Design, Synthesis, and Use of MMP-2 Inhibitor-Conjugated Quantum Dots in Functional Biochemical Assays

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ABSTRACT: The development of chemically designed matrix metalloprotease (MMP) inhibitors has advanced the understanding of the roles of MMPs in different diseases. Most MMP probes designed are fluorescent substrates, often suffering from photo- and chemical instability and providing a fluorescence signal of moderate intensity, which is difficult to detect and analyze when dealing with crude biological samples. Here, an MMP inhibitor that selectively inhibits MMP-2 more strongly than Galardin has been synthesized and used for enzyme labeling and detection of the MMP-2 activity. A complete MMP-2 recognition complex consisting of a biotinylated MMP inhibitor tagged with the streptavidin-quantum dot (QD) conjugate has been prepared. This recognition complex, which is characterized by a narrow fluorescence emission spectrum, long fluorescence lifetime, and negligible photobleaching, has been demonstrated to specifically detect MMP-2 in in vitro sandwich-type biochemical assays with sensitivities orders of magnitude higher than those of the existing gold standards employing organic dyes. The approach developed can be used for specific in vitro visualization and testing of MMP-2 in cells and tissues with sensitivities significantly exceeding those of the best existing fluorogenic techniques.

INTRODUCTION

Studying biological processes at the molecular level within a living cell is a major challenge for cell biology investigations. Similarly, spatiotemporal tracking of small molecules in ex vivo or in vivo environments gives better insight into their interactions with proteins, rendering target identification more efficacious and, in fine, accelerating drug development. The existing techniques based on spectroscopic and biooptical methods using organic dyes are mostly limited by the difficulties in imaging individual molecules in the optically noisy cellular environment and accessing directly the interior of living cells. As promising alternative tools, semiconductor nanocrystal quantum dots (QDs) have emerged for numerous biomedical applications, such as cellular labeling, biochemical sensing, probing biocatalyzed reactions, and drug delivery. QDs are characterized by their narrow composition and size dependent emission wavelength, extreme brightness, rock-solid photostability, and chemical robustness. QD fluorescence covers the optical spectrum from the near-UV to the IR, which provides a unique possibility for multiplexing, unlike with the use of organic dyes. Even if QDs possess optical properties for biological applications, nanoparticles need to be functionalized to achieve a convenient solubility in aqueous media, and in fine, hydrophilic ligands may be used as anchor points for biomolecules such as peptides, DNA, or drugs. The hydrophobic outer shell of QDs may be exchanged with bi- or multifunctional molecules containing capping ligands, cationic polymers, or liposomes bearing a cell penetrating peptide (CPP), or with amphiphilic polymers. Biomolecules may be attached to
Figure 1. Structure of highly efficient inhibitors: batimastat, marimastat, and ilomastat.

Batimastat

Marimastat

Ilomastat (Galdarin®)

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114 with addition of a C12 alkyl chain as the P1 glutamate moiety of the inhibitor for MMP-2 in comparison to MMP-1, S1 > S2, S3 > S1 > S2.22,23. Thus, attempts at obtaining selectivity among MMP enzymes have concentrated on the S1 subsite, called the specificity pocket.24,25. The S1 pocket of MMP-2 is hydrophobic, forms a large, nearly bottomless channel, and the S1 loop is flexible to accommodate more bulky ligands.26. Thus, modifications of the P1 group in MMP inhibitors (MMPIs) ensure selectivity among MMP-family members. Since the succinyl hydroxamate backbone is a common feature in the most efficient first-generation MMPI binders,27 pharmacomodulation was carried out at this structural unit.

For this purpose, Miller et al.28 observed the maximum specificity of the inhibitor for MMP-2 in comparison to MMP-1 with addition of a C12 alkyl chain as the P1′ group into the structure of batimastat (IC50 = 1 and 50 000 nM, respectively). Broadhurst et al.29 have described a marimastat analogue containing a C9 linear chain displaying a good MMP-2 inhibitor activity (IC50 < 0.15 nM).30 However, Miller et al.28 described the best selectivity for MMP-2 versus MMP-1 with a compound containing a C16 alkyl chain (IC50 = 0.6 and 12 000 nM, respectively). Levy et al.31 modified ilomastat by elongating its alkyl chain to eight carbon atoms. Neither inhibitory activity nor selectivity was ameliorated.

MMP inhibitors contain hydroxamic acid as a zinc-binding group (ZBG). Although this function has proved to be a very strong ZBG, toxicity, low bioavailability, and in vivo instability of this chemical entity associated with severe side effects have resulted in the development of alternative ZBG such as 2-carboxylic acid, a precursor of the hydroxamate group.29 Batimastat and marimastat analogues bearing a C16 alkyl chain in the P1′ position with carboxylic acid type ZBG showed the lowest MMP-2 inhibitory activity (IC50 = 50 and 30 nM, respectively).26

Reliable techniques are crucial for hit detection, biological evaluation of compound collections, or monitoring enzyme inhibitory activities. In the past, conventional techniques including gel electrophoresis,32 fluorescence (near-infrared),33−36 magnetic-resonance-based approaches,41,42 were used to detect MMPs activity, but recently, much attention has been paid to Förster resonance energy transfer (FRET) for assay of proteases.43−46

QD-FRET-based protease sensors exploiting QDs as donor and rhodamine or gold nanoparticle as acceptor have also been developed to survey collagenase activity47−49 in normal and cancer cells.50 QDs can also act as energy acceptors in bioluminescence resonance energy transfer (BRET) from a protein energy donor, such as a mutant form of Renilla luciferase, to detect protease activity.51,52 QD-based methods have also been used for multiplexed detection and imaging of several MMPs (MMP-2 and MMP-7)53,54 and multiplexed protease inhibition and competition (folic acid and MMP-7)55 assays.55 In these methods, the MMP activity is identified by changes in QD fluorescence resulting from the cleavage of a specific enzyme-recognizing peptide attached to the QDs. Other approaches employ QDs coupled to antibodies or MMP-9 siRNA to study the enzymatic activity of MMP-956 or the MMP-9 gene expression in the brain.57

