Discovery of a new inhibitor lead of adenovirus proteinase: steps toward selective, irreversible inhibitors of cysteine proteinases

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Abstract Using the computer docking program EUDOC, in silico screening of a chemical database for inhibitors of human adenovirus cysteine proteinase (hAVCP) identified 2,4,5,7-tetranitro-9-fluorenone that selectively and irreversibly inhibits hAVCP in a two-step reaction: reversible binding (K_i = 3.09 μM) followed by irreversible inhibition (k_i = 0.006 s^{-1}). The reversible binding is due to molecular complementarity between the inhibitor and the active site of hAVCP, which confers the selectivity of the inhibitor. The irreversible inhibition is due to substitution of a nitro group of the inhibitor by the nearby Cys122 in the active site of hAVCP. These findings suggest a new approach to selective, irreversible inhibitors of cysteine proteinases involved in normal and abnormal physiological processes ranging from embryogenesis to apoptosis and pathogen invasions. © 2001 Published by Elsevier Science B.V. on behalf of the Federation of European Biochemical Societies.

Key words: Computational screening; In silico screening; Structure-based drug design; Molecular docking; Antiviral agent; Cysteine proteinase inhibitor; Drug discovery; Development

1. Introduction

Cysteine proteinases are involved in a wide variety of normal and abnormal physiological processes ranging from embryogenesis [1] to programmed cell death [2,3] and in virulence or replication of many viruses, bacteria, and parasites [4]. Human adenovirus cysteine proteinase (hAVCP) is one such proteinase and is responsible for the production of infectious viral particles [5,6]. Selective inhibitors of hAVCP could potentially be used as effective antiviral agents. Novel approaches to selective inhibitors of cysteine proteinases are therefore highly desirable for developing effective tools to study functions of cysteine proteinases and therapeutic agents.

Several classic cysteine proteinase inhibitors such as iodoacetate, dithiodipyridine, and *p*-chloromercuribenzoate are effective in inhibiting hAVCP, however, they are non-selective and can not be used as antiviral agents [7,8]. Although the X-ray structure of hAVCP was reported in 1996, to date, no selective and irreversible inhibitors of hAVCP have been reported despite various reported methods for developing cysteine proteinase inhibitors, in particular, those aiming at reactions with the nucleophilic cysteine residue in the active site of drug targets [9,10].

To develop antiviral agents for adenovirus infections [11–15] and to develop novel approaches to selective inhibitors of cysteine proteinases, we have performed in silico screening of a chemical database for inhibitors of hAVCP and identified 2,4,5,7-tetranitro-9-fluorenone (TNFN, see Fig. 1) as a new inhibitor lead that selectively and irreversibly inhibited hAVCP in a two-step reaction. The mechanism of selective and irreversible inhibition of hAVCP by TNFN proposed in this report suggests a new approach to selective, irreversible inhibitors of cysteine proteinases.

2. Materials and methods

2.1. Materials

Tris–HCl, HEPES, and papain were purchased from Sigma (St. Louis, MO, USA). TNFN was purchased from Pfaltz and Bauer (Waterbury, CT, USA). Other chemicals were purchased from Aldrich Chemical (Madison, WI, USA) or synthesized as described below. pVIc (GVQSLKRRRCF) was obtained from Research Genetics (Huntsville, AL, USA). The fluorogenic substrates (Leu-Arg-Gly-Gly-$NH)_2$-Rhodamine for hAVCP and (Phe-Arg-$NH)_2$-Rhodamine for papain were synthesized and purified as described previously [8,16,17].

2.2. Enzymes

Recombinant hAVCP was purified as described previously [16,18]. hAVCP–pVIc complexes were formed by incubating 100 μM hAVCP with 150 μM pVIc in 10 mM Tris (pH 7.5), 5 mM NaCl, and 1 mM EDTA at 4°C for 16 h.

2.3. In silico screening

The 1998 release of the Available Chemicals Directory obtained from MDL Information Systems (San Leandro, CA, USA) was used in the in silico screening. Compounds in the Available Chemicals Directory that had shape and charge complementarity to the X-ray structure of the active site in hAVCP [19] were identified by using a computer docking program, EUDOC [20], according to a published protocol [21].

