# Cell-cell interactions in the gastrointestinal tumour-microenvironment

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*Chapter 9*

# Efficient degradation-aided selection of protease inhibitors by phage display

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*Biochem.Biophys.Res.Commun. 2007; 364, 549-555.*

**Abstract**

In this report, we describe a novel phage display strategy for the identification of dedicated protease inhibiting peptides, based on degradation-aided enrichment of protease resistant phages. Phages were directly incubated with a range of phage-degrading proteases, after which non-degraded phages were used for the next selection round. For proteinase-K we identified after only four selection rounds a peptide (VLIMPVLLGIPLLC) that inhibits proteinase-K activity with an inhibition constant of 4 μM. In analogy, we identified a peptide capable of inhibiting substrate degradation by cathepsin-S (VWNCERITISRLIN), which showed functional inhibition of cathepsin-S induced sprouting of endothelial cells. We envision that the pursued strategy of degradation-aided selection of protease inhibitors (DASPI) represents an effective approach in the design of new protease inhibitors but also of new strategies to render gene and drug vectors protease resistant.

## *Introduction*

Proteases play essential roles in biological processes as non-specific mediators of protein degradation and in specific cleavage events. Especially imbalanced regulation of protease activity leading to basement membrane degradation and excessive release of bioactive molecules (e.g. growth factors, chemoattractants, angiogenic molecules) is of major importance in inflammatory disease and cancer progression[1-5]. Furthermore it also poses a major hurdle in drug and gene delivery protocols[6].

Therefore, design of selective protease inhibitors constitutes a major scientific and pharmaceutical challenge. Several approaches including combinatorial chemistry[7,8], positional-scanning of synthetic libraries[9] and oriented synthetic peptide libraries[10] have been used to develop protease inhibitors. Furthermore combinatorial repertoire cloning and peptide/protein phage display have shown to hold great promise in the design of peptide ligands[11-13] such as caspase inhibitors[14] and DNaseII inhibitors[15]. Generally, identification of binding peptides/proteins is based on affinity-selection for a selective agent (antigens or cells) that is immobilized on a solid support[16-18]. A possible disadvantage of these selection procedures is their rather unbiased nature that generally favours the selection of binders rather than functional antagonists.

In this study, we present a new selection strategy to identify functionally active protease inhibitors: degradation-aided selection of protease inhibitors (DASPI, Figure 1). In DASPI, phages are directly incubated with the target protease and selection pressure is based on the extent of phage degradation. We show the effectiveness of DASPI in the identification of peptide sequences inhibiting proteinase-K and cathepsin-S, which could further be chemically optimised.

## *Materials and methods*

*Materials*

Phagemid libraries pComb8, with a constrained 6 (CX6C) or 15 amino acid ($CX_{15}C$) insert and pComb3 with a linear 6 ($X_6$) or 15 amino acid ($X_{15}$) insert were generous gifts from Prof. H. Pannekoek, Amsterdam Medical Center, Amsterdam, The Netherlands. MOPS was from Boehringer Mannheim, Mannheim, Germany. BSA was obtained from Sigma, St Louis, MO, USA. Phenyl Methyl Sulphonyl Fluoride (PMSF) and E64 cysteine protease inhibitor were supplied by Sigma-Aldrich, Steinheim, Germany. VCSM13 was from Stratagene, La Jolla, CA, USA. Cathepsin-S (bovine spleen and human spleen), cathepsin B (human liver), calpain

I (human erythrocytes) and cathepsin inhibitor I were purchased from Calbiochem, San Diego, CA, USA. The specific cathepsin-S substrate (Z-Val-Val-Arg-AMC) was purchased from Bachem, Weil am Rhein, Germany.

*Determination of colony forming units (CFU)*

*Escherichia coli* (XL-1-blue) was grown at 37°C to mid-log phase ($OD_{600}$ = 0.6-0.8) in Super Broth (SB) medium (30 g/l tryptone, 20 g/l yeast, 10 g/l MOPS) containing 20 μg/ml tetracycline (tet). M13-bacteriophage libraries containing an ampicilin (amp) resistance gene, were diluted in TBS and allowed to infect XL-1-blue (100 μl, 30 min, 37°C). Infected bacteria were plated on amp (100 μg/ml) containing LB agar, incubated overnight (o/n) at 37°C and CFU were counted.

