:

$$\text{Cytotoxicity} (\%) = \left(1 - \frac{\text{Number of viable cells in the experimental group}}{\text{Number of viable cells in the control group}} \right) 100$$

The type of cells tested are shown in Table 1.

Cytogenetic analysis

In the cases of mixed cell lines, of malignant and normal diploid cells, the cells that survived CANP-I treatment, were allowed to grow for cytogenetic analysis. The technique applied for the chromosome preparation was the one described by Boue (1981). Chromosome analysis was performed by standard Giemsa and RhG banding. One hundred metaphases were studied.

Use of CANP-I on the viability of normal and malignant urothelium

Tumor (from 5 patients) and normal (from 5 persons) tissue pieces of human urothelium of 2 mm x 2 mm x 2 mm were rinsed in complete RPMI-1640. One piece from each type of tissue was immersed in complete RPMI-1640 (control) and one piece in the CANP-I solution (10 U/ml) in polypropylene tubes and incubated at 37 °C for one hour in the humidified, 5% CO$_2$ incubator. All tissue species were then rinsed carefully in complete medium and were immersed in polypropylene tubes (1 piece/tube) containing 2 ml complete RPMI-1640, and then incubated for 4 days at 37 °C. The tissue

Table 1. Categories of cases with various cells tested.

TISSUE ORIGIN	DESIGNATION	MALIGNANT CLONE	NORMAL DIPLOID CLONE
Bladder transitional cell carcinoma	Pa-cells	+	
	Br-cells	+	+
	S-cells	+	+
	IG-cells	+	
	R-cells	+	+
Lung carcinoma	M-cells	+	+
	P-cells	+	+
	B-cells	+	+
Melanoma	Ha-cells	+	
Chronic myeloid leukemia	BM-cells	+	
Walker rat tumor	W-cells	+	
Normal liver	Li-cells		+
Normal urothelium	N-cells		+
White blood cells (5 specimens)	WBC		+
Human amniotic embrionic cells (5 specimens)			
Human fallopian cells (F-cells)			+

+: indicates the cytogenetic state of each cell type.

pieces were then fixed in formaldehyde, embedded in paraffin and tissue sections were stained with haematoxylin-eosin. The exfoliated cells in the tubes with the malignant tissue pieces were allowed to settle in a conical polypropylene tube for 10 min, then smeared on glass slides, fixed with cytospray, and stained by the Papanicolaou method.

Cytotoxicity of CANP-I on rat tumors

One Walker tumor was excised 2 weeks following the subcutaneous implantation of tumor tissue in male Wistar rat. Wistar rats with Walker tumors were donated by Dr. S. Kotarides, Hellenic Anticancer Institute. Tumor cell suspension for injection was prepared as described by Fisher and Fisher (1959). Ten male Wistar rats, weighting 100 g each, were injected with 10 x 10^6 Walker tumor cells subcutaneously in the left foot pad. The rats were then divided into two groups, one control and one treated. Treatment was initiated when tumors had reached a measurable size of 70 mm^3. The CANP-I solution was injected into each rat intraperitoneally, at 50 U/2.5 ml every day (6.45 mg/gr body weight/day), for a period of 5 days. Control rats were each injected with 2.5 ml RPMI-1640 medium with 25 mM HEPES (placebo). Tumor diameters were measured every day with calipers, and tumor volumes were calculated by the formula

$$V = \pi \times \frac{Dd^2}{6}$$

where D is the largest diameter and d the smallest.

Treatment evaluation was based on tumor volume analysis and statistical evaluation was performed using the Student's t-test.

All rats were sacrificed one day after the last treatment because the legs of the control group were all covered with tumors including up to the shoulder blade and made accurate tumor measurements impossible. The tumor-bearing legs and organs from all rats were excised, fixed in formalin and embedded in paraffin for histological studies.

Results

Cytological changes of cell cultures in the continuous presence of the inhibitors

Trypsin-chymotrypsin inhibitor, aprotinin, E64 and leupeptin did not affect the growth and cytology of M- or P-cells when compared to control cell cultures (Fig. 1 inset).

