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An International Collaborative Program To Discover New Drugs from Tropical Biodiversity of Vietnam and Laos

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Abstract – An International Cooperative Biodiversity Group (ICBG) program based at the University of Illinois at Chicago initiated its activities in 1998, with the following specific objectives: (a) inventory and conservation of of plants of Cuc Phuong National Park in Vietnam and of medicinal plants of Laos; (b) drug discovery (and development) based on plants of Vietnam and Laos; and (c) economic development of communities participating in the ICBG project both in Vietnam and Laos. Member-institutions and an industrial partner of this ICBG are bound by a Memorandum of Agreement that recognizes property and intellectual property rights, prior informed consent for access to genetic resources and to indigenous knowledge, the sharing of benefits that may arise from the drug discovery effort, and the provision of short-term and long-term benefits to host country institutions and communities. The drug discovery effort is targeted to the search for agents for therapies against malaria (anti-malarial assay of plant extracts, using *Plasmodium falciparum* clones), AIDS (anti-HIV-1 activity using HOG.R5 reporter cell line (through transactivation of the green fluorescent protein/GFP gene), cancer (screening of plant extracts in 6 human tumor cell lines - KB, Col-2, LU-1, LNCaP, HUVEC, hTert-RPE1), tuberculosis (screening of extracts in the microplate Alamar Blue assay against *Mycobacterium tuberculosis* $H_{37}Ra$ and $H_{37}Rv$), all performed at UIC, and CNS-related diseases (with special focus on Alzheimer's disease, pain and rheumatoid arthritis, and asthma), peformed at Glaxo Smith Kline (UK). Source plants were selected based on two approaches: biodiversity-based (plants of Cuc Phuong National Park) and ethnobotany-based (medicinal plants of Cuc Phuong National Park in Vietnam and medicinal plants of Laos). At UIC, as of July, 2001, active leads had been identified in the anti-HIV, anticancer, antimalarial, and anti-TB assay, after the screening of more than 800 extracts. At least 25 biologically active compounds have been isolated, 13 of which are new with anti-HIV activity, and 3 also new with antimalarial activity. At GSK of 21 plant samples with a history of use to treat CNS-related diseases tested to date, a number showed activity against one or more of the CNS assay targets used, but no new compounds have been isolated. The results of the drug discovery effort to date indicate that tropical plant diversity of Vietnam and Laos unquestionably harbors biologically active chemical entities, which, through further research, may eventually yield candidates for drug development. Although the substantial monetary benefit of the drug discovery process (royalties) is a long way off, the UIC ICBG program provides direct and real-term benefits to host country institutions and communities.

Keywords – ICBG; Vietnam; Laos; Drug discovery; AIDS; Cancer; Malaria; Tuberculosis; CNS diseases; Plants; Biodiversity; Medicinal Plants

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Introduction

The interrelationship between drug discovery, economic growth and conservation of biodiversity is the philosophical foundation of a program called ICBG (International Cooperative Biodiversity Groups) funded by the US Government in partnership with the industry, established in 1993 and administered through the Fogarty International Center (FIC), National Institutes of Health (NIH), USA (Suffness *et al.*, 1995; Rosenthal, 1999; Rosenthal *et al.*, 1999). As a result of the first 5-year cycle of funding competition in 1992, awards were made to five research groups in 1993. The second 5-year cycle was re-competed in 1997, with funding awards announced in mid-1998, and six research groups (three incumbents, three new groups) began their operation on October 1, 1998. An ICBG based at the University of Illinois at Chicago (UIC), referred to as UIC ICBG, with member-institutions located in Vietnam and Laos, and with an industrial partner located in the UK, is one of these six second-generation ICBGs. Program goals, organization details and philosophy, and research plan of UIC ICBG have been presented in a previous paper (Soejarto et al., 1999) and will be mentioned in the present paper only where relevant to the discussion on hand.

The specific aims of the UIC ICBG are : **(a)** to produce a documented inventory of tropical forest plant diversity of Vietnam and Laos, specifically, the seed plants of the Cuc Phuong National Park (Vietnam), and the medicinal plants of Laos; **(b)** to discover novel, biologically active molecules from plants of Vietnam and Laos as possible candidates for drug development for the treatment of malaria, viral (including AIDS), CNS-related diseases, cancer, and tuberculosis; **(c)** to improve the standard of living of members of the communities who participate in the ICBG studies, through community education and biomass production of plants that have been identified as having promising economic potential, in particular medicinal potential, and to support the development of human resources and the institutional strengthening of research facilities of colleagues at host countries in Vietnam and Laos.

In order to achieve the three-pronged goals of UIC ICBG, five program components, called Associate Programs 1-5 (AP1-5), work in close collaboration under the coordination of a Group Program (Central Operations Office) based in Chicago and directed by the UIC ICBG Principal Investigator (D.Doel Soejarto). Each Associate Program is led by a Project Leader. AP1 (based at Cuc Phuong National Park/CPNP, Vietnam; Soejarto, Project Leader) has the responsibility of carrying out plant collection and inventory and of improving biodiversity conservation strategy at the park. AP2 (based at Traditinal Medicine Research Center/ TMRC, Laos; Boun Hoong Southavong, Project Leader) has the responsibility of carrying field ethnobotanical and laboratory studies and conservation of medicinal plants of Laos, and of promoting economic development of communities where UIC ICBG project operates. AP3 (based at the Program for Collaborative Research in the Pharmaceutical Sciences/PCRPS-UIC, Chicago; John M. Pezzuto, Project Leader) has the responsibility of carrying out biological assays in cancer, AIDS, malaria, and tuberculosis disease targets, and of isolating, identifying and elucidating the chemical structures of compounds responsible for biological activity in the target diseases. AP4 (based at Institute of Biotechnology/IBT, Vietnam; Le Thi Xuan, Project Leader) has the responsibility of carrying out ethnobotanical field studies, determining the market potential of medicinal plants of Cuc Phuong, propagation of promising species, and economic development of communities who take part in the ICBG project. AP5 (based at Glaxo Smith Kline/GSK, formerly Glaxo Wellcome/GW, in Stevenage, UK; Melanie J. ONeill, Project Leader) has the responsibility of carrying out biological assays of samples with ethnomedical use to treat CNS-related diseases, in assays to detect activities against Alzheimers disease, rheumatoid arthritis, asthma, and pain, and to follow up development of promising compounds generated by either the UIC group (AP3) or by Glaxo group (AP5 itself). The complex interactions between these Associate Programs are described in a previous paper cited above (Soejarto *et al.*, 1999).

The present paper deals only with the drug discovery aspect of this UIC ICBG, but *specifically*, with the drug discovery work of Associate Program 3 based in Chicago, as the primary site, and in Hanoi (Institute of Chemistry/ ICH of the National Center for Science and Technology/ NCST), as the secondary site.

Issues on Property/Intellectual Property Rights and Benefit Sharing

As its operating principles, the UIC ICBG recognizes ownership of the genetic material by the host countries in Vietnam and Laos, and traditional medical knowledge held by the people or individual members of the communities, in line with the terms of the UN Convention on Biological Diversity (http://www.biodiv.org).