*Phage amplification*

The selected phage pool (10 μl) was incubated with 100 μl bacterial culture in the mid-log phase (30 min). Infected bacteria were plated on amp-containing agar plates and incubated o/n at 37°C. Colonies were dissolved in 10 ml SB/tet/amp medium and incubated for 30 min at 37°C. 200 μl was transferred to 10 ml SB/tet/amp (30 min, 37°C) and helper phage VSCM13 was added in a 20-fold excess (2 hours, 37°C). Kanamycin (125 μg/ml) was added and the culture was incubated o/n. Bacteria were centrifuged (5,000 rpm, 20 min) and phage containing supernatant was precipitated by addition of 2.5 ml 20% PEG/23.4% NaCl and 2 hour incubation on ice. Phages were centrifuged (10,000 rpm, 20 min) and the pellet was washed in TBS (30 min at 37°C).

*Phage selection*

Phage libraries ($10^{10}$ CFU, 1 μl in TBS) were incubated with 100 μg/ml proteinase-K at 0°C. After 1 hour, proteolysis was quenched by addition of 50 μl of buffer containing 1% BSA, 10 μM PMSF, 10 mM EDTA to inactivate proteinase-K. For bovine cathepsin-S selection, phage libraries ($10^{10}$ CFU in TBS) were incubated with 20 pmol enzyme for 24 hours at 37°C in 100 μl protease buffer (50 mM NaAc, 1 mM dithiothreitol (DTT), 1 mM EDTA, pH 5.5). Selection was stopped by adding 10 μl cathepsin inhibitor I (20 pmol in DMSO). Protease resistant phages were incubated with 100 μl *E.coli* XL-1 blue log culture (30 min, 37°C) and the suspension was incubated o/n at 37°C on LB/amp plates. Phages were titrated, amplified and purified as described[12]. For DNA sequencing, plasmid DNA was isolated from single

colonies using the Wizard Plus SV miniprep DNA purification System (Qiagen, Westburg, Belgium). DNA sequencing was conducted at the Leiden Genome Technology Centre (LGTC), Leiden, The Netherlands, using a standard M13 primer[12].

*Evaluation of proteinase-K inhibition by SDS-PAGE*

Phages ($10^9$ CFU) were incubated with BSA (10 μg) and proteinase-K (50 μg) at 0°C (in PBS, 100 μl). After 1 hour PMSF (1 mM, 5 μl) was added. Degradation of BSA was evaluated by SDS-PAGE (15%). Proteins were stained with Bio-Safe Coomassie brilliant blue G250 (Bio-rad laboratories, Hercules, CA, USA).

*Peptide synthesis*

Peptide sequences (K1: VLIMPVLLGIPLLCY; K1-C: VLIMPVLLGIPLLY, S1: VWNCERITISRLIN; S2: c-LRNSPRKQADRIL-c) were synthesized according to standard Fmoc solid phase chemistry. The terminal tyrosine was added to allow (radio) labelling of the peptide. After cleavage from the resin and simultaneous deprotection in 94.5% trifluoroacetic acid, 2.5% ethane-dithiol, 2.5 % $H_20$ and 0.5% tri-isopropylsilane, the crude peptides were precipitated by diethylether and purified on a reverse phase C18 column (Alltech, Deerfield, IL, USA) using a 20-90 % acetonitril/water gradient. Peptide mass and purity (> 70% for all peptides) was verified by LC-Mass Spectrometry.

*Evaluation of protease inhibition*

For the proteinase-K degradation assay, $^{125}$I-BSA (1 μg), iodated according to McFarlane[19], was incubated with proteinase-K (50 μg) in the presence or absence of K1 and K1-C peptide. Samples were incubated for 30 min at room temperature in triplicate. The reaction was quenched by addition of excess BSA (800 μg) and trichloroacetic acid (35% v/v; 200 μl) to precipitate BSA. After centrifugation the percentage degradation was calculated from the radioactivity in the supernatant (degraded BSA) relative to a control sample without peptide.

