Microtiter plate based chemistry and *in situ* screening: a useful approach for rapid inhibitor discovery

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The use of libraries extracted from nature or constructed by combinatorial chemistry, have been widely appreciated in the drug discovery area. In this perspective, we present our contribution to the field of enzyme inhibitor discovery using a useful approach that allows diversification of a common core in a microtiter plate followed by *in situ* screening. Our method relies on an organic reaction that is highly selective, high yielding, amenable to the microscale and preferably can be performed in water. The core can be a designed molecule based on the structural and mechanistic information of the target, a compound with a weak binding affinity, or a natural product. Several reactions were found useful for this approach and were applied to the rapid discovery of potent inhibitors of representative enzymes.

Introduction

In the drug discovery area, the focus initially is on the discovery of "hits", which are active compounds that meet certain criteria. Improvement steps are then followed for the discovery of "lead" compounds with acceptable potency, selectivity, pharmacokinetics, physicochemical properties and absence of toxicity. The discovery journey of "hits" goes through a screen of a large collection of compounds (*i.e.* libraries) extracted from natural products or constructed chemically in a combinatorial fashion. Generally, these processes are aided by the structural and mechanistic information of the target (Fig. 1).

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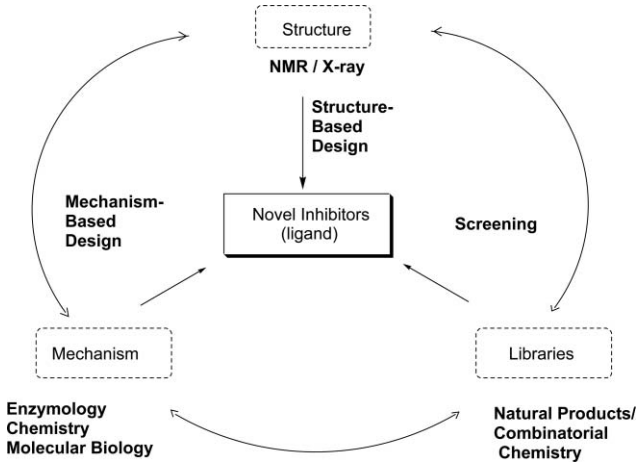

Fig. 1 General scheme of the various elements that are involved in the discovery process of novel inhibitors/ligands. These elements (*i.e.* structure-, mechanism-, and library based approach) are interrelated and often used together for lead identification and optimization.

Nature with its large biodiversity and complexity of molecules has supported and inspired the discovery of most of the drugs on the market today. Indeed, 61% of the small molecules introduced as drugs worldwide during the last two decades can be traced back to nature or have been inspired by natural products.[1] Yet, chemistry with its wide range of tools provides us with a large number of compounds that can be "bench made" and accessible through combinatorial methods. Traditionally, combinatorial chemistry of small molecules is carried out on a solid support by applying lessons learned from solid phase peptide and oligonucleotide synthesis.[2,3,4,5,6] With the advances in traditional organic synthesis, combinatorial chemistry in solution phase has emerged as a robust approach for parallel synthesis to expand the structure diversity of hit compounds. In many aspects, these two approaches are complementary and are often used to produce libraries in the industry and academic institutes. However, while the purification of the library is a straightforward step in solid phase, it becomes a major obstacle and a rate-determining step for parallel synthesis in solution. Several protocols have been introduced in order to overcome the problems associated with parallel purification in solution and are discussed in many reviews.[7,8] In this perspective we do not intend to review either one of these strategies, as excellent reviews on the subject have already been published.[2,8] However, we will be guided by some examples of combinatorial chemistry on a solid support and in solution phase for lead optimization to better illustrate our contribution to the field of rapid inhibitor discovery.

Solid phase combinatorial libraries

In the last two decades, small molecule (300–700 Da) combinatorial chemistry has emerged as a useful tool for rapid generation of libraries that are used in drug discovery.[9] Different strategies have been developed for the synthesis and evaluation of libraries, particularly parallel syntheses for the preparation of discrete compounds and split syntheses for the preparation of compound mixtures have been used to prepare different classes of molecules such as isoquinolinones and imidazoles.[2,5] The following example of the diversification of indinavir on a solid support is given to illustrate the value of combinatorial chemistry in the drug discovery area.

Indinavir, one of the seven FDA-approved HIV protease drugs, is rapidly metabolized, mainly by *N*-dealkylation of the pyridyl methyl moiety and hydroxylation of the aminoindol (Fig. 2A). In order to replace the aminoindol at the P2′ position in this inhibitor, a solid phase combinatorial approach was sought. Thus, the group at Merck developed a new strategy for the solid phase synthesis of indinavir and constructed a library of 902 compounds of indinavir analogues on solid support. Evaluation of these compound found that 2,6-dimethyl-4-hydroxy phenol is a good replacement for the aminoindanol moiety (Fig. 2B).[10]

Solution phase combinatorial libraries

Traditionally, combinatorial chemistry is carried out on a solid support. However, during the last decade combinatorial chemistry in solution has emerged as the method of choice in many pharmaceutical companies and academic research groups.[7,8] The advantage of solution phase over solid phase is that the accumulated knowledge of organic chemistry can be applied directly without the extra effort that is needed to optimize the reaction conditions for solid supports.[11] Yet, parallel purification in solution is the difficult element of the synthetic process compared to a 'routine washing' when the parallel synthesis is carried out on solid support. Purification and synthetic strategies such as the use of solid-supported scavengers, fluorous synthesis, liquid–liquid extraction, and several other techniques have been developed to aid the purification of intermediates and final compounds generated from parallel solution chemistry.[12] The following example, which is related to targeting human rhinoviruses, is given to illustrate the power of solution phase combinatorial chemistry in the drug discovery area.

The human rhinoviruses (HRVs) are members of the picornavirus family and are the single most causative agent of the common cold. The HRV 3C protease (HRV 3CP) has been considered as a drug target for this virus. The protease belongs to the cysteine protease family and Michael acceptor-containing HRV 3C protease inhibitors such as **1**, (Fig. 3) have been developed. In order to develop a new derivative of **1**, with better physical properties (less peptide like), the group at Pfizer sought to replace the P2–P4 portion of **1** with smaller, nonpeptidic substituents. To achieve this goal, the core molecule **2** was selected and a library of carboxylic acids was coupled to **2** using polystyrene-bound carbodiimide. Direct evaluation of the library against 3C protease, furnished the potent inhibitor **3** (EC_{50} of 0.16 μM), with a better antiviral activity when compared to the peptidic inhibitor **1**.[13]

Combinatorial chemistry in drug discovery has grown to a mature level both in solution and on a solid support. While the synthesis of libraries in solution has reached maturity, the need for some form of purification makes it more difficult to construct libraries due to the high cost of instruments and the need for specialists to run the instruments. Many labs are trying to develop and improve purification methods so chemists can use simple and affordable strategies for library construction and purification. Our lab took another route for such a goal. The next paragraphs will deal with our newly developed approach, that is microtiter plate-based chemistry and screening *in situ* for rapid inhibitor discovery.