Memorandum of agreement/MOA – In the functioning of UIC ICBG, a Memorandum of Agreement (MOA) signed [last signature was affixed on June 28, 1999] by UIC, NCST, CPNP, TMRC, and GSK, binds these five

member-organizations to work together to achieve the specific aims of the program. This 5-way MOA defines the obligations of each party and their joint obligations. These include issues on property rights and intellectual property rights for plant genetic material to be used in the studies, within the framework of the ICBG. All members agree that property of the plant genetic material (plant species) belongs to the country where that genetic material originates, and that any discovery or invention that may arise as a result of research under the framework of the ICBG, and new technology to be developed based on this genetic material should be protected, and that the benefits of the discovery be shared in an equitable manner between the participating members. Benefit-sharing also includes the assignment of authorship of publications and new technology that may result from the ICBG's efforts. GSK, however, waives its rights to any share of monetary benefits that may result from a royalty stream.

Two schemes of benefit-sharing were created. In the first, a commercialized compound may have been derived from a discovery or invention made at UIC. In the second, the discovery or invention of a compound that results in the commercialization of a product may have been made at GSK. In the development phase of a compound, GSK has the right of first refusal, in which case a different, non-ICBG company develops the drug.

The benefit-sharing schemes include the establishment of a Trust Fund (s), and the financing of the Trust (s) from a portion of the fund derived from the royalty income. As of July 31, 2001, plans for the establishment of three Trust Funds (Global Biodiversity Fund based at UIC; Vietnam Biodiversity Fund based in Vietnam; and Lao PDR Biodiversity Fund, based in Laos) were in place, but their actual implementation still awaits a donation, which is being sought. The full schemes of the UIC ICBG benefit sharing plan reflect the benefit sharing policy in natural product research of the University of Illinois at Chicago (Soejarto *et al.*, in press). In these schemes, beneficiaries include communities that participate in the project and educational organizations involved in research, education and conservation activities. Overall, more than 51% of the total royalty income that may be derived as a result of the commercialization of a plant-derived compound, either in its native form or its synthetic derivative, will go back to the source country. Other participating institutions located in the country from which the source materials did not originate will also receive a portion, albeit smaller, of the royalty income.

Under the blanket of the 5-way MOA, a bilateral subcontractual agreement was set-up between UIC and each of the institution member in host countries, namely, NCST (IBT/IEBR/ICH) and CPNP in Vietnam, and TMRC in Laos. Such an agreement provides the mechanism by which the work to be performed is defined and the transfer of funds is effected.

Permit-giving organization (s) in host country – In Vietnam, access to genetic resources is provided by the Ministry of Agriculture and Rural Development (MARD).

In Laos, access to genetic resources is provided by is provided by the Cabinet of the Prime Minister.

Informed consent – Informed Consent (IC) is defined as the consent or permission given by a governmental or a non-governmental organization or by a community, including indigenous communities, or by individuals, following the presentation of a request to access physical things, such as plants or genetic material, or knowledge or information, such as information on uses of medicinal plants, owned by or under the control of such an organization, community or individuals.

In the context of the ICBG, a signed collecting permit signifies that a consent has been given by a government body that regulates the collection and export of plant (genetic) material of the host country, for use in the ICBG study. Thus, in the case of UIC ICBG, signed permits were obtained from the Ministry of Agriculture and Rural Development of Vietnam (September 15, 1998) and from the Cabinet of the Prime Minister of Laos (September 18, 1998), respectively. Similarly, a written permit from the Cuc Phuong National Park, an entity directly under MARD, for the collection of plants from the Park, for use in the ICBG research was also granted (September 15, 1998). Permits or informed consent by specific community or individuals, for interviews and for the collection of plants within the territory owned by or under the control of such a community or individuals were obtained in all interviews carried out in the UIC ICBG research. Details of this aspect are presented in a separate paper (Gyllenhaal *et al.*, in press).

Selection of plants for study – The target of the drug discovery and development research of the UIC ICBG are plants of the Cuc Phuong National Park (CPNP) in Vietnam, with or without a history of medicinal use, and the medicinal plants of Laos. Thus, plants to be investigated to date were selected for collection based on two approaches.

The first approach is biodiversity-based collection (Soejarto, 1996) of plant samples from the 22,000-hectare CPNP, with a goal to maximize taxonomic diversity (sometimes referred to as a "random" collection), for submission to biological evaluation. The target of this selection approach is the more than 1,700 angiosperm species (distributed in 911 genera and 219 families) found

on limestone hills and valleys inside the park, as listed in existing catalogues (Anonymous, 1996; Anonymous, 1997; Nguyen Nghia Thin, 1997). This Park, which was officially recognized as a National Park by the Vietnamese Government on July 7, 1962 (Thu *et al.*, 1992; Quy *et al.*, 1996), is located in North Vietnam in the intersection of three provinces (Ninh Binh, Thanh Hoa, and Hoa Binh), about 100 km southwest of the Vietnam's capital city, Hanoi. It was felt that this number (>1,700 species) would provide an adequate array of chemical diversity as a basis for our drug discovery program. Since the existing catalogues do not provide material documentation of the species listed, which would substantiate the taxonomic identification of species listed, an effort to re-inventory these species through field collection and identification is being performed by AP1 and represents one of the specific aims of UIC-ICBG. Those who wish to view a species list and some images of plants of Cuc Phuong National Park may visit the Website of UIC ICBG at <http://www.uic.edu/pharmacy/research/icbg/ICBG.htm> and click the link to "Atlas of Seed Plants of Cuc Phuong National Park".

In our research design, 500 fully documented plant samples are being targeted for collection annually in this biodiversity-based selection approach for testing in anticancer, anti-HIV, antimalarial, and anti-TB assays. Samples collected using this approach were not submitted to CNS-testing.

The second selection approach is an ethnobotany-based approach (Soejarto, 1996), whereby plants were selected for collection and biological evaluation based on previous history of medicinal use, specifically, medicinal use related to the disease target. Since the full ethnobotanical account of UIC ICBG is being published elsewhere (Gyllenhaal *et al.*, in press), only the salient features of this aspect as they relate to selection of plants for biological evaluation are discussed below.

In Vietnam, this approach is targeted to plants found growing in the Cuc Phuong National Park and environs. When Cuc Phuong National Park was established as a National Park, a number of human settlements, called hamlets, of the Muong tribal people were found in the park, located primarily along the Buoi River on the western section of the park, and along the 20-km valley that forms the northwest-southeast axis of the park. A 20-km paved road now runs along this valley, connecting the park headquarters in the southeastern tip and the Parks Center or "Bong", located about halfway along the park's long axis. Starting in 1990, the Vietnamese Government (Ministry of Agriculture and Rural Development) initiated a move to relocate the Muong population from inside the park to locations outside of the park. Approximately 3,000 Muong people were fully relocated outside the park by 1999, and the entire section of the park east of the Buoi River is now practically empty of human settlement. Following relocation of these peoples, and to this day, cases continued to be recorded of people from villages surrounding the park, either from the new or from the already established communities, made incursions into the park to hunt animals and to collect plants to satisfy their day-to-day needs, including plants used for their primary health care. Obviously, the Muong who formerly occupied the park had developed knowledge on the medicinal properties of plants found in the park through their long period of settlement there since early times. To document such plants, field interviews were conducted with the Muong people (healers and adult population). Samples were collected for submission to bioassays against malaria, viral/HIV, cancer, tuberculosis, and CNS disease targets.

