Novel Strategies for the Discovery of Plant-Derived Anticancer Agents

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Kinghorn, A.D.; Farnsworth, N.R.; Soejarto, D.D.; Cordell, G.A.; Swanson, S.M.; Pezzuto, J.M.; Wani, M.C.; Wall, M.E.; Oberlies, N.H.; Kroll, D.J.; Kramer, R.A.; Rose, W.C.; Vite, G.D.; Fairchild, C.R.; Peterson, R.W.; and Wild, R. (2003) Novel Strategies for the Discovery of Plant-Derived Anticancer Agents *Pharmaceutical Biology*, 41 (Supplement), 53–67.

Abstract:

Work has continued on the investigation of plants, collected mainly from tropical rainforests, as potential sources of new cancer chemotherapeutic agents. About 400 primary samples are obtained each year, with the chloroform-soluble extract of each being screened against a battery of *in vitro* assays housed at the three consortial sites in our current National Cooperative Drug Discovery Group (NCDDG) research project. An HPLC-MS dereplication procedure designed to screen out "nuisance" compounds has been refined. Several hundred secondary metabolites that are active in one or more of the primary assays utilized have been obtained in the project to date, and are representative of wide chemical diversity. Some of these are also active in various *in vivo* assays, inclusive of the hollow fiber assay, which was installed recently as part of our collaborative research effort. A number of bioactive compounds of interest to the project are described. **Keywords:** Plants, secondary metabolites, anticancer activity, biological testing, activity-guided fractionation, dereplication.

Article:

Introduction

Cancer remains a major obstacle to overall public health, and is responsible for one in every four deaths in the United States. It was estimated by the American Cancer Society that in 2003 there would be 1,334,100 new cases of invasive cancer diagnosed, and 556,500 deaths from this cause. In addition, over one million additional people will be diagnosed with basal and squamous cell skin cancers (Jemal et al., 2003). However, as a contribution towards the chemotherapy of cancer, natural product secondary metabolites from plants and microbes in particular play a very important role in the amelioration of this group of diseases (Shu, 1998; Cragg & Newman, 2000; Cragg et al., 2000; Newman et al., 2000). In a recent review dealing, in part, with an analysis of the antineoplastic drugs available in western countries and Japan, of 140 compounds in total, a majority (54%) are either natural products (14%), natural product derivatives (26%), or compounds made by total synthesis, but modeled on natural product leads (14%) (Newman et al., 2003). Accordingly, there is a considerable scientific and commercial interest in the continuing discovery of new anticancer agents from all natural product sources, inclusive of plant secondary metabolites (Mann, 2002).

As one of several groups funded through the National Cooperative Drug Discovery Group (NCDDG) mechanism (Suffness et al., 1995; Cragg et al., 2000), the College of Pharmacy, University of Illinois at Chicago, Chicago, IL and the Natural Products Laboratory, Research Triangle Institute, Research Triangle Park, NC have collaborated for nearly 15 years. Our current industrial partner in this NCDDG project is Bristol-Myers Squibb, Pharmaceutical Research Institute, Princeton, NJ (1995–present). During the period 1990–1995, Glaxo Wellcome Medicines Research Center, Stevenage, U.K. were involved in our NCDDG program as the industrial partner. The major objective of our joint research program is the discovery of new antitumor agents from plants. At present, four classes of anticancer agents used clinically in the United States are plant-derived,

namely, the Vinca alkaloids, the epidodophyllotoxin, the taxanes, and the camptothecin derivatives (Newman et al., 2000), with two co-authors of this review having co- discovered camptothecin and paclitaxel (Wall & Wani, 1996; Wall, 1998). In the present review, the general botanical, biological testing, and phytochemical aspects of the program will be discussed in turn, followed by examples of bioactive compounds investigated recently. In prior literature contributions, our NCDDG program was reviewed in terms of both the organizational aspects and the plant secondary metabolite leads obtained (Cordell et al., 1993; Kinghorn et al., 1995, 1998, 1999).

Plant collections

The major focus of the plant collection aspects of our NCDDG program has been on plants from the world's tropical rainforests. We have chosen to work on tropical rainforest plants for several reasons. First, the tropical forests sustain considerable biodiversity, and, in some of these, more tree species are found in a 0.5 km^2 area than all of North America (Burslem, 2001). Second, there is justifiable concern about the impending loss of all tropical species, so some of these species may not be available to future generations of natural product drug discovery scientists (Cox, 2000). Thus, it has been estimated that of the total number of higher plants on earth (310,000 to 422,000), about 120,000 of these are tropical endemic species. Moreover, some biodiversity "hot spots" with high floristic diversity are undergoing major habitat loss (Myers et al., 2000; Pitman & Jørgensen, 2002).

In our collaborative work, initial specimens of about 400 plant samples are collected annually, mainly from tropical rainforest regions. Over the period 1990–2003, 5886 total plant accessions were obtained, from a total of 34 countries. These comprise 2528 species, 1358 genera, 213 families and represent an increasingly valuable project resource. In recent years, large proportions of our samples have come from Indonesia and Peru. Priority is given to species endemic in the country of collection, and, for many of these, there are no previous phytochemical reports available in the literature. Taxonomic authentification is carried out primarily in the countries where the collections are made, and are verified by one of us (D.D.S.) at the Field Museum of Natural History, Chicago. Plant collections are made only in countries where there is a previously negotiated, formal written collection agreement. Much time has been spent in formulating such "benefit sharing" policies over the years, in order that institutions in the source countries of the plants concerned receive equitable compensation, such as a fair share of any licensing agreement and/or royalty payments (e.g., Soejarto et al., 2002).