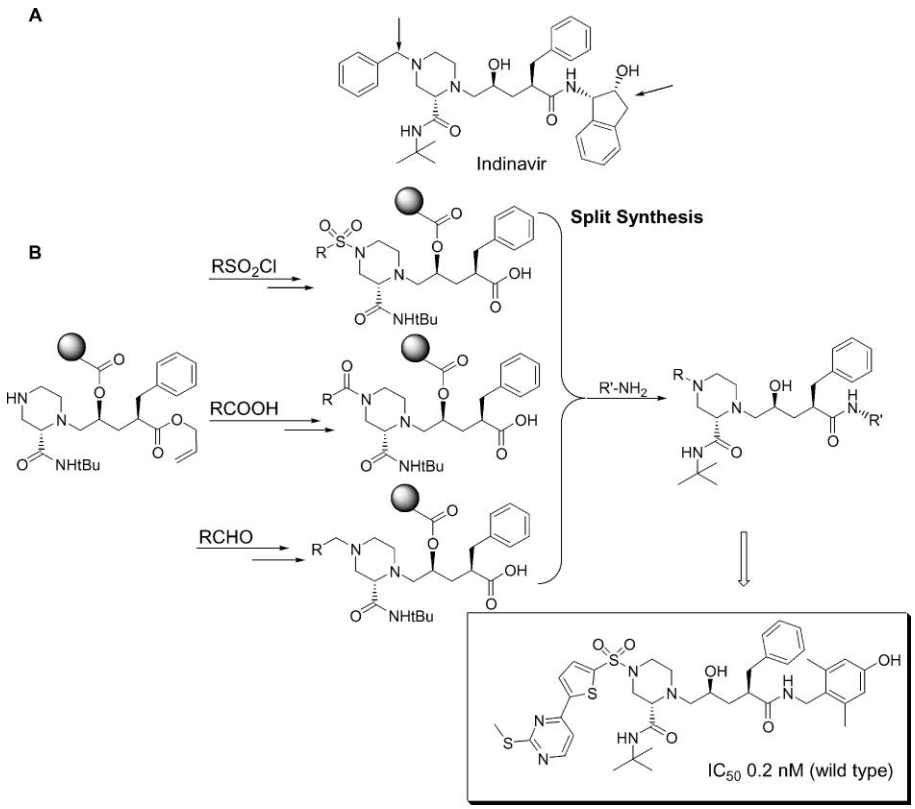

Fig. 2 Synthesis of indinavir analogues using solid phase combinatorial chemistry.

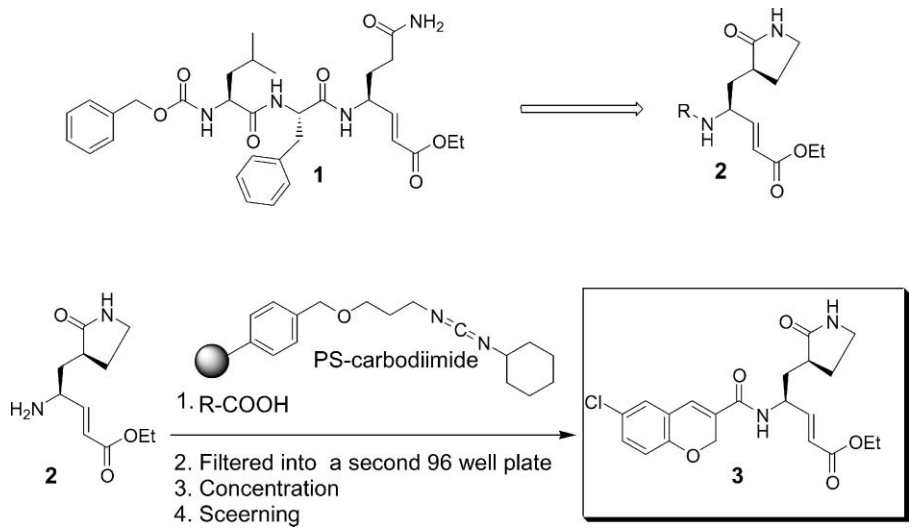

Fig. 3 The use of resin bound reagents for parallel synthesis and lead optimization of HRV 3C protease inhibitors.

The principle, application, and future directions of this approach will be discussed.

Microtiter plate based chemistry and *in situ* screening

During our work in inhibitor discovery we developed a useful strategy for rapid identification and optimization of enzyme inhibitors using microtiter plate based reactions and *in situ* screening (Fig. 4). This method utilizes high yield organic reactions with high selectivity starting from a core (weakly binding molecule, natural product, transition state analogue) and a set of building blocks to generate a focused library in a microtiter plate, in which a single compound is formed in each well. The chemistry should also be amenable to micro scale reactions and be carried out without protecting group manipulation. Preferably, the reaction should be performed in water or water miscible non-toxic solvents, so that the final products can be screened directly without isolation or purification.

For the best assessment of inhibition results, reaction wells are normally subjected to LC-MS and TLC analysis for determination

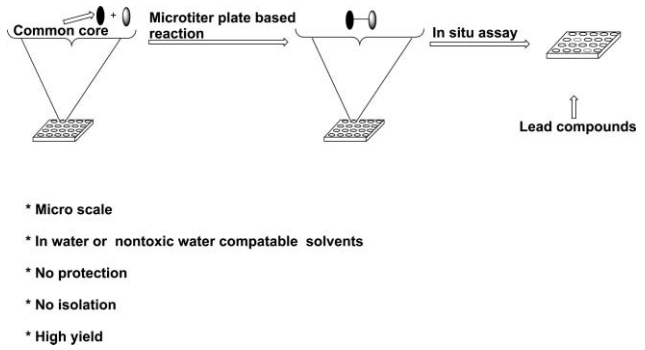

Fig. 4 General scheme for microtiter plate based reaction and *in situ* screening.

of the product yield. Reactions are usually carried out at a high concentration (>5 mM) and upon completion the plate will first go through a round of dilution (micromolar range). The assay will always be tested under the reaction condition to verify that there is no interference of these conditions with the screening. Wells that show more than 50% inhibition will go through a second round of dilution (nanomolar range) from which 1–2 compounds may emerge as the best inhibitors from the library. The compounds will then be synthesized on larger scales that are purified, and analyzed for structural integrity, resulting in the determination of the inhibition constant of the pure compounds. Our experience shows that once a good starting point is selected, the diversification process (50–100 compounds), coupled with *in situ* screening, can be accomplished within a day. In the following sections, we will summarize the reactions that were found useful for this approach to identify potent inhibitors that are targeting various enzymes.

Microtiter-plate based amide bond forming reaction and *in situ* screening

A vast amount of information is available regarding the amide bond forming reaction, both in solution and on a solid support. Several activation reagents in different solvents have been found to give nearly quantitative yields in a very short time. For example, analogues of phosphonium salts, in particular *N*-[(1-*H*-benzotriazole-1-y)(dimethylamino)methylene]-*N*-methylmethanaminiumhexafluorophosphate *N*-oxide (HBTU), have been found to be very efficient and convenient reagents to activate carboxylic acids for coupling with primary and secondary amines. Initially, the microtiter plate based amide bond forming reaction coupled with *in situ* screening was tested against HIV-1 protease (HIV-1 PR) and drug resistant HIV-1 PR.[14]

The enormous efforts over the past two decades to develop effective molecules that inhibit the HIV-1 PR have resulted in the discovery of drugs that dramatically improved the quality of life and survival of patients infected with HIV-1.[15] Unfortunately, many drug-resistant and cross-resistant mutant HIV-1 PRs have emerged. The development of second-generation protease inhibitors, which are efficacious against both the wild type and drug resistant HIV-1 PR, less prone to development of resistance, and with fewer side effects, is urgently needed.