In Laos, the ethnobotany-based selection approach is targeted to Lao medicinal plants, which form part of the rich Lao heritage of herbal medicine. Lao forest resources have provided an appropriate *materia medica* whose effectiveness in the prevention and treatment of diseases was discovered since time immemorial by the Lao people, and this knowledge has been handed down from generation to generation in Lao traditional culture, a process which continues to the present day. Part of this heritage in the form of Lao medicinal prescriptions is recorded in palm leaf scripts in repository in Buddhist monasteries, and part in Alfred Petelot's book "Les Plantes medicinales du Cambodge, du Laos et du Vietnam" (Petelot 1952-1954). Recognizing the importance of traditional medicine, the Ministry of Health of Laos established the "Traditional Medicine Research Center/TMRC" in 1976. It is the only Institute of this kind in the country and is conducting adaptive research on medicinal plants and Traditional Lao Medicine. In addition to identifying, cataloguing, studying, and preserving knowledge of medicinal plants and traditional prescriptions, and transferring TMRC's achievement to pharmaceutical factories for large-scale production, TMRC provides technical assistance to more than ten provincial Traditional Medicine Stations located throughout the country. Each TMS consists generally of one or more buildings, one of which is a dispensary of Lao traditional medicines, with a staff of three to five. Heads of Stations include either university graduates from a College of Pharmacy, or graduates of secondary medical technical schools. Other TMS staff members may include senior traditional healers and monks. These TMSs are the bedrock of the ability of TMRC to conduct research in traditional herbal medicine among rural populations. Thus, in our ICBG project, field

interviews were conducted through the TMSs, which enable TMRC staff to effectively and efficiently survey medicinal plants from the entire country, in a variety of different vegetational zones and ethnic subgroupings of the Lao Loum, the dominant ethnic element in Laos, comprising 68% of the population (Schenck Sandbergen and Choulamany-Khamphoui, 1995). Utilizing this methodology, a substantial portion of the Lao medicinal plants, especially, those with a history of medicinal use to treat CNS-related diseases were being collected, sorted and submitted for biological evaluation within the framework of the ICBG.

Aside from primary data derived from field interviews, literature analysis, primarily based on data from the NAPRALERT database (Loub *et al.*, 1985; Farnsworth, 1996), was also performed to identify species of plants of Vietnam and Laos with a history of medicinal use for therapy against diseases relevant to therapeutic categories of interest to UIC-ICBG. Guided by this information, field interviews were undertaken to seek confirmation of uses, as basis for collection of such species.

Methodology

Plant collection and identification – Plant collection and documentation methods followed those previously published (Soejarto, 1993; Soejarto *et al.*, 1996). In the biodiversity-based collection process, whenever possible, three different types of samples were collected, usually, leaf + twig sample, stem bark sample, and root sample; occasionally, a branch and/or other types of sample were also collected. In the ethnobotany-guided collection, only plant parts specifically cited by informants (interviewees) as the parts used medicinally were collected. For each plant sample, 100-300 g dry weight of raw material was acquired. Samples were dried at the herbarium facilities of Cuc Phuong National Park, and dried samples were forwarded to the Vietnam-based personnel of Associate Program 3 (at ICH, Hanoi) for extraction.

In Vietnam, taxonomic identification of plants collected was first performed at the Herbarium of CPNP, but the primary site for taxonomic identification has been the Institute of Ecology and Biological Resources/IEBR of the National Center for Science and Technology, Hanoi, under the coordination of AP1 Co-Project Leader, Nguyen Tien Hiep. A third identification site is at the Herbarium of the Field Museum of National History, Chicago. Assistance of taxonomic specialists from other institutions (Vietnam-based or overseas) was also sought. In Laos, initial taxonomic identification of plants collected was performed at the herbarium facilities of TMRC; re-identification or confirmation of identification was performed at IEBR and at the Field Museum, respectively.

Dispatch of samples for bioassays – Only crude extracts (see below) of plant samples collected in Vietnam were dispatched to Chicago (AP3 laboratories). Plant samples collected in Laos were initially shipped to Chicago and GSK (AP5 laboratories) in their raw material form (as bulk samples). Today, only crude plant extracts are dispatched to Chicago, although bulk samples continue to be shipped to GSK, both from Vietnam and Laos, through the request of GSK.

All the necessary permits and their documentation were obtained from respective permit-giving Government offices both in Vietnam and Laos for the dispatch of the plant materials and plant extracts.

Extraction of plant samples – All samples to be tested at UIC were extracted using a standardized protocol, as follows (see also: Zhang *et al.*, 2001). Samples of 100 g of dried and milled plant material was percolated overnight with 90% methanol, concentrated *in vacuo*, and subsequently defatted with n-hexane and partitioned with $CHCl_3$ to yield $CHCl_3$-soluble extract. The combined chloroform fraction from multiple extractions was then evaporated to dryness below 40°C *in vacuo*, and the remaining aqueous extract was lyophilized after the last traces of organic solvent had been removed. One-half of the dried chloroform soluble fraction was submitted for anti-HIV, antimalarial, anti-TB, and anticancer assays, while the other half set aside for future use.

For all plants with a history of use to treat CNS-related diseases, crude extracts were prepared using a standard protocol to generate a methanol extract which was treated with DE52 to facilitate tannin removal. Extracts were filtered, the methanol evaporated and the residue re-suspended in DMSO for storage at –20°C in controlled humidity. For the generation of fractions, a DE52-treated methanol extract was evaporated, reconstituted in DMSO and diluted with 0.1% aqueous formic acid. The extract was then passed through a C18 bond-elut column, and eluted with increasing ratios of acetonitrile/formic acid mixtures. Fractions were dried, weighed and adjusted to a 2 mg/mL stock concentration, and submitted for bioassay in proprietary assay systems developed at GSK to detect analgesic activity and activity against Alzheimers disease target.

Bioassay-guided isolation process – Once an extract of a particular species had demonstrated activity in a particular bioassay, a larger amount of sample was requested by AP3 team for recollection in quantities of 3-5 kg dry weight, for isolation studies. The recollection work was performed by the AP1 team. Bioassay-guided fractionation

and isolation of biologically active compounds were undertaken by members of AP3. The $CHCl_3$-soluble extract obtained as described above was chromatographed over a Si gel column, which was developed by gradient elution with $CHCl_3$ and increasing concentrations of Me_2CO to afford fractions. A bioassay localized the active fractions, which were subjected to flash chromatography on C-18 reverse phase (RP-18) columns (elution with Me_2CO and H_2O) and led to further concentrated active fractions. Preparative HPLC separation on C-18 reverse phase (RP-18) columns resulted in the isolation of final active compounds.

To assist in the isolation of novel and biologically active compounds, occasionally, the LC/MS technique was employed to detect the presence of target compounds in the extracts and fractions. In cases where anti-HIV, antimalarial or anticancer compound (s) had already been reported from either the plant under investigation or from a chemotaxonomically related species, the active fraction was subjected to a LC/MS/MS dereplication study to prevent the re-isolation of such constituent (s).

