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Design and synthesis of an affinity probe that targets caspases in proteomic experiments

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Abstract—The field of proteomics aims to study all proteins in the human proteome. This huge task may be accelerated by using active-site directed probes which profile proteins in an activity-dependent manner. Herein, we have developed a fluorescently-labeled affinity probe containing chemical reactivity specific towards caspases. Preliminary assays and proof-of-concept experiments demonstrated that this probe exhibits strong chemical reactivity towards caspase-1 over other enzymes, capable of covalently labeling caspapse-1 over other non-caspase enzymes. This thus demonstrates its selectivity and potential in high-throughput screenings of other unknown caspases in a large-scale proteomics experiment.

The completion of the Human Genome Project has opened up new and exciting possibilities in the understanding of the workings of the cell and diseases. However, genes alone do not tell the whole story of cellular functions. Proteins are ultimately responsible for most processes that take place within the cell. Hence, the onus is now on scientists, equipped with the sequence of 30 000–40 000 genes,[1] to elucidate the human proteome. Only then can the wealth of information gleaned from the genome project be translated into tangible benefits in medicine. Researchers expect the 30 000–40 000 genes in humans to produce more than 100 000 different proteins in each cell.

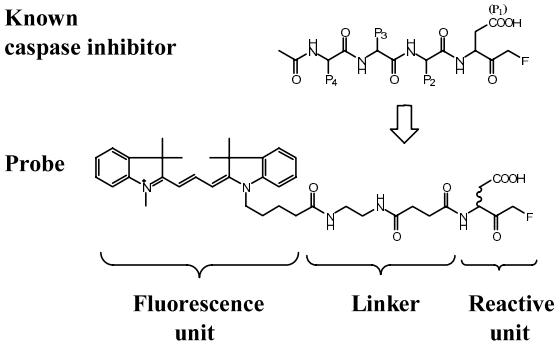

Figure 1. Structure of a typical tetrapeptide fmk inhibitor of caspase and the generalized probe used in target caspases. Note that amino acids are (L)-configured unless otherwise indicated.

To help cope with the enormity of the task, rapid technological advances in the techniques and methods involved in proteomic studies would be necessary. High-throughput screening methods and automation would enable scientists to analyze proteins on a global scale quickly and efficiently. Already researchers are developing new methods to simplify protein analysis and allow high-throughput screenings.[2–6] The activity-based, affinity tag approach can potentially filter out proteins that are not of interest and focus on certain classes of proteins (e.g. based on activity),[2] while protein chips and microarrays offer the attractive prospect of screening thousands of proteins simultaneously.[3–6] Unknown proteins could be spotted on arrays and incubated with potential ligands, which bind and allow the detection of the target proteins. The whole process of separating and identifying proteins in a complex mixture hence may be simplified, by zooming in only onto specific target proteins.[2]

Caspases are enzymes which play a key mediating role in apoptosis, or programmed cell death. All members of this protease family are characterized by their unique requirement for an aspartic acid at the P_1 position of the substrate cleavage site. Insufficient caspase activity promotes cell accumulation implicated in cancer and

