ORIGINAL ARTICLE

Gelatin degradation assay reveals MMP-9 inhibitors and function of O-glycosylated domain

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Abstract

AIM: To establish a novel, sensitive and high-throughput gelatinolytic assay to define new inhibitors and compare domain deletion mutants of gelatinase B/matrix metalloproteinase (MMP)-9.

METHODS: Quenched Dye-quenched (DQ)™-gelatin was used as a substrate and biochemical parameters (substrate and enzyme concentrations, DMSO solvent concentrations) were optimized to establish a high-throughput assay system. Various small-sized libraries (ChemDiv, InterBioScreen and ChemBridge) of hetero-cyclic, drug-like substances were tested and compared with prototypic inhibitors.

RESULTS: First, we designed a test system with gelatin as a natural substrate. Second, the assay was validated by selecting a novel pyrimidine-2,4,6-trione (barbiturate) inhibitor. Third, and in line with present structural data on collagenolysis, it was found that deletion of the O-glycosylated region significantly decreased gelatinolytic activity (k_{cat}/k_M ± 40% less than full-length MMP-9).

CONCLUSION: The DQ™-gelatin assay is useful in high-throughput drug screening and exosite targeting. We demonstrate that flexibility between the catalytic and hemopexin domain is functionally critical for gelatinolysis.

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INTRODUCTION

Matrix metalloproteinases (MMPs) constitute a family of

more than 25 soluble or membrane bound Zn^{2+}-dependent proteases involved in remodeling of the extracellular matrix, and in regulation of the function of bioactive molecules. MMPs are secreted as latent pro-enzymes and become activated after disruption of the coordination between the cysteine of the propeptide and the catalytic zinc (Zn^{2+}) in the active site, for example by proteolysis[1]. This process is described as the cysteine switch model[2]. During normal physiological processes, such as embryogenesis, vasculogenesis, wound healing and stem cell mobilization, MMP activities are regulated by transcriptional regulation, activation and by endogenous inhibitors, such as the tissue inhibitors of metalloproteinases. Disturbance of this essential balance between proteinases and natural inhibitors leads to uncontrolled MMP activities which results in pathological conditions such as tumor progression and metastasis, inflammation, neurodegenerative, cardiovascular and autoimmune diseases[3-6].

MMP inhibitors (MMPIs) have been considered as potential therapeutics for diseases in which excess MMP activity is detrimental. The MMPIs, all sharing a zinc binding group, are categorized into various classes[6], such as the hydroxamate based MMPIs[7] (e.g. batimastat), the non-hydroxamate based MMPIs[8] (e.g. SB-3CT), novel MMPIs[6] (barbiturates), synthetic peptides and pseudopeptides[9] (e.g. Regasepin 1) and biotechnological and macromolecular inhibitors of MMPs[10] (e.g. REGA-3G12). Bioavailability and MMP-specificity are major bottlenecks in designing MMPIs. The limited success of broad spectrum inhibitors in clinical trials stimulated research towards the development of highly sensitive assay methods to screen for specific MMP activities and to search for selective inhibitors[6,11,12].

One of the most studied and structurally most complex members of the MMP family is MMP-9 or gelatinase B. In contrast to the constitutively expressed MMP-2 or gelatinase A, MMP-9 expression is induced by various agonists. After neutrophil activation, MMP-9 is released from preformed granules[13]. Since many disease states, e.g. acute inflammation, autoimmunity and invasive cancer, are associated with excess gelatinase B activation, this enzyme is an interesting and important target for inhibition[6,13,14].

Here we describe a novel, fast and highly sensitive method for the screening of MMP-9 inhibitors. Dye-quenched (DQ)™-gelatin consists of quenched FITC-labeled gelatin which, upon gelatinolytic activity, is converted into bright fluorescent peptides. This reaction is conveniently used for *in situ* zymography techniques[15] and the substrate conversion was parametrically studied in this work. In contrast to all other MMPs, only gelatinases have a gelatin-binding fibronectin domain[16]. Hence, compared to the small fluorogenic peptide (FP) (7-methoxycoumarin-4yl)Acetyl-Pro-Leu-Gly-Leu-[3-(2,4-dinitrophenyl)-L-2,3 diaminopropionyl]-Ala-Arg-NH₂ described by Knight *et al*[17], DQ™-gelatin mimics the natural substrate to measure (MMP-9/MMP-2) gelatinolytic activity with high sensitivity. We studied the catalytic parameters of DQ™-gelatin conversion by human MMP-9, on the basis of which a high-throughput assay for rapid screening of MMP-9

inhibitors was established. With this assay we screened libraries (ChemDiv, InterBioScreen, ChemBridge) of small molecules for MMP-9 inhibition. Out of 1612 compounds, 5 inhibited MMP-9 by more than 50% at concentrations below 40 μmol/L. The best selected novel MMP-9 inhibitor was structurally analogous to an already described MMPI, RO-28-2653, which belongs to the class of pyrimidine-2,4,6-triones (barbiturates)[18]. Finally, it was demonstrated that this assay is useful for MMP exosite studies, because deletion of the O-glycosylated domain resulted in significantly reduced catalysis of DQ™-gelatin, in comparison with the activities of the intact MMP-9/gelatinase B.

MATERIALS AND METHODS

Proteins and reagents
Recombinant human full-length proMMP-9 (MMP-9 FL, 92 kDa) as well as mutants lacking the O-glycosylated domain (MMP-9ΔOG), or the hemopexin domain (MMP-9Δ Hem), or both the O-glycosylated and hemopexin domain (MMP-9ΔOGHem) and a mutant with a point mutation in the active site (the catalytic Glu₄₀₂ is mutated into Ala, rendering the enzyme inactive) and a point mutation in the OG domain (Cys₄₆₈ is mutated into Ala) (MMP-9 MutEC) were expressed in Sf9 insect cells and purified by gelatin-Sepharose chromatography. Subsequently, the enzymes were activated by incubation with the catalytic domain of stromelysin-1/MMP-3. These techniques were performed as described previously[13,19,20]. The enzymes were always used in the assays at a concentration of 0.1 nmol/L unless mentioned otherwise.

For the fluorogenic gelatin assay, DQ™-gelatin was purchased from Invitrogen (Carlsbad, CA, USA) and dissolved in water at 1 mg/mL. For this assay, all solutions and dilutions were prepared in assay-buffer (50 mmol/L Tris-HCl pH 7.6, 150 mmol/L NaCl, 5 mmol/L CaCl₂ and 0.01% Tween 20). In all experiments, DQ™-gelatin was used at a concentration of 2.5 μg/mL, unless mentioned otherwise.

