J|A|C|S
COMMUNICATIONS

Published on Web 09/09/2003

Facilitated Forward Chemical Genetics Using a Tagged Triazine Library and Zebrafish Embryo Screening

Sonya M. Khersonsky,[†] Da-Woon Jung,[†] Tae-Wook Kang,[†] Daniel P. Walsh,[†] Ho-Sang Moon,[†] Hakryul Jo,[‡] Eric M. Jacobson,[§] Vivekananda Shetty,[||] Thomas A. Neubert,[||] and Young-Tae Chang*,[†]

Department of Chemistry, New York University, New York, New York 10003, Whitehead Institute, Massachusetts Institute of Technology, Cambridge, Massachusetts 02142, Developmental Genetics Program, Department of Cell Biology, and Department of Pharmacology, Skirball Institute of Biomolecular Medicine, New York University School of Medicine, New York, New York 10016

Received March 26, 2003; E-mail: yt.chang@nyu.edu

Forward chemical genetics is an emerging field that offers powerful tools to search for novel drug candidates and their targets.[1] It differs from classical genetics by substituting small molecules for mutation-inducing agents or X-ray irradiation. Using combinatorial techniques,[2] one is able to rapidly screen a large number of small molecules and identify those that induce a novel phenotype in a cellular or embryonic system. Once a phenotype effect is found, the next step is to identify the biological target using an affinity matrix made of the immobilized hit compound. However, the synthesis of an efficient affinity matrix without the hit compound's activity loss has been shown to be challenging, or sometimes totally impossible, due to the difficulties of adequate linker attachment. In this paper, we demonstrate a novel tagged library approach to accelerate the conversion of a hit compound to an efficient affinity matrix, thus making forward chemical genetics a more systematic strategy (Figure 1).

The design of our tagged library was based on a triazine scaffold due to its ease of manipulation and structural similarity to purine and pyrimidine, which have already been demonstrated to be active in various biological systems.[3] In addition, the triazine scaffold has three-fold symmetry, and the positional modification is much more flexible than that in the purines or pyrimidines. In our previous report, we described an orthogonal solid-phase method to synthesize a triazine-based combinatorial library[4,5] and demonstrated antimicrotubule activities among the library entities.[4] A similar chemistry has been applied to construct a novel tagged triazine library, where three building blocks were prepared separately and assembled orthogonally to yield 1536 highly pure compounds (see Supporting Information). Each library compound contains one of a variety of triethyleneglycol (TG) linkers at one of the diversity sites of the triazine scaffold. Traditionally, selected and modified active molecules, after biological screening, are fitted with a linker, to provide an attachment point to the affinity bead. In many cases, this can lead to activity loss, and thus a time-consuming and laborious structure−activity relationships (SAR) study is required. The incorporation of the linkers, before biological screening, provides for a straightforward method of isolation of the target protein without compromising the lead compound's activity or performing further SAR experiments.

Two possible problems may be envisioned. One is that the linker may interfere with the biological activity and we may miss the active hits. However, this negative selection will be favorable for a researcher, as it will reduce unfruitful efforts to modify the hit compounds later. The other possibility is that the linker itself may play an important role in the biological activity. However, this can be easily probed by detaching or modifying the linker. It should be noted that removing an already existing linker is much easier than adding a new one into the molecule via SAR.

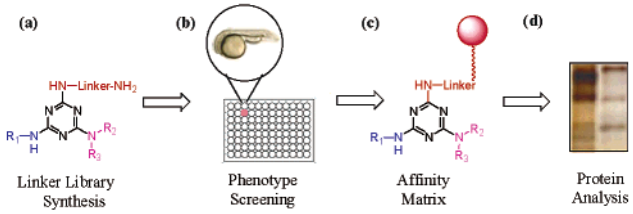

Figure 1. Tagged library approach for forward chemical genetics. (a) Synthesis of the linker library, (b) screening for a novel phenotype, (c) affinity matrix step facilitated by linker, and (d) protein analysis and identification.