Herein, we describe the synthesis and preliminary biological evaluation of a range of ilomastat derivatives bearing carboxylate and carboxylic acid as a ZBG and alkylidene chains of different lengths at the P1′ position. Additional pharmacomodulations were made at the C-2 position of the indole ring to evaluate the impact of a bulky phenyl substitution, P2′ group, on MMP inhibition activity. Among these derivatives, the most selective MMP-2 inhibitor (4k) was conjugated to QDs to use it as probe to identify MMP-2 enzyme activity. The advanced optical
properties of streptavidin-QD conjugates, the high binding constant for streptavidin–biotin interaction and the technical simplicity of mixing streptavidin-QD nanoparticles with the biotinylated ligand (4k) make this approach an attractive tool to generate imaging probes with potential in vitro applications in advanced biochemical MMP-2 assays (Scheme 1).

Scheme 1. Noncovalent Coupling of a Biotinylated Compound and a Fluorescent QD-Streptavidin Conjugate

RESULTS AND DISCUSSION

Influence of the P′1 Part of Ilomastat on Gelatinase Inhibition. For a decade, we have been involved in the pharmacomodulation of ilomastat, focusing recently our efforts on designing analogue derivatives with a modified P′1 succinic component.

First, incorporation of one unsaturation at the P′1 position aiming at increasing the hydrophobicity and conformational rigidity was considered. Replacement of the isobutyl group of ilomastat with an isobutylidene function 1 with the E geometry improved the selectivity for MMP-2 versus MMP-3 (IC50 = 1.3 and 179 nM, respectively) (Figure 2).57 Pursuing these pharmacomodulations, we synthesized other dehydro and didehydro analogues (2a–d).58 The inhibitory activities of these analogues for all MMPs were decreased as compared to ilomastat (Galardin). However, analogue 2a with a C7 alkyl chain displayed an interesting selectivity for MMP-2 compared to MMP-9 (IC50 = 123 and >104 nM, respectively) indicating that the S′1 pocket of MMP-2 is sufficiently deep to accommodate long alkyl chains.

Influence of the P′2 Part of Ilomastat on Gelatinase Inhibition. Introduction of an amino-alkyl chain at the C2 position of the indole ring leads to a decrease in the MMP inhibitory activity but greatly improves the selectivity for MMP-2.57 In this line, a phenyl group was introduced at the C2 position through the Suzuki reaction59,60 (Figure 3), and the obtained analogue 3 was shown to have enhanced potency and selectivity for MMP-2 versus MMP-1 (IC50 = 0.092 and 0.244 nM, respectively).61

In order to clarify the mode of interaction between MMP-2 and analogue 3, molecular docking computations have been carried out using AutoDock software.62,63 As expected, the hydroxamate group chelates the Zn atom in the lowest-energy binding mode (Figure 4a). Moreover, the carbonyl function of the hydroxamate group is involved in H-bond interaction with Ala192. The isobutyl group is located in the S′1 subsite forming van der Waals interactions with Leu191, Ala192, Val400, His403, Pro423, and Tyr425. The modified indole ring is located in the S′2 subsite and is oriented toward the solvent. In addition, four H-bonds between compound 3 and MMP-2 amino acid residues located in the S′3 subsite, namely, Gly189, Leu191, and...
It is well known that the large and solvent-exposed S’2 pocket is flexible to accommodate bulky and hydrophobic groups, and aromatic groups are in agreement with this model as supported by in vitro activity.

**Influence of the P’1 and P’2 Parts of Ilomastat on Gelatinase Inhibition.** In order to further improve the selectivity for MMP-2, the alkyl chain was elongated by 8 to 20 carbon atoms using a method described previously, and a phenyl group was also introduced at the C2 position of the indole part through the Suzuki reaction (Figure 5). Replacement of the very strong hydroxamic acid Zn-chelating group by a less complexing carboxylate function may also favor selectivity to the detriment of affinity. For comparison, ilomastat and compound \(3b\) were also included in the series of biological evaluations (Table 1). As shown in Table 1, the carboxylate counterpart \(3b\) is more selective for MMP-1 (among other MMPs) than ilomastat, although its inhibitory activity is decreased.

In agreement with the literature data, the inhibitory activity values for compounds \(4a-p\) are detrimental to affinity compared to ilomastat (Galardin) but remain of the same order of magnitude as that found for the carboxylic-acid-type ilomastat analogue \(3b\).

**Table 1. P’1- and P’2-Modified Ilomastat Inhibitors**

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<th>R</th>
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aThe IC\(_{50}\) values are expressed in nanomolar. bUnpublished results.
invasion (Figure 9), while ilomastat inhibited cell migration by 25%.

During the invasion process, HT-1080 cells produce different types of MMPs (MMP-14, MMP-2, etc.) and serine proteinases (u-PA, t-PA, etc.), which are involved in cell migration. Evidently, compound 4k mainly blocks the MMP-2 activity and is a selective MMP-2 inhibitor, but its involvement in the migration process is probably insignificant, which explains the absence of inhibitory effect on HT-1080 cell invasion. Ilomastat (Galardin) inhibits different types of MMPs, including MMP-14, which is known to degrade the extracellular matrix, to activate proMMP-2, and to greatly favor tumor cell invasion. This could explain the observed inhibitory effect on HT-1080 cell invasion (25%).

**Synthesis of a Biotinylated Inhibitor 4k.** In line with our goal for providing tools for imaging, we investigated the feasibility of MMP inhibitor-QD conjugates. The selected

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Figure 6. Docking of analogue 4k in the structure of MMP-2. (a) The binding mode of analogue 4k with MMP-2. (b) A detailed view of the H-bonds enabling model stabilization.

Figure 7. Docking of analogue 4k in the structure of MMP-9. (a) The binding mode of analogue 4k with MMP-9. (b) A detailed view of the H-bonds enabling model stabilization.

