Photodynamic therapy combined with a cysteine proteinase inhibitor synergistically decrease VEGF production and promote tumour necrosis in a rat mammary carcinoma

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Abstract. *Objectives:* Photodynamic therapy (PDT) and inhibition of cathepsin B proteases by cystatin (cysteine proteinase inhibitor, CPI) are potential new tumour treatment modalities. We have investigated the efficacy of PDT and CPI alone and in combination on a solid mammary carcinoma transplanted into Wistar rats. *Materials and Methods:* Intraperitoneally injected single doses of chlorine e6 or HpD as photosensitizers were excited at 630 nm (90 J/cm²). CPI (500 µg per animal) was injected around the tumour daily during the 8-day treatment. Inoculation of tumour was either on day 1 of the protocol, or 8 days before. On day 8, tumour size was measured, tumour necrosis and vascularization were determined based on haematoxylin and eosin (H&E)-stained sections and serum vascular endothelial growth factor (VEGF) levels measured using an enzyme-linked immunosorbent assay kit. *Results:* No differences (two-way ANOVA) were found for treatments started with various time lags. At doses where CPI or PDT alone had no or negligible effect, their combination caused a marked ($P < 0.001$) decrease in serum VEGF, paralleled by a significant decrease in tumour size and number of capillary vessels, and a significant increase in necrosis (up to 80% of the tumour tissue). *Conclusions:* The combination of PDT and CPI could be a useful approach in tumour therapy as the two agents appear to be synergistic and probably decrease VEGF production by the tumour tissue.

INTRODUCTION

In spite of the wide spectrum of tumour treatment agents available, anticancer therapy is still a major clinical challenge and none of the current therapies can be considered fully effective when

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applied alone. In most cases, chemotherapy has been proven to be powerful, and is often combined with radiotherapy and surgery. In spite of radical surgery, tumour cells may remain in the surrounding tissue, which often require the application of post-operative adjuvant therapy. Both chemotherapy and irradiation can have various undesired side effects.

A relatively new therapeutic modality for both neoplastic and non-neoplastic diseases is photodynamic therapy (PDT), which involves light-driven activation of photosensitizers (PS) to produce molecular oxygen and other free radicals (Huang 2005). PDT can cause damage to malignant cells and/or to tumour vasculature, which leads to necrosis and/or apoptosis of tumour cells (Plaetzer *et al.* 2005). Because most cancer cells selectively accumulate the PS, the technique is meant to be somewhat selective for the destruction of tumour cells. Although the serum level of various biologically active molecules is known to change after PDT (Ziolkowski *et al.* 1996; Ziolkowski *et al.* 1997; Ziolkowski *et al.* 1999), the appropriate design of the therapy (Sibata *et al.* 2000) should help avoid serious side effects. Hence, it is a potentially effective and relatively safe adjuvant therapy in complementing surgery, radiation and chemotherapy (Ris 2005).

Cysteine proteinases of tumour cells play a crucial role in the proteolytic cascade, which results in the destruction of neighbouring extracellular matrix components such as laminin, collagen and elastin (Buck *et al.* 1992). Increased activity of cathepsin, a member of the cysteine proteinase family, is associated with vascularization and an invasive front of carcinomas (Foekens *et al.* 1998; Joyce *et al.* 2004). Inhibitors of these proteinases (cysteine proteinase inhibitor, CPI) produced by the organism participate in the mechanisms of defense against cancer progression (Lah & Kos 1998). The balance between activity of cysteine proteinases and their inhibitors may determine the aggressivity of cancer cell metastasis (Lah *et al.* 1997). In normal tissues, extracellular proteolysis is regulated by endogenous inhibitors; in pathogenic processes, the balance between proteinases and inhibitors of proteinases is believed to be disturbed (Nyberg *et al.* 2005). Cystatin isolated from egg white was one of the first inhibitors of cysteine proteinases to be identified and characterized. It belongs to family II of cystatins and consists of 120 amino acids with two disulphide bridges (Herszenyi *et al.* 2000). Because cystatins isolated from egg white effectively inhibit cysteine proteinases from cancer cells, these molecules are potential new-generation drugs in 'inhibitor therapy' (Lah & Kos 1998).