Our previous efforts towards the development of protease inhibitors efficacious against both HIV and drug resistant mutants were focused on the systematic analysis of the S3–S3′ subsite specificities of the enzymes using a series of C2-symmetric inhibitors containing (1*S*,2*R*,3*R*,4*S*)-1,4-diamino-1,4-dibenzyl-2,3-butandiol **4**.[16] Although various inhibitors based on this core were found to have high potency against HIV-1 PR *in vitro*, the evaluation of other residues at these positions is a tedious process and is restricted by the time required for the syntheses and purification of new compounds for testing.

The C2-symmetrical diaminodiol **4** core was reacted with 60 acids in solution, as shown in Fig. 5. Each carboxylic acid was added to a 200 μL solution of a 96-well microtiter plate containing HBTU and DIEA in DMF followed by the addition of core **4**. The mixture was kept at room temperature for 30 min. The reaction mixture in each well was diluted to a final concentration of 100 nM, based on a complete conversion of the starting material to product, and screened for HIV-1 PR inhibition. Wells that inhibited more than 50% of the HIV-1 PR activity were diluted to 10 nM and screened again.

Compound **5** was found to be the most potent compound from this library with K_i values of 2 nM against wild type protease and 5 and 12 nM against V82F and G48V mutants, respectively. Moreover, this inhibitor was active in tissue culture at 1.3 μM. We have found that up to a 200 μM concentration of HBTU and DIEA does not interfere with the activity of HIV-1 PR. In addition, the measured IC_{50} values for the crude products were similar to the purified compounds. We were able to carry out the whole process of activation, coupling, and *in situ* screening within 1 h.[14] As a control experiment, we prepared and screened *in situ* a well studied inhibitor in our lab named TL-3. Our results showed that the well that contains this inhibitor emerged with very similar IC_{50} value when compared to the pure compound.[14]

A second core derived from amprenavir was diversified using the same strategy. Interestingly, from the 100 different acids that were used, the P2 residue (3-hydroxy-2-methylbenzoic acid) that was selected is the same residue found in the drug nelfinavir (Fig. 6). This amprenavir–nelfinavir hybrid inhibitor showed a good correlation with the *in vitro* enzymatic assay and *in vivo E. coli*-based system for high-throughput screening.[17] The IC_{50} of this inhibitor against I84V (a key amprenavir resistant mutant that resides at the S2 pocket) in the *E. coli* based system was determined to be 140 μM compared to IC_{50} of 280 μM of amprenavir. Following the success with the HIV protease, we extended the microtiter plate based amide bond forming reaction and *in situ* screening to the inhibition of three different enzymes: β-aryl sulfotransferase, α-fucosidase, and SARS 3CL protease.

Sulfotransferases (STs) catalyze the sulfuryl group transfer from the donor adenosine 3′-phosphate-5′-phosphosulfate (PAPS) to a range of acceptor substrates. This transformation is involved in a variety of biological processes and various STs have been implicated in the development of disease states. For instance: estrogen ST helps regulate estrogen concentration relating to breast cancer and tyrosylprotein STs (TPSTs) sulfonate CCR-5 and the *N*-terminus of P-selectin glycoprotein-1 (PSGL-1) leading to the cell entry of HIV and to diseases of chronic inflammation.[18] Therefore, these enzymes have emerged as promising therapeutic targets and the search for potent inhibitors is of interest.

We have used the enzyme β-arylsulfotransferase-IV (β-AST-IV) to study the requirements for ST inhibition. To facilitate screening, we developed a simple and sensitive high-throughput assay for arylsulfotransferase (Fig. 7A), which was used to screen 35000

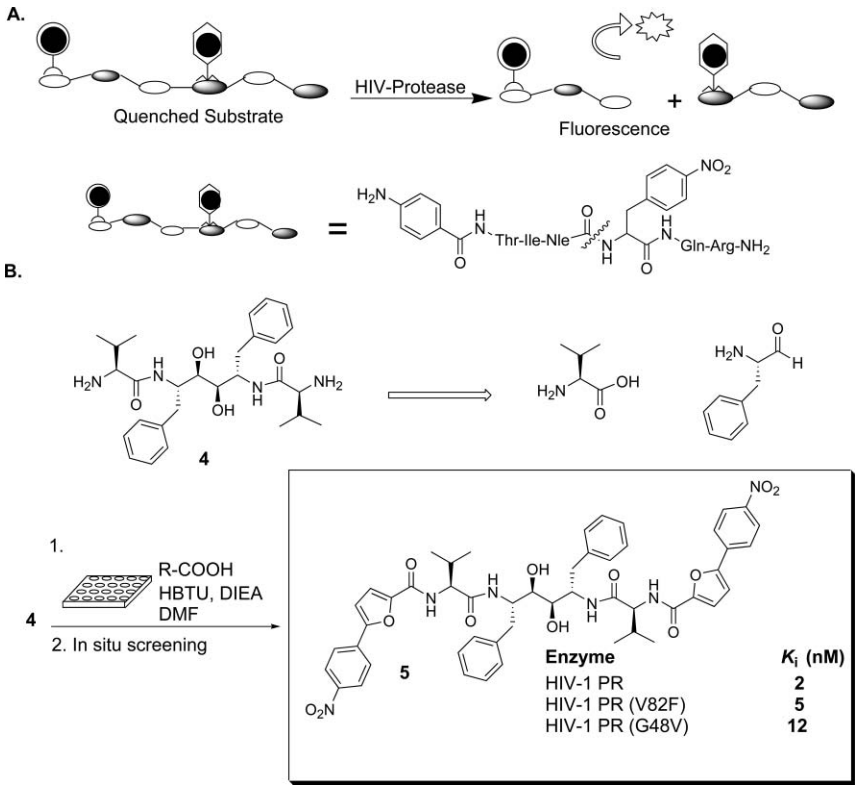

Fig. 5 A. Fluorescent substrate for the screening of HIV protease inhibitors (FRET assay). B. Microtiter plate based amide bond forming reaction and *in situ* screening for the discovery of HIV protease inhibitors.

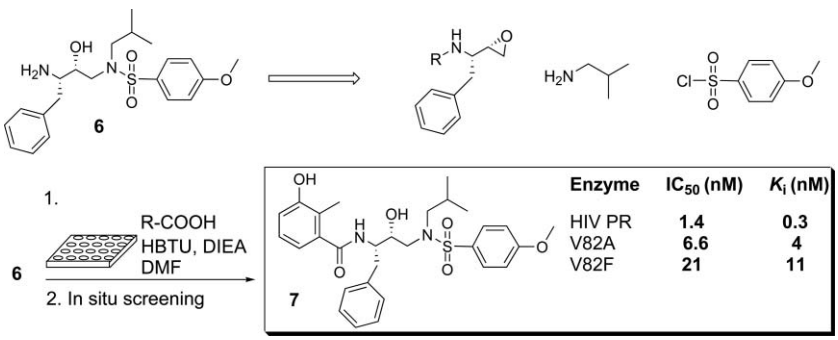

Fig. 6 Microtiter plate-based amide bond forming reaction and *in situ* screening for the discovery of HIV protease inhibitors.

purine and pyrimidine analogues, a study that resulted in the discovery of compound **8** with a K_i of 96 nm (Fig. 7B).[19] In order to improve the effectiveness of compound **8**, core **9** was designed and reacted in microtiter plates with a library of acids. *In situ* screening of this library furnished inhibitor **10** with a K_i of 30 nM (Fig. 7C).[20] Next, we sought to improve this inhibitor through replacement of the adenine moiety in the inhibitor. A reverse library was synthesized based on the 4-fluro-3-hydroxy benzoic acid group coupled to a 12-carbon linker functionalized with amine. *In situ* screening of a second library yielded compound **12** with a K_i of 5 nM (Fig. 7D).[20] This compound represents the most potent inhibitor of β-AST-IV developed to date.

