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# High throughput testing of drug library substances and monoclonal antibodies for capacity to reduce formation of cystatin C dimers to identify candidates for treatment of hereditary cystatin C amyloid angiopathy

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## Abstract

**Objective.** To establish a high-throughput system for testing the ability of drugs or monoclonal antibodies to reduce the *in vitro* formation of cystatin C dimers to identify substances potentially useful for treatment of patients with hereditary cystatin C amyloid angiopathy (HCCAA). **Methods.** Various combinations of incubation temperature, time period, guanidinium chloride concentration and concentration of cystatin C monomers were tested in low-volume formats to induce dimer formation of recombinant cystatin C. The extent of dimerization was analysed by gel filtration chromatography and agarose gel electrophoresis. **Results.** A high-throughput system based upon agarose gel electrophoresis was developed and used to test 1040 drugs in a clinical drug library for their capacity to reduce cystatin C dimer formation *in vitro*. Seventeen substances reducing dimer formation by more than 30% were identified. A similar system for testing the capacity of monoclonal antibodies against cystatin C to reduce the *in vitro* formation of cystatin C dimers was also developed and used to test a panel of 12 monoclonal antibodies. Seven antibodies reducing dimer formation by more than 30% were identified and the two most potent, Cyst28 and HCC3, reduced dimerization by 75 and 60%, respectively. **Conclusion.** We constructed a simple high-throughput system for testing the capacity of drugs and monoclonal antibodies to reduce the *in vitro* formation of cystatin C dimers and several candidates for treatment of HCCAA could be identified.

**Keywords:** Amyloidosis, brain hemorrhage, drug library screening

## Introduction

Human cystatin C is a low molecular mass protein (13.3 kDa) belonging to the cystatin superfamily of inhibitors of cysteine proteases of the papain and legumain families [1]. It is produced and secreted by all nucleated cells and is present as monomers in all body fluids [1]. In the dominantly inherited disease, hereditary cystatin C amyloid angiopathy (HCCAA), a leucine 68 to glutamine variant of cystatin C (L68Q-cystatin C) is deposited as amyloid in the cerebral arteries, resulting in brain hemorrhage and death in young adults [2]. Although carriers of the allele for L68Q-cystatin C may easily be identified by a polymerase-chain-reaction based diagnostic procedure [3, 4], no treatment to avoid early death by brain hemorrhage is available [2]. It has been suggested that the cystatin C amyloid fibrils are generated by propagated domain swapping between cystatin C monomers in which N- and C-terminal parts of the monomers are exchanged, forming long protein chains [5, 6]. Whereas L68Q-cystatin C easily forms dimers and amyloid fibrils *in vivo* and *in vitro* [7, 8], wildtype cystatin C requires mild denaturing conditions to form dimers and amyloid fibrils *in vitro* [6, 9]. Importantly, if variants of wildtype cystatin C and L68Q-cystatin C with intra-chain stabilizing disulfide bonds preventing domain swapping are produced, these variants cannot be induced to form neither dimers nor amyloid fibrils [6, 9]. This suggests that substances preventing domain swapping of cystatin C might be used for treatment of HCCAA. The objective of this investigation was to establish a simple high-throughput system for testing the ability of substances in a drug library or in a panel of monoclonal antibodies to reduce the *in vitro* formation of cystatin C dimers to identify agents potentially useful for treatment of patients with HCCAA.

