

A new paradigm for drug discovery in tropical rainforests

Todd L. Capson, Phyllis D. Coley, and Thomas A. Kursar

Tropical rainforests contain one-half to two-thirds of the world's flowering plants and should be a particularly rich source of pharmaceutical agents¹. In comparison with plants from temperate regions, rainforest plants are subject to greater levels of herbivory and disease, and have therefore evolved both higher levels and a greater diversity of defensive compounds²⁻⁴. It is estimated that less than 1% of rainforest plants have been studied for their pharmaceutical potential⁵.

Recent developments are changing markedly the nature of drug discovery programs in which resources from biologically rich developing nations primarily have benefited developed countries⁶. Because of the Convention on Biological Diversity, signed in Rio de Janeiro in 1992, as well as a greater appreciation of the value of biodiversity, the issues of ownership of and access to genetic resources have received greater attention. The 150 nations that signed the convention recognized that biological diversity is a sovereign national resource, and that both suppliers and receivers should benefit economically.

Here we describe a model for drug discovery that incorporates a number of innovations that may prove broadly applicable to other programs. With the support of the government and local scientists, we are carrying out most of the drug discovery process in Panama.

Our program incorporates the following features. First, we are using ecologically informed searches for plants with biologically active compounds. Our searches are based on ecological hypotheses that we have developed over the past twenty years of research into the chemical defenses of rainforest plants. Second, we extract compounds from fresh rather than dried plant samples to increase the yields of active compounds. Third, plant extracts and purified compounds are assayed in Panama for activity against a variety of diseases important to both developed and developing nations.

Young leaves from rainforest plants are a promising source of biologically active compounds. Drug discovery programs in the tropics often focus on readily accessible mature leaves. The primary defenses of mature leaves

are tannins and toughness, non-specific defenses that are of little therapeutic value. Our results suggest that young, expanding leaves have higher concentrations and a greater diversity of chemical defenses than do mature leaves. Furthermore, analysis of the developmental traits of leaves reveals that simple characters may serve as good field indicators of chemically well-defended species⁷. As well-defended species are not related taxonomically, they may also provide a diversity of novel compounds.

We have also found that sample preparation is crucial for the detection of biologically active compounds. For expanding young leaves, antibacterial activities were 50% higher for extracts from fresh versus air-dried leaves. In mature leaves, drying did not affect the activity. Presumably the biologically active compounds that were sensitive to air-drying in the young leaves are either absent or in negligible quantities in mature leaves. Hence, by using ecological insight to target searches and by extracting fresh samples, we expect an increased yield of diverse, biologically active compounds.

As are many advantages to a drug discovery program based in the source country. Conventional plant-based drug discovery programs often call for kilogram quantities of plant material that are dried and shipped to laboratories distant from the site of collection. In our plant collection strategy, fresh, expanding leaves are extracted and stored within hours after harvesting. Initially, we need to collect only gram quantities of each species because the recollection of samples with biological activity is easily accomplished.

As we rely on vegetative identification of the plant species, recollections can be made at any time of year. The facile recollection of interesting plants vastly minimizes the serious difficulties that many Northern-based drug discovery efforts encounter during recollection. Although young leaves are both smaller and less abundant than mature leaves, the higher concentrations of compounds in young leaves facilitates extraction and purification. For example, ampelopsin (dihydromyricetin), is an alkaloid found in the rainforest tree, *Licania platypus*. In expanding leaves, ampelopsin is surprisingly abundant, comprising about 14% of the dry weight, but is present in only trace amounts in the mature leaves.

The selection of assays used to detect biological activity in plant extracts and purified compounds can be varied to accommodate the facilities at hand. Straightforward assays based

on brine shrimp lethality, antibacterial and antifungal activity are widely used⁸⁻¹⁰. More complex assays require tissue-culture facilities, such as sterile hoods and autoclaves. Even for the latter assays, the infrastructure and expertise requirements are modest.

We are currently collaborating with three Panamanian institutions involving five different laboratories. Plant extracts will be assayed in Panama for a wide variety of activities, including anticancer, antibacterial, antifungal and antiviral activity. Local health concerns will be addressed with assays to detect activity against diseases such as dengue. The assays will then be used in activity directed purification. At present, some components of the project, such as structural elucidation and mechanism-based assays, will be accomplished in our laboratories in Utah. Benefits to the source country generate incentive for rainforest preservation. The provision of royalties to the host country is frequently cited as a means of providing incentive for rainforest preservation⁶. In reality, the possibility of royalties may not provide sufficient incentive for the promotion of conservation. As many as 10,000 compounds are screened for every commercially valuable pharmaceutical agent that is discovered⁶. A more likely guarantee for a lasting host-country benefit is the enhancement of host country infrastructure through education, technology transfer, and the provision of specialized equipment. Panama has a large pool of highly qualified scientists and students able and willing to participate in a drug discovery program. We provide salary and tuition for graduate study in Panama for the students involved in our program. By helping provide the capability for a permanent host country drug discovery program, the prospects for the eventual discovery of novel, commercially viable pharmaceutical agents are greatly enhanced.

