DOI: 10.1002/anie.200604408

Functional Proteomics

Cross-Linking Yield Variation of a Potent Matrix Metalloproteinase Photoaffinity Probe and Consequences for Functional Proteomics**

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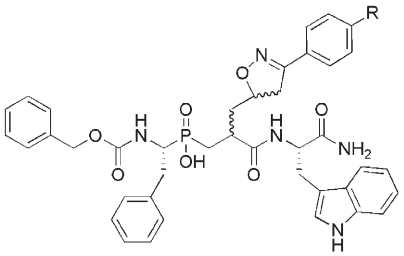
Recent developments in proteomic technology offer tremendous potential to provide novel biomarkers for diagnosis of early-stage diseases or identification of patients at higher risk of dying.[1] Among the new proteomic approaches recently developed, activity-based protein profiling (ABPP) technologies use active-site-directed probes to profile the active forms of enzymes in complex proteomes.[2,3] ABPP probes have been shown to selectively label active enzymes but not their zymogen or inhibitor-bound forms. Thus changes in enzyme activity can be detected by these probes, independently from posttranslational controls.[2,3] The ABPP approach has been successfully applied to profile serine proteinases with fluorophosphonates[4] and cysteine proteinases with epoxides[5] as the irreversible inhibitors. To profile zinc proteinases, the largest family of proteases in humans (159 members),[6] photolabile groups have been incorporated into the ABPP probes for covalent modification of these enzymes, due to a lack of conserved nucleophiles in their active sites; this has led to the discovery of some elevated zinc proteinase activities in disease states.[7–9] However, in the specific case of matrix metalloproteinases (MMPs, a group of 23 closely related members in humans[10]), this approach failed to detect active forms of MMPs produced by cancer cells or in tumors,[8,9] despite the over-expression of MMPs that has been reported in these contexts.[11] It has been argued that MMPs are mostly present in inactive forms (zymogen and inhibitor-bound forms). Thus the active forms of MMPs may exist in complex proteomes at levels below the current detection limits of ABPP technologies.[9] Therefore, ABPP probes able to detect low levels of the MMP active forms (in the femtomolar range) are still required and the factors controlling probe sensitivity should be further investigated. The present study was designed first to address the sensitivity issue and also to determine how the yields of MMP covalent modification by a specific ABPP probe affect the detection threshold for these enzymes.

Prior studies have shown that phosphinic peptides containing isoxazole or isoxazoline side chains in their P_1' position behaved as potent MMP inhibitors.[12–14] This led us to design and synthesize the photolabile compound **1** for the present project.



compound **1**, R = N_3
compound **2**, R = H

As shown in Table 1, compound **1** displayed high potency toward several MMPs. The isoxazoline side chain in compound **1** is expected to fill the S_1' subsite of the MMPs, thereby placing the azido group inside a very deep cavity conserved in most MMPs. Compound **2**, which lacks the azido group, exhibited similar potency toward the MMPs, a result showing that the azido group in **1** is well tolerated by the MMP S_1' cavities.

Table 1: Potencies of compounds **1** and **2** toward human MMPs.

	MMP							
	2	3	8	9	11	12	13	14
K_i with $\mathbf{1}_{F1}$ [nM][a]	0.7	13	0.8	0.75	0.2	0.65	0.4	3.5
K_i with $\mathbf{2}_{F1}$ [nM][a]	1.1	215	1.2	0.9	1	0.4	0.45	5

[a] The diastereomers of compounds **1** and **2** were resolved by HPLC. The reported inhibition constant (K_i) values are those of the most potent isomers (F_1).

Compound **1** was radiolabeled with tritium. This radioactive tag was preferred to a fluorescent one, as the latter involves chemical modification of the probe with bulky and hydrophobic groups. Moreover, the radioactive specificity of probe **1** (8 Ci mmol^{-1}) allows covalent MMP–probe complexes to be detected with high sensitivity and the radioactive signal provides a means to quantify the cross-linking efficiencies of the probe with different MMPs.

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[**] This work was supported by funds from FP6RDT (LSHC-CT-2003-503297).

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As shown in Figure 1 A, incubation of compound **1** with MMP-12, followed by UV irradiation, led to radioactive labeling of MMP-12. By contrast, no labeling was observed without the UV irradiation. These results indicate that the

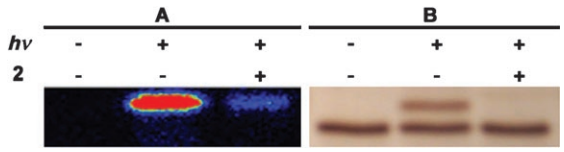

Figure 1. Covalent labeling of MMP-12 by compound **1**. A) MMP-12 (50 ng) was incubated with compound **1** (2 μM) for 10 min, either with or without compound **2** (11 μM), before UV irradiation (*hv*, 2 min). The MMP-12 complex was resolved by 1D sodium dodecylsulfate PAGE electrophoresis, then proteins were transferred onto a polyvinylidene fluoride (PVDF) membrane that was analyzed with a radioimager. B) Results of the same experiments but with the protein visualized on the gel by silver staining (MMP-12, 100 ng).