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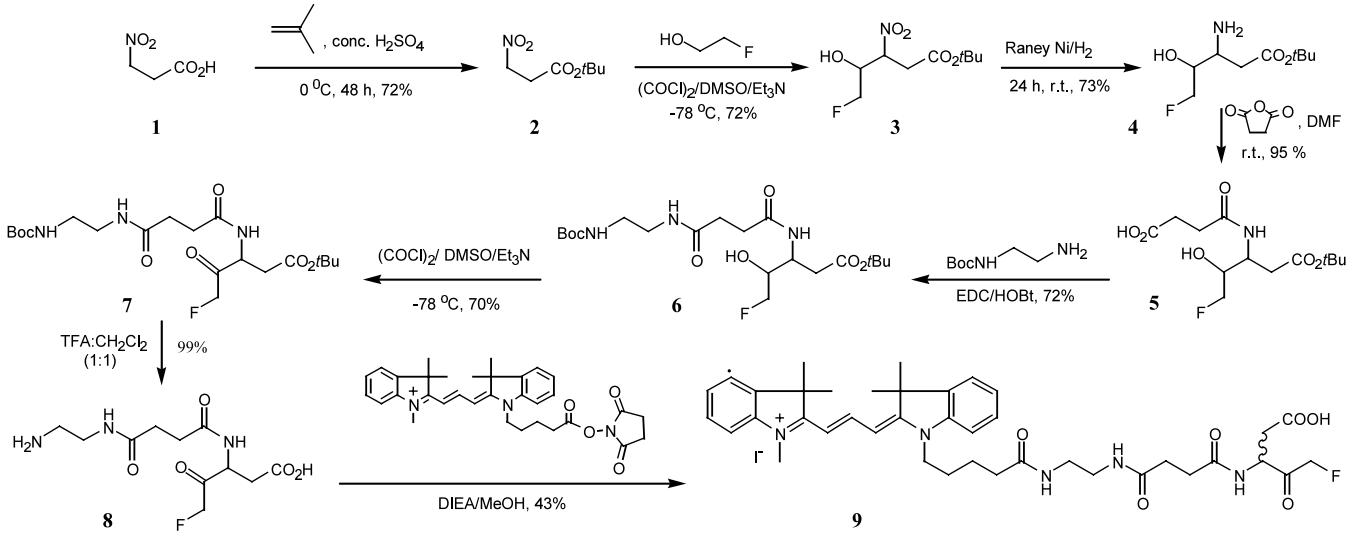
autoimmune diseases, while excessive caspase activity could lead to accelerated cell death, believed to result in neurodegenerative disorders such as Huntington's and Alzheimer's diseases.[7–9] Given their pivotal role, caspases are attractive therapeutic targets for treatment of these conditions. It is hence in our interest to discover new caspases, and to design therapeutic drugs to target caspases and their regulation.

Herein, we aim to utilize the affinity tag approach[2] to focus on specific classes of proteins, one class at a time. The ultimate goal is to develop fluorescently-labeled affinity probes containing chemical reactivity specific towards different classes of proteins, such as kinases, serine proteases and caspases. These probes, combined with standard gel-based proteomic experiments, or with newly developed protein array-based strategy,[10] may serve as a high-throughput tool to study the human proteome.

The general structure of our affinity probes consist of three units—the reactive unit which may be fine tuned to target different classes of proteins based on their enzymatic activity, the linker unit and the fluorescence unit which facilitates the detection of proteins upon labeling with the probe. In this report, we have successfully developed a fluorescently-labeled affinity probe capable of selective labeling of caspases. Fluoromethylketone (fmk)-containing tetrapeptides (Fig. 1) are well-documented examples known to covalently react with the active site residues in many cysteine proteases.[11,12] It was therefore chosen as the electrophilic moiety in the Asp-mimic reactive unit in our probe structure (Fig. 1). Other classes of cysteine proteases may also be targeted by simply replacing the Asp portion with other structures. Cyanine dye, Cy3, has many outstanding properties, and was thus chosen as the dye unit.[13,14] The linker unit decides the probe's specificity towards individual caspases. Since our goal in this work was to design a general caspase probe capable of detecting caspases over other non-caspase enzymes, a linker unit consisting of a simple alkyl chain, as shown in Figure 1, was chosen, which is approximately the length of the P_2–P_4 tripeptide sequence in a typical caspase inhibitor and, at the same time, does not impose any specificity upon a particular caspase. Other linkers may be chosen when needed to fine-tune the specificity of the probes against their targeting enzymes.

