Drug Design

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Inhibitors of HIV-1 Protease by Using In Situ Click Chemistry**

Matthew Whiting, John Muldoon, Ying-Chuan Lin, Steven M. Silverman, William Lindstrom, Arthur J. Olson, Hartmuth C. Kolb, M. G. Finn, K. Barry Sharpless, John H. Elder, and Valery V. Fokin**

The global AIDS epidemic has claimed the lives of more than 20 million people since 1981. Another 10 million are now living with HIV and most of these are likely to develop AIDS over the course of the next decade. In spite of the various treatment protocols available, including the mainstream

[*] Dr. Y.-C. Lin, Dr. W. Lindstrom, Prof. A. J. Olson, Prof. J. H. Elder
Department of Molecular Biology
The Scripps Research Institute
10550 North Torrey Pines Road, La Jolla, CA 92037 (USA)
Fax: (+1) 858-784-2750
E-mail: jelder@scripps.edu

Dr. M. Whiting, Dr. J. Muldoon, S. M. Silverman, Prof. H. C. Kolb, Prof. M. G. Finn, Prof. K. B. Sharpless, Prof. V. V. Fokin
Department of Chemistry and
The Skaggs Institute for Chemical Biology
The Scripps Research Institute
10550 North Torrey Pines Road, BCC-315, La Jolla, CA 92037 (USA)
Fax: (+1) 858-784-7562
E-mail: fokin@scripps.edu

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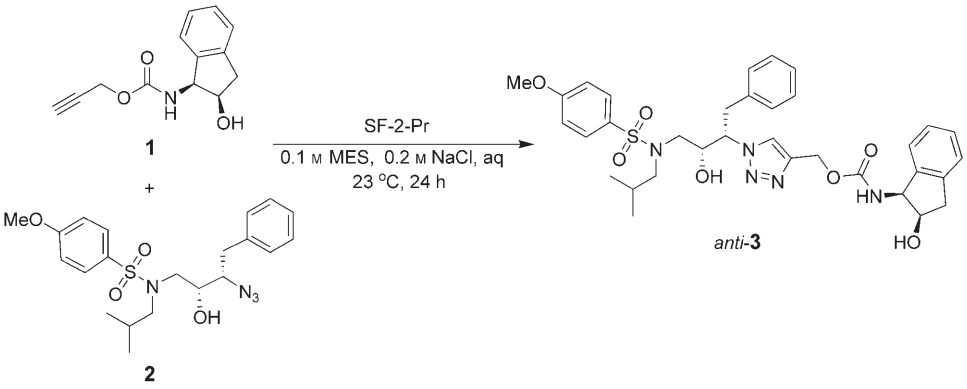
highly active antiretroviral therapy (HAART),[1] the number of people infected with HIV continues to rise. The most recent UNAIDS (United Nations Programme on HIV/AIDS)/world health organization estimates show that, in 2004 alone, 4.9 million people were newly infected with HIV.[2]

HIV-1 protease (HIV-1-Pr)[3] has been recognized as an important target for inhibition of viral replication. Although seven inhibitors have been approved by the food and drug association since 1995 and a number more are currently undergoing clinical evaluation, their success has been undermined by rapid mutation of the virus.[4] The alarming rate at which strains of HIV-1 that are resistant to the currently available drugs and their combinations emerge underscores the urgent need for new, broad-spectrum protease inhibitors that are effective against the new mutants as well as the wild-type virus.

Herein we describe the early results of our investigation into the application of a multidisciplinary, chemistry-driven approach for the rapid discovery of bioactive molecules, termed in situ click chemistry, for the elucidation of novel HIV-1-Pr inhibitors. The goal of in situ click chemistry is to accelerate the identification of novel pharmaceutical candidates through involvement of a biological target in the selection and covalent assembly of its own inhibitors. Although the concept has been previously demonstrated by several researchers,[5] the in situ click-chemistry approach[6] is unique in that it relies on the completely bio-orthogonal 1,3-dipolar cycloaddition of organic azides and alkynes.[7] This highly exergonic reaction produces five-membered nitrogen heterocycles, 1,2,3-triazoles, which are exceedingly stable to acidic and basic hydrolysis as well as severe reductive/oxidative conditions. At the same time, the triazoles produced are capable of active participation in hydrogen bonding as well as dipole–dipole and π-stacking interactions.