2.4. Enzymatic assays

To characterize the irreversible inhibition of hAVCP–pVIc by TNFN, a competition was set up between the substrate and the in-

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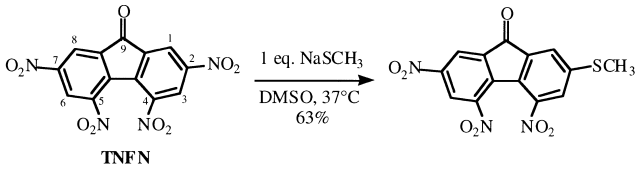

Fig. 1. A model reaction of aromatic nucleophilic substitution.

hibitor for the active site of the enzyme [22]. The results were then used to calculate the equilibrium dissociation constant for reversible binding by the inhibitor and a first-order rate constant for irreversible inhibition by the inhibitor.

The interaction between hAVCP–pVIc and the substrate can be characterized by:

$$E + S \overset{K_m}{\leftrightarrow} E{\cdot}S \xrightarrow{k_{cat}} E + P \quad (1)$$

where E is enzyme hAVCP–pVIc; S is the substrate (Leu-Arg-Gly-Gly-NH)$_2$-Rhodamine; $E{\cdot}S$ is the adsorptive enzyme–substrate complex; P is the product (Leu-Arg-Gly-Gly-NH)$_2$-rhodamine; K_m is the Michaelis constant; k_{cat} is a first-order rate constant.

The interaction between hAVCP and the inhibitor can be characterized by:

$$E + I \overset{K_i}{\leftrightarrow} E{\cdot}I \xrightarrow{k_i} EI' \quad (2)$$

where I is the irreversible inhibitor TNFN; $E{\cdot}I$ is an adsorptive enzyme–inhibitor complex; EI' is the irreversibly inhibited enzyme–inhibitor complex; K_i is an equilibrium dissociation constant; k_i is a first-order rate constant.

Assays with hAVCP–pVIc complexes contained 10 mM Tris (pH 8.0) and 5 mM octylglucoside. Assays with papain contained 25 mM NaAc (pH 5.0). Papain was activated just prior to an assay by preparing a 1:50 dilution of a stock solution of 5 mg ml^{-1} in 25 mM NaAc (pH 5.0) into 50 mM NaAc (pH 5.0) containing 5 mM dithiothreitol and incubating at 37°C for 4 min. Assays, usually started with the addition of enzyme, were performed in an ISS spectrofluorometer (Urbana, IL, USA) equipped with a 4-position cuvette holder. Fluorescence readings were taken continuously for 40 min. The excitation wavelength was 492 nm, and the emission wavelength was 523 nm, both with a 4 nm bandpass filter.

2.5. Determination of K_i and k_i

Competition assays between the substrate and TNFN for the active site of hAVCP–pVIc complexes contained 100 nM hAVCP–pVIc, 3 μM (Leu-Arg-Gly-Gly-NH)$_2$-Rhodamine and 0.3, 0.5, 0.7, 1.0, 2.0, 3.0, 4.0, 5.0, 6.0, 7.0, 8.0, and 9.0 μM TNFN. Competition assays between the substrate and TNFN for the active site of papain contained 8.7 nM papain, 20 μM (Phe-Arg-NH)$_2$-Rhodamine and 2.5, 5.0, 10, 30, 50, 60, 70, 80, and 100 μM TNFN.

2.6. Data analysis

For the experiments to determine K_i and k_i, the primary data were imported into the program TableCurve from Jandel Scientific (San Rafael, CA, USA) and plotted as amount of product (P) formed versus time (t). The program determined an equation that best described the data points and then, using that equation, drew a best-fit curve through the data points.