Angiogenesis, the formation of new blood vessels from pre-existing vasculature, is essential for the growth of solid tumours and for the appearance of metastases. This process can be stimulated by hypoxia. Several types of cells respond to hypoxia by producing different angiogenic factors, such as tumour necrosis factor (Fajardo *et al.* 1992), fibroblast growth factor (Hayrabedian *et al.* 2005) and vascular endothelial growth factor (VEGF) (Casanovas *et al.* 2005). After binding of VEGF to its type 2 receptor, endothelial cell migration and proliferation are induced, whereas activation of VEGF receptor-1 by this factor enhances cell migration and secretion of tissue factor by endothelial cells (Ferrara *et al.* 2003). Over-expression of VEGF has been associated with tumour progression and poor prognosis in several tumours (Wey *et al.* 2004). The best characterized of the VEGF family members, VEGF-A (also known as vascular permeability factor), is associated with key events in physiological angiogenesis. VEGF is a potent vascular permeabilizing agent, and enhances microvascular permeability. The hyper-permeability of tumour vessels to plasma proteins is attributable to VEGF secretion by tumour cells, which leads to the transformation of normally anti-angiogenic stroma of normal tissues into a pro-angiogenic environment. VEGF has been shown to inhibit necrosis and to up-regulate anti-apoptotic proteins such as Bcl-2 and to stimulate the phosphatidylinositol 3'-kinase/Akt pathway that promotes endothelial cell survival despite apoptotic stimuli (Dvorak 2002).

In this work, on the efficacy of the combined application of photodynamic therapy and cysteine treatment has been investigated on solid mammary carcinoma transplanted into Wistar rats. The combined therapy was significantly more effective than either treatment alone, and not only caused the decrease of tumour size by induction of necrosis and inhibiting cysteine proteinases but it also diminished the level of neo-vascularization, partly by inhibiting VEGF production.

MATERIALS AND METHODS

Chemicals

Cysteine proteinase inhibitor (CPI, cystatin) was isolated from eggs as previously described (Saleh *et al.* 2003), and was diluted in 0.5 ml physiological saline prior to injection. The photosensitizers (PS), haematoporphyrin derivative (HpD) and chlorine e6 (Ce6) were from Frontier Scientific (formerly Porphyrin Products), Inc., Logan, UT. Both were dissolved in physiological saline at pH 7.2. All other chemicals were of analytical grade.

Animal model

Female Wistar rats, aged 3–3.5 months, average weight 200 g, were kept according to the 'Guidelines for the Care and Use of Animals' (2002). The transplantable solid mammary carcinoma was obtained from the Institute of Oncology, Gliwice, Poland (Saleh *et al.* 2001). The tumour was found to be Her-2 negative using HercepTest immunohistochemistry (Dako, Glostrup, Denmark, data not shown).

Light source

A Penta lamp (Teclas, Sorengo, Switzerland) was applied as a light source 24 h after photosensitizer administration. Excitation wavelength ranges were selected with bandpass filters: 654 ± 20 nm for chlorine e6 and 630 ± 20 nm for HpD. Light intensity was adjusted to 230 mW/cm^2 , and total light dose was 90 J/cm^2 . Tumours were irradiated for 7 min without masks.

Treatment protocols

In Protocol I, the homogenized ground tumour tissue (1 ml) was diluted with 0.5 ml physiological saline containing $500 \mu\text{g}$ CPI and was implanted subcutaneously into the left abdominal region of the rat (day 0). On the same day, HpD was injected intraperitoneally at a dose of 10 or 20 mg/kg . The next day (day 1), $500 \mu\text{g}$ CPI per animal was administered between the normal and cancerous tissues, and the site of inoculation was light-irradiated. During the following 6 days, CPI was injected around the tumour every day. After an 8-day treatment, rats were sacrificed (day 7), $200 \mu\text{l}$ blood was collected by heart puncture and was left to clot in Eppendorf tubes for 2 h. Samples were centrifuged for 10 min at 1000 g and sera were stored at -70°C until the determination of VEGF levels by enzyme-linked immunosorbent assay (ELISA). Tumours were excised, formaldehyde-fixed and paraffin-embedded. In this protocol, 30 animals were used.

Protocol II was similar to protocol I, except HpD was injected on the day after the inoculation (day 1) and tumours were irradiated on day 2. In this protocol, 10 animals were used.

Protocol III differed from protocol I in that tumour implantation took place 7 days before the start of treatments on day 0 and that the photosensitizer used was Ce6. In this protocol, 10 animals were used.