Initial attempted synthesis of the probe indicated that the fmk group in the probe is a potential complication as the presence of fluorine increases the electrophilicity of the carbonyl carbon.[15] Together with its acidic α-proton, the fmk is rather susceptible to reactions involving bases and nucleophiles, which can lead to dimerization, epimerization and Schiff base reactions.[16] Therefore, a synthetic route (Scheme 1) was devised, in which reactions involving the use of potential nucleophiles and strong bases were intentionally avoided. Strategically, the oxidation of the alcohol to generate the fmk group was left to the final steps. Briefly, the *t*-butyl ester of 3-nitro-propionic acid, **2**, was generated from **1** using the classical isobutene/concentrated acid approach.[17] Fluoroacetaldehyde, generated in situ by oxidation of fluoroethanol under Swern Conditions,[18] was then added to **2** to yield the key intermediate **3** via a nitro-aldol reaction.[19] Hydrogenation[19] of **3** over freshly prepared Raney nickel catalyst[20] gave amino-alcohol **4** in 73% yield. The amino-alcohol was reacted with succinic anhydride to give the acid **5**. The singly Boc-protected ethylenediamine, prepared as previously reported,[21] was coupled to **5** using standard coupling reagents. Swern oxidation of the resulting compound **6** yielded **7**, which was subsequently treated with 50% TFA to give **8**. Conjugation of the dye molecule, Cy3, to the probe was carried out by coupling **8** with the NHS ester of Cy3, which was synthesized from Cy3 using standard coupling methods.[22] Overall, the probe **9** was obtained in eight steps with yield of ~10%.[23]



Scheme 1.

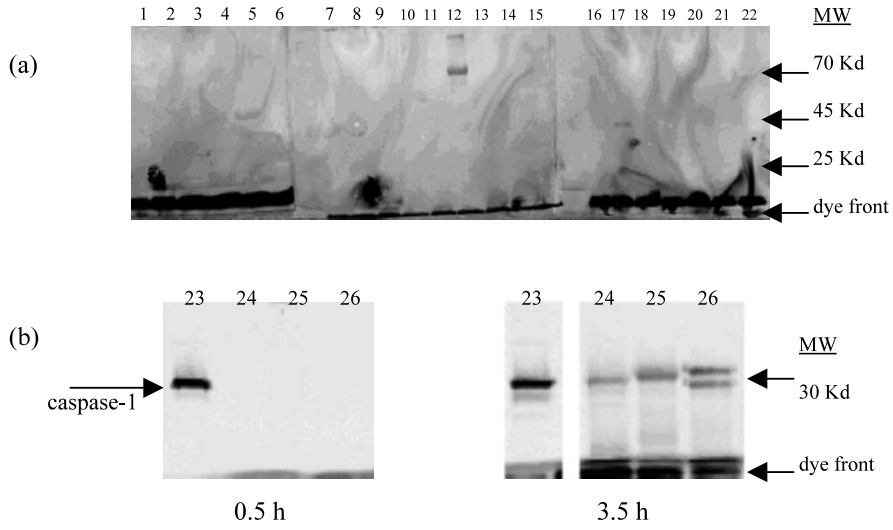


Figure 2. List of enzymes (Sigma Catalog #). (a): (1) α-chymotrypsin (C-4129), (2) β-chymotrypsin (C-4629), (3) γ-chymotrypsin (C-4754), (4) chymotrypsinogen (C-4879), (5) pepsin (P-6887), (6) proteinase K (P-6556), (7) subtilisin (P-5380), (8) trypsin inhibitor (T-9378), (9) trypsinogen (T-1143), (10) trypsin (T-1426), (11) thrombin (T-4648), (12) BSA (A-2512), (13) lysozyme (L-6876), (14) phosphatase, acid (P-3627), (15) phosphatase, alkaline (P-7640), (16) phosphatase, alkaline (P-2265), (17) phosphatase, alkaline (P-4002), (18) lipase (L-1754), (19) lipase (L-3001), (20) lipase (L-9031), (21) protease (P-6911), (22) phosphatase, acid (P-3752). (b): (23) Caspase-1, (24) papain (P-4762), (25) chymopapain (C-8526), (26) bromelain (B-4882).

Next, the probe was tested for selective labeling of proteins based on their enzymatic activity. A panel of 22 commercially available proteins was used, none of which were caspases. Upon incubation with the probe for 30 min under appropriate conditions,[24] the proteins were first separated using SDS-PAGE (Fig. 2a), followed by detection of fluorescence labeling using a fluorescence-based gel scanner. None of these proteins gave any significant fluorescent bands, except for BSA, which is known to bind to many molecules non-specifically.

