QSAR analysis of the behavior of such analog-containing RNAs can reveal chemical principles that are not otherwise obvious. Future progress toward total synthesis of proteins containing nonnatural residues will undoubtedly yield similarly surprising insights into the function of protein enzymes.

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Finding Cinderella after the Ball: A Three-Hybrid Approach to Drug Target Identification

A major bottleneck in drug discovery is identifying the targets of small molecules. The yeast three-hybrid assay extends the two-hybrid approach to screen for protein-small molecule interactions. In this issue of *Chemistry & Biology*, GPC Biotech reports the first application of this promising assay [11].

Drug developers face a problem similar to that of the Prince in the Cinderella story. They have a small molecule that produces the desired physiological response, but they do not know the protein target to which the small molecule binds, the Cinderella who fits the glass slipper. Traditionally, the protein targets of small molecule ligands have been identified using in vitro methods such as affinity chromatography and photoaffinity labeling. The ligand is derivatized so that it can be linked to a resin or photoreactive group. The modified ligand is then incubated with a crude cell lysate, and the labeled protein is finally identified by N-terminal sequencing. These methods have been integral to target discovery, but they are laborious and subject to low protein expression levels, protein degradation during cell lysis, or insufficient affinity for the small molecule ligand.

Genomics and proteomics are beginning to provide real alternatives to these traditional methods for drug target identification. Expression profiling using DNA microarrays enabled the identification of the microbial protein and pathway targets for isoniazid, the primary drug used to treat tuberculosis [1], and potential mechanisms of resistance. A complementary approach using a "synthetic lethal" small molecule screen allowed for the identification of proteins and pathways in yeast that are involved in DNA synthesis and repair [2]. Borrowing from a traditional genetic approach, the small molecules camptothecin and hydroxyurea were screened for lethality against a library of single-knockout mutants in yeast. Uncharacterized open reading frames involved in DNA repair were then inferred by comparing the small molecules' patterns of lethality to those of all double mutants. Alternatively, Snyder and coworkers used protein chip technology to print the >6000 proteins found in *S. cerevisiae* on glass slides and assay them for binding to phospholipids [3].

In 1996, Licitra and Liu extended high-throughput yeast two-hybrid methods to small molecule target identification [4]. In the two-hybrid assay, protein-protein interactions are detected as reconstitution of a transcriptional activator from its DNA binding (DBD) and activation domains (AD) [5]. The assay can be run on a genome-wide scale by creating a library of activation domain-cDNA clones [6]. The three-hybrid assay extends this approach to small molecule-protein interactions by dimerization of two receptor proteins via a bridging heterodimeric ligand [7] (Figure 1). One ligand-receptor pair serves as an anchor, while the other ligand-receptor pair is the small molecule-protein interaction of interest. As a proof of principle, Licitra and Liu used a dexamethasone-FK506 heterodimer to isolate FK506-binding protein 12, the known target of FK506, from a Jurkat cDNA library. This approach allows the cDNA clones to be expressed at uniformly high levels and without the need for purification, and the target protein's identity to be simply read out from the cDNA-AD sequence at the end of the selection. Despite obvious applications for drug discovery, however, there were no further reports of using the three-hybrid assay for target discovery beyond this proof of principle experiment.