The inhibitory capacity of the S1 and S2 peptides was assessed by a fluorometric assay. Human cathepsin-S (5nM) was incubated in 100 mM NaAc, 2 mM EDTA and 1 mM DTT in 0.1% Brij 35, pH 5.5 in the presence or absence of peptide (0-70 μM; 0.25% DMSO/100 mM NaAc final concentration) for 15 min at RT. Cathepsin-S specific substrate (Z-Val-Val-Arg-AMC; 10 μM in 1% ethanol/0.002% DMSO/100 mM NaAc) was added and the A360/480 was monitored at 28°C during 60 minutes[20]. As a measure of residual enzymatic activity, the

slope in Relative Fluorescence Units/time (ΔRFU/min) was calculated for all individual samples. Percentage degradation was calculated relative to the control (in 0.25% DMSO/100 mM NaAc).

All $IC_{50}$ values were determined by non linear regression analysis for a single site binding model (Graphpad-prism 4.0).

*Cell culture and spheroid formation*

HT29 colon carcinoma cells were grown in DMEM/F12 (Invitrogen) supplemented with 10% heat inactivated Fetal Calf Serum (FCS, Perbio Science, Belgium), 10 mM HEPES, 50 μg/ml gentamycin, 100 U/ml penicillin and 100 μg/ml streptomycin (all Invitrogen). HT29 spheroids (2,500 cells/well) were generated by the agarose liquid overlay technique as described before[21], collected after 48 hour and incubated in 500 ng/ml human spleen cathepsin-S containing serum free (SF) DMEM/F12 in an agarose coated 48 well plates (10 spheroids/well in 100 μl). After 24 hour incubation conditioned medium (CM) was collected and applied to the HUVEC sprouting assay. Inhibition studies for the S1 peptide (stock 1 mg/ml in SF DMEM/F12) and the cysteine protease inhibitor E64 (in PBS, stock 1 mg/ml) were performed by pre-incubating cathepsin-S with the peptide inhibitors for 15 minutes at room temperature before addition to the HT29 spheroids.

Human umbilical vein endothelial cells (HUVEC) were isolated according to Jaffe *et al.*[22]. Cells (passage 6-9) were grown in fibronectin coated flasks (0.05 mg/ml) in M199 medium (Invitrogen) supplemented with 20% FCS, 100 U/ml penicillin and 100 μg/ml streptomycin, 2 mM glutamin (Invitrogen), 0.05 mg/ml heparin and 12.5 μg/ml endothelial growth factor supplement (Sigma-Aldrich).

*HUVEC sprouting assay*

HUVEC spheroids were prepared as described before[23]. In brief, HUVEC cells were suspended in complete M199 medium containing 0.1% carboxymethylcellulose (Sigma-Aldrich) and seeded into 96 well U-shape suspension culture plates (Greiner Bio-one, The Netherlands, 750 cells/well). After 24 hour incubation HUVEC spheroids were embedded in 33% collagen type I (1 mg/ml, Vitrogen 100, Nutacon, CA, USA). The HUVEC spheroids were treated with HT29 spheroid CM. Sprout formation was determined after overnight incubation and scored on a 0 (absent) - 5 (abundant) scale in a double blinded manner.

Experiments were performed in triplicate. Representative photomicrographs were made using a Zeiss Axiovert 200 microscope.

## *Results*

*Screening for protease inhibitors*

We developed a method to select potential protease inhibitors by the use of the phage degrading capacity of proteases. Excess of protease is used to create the highest possible selection pressure. Only phages displaying peptides capable of resisting protease treatment survive selection (Figure 1).