We next investigated the selective labeling of the probe against its targeting enzyme, caspase. Caspase-1 was incubated with probe for differing lengths of time and the resulting labeled reaction was analyzed by SDS-PAGE and fluorescence detection. In order to unambiguously confirm the selectivity of the probe against ONLY caspases, three other non-caspase cysteine proteases were used as controls and analyzed simultaneously. As shown in Figure 2b, only caspase-1 was selectively labeled by the probe following 0.5 h incubation. No labeling was observed for the other three non-caspase cysteine proteases, indicating the high specificity of this probe for the detection of caspase activity. As the incubation time of the labeling reaction increases (3.5 h), other non-caspase cysteine proteases started to react with the probe, as evidenced by appearance of some fluorescence bands on the gel. However, a comparatively much stronger band was still observed for the caspase reaction. In order to further confirm that the fluorescence labeling of caspase-1 by the probe is due to its enzymatic activity, the enzyme was first inactivated by heat, then treated with the probe followed by SDS-PAGE analysis. No labeling of caspase-1 was observed, indicating the enzymatic activity of caspase-1 is a prerequisite for the labeling reaction to occur.

In conclusion, we have successfully designed, synthesized, and tested a fluorescent, small molecule probe capable of labeling caspases in a highly specific, activity-based fashion. Our studies clearly demonstrated the potential of this probe in the selective detection of caspases. The selectivity of this probe towards caspase-1, over non-cysteine protease enzymes, as well as other cysteine proteases, might enable the selective detection of caspase-1 in a complex protein sample. Subsequent experiments with this probe would involve other caspase members as well as in vivo samples to further verify the versatility of the probe. Furthermore, our probe may be readily modified to target other classes of cysteine protease, or a specific cysteine protease.

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References

1. Venter, J. C.; et al. *Science* **2001**, *291*, 1304–1351.
2. Cravatt, B. F.; Sorensen, E. J. *Curr. Opin. Chem. Biol.* **2000**, *4*, 663–668.
3. http://www.ciphergen.com