Evaluation of histological sections

Four micrometre sections were prepared from paraffin-embedded tumour tissue and stained with haematoxylin and eosin (H&E). Extent of necrosis was determined from examining four complete sections of each tumour at 200× magnification. The ratio of damaged cells to all cells was calculated after counting, applying the usual pathological criteria: cells showing deteriorated nuclear morphology (such as signs of pyknosis, karyorrhexis or karyolysis) in addition to cytoplasmic changes (coagulation or, at later stages, liquefaction), as well as instances of shrunken, intensively stained nuclei inside similarly shrunken, condensed cytoplasm, which is characteristic of apoptosis, were considered as dying or damaged cells. Blood vessels were counted in four complete sections and their average number per field of view at 10× magnification for each tumour was calculated.

Determination of VEGF levels in rat sera

Determination of serum VEGF levels was carried out according to the manufacturer's instructions using a mouse VEGF immunoassay kit (MMV00 Quantikine, R&D Systems, Wiesbaden-Nordenstadt, Germany), which also recognizes rat VEGF. Five-fold diluted samples were compared to 0, 7.8, 15.6, 31.2, 62.5, 250 and 500 pg/ml standards in a 96-well microplate assay based on peroxidase and the stabilized chromogen tetramethylbenzidine. OD was measured at 450 nm.

Statistical analysis

Because some data were non-normally distributed, differences among all treatment groups and all protocols were tested using two-way ANOVA on ranks, followed by testing the difference between each treatment group and the control (Holm-Sidak method). Differences between various treatments were compared using one-way ANOVA, followed by pairwise *t*-tests. Correlation between readout parameters (tumour size, necrosis, neovascularization, VEGF levels) was determined from Pearson's product moment correlation coefficient (*R*).

Percentage improvement of combinations, upon the mere addition of the effect of the two components, was calculated as follows. The parameter in question is considered baseline in the control group. Relative changes (in percentage) are calculated for the parameter normalizing the difference to the baseline value. Relative percentage changes are added for the two single treatments to be combined, and the difference between this sum and the relative percentage change for the combination itself calculated. This difference then is normalized to the sum generated for the two monotherapies and expressed in percentage improvement gained by the combination over the sum of individual monotherapies.

RESULTS

Assessment of necrosis

Microscopic images taken of tumour samples showed that up to 85–90% of the tumour tissue had died by necrosis after PDT with 20 mg/kg PS combined with CPI injections (Fig. 1b), compared to around 28% in the untreated control (Fig. 1a). The application of PDT or CPI alone caused less severe tumour damage resulting in about 35–45% necrosis. Assessment of necrosis indicated that the most effective treatment was obtained with experimental protocol I (Fig. 2). Nonetheless, in combined therapy, all protocols had caused significant ($P < 0.001$) necrosis. In combined therapy, the higher dose of photosensitizer was somewhat more effective in killing cells by necrosis than the lower one ($P = 0.06$). In the case of PDT or CPI monotherapies, 10 mg/kg dose of PS had no effect ($P = 0.58$), but the higher, 20 mg/kg dose of PS as well as

Figure 1. PDT and cystatin together cause extensive tumour necrosis. (a) Mammary solid adenocarcinoma from the control group; 25–30% is necrotic (H&E staining, 200×). (b) After HpD (20 mg/kg), CPI (500 µg/animal) and PDT (light dose: 90 J/cm²), 85–90% of the tumour tissue was necrotic (H&E staining, 200×).

Figure 2. Quantitative analysis of necrosis. PDT or CPI caused a modest increase of tumour necrosis, whereas their combination was far more effective. PS, photosensitizer; CPI, cystatin; *significant changes ($P < 0.001$) relative to control.

CPI caused a measurable increase in necrosis ($P = 0.01$ and $P = 0.06$). Nonetheless, the efficiency of either monotherapy alone was inferior both to the combination using low-dose PS ($0.03 < P < 0.1$), and especially to the combination with high-dose PS ($P = 0.001$). In fact, whether the combination therapy was with low or with high-dose PS, it proved to be synergistic,

Figure 3. Changes of tumour volumes. Tumour volumes were significantly reduced by high-dose PDT or its combination with CPI. The use of higher concentrations of photosensitizer caused greater decreases in tumour volumes. PS, photosensitizer; CPI, cystatin; *significant changes ($P < 0.001$) relative to control.

improving, in protocol I, by 429% and 200%, respectively, the expected total rate of necrosis estimated as the sum of effect caused by the two agents alone.