Figure 8. MMP-2 proteolytic activity measurement. HT-1080 cells were treated overnight with concanavalin A. Cells were then preincubated with or without the effector (100 μM 4k or 10 μM Galardin) for 1 h and then with dye quenched substrate (DQ gelatin) (500 ng/300 μL per well). Fluorescence was measured at 525 nm. *: p < 0.05. **: p < 0.01.

Figure 9. HT-1080 cell invasion rate estimated by the ability of cells to migrate through Matrigel-coated membranes upon treatment with compound 4k or ilomastat (Galardin). (a) A representative photomicrograph showing cell invasion after 6 h of incubation. (b) A representative bar graph quantifying cell invasion. *: p < 0.1. Scale bar, 50 μm.
compound 4k was modified at the indole NH group in order to insert the biotin compound into the inhibitor structure (Figure 10). Biotin interacts noncovalently with streptavidin with an extremely high affinity ($K_D \approx 10^{15}$ M$^{-1}$), resulting in the formation of a highly stable specific biotin–streptavidin complex, which is widely employed in numerous biological approaches.$^{70,71}$

Modification of compound 4k with biotin included several steps. First, the tryptophan derivative 8 obtained from derivative 7 using the Suzuki reaction was treated with butyl acrylate (Michael reaction) to obtain compound 9 with a yield of 90%. The obtained functionalized indole derivative 9 was subjected to transamidation and deprotected with a yield of 80% and coupled to compound 11 to obtain compound 12 with a yield of 82% (Scheme 2).

The biotinylated diaminoalkyl spacer 16 was obtained by coupling monoprotected diaminopropan 14 and biotin 15 with a yield of 74% (Scheme 3).

In the next step of the synthesis, the deprotected pseudodipeptide 13 was reacted with the deprotected biotinylated diaminoalkyl spacer 17 to obtain compound 18 with a yield of 38% in two consecutive steps. From compound 18, the allyl-protecting group was removed upon treatment with the catalyst Pd(PPh$_3$)$_4$ to obtain compound 5 with a yield of 72% (Scheme 4).

Compound 5 was also submitted to the MMP-2 inhibition assay by using classical gelatin zymography made under the same conditions as described for compound 4k. A comparative study showed that compound 5 ensured a 34% inhibition of the enzyme versus 45% inhibition with compound 4k, indicating that biotinylation only slightly decreased the inhibitory activity toward MMP-2 (Figure 11). Subsequently, compound 5 served as a basis for our further experiments.

MMP-2 Inhibition with the Designed Inhibitor 5. The MMP-2 activity was estimated in standard gelatinase/collagenase assay based on digestion of an internally quenched fluorescent substrate with an activated enzyme. The efficiency of substrate digestion resulting in an increase in dye fluorescence was measured continuously at multiple time points. The fluorescence of the digested product increased proportionally to the enzyme proteolytic activity and the amount of activated MMP-2 (Figure 12a). In the presence of the designed inhibitor 5, the protease gelatinolytic activity decreased proportionally to an increase in the concentration of inhibitor, resulting in quenching of the fluorescence of the undigested substrate (Figure 12b). We have found that addition of 100 ng of compound 5 to 50 ng of MMP-2 reduces the protease activity by 50%. Increasing the amount of the inhibitor leads to stronger enzyme inactivation.

We have further coupled the MMPI biotinylated compound 5 to the fluorescent QD-streptavidin conjugate in order to detect MMP-2 in different in vitro assays and to compare QD-based assays with those based on organic dyes.

MMP-2 Ultrasensitive Detection with QD-Tagged Compound 5. According to the manufacturer’s (Invitrogen) protocol, the number of streptavidin molecules per QD may be estimated as 5 to 10, depending on the QD batch, emission wavelength, and steric effects of the bound streptavidin. Typically, a streptavidin molecule contains four biotin binding sites enabling coupling from 20 to 40 biotinylated compounds per QD-streptavidin conjugate. However, it was shown that, due to the steric effects, only one or two binding sites are easily accessible for biotin on the surface of a QD-streptavidin conjugate.$^{73,74}$

**Dot-Blotting of the Biotinylated MMP-2 Inhibitor.** Formation of an effective complex of compound 5 with the streptavidin-QD565 conjugate was visualized using the dot-blot technique. Biotinylated compound 5 diluted in methanol was severely damaged and dissolved the nitrocellulose membrane and was only detectable in amounts above 2 ng. Depositing these samples on a polyvinylidene difluoride (PVDF) membrane allowed the detection of 1 ng of the inhibitor as the lowest detection limit (data not shown). When dissolved in dimethyl sulfoxide (DMSO) and subsequently diluted in phosphate buffered saline (PBS), the inhibitor can be loaded on a nitrocellulose membrane without any damaging effects and is detectable in amounts of 0.2 to 1 ng as the lowest detection limit (Figure 13).

**Scheme 2. Preparation of the Substituted Analogue 13**
In Vitro Sandwich-Type Assay for MMP-2 Detection Using the Designed QD-Coupled Inhibitor 5. The formation of a complete complex consisting of the MMP-2 enzyme, biotinylated inhibitor 5, and fluorescent conjugate of streptavidin with QD800 nm was performed by means of a solid-state sandwich-type analytic biochemical assay. Here, 50 ng of MMP-2 was used in MMP-2 activity assays since this amount of active enzyme was estimated to efficiently and quickly digest the gelatin substrate. The proenzyme was activated with p-amino phenylmercuric acetate before adsorption in order to provide appropriate conformation of the inhibitory binding sites. Activated MMP-2, MMP-2, or the same quantity of BSA protein control were adsorbed on a treated 96-well plate in PBS (pH 7.4). Increasing amounts of biotinylated compound 5 and the QD-streptavidin conjugate were subsequently incubated with the adsorbed MMP-2. The QD fluorescence intensity increased proportionally to the biotinylated inhibitor amount in the range from 0 to 100 μg (Figure 14). Thus, the complex of MMP-2 with the designed biotinylated compound 5 can be quantitatively detected and visualized using compact and bright QD-streptavidin conjugates.