## Materials

Chromatography columns were obtained from GE Healthcare (Uppsala, Sweden). Ion exchange using Q-sepharose Fast Flow was performed in a Pharmacia chromatography system run by a peristaltic pump. MonoQ-columns (MonoQ HR 10/10) and preparative gel filtrations (Superdex 75 HR 10/30) were run in an ÄKTA Fast Protein Liquid Chromatography (FPLC) system from GE Healthcare (Uppsala, Sweden). Analytical gel filtrations were performed on a Superdex 75 PC 3.2/30 column in a High-Pressure Liquid Chromatography (HPLC) system from Waters Sweden AB (Sollentuna, Sweden). Agarose (SeaKem LE) was purchased from BioWhittaker Molecular Applications (Rockland, ME, USA). Ultrafiltration devices and membranes (Diaflo 5 kDa CO) were from Amicon (Bedford, MA, USA). Microtiter plates (384-well, shallow, #264340) and polyolefin sealing tapes were from Nunc A/S (Roskilde, Denmark). Polypropylene tubes (0.5 mL) were purchased from Applied Biosystems (Carlsbad, CA, USA). All chemicals were of at least reagent grade and, unless specified, obtained from Sigma-Aldrich (Stockholm, Sweden).

### *US Drug Collection*

The US Drug Collection was obtained from Microsource Discovery Systems (Gaylordsville, CT, USA). It is a substance library with 1040 drug compounds already approved for use in humans. All compounds are either clinically used drugs or have reached clinical testing stages and 95% of the drugs are currently off patent. The US Drug Collection was supplied as 10 mmol/L stock solutions in dimethyl sulfoxide (DMSO) and stored at -80°C until used.

### *Monoclonal antibodies*

Twelve different murine monoclonal antibodies raised against human cystatin C, were tested for effects on cystatin C dimerisation. Eleven of the antibodies, denoted Cyst10, Cyst12, Cyst13, Cyst16, Cyst18, Cyst19, Cyst23, Cyst24, Cyst27, Cyst28 and Cyst29 are commercially available antibodies (HyTest Ltd, Turku, Finland) and one, HCC3, was prepared as described previously [10].

## **Methods**

### *Isolation of monomeric cystatin C*

Recombinant human wildtype cystatin C was expressed in *E coli* MC1061 and purified using a novel three-step protocol, based on a previously described two-step procedure [11]. Periplasmic extracts from heat-induced *E coli* MC1061 were purified by two subsequent anion exchange chromatography systems, using 20 mmol/L ethanolamine adjusted to two different pH, with 1 mmol/L of the protease inhibitor benzamidine chloride, as running buffers. Cystatin C-containing flow-through fractions from the first run at pH 9.0 (Q-sepharose Fast Flow) were identified by agarose gel electrophoresis, pooled, dialysed and concentrated by pressure ultrafiltration using concentrators with a cut-off of 5 kDa and subsequently applied to a second ion exchange column at pH 10.3 (MonoQ HR 10/10). The bound proteins were eluted using a linear salt gradient (0 – 20% of 1.0 mol/L NaCl in the running buffer) developed with 8 column volumes followed by a steeper linear salt gradient (20 – 100% of 1.0 mol/L NaCl in the running buffer) developed with one column volume. Eluted fractions were analysed by agarose gel electrophoresis and those containing cystatin C were again pooled and concentrated. Gel filtration, using a column of Superdex 75 HR 10/30 with a running buffer of 50 mmol/L phosphate-buffer, pH 7.4, with 50 mmol/L NaCl and 1 mmol/L benzamidine chloride (PBS), was employed as the final isolation step. By pooling and concentrating samples from several expression and isolation runs, a large batch of pure monomeric cystatin C in PBS was obtained. Its protein concentration was measured by UV absorption spectroscopy at 280 nm, using a molar extinction coefficient of  $\epsilon = 11,100 \text{ M}^{-1} \text{ cm}^{-1}$  ( $A_{280,0.1\%} = 0.83$ ) [12] and found to be 2.2 mg/mL. It was stored in 100-microlitre aliquots at  $-80^{\circ}\text{C}$  until used.