Recently, we reported several potent inhibitors against α-fucosidase by using the microtiter plate-based amide-forming reaction and *in situ* screening.[24,25] α-Fucosidase is a member of the glycosidase family and is involved in the removal of nonreducing terminal L-fucose residues that are connected to oligosaccharides *via* several different linkages. An aberrant distribution of α-fucosidase has been reported relevant to inflammation, cancer, and cystic fibrosis.[21,22,23] These enzymes have been recognized as diagnostic markers for the early detection of colorectal and hepatocellular cancers because of the presence of α-fucosidase in these patients' sera. Potent α-fucosidase inhibitors may be used to study their functions and to develop potential therapeutic agents.

The 1-aminomethylfuconojirimycin **13** was designed as a transition state mimic for the fucosidase reaction (Fig. 8). Thus, we used this core as a starting point for the diversification process with various acids in microtiter plates and screened them *in situ* against α-fucosidase from bovine kidney. Two potent inhibitors **14** and **15** were selected and their K_i values were determined to be 0.5 and

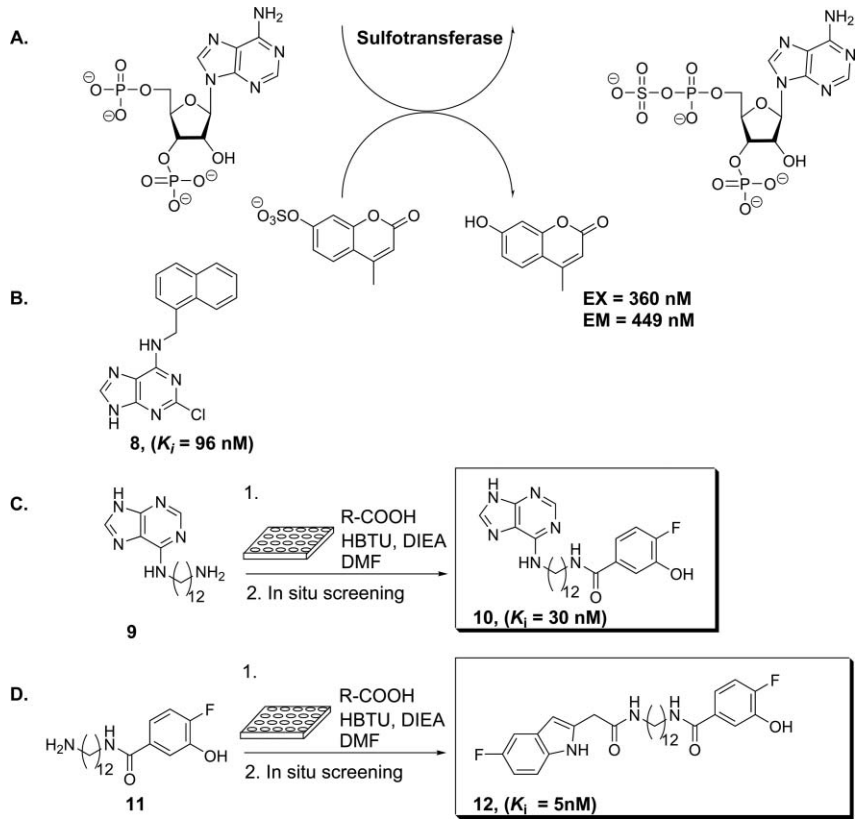

Fig. 7 A. Fluorescence-based assay used for HTS for of β-arylsulfotransferase-IV inhibitors. B. Purine based inhibitor discovered from screening a library of 35000 purine and pyrimidine analogues. C. Microtiter plate based amide bond forming reaction and *in situ* screening for the discovery of β-arylsulfotransferase-IV inhibitors. D. Follow-up library replacing the purine group.

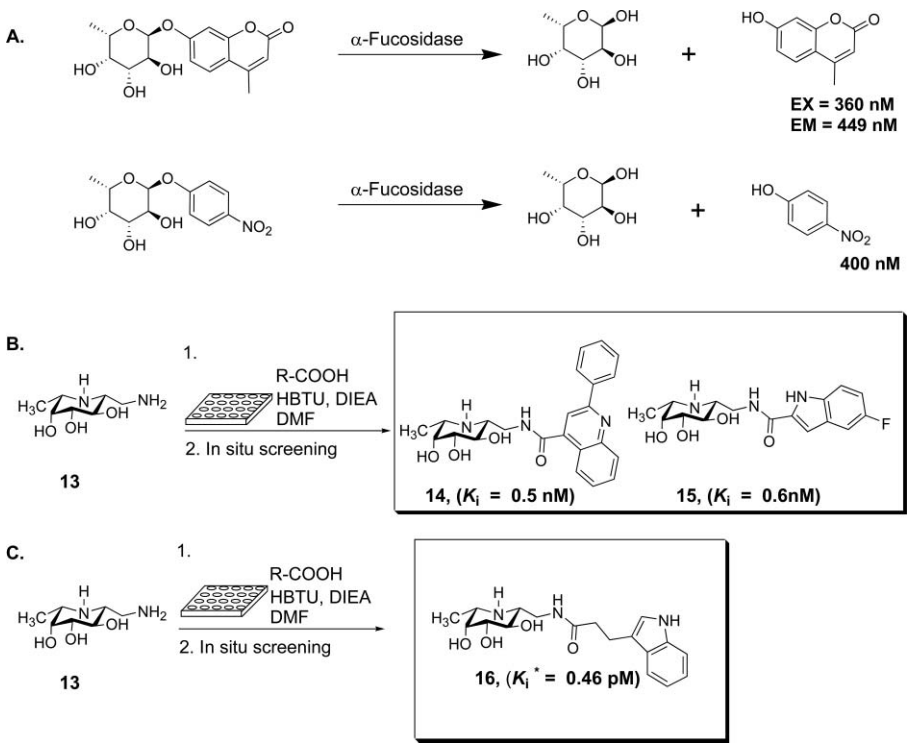

Fig. 8 A. High throughput assays that are used for the screening of α-fucosidase inhibitors. B. Microtiter plate based amide bond forming reaction and *in situ* screening for the discovery of α-fucosidase inhibitors. C. Microtiter plate based amide bond forming reaction and *in situ* screening for the discovery of slow binding α-fucosidase inhibitors.