We performed additional experiments in order to explore the photostability of QDs enabling long-term accumulation of their fluorescence signal accompanied by order-of-magnitude improvements of the signal-to-noise ratios in a fluorogenic assay and, consequently, increasing the sensitivity for application of QD-based conjugates to MMP-2 detection in vitro and to compare the sensitivity of QD-based assays with those based on organic dyes.

Figure 14 shows that accumulation of the signal from the fluorogenic assay employing strepta-QD conjugate for visual-
417 ization of MMP-2-inhibitor complexes leads to an increase in 418 the signal-to-noise ratio in the given detection scheme by more 419 than 2 orders of magnitude and, hence, the corresponding 420 increase in the sensitivity of detection of the inhibitor-MMP-2 421 complex. This increase may reach even higher values if longer 422 signal accumulation times and optimized detection schemes are 423 employed. The result shown in Figure 15 was achieved due to 424 the rock-solid photostability of QDs enabling long-term QD 425 fluorescent signal accumulation without significant photo- 426 bleaching of the nanocrystals. Such a photostability is not 427 characteristic of the organic dyes used in currently available 428 fluorogenic assays. Therefore, we have performed an additional 429 comparative study of the model biochemical assays employing 430 different organic dyes in parallel with the assays employing 431 strepta-QD conjugates in order to compare the changes in their 432 state during long-term signal detection and accumulation. 433 As seen from Figure 16, accumulation of the signal from 434 fluorogenic assays employing strepta-QD for revealing the 435 (compound 5)-MMP-2 complex during 10–20 min may result 436 in an increase in the intensity of the signal by more than two 437 orders of magnitude, whereas the signals of all typical organic 438 dyes recorded under exactly the same conditions are photo- 439 bleached. However, the most important fact is that the 440 fluorescent signal from the assay employing the strepta-QD 441 obtained in the course of signal accumulation is more than 2 442 orders of magnitude stronger than that for the maximal signals 443 that may be obtained for typical fluorogenic assays employing 444 the best organic dyes under optimal conditions. This shows that 445 the use of QD-based labels in typical fluorogenic assays is 446 decisively advantageous.

Panels a and b show variations of the fluorescence signals as 447 a result of their accumulation at permanent signal-to-noise ratios for 448 typical in vitro biochemical sandwich-like fluorogenic assays employing 449 two different concentrations of the fluorescent streptavidin-QD800 450 conjugate and the most popular organic dyes.
CONCLUSIONS

Proteolytic enzymes of the MMP family, in particular, MMP-2, are overexpressed during cancer pathological processes. Activated MMPs have been detected in tissues, plasma, serum, and urine of cancer patients at increased levels; they are also positively correlated with the severity of metastatic tumors.75–77 Thus, MMPs can serve as selective and specific tumor markers for clinical applications.

Because of the unique potency to combine monitoring and therapy, nanoparticle-based techniques can provide an advantage over standard diagnostic and therapeutic tools with respect to disease diagnosis and monitoring, drug delivery, and release. Moreover, nanoparticle-based diagnostic tools can be easily modified, combined, and improved to ensure multiplexed detection and advanced diagnostics sensitivity. Thus, nanoparticles (e.g., fluorescent nanocrystals or QDs) are considered a useful and indispensable tool for advanced clinical diagnostics, imaging, and therapeutic applications.78

In this study, a series of new MMP-inhibitors, ilomastat derivatives, have been prepared and subjected to preliminary biological evaluations in experiments with the main matrix metalloproteinases (MMP-1, MMP-2, MMP-9, MMP-13, and MMP-14). A systematic enzyme inhibition/selectivity study was carried out, with carboxylic acid serving as a "ZBG, by introducing a phenyl group to the C2 carbon atom of the tryptophan indole ring and by incorporating alkylidene chains of different lengths at the P’1 position of the succinic acid moiety. As a result of structure–activity relationship (SAR) studies, we have identified the most selective MMP-2 inhibitor with high inhibition potency (IC50 = 80 nM) and biotinylated it for use in assays employing the QD-streptavidin conjugate for visualization. The specific fluorescent complex of compound 8-biotin/streptavidin-QD was tested and evaluated for MMP-2 inhibition in model in vitro sandwich-type analytic biochemical assays and compared with similar model assays employing typical organic dyes. The data show that the fluorogenic assays employing QDs allows the detection signal level to be increased by more than 2 orders of magnitude compared to that of typical organic dyes, thus paving the way to a considerable increase in the sensitivity of MMP detection in in vitro assays. The described ultrasensitive QD-based detection approach, exemplified here by MMP-2 detection, is by no means limited to it. Designing and using broad-spectrum inhibitors will allow this strategy to be efficiently modified for detecting both other MMPs and other hydrolytic enzymes for different substrates.

EXPERIMENTAL PROCEDURES

General Synthesis Methods and Materials. Standard solvents were purchased from commercial sources and were dried by standard procedures and redistilled under N2 prior to use. Reactions and products were routinely monitored by thin layer chromatography (TLC) on silica gel (KIESSELGEL 60 PF254, Merck). HBTU and 2,4,6-collidine were purchased from commercial sources. Pure products were obtained by means of flash chromatography using Merck Gelura SI silica gel 60 (70–230 mesh ASTM). The melting points were determined using a Reichert Thermovar hot-stage apparatus and are uncorrected. The NIR-FT spectra (KBr or NaCl film) were measured using a PerkinElmer Spectrum BX FTIR instrument. The 1H NMR (300 MHz) and 13C NMR (75 MHz) spectra were recorded by means of a Bruker AC 300 spectrometer using TMS as an internal standard; the chemical shifts δ were expressed in ppm; the following abbreviations are used: singlet (s), doublet (d), doublet of doublets (dd), triplet (t), and multiplet (m). Coupling constants J were expressed in hertz. Mass spectra were recorded by means of an MSQ ThermoFinnigan apparatus using the chemical ionization (CI) method. Electrospray ionization mass spectrometry experiments were carried out using a hybrid tandem quadrupole/time-of-flight (Q-TOF) instrument equipped with a pneumatically assisted electrospray (Z-spray) ion source (Micromass, Manchester, UK) operated in the positive mode. Optical rotations were measured on a PerkinElmer 241 polarimeter (Na lamp, λ = 589 nm).

