Award Review

Introduction of New Tools for Chemical Biology Research on Microbial Metabolites

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Online Publication, June 7, 2010
[doi:10.1271/bbb.100061]

Recently, high-throughput screening (HTS) has become the mainstream technique for drug discovery. Compounds that are synthesized by combinatorial chemistry might be more suitable than natural products to apply to HTS, because the purification procedure is a drawback of using natural products. Nevertheless, natural products remain an extremely important source of drugs. To overcome the demerits of natural products, we are constructing the RIKEN Natural Products Depository (NPDepo) that is focused primarily on microbial metabolites. In this review, I describe (i) engineering pathways for biosynthetic gene clusters of microbial metabolites, (ii) construction of fraction libraries of microbial metabolites, and (iii) the development of a new screening system using a chemical array and a protein library produced by GLORIA.

Key words: microbial metabolites; chemical library; fraction library; chemical array; screening

Chemical biology is directed toward solving the mystery of complex biological systems from the viewpoint of organic chemistry. This is consistent with the concept of agricultural chemistry: “explaining agricultural phenomena on the basis of chemistry.”

One of the achievements of the society is a series of studies on gibberellins isolated from a fungal strain of rice bakanae disease (abnormal rice seedling elongation). This research is the origin of subsequent plant-hormone studies. For another example, the society issued a paper on a fungal metabolite, kojic acid, written by Professor Yabuta, in the first issue of the society’s journal. Natural products chemistry, especially identification of the chemicals that govern important biological phenomena, is a specialty of the society.

As a graduate student, I was interested in antagonism among microorganisms, and studied the mechanisms of colicin E2 activity. After obtaining my degree, I joined the Antibiotics Laboratory at RIKEN. Since then, I have been fascinated by the bioactive products of microorganisms because of their diverse chemical structures and biological activities.

When I started research on microbial metabolites in the 1980s, this field prospered not only in academia but also in the pharmaceutical industry. Many antitumor compounds, cholesterol synthesis inhibitors, and immnosuppressive agents were discovered one after another from microbial metabolites.

Drug screening using microbial metabolites, however, has decreased slightly since the mid-1990s. Back then, pharmaceutical companies adopted high-throughput screening (HTS) methods, and they believed that compounds synthesized by combinatorial chemistry were more suitable for HTS than compounds isolated from microbial metabolites. This trend hampered natural product chemistry. However, I believe that microbial metabolites can be used to screen drug candidates. In this review article, I describe new methods for the utilization of microbial metabolites in drug discovery.

The disadvantage of using natural products as a screening source is the difficulty in supplying many compounds for HTS. To overcome this obstacle, we took three approaches as described below.

I. Pathway Engineering

Recent advances in the biosynthesis study of microbial products have made it possible to generate many kinds of derivatives. Often, the microorganisms that are used to make the products grow slowly and are genetically unstable, in which case it is necessary to clone entire biosynthetic gene clusters and to engineer them. Moreover, it might be necessary to express the gene cluster in other suitable hosts. The heterologous expression makes possible the production of the compound of interest as well as the synthesis of new derivatives. In collaboration with foreign researchers, we have cloned actinomycetes gene clusters of phospholactomycins (PLMs), tautomycin, and RK-682, and a fungal gene cluster of fumitremorgins.