Comparison of treatment protocols

Because the solid mammary carcinoma grew very rapidly, injection of CPI directly after tumour implantation was chosen as the main approach (protocol I) to test. Here, 500 µg of CPI/animal and/or 10 or 20 mg/kg HpD were injected on the day of tumour implantation and was irradiated 24 h later. In the case of combined CPI-PDT therapy, where protocol I caused almost complete necrosis, the other protocols (protocol II with 1 day delay in PDT and protocol III with tumour implantation 8 days prior to treatment) resulted in poorer responses, and were significantly ($P < 0.05$) inferior to protocol I (Fig. 2). However, in combined therapy, all three protocols had a significant ($P < 0.001$) effect of causing necrosis. Also, two-way ANOVA revealed no significant differences amongst the three protocols. Furthermore, pairwise comparison of parameters showed no significant difference in the efficacy of various protocols for any single or combined therapy, except for the aforementioned difference in necrosis after combined therapy. Thus, in subsequent analysis, efficiency of various treatments was also compared using data pooled for all three protocols.

High-dose PDT or its combination with CPI decreases tumour volumes most efficiently

Tumour volumes were determined by measuring three diameters of the tumour dissected on day 8 (Fig. 3). Combination of CPI and high-dose PS, which was most effective in inducing necrosis, caused a reduction in tumour volumes to approximately 2.5 cm³, compared with the untreated control, which varied from 4.5 to 6 cm³ ($P < 0.001$). Combination therapy with the smaller dose

Figure 4. VEGF levels in rat sera. Serum VEGF levels were lower in rats treated with PDT and significantly lower after combination of PDT and CPI. PS, photosensitizer; CPI, cystatin; *significant changes ($P < 0.001$) relative to control.

of PS was also of beneficial effect, and significantly ($P = 0.003$) reduced tumour size to an average of about 3.5 cm^3 . Application of PDT alone caused a lesser, but still significant, decrease in tumour volume to about 4.5 cm^3 ($P = 0.03$, and $P = 0.004$ for the lower and higher doses). CPI alone caused the smallest reduction in tumour volume ($P = 0.19$). Combination therapy was more effective than either of the monotherapies, for low-dose PS $0.003 < P < 0.06$, for high-dose PS $0.001 < P < 0.003$. Combination therapies, both with low- and high-dose PS, were equally effective ($P = 0.41$), and proved to be synergistic, improving by 118% and 33%, respectively, the expected total rate of volume decrease estimated as the sum of effect caused by the two agents alone.

Serum VEGF levels are lowest in combined PDT and CPI

Because over-expression of VEGF has been associated with tumour progression and poor prognosis, VEGF serum levels were measured upon termination of the experiments. In healthy animals not transplanted with tumour, serum VEGF level was $106.5 \pm 7.2 \text{ pg/ml}$, and this was elevated to a range of $134.8\text{--}170.4 \text{ pg/ml}$ in carcinoma-bearing non-treated rats. After treatment with PDT, VEGF levels dropped only mildly ($P > 0.15\%$), whereas CPI injection even seemed to elevate serum VEGF ($P = 0.02$, Fig. 4). Concentration of VEGF was lowest in sera obtained from rats treated with PDT using 20 mg/kg PS combined with CPI, but the smaller dose of PS with CPI was also enough to reduce the level significantly compared to controls ($P < 0.001$ in both cases). Combination treatments were significantly more effective than monotherapies ($P < 0.001$ for all comparisons), and showed a most striking synergy, changing net elevation of 23%, estimated from the sum of treatments, into a drastic decrease of 86% in the case of 20 mg/kg PS, the corresponding proportions being 17% and 56% for 10 mg/kg PS. The higher dose of PS combined with CPI caused a significantly greater decrease of VEGF than the lower one ($P = 0.001$).

Figure 5. Tumour vascularization as a function of therapy. Although 20 mg/kg photosensitizer combined with CPI proved to be the most powerful in decreasing the number of blood vessels in the tumour, all treatments and protocols were effective. PS, photosensitizer; CPI, cystatin; *significant changes ($P < 0.001$) relative to control.

Table 1. Correlation of tumour volumes, necrosis, vascularization and serum VEGF levels

	Tumour volumes	VEGF levels	Number of vessels
Extent of necrosis	-0.661	-0.691	-0.625
Tumour volumes		0.713	0.734
VEGF levels			0.568

Pearson's product moment correlation values (R) are shown at $P < 10^{-4}$.