It has been the usual practice of our research program to collect up to four anatomical plant parts for each species, if readily available. In a recent investigation on the plant, *Picramnia latifolia* Tul. (Simaroubaceae), collected in Peru, both the roots and leaves were studied in detail by activity- guided fractionation, using two cancer cell lines to monitor purification. While two known anthraquinones were found in both plant parts, anthraquinone glycosides and benzanthrones were present only in the roots, and some new anthrone- and oxanthrone *C*-glycosides occurred exclusively in the leaves of *P. latifolia* (Díaz et al., 2004b). In a similar manner, different profiles of xanthone secondary metabolites were obtained from the leaves, stem bark, and twigs of *Cratoxylum sumatranum* Blume (Clusiaceae) collected in Indonesia, as described in more detail later in this review, together with some unusual, but inactive, anthraquinobenzophenones found only in the stem bark (Seo et al., 2002). Therefore, the chemical diversity available to the overall investigation can be enhanced if more than one plant part is collected (Seo et al., 2002; Díaz et al., 2004b). Even for the same plant part collected in the same country, the local conditions at the collection site appear to be important. For example, when samples of the bark of *Diospyros maritima* Blume (Ebenaceae) were collected from two distinct regions and habitats in Indonesia, different phytochemical profiles were encountered. One of these samples was collected in West Java at sea level, in which naphthoquinone dimers predominated, and the other obtained in Lombok Island at ca. 350 meters in altitude, where methoxylated and methylated naphthoquinone monomers were prevalent (Gu et al., 2003).

Previously, members of our group have utilized plot-based plant collection as a strategy in natural products drug discovery projects (Calderon et al., 2000; Soejarto, 2000). In addition to collecting samples from the tropical rainforests in the last few years, we have also performed a pilot project in which plot inventories have been established in three locations of hardwood forests in southern Florida, known as "hammocks". From these

collections, 71 species from 46 plant families were collected, and the extracts prepared from these species were subjected to biological screening. Appropriate local permission was obtained before the initiation of this study in 1999, and we agreed not to collect the bark and roots of any species of interest. A number of the species collected are native to Central America and the Caribbean region and were introduced into southern Florida as a result of previous hurricane activity (Lobo-Echeverri, 2003).

For promising leads, it is often necessary to obtain a plant recollection (1–5kg), for further biologial and chemical investigation. In the project to date, nearly 200 recollections have been made. Care is taken during recollections to obtain, as far as possible, the same plant part, at the same time of the year, and from the same geographical location as the initial collection. The plot-based approach to plant collection mentioned above makes it relatively easy to perform recollections of active plant leads, which are obtained from marked specimens within the plot, and serve as a living reference samples (Soejarto, 2000).

In order to guide the selection of future plant collections, a survey using the NAPRALERT^{SM} database has been conducted of plants used ethnomedically against cancer. Altogether, 350 species in over 280 genera and 100 families were listed that were not included in the earlier seminal series of reviews published in the journal *Lloydia* (now the *Journal of Natural Products*) by Jonathan Hartwell, entitled "Plants used against cancer: a survey" (Graham et al., 2000).

Biological testing

Our project has continued to evaluate the crude chloroform- soluble extracts of plants in a diverse battery of cell-based and mechanism-based *in vitro* assays, which are now performed mainly using 96-well plates. This approach has been described in previous reviews of our NCDDG program (Cordell et al., 1993; Kinghorn et al., 1995, 1997, 1999). A key aspect of the program is the need to select *in vitro* active leads for activity-guided fractionation, and this is accomplished through several strategies, including chemical and biological dereplication, which is described in a subsequent section. Active extracts for which there is no obvious chemical class of bioactive principle may be subjected to evaluation in an Oncology Diverse Cytotoxicity Assay panel at Bristol-Myers Squibb (Cui et al., 1999; Seo et al., 2002). Plant extracts with selective and potent cytotoxicity in this panel may then be evaluated directly in the P388 *in vivo* murine leukemia model (Rose et al., 1988; Peraza-Sânchez et al., 2000; Seo et al., 2002). P388 *in vivo*-active extracts that do not contain common plant secondary metabolites likely to be responsible for their activity are assigned for activity-guided fractionation with high priority. Once isolated in pure form, active compounds are evaluated in all of the *in vitro* assays available to the program. We have recently introduced the *in vivo* hollow fiber assay at the College of Pharmacy, University of Illinois at Chicago, and a summary of how this is used to select compounds for additional biological testing is presented in the following paragraphs.

The *in vivo* hollow fiber assay for the evaluation of potential anticancer agents was developed originally at the National Cancer Institute (Casciari et al., 1994; Hollingshead et al., 1995; Hall et al., 2000). Using this method, confluent layers of cells from a tumor model of interest are harvested, collected by centrifugation, and resuspended in the condi tioned medium at a concentration of 10^6 or 5 x 10^5 cells per ml. Polyvinylidine fluoride hollow fibers filled with cell suspensions are then incubated in 6-well plates overnight at 37'C in a 5% CO2 atmosphere. Female athymic NCI nu/nu mice, obtained from the National Cancer Institute-Frederick Cancer Research Facility (Frederick, MD) are then implanted with up to six hollow fibers cultured in one of two compartments, either intraperitoneal (i.p.) or subcutaneous (s.c.). For i.p. implants, a small incision is made through the skin and musculature of the dorsal abdominal wall of the mouse, with the fibers inserted into the peritoneal cavity in a craniocaudal direction, and the incision closed with skin staples. In the case of the s.c. implants, a small skin incision is made at the nape of the neck to allow the insertion of an 11 -gauge tumor impant trochar containing the hollow fibers. This trochar is inserted caudally through the subcutaneous tissues, with the fibers then deposited during the withdrawal of the trochar, and the incision is closed with skin staples (Hollingshead et al., 1995; Mi et al., 2002c). The test mice are then randomized into control groups and compound treatment groups, with test compounds administered by i.p. injection from days 3–6 after implantation. Body weights are determined daily. On day 7, mice are sacrificed, and the fibers are retrieved.