We have found that PLMs are potent and selective inhibitors of serine/threonine phosphatases. Although PLMs are desirable for their antitumor and antiviral activities, difficulties that arise due to the existence of multiple analogs and low titers in the fermentation process have restricted the development of this class of natural products. To circumvent these disadvantages, the entire 75-kb PLM biosynthetic gene cluster of Streptomyces sp. HK-803 was cloned, sequenced, and analyzed (Fig. 1). The loading domain and seven extension modules of the PLM polyketide synthase form an unusual linear, unsaturated polyketide chain that con-
contains E- and Z-double bonds from a cyclohexanecarboxylic acid (CHC) primer. Hydroxylation of the CHC-derived side chain of the resulting PLM-B by plmS2 and subsequent esterification yielded the remaining PLM analogs. The plm2 deletion mutant selectively produced PLM-B at 6-fold higher titers than the wild-type strain. This mutant and the biosynthetic gene cluster should facilitate the engineered microbial production of improved hybrid PLMs. Moreover, we purified a new derivative, deaminohydroxy PLM-B, from the culture broth of the original strain. This derivative inhibited PP2A activity and induced the differentiation of acute myeloid leukemia cells (HL-60) into granulocytes/monocytes. In contrast, known PP2A inhibitors, such as okadaic acid, did not induce HL-60 cell differentiation.

Fumitremorgin C, a diketopiperazine mycotoxin produced by Aspergillus fumigatus, is a potent and specific inhibitor of breast cancer resistance protein (BCRP). We cloned the gene cluster of fumitremorgins of Aspergillus fumigatus and isolated various derivative of the fumitremorgin family (Fig. 2). Initially, we cloned a 27-kb DNA fragment that encompassed the fim gene cluster, containing a dimodular type nonribosomal peptide synthetase gene (ftmA), two prenyltransferase genes (ftmB, ftmH), three P450 genes (ftmC, ftmE, ftmG), and a methyltransferase gene (ftmD). A structure-activity relationship study of the fumitremorgin derivatives and inhibitory activity against BCRP revealed that the cyclization that formed fumitremorgin C was the most critical step for it to exert inhibitory activity. To accumulate the various fumitremorgin derivatives, targeted gene inactivation and heterologous overexpression of the ftm genes were performed. Pathway engineering of the fumitremorgin biosynthetic gene cluster enabled us to generate many derivatives of diketopiperazine and to measure their biological activities.

II. Fraction Library

We collected semi-purified samples of microbial fermentation broth instead of pure compounds systematically and called them the fraction library. This contained all of the eluted fractions from HPLC (Fig. 3). Each fraction was partially purified, and some compounds might have coexisted (over 50% purity expected). All of the fractions were analyzed on an
HPLC-mass spectrometer with a photodiode array system, making it possible to obtain greater amounts of the same sample from the fermentation broth according to the systematic purification.

Before purification of the natural products, we often found that co-existing compounds masked the biological activity of the target compound. The use of fraction libraries can overcome the drawbacks encountered in screening natural products. We are now constructing a database of fraction libraries that stores physicochemical properties (retention time, UV, and molecular weight) and biological activities. Using this database, we should be able to identify the active principal without purification and to avoid isolating duplicates. The fraction libraries are used to screen new drug candidates using a conventional bioassay and a newly developed chemical array method (Fig. 4).

III. Chemical Array

The chemical array technique represents a new platform for drug screening to save microbial products. The principle of chemical array is derived from DNA microarray. If thousands of compounds are arrayed on a slide glass and used on a screening to detect small molecule-protein interactions, chemical array might prove a revolutionary method of screening drugs.

When we began our study of chemical array, researchers at Harvard University had launched a similar study. They used diazobenzilidene-functionalized glass slides to capture acidic compounds, such as phenols. However, we wanted to develop a new method to capture a small molecule of any type on a slide glass without depending on the functional group. Although small molecules can be attached to the glass slide, part of the small molecule is used to link it to a spacer. If the binding protein recognizes the portion that is used for linking, the binding protein cannot bind to the small molecule on the glass slide. Because it is difficult to predict the binding site between small molecules and unknown proteins, this is a disadvantage, especially in attempting to discover small molecules for new target proteins by chemical array.

