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Recent advances in solution-phase derived synthetic libraries and rapid bioassays on microtiter plates

ABSTRACT

Compound library derived from solution-phase synthesis via amide or 1,3-dipolar cycloaddition coupled with an in-situ enzymatic screening has yielded a fast discovery of several potent inhibitors. Novel compounds against hydrolases including exo- and endo hydrolases and transferases has been found by using the probing substrates such as chromophore-, fluorophore- and FRET-tagged compounds, or indirect sensing methods such as fluorescence. The dilution method minimize the disturbing effects arisen from residual reagents thereby making the assay attractive to the medicinal chemistry. Aspects regarding chemical reaction, solvent effect, and probing methods are briefly reviewed.

Table of Abbreviations
FRET = Fluorescence resonance energy transfer
HTS = High throughput screening
DIEA = N, N-diisopropylethylamine
HBTU = 2-(1H-Benzotriazole-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate
DMF = N, N-dimethylformamide
DMSO = Dimtheyl sulfoxide
IC50 = 50 % inhibition concentration
HIV = Human immunodeficiency virus
CD4 = Cluster determinant 4
HIV-1 = Human immunodeficiency virus type 1
LST-03 lipase = Pseudomonas aeruginosa LST-03 lipase
PST-01 protease = Pseudomonas aeruginosa PST-01 protease
Ex = Excitation wave length
Em = Emission wave length
3CL-protease = 3C-like protease
HSV TK = Herpes Simplex Virus Thymidine Kinase

INTRODUCTION

Combinatorial chemistry has become a useful tool in drug discovery area. In general, the lead or hit compound may be discovered through this approach. The reaction is mainly carried out in solid support or solution phase. After construction and purification of the compound libraries, the product mixture are submitted to a high throughput screening (HTS) procedure to evaluate their bioactivity on an enzymatic or a cellular level. Although the solid-phase approach has gained many expertise in both chemical synthesis and biological assay, the inherent weakness associated with the requirement of a linker for attaching the starting compounds onto the solid support and the low specific capacity of the support makes the subsequent purification and bioassay a tedious task (1, 2). Take an example, while the unreacted reagents and solvents could be completely removed after washing, removal of the linker resulted in undesired contamination of products which needed to be further purified (3). On the other hand, the capacity for a bioassay carried out in the microtiterplate with a volume of 200 μL for each well could be easily achieved using only 5 μmol of the starting material in the solution phase. However, at least a 10-times reservoir for the solid support is needed to obtain a same concentration (4). The solution-phase chemistry possesses many advantages except purification. In the past development of drug discovery, the rate determining step rests neither on the stage of high-throughput screening nor on the combinatorial chemistry itself, but on the stage inbetween, i.e. how to bridge the two platforms in a smooth and efficient way.
Recently, Wong and co-workers developed a methodology combining a rapid solution-phase synthesis and an in-situ bioassay in microtiterplate. As a review on this topic has been recently reported by Wong and co-workers (5), we wish to present our aspect more on the screening method. Issues will be covered including the solvent effect and various probing methods. In addition, novel approaches other than enzymatic assay will be briefly discussed.

RAPID LIBRARY CONSTRUCTION AND IN-SITU HIGH-THROUGHPUT SCREENING

This methodology was first reported by Wong and co-workers in 2002 (6). As a typical example, the compound library was constructed through an amide-bond formation of a core amino compound with various carboxylic acids in solution phase (6-11) or through 1,3-dipolar cycloaddition of a core ethyne derivative with various substituted azides (12-21).
The reaction was performed either in water or water-miscible solvents such as DMF or DMSO (5). The appropriate core compound could be a weakly binding molecule, a natural product or a transition-state analog from mechanistic consideration. The typical coupling yield is in general greater than 60 percent, mostly dependent on the structure and the reactivity of the core amines. Since the products could be obtained in a significant high

yield, a bioassay of this crude mixture should represent a certain fraction of the bioactivity responsible for the desired products. Generally, the compounds of interest should meet a criterion of IC50 smaller than micromolar range. Hence, the working concentration for bioassay requires a 1000-times dilution. Dilution of the crude mixture should minimize the disturbance by the unreacted starting materials, the salts formed and the organic solvents. If the compound was significantly bioactive, the assayed results will reflect this characteristic. Furthermore, these potential compounds will go through a series of dilution to determine the apparent IC50 values. Although, traditionally, the bioassay experiment requires a high purity of compound, Wong's protocol employs the concept of dilution to make the disturbing effects by concomitant impurities negligible.