The fibers are then placed into 6-well plates, with each well containing culture medium and allowed to equilibrate for 30 min at 37'C. The viable cell mass contained within each hollow fiber is determined with a MTT [3-(4,5-demethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] dye conversion method. After incubating suitable aliquots of the culture medium and the MTT solution for 4 hours, the culture medium is removed, and 2.5% protamine sulfate solution added, with the plates stored at 4°C for 2–4h. To assess the optical density of the samples, fibers are transferred to 24- well plates, cut in half, and dried overnight. Formazan is then extracted from each sample with DMSO for 4 h on a rotation platform. Aliquots of the extracted formazan are then transferred to individual wells of 96-well plates and assessed for optical density at 540 nm. The effect of the treatment regimen is determined by the net growth percentage of cells relative to changes in body weight (Hollingshead et al., 1995; Mi et al., 2002c).

The feasibility of using this *in vivo* hollow fiber assay as a secondary biological discriminator to select promising *in vitro*-active natural products for additional *in vivo* biological testing has been examined in some detail. Growth conditions have been established for over ten cancer cell lines that can be implanted into the i.p. and s.c. compartments of athymic mice. Several model cytotoxic agents were evaluated in this test system, and this method can provide a preliminary indication of the potential therapeutic efficacy of natural products quickly and at a relatively low expense, with comparatively small amounts of test compound required (e.g., 25 mg). Compounds which give positive responses at the s.c. site seem to be of more importance for follow-up testing than those showing activity only at the i.p. site in the *in vivo* hollow fiber assay (Mi et al., 2002c).

To exemplify the use of this assay, two compounds will be considered from our NCDDG program. The first of these is 13-hydroxy-15-oxozoapatlin, which was obtained initially in our program as a new compound from the root bark of the plant, *Parinari curatellifolia* Benth. (Chrysobalanaceae), collected in Zimbabwe (Lee et al., 1996). A larger quantity of this broadly cytotoxic compound was provided from a collaborative isolation study on *Parinari sprucei* Hook. f., collected in Venezuela (Braca et al., 2004). Thus, 13- hydroxy-15-oxozoapatlin was examined in the hollow fiber model at doses of 25, 50, 75, and 100mg/kg, after it was shown to be cytotoxic toward cultured KB (nasopharyngeal), LNCaP (human hormone-dependent prostate), and Lu1 (human lung) cells, with ED50 values of 1.2, 1.5, and 5.1 mg/ml, respectively. It showed more than 50% growth inhibition with KB and LNCaP cells (up to 68.7 and 87.7% respectively at the i.p. site, but no significant growth inhibition was observed with Lu1 cells (Fig. 1, panel A). There was no evidence of activity at the s.c. site for any of the cell types studied. In addition, this compound produced no indication of body weight loss in the hollow fiber assay, suggesting that it would be worthy of additional biological testing in an *in vivo* model (Braca et al., 2003). Mechanistically, 13-hydroxy15-oxozoapatlin was found to be a G2 DNA damage checkpoint inhibitor and to exhibit antimitotic activity (Rundle et al., 2001).

The second compound chosen as an example after evaluation in the hollow fiber assay is plumbagin, which was isolated in our investigation of the bark of *Diospyros maritima*, collected in Indonesia (mentioned above). Plumbagin was the most potent cytotoxic naphthoquinone found in our study (Gu et al., 2003), and is well-known as a bioactive principle of many medicinal plants (Shen et al., 2003). This compound was shown to be cytotoxic towards cultured KB, LNCaP, and Lu1 cells with ED50 values of 0.1, 0.8, and 0.3 mg/ml, respectively. Accordingly, plumbagin was evaluated in the hollow fiber assay against these same three cell types at doses of 1.25, 2.5, 5.0, 7.5, and 10 mg/kg. However, the two highest doses were lethal to one or more mice, and a dose of 5 mg/kg caused significant weight loss. Since no significant growth inhibition was observed at either the i.p. or the s.c. sites with KB, LNCaP, and Lu1 cells for all of the non-toxic doses (Fig. 1, panel B), this compound is not regarded as a very promising lead for further development as a cancer chemotherapeutic agent (Gu et al., 2003).

Providing compound quantities permit, and the compounds elicit sufficient interest from the project senior investigators after evaluation in the Bristol-Myers Squibb Oncology Diverse Cell Assay and/or the hollow fiber assay at UIC, pure plant-derived compounds found to be active are then evaluated in the P388 *in vivo* assay at B-MS, using both the i.p. and s.c. routes of administration (Rose et al., 1988; Peraza-Sánchez et al., 2000; Seo

et al., 2002). Other *in vivo* test systems that may be used are the M109 murine lung carcinoma and the HCT116 human colon carcinoma models (Cui et al., 1999).

For active compounds of particular interest to the program, we conduct preliminary mechanistic studies on cell-cycle arrest and apoptosis induction (e.g., Cui et al., 1999). In addition, compounds may be submitted to the National Cancer Institute for mechanism of action evaluation in the 60 cell-line panel via COMPARE analysis (Lee et al., 1994). More detailed mechanism of action work of active compounds isolated in our NCDDG project has also been carried out at the University of Illinois at Chicago. For example, the common plant triterpenoid, betulinic acid, was isolated several years ago in our work on *Ziziphus mauritiana* Lam. (Rhamnaceae), and found to exhibit melanoma-specific cytotoxicity caused by the induction of apoptosis, and to exhibit an antitumor effect for human melanoma xenografts carried by athymic mice (Pisha et al., 1995). Recently, continued mechanistic studies have shown that treatment of UISO-Mel-1 human melanoma cells with betulinic acid leads to the activation of p38 and stress-activated protein kinase/cJun NH2-terminal kinase (Tan et al., 2003).