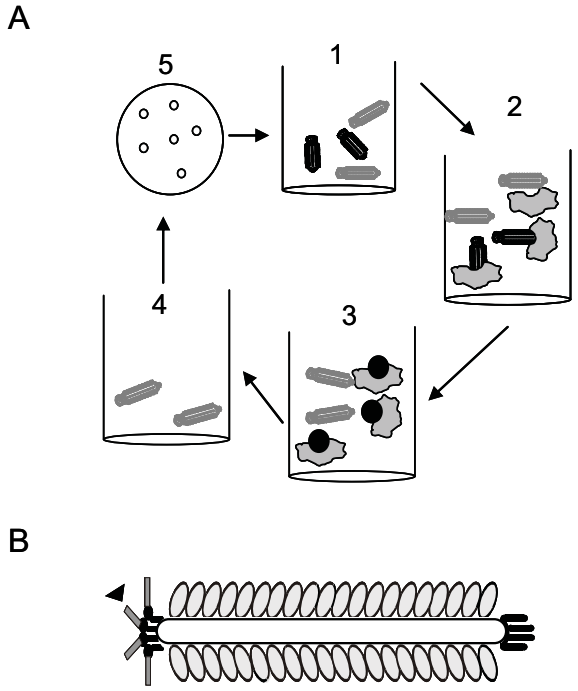


*Figure 1. Concept of degradation-aided selection of protease inhibitors (DASPI). The phage library displaying different peptides (step 1) is incubated with a protease (step 2) to degrade non-inhibiting phages. A protease inhibitor is added to the mixture (step 3) to stop the selection procedure by inactivating the protease. In step 4 and 5 the non-degraded phages are recovered and phages are amplified. Amplified phages are then subjected to a second selection round and the procedure is repeated until sufficient enrichment is achieved. (B) Schematic presentation of the pComb3 phage library with displayed peptides (arrowhead).*

We incubated proteinase-K or cathepsin-S with the pComb8 (CX15C) library and observed more than 99.9% of the phages being degraded by proteinase-K and 99.8% by cathepsin-S. Other proteases including cathepsin B and calpain, although less potent (respectively 99.7% and 99.6% phage degradation), still were capable of degrading phages and therefore creating selection pressure.

For selection, all phage libraries were incubated with proteinase-K (proof of principle), or, therapeutically interesting, cathepsin-S. After proteolysis, non-degraded phages were isolated, amplified, and used as template for a subsequent selection round. After four selection rounds the 6-mer libraries did not show any enrichment during the selection.

In contrast, the pComb3 15-mer libraries showed that input-to-output ratios were increased by 40,000-fold for proteinase-K (Figure 2A) and 3,500-fold for cathepsin-S (Figure 2B), reflecting a substantial enrichment of protease resistant phages.

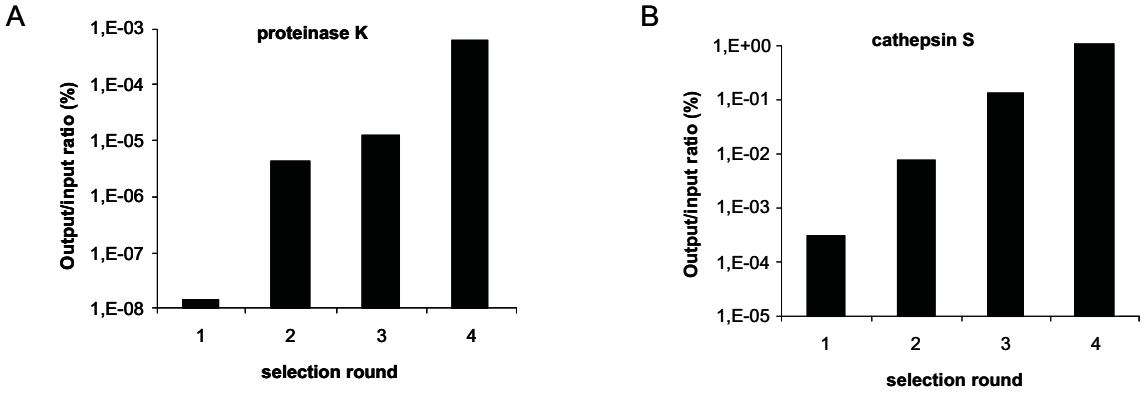


*Figure 2. A) Selection for proteinase-K resistant phage clones in the pComb3 library resulted in a 40,000-fold enrichment of proteinase-K resistant phage after 4 selection rounds. B) Selection for cathepsin-S resistance resulted in a 3,500-fold enrichment of cathepsin-S-resistant phages in the 4th selection round, using the pComb3 library. All values are expressed as the ratio between output and input titer (%).*

DNA sequence analysis revealed for proteinase-K, that 70% of the phage clones contained the VLIMPVLLGIPLLC (K1, table 1) sequence, suggesting that this sequence may be instrumental in proteinase-K resistance. Furthermore, two phage clones, K2 and K3 were identified with highly homologous peptide inserts (10 out of 15 amino acids, table 1).
Selection of the pComb3 and pComb8 libraries for cathepsin-S resistant clones revealed two major peptide sequences (S1: VWNCERITISRLIN; 50% and S2: c-LRNSPRKQADRILN-c; 60%; table 1).