### *Assay optimization*

Aliquots of the cystatin C stock solution (2.2 mg/mL) were added to solutions of guanidinium chloride in PBS, giving final concentrations of guanidinium chloride varying from 0.25 to 1.0 mol/L and final cystatin C concentrations varying from 0.1 to 0.5 mg/mL. Portions of 10  $\mu\text{L}$  of the mixtures were distributed into the 25-microlitre wells of 384-well microtiter plates. DMSO, the vehicle of the US Drug Collection, was added to each well to a final concentration of 3.8% (v/v), the plates were sealed with sealing tapes and incubated for different time periods at 35, 37, 40 or  $42^{\circ}\text{C}$ . For each time point, the solutions of two wells were pooled in 0.5 mL tubes, centrifuged at 10,000 g for 10 minutes and samples of 20  $\mu\text{L}$  analysed in a HPLC system using a Superdex 75 PC 3.2/30 column for gel filtration, run in 150 mmol/L  $\text{NH}_4\text{HCO}_3$ , pH 7.4, with a flow rate of 0.1 ml/min. The eluate was monitored by its absorption at 280 nm and the dimeric fraction of each sample calculated from its chromatographic peak area expressed as a percentage of the total peak areas of monomeric and dimeric cystatin C (Figures 1 and 2).

To allow high-throughput studies of the capacity of many substances to reduce dimerisation of cystatin C, an agarose gel electrophoresis system was developed. Agarose gel slabs (1% w/v) with the dimension 1 x 100 x 200 mm were cast in 75 mmol/L barbiturate buffer, pH 8.6, with 2 mmol/L ethylenediaminetetraacetate (EDTA) as described [13] and stored in a closed box at 4°C until used. Forty 10-microlitre samples were applied in four rows and electrophoresis was run at 250 V for 40 minutes. After protein precipitation using picric acid, drying of the gel slabs, staining with Coomassie Brilliant Blue R-250, destaining and drying, protein band patterns were digitized by optical scanning at high resolution (600 dpi) in grayscale mode using an EPSON 1680 Pro flatbed scanner. Individual protein bands were quantitated by densitometric analysis using ImageJ software [14]. Dimerisation of cystatin C results in an increased anodal mobility of the protein so that a mixture of monomeric and dimeric cystatin C results in two protein bands after electrophoretic separation under native conditions in an agarose gel [9]. Figure 3 shows the application arrangement of the drug compounds on the agarose gels. Each gel could be used for analysing 40 drug compounds of the drug library used. The dimeric content in each sample was calculated from the intensities of the two bands.

#### *High-throughput screening assay*

The stock solution of monomeric cystatin C was diluted to a final concentration of 0.21 mg/mL with a guanidinium chloride solution in PBS to a final guanidinium chloride concentration of 1 mol/L. Portions of 10 µL of this mixture were pipetted into each well of the 384-well microtiter plates and then DMSO-solutions (0.4 µL) of the drug library or pure DMSO were added. The library compounds were added to final concentrations of 385 µmol/L, corresponding to a molar ratio to monomeric cystatin C of about 25:1, and producing a final concentration of DMSO of 3.8% (v/v). The final concentrations of cystatin C and guanidinium chloride in the incubation mixture were 0.2 mg/mL and 0.96 mol/L, respectively. To reduce sample evaporation, the plates were sealed with sealing tapes before incubation at 40°C for 24 hours. The same incubation conditions were used for monoclonal antibodies. The incubation temperature of 40°C was selected, because it was the lowest of those tested allowing use of a relatively short incubation period of 24 hours to achieve a cystatin C dimerisation of 60% in solutions without additions of drugs or monoclonal antibodies. All antibodies were added from stock solutions in PBS (concentration range from 2.4 to 6.1 mg/mL) directly into wells containing cystatin C (final concentration 0.4 mg/mL) yielding a final antibody concentration of 1.25 mg/mL. Inhibition of dimer formation by each substance or antibody was calculated using the equation:

$$\% \text{ Inhib} = 100 \times \left[ 1 - \left( \frac{\frac{\text{Dimers}_{\text{Sample}}}{\text{Dimers}_{\text{Sample}} + \text{Monomer}_{\text{Sample}}}}{\frac{\text{Dimer}_{\text{Control}}}{\text{Dimer}_{\text{Control}} + \text{Monomer}_{\text{Control}}}} \right) \right]$$

The determinations of the inhibitions of dimer formation by the monoclonal antibodies were repeated 5 times for each antibody and the standard deviation of the percentages of inhibition was below 5% for all antibodies, except for one antibody (Cyst10), which partially precipitated during incubation.