2.7. Synthesis of 2-methylsulfanyl-4,5,7-trinitro-9-fluorenone

Sodium thiomethoxide (30 mg, 0.43 mmol) was placed in a 25 ml round bottom flask that had been purged with nitrogen and equipped with a stir bar. Anhydrous DMSO (10 ml) was added, followed by 154 mg (0.43 mmol) of 2,4,5,7-tetranitro-9-fluorenone, while maintaining a constant flow of nitrogen. The flask was then sealed with a septum. Immediately upon addition of the 2,4,5,7-tetranitro-9-fluorenone, the solution in the flask became dark blue in color. After stirring at 37°C for 18 h, the DMSO was removed under reduced pressure. The residue was partitioned between 25 ml of water and 10 ml of CH_2Cl_2. The aqueous phase (dark red) was extracted with 2×10 ml CH_2Cl_2. The combined organic phases were dried over $MgSO_4$ and the solvent was removed on a rotary evaporator. This crude product was chromatographed using a CombiFlash system from Isco (Lincoln, NE, USA) equipped with a 20×300 mm column packed with 35–75 μM Geduran silica gel 60 obtained from EM Science (Gibbstown, NJ, USA) using a gradient of 0–10% methanol in chloroform as eluant. The fractions containing product were combined and the solvent was removed using a rotary evaporator to give 98 mg (63%) of product as orange needles. M.P. 222–223°C (uncorrected). Found C, 46.54; H, 2.09; N, 11.39%; $C_{14}H_7N_3O_7S$ requires C, 46.54; H, 1.95; N, 11.63%. ^{1}H-NMR (600 MHz, DMSO-d_6) δ 8.86 (d, 1H, J = 1.9 Hz), 8.59 (d, 1H, J = 2.0 Hz), 8.05 (d, 1H, J = 1.6 Hz), 8.02 (d, 1H, J = 1.6 Hz), and 2.72 (s, 3H). ^{13}C-NMR (600 MHz, DMSO-d_6) δ 185.8, 148.6, 148.2, 146.1, 144.4, 138.2, 137.7, 137.5, 126.6, 125.8, 125.5, 124.6, 122.1, and 14.4. Assignment of structure was based upon the ^{1}H-NMR spectral data. Of the two possible isomers that could be formed by the aromatic nucleophilic substitution of thiomethoxide for a nitro group in the starting material, only one isomer would be expected to have similar chemical shifts for H_a and H_b (Fig. 2).

2.8. Synthesis of bis-(3,5-dinitrophenyl)-methanone

Benzophenone (5.00 g, 27.4 mmol) was added to 25 ml concentrated sulfuric acid cooled in an ice water bath. This solution was added to a solution of 80 ml concentrated sulfuric acid in 35 ml concentrated nitric acid cooled in an ice water bath. This solution was warmed to room temperature for 60 min then slowly heated to 90°C over 75 min. The solution was maintained at 90°C for 105 min. After cooling the solution in an ice water bath, it was poured over 600 g of crushed ice. The resulting yellow precipitate was recovered by vacuum filtration and washed with water, saturated sodium bicarbonate solution and additional water. ^{1}H-NMR indicated a mixture of nitration products. This solid was dissolved in 25 ml concentrated sulfuric acid and was added to a solution of 75 ml concentrated sulfuric acid mixed with 25 ml concentrated nitric acid. This solution was heated at 140°C for 60 min then worked up as described above. Recrystallization from acetone gave 2.03 g (20% yield) of white needles. M.P. 242–243°C (uncorrected). Found C, 43.18; H, 1.67; N, 15.65%; $C_{13}H_6N_4O_9$ requires C, 43.11; H, 1.67; N, 15.47%. ^{1}H-NMR (600 MHz, DMSO-d_6) δ 9.09 (t, 2H, J = 2.0 Hz) and 8.84 (d, 4H, J = 2.0 Hz). ^{13}C-NMR (150 MHz, DMSO-d_6) δ 189.2, 148.2, 138.3, 129.5, and 122.2.