Phytochemical procedures

For several years, we have used a simple solvent extraction scheme for all new primary plant collections. Thus, a small quantity (20 g) of each dried and powdered plant is extracted into methanol-water (9: 1), then defatted with hexanes. The resultant aqueous methanol partition is then extracted with chloroform. Each chloroform extract is then washed with 1% sodium chloride, which enables it to be relatively free from plant polyphenols, which are known to interfere with certain *in vitro* bioassays (Wall et al., 1996). Bioactive compounds are isolated by activity-guided chromatographic fractionation using standard techniques, and their structures are determined by spectral data interpretation. When the need arises, the compound structures are confirmed using single-crystal X-ray crystallography (e.g., Ito et al., 2000; Chávez et al., 2001). To facilitate the determination of absolute stereochemistry of natural product isolates containing a secondary hydroxyl group, a convenient technique has been developed recently at the University of Illinois at Chicago. This involves preparing Mosher esters directly in NMR tubes, which are then directly measured, thus obviating the need to purify these derivatives chromatographically (Su et al., 2002).

To avoid reisolating known bioactive compounds as far as possible, we have used a LC-MS dereplication procedure in our NCDDG program for several years (Constant & Beecher, 1995; Cordell et al., 1997; Cordell & Shin, 1999; Kinghorn et al., 1999). Previously, we developed an integrated on-line LC-MS-bioassay protocol combined with database searching, which enabled the molecular ions of cytotoxic compounds to be detected. In this manner, it is possible to rank extracts for subsequent activity-guided fractionation, with a higher priority accorded to extracts for which the active region of a chromatogram is not associated with any known cytotoxic compound. On the other hand, when there is a high

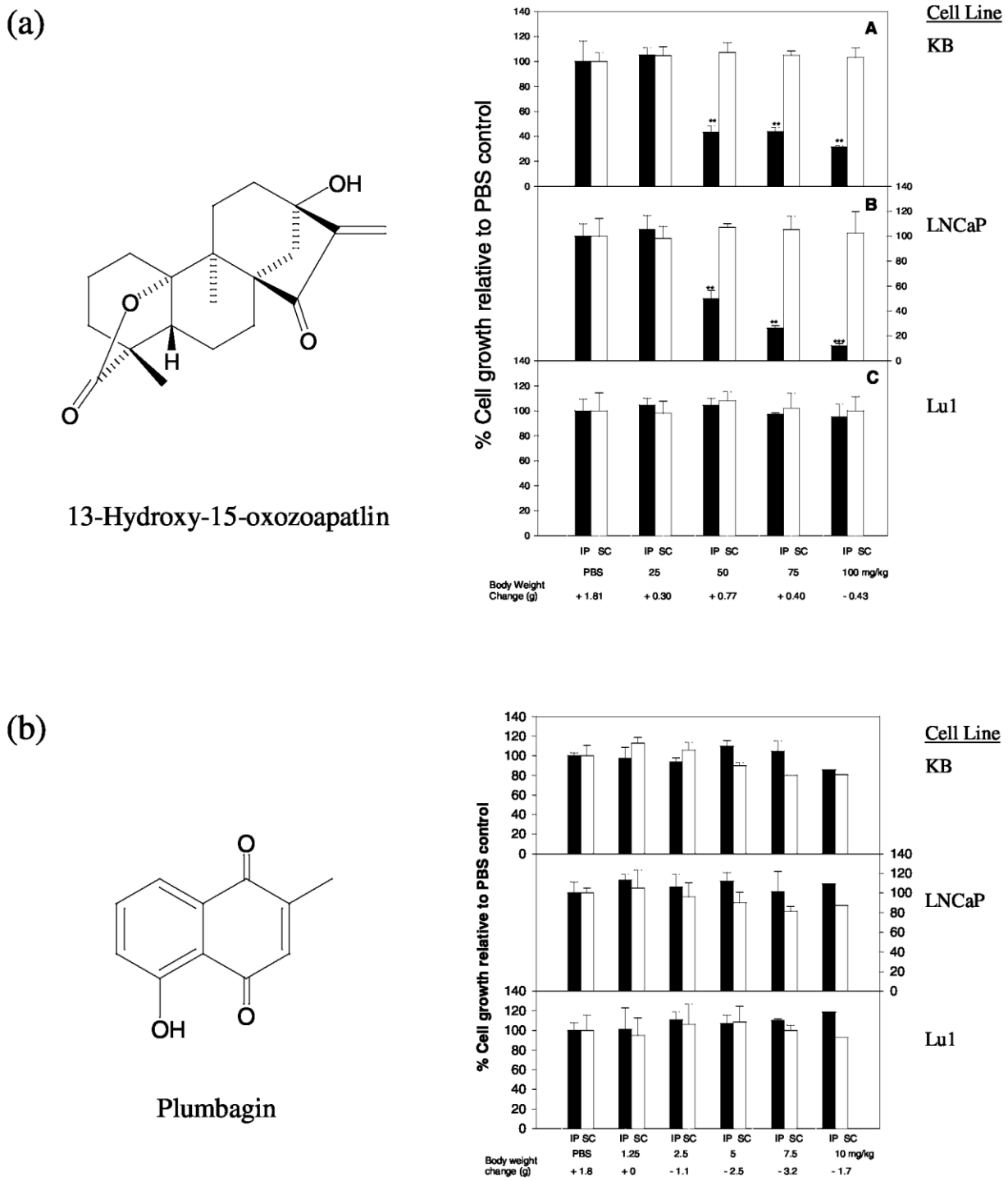


Figure 1. Effects of 13-hydroxy-15-oxoxapatlin and plumbagin on the growth of KB, LNCaP, and Lu1 cells in the hollow fiber assay.

probability that a known active compound is responsible for the cytotoxicity observed, extracts of this type are given a lower priority for phytochemical fractionation (Cordell et al., 1997; Kinghorn et al., 1999).