## Results

### *Development of a high-throughput testing system*

In order to develop a high-throughput testing system to identify substances which reduce dimer formation of cystatin C and requires only minimal amounts of isolated monomeric cystatin C, we modified a system previously used to induce dimer formation of cystatin C [9]. To ensure reproducibility during optimization and screening experiments, a large homogenous batch of purified recombinant monomeric cystatin C was produced and used for all experiments. An additional ion exchange step was used during protein isolation, as described in *Methods*, to remove trace amounts of contaminating proteins with isoelectric points just below that of cystatin C (pI 9.3). The conditions used to dimerise cystatin C were optimized and selected so that about 60% of the protein dimerised after incubation for 24 hours and analytical gel filtration was used to measure the degree of dimerisation (Figures 1 and 2). However, gel filtration is not suitable for high-throughput analysis and was therefore replaced by an agarose gel electrophoresis system followed by densitometric scanning for assessment of dimerisation (Figures 3 and 4). This system allows determination of the dimer fraction in 40 samples in about 2 hours, when one agarose gel slab is run. Several gel slabs may be run simultaneously, thus increasing the analytical capacity accordingly. The amount of cystatin C required in the new dimerisation system was reduced compared to that of the previous system [9] by use of a lower cystatin C concentration (0.2 vs 0.5 mg/mL) and a smaller incubation volume of 10.4  $\mu$ L. To test the capacity of a substance to reduce dimerisation of cystatin C, using the new system, thus requires only about 2  $\mu$ g of isolated cystatin C.

### *Screening of a drug library*

A clinical drug library containing 1040 drugs, approved by the US Food and Drug Administration for use in humans, was screened for substances reducing cystatin C dimerisation. An approximately 25-fold molar excess of drug was used in the incubation mixtures (385  $\mu$ mol/L of drug and 15  $\mu$ mol/L of monomeric cystatin C) and controls containing only the drug vehicle DMSO were run in parallel on each agarose gel slab (Figure 3). Thirty of the drugs reduced dimer formation by more than 20% (Table I). The most potent compound identified, dobutamine hydrochloride, reduced dimer formation by 74%. Six compounds showed more than 50% inhibition of dimer formation, namely dobutamine hydrochloride, bithionate sodium, norepinephrine, carboplatin, hydroquinone and meclocycline sulfosalicylate. Among the 17 drugs reducing dimer formation by more than 30% were two catecholamines (dobutamine hydrochloride, norepinephrine), two proton pump-inhibitors (lansoprazole, pantoprazole), two platinum-based compounds (carboplatin, cisplatin) and four tetracycline analogues (meclocycline sulfosalicylate, oxytetracycline, methacycline hydrochloride, doxycycline hydrochloride). Four drugs (benzerazide, cefpodoxime proxetil, vinblastine sulfate, erythromycin estolate) increased dimer formation by more than 20% with the 44% increase induced by erythromycin estolate as the highest value. In some mixtures (about 5%) precipitation phenomena or complex formations occurred and distorted the electrophoretic pattern so that no evaluation of the concentrations of dimers/monomers could be done (see Figure 3).

### *Screening of a panel of monoclonal antibodies against cystatin C*

A panel of 12 different monoclonal antibodies against cystatin C was also screened for antibodies reducing cystatin C dimer formation using the high-throughput testing system described above with small modifications. No DMSO was used in the system

and the antibodies, in PBS, were tested at a final concentration of 1.25 mg/mL with a final concentration of cystatin C of 0.4 mg/mL, which corresponds to an approximate antibody:cystatin C molar ratio of 1:4. According to the densitometric scanning of the agarose gel electropherograms, the monoclonal antibodies displayed from 4 to 75% inhibition of dimer formation (Figure 4). One of the antibodies (Cyst10) precipitated during incubation. Four antibodies displayed more than 50% inhibition of dimer formation (Cyst28, HCC3, Cyst16, Cyst23). The two most potent antibodies, Cyst28 and HCC3, reduced dimer formation by 75 and 60%, respectively.