0.6 nM, respectively (Fig. 8B).[24] Both inhibitors showed high selectivity for α-fucosidase among other glycosidases. Next, we used the same library based on core **13** against different α-fucosidases and searched for slow binding inhibitors. We were pleased to discover that compound **16** showed time-dependent inhibition against α-fucosidases from *corynebacterium* sp. (Fig. 8C).[25] The progressive tightening of enzyme-inhibitor **16** complex from a low nanomolar K_i to K_i* of 0.46 picomolar represents the most potent glycosidase inhibitor to date.

By applying the microtiter plate based amide bond forming reaction and *in situ* screening, we were able to identify new inhibitors against the severe acute respiratory syndrome (SARS). SARS is a newly emerged infectious disease that has affected more than 8000 individuals across 32 countries and has resulted in more than 800 fatalities.[26,27] To date, no efficacious therapy for SARS is available. This disease is caused by an infection with a novel human coronavirus (SARS-CoV). Therefore, a search for effective antivirals for the SARS-CoV is of current interest. SARS coronavirus is a positive-strand RNA virus that encodes two polyproteins pp1a and pp1ab.[28] Extensive proteolytic processing of these nonstructural polyproteins is required to provide the functional proteins for viral propagation. These processes are mediated primarily by the main protease (M^{pro}), which is also known as dimeric chymotrypsin-like protease ($3CL^{pro}$).[29] The active site of $3CL^{pro}$ contains Cys145 and His41 to constitute a catalytic dyad, in which the cysteine thiol functions as the common nucleophile in the proteolytic process. Due to its essential role in viral replication, the $3CL^{pro}$ is an attractive target for the development of therapeutics against SARS.

We used a cell-based assay to screen more than 10,000 compounds.[30] We found that HIV protease inhibitor Lopinavir inhibits $3CL^{pro}$ with IC_{50} *ca.* 50 μM, (Fig. 9B). In order to find more potent $3CL^{pro}$ inhibitors, a library of Lopinavir-like compounds was assembled using either diamine **17** or amine **18** as the core structure for reactions with various acids in microtiter plates followed by screening *in situ* (Fig. 9,C). In this study, we found that the products derived from 2-aminobenzoic acid, 4-(methylamino)benzoic acid, 4-(dimethylamino)benzoic acid and 4-(diethylamino)benzoic acid showed the best inhibition.[31]

In order to characterize these inhibitors and measure their inhibition constants, we attempted to prepare the pure amide derivatives; however we found that the amide formation was very slow, and the intermediates benzotriazole esters **19**–**22** were isolated as major products (Fig. 9D). To our surprise, all the Lopinavir-like compounds showed only modest inhibitory activities toward $3CL^{pro}$ ($IC_{50} \geq 10\,\mu M$), whereas the benzotriazole esters **19**–**22** showed high inhibition activities (Fig. 9D).[31] These compounds were stable in pH 5.0–8.0 over 24 h at room temperature. This study showed for the first time a new class of stable benzotriazole esters as mechanism based irreversible inhibitors of $3CL^{pro}$. These inhibitors showed no cytotoxicity, and among which compound **19** is the most effective with an inactivation constant $0.0242\ min^{-1}$ and inhibition constant K_i of 21.0 nM, respectively.[31] Mechanistic investigations of the mode of action using kinetic and mass-spectrometry analyses indicates that the active-site Cys-145 is the acylated residue.

Microtiter-plate based 1,2,3-triazole forming reaction and *in situ* screening

Click chemistry has emerged as a new strategy for the rapid and efficient assembly of molecules with diverse functionality.[32] Among these reactions is the copper(I)-catalyzed 1,2,3-triazole synthesis. It guarantees reliable synthesis of 1,4-disubstituted 1,2,3-triazole compounds in high yield, regioselectivity and purity.[33] Moreover, the reaction works best in aqueous media and tolerates virtually all functional groups without protection. This makes it ideal for achieving desired diversity of a starting core in microtiter plates followed by direct screening without any purification.

Fucosyltransferases (Fuc-T) catalyze the final glycosylation step in the biosynthesis and expression of many important saccharides, such as sialyl Lewis x (sLe^x) and sialyl Lewis a (sLe^a) of cell-surface glycoproteins and glycolipids. These and other fucosylated oligosaccharide structures are central to cell–cell interactions and cell migration in connection with physiological and pathological processes such as fertilization, embryogenesis, lymphocyte trafficking, immune responses, and cancer metastasis.[34,35] The terminal step in the biosynthetic pathway of these fucose-containing saccharides is the transfer of L-fucose from guanosine diphosphate-L-fucose (GDP-fucose) to the corresponding glycoconjugate acceptor.[34] The rational design of potent inhibitors against this enzyme has been difficult due to the lack of structural data for Fuc-Ts and the best inhibitors are in the micromolar range (5–100 μM).

We were able to find a highly selective inhibitor for human α-1,3-fucosyltransferases using microtiter plate based 1,2,3-triazole forming reaction coupled with *in situ* screening.[36] Our approach was based on the use of a GDP moiety as a starting core since the majority of the binding energy for Fuc-T lies at this moiety.[37,38] Hence, GDP alkyne core **23** was prepared and a library of azide molecules was reacted with this core in a microtiter plate to give 85 triazole candidates (Fig. 10). The GDP-traizole compounds were screened for inhibitory activity directly in microtiter plates from which compound **24** emerged as the best inhibitor with a K_i of 62 nM (Fig. 10). The inhibition of **24** is an 800-fold improvement over GDP-alkyne **23** ($K_i = 47\,\mu M$). This inhibitor represents the first nanomolar and most potent inhibitor of Fuc-Ts. Moreover, it shows no inhibition against two known galactosyltransferases, catalytically promiscuous pyruvate kinase, and has lower inhibition properties against other Fuc-Ts known to date.[36]

In a similar manner we applied a microtiter plate-based 1,2,3-triazole forming reaction, coupled with *in situ* screening, for the discovery of HIV proteases inhibitors.[39] In this study, we used two azide cores **26**, **27** derived from amprenavir prepared from the same epoxide precursor (Fig. 11). A library of alkynes was then reacted with each core in microtiter plates and were screened "as is" against HIV protease and several resistant mutants. While the triazole compounds derived from core azide **26** did not show any good inhibition at 100 nM, compound **28** generated from core **27** showed excellent inhibition properties against wild type and several other mutants. This compound was synthesized on a larger scale and K_i values were determined for wild type and HIV resistant mutants (Fig. 11). Further studies on this inhibitor by computational docking and X-ray crystallography have revealed that the 1,2,3-triazole acts as an amide bond surrogate (Fig. 12).[40]