The CANP-I, in cultures with medium #1 and #6, caused great exfoliation of cells and extracellular matrix (ECM) in the culture medium, after 72 hours of continuous presence in cultures. All exfoliated cells were necrotized, consisting of hyperchromatic, pyknotic nuclei and little cytoplasm. On the culture dish surface, a

few, countable per field, attached endothelial cells remained vital, cytologically normal and cytogenetically normal diploid (Fig. 1). All other cell culture dishes and the control ones (Fig. 1 inset) were full of cells, without cell exfoliation. Following Papanicolaou staining macroscopically apparent green, fibrillar, translucent ECM and GSG sacs were observed. These observations were persistent after 120 hours of continous presence of the CANP-I cell cultures, except that the surviving endothelial cells had grown despite the presence of the inhibitor.

CANP-I treated postconfluent M- and P-cell cultures exhibited cells with vacuolated cytoplasm and degenerated nuclei of different sizes with and without tails. The rounded up, detached, dead cells were holding to each other on the culture dish surface by a network of haematoxylinophilic membranes, visible microscopically. The abundant ECM and GSG sacs had disappeared, but large masses of haematoxylinophilic granules were present (Fig. 2).
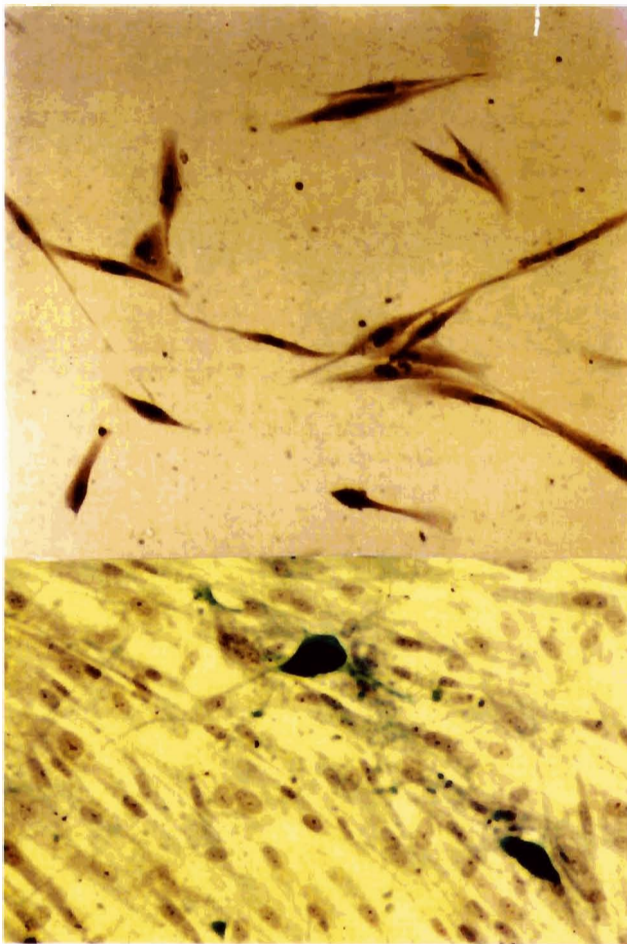
Cytotoxicity of CANP-I in vitro

CANP-I was cytotoxic to all types of malignant cells tested. Normal cells, however, within the same or separate culture were able to grow and propagate (Table 2). Cytotoxicity was dose-dependent and the optimum concentration of 4 U/ml CANP-I induced selective malignant cell killing. At this concentration, the inhibitor induced 100% cytotoxicity to all malignant cell lines tested except melanoma, bladder Pa and embryonic cells, which required 5 U/ml. The inhibitor was not cytotoxic to normal diploid cells, including liver cells and resting WBCs, but it was cytotoxic to normal diploid embryonic cells. The other CANP inhibitors, leupeptin and E64 did not induce cytotoxic effects on cells.