## Discussion

Systems for high-throughput testing to identify agents reducing cystatin C dimerisation and amyloid fibril formation *in vitro* are important tools for selecting substances, which might be used as drugs for prevention of the amyloid formation and lethal brain hemorrhage of persons with HCCAA. The present system was constructed to produce about 60% dimerisation of cystatin C in the absence of additions to the system, since influences of added substances will be easier to identify, whether dimerisation is reduced or increased, compared to systems inducing 100% dimerisation in the absence of additions. The selection of a 60% dimerisation endpoint was also influenced by the observation that a higher degree of dimerisation would require an incubation period considerably longer than 24 hours (Figure 2). The testing system for reduction of dimerisation developed in the present investigation was not only quicker than previously described testing systems [9] but also required much less cystatin C. Analysis of the capacity of the 1040 substances in the US Drug Collection and of 12 monoclonal antibodies to reduce dimerisation of cystatin C required only about 2.2 mg cystatin C.

The reason that wildtype cystatin C, rather than L68Q-cystatin C, was used in the present study is that it is extremely difficult to produce sufficient amounts of monomeric L68Q-cystatin C for extensive experiments [2,7] and that previous investigations have proven that wildtype cystatin C, under slightly denaturing conditions, shows similar unfolding properties as L68Q-cystatin C under physiological conditions [6,9]. Nevertheless, before *in vivo* studies can be performed, it is required that the most promising drug candidates are also tested using L68Q-cystatin C. Pilot studies of a few drugs and antibodies, reducing formation of dimers of wildtype cystatin C at the conditions employed in this study, were therefore undertaken and demonstrated that they also reduced the spontaneous formation of dimers of L68Q-cystatin C at 37°C in non-denaturing buffers at neutral pH. This suggests that the present *in vitro* results are relevant for *in vivo* conditions.

One reason to select the US Drug Collection for testing of substances capable of reducing dimerisation was that most substances in this collection are already clinically used and therefore already have been extensively tested for adverse side effects in humans and animals. The decision to additionally test a panel of murine monoclonal antibodies was based upon the successful present use of many monoclonal antibodies for treatment of several human diseases [15]. The complete mRNA sequences coding for the light and heavy chains of one of the two most potent antibodies (HCC3) have recently been determined (unpublished results), facilitating the development of a humanised similar monoclonal antibody with less antigenicity in human subjects.

Among the 17 drugs reducing dimer formation by more than 30% in this assay, are several catecholamines, proton pump-inhibitors, platinum-based compounds and four different tetracycline analogues. It is encouraging that all of these drugs already have been approved for use in humans and some may be administered at high doses for long periods without adverse side effects. Interestingly, previous *in vitro* and *in vivo* studies suggest that tetracycline and structural analogues may substantially reduce abnormal protein accumulation in various other forms of amyloidoses with different monomeric protein precursors involved in the formation of amyloid. Recent examples include reduction of *in vitro* fibril formation by  $\beta_2$ -microglobulin (dialysis-related amyloidosis) [16], transthyretin (familial amyloidotic polyneuropathy) [17], prion protein (Creutzfeldt-Jakob disease) [18], amylin/IAPP (diabetes mellitus type II) [19],  $\alpha$ -synuclein (Lewy body dementia and Parkinson's disease) [20] and amyloid- $\beta$  (Alzheimer's disease) [21]. In addition, tetracycline administration has been shown to delay diabetes onset and suppress disease progression in a transgenic hAmylin/IAPP mouse model of diabetes mellitus type II [22].