Compound identification and structural elucidation – The identification of known compounds, or the structural elucidation of novel active isolates was accomplished by the measurement and interpretation of their physical (TLC R_f value, HPLC retention time, melting point, and mixture melting point) and spectroscopic (UV, IR, NMR and MS) data. In the verification of a known active isolate, the comparison of its physical properties (melting point, optical rotation) and spectral characteristics (UV, IR, ^{1}H- and ^{13}C-NMR and low resolution MS), in addition to its TLC and/or HPLC R_t values to those reported in the literature, or to those obtained for reference standards is sufficient. For the elucidation of the structures of novel active compounds, the molecular formula was obtained by the measurement of high resolution mass spectra, and the structure elucidation and relative stereochemistry were determined by the use of 1D and 2D NMR (^{1}H NMR, ^{13}C NMR, DEPT, ^{1}H-^{1}H COSY, HMQC, HMBC, ROESY, NOESY, etc.) spectra, Mass spectra (EI, FAB, TOF, LC-MS, etc.), IR and UV spectra, and chemical reactions. CD spectra and Moshers esterification were used for determination of an absolute stereochemistry. In cases of compounds that had novel carbon skeleton or that pose difficult stereochemistry problems, single crystal X-ray crystallographic analysis was carried out where possible.

Bioassay protocols – The following bioassays were performed to evaluate the pharmaceutical potential of plants acquired within the framework of the UIC-ICBG:

Assay for the inhibition of HIV-1 infectivity : The inhibition of HIV-1 infectivity in the present study is conducted using green fluorescent protein (GFP) reporter cell lines HOG.R5 (Tan *et al.*, 1997). This reporter cell line for the quantitative assay of HIV infectivity was developed using HOS (human osteosarcoma) cells rendered susceptible to HIV infection by the transfection of genes for CD4 and CCR5 as the co-receptor for M-tropic HIV-1 isolates. This microtiter assay is based on the transactivation of a stably integrated HIV-1 LTR-GFP transcription unit. Upon HIV-1 entry into these HOS target cells, Tat expression increased the HIV LTR-directed transcription of the GFP gene as demonstrated by the increased fluorescence of detergent lysates of infected cells relative to that of uninfected controls. By virtue of the low level expression of endogenous CXCR4, HOG.R5 cells also efficiently supported infection by a diverse array of T-tropic viruses, in addition to M-tropic and clinical isolates.

The assay's utility and biological relevance were demonstrated by the sensitive and accurate assessment of several HIV neutralizing monoclonal antibodies (e.g., C108G, C311E, 4117C, IgG1b12, 2G12, and 2F5) and anti-HIV pharmacological agents (e.g., 3TC, ddI, ddC, delavirdine mesylate, recombinant soluble CD4, dextran sulfate, and (+)-calanolide A), which gave predictable responses that paralleled those obtained in traditional peripheral blood mononuclear cell (PBMC) systems (Tan et al., 1997). The short duration of the assay and its 96-well format allow for rapid throughput with minimal quantities of reagents in addition to minimal handling of infectious HIV (minimal biohazard). Thus, the HOG.R5 assay provides a novel, sensitive, reproducible and standardized means for the large-scale *in vitro* evaluation of anti-HIV agents.

The anti-HIV activity of pure compounds and plant extracts (max. conc. 20 μg/ml) were assessed by the addition of test samples to cells in triplicate just before the addition of virus. The initial virus inoculum and the duration of assays have been optimized for various virus isolates with respect to the linearity and level of signal enhancement, and the integrity of the host cell monolayer. In general, HIV-1_{IIIB} (5 ng p24/ml) was used for the preliminary evaluation of plant extracts. Relevant controls consisting of infected cells which have not been treated with the test material were included. Infected cultures were incubated for 4 days. At the end of the assay as determined by the presence of syncytia, the media was thoroughly removed and 200 μl of 0.5% (v/v) Nonidet P-40 in phosphate buffered saline (PBS) was added to each well. The contents were mixed by repeated pipetting, and GFP fluorescence signal was quantitated as Relative Fluorescence Units (RFUs) at excitation and emission wavelengths of 485 nm and 535 nm, respectively, using a laboratory fluorometer.

The background fluorescence of uninfected cells was subtracted from the fluorescence signal of all infected cultures, after which the percent remaining fluorescence output of test wells (% Control Infection) were determined relative to those of untreated control cultures. The median inhibitory concentration (IC_{50}) was computed from a linearly regressed dose-response plot of % Control Infection versus concentration or log concentration of compound, utilizing at least five concentrations of each compound. The positive control compound used was 3TC (Lamivudine) which had an IC_{50} value of approximately 1.2 μM in the HOG.R5 system utilizing the assay conditions described above. This nucleoside reverse transcriptase inhibitor and the virus stock of HIV-1_{IIIB}/H9 were obtained through the AIDS Research and Reference Reagent Program, Division of AIDS, NIAID, NIH.

The toxicity of pure compounds and plant extracts to HOG.R5 cells in 96-well microtiter plates were evaluated in parallel assays where virus is omitted. The level of basal GFP expression (approximately 4,000 RFU) demonstrated by HOG.R5 cells has afforded an acceptable dynamic range for the determination of cellular viability. A decrease in the basal level of fluorescence of cell lysates coupled with microscopic evidence of cell death in the presence of the test agent were indicative of an adverse effect on cell growth. Untreated cells served as controls. All compound and extract concentrations that were devoid of cellular toxicity were employed for generating useful dose responses. The median cytotoxic concentration (CC_{50}) was also considered in the calculation of Selectivity Indices (SI), where $SI = CC_{50}/IC_{50}$. Generally, extract toxicity identified with the HOG.R5 reporter cell line (measured utilizing the constitutive fluorescence output of cells) correlated well with that observed in cytotoxicity assays utilizing tumor cell lines (quantitated by the measurement of total protein content with sulforhodamine dye) as described below.

Our ICBG-funded drug discovery efforts over the past 3 years have resulted in the availability of a large database of information for retrospective analysis. Based on information in the database, the criteria for determining priority leads, as well as cut-offs for activity have now been defined. General guidelines and strategies for screening have also been established. It was ascertained that extract toxicity was encountered with much less frequency when the initial cell concentration used to seed microtiter plates prior to each assay was greater than or equal to 4×10^4 /ml. Toxic effects were diluted out in most cases by the increased cell number while still preserving the kinetics and dynamic range of this cell-based anti-HIV assay. This modification was subsequently incorporated into the assay protocol. For all practical purposes, extracts that are highly toxic will be dismissed from further testing as potentially useful anti-HIV agents.

The assay protocol and strategy for screening have been further refined as follows: All chloroform or methylene chloride extracts were prescreened for toxicity and anti-HIV activity at 20 and 10 μg/ml in triplicate. Assays for cytotoxicity and anti-HIV activity were performed concurrently in an attempt to achieve exactly matched and standardized conditions that would validate subsequent comparisons and cross-analyses. The examination of initial samples at two concentrations provided a preliminary indication of the concentration dependence, and hence, legitimacy of the observed antiviral activity. Anti-HIV extracts that do not demonstrate toxicity during initial testing were fractionated. However, fractions often demonstrated toxicity when tested at 20 μg/ml due to the more concentrated nature of the constituents. In an attempt to gain further insight into the selectivity of the antiviral effect of fractions which also demonstrated toxicity, selected series of fractions were also assayed at 20, 10, 5 and 2.5 μg/ml. Changes in selectivity can then be monitored by comparing toxicity and anti-HIV profiles at diminishing concentrations of the extract. The ideal scenario would be a complete separation of peaks representing the two distinct activities indicating successful chromatographic resolution of antiviral and toxic components. This overall approach has proven to be most effective and practical in the prioritization of plant materials for recollection.