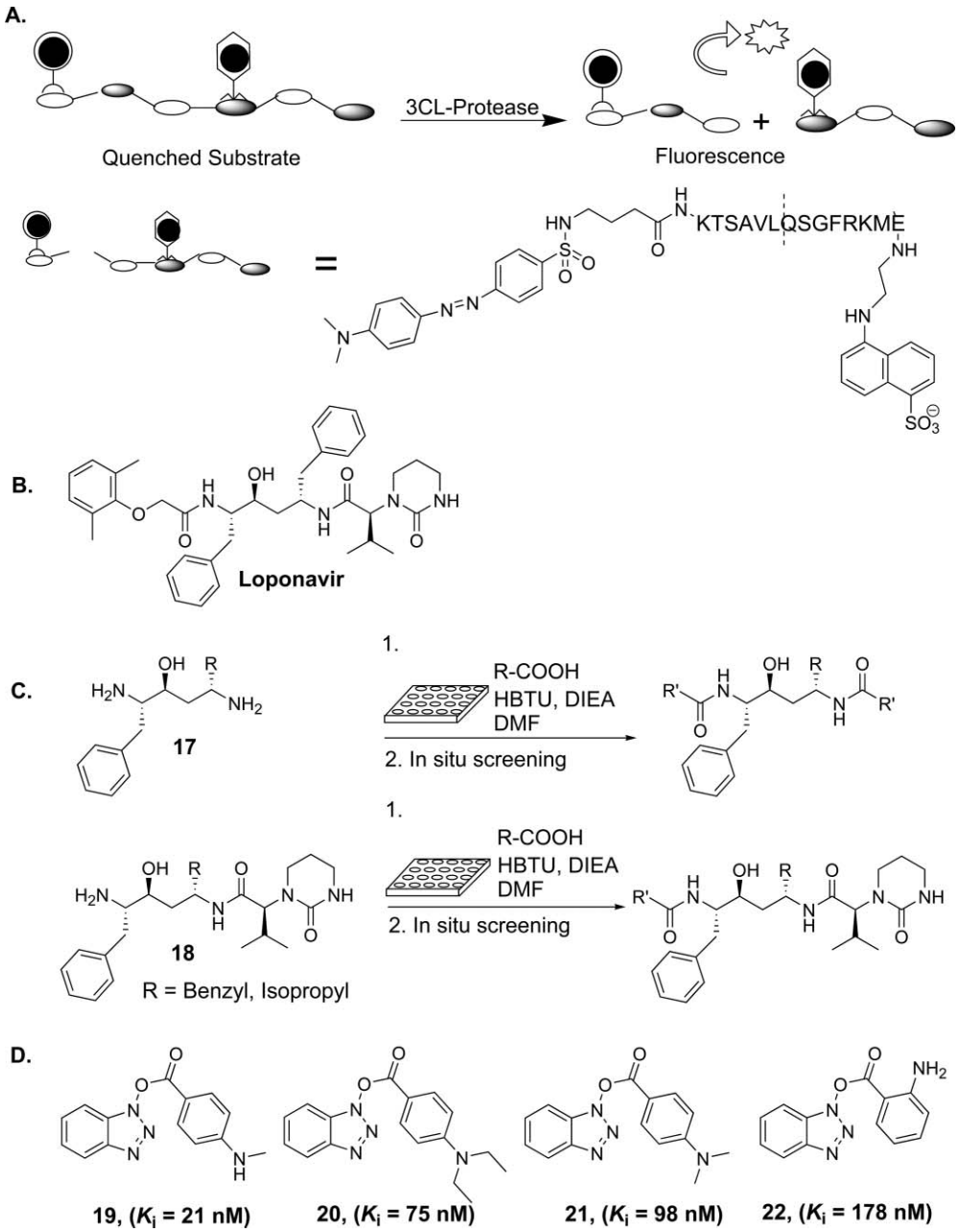

Fig. 9 A. High throughput assay that is used for the screening of $3CL^{pro}$ inhibitors. B. Loponavir structure. C. Microtiter plate based amide bond forming reaction and *in situ* screening for the discovery of $3CL^{pro}$ inhibitors. D. A new class of stable benzotriazole esters as mechanism based irreversible inhibitors of $3CL^{pro}$.

Microtiter plate-based Pictet–Spengler reaction and screening for the discovery of novel anthrax lethal factor inhibitors

Anthrax lethal factor (LF) is a zinc dependent metalloprotease produced by *Bacillus anthracis*, and is the causative agent of anthrax. LF and two other plasmid encoded proteins known as edema factor (EF) and protective antigen (PA) are responsible for the virulence of *Bacillus anthracis*.[41,42,43] LF cleaves the *N*-terminal fragment of mitogen-activated protein kinase (MAPKK) that triggers a cascade of events that lead to the apoptosis of the host cell. An efficient treatment of *Bacillus anthracis* in the late stage of the infection requires blocking the activity of LF. Thus, the identification of potent inhibitors against LF has become the focus of several studies.

Recent work by Dell'Aica *et al.* showed that some galloyl derivatives extracted from green tea are able to inhibit the LF.[44] Inspired by this study, we decided to diversify 5-hydroxydopamine **29** as a core structure with an aldehyde library using the Pictet–Spengler reaction.[45] This reaction allows the formation of tetrahydroisoquinoline derivatives through condensation of β-arylethylamines with carbonyl compounds and cyclization of the Schiff bases formed under acidic aqueous media. Over 60 aldehydes were reacted with 5-hydroxydopamine in microtiter plate and

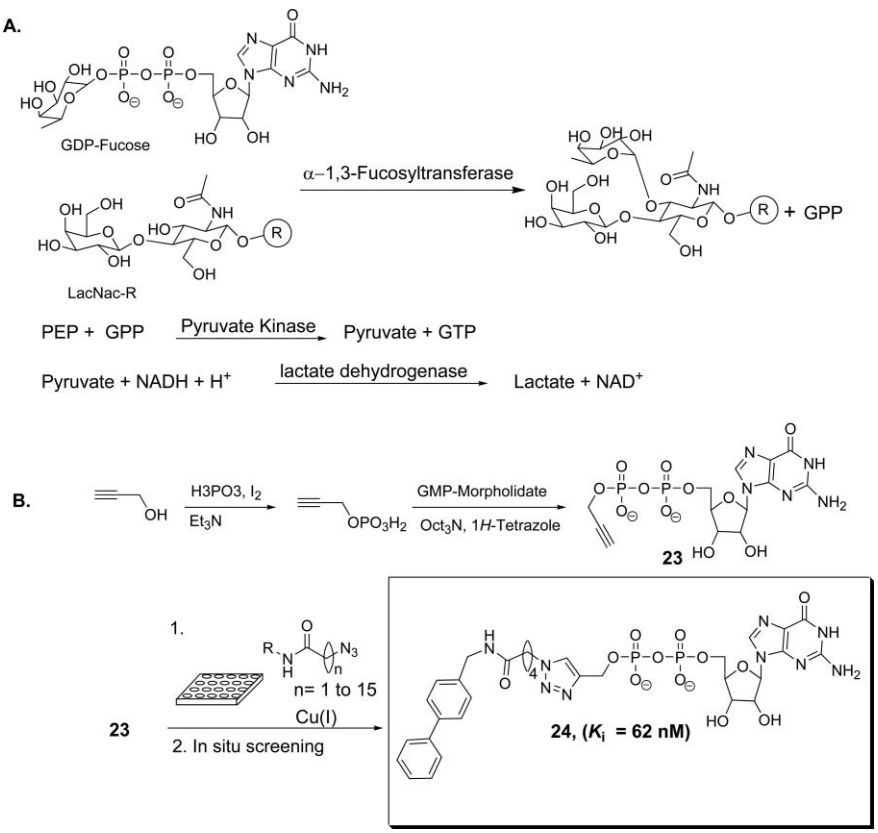

Fig. 10 A. High throughput assay that is used for the screening of α-1,3-fucosyltransferase inhibitors. B. Microtiter plate based 1,2,3-triazole forming reaction and *in situ* screening for the discovery of 1,3-fucosyltransferase inhibitors.