Although the present study shows that the formation of cystatin C dimers can be reduced by some drugs and monoclonal antibodies against cystatin C, the molecular mechanisms involved are unknown. For example, dimer production may be inhibited by stabilisation of monomeric cystatin C so that its propensity for domain swapping is reduced. Another possibility is that certain drugs and antibodies precipitate dimeric cystatin C directly after its formation, or convert dimeric cystatin C into monomers. Further steps in the development of treatment options for HCCAA will therefore have to comprise not only selection of the most promising drugs and monoclonal antibodies of the present work and studies of their dose response curves for reduction of amyloid formation and dimerisation of wildtype- and L68Q-cystatin C both *in vitro* and *in vivo*, but also elucidation of the molecular mechanisms involved. Interestingly, drugs reducing formation of dimers of amyloid-producing proteins, also have the potential to reduce formation of toxic oligomers known to occur in several types of amyloidosis [6, 23, 24].

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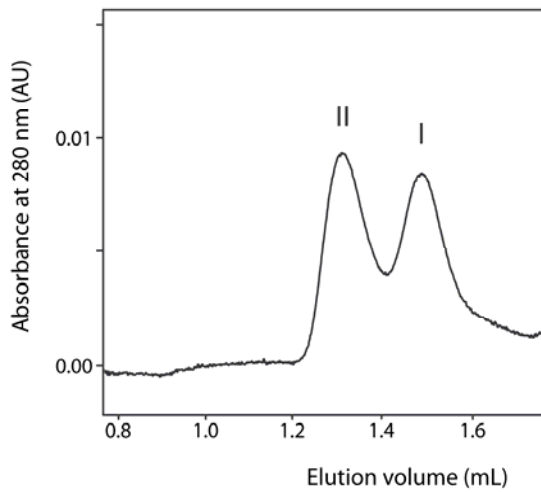
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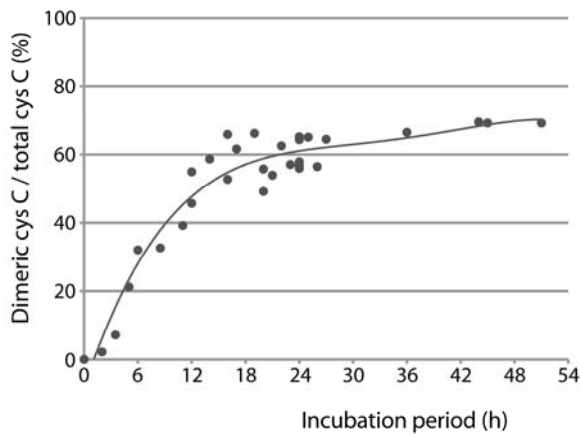