A new off-line dereplication system has been developed in the last two years, and is comprised of separate fractionation, bioassay, and mass spectral analytical steps. In brief, extracts that have shown bioactivity in preliminary screening are chromatographed by reversed-phase HPLC using a standard 30-minute gradient, and fractions distributed onto 96- well plates. One or more of these plates is submitted for biological testing in the same assay in which activity is shown initially, and a duplicate plate serves as a fraction library for the LC-ms

analysis of the wells corresponding to the active fractions. An Agilent Series HPLC-MSD instrument, with an auto-sampler, an electrospray ionization (ESI) source, a single quadrupole analyzer, and a photodiode array detector is used. Database searching primarily with the NAPRALERT^{SM} and SciFinder® Scholar databases, for reported cytotoxic compounds, using taxonomic restrictions (usually in the same family, genus, and/or species), yields a list of possible matches. Masses measured for the active fractions are compared with the masses of reported cytotoxic compounds. If the active compounds match one another, the extract under consideration is given a low priority for subsequent activity-guided fractionation. This new method allows greater convenience and flexibility than previously, including the capability to perform accurate mass and MS/MS determinations (Jones et al., 2003).

As part of a method-validation study, this procedure was used to evaluate separate extracts of the leaves and twigs of an undetermined *Elaeocarpus* species collected in Indonesia. As a result, the known cytotoxic agents cucurbitacins D and F were determined as being present in both extracts, so this species was not selected for phytochemical study. It was also deemed unnecessary to identify this plant acquisition to the species level (Jones et al., 2003). We have previously examined in some detail the cucurbitacin and ellagic acid constituents of *Elaeocarpus mastersii* King (Elaeocarpaceae) (Ito et al., 2002).

Examples of bioactive compounds recently isolated and evaluated

The structures of a number of active compounds isolated and characterized in our recent work in our NCDDG project are shown in Figure 2. The examples shown have been chosen to complement those included in our most recent review on this project (Kinghorn et al., 1999).