The fluorogenic DQ™-gelatin assay
The following general protocol was used for the setup of a fluorogenic DQ™- gelatin assay. To a 96-well plate (Macro-assay plate (chimney, 96-well, black, clear bottom, Greiner Bio-one, Frickenhausen, Germany), 0.1 nmol/L (for a final volume of 100 μL) of the enzyme was added. For inhibitor tests, the required amount of inhibitor was added and the plate was incubated for 30 min at 37℃ (note that in this case the actual concentrations of enzyme and inhibitor were 1.7 times higher during this incubation period than in the interval used for substrate conversion). Subsequently, DQ™-gelatin at a final concentration of 2.5 μg/mL was added. Immediately thereafter, the plate was placed in the fluorescence reader (FL600 Microplate fluorescence reader, Biotek, Highland Park, IL, USA) and fluorescence was measured every 10 min for 2 h at 37℃ (ex. 485 nm/em. 530 nm). In each experiment, both positive (no inhibitor) and negative (no enzyme) controls were included. All data were corrected by subtraction of their

Table 1 Set of used protease inhibitors in control experiments

Inhibitor (% inhibition)	Alternative name	MW (g/mol)	Target	Mechanism	Ref.
Aprotinin (5%)	Bovine pancreatic trypsin inhibitor	Approximately 6500	Serine proteases including plasmin, tissue plasminogen activator, kallikrein and thrombin	Nonspecific protease inhibitor	[33,34]
Batimastat (94%)	BB-94; [4-(N-hydroxyamino)-2R-isobutyl-3S-(thiopen-2-ylthiomethyl)succinyl]-L-phenylalanine-N-methylamide	478	MMP-1, -2, -3, -7 and -9 TACE (MMP IC_{50} = 10-30 nmol/L)	Peptide backbone similar to the cleavage site in collagen (= peptidomimetic inhibitor)	[6,9,35-37]
Benzamidine (0%)	-	120	Trypsin, plasmin and thrombin	Competitive inhibitor	[36]
Bestatin (0%)	[(2S,3R)-3-amino-2-hydroxy-4-phenylbutanoyl]-L-leucine, Ubenimex	308	Aminopeptidase N	Slow-binding competitive inhibitor	[38-40]
Chymostatin (0%)	-	608	Proteinases including Serine, thiol, and carboxyl endopeptidases serine proteinases, chymotrypsin and Streptomyces griseus proteinase A, and several cysteine proteinases	Tetrapeptide analogue, formation of a hemiacetal or hemithioacetal adduct with the nucleophilic hydroxy or thiol group of the serine and cysteine proteinases	[41,42]
E-64d (0%)	Aloxistatin, EST, [2S,3S-trans-(Ethoxycarbonyloxirane-2-carbonyl)-L leucine-(3-methylbutyl) amide]	342	Specific thiol protease inhibitor such as papain and cathepsin B	Interaction with active thiol group	[43]
EGCG (33%)	Epigallocatechin-3-gallate	458	Multiple targets including MMP-2 and MMP-9 (MMP IC_{50} = 8-50 μmol/L)	Blocking the activation mechanism of MMP-2 induced by concanavalin A Other exact molecular targets remain unknown	[44-47]
Pefabloc (0%)	AEBSF; 4-(2-Aminoethyl) benzenesulfonyl fluoride hydrochloride	239	Serine protease inhibitor	Irreversible inhibition by covalent interaction with the active-site serine	[48,49]
Pepstatin (0%)	Isovaleryl-L-valyl-L-valyl-4-amino-3-hydroxy-6-methylheptanoyl-L- alanyl-4-amino-3-hydroxy-6-methylheptanoic acid	686	Pepsin and gastricsin (acid proteinase activity)	-	[50]
PMSF (0%)	Phenylmethylsulfonyl fluoride	174	Serine protease/carboxylesterase inhibitor	Covalent binding to the serine residue of the catalytic Ser-His-Asp triad	[51]
SB-3CT (91%)	-	306	MMP-2 and MMP-9 (MMP IC_{50} = 185-290 nmol/L)	Competitive, mechanism-based, thiirane-opening mechanism	[8,52]

The chemical compounds were drawn with ACD/ChemSketch Freeware Software and the structure of Aprotinin was obtained from the Protein Data Bank (PDB ID: 3LDI). The inhibition percentages are shown below the compound names, as obtained in the initial screen with the compounds at 20 μmol/L. MMP: Matrix metalloproteinase.

respective negative controls. Graphs and calculations were obtained with Prism 5 (GraphPad Software, Inc.). For the calculation of substrate molarities we used an approximate molecular weight of 100 000 g/mol.

Optimization of the fluorogenic gelatin assay

Concentration ranges of both the full length enzyme (MMP-9 FL) and substrate (DQ™-gelatin) were tested. MMP-9 FL was serially diluted 1/3 starting with a concentration of 4 nmol/L. The substrate was diluted by 1/2, starting with 40 μg/mL (0.4 μmol/L) DQ™-gelatin. As a negative control, each substrate dilution, without enzyme and in assay buffer, was always included as a control for spontaneous substrate conversion.

Analysis of enzyme kinetics of MMP-9 FL and MMP-9 mutants using DQ™-gelatin

MMP-9 FL, MMP-9 ΔHem, MMP-9 ΔOG, MMP-9 Δ OGHem and MMP-9 mutEC were used at a concentration of 1 nmol/L. Each enzyme form was tested at a range of substrate concentrations (40 μg/mL to 0.075 μg/mL in a 1/2 dilution series). For each enzyme variant, the corresponding kinetic parameters and kinetic graphs were calculated.

Assay validation with a range of known protease inhibitors

A random set of available protease inhibitors was tested for their potential MMP-9 FL inhibition in our fluorescent gelatin assay. Details of the used inhibitors are summarized in Table 1. A first screening was carried out with all compounds at a concentration of 20 μmol/L. After the initial screening, the active compounds were tested in a 1/2 dilution series starting at the highest concentration of 20 μmol/L.

The influence of DMSO on the fluorogenic gelatin assay

In view of the fact that hydrophobic compounds are often dissolved in DMSO, and 10% DMSO disrupts the interaction between gelatin and MMP-9[13], we evaluated the highest concentration of DMSO that may be used without interfering with the test system. Prior to the enzymatic tests, a series of DMSO dilutions were added to the 96-well plate containing MMP-9 FL and DQ™-gelatin. Negative controls were included, containing the used DMSO concentration and 2.5 μg/mL DQ™-gelatin.