2.9. Synthesis of (3,5-dinitrophenyl)-(3-methylsulfanyl-5-nitrophenyl)-methanone

Procedure was identical to that for 2-methylsulfanyl-4,5,7-trinitro-9-fluorenone. Upon addition of the bis-(3,5-dinitrophenyl)-methanone to the sodium thiomethoxide solution, the reaction mixture turned dark red. Subsequent work-up gave rise to 47 mg (30%) of the desired product as yellow needles. M.P. 177–178°C (uncorrected). Found C, 46.13; H, 2.49; N, 11.44%; $C_{13}H_6N_4O_9$ requires C, 46.28; H, 2.50; N, 11.57%. ^{1}H-NMR (500 MHz, DMSO-d_6) δ 9.07 (t, 1H, J = 2.0 Hz), 8.82 (d, 2H, J = 2.1 Hz), 8.32–8.30 (m, 1H), 8.23–8.21 (m, 1H), 8.02–8.00 (m, 1H), and 2.64 (s, 3H). ^{13}C-NMR (125 MHz, DMSO-d_6) δ 190.3, 148.4, 148.2, 142.7, 138.8, 132.0, 129.4, 123.4, 121.8, 120.2, and 14.5.

3. Results and discussion

In order to develop effective antiviral agents, peptides and

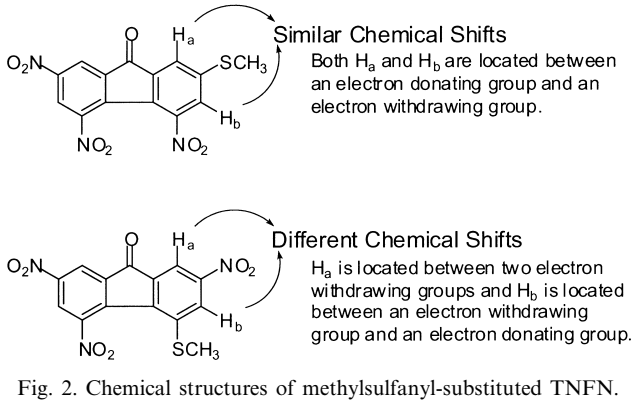

Fig. 2. Chemical structures of methylsulfanyl-substituted TNFN.