One thought was that the affinity of the ligand-recep-

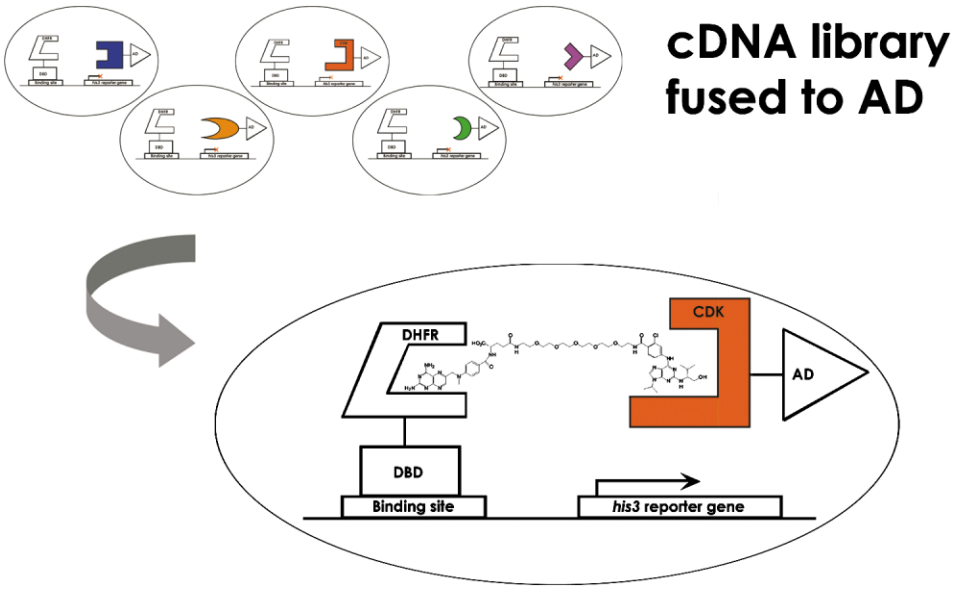

Figure 1. Three-Hybrid Assay

The three-hybrid assay offers a potentially powerful tool for drug target identification. Two- and three-hybrid assays detect protein interactions based on reconstitution of a transcriptional activator from a DNA binding (DBD) and activation (AD) domain. The three-hybrid assay extends this approach to small molecule-protein interactions by dimerization of two receptor proteins via a heterodimeric ligand. For drug target identification, as illustrated here, one ligand-receptor pair (methotrexate and DHFR) serves as the anchor, while the other is the drug target of interest (PurB and CDK). The heterodimeric ligand (Mtx-PurB) can then be screened against a library of AD-cDNA clones. The reporter gene will only be activated in cells expressing a cDNA to which the drug (PurB) binds.

tor anchor pair (dexamethasone-glucocorticoid receptor in Licitra and Liu [4]) was limiting the sensitivity of the three-hybrid assay and hence its ability to detect a variety of ligand-receptor interactions. Thus, several groups developed ligand-receptor anchors intended to increase the sensitivity of the assay. Our laboratory developed an anchor based on the low picomolar interaction between methotrexate and dihydrofolate reductase [8]. Peterson has now developed anchors based on estradiol-estrogen receptor and a mutant biotin-streptavidin [9]. Most recently, Johnsson and coworkers reported a covalent anchor based on a nucleotide analog and a DNA repair enzyme [10].

In this issue of *Chemistry & Biology*, Becker et al. report the first successful application of the three-hybrid assay to discover novel drug targets, specifically, targets of cyclin-dependent kinase (CDK) inhibitors [11]. Using roscovitine (CYC202, currently in phase I/II clinical trials), Purvalanol B, and indenopyrazole linked to methotrexate, they isolate not only known CDK targets of these drugs but also several additional kinases as potential new targets. These new targets may be contributing to the efficacy of these CDK inhibitors or represent crossreactivity and potential toxicity. Further, a subset of these novel targets is characterized in vitro for validation. Their study establishes that the three-hybrid assay can be used to detect novel targets with interactions as weak as low micromolar. As such, this work represents an important step toward making the three-hybrid assay a real tool for drug discovery. What remains is to show that diverse classes of ligands and receptors are compatible with this method and to further understand how the transcription readout relates to the strength of the ligand-receptor interaction. Drug discovery happens not through a single technique, but by bringing together a host of techniques to bear on the problem. Perhaps the three-hybrid assay can add to the existing methodologies and speed the search for Cinderella.

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What Makes Epothilones Stick?