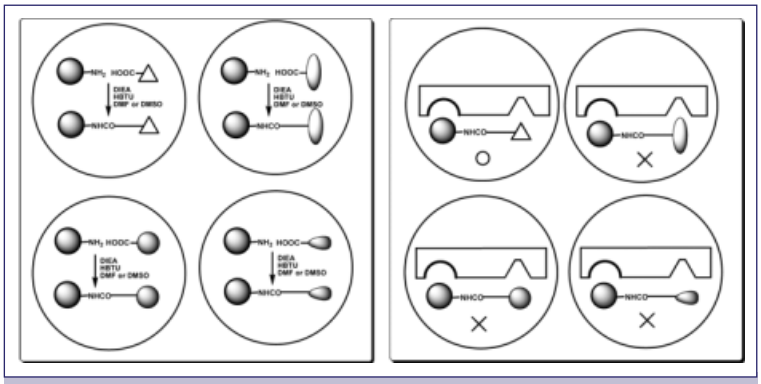

Figure 1. Panel A. Construction of library from a core amine with carboxylic acids in microtiterplates. Panel B. Transfer of the product mixtures to a new plate for high-throughput screening.

Under the dilution environment, the conformation of the protein preserves well, thereby providing a functional active site to be competed for binding by the coupling products as well as the various concomitant species. To confirm the data abstracted from impure state, a preparative amount of the target compounds can be obtained by using a traditional experiment in a flask followed by purification with chromatography. The intrinsic IC50 value obtained aforementioned is generally in consistent with the apparent data. This method has soon shown a wide application in the discovery of inhibitors against various enzymes such as hydrolases and transferases. In these screenings, two types of hydrolases: exo- and endo-hydrolases e.g. fucosidase (8, 9) and HIV protease, respectively (6, 11), have been employed as the target enzymes. Some potential compounds exerting excellent activity with IC50 of nanomolar were thereby discovered. Additionally, some novel compounds were also discovered to show an excellent inhibition against transferases such as b-arylsulfortransferase (7), α-1,3-fucosyltransferase (12), and sulfotransferase in IC50 of nanomolar.

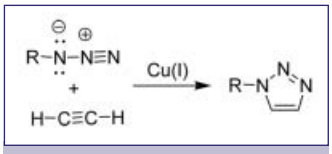

Figure 2. Construction of library from the ethyne core with various azides.

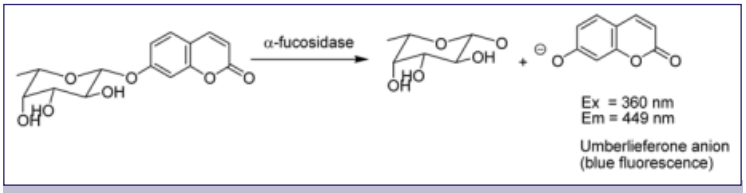

Figure 3. Hydrolysis of the labeled substrate to release the fluorescent byproduct (5).

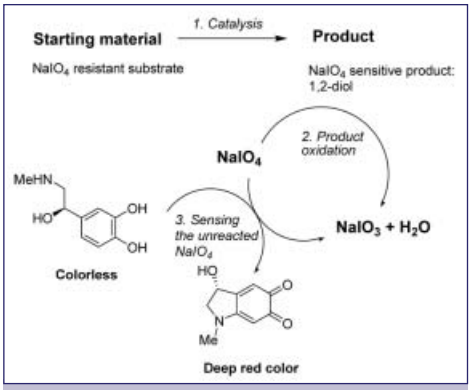

Figure 4. Indirect sensing of the $NaIO_4$-resistant substrate during enzymatic reaction (29).

ASPECT ON THE CHEMICAL REACTION

In addition to the amide-bond formation used in the in-situ bioscreening mentioned above, a recent report of the construction of a library from a core sugar-conjugated sphingosine analog, a so-called alpha-gal ceramide, was also impressive (22). This type of glycolipid has been demonstrated to be a potential immune-response enhancer through a ligand-receptor interaction mediated by the cluster determinant 4 (CD4) molecules on helper T cells of the host. Furthermore, numerous publications have addressed their significance on apoptosis and the inducing of stem-cell proliferation. In spite of the acceptable coupling yield, the bioassay was not performed in an in-situ manner. Library construction through click chemistry developed by Sharpless also arose attention with respect to their potency in the in-situ screening, since the chemical synthesis was performable in water (23, 24). With this inherent advantage, a core alkyne coupled with various azide-containing building blocks via the 1,3-dipolar cycloaddition provided another approach to the in-situ bioassay (12-21). This method has been firstly demonstrated by Wong and co-workers (12, 13) and then adopted by Yao and co-workers (14, 15) and Chmielewski and co-workers (11) for the investigation of inhibitors against protein tyrosine phosphatase and HIV-1 protease dimerization, respectively. In general, the dilution methodology is quite well suited for the chemical reactions in water-miscible organic solvents.

target enzymes			types of the sensing method	
			labeled substrates	indirect sensing the reaction of unmodified substrates
hydrolases	exo-	α-fucosidase[8,9]	fluorophore, chromophore	N.A.
	endo-	HIV protease[6,11,13,16]	FRET	N.A.
		3CL-protease[10]		
transferases		β-arylsulfotransferase[7]	fluorophore	N.A.
		tyrosine phosphatase[14]	fluorophore	
		α-1,3-fucosyltransferase[12]	N.A.	fluorophore
miscelleneous		Oxidoreductases[12]	N.A.	N.A.
		lyases		
		isomerases		
		ligases		

Table 1. Enzymatic probes which has been and to be developed in the in-situ assay.