By carrying out a highly directed search for bioactive compounds and working with extant facilities and research programs, we optimize the use of personnel, materials and financial resources. We are working closely with governmental and nongovernmental institutions to ensure that our program is consistent with local conservation efforts. By maximizing the degree of source country involvement, the connection between drug discovery and rainforest preservation will become more apparent in developing and developed countries, and provide a more effective means of promoting conservation.

Todd Capson, Phyllis Coley, and Thomas Kursar are at the department of Biology, University of Utah, Salt Lake City, UT 84112. Thomas Kursar is also at the Smithsonian Tropical Research Institute, Apartado 2072, Balboa, Republic of Panama.

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Nature Biotechnology
345 Park Avenue South
New York, NY 10010-1707, USA
Fax: +1 212 696 9635
E-mail: m.francisco@natureny.com

Abundance and competition in PCR

To the editor:

It is interesting to note that while there is an awareness that the abundance distribution of mRNAs within a cell creates a problem for the sequencing of all cDNAs, there does not seem to be an awareness of the problems that abundance distribution creates for displaying lower abundance mRNAs in differential display PCR^{1,2}. These problems are created by competition in PCR, whereby more abundant templates compete for reagents with less abundant ones such that the products from less abundant mRNAs are not detected³. This problem is particularly acute because many RNA species are rare and are present at ~0.004% of total mRNA.

Using computer simulations, López-Nieto and Nigam suggest that the use of 30 designed primers (in 370 primer combinations) allows the detection of about 75% of known human protein coding regions. However, their computer simulation does not take into account the effect of competition in PCR. The effect of competition will be to reduce the percentage of mRNAs represented by their method because a proportion of the PCR products predicted by their computer program will not be visible on the display gels. The severity of the competition can be ascertained by comparing the number of PCR products calculated in their computer simulation with the number of bands actually visible. The computer simulation shows how the use of these 30 primers gives an average of ~5 PCR products per mRNA. Therefore, using all of the 870 primer combinations on the mRNAs from a mammalian cell should produce ~56,000 bands (15,000 x 0.75 x 5) or ~65 bands per primer combination. Their figure of

three typical displays (Fig. 4B) shows that the number of PCR products actually visible per primer combination is about 30 (excluding closely grouped bands that represent a single PCR product⁴). Therefore, competition in PCR seems to be responsible for the loss of about half of the PCR products predicted in their computer model. It is worth noting that this estimate of “lost PCR bands” is conservative, because the assumption of high stringency in PCR for the computer simulations, an assumption which López-Nieto and Nigam themselves recognize will not be realized in real PCR.

In summary, although the method described by López-Nieto and Nigam seems to be a large improvement on standard differential display, it is probable that they have made a considerable overestimate of the percentage of mRNAs that can be displayed using combinations of 30 primers.

Ursula H.A. Schlichter David Bertioli
Max-Planck-Institut für Area de Biocologia
Züchtungsforschung CENARGEN/EMBRAPA
Köln, Germany 70770-Brasília DF, Brazil

López-Nieto and Nigam reply:

Competition in PCR when multiple products are being amplified is an important issue. As pointed out in the second-to-last paragraph of our paper¹, the ability to amplify a message is a function of the message abundance and specificity of primer-template interactions. Bertioli et al.² have shown one of the problems of standard differential display is a limited ability to detect rare messages due to competition. They also argue that 90–95% of all messages fall into this category. From our published gel, they suggest that we are detecting slightly less than half the predicted number of bands. Thus, it follows that we must be detecting a significant number of rare messages.

Of course, these kinds of arguments have obvious limitations, and directly extrapolating from their study to ours is difficult. Our method, based on coding-region selective oligonucleotides, uses longer primers and higher annealing temperatures, so we antici-

pate that more specific interactions between templates and primers would improve the detection rate of rare messages. It should also be emphasized that the roughly 30 bands per lane they have counted were from experiments with human primers, the conditions for which we did not put the same effort into optimizing (for reasons described in the paper).

The number of bands obtained per primer set may turn out to be greater under more stringent PCR conditions. Under optimal priming conditions, products for abundant messages are likely reach plateau levels, while the amplification of products for rare messages may continue. In our experiments, we have seen gel bands (PCR products) not present under low stringency conditions that start to appear as the PCR annealing temperature is increased. This finding suggests that, while competition under low stringency (similar to conditions used in differential display) may limit the ability to detect rare messages, as annealing temperature increases, message abundance may be less of an issue relative to priming specificity, and, as a consequence, messages that were previously below the limit of detection can be effectively amplified. That said, the actual degree to which competition and message level limit the gene detection rate with our method remains to be established experimentally.

Carlos López-Nieto and Sanjay K. Nigam
Brigham and Women's Hospital/
Harvard Medical School, Cambridge MA

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Erratum

Collaborative Laboratories was incorrectly identified as filing a \$40.5 million IPO in the August issue (*Nature Biotechnology* 14:930, 1996). The correct company is Collaborative Clinical Research, Inc.