High-throughput screening for MMP-9 inhibition with the use of the fluorogenic gelatin assay

The compound library: The compound library con-

tained in total 1612 small-molecule compounds (MW approximately 300 g/mol). 555 were purchased from Chem-Div (San Diego, CA, USA), 360 from InterBioScreen Ltd. (Moscow, RUS) and 697 from ChemBridge Corporation (San Diego, CA, USA). All compounds were first dissolved in DMSO (concentration of 10 mmol/L). The compounds were prediluted in assay buffer.

Initial screening: All compounds were tested at a final concentration of 20 μmol/L. For each compound a negative control was included (the enzyme was replaced by assay buffer). For each plate a positive enzyme control was included (no inhibitor but an equivalent amount of DMSO; 0.2%). The data for each compound were corrected with its negative control and compared with the positive control, giving a percentage decrease in fluorescence. The compounds which showed more than 20% inhibitory activity were tested twice more for corroborations. Inhibition percentages were calculated based on the fluorescence measurement after 2 h.

Dose response: All active compounds were tested again but at multiple concentrations (1/2 dilution starting at a concentration of 40 μmol/L and ending at a concentration of 0.312 μmol/L). For each compound the IC$_{50}$ was calculated and a dose response plot was drawn.

FP assay

If necessary, extra information on catalysis by MMP-9 was obtained by using a second FP substrate; {DNP-Pro-Cha-Gly-Cys(Me)-His-Ala-Lys(N-Me-Abz)-NH$_2$ (ex. 365 nm/em. 450 nm, MM: 1077.2/EMD/Calbiochem®, Darmstadt, Germany). This substrate is cleaved to a single cleavage product, Dnm-Pro-Cha-Gly. It can be used for the evaluation of MMP-9 inhibitors in a fluorescent plate reader[21]. MMP-9 FL was used at a concentration of 1 nmol/L (vs 0.1 nmol/L in the fluorogenic gelatin assay) and the FP was used at a concentration of 10 μg/mL (vs 2.5 μg/mL in the fluorogenic gelatin assay). Fluorescence was measured every 10 min for 2 h with the fluorescence reader (FL600 Microplate fluorescence reader, Biotek, Highland Park, IL, USA).

RESULTS

Assay optimization and validation

Development of the fluorescent gelatin assay: An enzyme assay was developed with DQ™-gelatin as substrate. By using different substrate/enzyme concentrations, we determined the sensitivity of the assay and the optimal substrate concentration. Figure 1 shows a 3D surface representation of the signal (measured fluorescence at the respective enzyme and substrate concentration) to noise (fluorescence measured in wells only containing DQ™-gelatin = spontaneous degradation) ratio at variable substrate and enzyme concentrations. At lower enzyme concentrations the signal-to-noise ratio dropped significantly. Based on a compromise between a good detection signal and minimal enzyme use, we selected the concentration

of 0.1 nmol/L as the enzyme concentration for further testing in high-throughput drug screening. The yellow line (Figure 1A) shows this optimal enzyme concentration and Figure 1B shows fluorescence as a function of gelatinase B concentration with a fixed substrate concentration of 2.5 μg/mL.

To determine the optimal substrate concentration, we made similar compromises and defined 2.5 μg/mL DQ™-gelatin as the optimal substrate concentration. This concentration is represented by the red line in Figure 1A and again in Figure 1C. By using only 2.5 μg/mL substrate, MMP-9 FL levels below 0.1 nmol/L (corresponding to 920 pg) could still be detected.

Standard: To determine the relationship between fluorescence and product formation a standard curve was constructed. A 1/2 dilution series of the substrate was prepared and ranged from 10 μg/mL DQ™-gelatin to 0.01 μg/mL DQ™-gelatin. In one dilution series, 0.2 nmol/L of MMP-9 was added. A negative control for spontaneous degradation was included. When all substrate was converted to product, when no more changes in fluorescence were observed, the fluorescence was measured. By using a linear regression analysis we determined that the fluorescence was proportional to the converted substrate concentration (in μmol/L) (Figure 2). 46 h later, another reading was done, which showed that fluorescence dropped slightly with time (less than 6%). With the use of a Wilcoxon signed rank test we found that the difference between both graphs was significant ($P = 0.0269$).

Enzyme kinetics of MMP-9 FL and MMP-9 mutants using DQ™-gelatin: MMP-9 FL, MMP-9 ΔHem, MMP-9 ΔOG, MMP-9 ΔOGHem and MutEC activity were tested using the fluorescent gelatin assay. The Michaelis-Menten curves and V$_{max}$ and k$_{cat}$/K$_M$ parameters are shown in Figure 3 and Table 2. Deletion of the Hemopexin or the Hemopexin and O-glycosylated domain seemed to have least influence on the enzyme efficiency. k$_{cat}$/K$_M$ was reduced by ± 10% (relative to the parameters obtained for MMP-9 FL). As expected, the inactive MMP-9 MutEC did not show any significant activity. Interestingly, the MMP-9 ΔOG was less active (k$_{cat}$/K$_M$ ± 40% less efficient) than the mutant lacking both O-glycosylated and hemopexin domains, suggesting an important role for the linker (OG) domain for MMP-9 gelatinolytic activity (vida infra). This OG-domain is a highly glycosylated and proline-rich sequence of approximately 64 amino acids. It links the active site and hemopexin domain, but its exact function remains elusive[19].