Cytogenetic analysis

Cytogenetic analysis of the survived cells (in mixed

Fig. 1. M-cell culture after 72 h of continuous treatment with CANP-I. The endothelial cells, which survived treatment, are cytologically normal and cytogenetically normal diploid. Papanicolaou. x 100. Inset: Untreated control M-cells with GSG-sacs. Papanicolaou. x 100
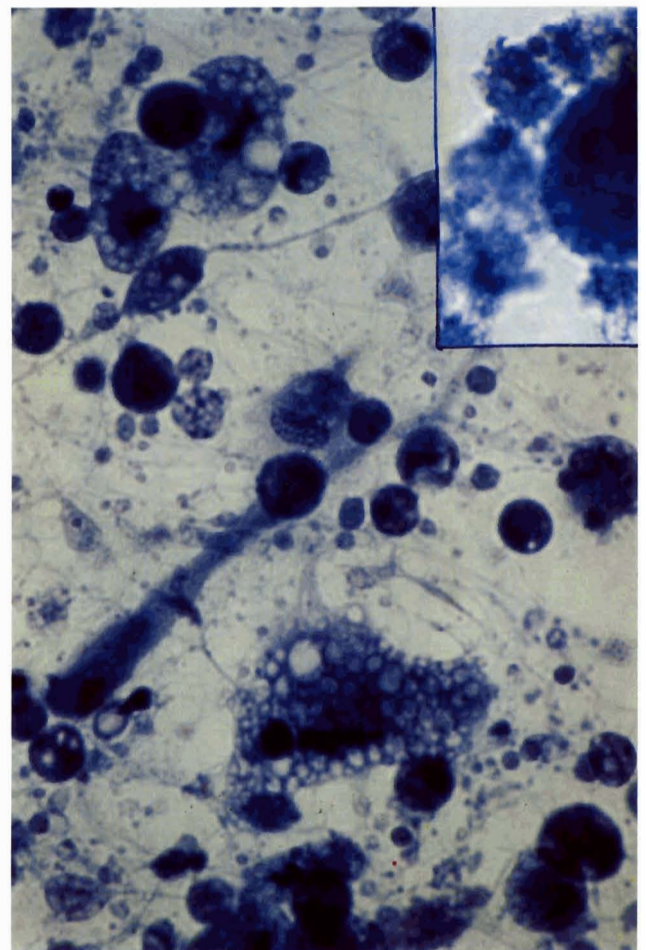

Fig. 2. CANP-I-treated post confluent M-cell culture, for 72 h. Vacuolated cytoplasm, pyknotic, hyperchromatic nuclei and hameatoxylinophilic membranous, granular ECM are noticeable. Papanicolaou. x 400. Inset: The blue granular texture of the ECM after treatment. Papanicolaou. x 1,000

cell lines) after the CANP-I treatment showed normal diploid karyotype, indicating that the inhibitor was mainly cytotoxic to malignant cells which had exhibited aneuploidies (Logothetou-Rella, 1992b).

Effects of CANP-I on the viability of normal and malignant urothelium

CANP-I induced massive cell exfoliation in the malignant tissues. Histological examination of the CANP-I treated malignant tissues exhibited bionecrotic to necrotic areas and large tissue areas consisting of eosinophilic extracellular matrix denuded of cells. The exfoliated cells were dead, with degenerated nuclei, and NV morphology, and separated from each other, lacking the green ECM (Fig. 3). Control malignant tissue in the absence of CANP-I remained intact (Fig. 4); the very few malignant tissue exfoliated cells showed compact cell masses in green ECM with indiscreet cell

Table 2. Sensitivity of cells to different concentrations of the inhibitor of CANP.

TESTED CELLS	INHIBITOR CYTOTOXICITY (%)		
	INHIBITOR CANP (U/ML)		
	1	4	5
M-cells	24	65	65
P-cells	45	82	
B-cells		86	87
Pa-cells	24	99	100
S-cells		34	
Br-cells		55	
R-cells		83	
IG-cells		100	
Ha-cells	21		100
Walker tumor cells		100	
Malignant bone marrow cells		100	
N-cells	0	0	0
Li-cells	0	0	0
WBCs	0	0	0
Embryonic cells	45	95	100
F-cells	0	0	0