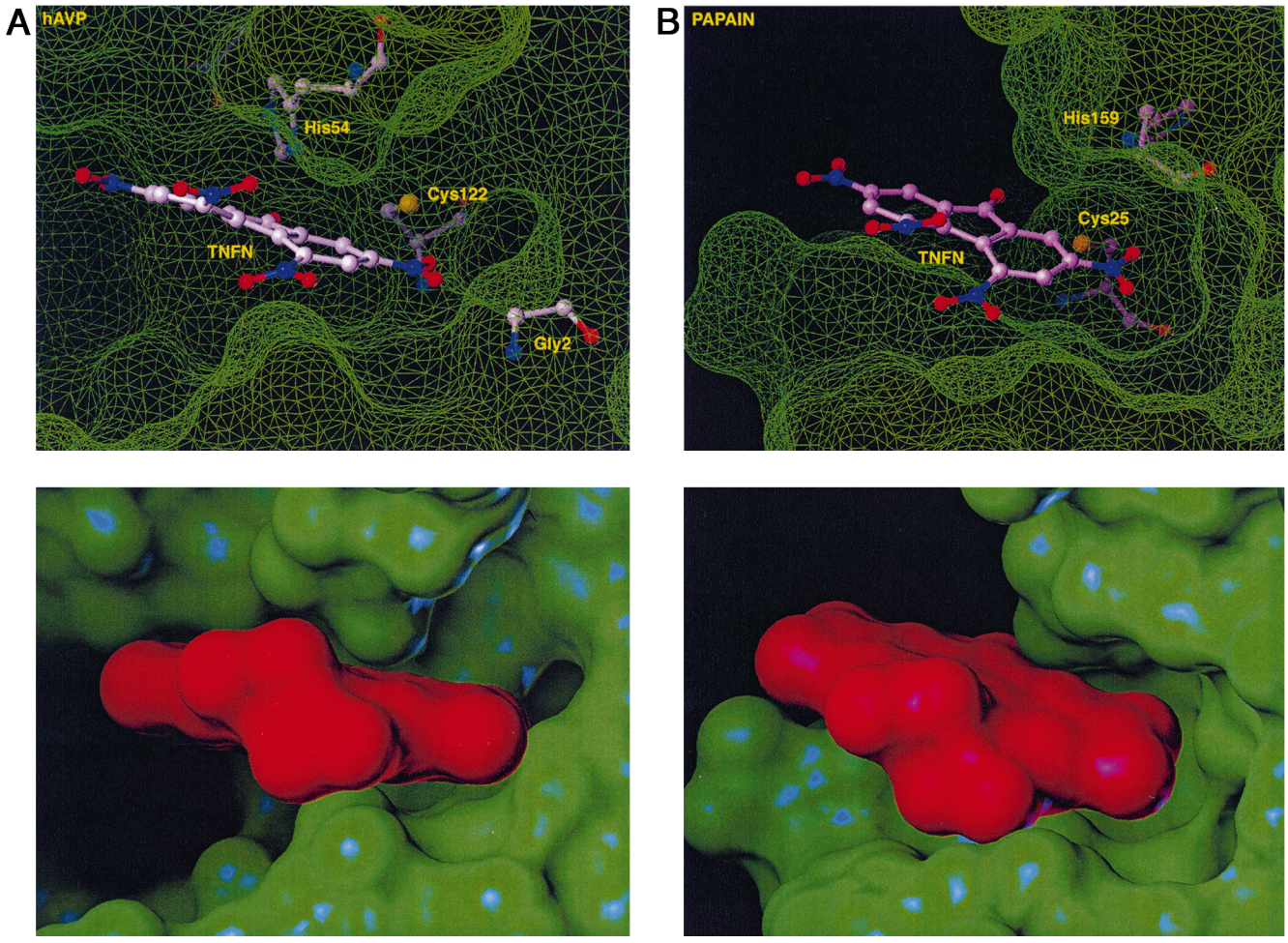

Fig. 3. The EUDOC generated most energetically stable complexes of TNFN–hAVCP (A) and TNFN–papain (B) showing shape complementarity between TNFN (red) and enzyme active site (green).

charged molecules were excluded in our in silico screening, since they were not generally considered to be druggable. Docking 67 928 neutral, non-peptidic compounds selected from the Available Chemicals Directory into the active site of the hAVCP crystal structure [19], the EUDOC program identified 30 compounds as inhibitor candidates. The top three candidates were purchased and tested experimentally. The top compound, TNFN, effectively inhibited hAVCP, while the other two were inactive. Kinetics experiments demonstrated that TNFN inhibited hAVCP in a two-step reaction: reversible association with an equilibrium dissociation constant, K_i, of 3.09 μM followed by irreversible inhibition with a first-order rate constant, k_i, of 0.006 s^{-1}. The irreversible inhibition was confirmed by a dilution experiment. When hAVCP was incubated with TNFN at the K_i concentration (3.09 μM), 50% of the enzymatic activity was inhibited. When the reaction solution was diluted by 10-fold, the degree of inhibition was still 50%. This indicated that hAVCP was irreversibly inhibited and that no dissociation occurred upon dilution of the inhibitor–enzyme complex.

To understand the two-step inhibition mechanism at the atomic level, we examined the most energetically stable TNFN–hAVCP complex generated by the EUDOC program and found shape and charge complementarity between TNFN and the active site of hAVCP (Fig. 3). The complementarity is indicated by the calculated relative intermolecular interaction energy of −39.9 kcal/mol that is composed of the steric interaction energy of −27.5 kcal/mol and the electrostatic interaction energy of −12.4 kcal/mol. In addition, the 2-substituted nitro group formed a hydrogen bond with the nitrogen proton of Gly2 and the carbonyl oxygen atom formed a hydrogen bond with the hydrogen atom attached to the delta nitrogen atom of His54 in the hAVCP complex. Furthermore, the nitro group at position 2 of TNFN was located close to Cys122 in the active site of hAVCP (Fig. 3A). The distance of carbon 2 in TNFN to the sulfur atom of Cys122 was 5.0 Å. We hypothesized that the reversible binding was due to the shape and charge complementarity, and that the irreversible inhibition was caused by aromatic nucleophilic substitution of the 2-nitro group by the nearby Cys122, namely, the nucleophilic thiolate added at position 2 of TNFN followed by elimination of the 2-nitro group.