Tumour vasculature is decreased by all treatment modes

The extent of vasculature was determined in samples stained with H&E. The average number of vessels per field of view (Fig. 5), as expected on the basis of decreased VEGF levels, was very much decreased by combination therapy ($P < 0.001$ for both PS concentrations). In contrast to the other parameters assessed, in the case of vascularization, monotherapies also had a significant decreasing effect ($P = 0.001$). Nonetheless, combination therapies both with low- and high-dose PS outperformed every monotherapy ($0.02 < P < 0.1$ for low and $0.001 < P < 0.02$ for high doses), but did not differ greatly from each other in their outcomes ($P = 0.08$).

Correlation of measured parameters

Table 1 shows Pearson's product moment correlation values calculated for the four quantified parameters. All correlations were significant at $P < 10^{-4}$. Tumour necrosis negatively correlated with tumour volumes, VEGF levels and the number of vessels present, whereas tumour volumes

exhibited positive correlation with VEGF levels and the number of vessels; VEGF levels positively correlated with the number of vessels.

DISCUSSION

In a previous study (Saleh *et al.* 2001), we have shown increased survival times by treating solid mammary adenocarcinomas with the combination of photodynamic therapy and CPI. In the present study, our aim was to characterize some of the pathological processes associated with both single and combined PDT and CPI treatment, and to use these characteristics to establish beneficial doses and determine the nature of interaction between the two drugs. As readout parameters, tumour size was measured, tumour necrosis and vascularization were determined based on H&E-stained sections, and serum VEGF levels measured using an ELISA kit.

Pairwise comparisons of treatments revealed that single application of either PDT or CPI resulted in increased tumour necrosis as compared to the untreated controls, and this was further increased by combining PDT with CPI in each of three time-schedule protocols. This means that the combination therapy using PDT and CPI may be a valuable clinical option.

In comparing the three time-schedule protocols in terms of necrosis caused, they provided similar results for all treatments except that protocol I was superior to the others in the case of 20 mg/kg PS with 500 µg CPI, causing almost complete necrosis of the tumours. This observation has possible clinical relevance, as it hints that CPI injected immediately after tumour removal combined to PDT could enhance the killing effect of PDT on leftover tumour cells better than delayed application, and thereby could be more efficient in preventing local recurrence or metastasis formation.

Therapeutic effects on necrosis were well correlated with decreases of tumour volumes, combination of CPI and PDT being the most effective. Interestingly, at the higher, 20 mg/kg dose of PS, combined therapy was more effective in further enhancing necrosis (beyond that caused by the lower dose) than in promoting the shrinkage of the tumour. However, it is quite possible that observation periods beyond our 8-day schedule would reveal a volume decrease proportional to necrosis with the higher dose combination treatment also.

Combination of PDT and CPI therapies proved to be highly synergistic, not only in terms of evoking necrosis, but also in their efficacy at reducing tumour volume. Photosensitizers accumulate in lysosomes and cause lysosomal disruption after irradiation (Caruso *et al.* 2004). Lysosomal photodamage can then initiate mitochondrion-mediated cell death by subsequent activation of Bid, pro-caspase-9 and -3, whereas pro-caspase-8 is not activated. Release of cytochrome C occurs simultaneously with Bid cleavage, which indirectly results in cell death (Reiners *et al.* 2002). One possible reason for CPI synergistically enhancing the effect of PDT is the inhibition of the activity of various proteases – especially cathepsins, legumain and calpain – by CPI. Cathepsins B and L play an important role in matrix degradation and cell invasion, thus contributing to the overall aggressivity of the tumour (Joyce *et al.* 2004). Legumain, an asparaginyl endopeptidase, was demonstrated in membrane-associated vesicles concentrated in invasion edges of lamellipodia of tumour cells and on cell surfaces, where it colocalized with integrins. Its abundance conveys enhanced migratory and invasive properties, possibly mediated by increased extracellular matrix degradation, resulting from activation of zymogens such as pro-gelatinase (Liu *et al.* 2003). Thus, administration of CPI inhibiting cathepsins and legumain can cause arrest of cell invasion into peritumoural stroma (Saleh *et al.* 2003). Lack of invasion and concomitant vascularization probably promote cell death in the tumour tissue, which is

further enhanced by PDT. Inhibition of calpain in myoblasts has been shown to hinder migration and cause rounded cell morphology, loss of membrane extensions, disorganization of stress fibers and major defects in new adhesion formation (Dedieu *et al.* 2004), as well as cell cycle arrest (Raynaud *et al.* 2004). Similar effects operating in our model could also contribute to synergism of the effects of CPI and PDT.