The cytotoxicity of the inhibitor in each specimen was obtained from mean of duplicate samples.
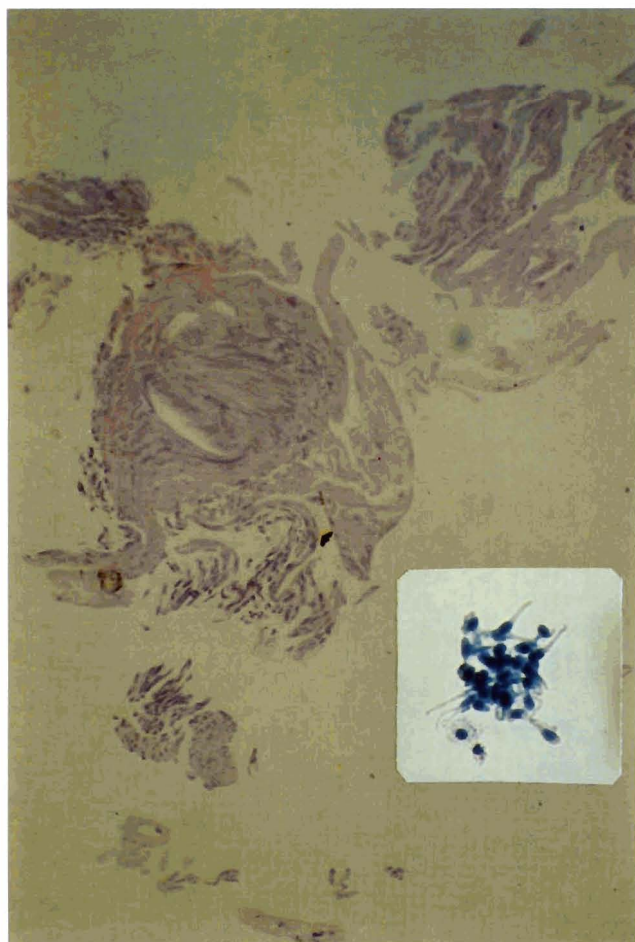

Fig. 3. Histological picture of bladder tumor tissue treated with CANP-I *in vitro*. Large tissue areas consist of eosinophilic ECM denuded of cells. H-E. x 100. Inset: Exfoliated necrotic NVs from the CANP-I-treated bladder tumor tissue. Papanicolaou. x 200
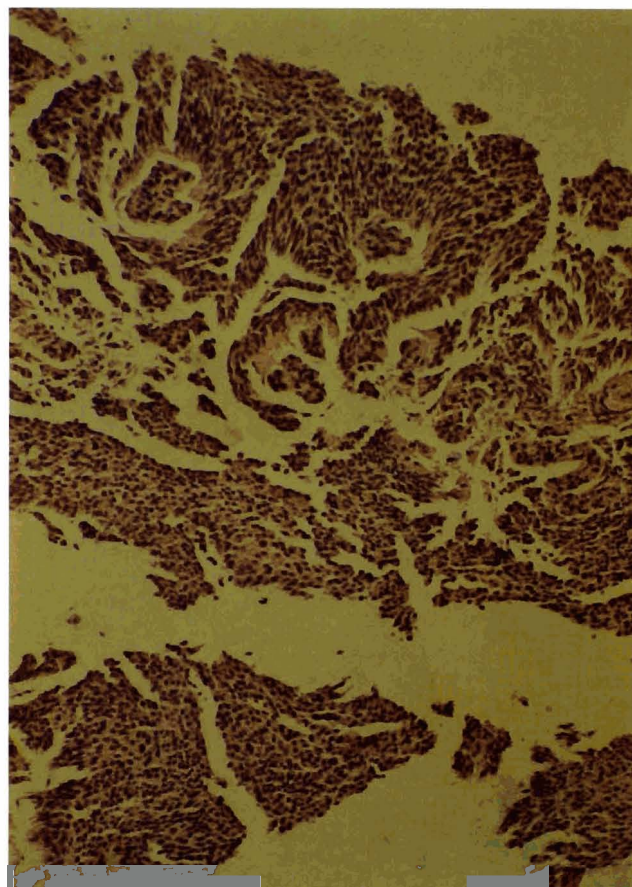

Fig. 4. Control bladder tumor tissue in the absence of CANP-I. The tissue is vital with very few exfoliated cells. H-E. x 100

boundaries.

Normal urothelial tissues were kept intact after treatment with the inhibitor (Fig. 5).

Cytotoxicity of CANP-I on rat tumors

The use of Walker rat tumor system was preferred to that of nude mice, for in this system the thymus and immune system is kept, thus representing more closely human cancer.

Treatment of rats, each with 6.45 mg CANP-I/g body weight/day for five consecutive days resulted in 90% tumor regression (Figs. 6, 7). Statistical testing of the difference between the placebo and treated group with respect to tumor size showed no difference at day 0 (p = 0.967) but significant difference at day 5 (p < 10^{-4}).