*Table 1. Peptide sequences identified by DASPI*

| Protease | Library | Enrichment** | Peptide sequences | Code | Occurrence (%) |
|---|---|---|---|---|---|
| Proteinase-K | pComb3 (X15) | 40,000 | VLIMPVLLGIPLLC* | K1 | 70% |
| | | | VXNCERITISRILN* | K2 | 10% |
| | | | VXNCERITISQNSKL | K3 | 10% |
| | | | RSLNHASSFGYSVIM | | 10% |
| Cathepsin-S | pComb3 (X15) | 3,500 | VWNCERITISRLIN* | S1 | 50% |
| | pComb8 C(X15)C | 200 | c-LRNSPRKQADRILN*-c | S2 | 60% |

***As compared to non-selected phage.* signifies a stopcodon, which is ignored and overran during transcription in XL-Blue and allows full display of the encoded peptide*

*Proteinase-K inhibition by K1*

To examine if clones K1, K2, and K3 were more resistant to proteinase-K degradation than non-selected phages, individual clones were amplified and tested for their ability to interfere with proteinase-K degradation. Selected phage pools withstood proteinase-K degradation better than non-selected phages, which were completely degraded (Figure 3A). Besides being more resistant to proteinase-K degradation, the phages also conferred protection of BSA to proteinase-K degradation (Figure 3B).