Nontoxic and tannin-free extracts demonstrating % Control Infection (%CI) of ≤ 50% were prioritized for further phytochemical analyses. These will consequently be re-collected. The $CHCl_3$ extracts that are being prepared and tested under the ICBG program are relatively free from the interfering effects of ubiquitous compounds such as polyphenolics. Nevertheless, confirmatory tests for the absence of polyphenolics has routinely been performed using $FeCl_3$ solution.

Assay for antimalarial activity : The antimalarial activity of plant extracts and pure compounds was assessed utilizing the *in vitro* radioisotope-incorporation method of Desjardins *et al.* (1979) with minor modifications (Likhitwitayawuid *et al.*, 1993). All test compounds were assayed in the presence of a suspension of *P. falciparum*-infected erythrocytes (0.5-1.0% parasitemia, 1.0% cell hematocrit), over a concentration range of 14 ng/ml to 10 μg/ml while extracts were pre-screened at a maximum concentration of 10 μg/ml. In addition, the known antimalarial drugs, quinine, chloroquine, mefloquine, and artemisinin were tested as controls in each experiment over a range of 0.3-250 ng/ml. Microtiter plates were incubated for 24

hours at 37°C in a sealed chamber under an atmosphere of 5% CO_2, 5% O_2 and 90% N_2. After this incubation period, 0.5 μCi of [^{3}H(G)]hypoxanthine was added to each well, and the microtiter plate returned to the sealed chamber at 37°C for an additional 18 hr incubation. The assay was terminated by harvesting the contents of each microtiter plate onto a glass fiber filtermat (Filtermat A) (Wallac) using a Tomtec 96-well harvester (Tomtec, Orange, Conn.). Filtermats were dried, and immersed in minimal nonaqueous scintillation fluid in a small heat-sealed plastic pouch. The radioactivity from wells were counted using the 1450 MicroBeta™ Liquid Scintillation Counter (Wallac Oy, Turku, Finland). Concentrations of both test compounds and positive controls which inhibited parasite-specific incorporation of [^{3}H]hypoxanthine by 50% (IC_{50}) were determined by non-linear regression analysis. Drug-free controls define 100% incorporation.

The relatively high throughput antimalarial screening system currently employed has the ability to test a diverse array of samples which act through any number of conceivable antimalarial mechanisms, and to identify bioactives without regard for selectivity. Therefore, the combination of this primary radioactivity-based antimalarial assay with secondary assays for general cytotoxicity is necessary in order to distinguish test material which exhibit specific inhibition of parasite growth from general cytotoxins. To that end, test substances active against cultured *P. falciparum* were subsequently evaluated for cytotoxic potential with a selected panel of tumor cell lines (KB, LNCaP, Col-2 and Lu1) according to the method described below. However, results with KB cells (human oral epidermoid carcinoma) are generally utilized for the experimental computation of the *in vitro* "Selectivity Index", a parameter that facilitates the discrimination between general toxins and samples which exert a more selective inhibitory effect on *Plasmodium*. Selectivity Index (SI) for antimalarial activity is, therefore, defined as CC_{50} (KB)/IC_{50} (*P. falciparum*). This parameter serves as an *in vitro* indicator of clinical significance in the absence of the more comprehensive *in vivo* "therapeutic index", and has proven to be of practical value in prioritizing active test samples, and in guiding bioassay-directed fractionation with the aim of isolating the most promising antiplasmodial compounds (Angerhofer *et al.*, 1992). KB cells were selected for the assessment of SI by precedent (Wright and Phillipson, 1990), and also because, in our experience, this cell line exhibits an intermediate sensitivity to a large number of cytotoxic agents when compared to cell lines derived from a variety of other human tumors.

In the course of screening samples collected by AP1 of this ICBG, only extracts demonstrating >90% inhibition of the chloroquine-sensitive strain (D6) at 10 μg/ml were selected for a full dose-response evaluation, in addition to further testing against the chloroquine-resistant strain (W2) of *P. falciparum*. The resultant database of information has allowed the following cut-offs to be established. All nontoxic extracts (defined as 100% KB viability at 20 μg/ml) demonstrating an $IC_{50} \leq 4$ μg/ml against *Plasmodium* were defined as active. Extracts toxic to the KB cell line will be dismissed from further testing. An activity cut-off of 400 ng/ml has also been adopted for pure compounds. Correlation between cytotoxicity in the KB cell line and activity in the antimalarial assay indicated a suboptimal SI value, implying that these extracts inhibited parasite growth not by a specific mechanism but rather through a general cytotoxic effect.

Assays for cytotoxicity and *in vivo* antitumor activity : A specific aim of the ICBG is to define the most promising starting materials as unrefined sources of viable antitumor agents. The majority of research programs dealing with the isolation and identification of potential antitumor compounds from plants have relied on cytotoxicity for bioassay-directed fractionation. In accordance with this widely-held approach, we have utilized a panel of well-established tumor cell lines for the evaluation of the potential antitumor activity of plant extracts. This panel which comprises Lu1 (lung), KB (human oral epidermoid carcinoma), Col-2 (ovarian) and LNCaP (prostate) was judiciously selected based on the statistical analysis of several thousand experiments that have been performed previously in our laboratories. The 4 cancer cell lines were adopted based on a new overall paradigm that supports the use of only the most representative cell lines in order to identify potential antitumor compounds. In addition, the HUVEC (human umbilical vein endothelial cells) line was also employed as a test system for identifying samples with potential antiangiogenic activity. hTERT-RPE1 (human telomerase reverse transcriptase-retinal pigment epithelial), a telomerase-immortalized normal human cell line, was introduced as a substitute for a primary cell line for determining toxicity to normal human cells.

Briefly, bioassay procedures involve treating cells with various concentrations of the test substance, and assessing cell growth after 72 hrs. A standard protocol for the assessment of cellular toxicity measures the ability of cultured cells to proliferate in the presence of a test extract or pure compound (NIH, 1984), and subsequently quantitates total protein content with sulforhodamine B dye as a measure of the percentage of surviving cells (Skehan *et al.*, 1990). The results are expressed as an ED_{50} (concentration required to inhibit cell growth by 50%), and the criteria for activity, as established by the National Cancer Institute (NCI), are ED_{50} values < 20 μg/ml for extracts, and < 4

μg/ml for pure compounds.