CANP-I, although proteinaceous and originating from rabbit skeletal muscle, was absorbed easily and did not induce any toxic effects in the animals. Histological examination of all organs and specially livers of the treated rats did not show any cytotoxic effects, as central venules and centrilobar parenchymal cells did not show any necrosis or cellular damage. The testis of all rats showed spermatogenetic arrest.

Among the treated group one rat developed metastatic abdominal wall focus and another metastatic hepatic focus. On palpation, the abdominal wall focus had disappeared 24 hours after the first dose. Histological examination showed necrosis of a large carcinomatous nodule with formation of abscess, necrosis of the overlying epidermis and ulceration (Fig. 8). The liver metastatic focus was necrotized, exhibiting necrotic material with nuclear debris in the centre and remnants of carcinomatous tissue with mitoses in its periphery (Fig. 9). Histology of the remaining foot pad tumors after treatment showed larger necrotic areas than those of placebo group with formation of micro-abscesses.

Discussion

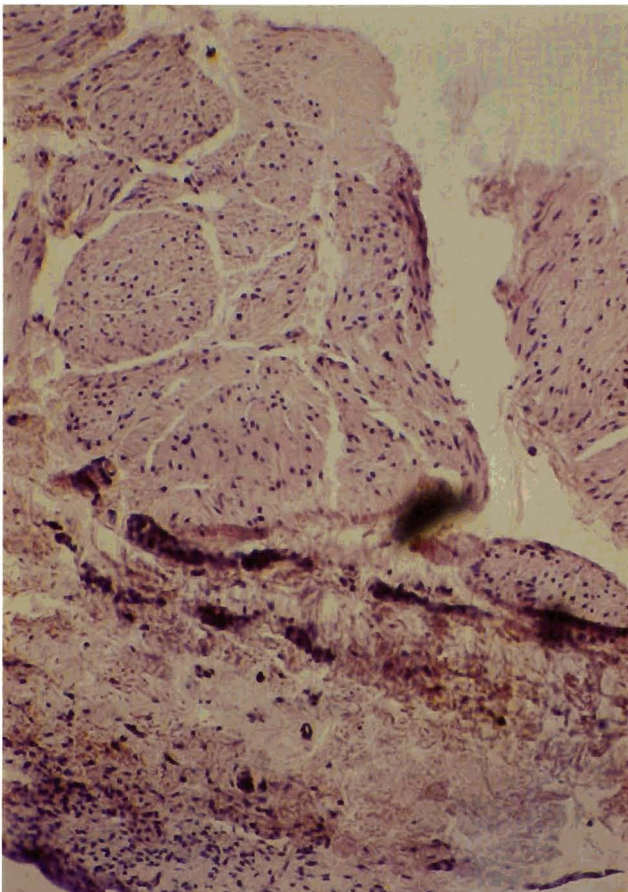The results of this study show that *in vitro* CANP-I

Fig. 5. Uneffected vital normal urothelium tissue treated with CANP-I. H-E. x 100
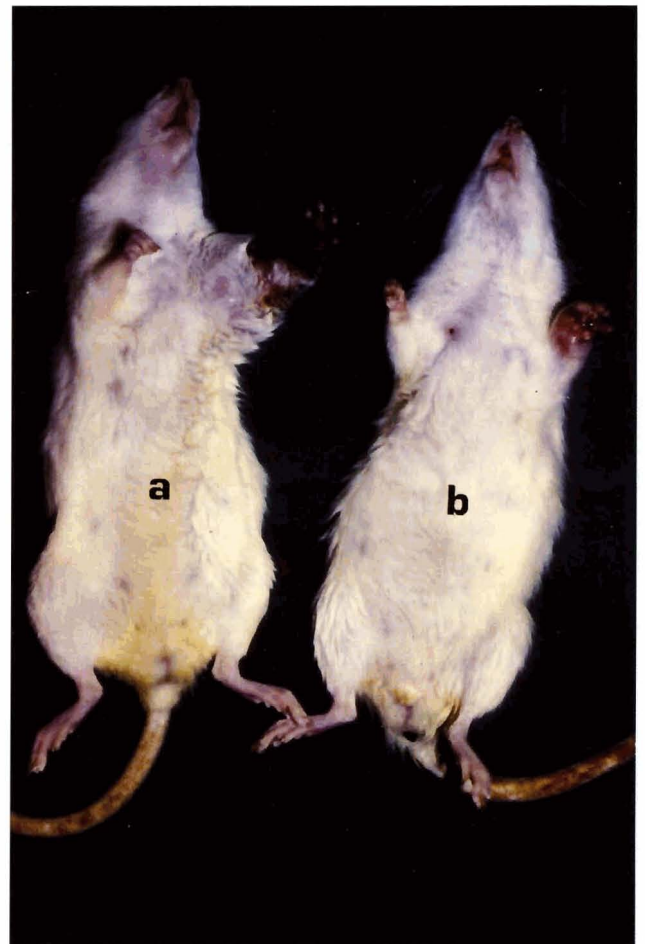