4. MacBeath, G.; Schreiber, S. L. *Science* **2000**, *289*, 1760–1763.
5. Zhu, H.; Bilgin, M.; Bangham, R.; Hall, D.; Casamayor, A.; Bertone, P.; Lan, N.; Jansen, R.; Dinlingmaier, S.; Houfek, T.; Mitchell, T.; Miller, P.; Dean, R. A.; Gerstein, M.; Snyder, M. *Science* **2001**, *293*, 2101–2105.
6. Lesaicherre, M. L.; Lue, R. Y. P.; Chen, G. Y. J.; Zhu, Q.; Yao, S. Q. *J. Am. Chem. Soc.* **2002**, *124*, 8768–8769.
7. Grutter, M. G. *Curr. Opin. Struct. Biol.* **2000**, *10*, 649–655.
8. Thornberry, N. A.; Lazebnik, Y. *Science* **1998**, *281*, 1312–1316.
9. Earnshaw, W. C.; Martins, L. M.; Kaufmann, S. H. *Annu. Rev. Biochem.* **1999**, *68*, 383–424.
10. Chen, G. Y. J.; Uttamchandani, M.; Zhu, Q.; Wang, G.; Lesaicherre, M. L.; Yao, S. Q., *J. Am. Chem. Soc.*, submitted for publication.
11. Otto, H. H. *Chem. Rev.* **1997**, *97*, 133–171.
12. Rasnick, D. *Anal. Biochem.* **1985**, *149*, 461–465.
13. Southwick, P. L.; Ernst, L. A.; Tauriello, E. W.; Parker, S. R.; Mujumdar, R. B.; Mujumdar, S. R.; Clever, H. A.; Waggoner, A. S. *Cytometry* **1990**, *11*, 418–430.
14. Ernst, L. A.; Gupta, R. K.; Mujumdar, R. B.; Waggoner, A. S. *Cytometry* **1989**, *10*, 3–10.
15. Linderman, R. J.; Jamois, E. A. *J. Fluorine Chem.* **1991**, *53*, 79–91.
16. Walter, M. W.; Adlington, R. M.; Baldwin, J. E.; Schofield, C. J. *J. Org. Chem.* **1998**, *63*, 5179–5192.
17. Valerio, R. M.; Alewood, P. F.; Johns, R. B. *Synthesis* **1988**, *10*, 786–789.
18. Mancuso, A. J.; Swern, D. *Synthesis* **1981**, *3*, 165–185.
19. Revesz, L.; Briswalter, C.; Heng, R.; Leutwiler, A.; Mueller, R.; Wuethrich, H. J. *Tetrahedron Lett.* **1994**, *35*, 9693–9696.
20. Mozingo, R. *Org. Synth.* **1941**, *21*, 15–17.
21. Beer, P. D.; Cadman, J.; Lloris, J. M.; Manez-Martinez, R.; Soto, J.; Pardo, T.; Marcos, M. D. *J. Chem. Soc., Dalton Trans.* **2000**, 1805–1812.
22. Korbel, G. A.; Lalic, G.; Shair, M. D. *J. Am. Chem. Soc.* **2001**, *123*, 361–362.
23. **Probe 9**. ^{1}H NMR (300 MHz, $CDCl_3$, 25°C) δ 8.40 (t, $J=13.2$ Hz, 1H); 7.45–7.36 (m, 4H); 7.30–7.28 (m, 2H); 7.21–7.15 (m, 2H); 6.39 (2 unresolved d, 2H); 4.84 (br m, 1H); 4.66–4.41 (m, 2H); 4.05 (br t, 2H); 3.63 (s, 3H); 3.38 (m, 4H); 2.90–2.87 (m, 2H); 2.57 (m, 4H); 2.33 (br t, 2H); 1.80 (m, 4H); 1.73 (s, 12H) ppm; ^{13}C NMR (75.4 MHz, $CDCl_3$, 25°C) δ? 203.1, 176.2, 174.3, 174.1, 173.4, 173.1, 172.7, 150.4, 142.4, 141.7, 140.3, 140.2, 129.0, 128.9, 125.6, 125.5, 122.0, 121.9, 111.3, 110.9, 103.2, 103.1, 85.5, 83.1, 52.3, 49.2, 49.0, 44.2, 39.5, 39.0, 35.3, 31.8, 31.3, 29.5, 28.0, 27.9, 26.6, 22.9 ppm; ^{19}F NMR (282.2 MHz, $CDCl_3$, 25°C) δ −156.41 (t, $J=38.1$Hz) ppm; MS (ESI) calcd for $C_{40}H_{51}FN_5O_6^+$ 716.3823; found 716.3822.
24. SDS-PAGE experiments: (1) *Non-cysteine proteases*. 2 μl of each non-caspase enzyme (10 μM) was first activated in 50 mM Tris, 2 mM ETDA, 0.31 mg/ml DTT (18 μl) for 1/2 h and then incubated at room temperature with 200-fold molar excess of **9** (0.2 μl, 2 mg/ml). After 1/2 h, 10 μl of each reaction mixture was pipetted into 10 μl of loading dye (β-mercaptoethanol and dye) and heated at 90°C for 10 min. After another 3 h, 10 μl of loading dye was added to each of the remaining incubated reaction mixtures and heated at 90°C for 10 min. The 0.5 and 3.5 h incubated samples were run on 10% SDS-PAGE gels. Each lane on the gel was loaded with 15 μl of the reaction mixture. The gels were then scanned using the Typhoon scanner (Amersham Biosciences, USA) at $\lambda_{ex}=532$ nm to detect Cy3 labeled enzymes. Each gel was finally stained with Coomassie Blue or silver stain to visualize the proteins; (2) *Cysteine proteases*. 0.5–2 μl of the enzyme (5–10 μM) was incubated at room temperature with 200–fold molar excess of **9** (0.2 μl, 2 mg/ml) in 50 mM Tris, 2 mM EDTA, 0.31 mg/ml DTT (total volume 20.2 μl). After 1/2 h, 10 μl of the reaction mixture was pipetted into 10 μl of loading dye (β-mercaptoehanol and dye) and heated at 90°C for 10 min. After another 3 h, 10 μl of loading dye was added to the remaining reaction mixture and heated at 90°C for 10 min. Both the 0.5 and 3.5 h incubated samples were run on 10% SDS-PAGE gels. Each lane on the gel was loaded with 15 μl of the reaction mixture. The gels were then scanned using the Typhoon scanner at $\lambda_{ex}=532$ nm to see if caspase-1 has been labeled by the Cy3 probe. Each gel was finally stained with Coomassie Blue or silver stain to visualize the proteins.