Furthermore, in order to assess the *in vivo* therapeutic efficacy of antitumor agents uncovered in the *in vitro* cell-based screens without the substantial expenditure of time, labor, and cost associated with conventional animal models and large-scale animal testing, the most active plant extracts uncovered in the prescreen will be evaluated in the hollow fiber assay. The hollow fiber assay was developed and introduced by the NCI as an intermediate screen through which compounds must pass before full evaluation in xenograft tumor models (Hollingshead *et al.*, 1995). The sensitive cell lines will be cultivated in biocompatible, semi-permeable PVDF (polyvinylidene fluoride) hollow fibers with subsequent implantation of these encapsulated tumor cells into the intraperitoneal and subcutaneous compartments of host NCr nu/nu mice. Subsequently, plant extracts or pure compounds will be administered daily from days 3 to 6 post-implantation to mice bearing the hollow fibers containing the replicating tumor cells by IP injection. Doses are selected based upon single mouse toxicity studies in which the Maximum Tolerated Dose (MTD) is identified. Fibers will be retrieved on day 7 for *ex vivo* quantification of viable tumor cells using the stable end point MTT (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide) dye conversion assay. The viable cell mass is determined in conjunction with parallel *in vitro* samples assessing sterility and *in vitro* growth potential. Also included are blank controls consisting of hollow fibers filled with complete medium which are handled in parallel to the cell samples. The viable cells in prepared fibers are also determined on the day of implantation to serve as an index for the day 0 cell mass. The antiproliferative effects of test agents can then be measured by comparison of treated and control samples.

This *in vivo* efficacy assay will be used to select and prioritize cytotoxic plant extracts for fractionation and isolation studies since it serves as a preliminary tool to assess the capacity of a test agent to reach tumor cells growing in two distinct physiological compartments at pharmacologically active concentrations. These prioritized plant extracts will then be subjected to bioactivity-guided fractionation using the hollow fiber assay itself to monitor the progress of the isolation. All pure compounds obtained will be tested for *in vivo* efficacy using the same hollow fiber system.

Screening of plant extracts for the induction of HL-60 cell differentiation : One proposed strategy for cancer chemotherapy involves the induction of differentiation of malignant cells into a phenotype with a limited capacity for proliferation. Therefore, differentiation inducers are expected to constitute a new class of anticancer agents. In light of this, the HL-60 cell line was introduced during year 3 of the project in an attempt to introduce additional molecular targets into the overall anticancer screening approach.

HL-60, a human promyelocytic leukemia cell line, provides a unique *in vitro* model for studying the processes of proliferation and differentiation in normal and leukemic cells. In particular, these cells can be triggered to become morphologically, histochemically and functionally mature granulocytes and monocytes/macrophages by treatment with agents such as retinoids, derivatives of vitamin D_3, phorbol esters and interferon.

The differentiated HL-60 phenotype is characterized by growth inhibition, increased adherence, loss of cell surface transferrin receptors, increase in monocyte surface markers, induction of ∀-naphthyl acetate esterase (NSE) staining and certain patterns of protein phosphorylation. Cell differentiation effects can, therefore, be measured by monitoring cellular properties such as nitroblue tetrazolium (NBT)-reducing activity, the appearance of nonspecific/specific acid esterases (NSE/SE), and the decrease in [^{3}H] thymidine incorporation.

The strategy presently adopted involves the prescreen of all plant samples for the inhibition of [^{3}H]thymidine incorporation as an indication of reduced cellular proliferation. Samples demonstrating ∃50% inhibition of [^{3}H]thymidine incorporation were then evaluated in secondary assays involving the measurement of cellular viability (trypan blue exclusion) and the detection of enzymatic markers of differentiation (NBT reduction and the appearance of NSE/SE).

The criteria for activity have been established. As alluded to above, extracts must demonstrate ∃50% inhibition of [^{3}H]thymidine incorporation, and ≥30% cellular viability by trypan blue exclusion in order to warrant further investigation. Subsequent to that, only extracts capable of demonstrating ≥20% and ≥10% cellular differentiation levels based on the NBT and NSE/SE procedures, respectively, were deemed active.

HL-60 cells were maintained in continuous suspension culture in RPMI 1640, supplemented with 10% heat-inactivated calf serum, 100 units/ml penicillin and 100 μg/ml streptomycin at 37°C in a humidified atmosphere of 5% CO_2 in air. Cells in the log phase of growth (approx. 1 $\times 10^6$ cells/ml) were diluted to 1.2×10^5 cells/ml and pre-incubated for 18 hr, after which the cell density achieved was approximately 20×10^4 cells/ml.

Samples (2 μl) dissolved in DMSO were prepared in 24-well plates and 2 ml of HL-60 cells at the above seeding concentration were added (final DMSO concentration, 0.1% v/v). Plant extracts were prescreened at a final

concentration of 4 μg/ml. Vitamin D_3 [1,25-di(OH) cholecalciferol, 10^{-9} M] was tested as the positive control differentiation inducer. After 4 days of incubation, cells were analyzed to determine the percentage exhibiting functional and enzymatic markers of differentiated cells utilizing the following assays:

a) Inhibition of [^{3}H]thymidine incorporation

A pre-screen was performed for the inhibition of [^{3}H]thymidine incorporation into DNA as a means of quantitating cellular proliferation. Sample-treated cells (100 μl) were transferred into 96-well microtiter plates and [^{3}H]thymidine in 100 μl of fresh media was added to a final specific activity of 65 Ci/mmol (0.5 μCi/ml). After 16 hr of incubation, cells were harvested and the filters washed using the TOMTECH® harvester. The level of [^{3}H]thymidine incorporated was expressed as a percentage relative to that of control cultures treated with pure DMSO.

b) Trypan blue dye exclusion

Cell viability (expressed as a percentage of the total number of cells examined) was determined by mixing 250 μl trypan blue solution, 150 μl Hanks balanced salt solution (HBSS) and 100 μl of cell suspension, and subsequently counting unstained cells under the microscope.

c) Nitroblue tetrazolium (NBT) reduction

This assay was used to evaluate the ability of sample-treated HL-60 cells to produce superoxide when challenged with 12-*O*-tetradecanoylphorbol-13-acetate (TPA). A 1:1 (v/v) mixture of cell suspension and freshly prepared TPA/NBT solution (PBS containing 2 mg/ml NBT and 1 μg/ml TPA) were incubated for 1 hr at 37°C protected from light. Cells were then smeared onto glass slides and air-dried. After fixation in methanol, cell smears were counterstained with 0.3% (w/v) safranin O in methanol for 7 min. Superoxide-producing cells are able to reduce NBT yielding intracellular blue-violet formazan deposits which can be visualized and counted under the microscope. Cells stained blue-violet represent those that have differentiated into monocytes/macrophages. Red-pink cells represent their nondifferentiated counterparts.

d) Nonspecific/specific acid esterase (NSE/SE) activity

Assays for the presence of ∀-naphthyl acetate esterase (nonspecific acid esterase, NSE) and naphthol AS-D chloroacetate esterase (specific acid esterase, SE) were performed using cytochemical kits from Sigma Chemical Co. [∀-Naphthyl Acetate (Non-Specific Esterase), Product No. 91-A and Naphthol AS-D chloroacetate (Specific Esterase), Product No. 91-C].

∀-Naphthyl acetate esterase (NSE) is detected primarily in monocytes, macrophages and histiocytes, and is virtually absent in granulocytes. Monocytes show black granulation if NSE is detected. Lymphocytes may occasionally exhibit enzymatic activity. Naphthol AS-D chloroacetate esterase (SE) is usually considered specific for cells of the granulocytic lineage. Sites of activity show bright red granulation. Activity is weak or absent in monocytes and lymphocytes.

The level of differentiation was assessed by microscopic enumeration of a minimum of 200 cells (in duplicate) for each test sample. Activity is expressed as a percentage of positive cells over the total number of cells examined.