We accordingly carried out a model reaction of TNFN with one equivalent of sodium thiomethoxide in dimethylsulfoxide at 37°C. This reaction produced 2-methylsulfanyl-4,5,7-trinitrofluorenone as the major product in 63% yield (Fig. 1). NMR spectroscopic analysis revealed that the substitution did occur at position 2 of TNFN, thus directly supporting the substitution mechanism for the irreversible binding of TNFN to hAVCP.

We also docked TNFN into the active site of the X-ray structure of another cysteine proteinase, papain [23], and

found that the 2-substituted nitro group of TNFN was located close to Cys25 in the active site of the most energetically stable TNFN–papain complex generated by EUDOC (Fig. 3B). The 2-position carbon atom of TNFN was at a distance of 4.9 Å from the sulfur atom of Cys25 (Fig. 3B). In contrast to the binding of TNFN to hAVCP, the 2-substituted nitro group of TNFN did not form a hydrogen bond with any residue of papain. The intermolecular interaction energy in the papain complex is 1 kcal/mol higher than that in the hAVCP complex. These results could suggest qualitatively that TNFN would first bind reversibly to papain but with a larger equilibrium dissociation constant than it exhibited in binding to hAVCP, and would then irreversibly inhibit papain. Kinetic experiments revealed that TNFN irreversibly inhibited papain in a two-step manner as well: reversible association with a larger K_i of 9.24 μM followed by irreversible inhibition with a slower k_i of 0.001 s^{-1}, thus further supporting the proposed irreversible inhibition mechanism.

To investigate the selectivity of agents that inhibit cysteine proteinases through the mechanism of the aromatic nucleophilic substitution, we synthesized an analog of TNFN, bis-(3,5-dinitrophenyl)-methanone. This analog differs from TNFN in that it has a non-planar structure whereas TNFN has a planar structure (Fig. 4). Consequently, this analog does not fit well into the active site of hAVCP, which has been confirmed by our docking study using the same procedure as used in docking TNFN. The calculated intermolecular interaction energy of TNFN was 3 kcal/mol lower than that of the TNFN analog. Experimental study revealed that the analog did not reversibly or irreversibly inhibit hAVCP at concentrations up to 5.0 μM (it precipitated at concentrations greater than 5.0 μM), despite our model reaction revealing

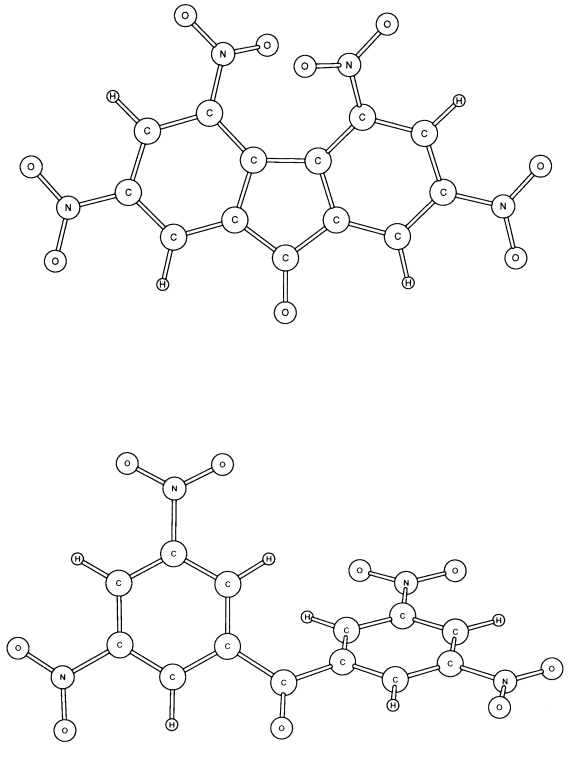

Fig. 4. Ball and stick models of TNFN (top) and bis-(3,5-dinitrophenyl)-methanone (bottom) with the non-planar conformation optimized by an ab initio calculation using the HF/6-31G* method.

that the nitro group of this analog could be replaced by the methylsulfanyl group when the analog was treated with one equivalent of sodium thiomethoxide in dimethylsulfoxide at 37°C. The results demonstrate that arylnitro-containing compounds can not alkylate the cysteine residue in the active site of proteins without their shape and charge complementarity to the active site, thus suggesting that inhibitors designed according to the aromatic nucleophilic substitution mechanism can be selective.