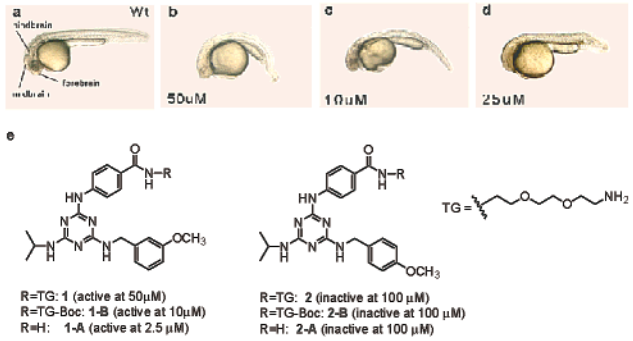

Figure 2. Zebrafish brain and eye morphogenesis upon treatment with derivatives of **1**. (a) Wild-type embryo; (b) 50 μM of **1**; (c) 10 μM of **1-B**; and (d) 25 μM of **1-B** treatment in 8-cell-stage embryos. (e) Structures of derivatives of **1**. The concentration in parentheses indicates MIC.

The library compounds were screened for brain/eye morphological changes in a zebrafish (*Danio rerio*) embryo assay using the 96-well plate format. Zebrafish has proven to be a powerful screening system for forward genetic research because of its small size and high fecundity.[4,6] Among the 1536 triazines we have tested, initially we found one library compound, **1**, to generate significant phenotype changes on zebrafish brain and eyes at 50 μM (Figure 2b). To see if the TG tag and amino end functionality are important for the activity, two derivatives **1-A** and **1-B** were synthesized and tested (Figure 2e). **1-B**, a Boc attached derivative of **1**, was found to be more effective than the mother compound, which shows that the amino end is not critical for the activity. The shape of the head region was flatter and smaller than that of wild-type, and also the eye development was retarded by 10 μM of **1-B** treatment (Figure 2c). At a higher concentration (25 μM) of **1-B**, eyes completely

[†] New York University.
[‡] Massachusetts Institute of Technology.
[§] Department of Cell Biology, New York University School of Medicine.
[||] Department of Pharmacology, New York University School of Medicine.

10.1021/ja035334d CCC: $25.00

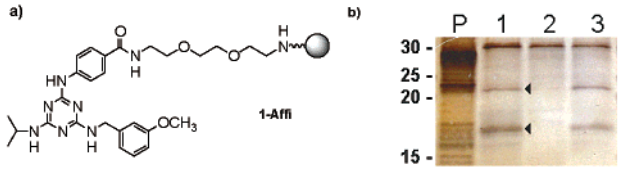

Figure 3. Isolation of **1**-specific proteins by competitive affinity chromatography. (a) Structure of **1**-immobilized agarose bead (**1-Affi**). (b) The proteins bound to **1-Affi**. Lane P: whole protein extract. Lane 1: **1-Affi** alone. Lane 2: **1-Affi** + **1-A** (50 μM). Lane 3: **1-Affi** + **2-A** (50 μM). Protein bands indicated by arrowheads were collected for further analysis.

disappeared (Figure 2d). It was also found that **1-A**, **1** with removed linker (Figure 2e), generated similar phenotype changes with even higher activity (MIC-minimum inhibitory concentration: 2.5 μM). Again, this clearly demonstrates that the TG linker moiety of **1** is not important for the activity. The specific effects of these compounds were confirmed by the inactivity of **2**, which is a regioisomer of **1** with a different methoxy group position (meta vs para). The corresponding derivatives **2-A** and **2-B** were also synthesized and shown to be inactive even at higher concentration (100 μM) and were further used as negative control compounds (Figure 2e).

We also tested **1-B** in zebrafish embryos at different time points: 1 cell (0.2 hpf: hour post fertilization), 8 cell (1.25 hpf), 1K (1000) cell (3 hpf), 50% epiboly (5.3 hpf), and 10 somite (10 hpf, postgastrulation) stages. Almost the same morphological changes were observed at 1, 8, and 1K cell stages, which are all pregastrulation, but not at the 50% epiboly and 10 somite stages (Figure 4, Supporting Information). This demonstrates that the target proteins of **1** play an important role in brain/eye development only before the gastrulation.