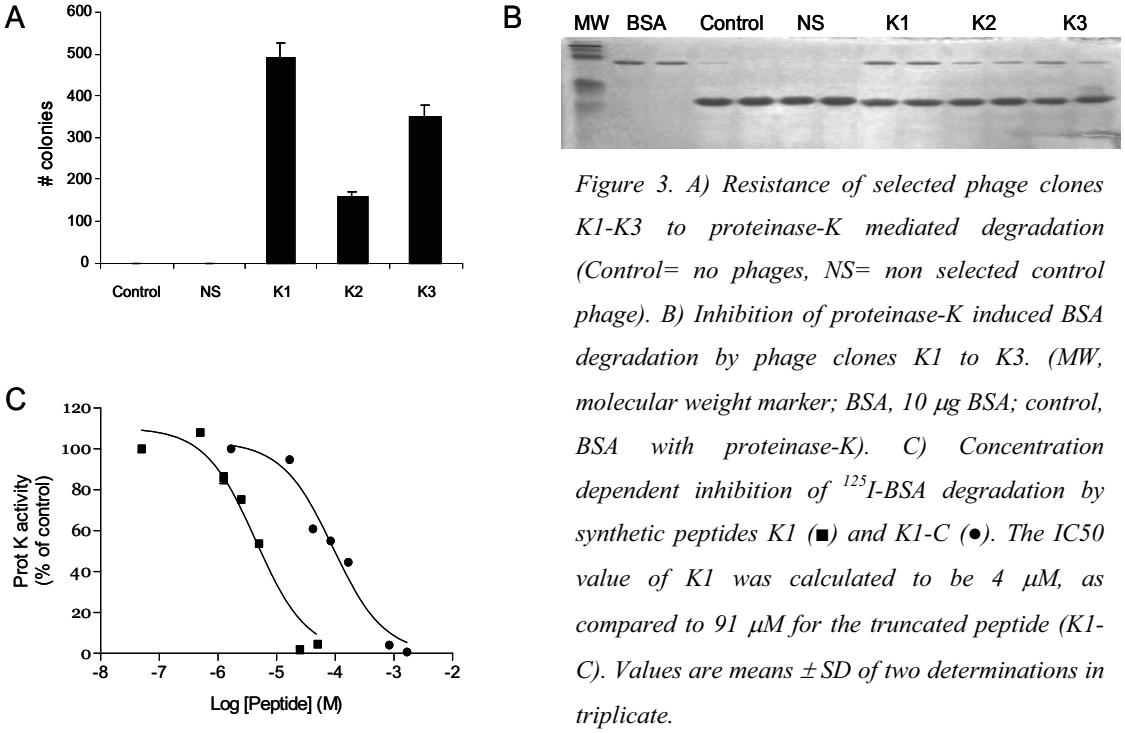


*Figure 3. A) Resistance of selected phage clones K1-K3 to proteinase-K mediated degradation (Control= no phages, NS= non selected control phage). B) Inhibition of proteinase-K induced BSA degradation by phage clones K1 to K3. (MW, molecular weight marker; BSA, 10 μg BSA; control, BSA with proteinase-K). C) Concentration dependent inhibition of $^{125}$I-BSA degradation by synthetic peptides K1 (■) and K1-C (●). The IC50 value of K1 was calculated to be 4 μM, as compared to 91 μM for the truncated peptide (K1-C). Values are means ± SD of two determinations in triplicate.*

Next, we investigated whether synthetic peptides corresponding with the peptide sequence insert of the selected phage clones also could inhibit protease activity. VLIMPVLLGIPLLCY (K1), a truncated K1 mutant lacking the C-terminal cysteine (K1-C), VWNCERITISRLIN (S1) and c-LRNSPRKQADRIL-c (S2) were synthesized. Both K1 and K1-C peptides were able to inhibit $^{125}$I-BSA degradation by proteinase-K in a dose-dependent manner (Figure 3C) with $IC_{50}$ values of 4 and 91 μM, respectively. Maximal inhibition was more than 95% at 50 μM [K1]. The considerably lower potency of K1-C suggests that the C-terminal cysteine may be important for proteinase-K inhibition.

*Functional cathepsin-S inhibition by S1*

The inhibitory capacity of the cathepsin-S peptides S1 and S2 was tested in a fluorometric activity assay. Both peptides showed concentration dependent inhibition of cathepsin-S with $IC_{50}$ values of 13 and 26 μM, respectively (Figure 4A). S1 gave a maximum inhibition of 75 % at 70 μM, as compared to 45% for S2.