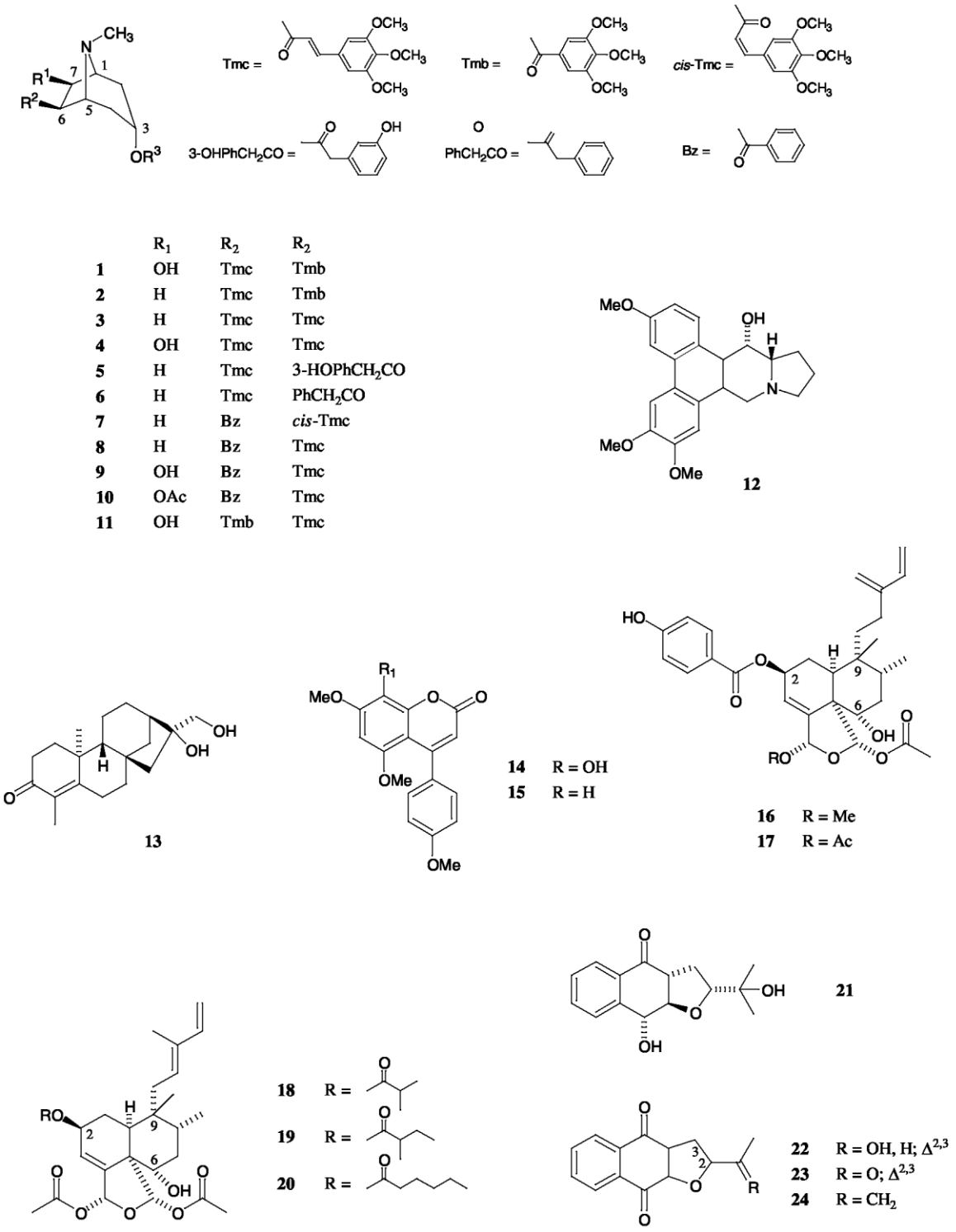
Alkaloids-tropane

As a result of the bioactivity-guided fractionation of a chloroform-soluble extract of the roots of *Erythroxylum pervillei* Baillon (Erythroxylaceae), collected in southern Madagascar, nine tropane alkaloid esters were isolated and characterized; seven of these were found to be new alkaloids. Six of the new compounds (pervilleines A–F, **1**–**6**) were found to reverse multidrug resistance (MDR), using a KB-V1 (vinblastine-resistant oral epidermoid carcinoma) cell line in the presence of vinblastine. The parent alkaloid, pervilleine A (**1**), was assigned structurally as 3a-(3,4,5-trimethoxybenzoyloxy)-60-(*E*)-(3,4, 5-trimethoxycinnamoyloxy)-70-hydroxytropane. Like pervilleine A (**1**), pervilleines B-F (**2**–**6**) also bear a trimethoxycinnamate ester group at position C-6, but exhibit minor structural variations in the nature of the aromatic ester unit affixed to C-3, and may or may not be hydroxylated at C-7. All of these six compounds were much less cytotoxic for normal KB cells and the other cancer cell lines in the UIC tumor panel than for KB-V1 cells in the presence of vinblastine; although some were more broadly cytotoxic than others. Confirmatory results were obtained for several of the pervilleines with MDR-resistant SKVLB human ovarian adenocarcinoma cells in the presence of adriamycin, and in a synergistic study performed with several cell lines from the NCI tumor panel (Silva et al., 2001). The *N*-oxide derivative of pervilleine A was also isolated in this investigation, but was shown to be devoid of inhibitory effects for either the KB-V1 or any of the other cell lines in the tumor panel (Silva et al., 2001).

Pervilleine A (**1**) was examined in more depth mechanistically, and was found to restore the vinblastine sensitivity of cultured KB-V1 and CEM/VLB100 (multidrug-resistant human leukemic lymphoblast CEM) cells with IC50 values of 0.36 and 0.02µM, respectively. In a similar manner, the chemosensitivity to colchicine of the KB-8-5 drug-resistant cell line, was restored by pervilleine A, with an IC50 value of 0.61 µM. Pervilleine A (**1**) inhibited competitively the binding of [^{3}H]vinblastine with isolated KB-V1 cell membrane vesicles. The effect of pervilleine A was then studied in the hollow fiber test, as described briefly as follows. It was established earlier in preliminary growth assays that a suitable concentration of hollow fibers for KB-3 (non-drug resistant) cells and KB-V1 cells was 7.5 x 10^{5} cells per ml. Also, it was determined that a dose of 150 µg/kg of vinblastine inhibited the growth of KB-3 cells without influencing KB-V1 cells, and that neither KB-3 nor KB-V1 cells were sensitive to the effects of pervilleine A at a dose of 0.136 mmol/kg. However, when vinblastine was co-administered with pervilleine A at these concentrations, a ca. 75% growth inhibitory effect was observed with KB-V1 cells implanted at the i.p. site. Equimolar doses under the same conditions of verapamil, a standard MDR inhibitor, were less effective than pervilleine A. This study was the first use of the

hollow fiber assay to evaluate MDR-reversing agents in combination with a cancer chemotherapeutic agent, and it was concluded that pervilleine A is an effective inhibitor of P-glycoprotein (Mi et al., 2001). The *in vitro* P-glycoprotein activities of pervilleines B (**2**), C (**3**), and F (**6**) were confirmed in the *in vivo* hollow fiber assay, when co-administered with vinblastine, in the same manner as described for pervilleine A (**1**) (Mi et al., 2002b, 2003). All four compounds showed similar levels of reversing activity in this assay, but it was noted that pervilleine F (**6**) did not cause any test animal body weight loss, in contrast to **1**–**3**, where slight body weight losses were observed (Mi et al., 2001, 2002b, 2003).

The stems of a second species in the genus *Erythroxylum* were collected in the Dominican Republic, and four additional tropane alkaloid aromatic esters were isolated from *E. rotundifolium* Lunan with the ability to reverse the multidrug-resistance phenotype (compounds **7**–**10**). Of these, alkaloids **8**–**10** were already known, and the new alkaloid **7** had an unusual *Z*-3,4,5-trimethoxycinnamyl ester unit affixed to C-3. Moreover, a second new alkaloid, **11**, was isolated from *E. robustifolium*, which is identical in structure to



	R_1	R_2	R_2
1	OH	Tmc	Tmb
2	H	Tmc	Tmb
3	H	Tmc	Tmc
4	OH	Tmc	Tmc
5	H	Tmc	3-HOPhCH$_2$CO
6	H	Tmc	PhCH$_2$CO
7	H	Bz	*cis*-Tmc
8	H	Bz	Tmc
9	OH	Bz	Tmc
10	OAc	Bz	Tmc
11	OH	Tmb	Tmc

Figure 2. Structures of bioactive compounds isolated from plants.

pervilleine A (**1**) in all respects except that the C-3 and C-6 aromatic ester substituents are interchanged. However, compound **11** proved to be broadly, but weakly, cytotoxic for the UIC tumor panel, with its specificity lost for the drug- resistant KB-V1 cell line (in the presence of vinblastine) (Chávez et al., 2002).

The pervilleines (A–C and F, **1**–**3** and **6**) are prominent among the compounds discussed in this review, since they are novel inhibitors of the MDR phenotype. In our recent work, it has been shown that these four aromatic

tropane alkaloids mediate potent activity with several *in vitro* and *in vivo* models, and that they meet the general structural