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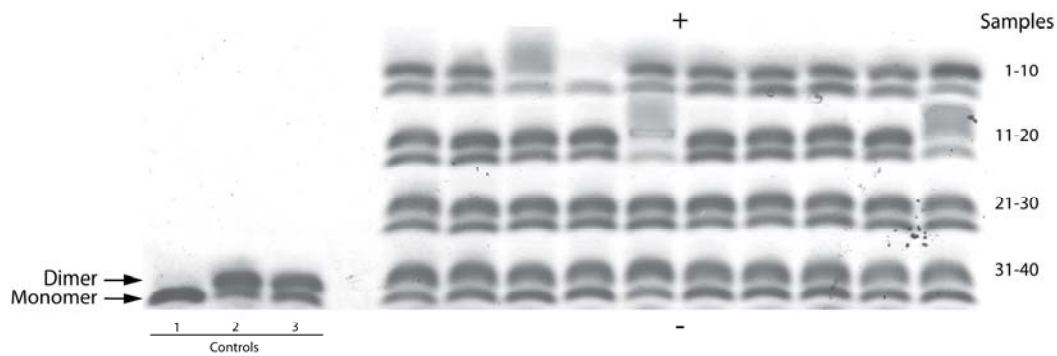
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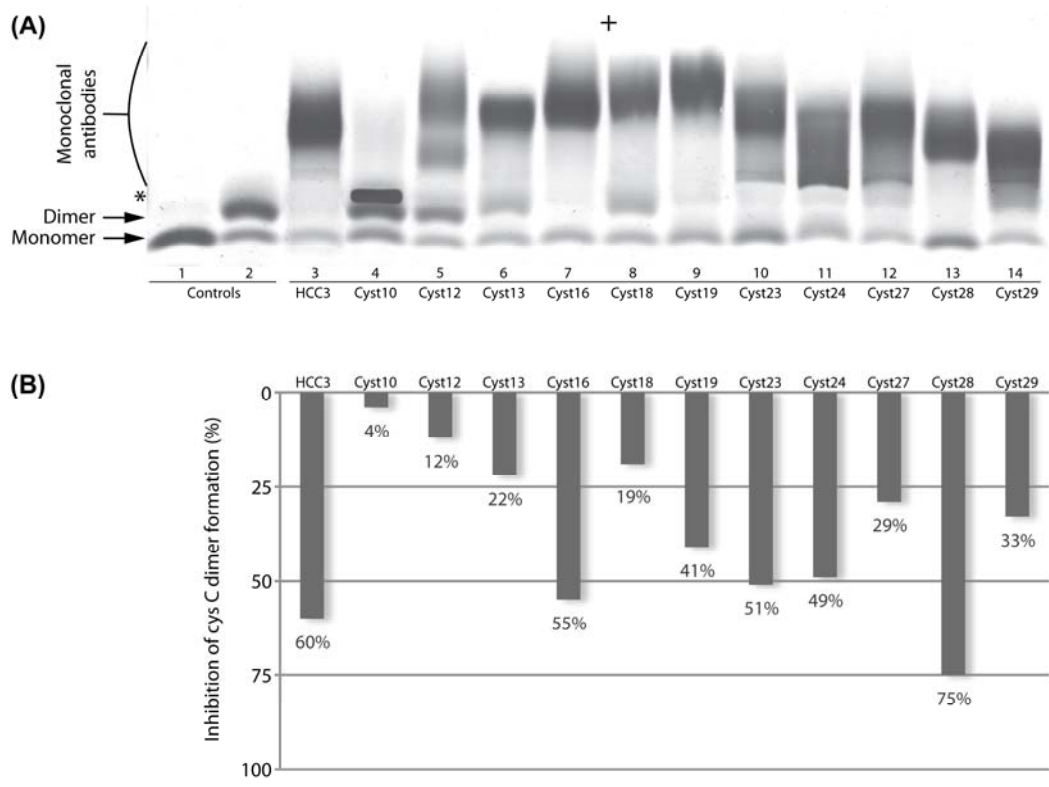
**Figure 1.** Gel filtration chromatography of a solution of monomeric and dimeric cystatin C. A solution of monomeric cystatin C, 0.2 mg/mL in PBS buffer with 0.96 mol/L guanidinium chloride and 3.8% DMSO (v/v), was heated to 40°C for 24 h and applied to the column. A Waters HPLC system was used to run the analytical Superdex 75 column in 150 mmol/L ammonium bicarbonate, pH 7.4, with a flow rate of 0.1 ml/min. The effluent was monitored for light absorption at 280 nm. The peaks containing monomeric and dimeric cystatin C are labelled I and II, respectively.



**Figure 2.** Cystatin C dimer formation. Solutions of monomeric cystatin C, 0.2 mg/mL in PBS buffer with 0.96 mol/L guanidinium chloride and 3.8% DMSO (v/v), were heated to 40°C for different periods and their content of dimeric cystatin C analysed by gel filtration. Each dot shows the results for one sample.



**Figure 3.** Agarose gel electropherogram showing the capacity of 40 drugs in a drug library to reduce dimerisation of cystatin C. Solutions of monomeric cystatin C, 0.2 mg/mL in PBS buffer with 0.96 mol/L guanidinium chloride, were incubated at 40°C for 24 h in the absence (controls) and presence of 385  $\mu\text{mol/L}$  of 40 drugs in the “US Drug Collection” and analysed by horizontal agarose gel electrophoresis. The controls are monomeric cystatin C before heat induction of dimerisation (1), the cystatin C solution after heat induction without (2) and with 3.8% (v/v) DMSO (3). The four application lanes display the capacity of the 40 drugs to reduce dimerisation of cystatin C. The drugs were added in DMSO to the solution of monomeric cystatin C and the final DMSO concentration was 3.8% (v/v). Sample number 4 contains dobutamine and shows a significant reduction of cystatin C dimerisation. Samples 3, 15 and 20 produce patterns which do not allow estimation of dimer/monomer concentrations. The anode (+) and cathode (-) are indicated.