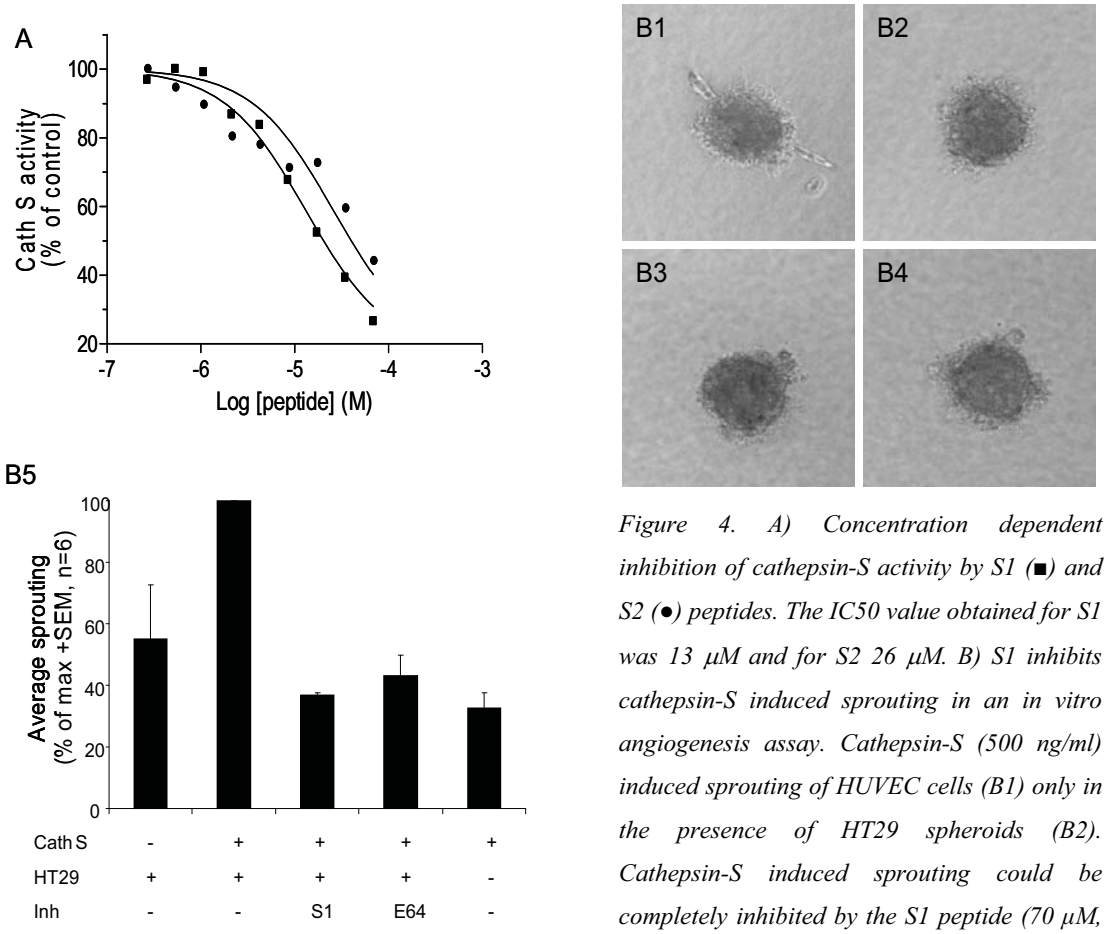


*Figure 4. A) Concentration dependent inhibition of cathepsin-S activity by S1 (■) and S2 (●) peptides. The IC50 value obtained for S1 was 13 μM and for S2 26 μM. B) S1 inhibits cathepsin-S induced sprouting in an in vitro angiogenesis assay. Cathepsin-S (500 ng/ml) induced sprouting of HUVEC cells (B1) only in the presence of HT29 spheroids (B2). Cathepsin-S induced sprouting could be completely inhibited by the S1 peptide (70 μM, B3) comparable to E64, a reference cysteine protease inhibitor (B4, 70 μM). Semi-quantitative analysis (B5) shows complete inhibition of cathepsin-S induced sprout formation by S1 and E64. Data are given as average percentage sprouting, ± SEM, n=6.*

The DASPI selected cathepsin-S inhibitors were tested for functionality in a HUVEC sprouting assay. Conditioned medium (CM) from cathepsin-S-treated HT29 spheroids induced HUVEC sprout formation in a dose dependent manner (0-1000 ng/ml, not shown). Cathepsin-S (500 ng/ml) induced sprouting (Figure 4B1/B5) depended on the presence of HT29 spheroids (Figure 4B2/B5), probably reflecting cathepsin-S induced release of pro-angiogenic factors. The S1 peptide (70 μM) completely blunted this cathepsin-S induced sprout formation (Figure 4B3/B5). CM from 0 ng/ml cathepsin-S treated HT29 spheroids

showed a minor increase compared to CM that was not incubated with HT29 spheroids, likely attributable to endogenous cathepsin-S secreted by the HT29 spheroids (cathepsin-S activity assay, not shown). As a control we included the cysteine protease inhibitor E64, which showed a similar dose-dependent inhibition (70 μM, Figure 4B4/B5) as observed for the DASPI selected inhibitor. These data illustrate that the DASPI selected S1 peptide is an effective cathepsin-S inhibitor, not only *in vitro* but also in a complex multi-cellular system.

## *Discussion*

The identification of culprit proteases and their role in the release of growth factors, chemoattractants and angiogenic molecules is critical to numerous (patho)-physiological processes. Specific protease inhibitors would greatly facilitate functional and therapeutic studies. The data presented in this study show that DASPI is an effective strategy, to select functionally active protease inhibitors. The mechanism of selection is based on inhibition of the protease by the phage displayed peptide. The pComb3 libraries display the peptide on the minor coat protein (Figure 1b), which makes it sterically possible to bind the active site of the protease. Although it seems unlikely that the displayed 15-mer peptide could prevent the whole phage from degradation there are several lines of evidence. In the first selection round 99.9% of the phages are degraded while after 4 rounds, 40,000 fold enrichment is observed and 70% of the phages contain an identical peptide sequence. These phages resist protease degradation much better than non-selected phages which are completely degraded by the protease. Secondly, synthetic peptides corresponding to the sequences obtained in selection show indeed to be potent protease inhibitors. Protease inhibition by the phage might be occurring via binding of the displayed peptide in the active site of the protease and thereby preventing its proteolytic activity. As the 6-mer libraries show no enrichment during selection, probably 6 amino acid peptides are too short to bind the active site.