Fig. 6. a. Control group, placebo-treated rat. The tumor has grown up to the shoulder blade. **b.** Tumor regression after 5-day CANP-I treatment.

was cytotoxic to malignant cells of different chromosomal abnormalities, tissue and species origin without affecting normal cell diploid genotype and phenotype. Sensitivity to CANP-I was not only shown by cells from solid and haematological tumors but also by chemoresistant tumor cells (lung P-cells and bladder Pa-cells) and NVs.

Embryonic cells, although of diploid karyotype, were also sensitive to CANP-I. This sensitivity constitutes an additional characteristic of resemblance between malignant and embryonic cells.

In this study, leupeptin and E64, although CANP inhibitors, did not affect growth and morphology of malignant cells *in vitro*. This indicates that CANP-I may inhibit a different CANP isozyme other than the one inhibited by leupeptin and E64.

Nishiura et al. (1979) have reported inhibition of the *in vitro* cell growth of malignant brain cells by leupeptin. However, leupeptin was not tested on the growth of normal brain cells for comparison. Hence, it is not clear in that study, whether CANP inhibited by leupeptin is associated with malignancy or brain cells themselves.

CANP-I was not cytotoxic to normal diploid cells including resting WBCs and hence normal cells either lack the CANP isozyme, inhibited by CANP-I, or do not dispose the appropriate substrate for CANP and CANP-I activity. Unaffected normal cells in the presence of the inhibitor, in co-cultivation with malignant cells, may support the lack of CANP isozyme in normal cells.

The inhibitor induced malignant cell cytotoxic effects, *in vitro*, in a dose-dependent manner. There appeared to be a small sensitivity difference to the degree of cytotoxicity shown by the various cell lines. The reason for this sensitivity is not currently known but may possibly have a relationship to the amount of CANP produced in the various cell lines. The inhibitor was cytotoxic to malignant tissue pieces indicating, indirectly, the presence of CANP and its substrate at the tissue level.

The use of CANP, *in vivo*, also shows its selective anticancer action. Treatment with 6.45 mg CANP-I/gr body weight/day for five days induced not only a 90% main tumor regression but also caused necrosis of metastatic foci without any toxic side effects. Also, the anticancer action of CANP-I on Walker rat tumor cells *in*
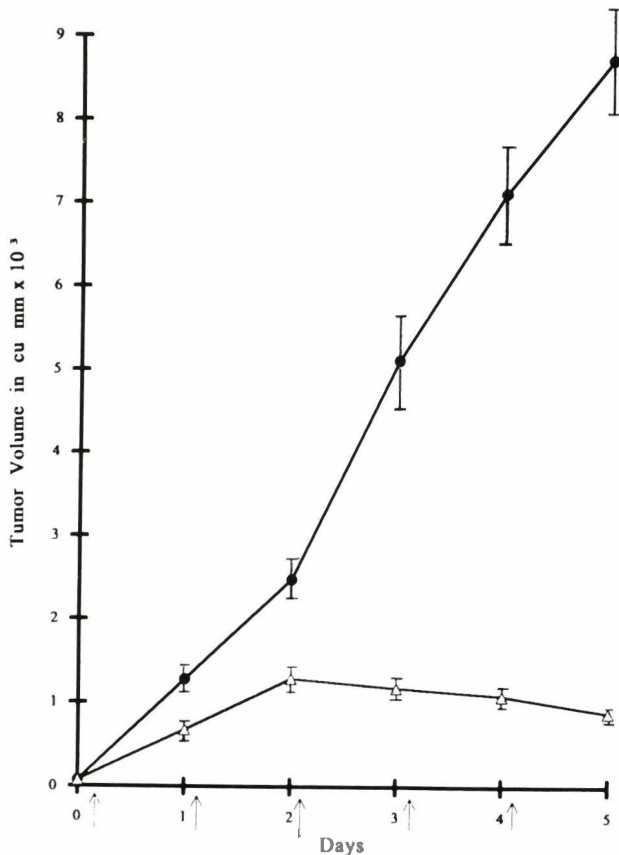