**Figure 4.** The capacity of 12 monoclonal antibodies to reduce dimerisation of cystatin C. (A) Solutions of monomeric cystatin C, 0.4 mg/mL in PBS buffer with 1.0 mol/L guanidinium chloride, were heated to 40°C for 24 h in the absence (lane 2) and presence of 12 different monoclonal antibodies against cystatin C and analysed by agarose gel electrophoresis. Lane 1 shows the solution of monomeric cystatin C before heat induction of dimerisation. Lanes 3 – 14 show the effects on dimerisation by 12 different monoclonal antibodies, denoted HCC3 and Cyst10 – 29. The antibodies show differing mobilities related to their isoelectric points. Antibody Cyst10 precipitated during incubation and the given percentage for its inhibition of dimer formation is therefore unreliable. Point of sample application (\*) and anode (+) are indicated. (B) Graph showing the percentage reduction of dimer formation produced by the different monoclonal antibodies, as determined by densitometric scanning of the electropherograms in A.

**Table I.** Reduction of cystatin C dimer formation by drugs in a clinical drug library. Ranking list of the 30 most potent substances of the 1040 present in the drug compound library “US Drug Collection”. All substances were tested at a concentration of 385  $\mu$ M in an assay volume of 10.4  $\mu$ L, as described in *Methods*. The inhibition percentages represent the means of two runs.

	<i>Drug name</i>	<i>Molecular mass (Da)</i>	<i>Drug action</i>	<i>Inhibition of dimer formation (%)*</i>
1	Dobutamine hydrochloride	338	Cardiotonic	74
2	Bithionate sodium	400	Antihelmintic, antiseptic	73
3	Norepinephrine	169	Adrenergic agonist	53
4	Carboplatin	371	Antineoplastic	52
5	Hydroquinone	110	Antioxidant	52
6	Meclocycline sulfosalicylate	695	Antibacterial	51
7	Cetylpyridinium chloride	340	Antiinfective (topical)	49
8	Oxytetracycline	497	Antibacterial	48
9	Lansoprazole	369	Antiulcerative	48
10	Methacycline hydrochloride	479	Antibacterial	48
11	Colistimethate sodium	1736	Antibacterial	48
12	Suramin	1429	Antiprotozoal, antiviral	44
13	Cephalothin sodium	418	Antibacterial	43
14	Cisplatin	300	Antineoplastic	37
15	Doxycycline hydrochloride	481	Antibacterial	36
16	Tannic acid	1701	Astringent	36
17	Pantoprazole	383	Antiulcerative	35
18	Minocycline hydrochloride	494	Antibacterial	30
19	Dibucaine hydrochloride	380	Anesthetic (local)	30
20	Ethylnorepinephrine hydrochloride	234	Bronchodilator	30
21	Pyrrvinium pamoate	770	Antihelmintic	30
22	Methyldopa	211	Antihypertensive	29
23	Enoxaparin sodium	1859	Antithrombotic	27
24	Gramicidin	1141	Antibacterial	25
25	Gallic acid	170	Antineoplastic, antibacterial	25
26	Trifluridine	296	Antiviral	25
27	Sulfisoxazole acetyl	309	Antibacterial	23
28	Cetrimonium bromide	364	Antiinfectant	22
29	Rolitetracycline	528	Antibacterial	21
30	Isoproterenol hydrochloride	248	Bronchodilator	21

\*In each assay run, control dimer formation was evaluated in the presence of drug vehicle (DMSO) only.