To identify target proteins, we immobilized **1** on activated agarose beads (Affi gel 10) to afford **1-Affi** (Figure 3a). After loading of **1**, the remaining active sites of the agarose bead were blocked by ethanolamine. Ethanolamine-only treated agarose beads were employed as a negative control matrix. Freshly prepared protein extracts, from 128 to 1000 cell stages, were loaded on the beads for binding to affinity matrices and gently rotated at 4 °C overnight. After extensive washing with bead buffer, the bound proteins were resolved by 14% SDS-PAGE and detected by silver staining.

Two strong bands (23 and 18 kDa) were identified from the **1-Affi** matrix, which were absent in ethanolamine-only resin. To confirm the specificities of the proteins selectively bound to the **1-Affi**, competition assays were performed using **1-A**, the strongest inhibitor, and **2-A** as a negative control. An addition of 50 μM of **1-A** to **1-Affi** beads caused a dramatic decrease of the two strong protein bands, indicated by arrowheads, but not by **2-A** (Figure 3b). Those two bands were excised from the gel, digested with trypsin, and the peptides were analyzed by LC-MS/MS using a nanoflow HPLC coupled directly to a Q-TOF mass spectrometer. The 23 kDa protein matched the 40S ribosomal subunit protein S5 (*Danio rerio*). Three different proteins were retrieved from the 18 kDa protein bands: 40S ribosomal subunit protein S18 (*Danio rerio*), *Danio rerio* EST (expressed sequence tags) sequence similar to human 40S ribosomal subunit protein S13, and mouse 60S ribosomal subunit protein L28 (Figure 5 in Supporting Information). 40S ribosomal subunit proteins S5, S13, and S18 have proven to have extraribosomal functions related to malignant transformation and development regulation in eukaryotics.[7] Furthermore, an early zebrafish development by insertional mutagenesis demonstrated that S5 mutant has a "small head/eye" and S18 mutation causes CNS necrosis phenotypes including inflated hindbrain or reduced forebrain.[8] Therefore, we suggest that **1** may interfere with the function of a protein complex which includes S5, S13, S18, and L28 and plays an important role for brain/eye development in the early zebrafish embryo.

In conclusion, we have successfully demonstrated the power of a tagged library approach for efficient forward chemical genetics, especially by expediting the connection of a hit compound to the affinity matrix by incorporating a linker directly to the library compounds prior to phenotypic screening. The current study elucidated the first novel small-molecule inhibitors for several ribosomal accessory proteins or their complex as the target, which are important for the early brain/eye development before gastrulation. A further study will be carried out to elucidate the exact binding mode of the small molecules and target proteins. The same tagged library approach will be applied to other biological systems to facilitate the general paradigm of forward chemical genetics.

Acknowledgment. We thank Dr. Victor Livengood and Dr. Yeounjin Kim at the Laboratory of Bioorganic Chemistry, NIDDK, NIH, for the high resolution mass spectrometry measurements. Funding support from Luminogene (www.luminogene.com) is acknowledged.

Supporting Information Available: Full experimental procedures and characterization data (PDF). This material is available free of charge via the Internet at http://pubs.acs.org.

References

(1) (a) Lokey, R. S. *Curr. Opin. Chem. Biol.* **2003**, *7*, 91−96. Tan, D. S. *Nat. Biotechnol.* **2002**, *20*, 561−563. (b) Specht, K. M.; Shokat, K. M. *Curr. Opin. Cell Biol.* **2002**, *14*, 155−159. (c) Schreiber, S. L. *Chem. Eng. News* **2003**, March 3, pp 51−61.

(2) (a) Jung, G. *Combinatorial chemistry: synthesis, analysis, screening*; Wiley-VCH: Weinheim; Cambridge, 1999. (b) Nicolaou, K. C.; Hanko, R.; Hartwig, W. *Handbook of combinatorial chemistry: drugs, catalysts, materials*; Wiley-VCH: Weinheim, 2002.