Even though both azides and alkynes are energetic species, their reactivity profiles are quite narrow, at least under physiological conditions. Furthermore, despite the large thermodynamic driving force for cycloaddition, the high kinetic barrier effectively hides the reactants until they are brought into close proximity by a biological template or activated towards each other by alternative means (e.g., catalysis). These features allow the target to sample numerous combinations of building blocks, but synthesize only the best binders. The efficacy of in situ click chemistry has already been demonstrated by the discovery of novel, highly potent inhibitors of acetylcholinesterase and carbonic anhydrase.[6] Previous studies have, however, always utilized building blocks with a high affinity for the target ($K_i < 37$ nM).[6c,d] In the HIV-1-Pr case, we do not have such a luxury and as such, we were pleased to find that the enzyme selectively formed an inhibitor from components that exhibited only weak binding to the target.

To probe the protease-templated reaction, alkyne **1** (500 μM, $IC_{50} > 100$ μM) and azide **2** (100 μM, $IC_{50} = 4.2 \pm 0.6$ μM) were incubated in the presence of the HIV-1,-Pr SF-2-WTQ7K-Pr,[8] (henceforth simply denoted SF-2-Pr; 15 μM) in 2-morpholinomethanesulfonic acid (MES; 0.1M)/NaCl (0.2M) buffer solution at 23 °C for 24 h (Scheme 1). Controls



Scheme 1. SF-2-Pr templated in situ click-chemistry formation of protease inhibitor *anti*-**3**.

were performed both in the absence of the enzyme, to confirm the background rate of the cycloaddition, and in the presence of bovine serum albumin (BSA), to determine any non-specific peptidic catalysis. Analysis of the crude reaction mixtures was carried out by HPLC and mass spectrometry in single-ion mode (LCMS-SIM), which was previously demonstrated to be a sensitive, medium-throughput method for the detection of a desired product.[6b] Comparison of the enzyme and background reactions showed that the triazole *anti*-**3**, which has previously been shown to be an inhibitor of the wild-type HIV-1-Pr ($IC_{50} = 6$ nM, $K_i = 1.7$ nM) and also of several mutant strains,[9] was indeed formed in an increased rate in the presence of the enzyme. In contrast to other systems,[6b,c] the background reaction was observable under our chosen conditions and produced a detectable amount of the regioisomeric triazoles *anti*- and *syn*-**3** in a 2:1 ratio, favoring the 1,4-disubstituted product *anti*-**3**. Surprisingly, the presence of BSA gave an increase in the observed rate of product formation, which led to a small increase in the final product concentration without affecting the regioisomeric ratio. However, a marked acceleration in the rate of formation of only one of the regioisomers was observed in the presence of the enzyme with an approximately tenfold increase in the overall product formation. The triazole product was formed with a much enhanced regioisomeric ratio of 18:1 in favor of the same 1,4-isomer, *anti*-**3** (Figure 1). In a further control experiment, a known HIV-1-Pr active-site ligand, TL3[10] (15 μM), was added to both the background and enzyme reactions prior to incubation with the azide and alkyne fragments. The added inhibitor showed no effect on the background reaction[11] but completely suppressed the increase in product formation in the presence of the enzyme,