Synthesis Procedures. (S)-Ethyl 3-[(1H-indol-3-yl)-2-(2,2,2-trifluoroacetoamido)propanoate 7. t-Tryptophan (5.0 g, 24.5 mmol) was dissolved in absolute ethanol (80 mL). Triionyl chloride (3.6 mL, 2 equiv, 48.9 mmol) was added dropwise over 15 min. The mixture was refluxed for 3.5 h; then, the solvent was evaporated. The white solid was dissolved in EtOAc, the organic phase was washed with an aqueous solution of NaHCO3 (5%), and then dried over MgSO4. After filtration and concentration of the solvent, a slightly yellow oil was obtained. Then, CH2Cl2 (110 mL), triethylamine (3.4 mL, 1 equiv, 24.5 mmol), and trifluoroacetic anhydride (8.2 mL, 2 equiv, 85.6 mmol) were added at 0 °C. The mixture was stirred for 3 h at room temperature overnight. The solvent was evaporated; the crude product was purified by flash chromatography (cyclohexane/CH2Cl2; 40/60) to afford the white solid of 46 (7.6 g, 72%). mp: 117–119 °C. [α]21D −21 (c 1.13, DMSO).1H NMR (DMF-d4, 300 MHz): δ 1.14 (t, 3H, J = 7.1 Hz), 3.20–3.35 (m, 2H, 2H), 4.12 (2H, J = 7.1 Hz), 4.52–4.60 (m, 1H), 7.02–7.10 (m, 2H), 7.18 (s, 1H), 7.37 (d, 1H, J = 8.1 Hz), 7.55 (d, 1H, J = 7.8 Hz), 9.95 (d, 1H, J = 7.5 Hz), 10.92 (s, 1H).13C NMR (DMF-d4, 75 MHz): δ: 14.1, 26.2, 54.0, 61.3, 109.4, 110.6, 111.7, 114.4, 118.2, 118.4, 118.7, 121.3, 121.4, 122.0, 127.1, 136.3, 155.9, 156.4, 156.9, 157.4, 170.5. IR (KBr): ν (cm−1) 3374, 3322, 1736, 1696, 1555, 1304, 1278, 1230, 1176, 1093, 1027, 873, 860, 741, 692. MS (CI): m/z: 328.96 [M+Na]+ (100), 311 (14), 283 (15), 255 (25), 242 (23), 238 (59). Anal. Calcd for C14H12N2O3F2: C 54.88, H 4.61, N 8.53%. Found: C 54.64, H 4.74, N 8.38%.

(S)-Ethyl 3-[(2-phenyl-1H-indol-3-yl)-2-(2,2,2-trifluoroacetoamido)propanoate 8. N-Bromosuccinimide (54 mg, 0.3 mmol) was added to a suspension of α-N-(trifluoroacetoxy)-t-tryptophan ethyl ester 7 (0.1 g, 0.3 mmol) in CCl4 (2 mL). The mixture was refluxed for 30 min under nitrogen, and the solvent was evaporated in vacuo. The filtrate was purified by flash chromatography (petroleum ether/EA; 9:1) to afford a brominated compound (118 mg, 96%). mp: 134–137 °C. [α]21D −14 (c 1.41, DMSO).1H NMR (CDCl3, 300 MHz): δ 1.12 (t, 3H, J = 7.1 Hz), 3.29–3.35 (m, 2H), 4.08–4.24 (m, 2H), 4.91 (dd, 1H, J = 13.8 Hz, J = 5.8 Hz), 6.99 (d, 1H, J = 7.1 Hz), 7.09–7.20 (m, 2H), 7.25 (d, 1H, J = 7.4 Hz), 7.46 (d, 1H, J = 7.7 Hz), 8.35 (s, 1H).13C NMR (CDCl3, 75 MHz): δ 13.8, 27.3, 52.9, 62.5, 109.0, 109.6, 109.8, 110.7, 113.6, 117.4, 117.7, 120.5, 121.2, 122.8, 127.5, 136.0, 156.0, 170.5.
Phenyboronic acid (52 mg, 1.5 equiv, 0.42 mmol) was dissolved in a mixture of toluene and ethanol (1/1) (4 mL), the brominated compound (115 mg, 0.28 mmol), NaHCO₃ (47 mg, 2 equiv, 0.56 mmol) dissolved in pure water (1 mL), and LiCl (36 mg, 3 equiv, 0.84 mmol); finally, catalyst Pd(PPh₃)₄ (32 mg, 0.1 equiv, 0.028 mmol) was added. The yellow solution was stirred under reflux for 2 h. The red mixture was concentrated; then, CH₂Cl₂ and an aqueous solution of NaHCO₃ (10%) were added. The aqueous phase was extracted twice with CH₂Cl₂. The organic phases were collected, dried over MgSO₄, filtered, and concentrated. The crude product was purified by flash chromatography (petroleum ether/EtOAc: 9/1) to afford compound 8 (105 g, 92%). mp: 172–174 °C. [α]D²¹ = −10.5 (c 0.84, DMSO). ¹H NMR (CDCl₃, 300 MHz): δ 1.02 (t, 3H, J = 7.1 Hz), 3.56–3.62 (m, 3H), 3.91–3.97 (m, 1H), 4.81 (dd, 1H, J = 13.6 Hz, J = 5.8 Hz), 6.68 (d, 1H, J = 7.4 Hz), 7.12–7.57 (m, 9H), 8.22 (s, 1H). ¹³C NMR (CDCl₃, 75 MHz): δ 13.7, 26.5, 53.4, 61.9, 105.6, 109.0, 111.0, 113.0, 117.0, 118.5, 120.2, 121.0, 122.7, 128.2, 128.3, 128.9, 129.0, 129.1, 132.5, 135.6, 136.3, 155.1, 155.7, 156.3, 156.9, 170.2. IR (KBr): ν (cm⁻¹) 3374, 3286, 1753, 1705, 1555, 1450, 1278, 1203, 1172, 1018, 787, 745, 697. MS (EI): m/z calculated for C₁₅H₁₄N₂O₃BrF₃: C 44.25, H 3.47, N 11.72, P 15.84, S 4.37. Anal. Calcd for C₁₅H₁₄N₂O₃BrF₃: C 44.25, H 3.47, N 11.72, P 15.84, S 4.37. Anal. Calcd for C₁₅H₁₄N₂O₃BrF₃: C 44.25, H 3.47, N 11.72, P 15.84, S 4.37. Anal. Calcd for C₁₅H₁₄N₂O₃BrF₃: C 44.25, H 3.47, N 11.72, P 15.84, S 4.37. Anal. Calcd for C₁₅H₁₄N₂O₃BrF₃: C 44.25, H 3.47, N 11.72, P 15.84, S 4.37. Anal. Calcd for C₁₅H₁₄N₂O₃BrF₃: C 44.25, H 3.47, N 11.72, P 15.84, S 4.37. Anal. Calcd for C₁₅H₁₄N₂O₃BrF₃: C 44.25, H 3.47, N 11.72, P 15.84, S 4.37.
tert-Butyl 3-(5-(2-oxo-hexahydro-1H-thieno[3,4-d]-imidazol-4-yl)pentamidino) propargylcarbomate 16. HBTU (232 mg, 1.5 equiv, 0.61 mmol) and 2,6-collidine (110 μL, 2 equiv, 0.81 mmol) were added to a solution of biotin 15 (100 mg, 0.41 mmol) in dry DMF (3 mL). The reaction mixture was stirred for 1 h at room temperature; then, a solution of compound 14 (107 mg, 1.5 equiv, 0.61 mmol) in dry CH₂Cl₂ (3 mL) was added, and the mixture was stirred overnight at room temperature. The solvent was evaporated, and the crude product was purified by flash chromatography (CH₂Cl₂/MeOH: 9/1, 8/2) to afford compound 16 (121 mg, 74%).