The active site structure of hAVCP is reportedly very similar to that of papain as a consequence of convergent evolution, although the two enzymes have different substrates [19]. When the central helix containing the active site nucleophile of papain is aligned with a similar helix in hAVCP, the three amino acids involved in catalysis in the active site of papain, Cys25, His159, and Asn175, juxtapose with Cys122, His54, and Glu71 of hAVCP, respectively [19]. Even the nitrogen atoms of Gln19 and Cys25 in the oxyanion hole of papain juxtapose with those of Gln115 and Cys122 in the oxyanion of hAVCP [19]. Despite the strong similarity in the active site topology of the two enzymes, TNFN, identified solely on the basis of a search using the hAVCP active site, is already an 18-fold more selective irreversible inhibitor of hAVCP according to the reported definition of selectivity. This selectivity is partly due to the fact that the hydrogen bond between the 2-substituted nitro group of TNFN and hAVCP is disabled in the papain–TNFN complex as suggested by the docking study [17]. While assays with other proteinases such as chymotrypsin and cysteine-containing non-proteinases such as glutathione would be appropriate to further evaluate the selectivity of TNFN, the present study does suggest that further structural modification of TNFN according to active sites of hAVCP and papain would lead to analogs that fit only the active site of hAVCP thus inhibiting hAVCP with improved selectivity.

Cocrystals of the hAVCP–pVIc complex with TNFN have been obtained which diffracted at a resolution of 3 Å. It is clear that extra density appears in the active site over Cys122, but at this resolution it is difficult to assign structure to this density (McGrath and Mangel, unpublished). However, on the basis of the reaction of TNFN with sodium thiomethoxide and the kinetics experiments described above, it is conceivable that effective inhibitors of cysteine proteinases can be developed by searching for bulky compounds that fit only the active site of the targeted cysteine proteinase and have an arylnitro group located within 5.0 Å proximity of the cysteine residue in the active site of the proteinase. Our recent survey reveals that 2% of launched drugs in the 2001 release of the MDL drug database (San Leandro, CA, USA) contain arylnitro groups, indicating that arylnitro groups are not toxic and the proposal of using such groups in the design of cysteine proteinase inhibitors is reasonable.

The advantage of the design of bulky, arylnitro-containing compounds is 2-fold. First, the inhibitors so developed are selective because these compounds are designed not to fit the active sites of unrelated enzymes. The chance for these compounds to react with the active site cysteine of the unrelated enzymes is remote because of the low chemical reactivity of the arylnitro group relative to thiol-containing compounds and iodoacetate analogs. Second, such inhibitors are irreversible because they undergo aromatic nucleophilic substitution due to the 'neighboring effect' in chemical kinetics only when

they bind at the active site of the targeted enzyme. This approach is novel in that a much less chemically reactive arylnitro group has, for the first time, been proposed to react with the active site cysteine residue to achieve irreversible inhibition instead of using reactive agents such as thiol-containing compounds and iodoacetate analogs [9,10]. The importance of this nucleophilic substitution method for developing selective and irreversible cysteine proteinase inhibitors rests on the fact that most irreversible inhibitors of cysteine proteinases are nonselective because they are chemically reactive and small in size [2,4,24]. The common alkylating agents such as iodoacetate or *N*-ethylmaleimide fit the active site of any cysteine proteinase and are thus able to alkylate any cysteine proteinase; nitric oxide will nitrosylate the sulfur atom of any cysteine residue involved in catalysis for the same reasons. Although recent studies have demonstrated that *S*-nitrosothiols can mask the catalytic cysteine residue with a large chemical group via disulfide bond formation [24–26], these compounds can not be used as selective inhibitors of cysteine proteinases, because *S*-nitrosothiols can inhibit other cysteine proteinases as nitric oxide, released by homolysis, can react with any cysteine proteinase.