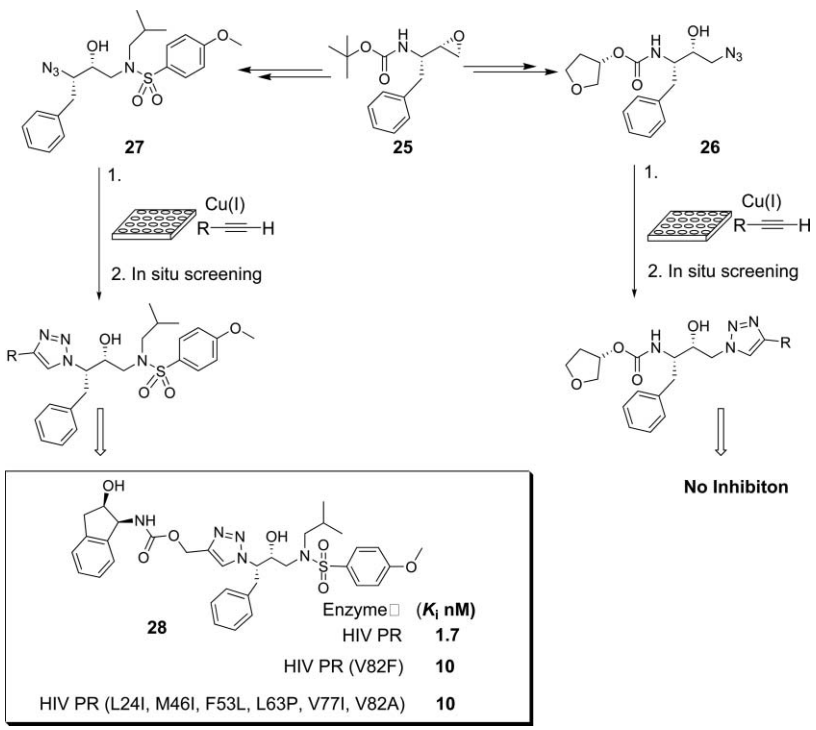

Fig. 11 Microtiter plate-based 1,2,3-triazole forming reaction and *in situ* screening for the discovery of HIV protease inhibitors.

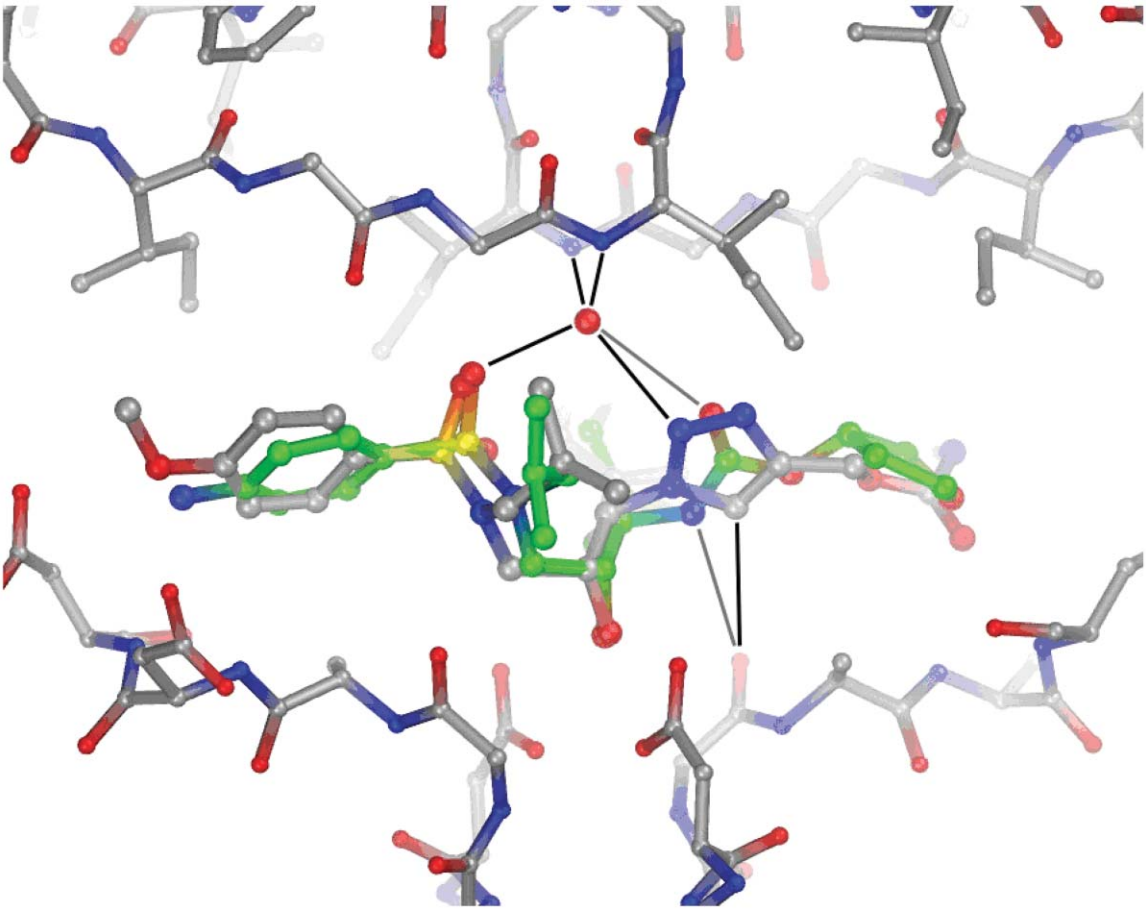

Fig. 12 Detail of hydrogen bonding interactions in compound **28**. A cross section through the active site is shown, with the protease flaps at the top, the two active site aspartates at the bottom, and the inhibitor running horizontally through the center. The position of amprenavir is also shown with carbon atoms in green. Key hydrogen bonds to the structural water and to the main chain of glycine 27 are shown with black lines.

the products were screened *in situ* against LF. Several compounds showed good activity at low micromolar concentration, in which compound **30** revealed to have the best inhibition activity with a K_i of 4.3 μM after purification (Fig. 13B).[45]

Tetrabutylammonium fluoride-assisted rapid alkylation in microtiter plates for the discovery of enzyme inhibitors *in situ*

Recently we reported that tetrabutylammonium fluoride (TBAF) is able to assist several alkylation reactions, including N^9-alkylation on purine rings, *N*-alkylation of sulfonamides, *O*-alkylation of carboxylates, and *O*-alkylation of hydroxy triazoles.[46,47] These high yielding and rapid reactions allowed for their use in microtiter plates for inhibitor discovery. As mentioned earlier, by screening 35000 purine and pyrimidine analogues against β-aryl sulfotransferase, we discovered compound **8** with a K_i of 96 nM. Using this compound as a starting core for reaction with a small library of organic halides in the presence of TBAF, we rapidly improved its inhibition properties to yield a new inhibitor **32** with a K_i of 9 nM (Fig. 14).[46] Similar chemistry was also successfully applied to the discovery of HIV protease inhibitors. Starting from sulfonamide core **31** and a library of organic halides we rapidly generated a new HIV protease inhibitor **33** with a K_i of 1.1 nM (Fig. 14).[47]