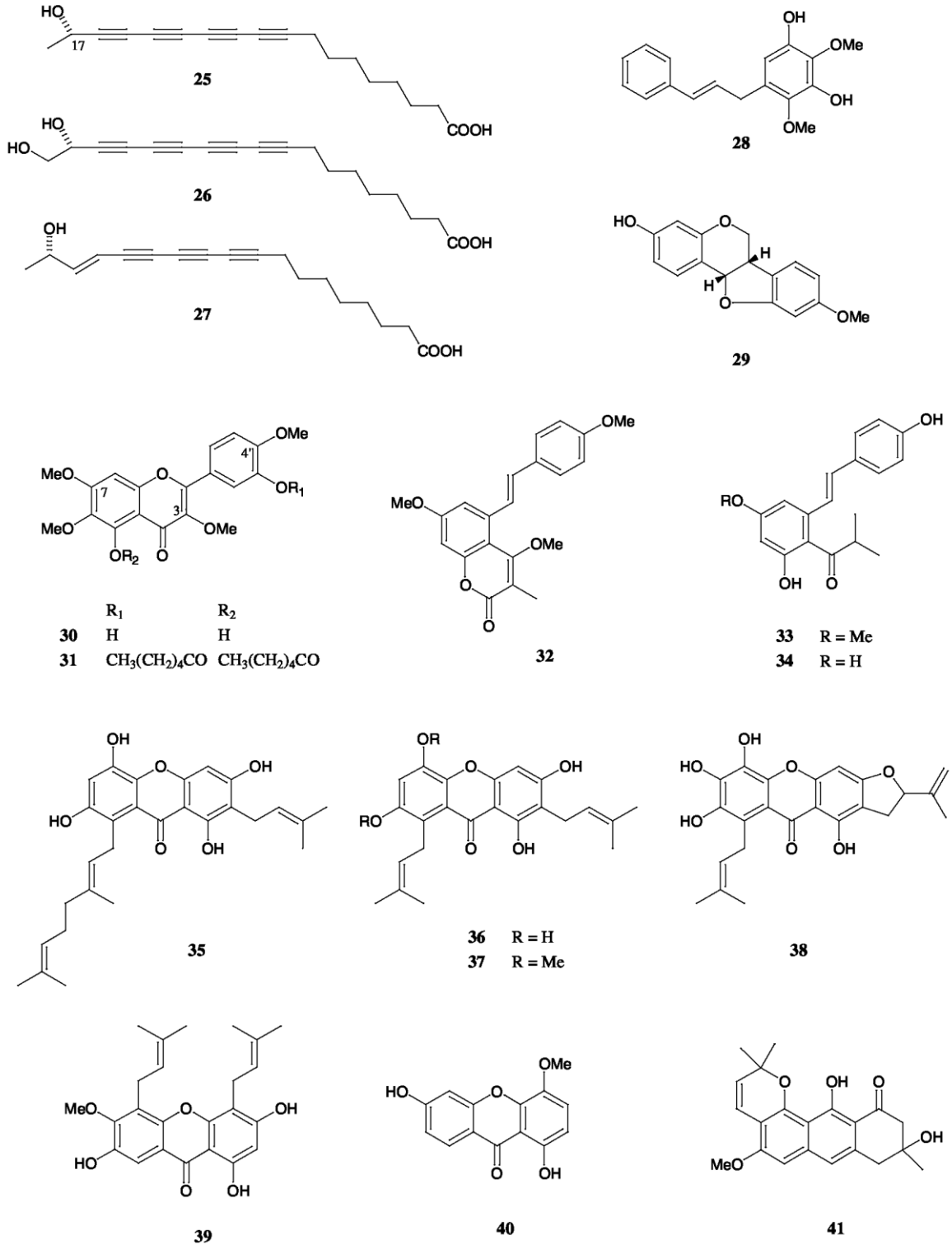


Figure 2. Continued

requirements of other MDR modulators in possessing planar aromatic rings and a nitrogen atom (Zamora et al., 1988; Mi et al., 2002a). Analysis of all of the MDR-inhibitory and inactive tropane alkaloid esters obtained from both *E. pervillei* and *E. robustifolium* has permitted a preliminary notion of the structural requirements needed for selective cytotoxicity for the KB-V1 cell line in the presence of vinblastine. Thus, optimal activity is

shown when there is an *E*-3,4,5-trimethoxycinnamyloxy (Tmc) *exo* substituent at C-6. A benzoate (Bz) ester is also permissible at this same position, but not a 3,4,5-trimethoxybenzoyl (Tmb) unit (Mi et al., 2002a). There was very little difference in the cytotoxicity profiles of compounds **7** and **8**, so the *trans*- or *cis*- stereochemistry in the Tmc ester unit appears to be of little biological consequence in this regard. There seems to be considerable structural latitude in the types of groups that can be substituted at C-3 ((x configuration) if a C-6 Tmc *exo* unit is present. MDR reversal persists with any of a Tmc, a Tmb, a phenylacetyl, or a 3-hydroxyphenylacetyl unit. An *exo* OH group at C-7 is also permitted, as in pervilleine A (**1**), but may be absent, as in pervilleines B (**2**), C (**3**), and F (**6**). On further probing their *in vitro* MDR modulating effects, pervilleines A–D (**1**–**4**) and F (**6**) were found to enhance the intracellular accumulation of a fluorescent dye, rhodamine 123, in drug-resistant MDR KB-V1 cells, as confirmed by visualization using confocal microscopy (Mi et al., 2002a). However, it was found that when ClogP and molecular refractivity data were determined for selected tropane alkaloids from *E. pervillei* and *E. robustifolium*, these values correlated only weakly with the propensity of these alkaloids to reverse MDR (Mi et al., 2002a).

In a more recent *in vitro* study performed on pervilleine F (**6**), this alkaloid was shown, at a non-toxic dose of 8 µM, to partially reverse the cross-resistance of clinically used or experimental anticancer agents, as follows: actinomycin (45.1-fold), baccatin III (>3.4-fold), daunomycin (>22.5- fold), ellipticine (1.9-fold), mithramycin A (42.5-fold), podophyllotoxin (1.6-fold), paclitaxel (32.2-fold), and vincristine (73.6-fold). No effect was observed with etoposide. The comparable figures for verapamil, when tested at the same dose, were: actinomycin D (82.2-fold), baccatin III (>5.9-fold), daunomycin (>32.8-fold), podophyllotoxin (2.1-fold), paclitaxel (20.8-fold), and vincristine (88.3-fold). In this case, no effects were found with ellipticine, etoposide, and mithramycin A. These evaluations were performed by determining the IC50 ratios of drug-resistant KB-3 cells and normal KB-3 cells. While generally potent in reversing cross- resistance to many anticancer agents, it may be pointed out that ellipticine, etoposide, and podophyllotoxin interact with topoisomerase II, an atypical multidrug resistance, rather than P-glycoprotein (Mi et al., 2003).