Fig. 7. Response of Walker rat tumor to CANP-I. Arrows indicate periods of treatment, each with 6.45 mg/g body weight/day. ●: control group; △: treated group.
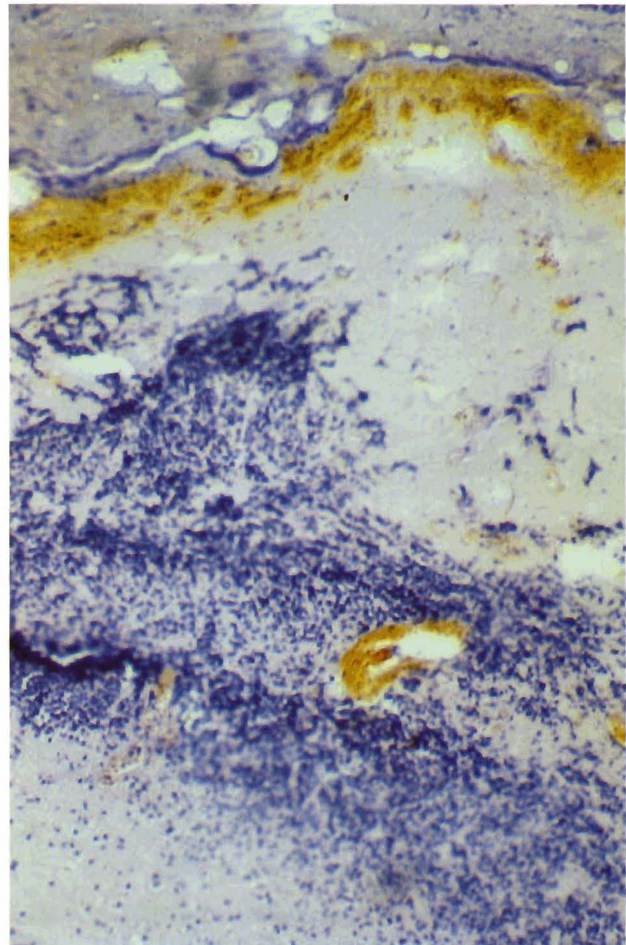

Fig. 8. Histological picture of a necrotic Walker abdominal metastatic focus after CANP-I treatment. H-E. x 125

vitro and *in vivo* strengthens its broad spectrum of activity, independent of the type and origin of the tumor. Such an activity is expected for a selective antineoplastic agent, since uncontrolled growth, the mechanism of dissemination of malignant cells and nuclear vlimata are common in all types of neoplasia (Logothetou-Rella, 1993a). The spermatogenetic arrest caused by CANP-I is achieved by cytotoxicity on the cells of the testis undergoing meiosis. This work is presently under investigation.

It has been reported that bladder (Logothetou-Rella et al., 1992a), lung tumor cells (Logothetou-Rella et al., 1992b) and tissues exhibit extracellular and intracellular GSG positive for α_1-chymotrypsin. However, these cells were not affected by the inhibitor of trypsin-chymotrypsin used in this study. The fact that CANP-I was cytotoxic to these cells, indicates that antisera against α_1-chymotrypsin may crossreact with CANP.

The biological role of CANP and its inhibitor, as well as the precise role of CANP-I, in regulating the activity of CANP is unknown. However, high activity of CANP in hyperplastic nodules and hepatocellular carcinomas in rats has suggested the involvement of this protease in the promotion stage of liver carcinogenesis (Enomoto et al., 1987). Also, the present results indirectly document CANP involvement in the viability and growth of malignant cells.

The cytological changes caused by the inhibitor on postconfluent malignant cells and GSG ECM strongly suggests that the active proteinase is bound to GSGs, a probable common substrate for both the proteinase and its inhibitor action. GSG-sacs and ECM are produced by host cells, *in vitro* and *in vivo*, as a defence mechanism against malignant cell invasion (Logothetou-Rella, 1994a). The inhibitor is a tetramer, of high molecular weight (240 KDa) and cannot pass the cell membrane unless dissociated into its subunits, in the presence of substrate. So far only casein or globin substrate have been tested (Melloni et al., 1984).