Assay validation with a range of known protease inhibitors: Initial screening at 20 μmol/L inhibitor concentration showed that only SB-3CT, BB-94 and EGCG significantly lowered MMP-9 activity (for inhibition percentages, Table 1). As also shown in Table 1, SB-3CT and BB-94 are two inhibitors known for their inhibitory activity against MMPs. In our assay, BB-94 and SB-3CT impaired MMP-9 FL gelatinolytic activity in the nmol/L range, with BB-94 be-
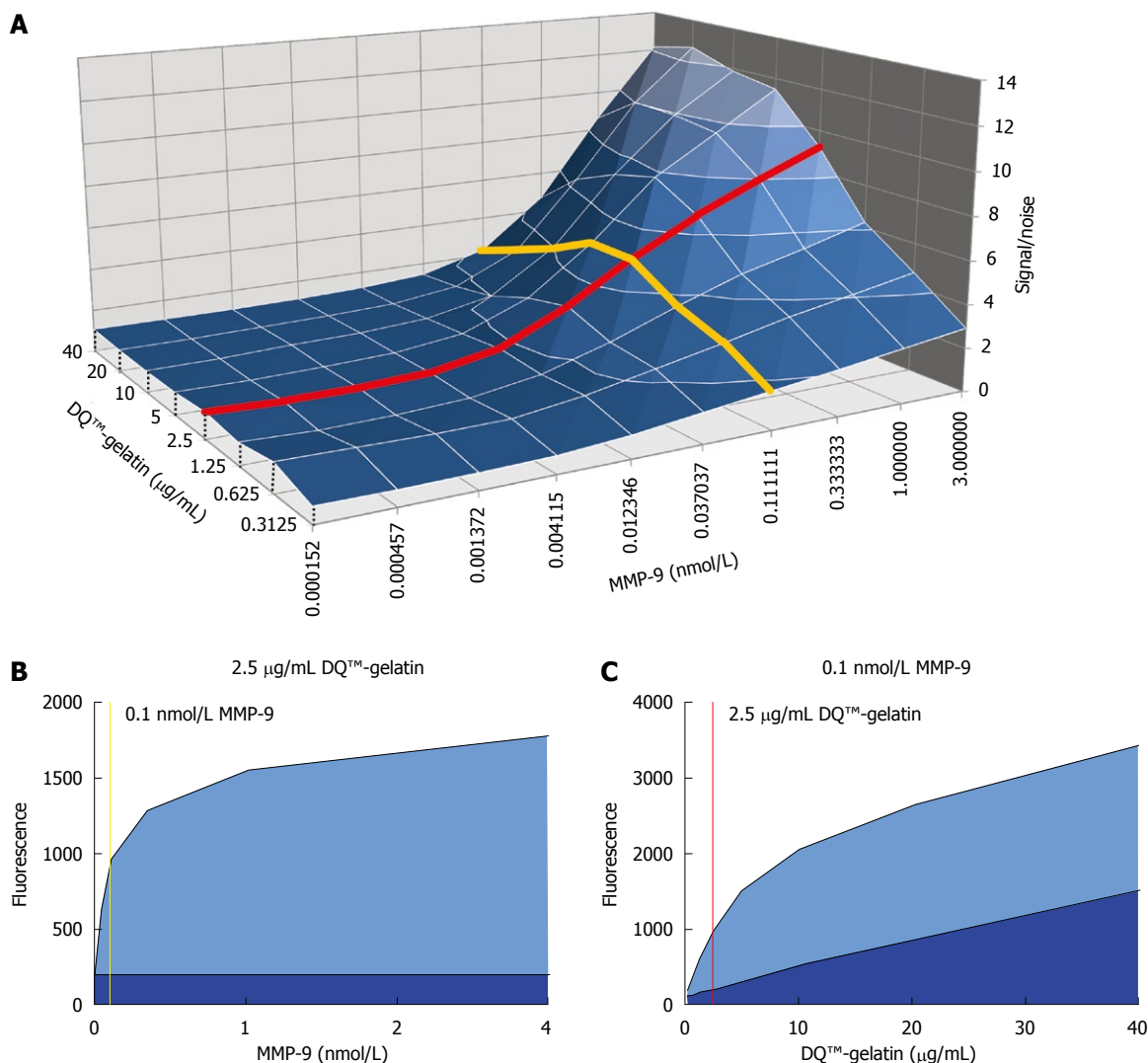
A

B   **C**

Figure 1 Optimization of enzyme and substrate concentrations. A: 3D surface representation of the signal fluorescence divided by the noise fluorescence (signal/noise) as a function of the enzyme [matrix metalloproteinase (MMP)-9 FL] and substrate (DQ™-gelatin) concentration. Data were obtained after an incubation period of 2 h. The red line represents the signal-to-noise ratio as a function of variable enzyme concentration and at a constant substrate concentration of 2.5 µg/mL. The yellow line shows the signal-to-noise ratio at variable substrate concentrations and at a constant enzyme concentration of approximately 0.1 nmol/L. These enzyme and substrate concentrations were chosen for further testing; B: The fluorescence signal (light blue surface) and noise fluorescence (dark blue surface) under different enzyme concentrations and at a constant substrate concentration of 2.5 µg/mL is shown; C: The fluorescence signal (light blue surface) and noise fluorescence (dark blue surface) under different substrate concentrations and at a constant concentration of 0.1 nmol/L MMP-9 FL is plotted.
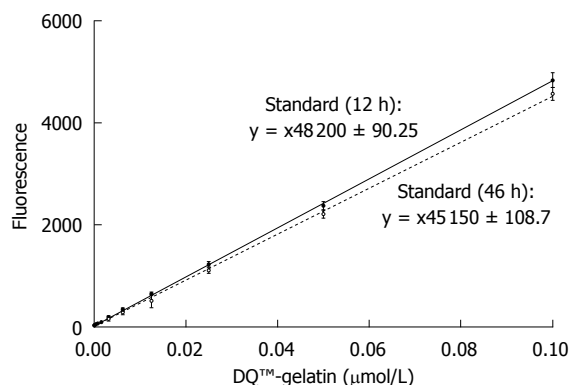

Figure 2 Standard curves of the correlations between fluorescence and product (DQ™-gelatin) concentration. The full line represents a linear regression of fluorescence data obtained after 12 h incubation. The dashed line represents a linear regression of the fluorescence data obtained after 46 h. The drop in fluorescence was significant ($P < 0.05$). Data represent mean ± SE (n = 32).

ing the best inhibitor (Figure 4). EGCG impaired MMP-9 gelatinolytic activity in the µmol/L range.

Influence of DMSO on the fluorogenic gelatin assay: Since most commercially available compound libraries are dissolved in DMSO, we tested whether DMSO had an influence on the assay. This was expected, since DMSO disrupts the binding of MMP-9 to gelatin[13]. Figure 5A shows the enzyme velocity as a function of DMSO concentration. With the used conditions, DMSO significantly inhibited the enzyme activity with an IC$_{50}$ of 56 mmol/L DMSO. Therefore, we tested different DMSO concentrations to define a low concentration at which the net inhibitory effect could still be measured (Figure 5B). At a concentration of 44 mmol/L DMSO (0.3% DMSO), the interference was ± 42% of the signal and at 22 mmol/L (0.15% DMSO), the DMSO interference was ± 24%.