(3) (a) Chang, Y. T.; Choi, G.; Bae, Y. S.; Burdett, M.; Moon, H. S.; Lee, J. W.; Gray, N. S.; Schultz, P. G.; Meijer, L.; Chung, S. K.; Choi, K. Y.; Suh, P. G.; Ryu, S. H. *Chembiochem.* **2002**, *3*, 897−901. (b) Verdugo, D. E.; Cancilla, M. T.; Ge, X.; Gray, N. S.; Chang, Y. T.; Schultz, P. G.; Negishi, M.; Leary, J. A.; Bertozzi, C. R. *J. Med. Chem.* **2001**, *44*, 2683−2686. (c) Armstrong, J. I.; Portley, A. R.; Chang, Y. T.; Nierengarten, D. M.; Cook, B. N.; Bowman, K. G.; Bishop, A.; Gray, N. S.; Shokat, K. M.; Schultz, P. G.; Bertozzi, C. R. *Angew. Chem., Int. Ed.* **2000**, *39*, 1303−1306. (d) Rosania, G. R.; Chang, Y. T.; Perez, O.; Sutherlin, D.; Dong, H. L.; Lockhart, D. J.; Schultz, P. G. *Nat. Biotechnol.* **2000**, *18*, 304−308. (e) Chang, Y. T.; Gray, N. S.; Rosania, G. R.; Sutherlin, D. P.; Kwon, S.; Norman, T. C.; Sarohia, R.; Leost, M.; Meijer, L.; Schultz, P. G. *Chem. Biol.* **1999**, *6*, 361−375. (f) Gangjee, A.; Yu, J.; Kisliuk, R. L.; Haile, W. H.; Sobrero, G.; McGuire, J. J. *J. Med. Chem.* **2003**, *46*, 591−600. (g) Baraldi, P. G.; Cacciari, B.; Spalluto, G.; Bergonzoni, M.; Dionisotti, S.; Ongini, E.; Varani, K.; Borea, P. A. *J. Med. Chem.* **1998**, *41*, 2126−2133. (h) Baraldi, P. G.; Cacciari, B.; Romagnoli, R.; Spalluto, G.; Monopoli, A.; Ongini, E.; Varani, K.; Borea, P. A. *J. Med. Chem.* **2002**, *45*, 115−126.

(4) Moon, H. S.; Jacobson, E. M.; Khersonsky, S. M.; Luzung, M. R.; Walsh, D. P.; Xiong, W. N.; Lee, J. W.; Parikh, P. B.; Lam, J. C.; Kang, T. W.; Rosania, G. R.; Schier, A. F.; Chang, Y. T. *J. Am. Chem. Soc.* **2002**, *124*, 11608−11609.

(5) Bork, J. T.; Lee, J. W.; Khersonsky, S. M.; Moon, H. S.; Chang, Y. T. *Org. Lett.* **2003**, *5*, 117−120.

(6) Peterson, R. T.; Link, B. A.; Dowling, J. E.; Schreiber, S. L. *P. Natl. Acad. Sci. U.S.A.* **2000**, *97*, 12965−12969.

(7) (a) SaeboeLarssen, S.; Lambertsson, A. *Genetics* **1996**, *143*, 877−885. (b) Wool, I. G. *Trends Biochem. Sci.* **1996**, *21*, 164−165. (c) Saeboe-Larssen, S.; Lyamouri, M.; Merriam, J.; Oksvold, M. P.; Lambertsson, A. *Genetics* **1998**, *148*, 1215−1224. (d) Weijers, D.; Franke-van Dijk, M.; Vencken, R. J.; Quint, A.; Hooykaas, P.; Offringa, R. *Development* **2001**, *128*, 4289−4299.

(8) Golling, G.; Amsterdam, A.; Sun, Z. X.; Antonelli, M.; Maldonado, E.; Chen, W. B.; Burgess, S.; Haldi, M.; Artzt, K.; Farrington, S.; Lin, S. Y.; Nissen, R. M.; Hopkins, N. *Nat. Genet.* **2002**, *31*, 135−140.

JA035334D