To overcome these drawbacks, we developed a non-selective universal coupling method to introduce small molecules onto a glass slide. The protocol is summarized in Fig. 5. Small-molecule solutions are first printed on a glass slide, that is coated with photoreactive linkers. Then the slides are dried to remove the solvent used in the printing process. Irradiation of the slides activates photoreactive groups, generating highly reactive carbenes into which small molecules can be inserted or to which they can be added. Immobilization should occur independently of functional groups. Using this method, some of the immobilized small molecules are expected to retain their affinity toward the possible binding proteins. If the slide is incubated with a fluorescent labeled protein, we can detect interactions between proteins and small molecules.

To screen many combinations of compounds and human proteins, Bradner et al. established a robust screening method using cell lysates. We modified their method and developed a unique, systematic ligand-screening strategy. The platform consists of a chemical array that bears compounds deposited in the Natural Product Depository (NPDepo) and the protein library generated from the Gene Library of the Osaka Laboratory of RIKEN for chemical array analysis (GLORIA). To use GLORIA for our chemical array system, we cloned 100 genes and engineered them to
express a fusion between red fluorescent protein (RFP) and the protein of interest. HEK293T cells were transfected with vectors that encoded RFP-fused proteins or RFP alone, and cell lysates were prepared. We used cell lysates instead of purified proteins for HTS because (i) cell lysates are easily and quickly prepared, and (ii) the target proteins must be folded in their natural conformation and modified properly.

To eliminate false positive signals on the array, merged display analysis was performed. First the positive signals on the glass slides after incubation with cell lysates that expressed RFP-conjugated proteins of interest and RFP-alone were colored red and green respectively on a computer. Next, the colored figures were merged into one map, which highlighted false positive signals in yellow, caused by the binding of ligands to RFP or by autofluorescent signals of the ligand. We reasoned that the results of this method should yield only real-hit compounds, and not false positive signals (Fig. 6).

Proteins that are submitted to GLORIA have various functions, such as transferase activity, hydrolase activity, and lyase activity, and they include nonenzyme proteins. For example, transferases contain protein kinases, which include clinical therapeutic targets (cf. aurora kinase B, and MEK1). Although many proteins are expressed in their full length, some were designed to be truncated, such as membrane proteins. I would like to emphasize the merit of using HEK293T cells. Many post-translational modifications exist in the proteins encoded in GLORIA, but such modifications do not occur when these proteins are expressed by E. coli. Post-translational modifications are important for the proper function and conformation of the proteins. Due to these
properties, the proteins in GLORIA are useful for our chemical array method.

I would like to highlight one example from GLORIA, carbonic anhydrase II (CAII), a ubiquitous zinc protein present in prokaryotes and eukaryotes. It regulates crucial physiological processes, such as glaucoma pathogenesis, through CO$_2$ hydration. Acetazolamide is a CAII inhibitor used as a clinical drug for glaucoma and seizure. But its side effects, such as depression and lowered levels of consciousness, necessitate a search for new CAII inhibitors.

To obtain novel ligands for CAII, we spotted approximately 10,000 compounds from NPDepo on five glass slides and prepared cell lysates from HEK293T cells that expressed RFP-fused CAII. After merged display analysis, we identified two hit compounds. To test the specificity of 1 and 2 for CAII, we prepared cell lysates from HEK293T cells that expressed 33 RFP-fused proteins, including CAII, from GLORIA. Among the 33 proteins tested, only the cell lysate that expressed RFP-fused CAII interacted with 1 and 2, suggesting that 1 and 2 are selective inhibitors of CAII.

To evaluate the binding affinities of 1 and 2 toward CAII, isothermal calorimetry (ITC) analysis was performed. Our results indicate that the Kd values of 1 and 2 for CAII were 115 nM and 324 nM respectively. Moreover, 1 and 2 showed strong inhibitory activity, equaling that of acetazolamide. In contrast, the sulfonamide group was masked in 3, which did not bind to the protein in the chemical array experiment and possessed weak inhibitory activity (Table 1). Substitution of the sulfonamide moiety at the meta-position of the phenyl ring resulted in a loss of potency against CAII as compared with hit compounds in which the sulfonamide moiety was at the para-position. Based on these observations, several derivatives were synthesized, and a new derivative, 4, showed the most potent inhibitory activity against CAII.