ν = 3.29 (m, 10H), 4.12 (q, 1H), 6.38 (s, 1H), 6.45 (s, 1H), 6.79 (t, 1H, J = 6.2 Hz), 7.03 (s, 2H), 7.56 (d, 2H, J = 6.8 Hz), 7.30 (t, 2H, J = 5.4 Hz). 13C NMR (CDCl₃, 75 MHz): δ 25.3, 27.8, 27.8, 28.1, 29.4, 35.6, 35.9, 37.0, 40.1, 55.3, 59.9, 61.6, 79.2, 156.7, 164.0, 174.1. IR (NaCl): ν (cm⁻¹) 3297, 2925, 1687, 1524, 1457, 1361, 1276, 1248, 1165, 842. HRMS (EI): m/z calcd for C₂₇H₂₅N₄O₂S 423.2034, found 423.2035 (+1.7 ppm). Anal. Calcld for C₂₇H₂₅N₄O₂S: C 57.58, H 4.02, N 13.24. Found: C 57.26, H 3.91, N 13.26.

N-(3-Aminopropyl)-5-(2-oxo-hexahydro-1H-thieno[3,4-d]-imidazol-4-yl)pentamidine 17. TFA (1 mL) was added to a solution of compound 16 (95.5 mg, 0.24 mmol) in dry CH₂Cl₂ (2 mL), and the mixture was sequentially stirred for 30 min at 0 °C and for 30 min at room temperature. After evaporation, an orange oil was obtained (144 mg, 100%). 1H NMR (CDCl₃, 300 MHz): δ 1.41–1.52 (m, 2H), 1.58–1.69 (m, 4H), 1.83–1.98 (m, 2H), 2.19–2.27 (m, 2H), 2.72–2.96 (m, 4H), 3.21–3.31 (m, 1H), 3.28–3.38 (m, 2H), 4.32–4.36 (m, 1H), 4.52–4.58 (m, 1H). 13C NMR (CDCl₃, 75 MHz): δ 25.0, 26.8, 27.6, 27.8, 35.0, 35.3, 36.5, 40.0, 55.3, 60.1, 61.9, 164.2, 175.5. IR (NaCl): ν (cm⁻¹) 3291, 2919, 2852, 1661, 1457. HRMS (EI): m/z calcd for C₂₇H₂₅N₄O₂S 423.2034, found 423.2035 (+1.7 ppm). Anal. Calcld for C₂₇H₂₅N₄O₂S: C 57.58, H 4.02, N 13.24. Found: C 57.26, H 3.91, N 13.26.