Table 2 Michaelis-Menten parameters for different enzyme variants

	MMP-9 FL	MMP-9 ΔHem	MMP-9 ΔOGHem	MMP-9	MMP-9 MutEC
V_{max} (nmol/L per minute)	3.643	2.686	2.314	1.117	-
k_{cat}/K_M (nmol/L per minute)	0.097	0.086	0.088	0.058	-
Goodness of fit (R^2)	0.9977	0.9900	0.9861	0.9470	0.7096
Difference from MMP-9 FL	-	$P = 0.0207$	$P = 0.0049$	$P = 0.0020$	$P = 0.0010$

The corresponding Michaelis-Menten curves are shown in Figure 3. The *P*-values were calculated with a Wilcoxon signed rank test. The V_{max} and K_M values could not be determined for the matrix metalloproteinase (MMP)-9 MutEC.
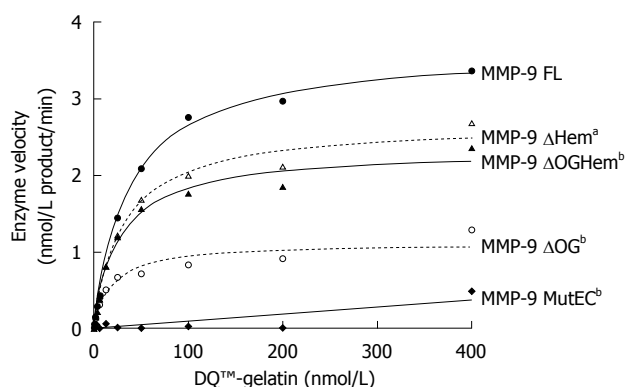

Figure 3 Enzyme velocity as a function of the amount of substrate (nmol/L DQ™-gelatin) (at a concentration of 1 nmol/L). Prism 5 (GraphPad Software, Inc) was used to fit the data with the corresponding Michaelis-Menten curve and to calculate the V_{max} and K_M values (Table 2). By using a Wilcoxon signed rank test we determined that all mutants had a significantly different activity from that of matrix metalloproteinase (MMP)-9 FL ([a]$P < 0.05$, [b]$P < 0.01$). The graphs are representative of three independent experiments.

Therefore, in subsequent experiments the DMSO concentration was always kept as low as possible. We recommend keeping the DMSO concentration at 0.2% or lower, if possible.

High-throughput screening for MMP-9 inhibition with the use of the fluorogenic gelatin assay

Initial screening: The results of the initial screening are shown in Table 3. Four hundred and fifty seven compounds reduced the fluorescence within a range of 1%-10% compared to the control with an equivalent amount of DMSO. We assumed that these small percentages were in the error-range of the assay. One hundred and twenty six compounds reduced the signal between 11%-20% and 37 compounds inhibited the fluorescence signal by more than 20%. The increase in fluorescence as a function of incubation time with and without an active compound is shown in Figure 6. All assays were replicated three times and inhibitory compounds were defined on the basis of thrice concordant results.

Further testing of active compounds: Out of the 37 MMP-9 inhibitors, 5 showed an IC$_{50}$ value below 40 μmol/L. The dose response graphs, IC$_{50}$s and molecular structures are shown in Figure 7. The two most active compounds had an IC$_{50}$ of 15 μmol/L and 19 μmol/L. One of these compounds was compound 6994210 (ChemBridge) or
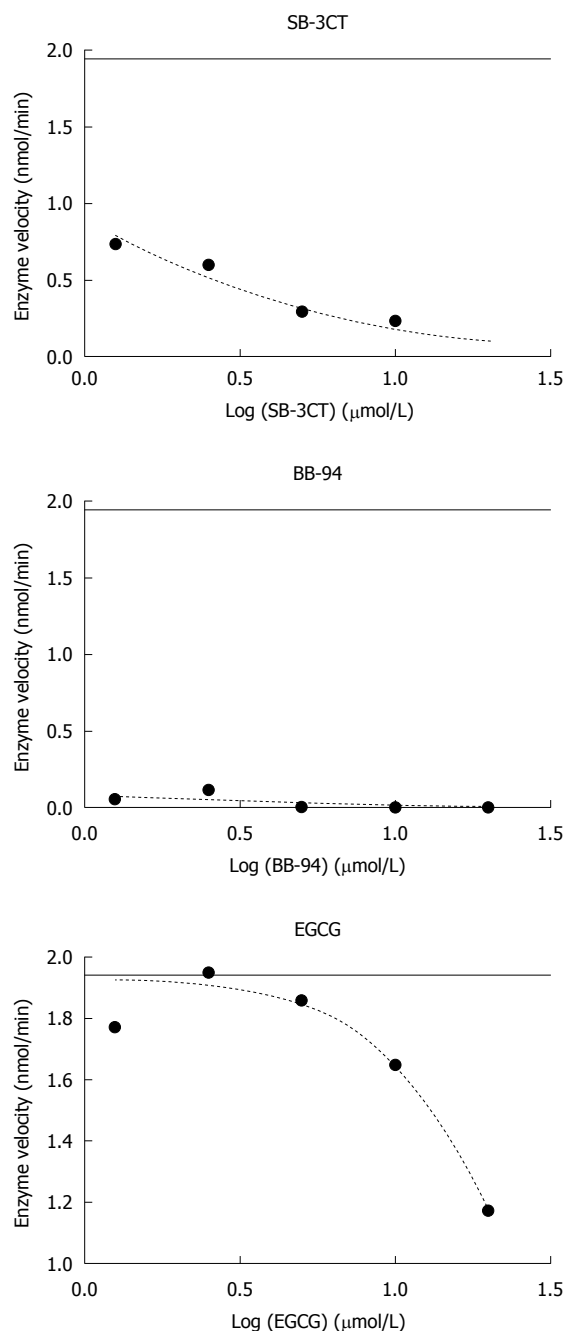