observed radioactive signal reflects only the presence of a covalent enzyme–inhibitor complex on the PVDF membrane, after electrophoresis separation. To demonstrate that this covalent modification only concerns the active site, MMP-12 was first preincubated with compound **2**, and this was followed by addition of compound **1** and UV irradiation. Under these conditions, no radioactive signal can be detected, a result indicating that compound **1** only labels the active site of MMP-12 when it is free of inhibitor. Interestingly, silver staining of a gel loaded with UV-irradiated MMP-12 in complex with compound **1** revealed two bands (Figure 1 B), whereas a single band was observed in the absence of irradiation. Comparison of these results with those obtained by the radioimagery analysis of the same samples indicates that the upper band visualized with the silver staining corresponds to the MMP-12 form covalently modified by compound **1**, while the lower band is the unmodified form of MMP-12. This interpretation is also supported by competition experiments. Indeed, when MMP-12 was preincubated with compound **2** before compound **1** was added and the photoirradiation took place, a single band was observed by silver staining (Figure 1 B). As a consequence, the ratio of the band intensities visualized by silver staining reflects the yield of MMP-12 cross-linking by compound **1**. This approach provides a cross-linking yield of $(42 \pm 5)\%$ for compound **1** with MMP-12 (average of three replicates).

The sensitivity of compound **1** for the detection of MMP-12 was determined by incubation of this compound with serial dilutions of MMP-12. As shown in Figure 2, as few as 50 pg of MMP-12 can be detected with compound **1** (2.5 fmol of MMP-12 at 100 pM concentration).

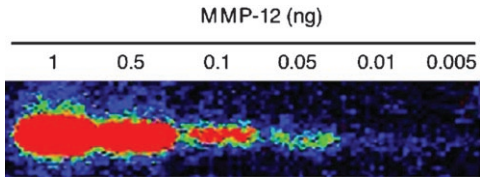

Figure 2. Sensitivity of compound **1** (2 μM) for the detection of MMP-12.

Finally, the yields of covalent modification of eight MMPs also possessing a deep S_1' cavity were determined with compound **1**. Experiments were performed by using the same number of active MMP catalytic units, as determined for each MMP by careful titration experiments with a highly potent MMP inhibitor. The results (Figure 3) demonstrate that compound **1** cross-links with all of the examined MMPs, as reflected by the radioactivity incorporation, but with a 40-fold difference in efficacy.

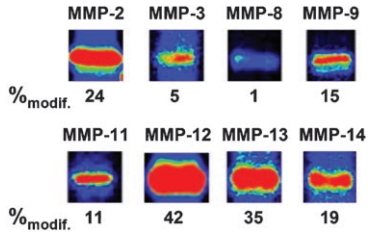

Figure 3. Covalent modification of various MMPs (1 pmol of active MMP) by compound **1** (2 μM). The percentage of modification was calculated by counting the radioactivity incorporated in each MMP, with the 42% yield determined for MMP-12 as a reference value.

From the above results, comparable sensitivity in detection can be expected for MMP-12, -13, -2, and -14 with compound **1**, but depending on their expression levels in complex proteomes, MMP-3 and MMP-8 might be hardly detected with the present probe. It is worth mentioning that cross-linking yields were not determined in previous reports on the development of ABPP probes to profile MMPs.[7–9] From the structure of these probes, it can be anticipated that the MMP residues possibly modified by these probes belong to surface solvent-exposed subactive sites,[7–9] a situation that should result in higher variability of the probe cross-linking yield with different MMPs. To avoid huge variation in cross-linking yields and possible failure to detect some MMPs, it seems mandatory to more systematically evaluate the influence of the photolabile-group type[15,16] and its position within the probe structure, in order to deliver a single probe able to modify each MMP with high yield. Whether such an ideal probe exists remains uncertain; however, an alternative possibility to resolve this hurdle would be to use a cocktail of MMP-optimized probes, instead a single one. Given the size of the MMP S_1' cavity, the development of compound **1** analogues containing benzophenone or diazirine photolabile groups may lead to a set of ABPP probes that will allow the sensitive detection of all MMP members.

In conclusion, we have developed a new MMP photoaffinity probe, which displays high potency towards MMPs and is able to covalently modify several members of this family. With a detection limit of 2.5 fmol for MMP-12, compound **1** is one of the most sensitive probes reported to date for MMPs. (A 60-fmol detection limit has been reported for MMP-2.[8]) MMP-12 has been implicated in several human diseases.[17–19] Thus the ABPP probe reported in this paper offers a simple and efficient strategy to detect the expression of MMP-12 active forms in biopsies or fluids relevant for these pathologies. Other MMPs may also be detected with

this probe, but with the limitations mentioned above. Preparation of a probe cocktail is underway to address this challenging issue.

Received: October 27, 2006
Revised: December 20, 2006
Published online: March 23, 2007

Keywords: inhibitors · metalloproteinases · photoaffinity probes · protein profiling · proteomics

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