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References

[1] Cho, W.L., Tsao, S.M., Hays, A.R., Walter, R., Chen, J.S., Snigirevskaya, E.S. and Raikhel, A.S. (1999) J. Biol. Chem. 274, 13311–13321.
[2] Zech, B., Wilm, M., Van Eldik, R. and Brune, B. (1999) J. Biol. Chem. 274, 20931–20936.
[3] Earnshaw, W.C., Martins, L.M. and Kaufmann, S.H. (1999) Annu. Rev. Biochem. 68, 383–424.
[4] Saura, M., Zaragoza, C., McMillan, A., Quick, R.A., Hohenadl, C., Lowenstein, J.M. and Lowenstein, C.J. (1999) Immunity 10, 21–28.
[5] Weber, J. (1976) J. Virol. 17, 462–471.
[6] Krausslich, H.G. and Wimmer, E. (1988) Annu. Rev. Biochem. 57, 701–754.
[7] Webster, A., Russell, W.C. and Kemp, G.D. (1989) J. Gen. Virol. 70, 3215–3223.
[8] McGrath, W.J., Abola, A.P., Toledo, D.L., Brown, M.T. and Mangel, W.F. (1996) Virology 217, 131–138.
[9] Veber, D.F. and Thompson, S.K. (2000) Curr. Opin. Drug Discov. Dev. 3, 362–369.
[10] Erlanson, D.A., Braisted, A.C., Raphael, D.R., Randal, M., Stroud, R.M., Gordon, E.M. and Wells, J.A. (2000) Proc. Natl. Acad. Sci. USA 97, 9367–9372.
[11] Downham, M.A., Gardner, P.S., McQuillin, J. and Ferris, J.A. (1975) Br. Med. J. 1, 235–239.
[12] Schepetiuk, S.K., Norton, R., Kok, T. and Irving, L.G. (1993) J. Med. Virol. 41, 316–318.
[13] Hendrix, R.M., Lindner, J.L., Benton, F.R., Monteith, S.C., Tuchscherer, M.A., Gray, G.C. and Gaydos, J.C. (1999) Emerg. Infect. Dis. 5, 798–801.
[14] Uchio, E., Matsuura, N., Takeuchi, S., Itoh, N., Ishiko, H., Aoki, K. and Ohno, S. (1999) Am. J. Ophthalmol. 128, 680–686.
[15] Itoh, N. et al. (1999) J. Med. Virol. 59, 73–77.
[16] Mangel, W.F., McGrath, W.J., Toledo, D.L. and Anderson, C.W. (1993) Nature 361, 274–275.
[17] Leytus, S.P., Patterson, W.L. and Mangel, W.F. (1983) Biochem. J. 215, 253–260.
[18] Mangel, W.F., Toledo, D.L., Brown, M.T., Martin, J.H. and McGrath, W.J. (1996) J. Biol. Chem. 271, 536–543.
[19] Ding, J.Z., McGrath, W.J., Sweet, R.M. and Mangel, W.F. (1996) EMBO J. 15, 1778–1783.
[20] Pang, Y.-P., Perola, E., Xu, K. and Prendergast, F.G. (2001) J. Comp. Chem., in press.
[21] Perola, E., Xu, K., Kollmeyer, T.M., Kaufmann, S.H., Prendergast, F.G. and Pang, Y.P. (2000) J. Med. Chem. 43, 401–408.
[22] Leytus, S.P., Toledo, D.L. and Mangel, W.F. (1984) Biochim. Biophys. Acta 788, 74–86.
[23] Pickersgill, R.W., Harris, G.W. and Garman, E. (1992) Acta Crystallogr. Sect. B 48, 59.
[24] Xian, M., Chen, X., Liu, Z., Wang, K. and Wang, P.G. (2000) J. Biol. Chem. 275, 20467–20473.
[25] Percival, M.D., Ouellet, M., Campagnolo, C., Claveau, D. and Li, C. (1999) Biochemistry 38, 13574–13583.
[26] Mohr, S., Hallak, H., De Boitte, A., Lapetina, E.G. and Brune, B. (1999) J. Biol. Chem. 274, 9427–9430.