Figure 4 Dose-response curves of the inhibitory activities of SB-3CT, BB-94 and EGCG. With GraphPad prism software, the IC$_{50}$ of SB-3CT and BB-94 was predicted to be in the nmol/L range and the IC$_{50}$ of EGCG in the μmol/L range. The data points correspond to inhibitor concentrations of: 1.25, 2.5, 5, 10 and 20 μmol/L, respectively. The horizontal line shows the enzyme velocity in the absence of inhibitor.
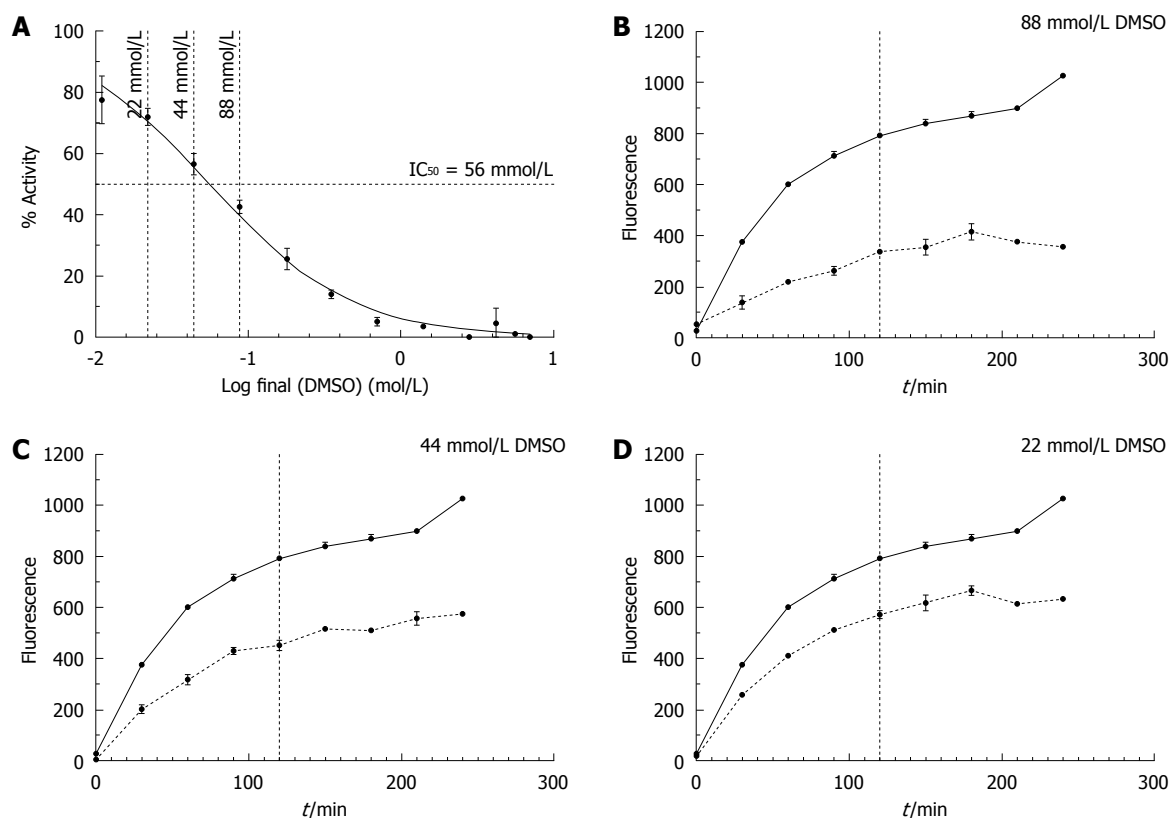
Figure 5 The influence of DMSO on the conversion of DQ™-gelatin into fluorogenic gelatin by matrix metalloproteinase-9. A: By using a non-linear fit, an IC$_{50}$ of 56 mmol/L DMSO (R^2 = 0.9867) (horizontal dotted line) was determined. The vertical striped lines represent the concentrations used in panels B, C and D; B: Influences of 88 mmol/L (0.6% DMSO), 44 mmol/L (0.3% DMSO) and 22 mmol/L DMSO (0.15% DMSO) on the fluorescence changes at different time points. The solid lines show the fluorescence evolutions measured in the absence of DMSO, the striped lines show the fluorescence measured in the presence of DMSO at the indicated concentrations. The vertical dotted lines represent fluorescence data measured after 2 h.

Table 3 Results of the initial screening of 1612 compounds

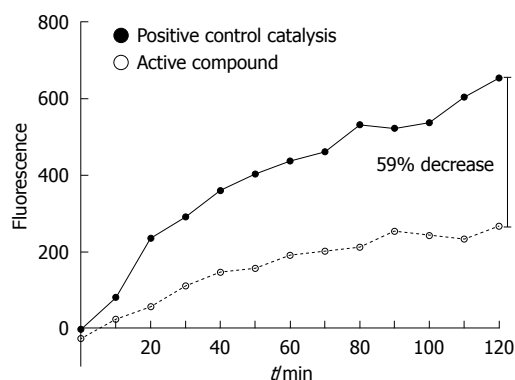|	Fluorescence decrease		
	1%-10%	11%-20%	> 20%
ChemDiv (555 compounds)	217	57	4 (Max = 33%)
InterBioScreen (360 compounds)	106	19	18 (Max = 100%)
ChemBridge (697 compounds)	134	50	15 (Max = 100%)

Figure 6 Typical increase in fluorescence (per time unit) between the positive control catalysis and in the presence of an active compound (ChemBridge, 6994210). The percentage inhibition was measured after 2 h.

5-[(2-hydroxy-6-methyl-3-quinolinyl)methylene]-2,4,-(1H,3H,5H)-pyrimidinetrione. Pyrimidine-triones have already been described as metalloproteinase inhibitors. They are known for their zinc-chelating activity and substituents have already been optimized to comparable inhibitory efficiency as batimastat (IC$_{50}$ = 10 nmol/L for MMP-2 and IC$_{50}$ of 12 nmol/L for MMP-9) and specificity for MMP-2 and MMP-9[18]. Therefore, the activity of compound 6994210 may be caused by its zinc-binding pyrimidine-trione group. Tochowicz *et al*[22] described the interaction of compound RO-206-0222 (a barbituric acid inhibitor) with the MMP-9 catalytic site (of an inactive E402Q mutant). This compound is a barbituric acid derivative with two substituents: a phenoxyphenyl and a pyrimidine-piperazine and gives a tight binding

in the active site of this MMP-9. The barbiturate ring chelates the catalytic zinc and orients both substituents into their respective subsites[22]. Intriguingly, compound 0204-5272 (ChemDiv) or N-[4-(6-methyl-1,3-benzothiazol-2-yl)phenyl]tetrahydrothiophene-2-carboxamide did not show any similarity with existing inhibitors.