In the present study the mechanism of the CANP-I cytotoxic effect on malignant cells as well as the mechanism of its selective action is not documented.

A possible mechanism of the inhibitor's cytotoxic action on malignant cells might be the dissociation of the inhibitor tetramer upon contact with extracellular GSG (substrate)-bound CANP, into monomers and formation of inactive inhibitor-proteinase complex. Then the following events may take place; the inhibitor monomers produced extracellularly may diffuse through malignant cell membrane and inactivate the endogenous CANP pools or the intracellular activated CANP diffuses extracellularly towards a lower concentration gradient (after the inactivation of the extracellular CANP) and is inactivated by the extracellular inhibitor monomers. Also both events might be taking place without excluding endocytosis of CANP-I. Cytoplasmic vacuoles observed in inhibitor-treated malignant cells support the diffusion of CANP-I monomers and CANP inactivation intracellularly.

Based on related work with lymphocytic NVs, the following hypothesis explains the CANP-I selective action. Activated lymphocytes produce NVs, identical to those of malignant cells, by meiosis (Logothetou-Rella, 1993b, 1994b). CANP-I is cytotoxic to lymphocytic and malignant NVs, products of meiosis, without affecting meiosis itself (Logothetou-Rella, 1994b). According to the mechanism of NV formation, NVs can pull along and guide the intracellular CANP and its substrate extracellularly. Normal cells are products of mitosis, do not exhibit the ECM of GSG-CANP (Logothetou-Rella et al., 1988b), do not form NVs and are not sensitive to CANP-I. It is feasible that CANP-I affects malignant cells via CANP produced by meiosis while it does not affect normal cells dividing by mitosis.

In conclusion, the endogenous CANP inhibitor seems promising as a new selective antineoplastic agent and
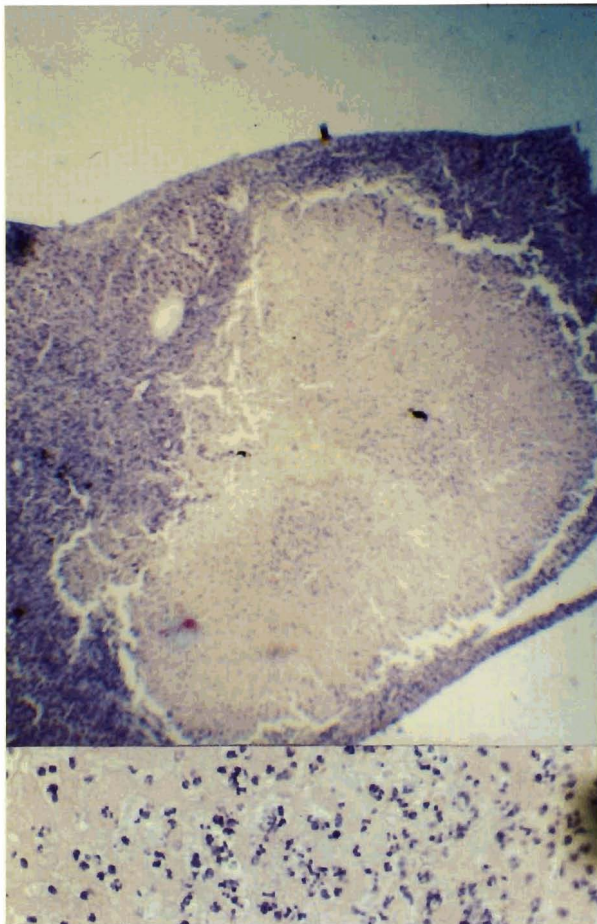

Fig. 9. Histological picture of a Walker rat tumor liver metastatic focus after CANP-I treatment. The largest area of the tumor is necrotized with only remnants of vital tumor tissue in its periphery. H-E. x 40. Inset: Necrotic liver tumor area exhibiting necrotic material with nuclear debris. H-E. x 200

more research is in progress to elucidate its action, and its use in human cancer treatment.

Since submission of this manuscript to the reviewers, it was documented that malignant cells divide and produce aneuploid NVs by meiosi involving CANP.

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