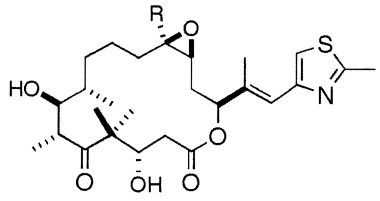
The potent epothilone tubulin polymerization promoters have been studied extensively by synthetic and SAR approaches. The paper by Buey et al. ([13], this issue of *Chemistry & Biology*) adds a new depth of mechanistic understanding by a careful analysis of the tubulin polymerization mechanism of the epothilones.

The discovery in 1979 of the ability of paclitaxel (Taxol) to promote the polymerization of tubulin heterodimers to microtubules [1] marked a turning point in the development of this compound as an anticancer agent, and it went on to become a blockbuster drug. Paclitaxel's success initiated a search for other anticancer agents that operate by the same mechanism, and several compounds that do this have been found, including discodermolide [2], eleutherobin [3], and several other natural products. Of all the "paclitaxel-like" compounds found to date, however, the epothilones have excited the most interest. Epothilones A (Figure 1[1]) and B (Figure 1[2]) were originally isolated as antifungal agents, but scientific interest in them was enormously stimulated by the discovery of their tubulin-polymerization activity in 1995 [4] and by the publication of their complete structure and stereochemistry [5]. Biological studies of the epothilones have shown that they stabilize microtubules in the same way that paclitaxel does but with somewhat higher potencies. Not only do they act as tubulin-polymerization agents in the same way that paclitaxel does, but they also compete for the same binding site on the polymer, since they act as competitive inhibitors of the binding of [^{3}H]paclitaxel to tubulin polymers [6]. In addition, epothilone B has been shown to be superior to paclitaxel in treating vinblastine-resistant CCRF-CEM tumors in the mouse [7], and desoxyepothilone B is curative against paclitaxel-resistant CCRF-CEM tumors [8]. Several total and partial syntheses of the epothilones and their analogs have been reported, and extensive SAR studies have been carried out. Their chemistry and biology have been reviewed, with two important reviews by one of the coauthors of the present paper [9, 10]. Epothilone B is the most potent antiproliferative agent among the naturally occurring tubulin polymerization promoters, with an activity from 2- to 10-fold greater than that of paclitaxel, and it is less susceptible than paclitaxel to Pgp- mediated MDR [11]. Epothilone B is in phase II trials by Novartis, and its lactam analog BMS-247550 is in phase II/III trials by Bristol-Myers Squibb. Deoxyepothilone B (Sloan-Kettering/Kosan/Roche) and C21-amino epothilone B (BMS) have also entered clinical trials [12].

Previous authors have made extensive studies of the SAR of the epothilones, as summarized in the reviews cited [9,10]. The study by Buey et al. [13] goes beyond these studies to provide an in-depth look at why certain structural modifications result in increased activity and why others give reduced activity. The authors do this by a careful analysis of the binding affinity of epothilone analogs to microtubules and by correlating this affinity with cytotoxicity. This paper is thus the first in which the microtubule binding of epothilones has been studied at a fundamental level.

The major problem with studies of the interaction of paclitaxel and paclitaxel-like compounds with tubulin is that they are too good at what they do. They bind to the polymerized form of tubulin and immediately induce further assembly so that it is not possible to study tubulin binding apart from microtubule assembly; the two reactions are inseparably linked. The present authors have previously developed a way around this difficulty by the use of microtubules stabilized by gentle crosslinking and by the use of fluorescent paclitaxel probes. These conditions allow the study of the ligand-microtubule interaction at low enough concentrations to obtain direct measurements of the binding affinity, and nonfluorescent ligands can be studied by competition with the fluorescent probes.

In the present study, the authors applied these methods to the study of the binding of epothilones A and B and various epothilone analogs to microtubules. Direct binding constants were calculated, and the reactions were carried out at various temperatures so that ΔH and ΔS values could be calculated for each ligand. The binding affinity results are presented graphically in a



1 R = H Epothilone A
2 R = Me Epothilone B

Figure 1. Epothilones A and B
Structures of Epothilone A and Epothilone B.