Compound 5805026 (ChemBridge) or N-(4-ethoxy-8-methyl-2-quinazolinyl)guanidine was the third most active compound (IC$_{50}$ = 25 µmol/L). Compound STOCK1S-82005 (InterBioScreen) displayed an IC$_{50}$ of 27 µmol/L
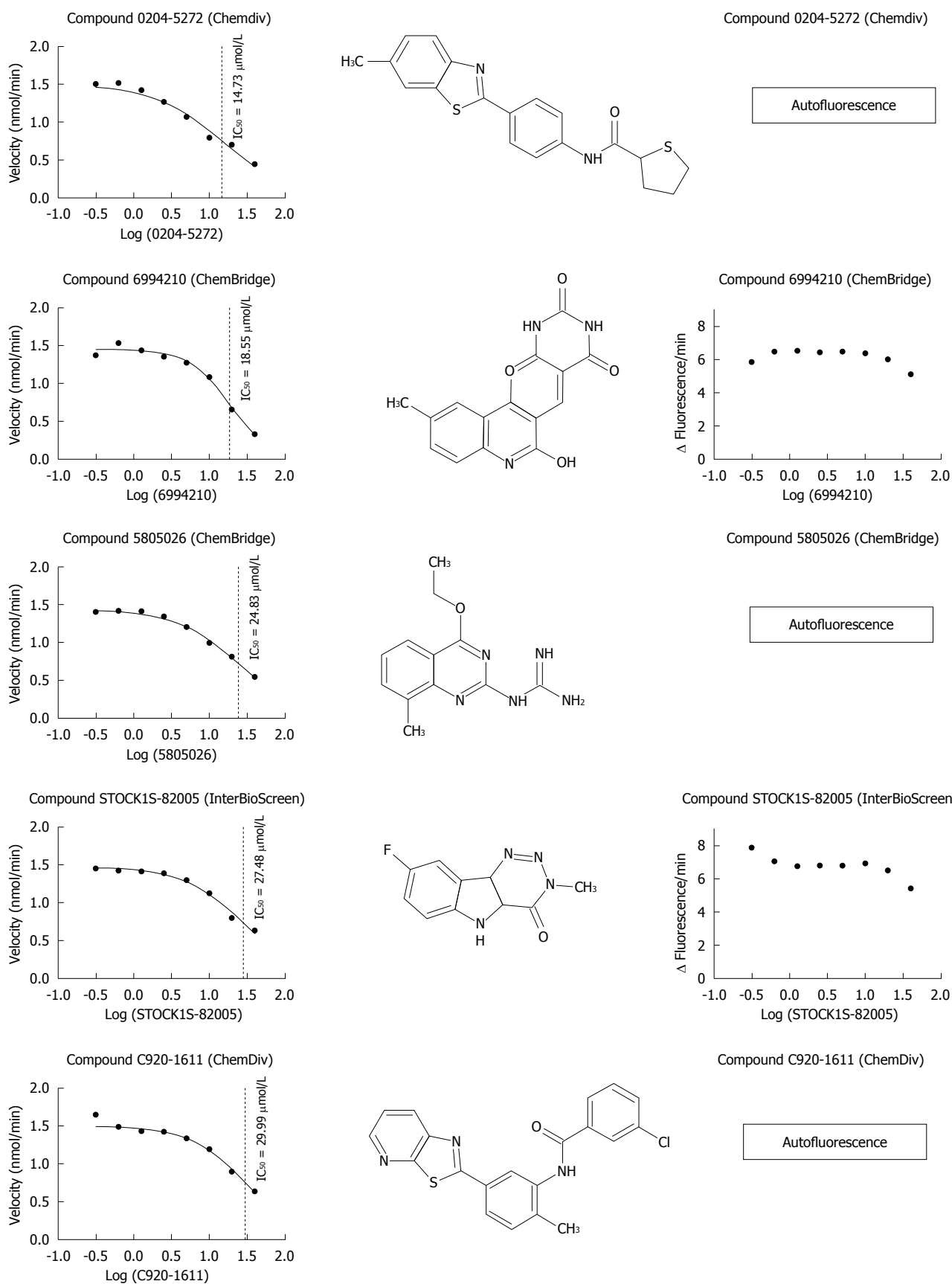
Figure 7 Dose-response graph, IC₅₀ and molecular structure of the 5 most active compounds (IC₅₀ < 40 μmol/L) on conversion of DQ™-gelatin and a fluorescent peptide by matrix metalloproteinase-9. The results obtained with the DQ™-gelatin assay (including the IC₅₀s) are shown in the left column. The chemical structures are shown in the central column. Data with the fluorescent peptide are shown in the right column.

and compound C920-1611 (ChemDiv) or N-(2,4-dimethylphenyl)-2-[(2-methyl-1,3-benzothiazol-6-yl)sulfonylamino] acetamide had an IC$_{50}$ of $\pm$ 30 μmol/L. For these compounds, no structural similarity could be found with existing MMP small-molecule inhibitors.

Inhibitor testing with the use of a small FP substrate:
As a comparison, we used a different assay with a FP substrate to test the inhibitory potential of our 5 newly discovered inhibitors. However, 3 of the 5 compounds (compound 0204-5272, compound 5805026 and compound C920-1611) were autofluorescent at the wavelengths required for this substrate (Figure 7 right column). In addition, no inhibition was detected for the other two compounds, illustrating the power of our new assay.

DISCUSSION

The DQ™-gelatin substrate was originally introduced for the fluorometric determination of gelatinolytic activity of cancer cells *in vitro*[23] but was, until now, mainly used for the *in situ* demonstration of gelatinolytic activity[15,24,25]. Here, we show that the DQ™-gelatin assay is a useful tool in many ways for the biochemical study of gelatinolysis of purified proteases e.g. MMP-9. With low amounts of substrate (2.5 μg/mL) and enzyme (0.1 nmol/L), MMP-9 activity was determined accurately. For comparison, with the fluorogenic peptide {DNP-Pro-Cha-Gly-Cys(Me)-His-Ala-Lys(N-Me-Abz)-NH$_2$, the optimal substrate and enzyme concentrations were 10 μg/mL and 1 nmol/L, respectively. Although {DNP-Pro-Cha-Gly-Cys(Me)-His-Ala-Lys(N-Me-Abz)-NH$_2$ was originally described as a good peptide for high-throughput screening efforts and has compatible emission and excitation spectra with most fluorescent plate readers[21], the fluorescent signal is not as sensitive and stable as with the DQ™-gelatin substrate.

In addition, the DQ™-gelatin substrate is a 'natural' MMP-9 substrate compared to short peptides. MMP-9 cooperatively binds gelatin with its fibronectin domain and catalytic site, thereby orienting the substrate into the catalytic site. The fibronectin domain is, therefore, also essential for the gelatinolytic activity[26]. With the use of gelatin as a natural substrate, the possibility exists of finding inhibitors targeting the fibronectin-like domain and exclusively impairing gelatinolytic activity, without having major implications on other MMP-9 proteolytic events. Previous clinical trials with MMPIs have been somewhat disappointing. One often invoked reason is the lack of specificity, since most existing MMPIs target the catalytic site, which is shown to be highly conserved and, therefore, similar amongst MMPs. Presently, attention is more focused on distal surface residues and accessory domains (called "MMP allosteric sites" or "exosites") which may allocate single or sets of MMPs and would, therefore, be good targets for specific MMP inhibition[16]. Some efforts in this direction have been made. Inhibitory peptides of the MMP-2 collagen binding domain (CBD) have been identified by Xu *et al*[27]. These peptides were also active against MMP-9.