(E)-3-(3-(1-Methylamino)-1-oxo-3-(3-oxo-3-(5-(2-oxo-hexahydro-1H-thieno[3,4-d]-imidazol-4-yl)pentamidino)propyl)propyl)-2-phenyl-1H-indol-3-yl)-propan-2-ylcarbamoyl)tridec-3-enoic Acid 5. Allyl ester 18 (64 mg, 0.07 mmol) was dissolved in THF (2 mL) in a nitrogen atmosphere and supplied with a catalyst (Pd(PPh₃)₄ 0.1 equiv) and morpholine (18.6 μL, 0.21 mmol) in the dark. After incubation for 30 min, the solvent was evaporated. The organic phase was filtered through the Celite filter agent, and the resultant solution was concentrated in vacuo. The concentrate was purified by silica gel column chromatography with the solvent CH₂Cl₂/MeOH: 7/3 to afford acid compound 5 as a white solid (44 mg, 72%). ν = 103–105 °C. 1H NMR (DMSO-d₆, 300 MHz): δ 0.87 (t, 3H, J = 6.8 Hz), 1.26–1.49 (m, 22H), 1.99–2.09 (m, 4H), 2.29–2.41 (m, 2H), 2.42 (d, J = 4.4 Hz, 2H), 2.77–3.10 (m, 11H), 4.11–4.40 (m, 5H), 6.14 (t, J = 7.0 Hz, 2H), 6.37 (s, 1H), 6.45 (s, 1H), 6.99–7.14 (m, 2H), 7.41–7.51 (m, 4H). 13C NMR (DMSO-d₆, 75 MHz): δ 14.2, 22.3, 25.5, 25.8, 27.8, 28.2, 28.4, 54.8, 55.6, 59.3, 61.2, 64.6, 109.2, 110.1, 117.5, 119.2, 119.3, 121.5, 128.0, 128.7, 129.1, 130.8, 131.3, 132.7, 135.9, 138.2, 138.3, 162.9, 167.3, 169.5, 170.4, 171.7, 172.1. IR (NaCl): ν (cm⁻¹) 3402, 3300, 2920, 2919, 2852, 1665, 1543, 1532, 1462, 1354, 1325, 1263, 842, 739. HRMS (EI): m/z calcd for C₃₅H₄₃N₇O₇SNa 948.5033, found 948.5041 (0.8 ppm). Anal. Calcld for C₃₅H₄₃N₇O₇SNa: C 82.94, H 6.87, N 9.48, S 3.09%. Found: C 82.98, H 6.97, N 9.48, S 2.94%.

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Molecular Docking. The AutoDock 4.0 software was used to perform the computational molecular docking. The AutoDockTools package was employed to prepare the input files necessary for the docking procedures and to analyze the results of docking. Figures were constructed using the PyMOL software (DeLano, W. L. (2002) PyMol Molecular Graphics System, Palo Alto, CA. http://www.pymol.org).

Initial Data on MMPs and Ligands. The structures from the Protein Data Bank (PDB) entries 1CK7 (for MMP-2) and 1GKC (for MMP-9) were used for docking simulations. While MMP atoms and the zinc ion in the catalytic site were retained, all of the other atoms were removed. Residues 31–109 corresponding to the pro-domain prohibiting the MMP-2 proteolytic activity have been also removed. The protonation states of all ionizable residues were computed using the PROPKA software. 11,12 The MMP side chains were kept fixed for all the docking computations. Ligands were built using the Marvin software (Marvin 5.3.2, 2010, ChemAxon: http://www. chemaxon.com). The AutoDock module AutoTors was used to determine the torsion angles of the ligands. All of the flexible torsions except amide bonds were allowed to rotate during the docking stage.

Molecular Docking Simulations and Calculations. Affinity grid maps were calculated for each atom type constituting 26.
MMP-2 with the use of the AutoGrid software. Grid maps were
centered on the MMP catalytic site, with 126 × 126 × 126 grid
definitions, and spacings of 0.291 and 0.225 Å between the grid
definitions for MMP-2 and MMP-9, respectively. Meher and
Solmajer’s distance-dependent dielectric permittivity was used
for the calculations of the electrostatic grid maps. Random
starting positions on the entire protein surface and random
orientations and tions were used for all ligands. The
AutoDock software, version 4.0, was used for docking
computations, with a Lamarckian genetic algorithm. Each
docking experiment was performed with four runs constituted
of a series of 250 simulations. Each docking simulation was
covered with an initial population of 250 individuals, a
maximum number of 2,500,000 energy evaluations and a
maximum number of 27,000 generations. The pseudo-Solis and
Wets modification methods were used with default parameters.
The docked conformations of the ligands were clustered with a
root-mean-square deviation (RMSD) cutoff of 0.5 Å.

**Biological Evaluations.** *Inhibition Studies.* The quenched
fluorescent substrates DNP-Pro-Cha-Gly-Cys(Me)-His-Ala-
Lys(N-Me-Abz)-NH2 for MMP-1 or MMP-9 inhibition
(when DNP is 2,4-dinitrophenyl; Cha is β-cyclohexylalanyl;
Abz is 2-aminobenzoyl(antraniloyl)) were purchased from
Calbiochem (VWR, Strasbourg, France), Mca l-Pro-Leu-Gly-
Leu-Dpa-Ala-Arg-NH2 for MMP-2, MMP-13, or MMP-14
inhibition (where Mca is (7-methoxycoumarin-4-yl); Dpa is
[31,2(4-dinitrophenyl)-l-2,3-diaminopropionyl]) and 6-(7-
nitro-benzo[1,2,5]oxadiazol-4-ylamino)-hexanoyl-Arg-Pro-Lys-
Pro-Leu-Ala-Nva-Trp-Lys(7 dimethylaminocoumarin-4-yl)NH2
for MMP-3 inhibition were from Bachem (Weil am Rhein,
Germany). The dye quenched fluorescent substrate (DQ-
gelatin) and EnzCheck Gelatnine/Collagenase assays were
purchased from Life Technologies (USA).

Human recombinant pro-MMP-1, pro-MMP-2, pro-MMP-9
and pro-MMP-13, and catalytic domains of MT1-MMP were
obtained from Calbiochem. The pro-enzymes (pro-MMP-2 or
pro-MMP-9) were freshly activated with 1–4 mM p-aminophenylmercuric acid (APMA, Sigma-Aldrich, Saint Quentin
Fallavier, France) at 37 °C for 1–2 h. Fluorescent conjugates of
strepavidin with QDs were purchased from Life Technologies
(USA).

*In Vitro Fluorogenic Substrate Digestion Assay.* Briefly, 1
nM of MMP-1, 0.95 nM of MMP-2, 0.89 nM of MMP-9, 0.6
nM of MMP-13, and 3.1 nM of MMP-14 catalytic domain were
incubated with increasing concentrations of the synthetic
compounds (from 1 to 5000 nM). Assays were initiated by
adding the respective fluorogenic substrate (1–10 nM). The
fluorescence was monitored with a PerkinElmer HT Soft 7000
plus spectrofluorimeter (PerkinElmer, Courtaboeuf, France).