Epoxide opening in water for rapid inhibitor discovery in microtiter-plate and *in situ* screening

Epoxides are versatile synthetic intermediates, which can be opened with amines using several known reagents. The resulting hydroxyethylamine products of epoxide aminolysis are important bioisosteres, which appear in several FDA-approved drugs. Their efficacy as transition-state mimics and as backbone replacements of amide bonds in the P1/P1′ position of aspartyl protease inhibitors have been well studied. Five out of the seven currently approved HIV protease inhibitors, amprenavir, nelfinavir, saquinavir, indinavir and atazanavir, contain the hydroxyethylamine core structures.[15] Epoxide opening reactions with amines have been reported to proceed in high efficiency using various methods with or without catalysts and mostly in organic solvents. Only a few examples reported the use of water as a solvent for epoxide opening.[48,49] Recently, we have shown that epoxide-opening reaction with an amine could be performed efficiently

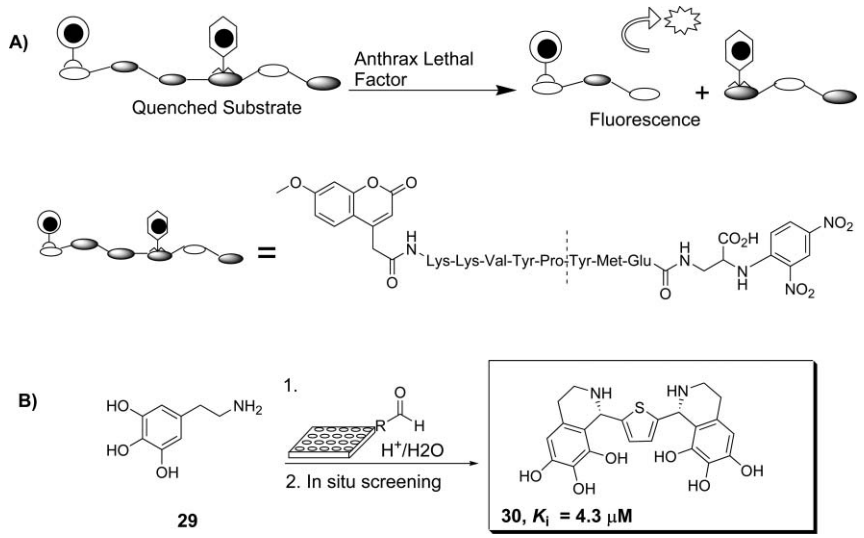

Fig. 13 A. Fluorescent substrate for the screening of anthrax lethal factor inhibitors protease inhibitors. B. Microtiter plate based Pictet–Spengler reaction and *in situ* screening for the discovery of anthrax lethal factor inhibitors.

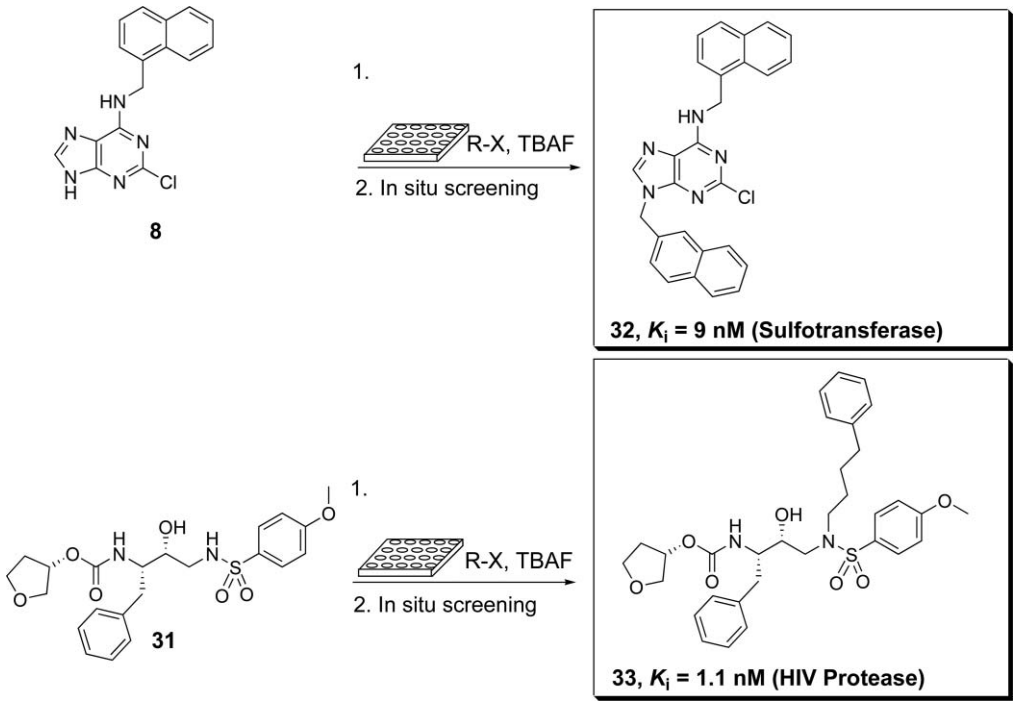

Fig. 14 Tetrabutylammonium fluoride-assisted rapid alkylation in microtiter plates for the discovery of enzyme inhibitors *in situ*.

in microtiter plates using water as a solvent,[50] which has the advantage of precluding water-insoluble compounds in the *in situ* screening. Core **34** was reacted in a microtiter plate with an amine library and screened *in situ* for the discovery of novel P1′ residues. The product in each well, which at this stage lacks the P2′ residue, was then diluted into another 96-well microtiter plate and assayed for its inhibition activity against HIV-1 PR and a mutant with the V82A mutation at S1/S1′ site. Compounds that showed the best activity were then coupled to the P2′ residue found in amprenavir, purified and their K_i's values were then determined (Fig. 15).

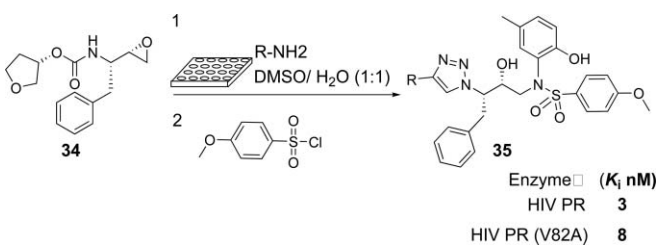

Fig. 15 Epoxide opening with amine library in microtiter plate for the discovery of HIV protease inhibitors *in situ*.

Summary

We have shown that microtiter plate based reaction coupled with *in situ* screening could serve as a useful method for rapid inhibitor discovery. Several reactions were found suitable for this approach, as we have demonstrated with the amide bond forming reaction for the discovery of potent inhibitors of HIV proteases, β-aryl sulfotransferase and α-fucosidases. In several examples, we were able to construct the library and complete the assay within a few hours. Moreover, our experience shows that once a good starting core is selected, a small library of building blocks is all that is needed to select a potent inhibitor. Future direction will be directed towards finding new organic reactions in water that can be applied to microtiter plates and allow for *in situ* screening. This method, which complements other combinatorial chemistry approaches, should find useful applications in the area of inhibitor discovery.

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