The possibility exists that the described inhibitory effect of DMSO is related to the fibronectin domain exosite. Indeed, recombinant MMP-2 CBD binds to gelatin and this complex dissociates in the presence of 2% DMSO[28]. Furthermore, 2% DMSO, which corresponds to 280 mmol/L, significantly reduced the gelatinolytic activity of MMP-2[29]. Our findings suggest that 2% DMSO has an even higher inhibitory effect (> 80% decreased activity) on MMP-9. This difference may be due to the known fact that MMP-9/gelatin binding (through the fibronectin domain) is dependent on cooperativity between the fibronectin type I and type II modules, whereas MMP-2 can bind gelatin without the need of cooperativity[28]. Also, in accordance with these findings for MMP-2, DMSO had no influence on MMP-9 processing of a small peptide substrate, suggesting that the CBD is not required for positioning such short peptide substrates relative to the active site[29].

A method for high throughput screening of potentially selective MMP-13 (collagenase) exosite inhibitors was developed by Lauer-Fields *et al*[30]. They used a triple-helical FRET substrate and found 34 active compounds including two pyrimidine-trione derivatives and new compounds which did not target the MMP-13 catalytic site. With the DQ™-gelatin assay we tested 1612 small-molecule compounds for their potential inhibition of MMP-9 FL gelatinolytic activity. We identified five compounds with an IC$_{50}$ below 30 μmol/L. One of these compounds (6994210) was a pyrimidinetrione derivative. Barbiturates have previously been identified as Zn^{2+}-binders[6]. We did not trace the other small-molecules in the existing literature, making these compounds additional candidates for further development towards MMPIs. The finding of an existing MMP-9 zinc binder by using the DQ™-gelatin assay endorses the suitability of this assay for high-throughput drug screening. In line with this, we were able to perfectly distinguish the three known MMP-9 inhibitors (SB-3CT, BB-94 and EGCG) out of a set of 11 other protease inhibitors with specificities for various (other) protease classes.

Besides the above-mentioned application, the DQ™-gelatin substrate was also useful in fundamental studies of MMP-9 action. We tested different MMP-9 mutants (MMP-9 ΔHem, MMP-9 ΔOG, MMP-9 ΔOGHem and MMP-9 MutEC) in the DQ™-gelatin assay. The fact that the MMP-9 ΔOG mutant form was $\pm$ 40% less efficient than the MMP-9 FL or the MMP-9 ΔOGHem form, suggests an important role for the OG-domain in MMP-9 gelatinolytic activity. This has been suggested by Rosenblum *et al*[31] on the basis of structural data. With the use of single-molecule imaging statistical analysis and small-angle X-ray scattering (SAXS), it was shown that MMP-9 FL is much more flexible than MMP-9 ΔOG. The OG domain thus lends the MMP-9 molecule flexibility, supporting multiple enzyme conformations[31]. With the use of atomic force microscopy, it was recently shown that MMP-9 FL can adopt an extended and a contracted conformation, addressed by the OG domain. Upon binding of collagen, MMP-9 changes from the extended into the

contracted form, thereby using the flexibility of the enzyme O-glycosylated domain to find an appropriate binding site[32]. Removal of this flexible linker may thus result in a rigid structure which has fewer degrees of freedom for interaction with the gelatin substrate. Removal of the OG domain, also results in direct contact between the catalytic and hemopexin domains. Our data on *in vitro* gelatinolysis demonstrate functionally the importance of the O-glycosylated domain in comparison with the hemopexin domain, and further underline the possibilities of the development of allosteric inhibitors.

We conclude that the DQ™-gelatin assay is useful in high-throughput drug screening and exosite studies of MMPs. The assay is easily applicable in multi-well plates and the substrate is compatible with emission and excitation spectra on most fluorescent plate readers. In addition, less autofluorescence of the compounds is measured at these wavelengths. Because of the high resolution of the assay, only small amounts of enzyme and substrate are necessary, which implies low costs. Besides the technological advancements, this study provides further insights into the MMP inhibitory role of DMSO mediated through the fibronectin domain and functionally defines the O-glycosylated domain as a crucial entity for gelatin substrate catalysis.

COMMENTS

Background

Matrix metalloproteinases (MMPs) are a family of Zn^{2+}-dependent multidomain enzymes, involved in pathological processes such as acute and chronic inflammation (e.g. rheumatoid arthritis and multiple sclerosis), cancer cell invasion and metastasis, periodontal diseases, liver and lung diseases. Historically, the MMPs were classified into gelatinases, collagenases, stromelysins, metalloelastases, matrilysins and membrane type MMPs (MT-MMPs), partially based on substrate conversion. Gelatinase A/MMP-2 and Gelatinase B/MMP-9 represent the gelatinases, having gelatins as natural substrates.

Research frontiers

Several MMP inhibitors (MMPIs) have been developed over the past 20 years. However, most clinical trials with MMPIs had poor outcomes and severe side-effects were observed. Many reasons have been postulated for these results, but one major problem was low selectivity of the used MMPIs. In order to increase selectivity, inhibitors that target the distal surfaces of MMPs in addition to the highly conserved catalytic site, may be more promising. New high-throughput screening assays which enable the identification of exosite inhibitors are therefore needed. Instead of commonly used small peptide substrates, we used high molecular weight gelatin in an attempt to mimic macromolecular interactions in order to probe exosite interactions.

Innovations and breakthroughs

The present study validates Dye-quenched (DQ)™-gelatin, a fluorogenic gelatin substrate, for high-throughput drug screening of MMPIs. The presented assay is easy, low cost and has a high resolution. In addition, this assay enables the identification of exosite inhibitors for gelatinases, since DQ™-gelatin mimics the natural substrate. Our study also stresses the crucial role of the O-glycosylated domain in gelatin catalysis and provides further insights into how DMSO inhibits MMP-9 through the fibronectin domain.

Applications

The gelatin degradation assay is useful in fundamental studies of gelatinase action and is applicable for high-throughput drug screening of MMPIs. It also has potential for the identification of exosite inhibitors.

Peer review

The experiments have been carefully performed and the manuscript is clearly written. A few issue need to be addressed before the paper should be published.

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