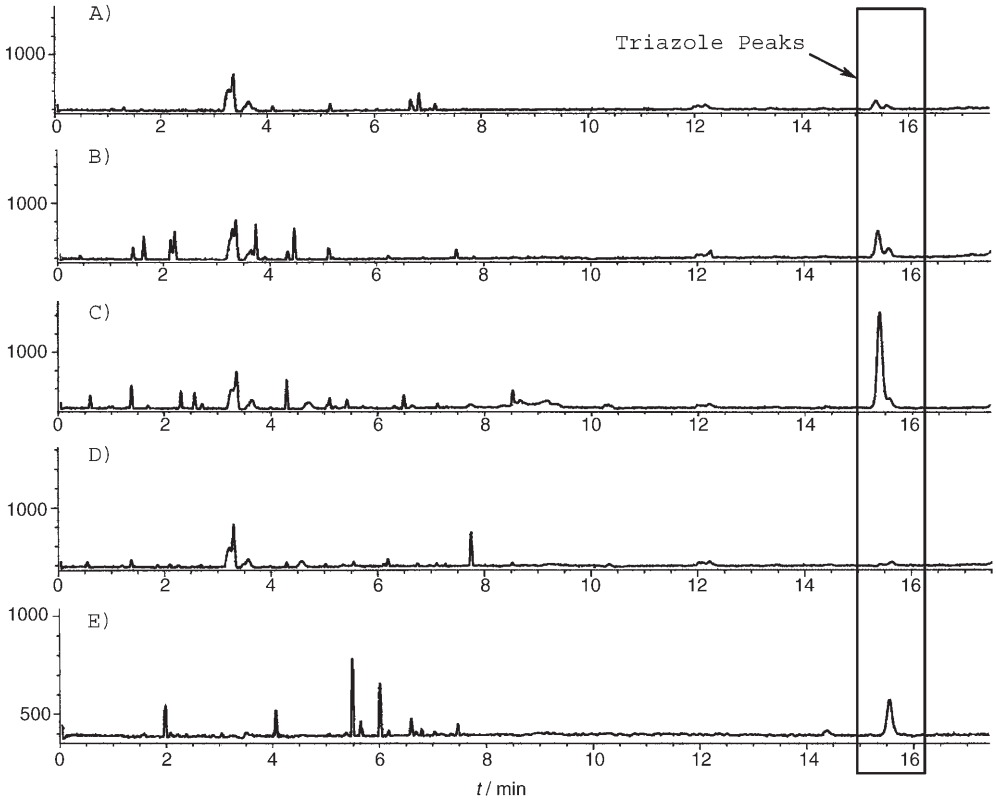
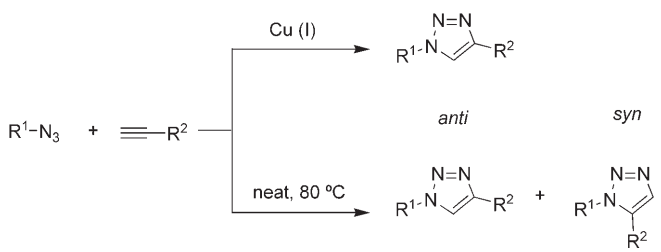


Figure 1. In situ formation of **3** determined by LCMS-SIM. A) Background reaction, alkyne **1** (500 μM) and azide **2** (100 μM) were incubated in MES (0.1 M) and NaCl aqueous buffer (0.2 M) for 24 h. B) Compounds **1** and **2** incubated in the presence of BSA (0.5 mg mL^{-1}, 7.5 μM). C) Compounds **1** and **2** were incubated in the presence of SF-2-Pr (15 μM). D) Compounds **1** and **2** incubated in the presence of SF-2-Pr (15 μM) and protease inhibitor TL3 (15 μM). E) Authentic sample of *anti*-**3**.

We next investigated whether the enzyme could select a pair of fragments that form a potent binder from a number of building blocks that are concurrently present in a single reaction mixture. In the context of a drug-discovery program, this would be a major advantage, enabling more-rapid screening and reducing the amount of enzyme required for each individual analysis. In the present system, azide **2** was known to be the tightest-binding fragment; we therefore hoped to show that it could recruit a complimentary fragment from an array of alkynes. Alkynes **4**–**7** (Figure 2) were chosen as test substrates owing to their similar molecular weight and degree of functionality when compared with **1**. It was also determined that, as expected, none of the individual fragments gave strong protease inhibition (IC_{50} of Alkynes **4**–**7** > 100 μM).

thereby demonstrating that the active site does indeed act as a template for the cycloaddition reaction. The reaction, performed in situ, was also carried out in the presence of the more clinically relevant HIV-1-Pr, NL4-3-WTQ7K-Pr,[12] which gave essentially identical results.

To determine the regiochemistry of the triazole formed by the enzyme, a mixture of both regioisomers, *syn*- and *anti*-**3**, was obtained by thermal cycloaddition, and the regioisomerically pure 1,4-disubstituted triazole product, *anti*-**3**, was prepared by a copper(I)-catalyzed reaction of the corresponding azide and alkyne blocks[13] (Scheme 2). Co-injection of the crude, enzyme-promoted reaction with both a mixture of the triazoles and the pure 1,4-regioisomer revealed that the enzyme had preferentially formed the expected 1,4-triazole, *anti*-**3**.[11]



Scheme 2. Preparation of regioisomeric triazoles by copper(I)-catalyzed and thermal cycloaddition reactions.