In summary, our screening method makes possible large-scale chemical array screening with a combination of a gene library (GLORIA) and a chemical library in NPDepo. To identify useful ligands against human proteins, we have increased the numbers of genes in GLORIA and the compounds in NPDepo, which should dissolve one of the bottlenecks in our quest for useful tools for modern chemical biology studies and drug discovery research.

### Table 1. CAII Inhibitors

<table>
<thead>
<tr>
<th>Name</th>
<th>Structure</th>
<th>Kd (nM)</th>
<th>IC$_{50}$ (nM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
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<td>24</td>
</tr>
<tr>
<td>2</td>
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<td>324</td>
<td>50</td>
</tr>
<tr>
<td>3</td>
<td><img src="image3.png" alt="Structure 3" /></td>
<td>NT</td>
<td>5400 ± 67</td>
</tr>
<tr>
<td>4</td>
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<td>66</td>
<td>6.8</td>
</tr>
<tr>
<td>Acetazolamide</td>
<td><img src="image5.png" alt="Acetazolamide" /></td>
<td>NT</td>
<td>34</td>
</tr>
</tbody>
</table>

Compound 4 was newly synthesized, based on structure-activity relationship research. Kd values were determined by measurement with an isothermal colorimeter. IC$_{50}$ values were determined by enzymatic reaction.

GLORIA and the compounds in NPDepo, which should dissolve one of the bottlenecks in our quest for useful tools for modern chemical biology studies and drug discovery research.

### IV. Future Prospects

We succeeded in developing a number of bioprobies. Recent progress in genomic science makes it possible to understand the structures and functions of genes and proteins, but there are still many unresolved problems, such as protein-protein interactions in cells. To elucidate this obscure mechanism, we intend to develop new inhibitors of protein-protein interactions. The chemical array platform, for example, is suitable for the screening of ligands for non-enzymatic proteins.
Although there are many convenient methods of identifying enzyme inhibitors based on their enzymatic activity, there are few efficient methods to find inhibitors of the protein-protein interaction. In this case, the chemical array makes it possible to detect the direct binders of the target proteins in a high-throughput manner. The development of new bioprobes to investigate unsolved biological phenomena should be a priority pursuit.26)

We would like to determine the reason microorganisms produce a variety of useful compounds called secondary metabolites. The latest advancements in genome research make it possible to challenge this as yet unsolved mystery. Now everybody can easily clone the biosynthetic gene clusters responsible for the production of secondary metabolites and analyze the regulatory mechanisms of the production process. Our future goal is to develop a system to awaken sleeping gene clusters and produce new bioprobes for potential drug discovery.

We intend to pay much attention to drug discovery because bioprobes are potential candidates for therapeutic medicines.

Acknowledgments

I would like to thank Dr. Teruhiko Beppu for his work as an excellent mentor since I was an undergraduate student, and Dr. Kiyoshi Isono for giving me continuous encouragement to pursue chemical biology research. And I would like to thank my collaborators in RIKEN, and express my sincere thanks to Dr. Naoki Kato, Dr. Yasuyo Sekiyama, Dr. Siro Simizu, Mr. Isao Miyazaki, Dr. Naoki Kanoh, Dr. Yasutomo Kondo, Dr. Tamio Saito, Dr. Toshihiko Nogawa, and all the members of my laboratory for their excellent work, described in this article. I am grateful to Dr. Kevin Reynolds, Dr. Ben Shen, Dr. Peter Leadlay, and Dr. James M. Cook for their collaboration. Financial support was provided in part by the Target Protein Research Program (MEXT), Grants-in-Aid for Scientific Research from Japan Society for the Promotion of Science, and from the Basic Science Research Project of RIKEN.

References