Proteinase-K-resistant phage clones K2 and K3, the less inhibiting sequences, did not exhibit significant homology to known protease inhibitors after discontinuous blast search. The most abundant and most inhibiting sequence K1 revealed a considerable homology (77%) with a short peptide stretch of the Kuniz-type serine-protease inhibitor from *C. elegans* (nine C-terminal amino acids including the terminal cysteine residue). Furthermore it showed homology to the phage coat protein D, but as non-selected phages are completely degraded by proteinase-K is it unlikely that non-specific inhibition by one of the phage coat proteins occurs. Furthermore the synthetic K1 peptide appeared to be a potent proteinase-K inhibitor

($IC_{50}$=4 μM). A truncated peptide lacking the C-terminal cysteine (K1-C) had an almost 25-fold lower inhibitory activity, suggesting that the C-terminal cysteine is critical to proteinase-K inhibition. These data show that DASPI is able to identify inhibiting sequences homologous to known protease inhibitors, but also new sequences.

Similarly, we have identified an inhibiting peptide sequence (S1) for cathepsin-S ($IC_{50}$=13 μM), which showed homology with serine-protease inhibitor 8 from *R. norvegicus.* Cathepsins are the major lysosomal proteases involved in degradation of incorporated drug- and gene delivery systems, as well as in cleavage of extracellular matrix components[24, 25]. Cathepsin-S is therapeutically of great interest because of its immuno-regulatory properties and involvement in atherosclerosis[26,27]. Furthermore it is regarded as a prognostic marker in cancer[28,29] and was shown to contribute to cancer progression by facilitating angiogenesis and cell migration leading to metastasis formation[5,28-30]. Several small inhibitors for cathepsin-S[20,31] have been devised and evaluated for therapeutic potential. A major hurdle in the design of inhibitors is the general lack of specificity, as the catalytic clefts of most serine- and cysteine-proteases are very similar in nature. A biased strategy towards identification of functional inhibitors rather than binders may facilitate the design of novel inhibitors with more favourable features in that regard. Further incorporation of optimized DASPI selected peptide inserts into gene or drug vehicles may reduce the susceptibility of these vectors to lysosomal elimination by endopeptidases[2] such as cathepsin-S. We demonstrate here that the S1 peptide is equally able to inhibit cathepsin-S in an endothelial sprouting model as the cysteine-protease inhibitor E64. This confirms that S1 not only inhibits at a biochemical level but also functionally inhibits cathepsin-S induced angiogenesis. DASPI selected peptides could therefore be valuable lead compounds in the design of specific inhibitors to reduce cathepsin-S activity in the aforementioned disorders.

In summary, we have shown that degradation-aided selection of phage display libraries for protease inhibitors is a rapid and powerful strategy to identify novel peptide sequences that confer resistance to and act inhibitory on the protease. We do not claim to have identified highly potent inhibitors for the mentioned proteases, but show that the pursued strategy may yield promising leads in the generation of dedicated protease inhibitors as it, unlike other methods, biases towards functionally active inhibitors. Moreover, DASPI is widely applicable to any protease, provided it is able to digest and/or inactivate phages. Proteases, which are not capable of degrading phages, may even serve as target after prior engineering of the phage libraries to render them susceptible to these proteases.

## *Ackowledgements*

We thank Prof. H. Pannekoek, Amsterdam Medical Centre, University of Amsterdam, the Netherlands for supplying the phagemid libraries and Dr. H.W. Verspaget, Leiden University Medical Centre, Leiden, the Netherlands for helpful suggestions and support. This work was supported by CW/STW project 349-4779 (SR) and the Netherlands Heart Foundation, project 2003T201 (EB).

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