SOLVENT EFFECT

It seems plausible that the vast amount of molecules in the life system are composed of organic compounds prepared by the catalysis through enzymes in aqueous environment (25). Enzymes perform their function by providing an active site to catalyze the chemical synthesis of their substrates. Enzymes maintain their structural conformations through both intramolecular hydrophobic interactions and intermolecular interaction with surrounding polar water. Upon the addition of an organic solvent, the outer water molecules could be replaced by the organic solvent, thereby forcing the hydrophobic domains to disperse and resulting in unfolding of the enzymes. Organic solvents are therefore seldom suggested to use in enzyme-catalyzed synthetic reaction. Nevertheless, some enzymes were found to have synthetic usefulness in organic solutions without significant loss of the bioactivity. It has been reported that LST-03 lipase and PST-01 protease as secreted from microorganisms are very resistant to organic solvents, e.g. DMSO and DMF (26). Impressive stability was shown with respect to the half-lifes of 36.2 d and > 50 d for LST-03 lipase and 9.5 d and 25.3 d for PST-01 protease in DMSO and DMF, respectively. Comparing to very rare enzymes available for synthesis in organic solvents, the reverse catalysis i.e. hydrolase is relatively stable in aqueous organic solvents, as exemplified by the subtle effect by DMF and DMSO on the reaction rate catalyzed by glucosidase (27). It was found that 90 percent and 80 percent of the relative reactivity of the enzyme were preserved in 20 percent aqueous solution of DMF and DMSO, respectively. An adequate condition for solvation of the sample is also important to determine the IC50 value. It was found that most organic compounds, except some organic salts and crystalline solids, were dissolvable in the buffer medium containing DMSO required for bioassay (28). Thus, DMF, DMSO or water used as the solvents in the library construction are quite suitable to the in-situ HTS.

PROBING METHODS

The current methods for probing enzymatic assays in HTS are either using labeled substrates or indirect sensing the reactions of unmodified substrates (29). The assaying protocols developed up to date are summarized in table 1.

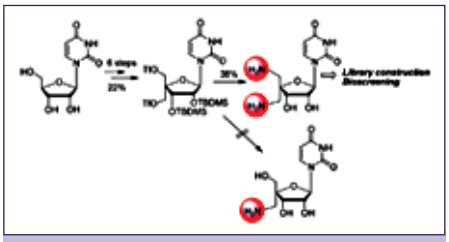

Figure 5. Development of amino uridine analogs as the core for construction of libraries.

The labeled substrates could be a chromophore or a fluorescent compound such as the derivatives of coumarin, nitrophenol, fluorescein, nitrobenzofurazane and rhodamine dyes (29-31).
This type of fluorophore possesses good leaving groups but stable enough under the condition of in-situ assay. There is still no relevant report of an in-situ assay taking the advantage of the indirect sensing the reaction. Since the oxidizing agent $NaIO_4$ is relatively tolerant by esterases, lipases, and phytases, we expect that in the future, the inhibitors against these enzymes may be discovered by using the above coupling strategy and the in-situ assay protocol (29).

OTHER APPROACHES DEVELOPED FOR THE IN-SITU ASSAY

It is interesting to develop novel applications other than the in-situ enzymatic assays. For example, development of an in-situ cell-line based assay would fascinate the medicinal chemists (32, 33). Especially, a compound candidate discovered throughout this screening process fulfills not only the requirement of an adequate fitting to the active sites of a target enzyme but also the qualification of in-vivo stability and preferable pharmaco characteristics or cytotoxicity. Due to these advantages, we are currently developing a compound library comprising a core amino nucleoside ready for coupling with various carboxylic acids for the in-situ screening of their cytotoxicities against Herpes Simplex Virus Thymidine Kinase (HSV) transfected cancer cells in cancer gene therapy (34, 35). Though the desired 4'-hydroxy-4'-amino uridine analog with the characteristics of preferential phosphorylation is not obtainable, the 4',4'-diamino uridine analog may achieve an alternative phosphorylation on one free amine followed by activating the suicide mechanism.
It is proposed that the chemical selectivity between these two amino groups is distinguishable during library construction. An in-vitro cellular assay for a compound library constructed from a core of 5′-amino uridine analog has been established and will be published in due course (36). Besides, another approach of developing the core amino-containing ceramide is also an interesting issue and is currently being under development (37).
In brief, this methodology combining a facile construction of compound library in solution phase and a rapid in-situ bioscreening in microtiterplate has opened a door to a simple approach for drug discovery. Employment of the adequate fluorophore- or chromophore-tagged substrate probes should pave the way for discovering more potential compounds against a vast amount of enzymes. In addition, a screening method by integrating the in-situ cell-line based assay with the solution-phase-derived compound library should be developed in the near future and will see a wide variety of applications.

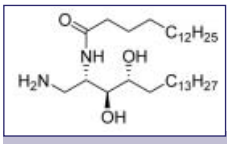

Figure 6. A potential core for screening the immune enhancing compounds.

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