NSE detection : Fast blue BB (1 ml) and sodium nitrate (1 ml) were mixed by inversion at room temperature for at least 2 min. The mixture was then added to 40 ml of pre-warmed (37°C) ddH_2O. TRIZMAL™ 7.6 buffer concentrate (5 ml) and α-naphthyl acetate substrate solution (1 ml) were then added. In the meantime, cell smears were fixed in Citrate-Acetate-Formaldehyde (CAF 15.6:40.6:5) at room temperature for 30 sec with vigorous agitation for a further 5 sec. Slides were rinsed thoroughly in running ddH_2O for 45-60 sec, and immediately incubated with the substrate mixture above in a Coplin jar for 30 min at 37°C. Slides were not allowed to dry prior to treatment with the substrate solution. At the end of the staining process, slides were rinsed in tap water for 2 min and air-dried.

SE detection : Fast Red Violet LB base solution (1 ml) and sodium nitrate (1 ml) were mixed by inversion at room temperature for at least 2 min. The mixture was then added to 40 ml of prewarmed (37°C) ddH_2O. TRIZMAL™ 6.3 buffer concentrate (5 ml) and naphthol AS-D chloroacetate substrate solution (1 ml) were then added. Slides were fixed and washed as described previously. They were then immersed in the substrate mixture as prepared above for 15 min, after which they were rinsed under tap water and air-dried.

Antituberculosis assay : Following the completion of our BSL-3 facilitites, we shifted from non-virulent to a virulent strain of *Mycobacterium tuberculosis*. Extracts were screened against this virulent but drug-sensitive strain ($H_{37}Rv$), and bacterial viability was determined by the microplate Alamar Blue assay (MABA) (Collins and Franzblau, 1997), following a one-week incubation with 50 ug/ml extracts at 37°C. Reduction of the Alamar Blue reagent was measured fluorometrically in a microplate fluorometer using excitation of 530 nm and emission of 590 nm. Extract activity was scored as percent inhibition of fluorescence relative to control wells with bacteria but without extract. Extracts effecting a reduction in bacterial viability (fluorescence) of 90% or greater were considered to have an MIC of ≤50 ug/ml. These extracts were re-tested at multiple concentrations to determine the actual minimum inhibitory concentration (MIC).

Assays to detect activity against CNS-related diseases :

These assays are performed by AP5 team at Glaxo Smith Kline (GSK) laboratories (UK). In view of the fact that the assays are proprietary in nature, no description of these assays can be given at this stage.

Results

As of July 31, 2001, biodiversity-based ("random") collection of samples from Cuc Phuong National Park yielded 2,467 plant samples, comprising approximately 700 species of angiosperms, while ethnobotany-based collection yielded 414 samples (162 samples from Vietnam, 252 from Laos). Recollection of samples of active species in Vietnam in the Cuc Phuong National Park yielded a total of 66 samples, comprising 52 species. Twelve (12) ethnomedical samples were also recollected, all of which were from Laos. In the laboratories, major extraction effort was performed at ICH in Hanoi by AP3 Vietnam personnel. By July 31, 2001, preparation of 2,006 extracts (from as many samples) out of 2,467 samples, plus 28 extracts from recollected samples was completed. In Chicago, Chicago-based AP3 personnel screened extracts prepared by the Hanoi-based AP3 and completed the screening of more than 800 extracts in HIV, malaria, cancer (cytotoxicity, HL-60 cell differentiation assays), and TB disease targets, and in Stevenage, AP5 personnel screened extracts of 21 ethnomedical samples in Alzheimer's disease and pain targets.

Out of the more than 800 extracts screened (Table 2), active samples were prioritized based on the degree of their biological activities in either anti-HIV, antimalarial and anticancer screen. A prioritized list of species (Table 1) was used to select species considered high priority for recollection and fractionation (isolation) studies. Such a list continued to be updated as new actives were uncovered.

At UIC, the overall screening results of our drug discovery work by July 31, 2001, are summarized in Table 2, while the details of compounds isolated from two active species, *Rhaphidophora decursiva* and *Litsea verticillata*, are presented in Table 3.

The following observations concerning Table 2 may be highlighted.

In all disease targets screened at UIC, initial hits of 5-10% were recorded. In the CNS assay, a higher percentage of hits were recorded, but the number of samples was small.

The largest number of actives (32 extracts) to date came from anti-HIV screen, consequently, the largest number of active (21) and new and active (13) compounds comprise this bioactivity. Some of these compounds are presented in Table 3, while representative structures are presented in Fig. 1.

Of particular interest is the structural novelty and

Table 1. High-priority species selected for fractionation[a]

Biological activity	Species	Sample Code	Plant part
Anti-HIV	Litsea verticillata Hance (Lauraceae)	SVA-0001	Leaf + twig
Anti-HIV	Ardisia maculosa Mez (Myrsinaceae)	SVA-0005	Leaf + twig
Anti-HIV	Vatica cinerea King (Dipterocarpaceae)	SVA-0039	Leaf + twig
Anti-HIV	Clerodendrum serratum (L.) Moon (Verbenaceae)	SVA-0008	Leaf + twig
Anti-HIV	Strychnos vanpruckii (Lganiaceae)	SVA-0246	Leaf + twig
Cytotoxicity	Litsea verticillata Hance (Lauraceae)	SVA-0001	Leaf + twig
Cytotoxicity	Vatica cinerea King (Dipterocarpaceae)	SVA-0038	Leaf + twig
Cytotoxicty	Clerodendrum seratum (L.) Moon (Verbenaceae)	SA-0008	Leafr + twig
Antimalarial	[b]Rhaphidophora decursiva Schott (Araceae)	SVA-5005	Leaf + stem

[a]These are species for which isolation studies have been completed and manuscripts communicating these results have been submitted for publication. Isolaton studies on other active species are on-going.
[b]Paper published: Zhang *et al.*, 2001.

Table 2. Overall results of all bioassay performed (mid-1999 - July, 2001)

Bioassay	Number of bioassay systems	Samples assayed	Initial hits (%)	Selected for fractionation	Active compounds	New compounds	Compounds in development
HIV	1	1164	65 (5.6)	32	21	13	0
Cytotoxicty	6	908	58 (6.3)	2	0	0	0
Malaria	2	860	89 (10.3)	20	7	3	0
HL-60	1	902	9 (1)	4	0	0	0
Tuberculosis	1	948	88 (7.3)	0	0	0	0
CNS	10	21	7 (33)	7	0	0	0

Table 3. Anti-HIV and antimalarial activities of compounds isolated/identified from two species