Upon cleavage of the fluorogenic peptide by MMP, the initial
rate of the peptide hydrolysis in the absence (V0) or presence
(V) of the synthetic molecule was determined. The IC50 was
calculated after plotting V/V0 as a function of the synthetic
molecule concentration by fitting with a nonlinear regression
(Grafit Computer software, R. Leatherbarrow, Ethicus
Software).

**MMP-2 Proteolytic Activity Measurement.** MMP-2 activity
was estimated in EnzCheck Gelatnine/Collagenase standard
commercial assays based on digestion of an internally quenched
fluorescent substrate with an activated enzyme according to the
manufacturer’s protocol. Briefly, 0.35–2 μg of recombinant
MMP-2 was first preactivated with 2.5 mM APMA in an
activation buffer (50 mM Tris-HCl, pH 7.5, 150 mM NaCl, 20
mM CaCl2, and 0.01% Tween-20) during 1.5 h at 37 °C. Upon
activation, 0–100 ng of MMP-2 was mixed with 50 μg/mL DQ
gelatin in a total volume of 200 μL of the activating buffer in a
black nontreated 96-well plate. The samples were incubated at
room temperature, while protected from light for 2 to 24 h. The
activity of MMP-2 was measured fluorometrically in a 969
fluorescence microplate reader (Infinite M200 Pro, Tecan, Switzerland). Since the reaction of quenched fluorescence substrate digestion with the activated enzyme is continuous, fluorescence was measured at multiple time points: 0, 20, 40, 60, 80, 100, 120 min, etc. The fluorescence of digested products of the DQ gelatin was induced at 488 nm and measured at 525 nm in a fluorescence microplate reader equipped with two
monochromatic scanners. Background fluorescence was
_corrected by subtracting the value derived from no-enzyme
substrate control. This protocol was also employed for the
detection of compound 5 (the enzyme inhibitor).

In this case, 50 ng of activated MMP-2 was preincubated with 0–
50 μg of compound 5 preliminarily dissolved in DMSO in a
total volume of 100 μL of the activating buffer. This enzyme-
inhibitor mixture was supplemented with 50 μg/mL DQ gelatin
in a total volume of 200 μL, and the inhibited MMP-2 activity
was measured fluorometrically at different time points.

*In Vitro Gelatinolytic Assays.* HT-1080 cells were grown to
a density of 80% in a 24-well plate and treated with 12.5 μg/mL
concanavalin A overnight to convert pro-MMP-2 into active
MMP-2. Cells were preincubated with or without effectors (100
μM 4k, 10 μM ilomastat (Galardin)) for 1 h and then with DQ-
gelatin (500 ng/300 μL per well). Fluorescence was measured at
525 nm.

*In vitro* invasion assay was performed in modified Boyden
chambers (tissue culture treated; diameter, 6.5 mm; pore size, 8
μm; Greiner-One, Courtaboeuf, France). Five × 104 HT-1080
were suspended in serum-free DMEM with 4.5 g/L glucose
containing 0.2% (w/v) BSA and seeded onto membranes coated with Matrigel (20 μg/well). The lower
compartment was filled with DMEM supplemented with 10% (v/v) FBS and 2% (w/v) BSA. After a 6-h incubation period,
the cells were fixed with methanol and stained with crystal
violet for 15 min. The cells remaining on the upper face of the
membrane were scraped. Crystal violet staining of the migrating
cells (on the lower face) was eluted with 10% (v/v) acetic acid,
and absorbance was read at 560 nm.

**Quantum Dot-Based Assays.** The streptavidin-coated
QDs used in this study were purchased from Invitrogen
Corporation.

**Dot-Blotting.** The formation of the complex of biotinylated
compound 5 with streptavidin-QD conjugate was visualized
using the dot-blot detection technique. Briefly, different
amounts (0–20 μg) of biotinylated compound 5 dissolved
either in methanol or in DMSO and supplied with PBS, pH 7.4,
were applied onto a nitrocellulose or PVDF membrane in a
total volume of 2 μL. The membrane was dried completely and
blocked from nonspecific binding of the fluorescent conjugate
with 5% (w/v) nonfat dry milk in PBS (pH 7.4) containing
0.05% (v/v) Tween 20 during 1 h at room temperature. Then,
the membrane was incubated with streptavidin-QD 565 nm
fluorescent conjugate (1:100 dilution in PBS (pH 7.4) containing
0.5% (w/v) casein from bovine milk) during 40–30
min at room temperature and washed thoroughly three times
with PBS (pH 7.4) containing 0.05% (v/v) Tween 20. The
fluorescence of the inhibitor-QD complexes of different
intensities was detected using a ChemiDoc MP Imaging system.
Bioconjugate Chemistry

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Bioconjugate Chem. XXXX, XXX, XXX–XXX

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**Notes**

The authors declare no competing financial interest.

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**ABBREVIATIONS**

APMA, p-aminophenylmercuric acetate; BRET, bioluminescence resonance energy transfer; BSA, bovine serum albumin; CPP, cell penetrating peptide; DMF, dimethylformamide; DMSO, dimethyl sulfoxide; DMEM, Dulbecco’s modified Eagle’s medium; FBS, fetal bovine serum; FRET, Förster resonance energy transfer; HBTU, O-(benzotriazol-1-yl)-N,N,N’,N’-tetramethyluronium hexafluorophosphate; IC50, the half maximal inhibitory concentration; MMP(s), matrix metalloproteinase(s); MMP1(s), matrix metalloproteinase inhibitor(s); PBS, phosphate buffered saline; PVDF, polyvinylidene difluoride; QD(s), quantum dot(s); TFA, trifluoroacetic acid; THF, tetrahydrofuran; TLC, thin layer chromatography; ZBG, zinc binding group.