Thus, instead of screening binary mixtures of azide and alkyne fragments, azide **2** (100 μM) was incubated with the five alkynes **1** and **4**–**7** (200 μM each) in the presence of both SF-2-Pr (15 μM) and BSA (to monitor the background reaction). After incubation for 24 h at 23 °C, the crude reaction mixtures were directly analyzed by LCMS-SIM (monitoring for MH^+ and MNa^+ of all potential products[14]). Comparison of the total ion count and the extracted-ion traces of each possible product from the enzyme and background reaction showed an increased amount of only one triazole product (**3**) in the presence of the enzyme (Figure 3).[15]

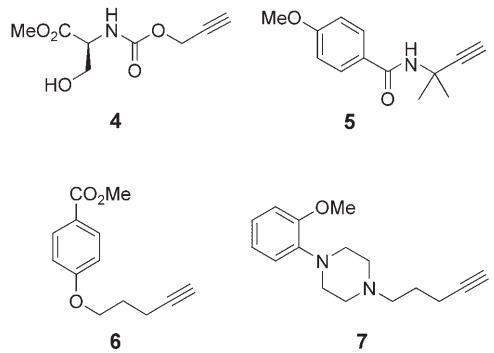


Figure 2. Alkynes **4**–**7**.

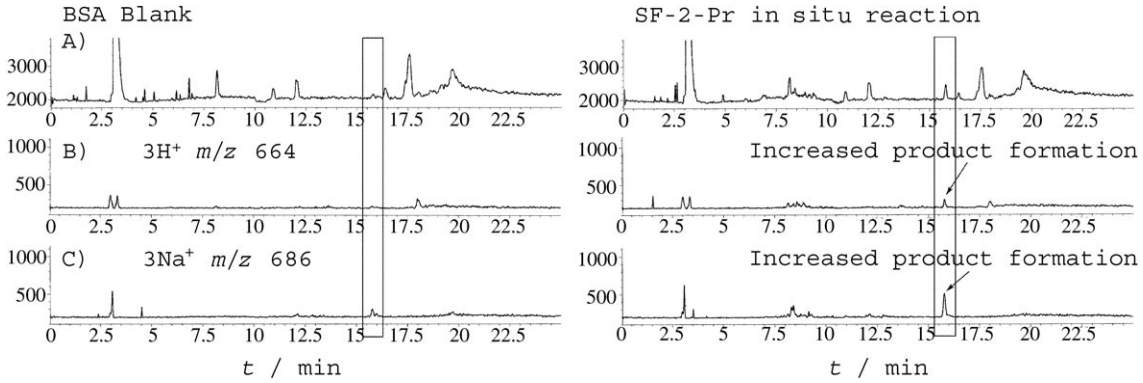


Figure 3. LCMS-SIM and extracted-ion traces for the in situ mixture experiment as described above. The combined traces (A) are shown as well as the extracted-ion traces for both **3**H^+ (B) and **3**Na^+ (C). The left-hand traces show the BSA background reaction and the right-hand traces show the corresponding in situ reaction with SF-2-Pr. Extracted-ion traces for all alternative-product masses showed no increased product formation.[11]

In summary, this work has demonstrated that the in situ click-chemistry approach can be successfully applied to the formation of HIV-1-Pr inhibitors. The protease itself acts as a template for the reaction and greatly increases the rate of formation of the 1,4-triazole product, *anti*-**3**. This rate acceleration is readily detected by simple comparison with the LCMS-SIM traces for the in situ reaction and a background reaction by using BSA in place of the enzyme. In this study, the use of building blocks with much lower affinities for the biological target than in previous examples should greatly facilitate the transfer of this technology to new systems in which the structures of high affinity binders are not known. Further studies are currently underway that apply this methodology to the discovery of entirely novel protease inhibitors by using even lower affinity fragments as well as efforts to directly employ drug resistant, mutant proteases in the in situ discovery process.

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[14] There is a possibility that potential products could be mass-spectrometry silent and thus would be left undetected if they had significantly different ionization energies. In the present study this was demonstrated not to be the case as the common azide fragment **2** gave a relatively large ESI-MS signal compared with alkyne **1** (see the Supporting Information for a more detailed explanation).
[15] One advantage of the in situ click-chemistry approach over the traditional drug-discovery techniques is that the need to independently synthesize each individual compound for screening is eliminated. Increased rate of formation of a triazole

product(s) indicates possible hits that can then be further investigated individually (again, very quickly thanks to the "guaranteed" nature of the cycloaddition reaction). To ascertain that no binders were overlooked in this particular case, we also carried out thermal cycloaddition reactions of each individual azide/alkyne combination and determined the product retention times by LCMS. This confirmed our initial determination that only one triazole was formed with an increased rate in the presence of the biological target, thereby illustrating that the enzyme was able to sample an array of potential inhibitor fragments and select only one productive combination, the one leading to the inhibitor **3**.