Synergistic action of PDT and CPI can also be a result of PDT enhancing the anti-invasive effect of CPI. According to previous studies (Osiecka *et al.* 2003), the amount of VEGF in tumour-bearing BALB/c mice was considerably decreased 24 and 96 h after PDT. Others, however, had reported short-term increases of VEGF production (Togashi *et al.* 2006). In our studies, VEGF level was elevated in carcinoma-bearing non-treated rats compared to healthy rats. After treatment with PDT, VEGF levels dropped only mildly, whereas CPI injection alone seemed even to slightly elevate serum VEGF, probably as a feedback response of the tumour mass to decreased invasion and neovascularization. However, when CPI was complemented with PDT, the concentration of serum VEGF dropped to levels even below that of healthy animals. These combination treatments were significantly more effective than PDT alone and showed very strong synergism. Also, the higher dose of PS combined with CPI caused a significantly greater decrease of VEGF than the lower one. Reduction in the level of VEGF is expected to result in decreased tumour neo-angiogenesis, an effect synergistic with CPI, which hampers local invasion, and the two globally leading to inhibition of tumour growth and progression. In support of this reasoning, our results clearly show the positive correlation between decreased VEGF levels and decreased vascularization, between VEGF levels or vascularization and tumour growth, and, conversely, the negative correlation between VEGF levels or vascularization and tumour necrosis.

As a further possible mechanism of synergy between CPI and PDT, one needs to consider lysosomal localization of cathepsin B, a main target of CPI inhibition (Joyce *et al.* 2004). Because photosensitizers are also accumulated in lysosomes, irradiation damages the lysosomes, resulting in reduced levels and activity of cathepsin B. As in tumours, increased activity of cathepsin B is associated with neo-angiogenesis and invasive fronts of carcinomas. Effective inhibition of its activity by the combined addition of CPI and PDT can be a major factor in preventing tumour growth.

Tumour vascularization was a highly sensitive parameter that decreased considerably after all treatments. Although combination therapies outperformed every monotherapy, the differences were not extremely large. Because the decrease of vascularization exceeded that of VEGF levels in PDT monotherapy, it is likely that the damaging effect of photodynamic therapy was not only manifested in cancer cells, but also in endothelial and mesenchymal cells forming new blood vessels (Peng & Nesland 2004). It is also possible that PDT decreases the production of factors driving vessel growth other than VEGF. Similarly, the disparate effect of CPI on VEGF levels and vascularization can be explained by the negative feedback of cathepsin inhibition-boosting VEGF production, whereas at the same time, CPI causing necrosis of tumour vasculature concomitant with necrosis of tumour cells themselves. The positive correlation of tumour volumes with serum VEGF levels substantiates the notion that at least part of the serum VEGF could originate from the tumour tissue. At the same time, the partly divergent effects of treatments with VEGF levels and vascularization explain why the correlation coefficient between these two parameters is relatively low.

It is interesting to note that the decrease by CPI of neovascularization alone could reduce the efficacy of PDT, and therefore other effects of CPI directly exerted on the tumour cells might also play a role in the synergy of CPI and PDT. Such an effect could be the antagonism of CPI against TGF β , which can decrease proliferation of mammary carcinoma cells (Sokol *et al.* 2005).

Taken together, our results indicate that cell damage caused by PDT as well as inhibition of tumour invasion by CPI could hinder tumour angiogenesis not only directly, but also via diminishing serum VEGF concentrations. As PDT and CPI acted very efficiently synergistically upon tumour volume, necrosis and VEGF levels at concentrations where they alone had little or no effect, we suggest that their combination could possibly gain clinical application, especially because during the treatment of animals no side effects were observed even at the most effective doses. Although PDT has already been established in clinical practice, the applicability of CPI is yet to be substantiated. However, some recent advances are encouraging. Cegnar *et al.* (2004) have optimized the uptake of cystatin into target cells using cystatin-loaded nanoparticles that effectively inhibited cathepsin B activity. Such an approach using paramagnetic particles could even provide for site-directed delivery of CPI using magnetic field gradients. It may also be possible in the future to enhance CPI expression using transfection approaches. Overexpression of the CPI stefin A in human oesophageal squamous cell carcinoma cells has been found to inhibit tumour cell growth, angiogenesis, invasion and metastasis (Li *et al.* 2005). Moreover, adenovirus-mediated cystatin C over-expression in host mice efficiently reduced lung metastasis of an experimental human fibrosarcoma (Kopitz *et al.* 2005). Nonetheless, caution also needs to be taken as cystatins have lately been found to have not only antiproliferative effects, but in a few cases also proliferative effects, depending on the system investigated (Keppler 2006).

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