Cpd. No.	K (isolate) No.	Species/Compound name	CC_{50} (μg/ml) HOG.R5	IC_{50} (μg/ml) HOG.R5	Comments
		Litsea verticillata Hance (SVA0001) Anti-HIV			
1	SVA0001-K001	(+)-epiexcelsin	Nontoxic @ 20 μg/ml	100% Control Infection @ 20 μg/ml.	Known INACTIVE
2	SVA0001-K002	(+)-demethoxyepiexcelsin	23.0	16.4 (SI = 1.4)	NEW ACTIVE
3	SVA0001-K003	Verticillatol	Nontoxic @ 20 μg/ml	34.5 (SI not determined)	NEW INACTIVE
4	SVA0001-K011	(±)-Litseaverticillol A	13.2	5 (SI = 3)	NEW ACTIVE
		Rhaphidophora decursiva Schott (SVA5005) - Antimalaria			
5	SVA5005-K001	Decursivine			NEW INACTIVE
6	SVB5005-K001	Polysyphorin	2000 ng/ml	D6: 404 (SI = 5) W2: 368 (SI = 5)	Known ACTIVE
7	SVA5005-K002	(±)-Glaberide I			Known INACTIVE
8	SVB5005-K002	Rhaphidecurperoxin	4000 ng/ml	D6: 540 (SI = 7) W2: 420 (SI = 9)	NEW ACTIVE
9	SVA-5005-K003	(+)-Dehydrovomifoliol			Known INACTIVE
10	SVB5005-K003	Rhaphidecursinol A	12,000 ng/ml	D6: 3,007 (SI = 4) W2: 1,736 (SI = 7)	NEW ACTIVE
11	SVA5005-K004	(-)-Loliolide			Known INACTIVE
12	SVB5005-K004	Grandisin	14,000 ng/ml	D6: 1,510 (SI = 9) W2: 1,485 (SI = 9)	Known ACTIVE
13	SVA5005-K005	(-)-Hydroxydihydrobovolide			Known INACTIVE
14	SVB5005-K005	Rhaphidecursinol B	10,000 ng/ml	D6: 5,372 (SI = 2) W2: 4,678 (SI = 2)	NEW ACTIVE
15	SVA5005-K006	*N*-butylbenzamide			Known INACTIVE
16	SVB5005-K006	Epigrandisin	16,000 ng/ml	D6: >10,000 (SI < 2) W2: 3,339 (SI = 5)	Known ACTIVE
17	SVA5005-K007	(+)-Medioresinol			Known INACTIVE
18	SVA5005-K008	(+)-Syringaresinal			Known INACTIVE
19	SVA5005-K009	(+)-Pinoresinol			Known INACTIVE

diversity of compounds isolated from *Litsea verticillata* (Table 3), a small tree belonging to the family Lauraceae, some of which showed anti-HIV activity (Zhang, *et al.*, submitted; Hoang, *et al.*, submitted). The following is a sampling of these Litsea compounds: (+)- demethoxyepiexcelsin (**2**; new, active) and verticillatol (**3**; new, inactive). A Litsea compound, litseaverticillol A, represents a new "litseane" sesquiterpene skeleton (structures are not given in this paper).

Another prolific species is *Rhaphidophora decursiva* (Table 3), a climbing epiphyte with a somewhat fleshy stem and large leaves belonging to the family Araceae. Seven (7) active antimalarial compounds were isolated from this species (Table 3), of which 3 are new (rhaphidecurperoxin, **8**; rhaphidecursinol A, **14**; rhaphidecursinol B, structure not presented) (Zhang *et al.*, 2001).

Work continues on the isolation and structure elucidation of active compounds from plant species collected from

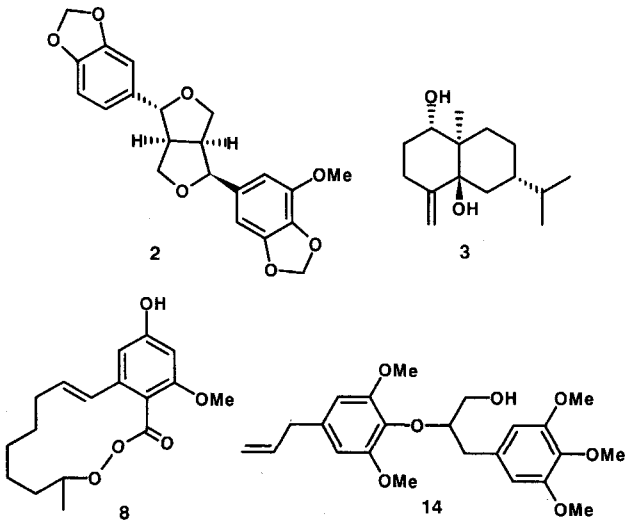

Fig. 1. Selected representatives of new and active compounds isolated from plants of Cuc Phuong National Park to date.

Cuc Phuong National Park.

It is immediately noticeable from Table 2 that no species have been prioritized for isolation in the anti-TB assay, even if a large number of species have been shown to be active. The reason for this is the fact that the anti-TB assay results came from *a*virulent (non-virulent) strain ($H_{37}Ra$) of *Mycobacterium tuberculosis*. Our biosafety level 3 (BSL-3) facilities to screen the virulent strain of M. tuberculosis $H_{37}Rv$ at the Institute of Tuberculosis did not become operational until June, 2001.

At GSK, 7 of the 21 species screened showed activities in Alzheimers disease and pain assays, but due to the nature of the active chemical compounds identified, no further work on these plants were undertaken.

V. Discussion and Conclusions

As a result of isolation studies of only half-a-dozen active species, a total of 25 biologically active (anti-HIV, cytotoxic, and antimalarial) compounds [not all of these are presented in this paper] have been isolated to-date from plants of Cuc Phuong National Park. Of the 25, fifteen (15) represent new and novel compounds. These results clearly demonstrate that the tropical rain forests of Cuc Phuong National Park represent a reservoir of chemical diversity with pharmaceutical potential. Continuing research on plants from these forests may eventually prove that one or more chemical compounds that are still hidden within the plant diversity inside this park may actually have potential for development toward therapy against one of the diseases for which we are now seeking a cure (cancer, AIDS, malaria, tuberculosis).

It is an accepted fact that the process of deforestation in the tropics is threatening the continuing existence of many plant species from the tropical rain forests, some of which may possess the capacity to produce bioactive compounds which have potential for development toward a new medicine. The disappearance of such species signifies the loss of our hope in discovering the cure of diseases which continue to afflict mankind. The UIC ICBG program provides a model in which scientists with diverse scientific expertise located in developed and in developing countries successfully work together in exploring the economic potential of biological diversity. Such a successful partnership has also been demonstrated by other ICBG set-ups in other geographic regions (Kingston *et al.*, 1999; Timmermann *et al.*, 1999; Schuster *et al.*, 1999). In a post-Earth Summit era of sensitivity to the access to genetic resources of a country, a close collaboration between institutions in biodiversity-rich countries and institutions in biotechnology-rich countries assures trust and goodwill, thus, facilitating the access to the genetic resources for scientific study and for biological evaluation toward their medicinal potential.

It is a well known fact that the substantial income that is generated as a result of the discovery and successful development of a new drug takes a long time to come (10-15 years), and in the majority of cases of newly discovered new compounds, it never comes. The ICBG program and research process offset such uncertainty and the false expectation it brings by actually providing direct and real-term benefits to the collaborating host country institutions and communities. These benefits come in the form of real research funding support, capacity building of the scientific personnel, strengthening of research facilities of colleagues, enrichment of the scientific contents of host country institutions, support for improving the standard of living of communities participating in the ICBG research process, and support for promoting and improving biodiversity conservation measures in the host country. In the case of this UIC ICBG, the benefits of the program in improving the standard of living of the communities and in conservation measures at the Cuc Phuong National Park have begun to be seen, which proves the point that drug discovery, economic growth and biodiversity conservation can successfully be linked.

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