Alkaloids – phenanthoindolizidine

Bioassay-directed study of the leaves and twigs of *Ficus hispida* L. f. (Moraceae), collected in Thailand, led to the isolation of a potent cytotoxic agent, *O*-methyltylophorinidine (**12**), a phenanthroindolizidine alkaloid of previously known structure (Govindachari et al., 1974). This alkaloid was highly cytotoxic, exhibiting ED50 values in the Col2 (human colon), KB, Lu1 (human lung), and LNCaP (human hormone-dependent prostate) cancer lines of 0.02, 0.02, 0.018, and 0.03 µg/ml, respectively. Unfortunately, compound **12** was isolated in too small an amount for follow-up biological testing (Peraza-Sânchez et al., 2002). Recently, Staerk and associates have also pointed to the high cytotoxic potency for cancer cell lines of several phenanthroindolizidine alkaloids in the low nanomolar range, and have suggested that the known neurotoxicity and other side effects of these alkaloids could be overcome by modern tissue- specific, drug targeting techniques (Staerk et al., 2002).

Diterpenoids – norkaurane

Exostema acuminata Urb. (Rubiaceae) is native to Central America and northern South America, and a sample of the roots was collected in the Dominican Republic. Activity-guided fractionation using the KB cell line led to the isolation of a new norditerpenoid, (16*S*)-*ent*-16,17- dihydroxy-19-nor-kaur-4-en-3-one (**13**), found to be weakly cytotoxic, with the best activity being against the LNCaP (hormone-dependent human prostate cancer) cell line (ED50 4.8 µg/ml). The structure of this compound was assigned as the 16*S* epimer of another new norditerpenoid, whose structure was determined by single-crystal X-ray crystallography [16*R*)-*ent*-16,17-dihydroxy-19-nor-kaur4-en-3-one (**13**)], which was not cytotoxic. In addition to these two compounds, six broadly cytotoxic phenylisocoumarins of previously known structure were isolated from *E. acuminata* roots. The two most abundant of these, 8-hydroxy-5,7,4'-trimethoxy-4-phenylcoumarin (**14**) and 5,7,4'-trimethoxy-4-phenylcoumarin (**15**), were evaluated in an *in vivo* P388 murine leukemia model (i.p.) at Bristol-Myers Squibb, but neither compound was deemed active, when evaluated at 135 and 24mgkg$^{\sim 1}$ per injection, respectively (Ito et al., 2000).

Diterpenoids – clerodane

Fractionation of the roots of *Licania intrapetiolaris* Spruce ex Hook. (Chrysobalanaceae) and of the leaves and stems of *Casearia sylvestris* Sw. (Flacoutiaceae), both collected in Ecuador, led to the isolation and characterization of five new clerodane-type diterpenoids, intrapetacins A (**16**) and B (**17**), and casearvestrins A-C (**18–20**). Although fundamentally very similar molecules, these diterpenoids differ in their type of C-20-substituted ester group and the C-9-affixed side-chain (Oberlies et al., 2001, 2002). Intrapetacins A and B were only moderately cytotoxic to the KB cell line, and were accompanied by the widely distributed plant cytotoxic agent, cucurbitacin B, in their plant of origin (Oberlies et al., 2001). The three clerodane diterpenoids from *C. sylvestris* were also shown to be active in the KB test system at RTI, and were then evaluated against the LX-1 (human lung), HCT116 (human colon), and A2780 (human ovarian) cell lines at B-MS, in which they exhibited comparable IC50 values for each cell line between 0.20 and 0.82µM. These compounds were not tested further because of the limited quantities available (Oberlies et al., 2002). When compared structurally against caesarins A-R isolated from other *Casearia* species earlier, compounds **18–20** would be representative of a group of compounds in a second tier of cytotoxic potency, and thus expected to be more potent cytotoxic agents if their C-6 P-hydroxy group were absent in each case (Itokawa and Takeya, 1993; Oberlies et al., 2002). Recently, other groups have also isolated and characterized cytotoxic clerodane diterpenoids from additional *Casearia* species (Beutler et al., 2000; Prakash et al., 2002).

Naphthoquinones

Along with three dihydronaphthalenone derivatives with new structures, which were not found to be biologically active in our work, four cytotoxic naphthoquinones of known structure (**21–24**) were isolated from the roots of another plant collected in the Dominican Republic, *Ekmanianthe longiflora* (Griseb.) Urb. (Bignoniaceae). One of these bioactive compounds, 2-(1 -hydroxyethyl)naphtho[2,3-*b*]furan-4,9- quinone (**21**) was found to be broadly cytotoxic in the UIC cancer cell panel (ED50 range 0.2–3.5 gg/ml). It was then evaluated in the 25-cell line Oncology Diverse Cell Assay at Bristol-Myers Squibb, representing a diverse group of murine and human tumors, fibroblasts, and normal bovine endothelial cells, and found to be active, with a mean IC50 value of 2.9gM. However, when subsequently evaluated in the P-388 *in vivo* system, injected i.p. at 72mgkg~[1], the com- pound was inactive (T/C !!9125%) (Peraza-Sânchez et al., 2000). This compound was found to be a cytotoxic agent for KB cells by Rao and Kingston, but it was not tested *in vivo* at that time (Rao & Kingston, 1982).

Polyacetylenes

The twigs of the southeast Asian tree, *Ochanostachys amentacea* Mast. (Olacaceae) were investigated, after a chloroform-soluble extract was found to exhibit significant cytotoxic activity when evaluated against the UIC tumor cell culture panel. Bioassay-guided fractionation using the LNCaP (human hormone-dependent prostate cancer) cell line, led to the isolation of three cytotoxic principles, namely, the known polyacetylene, minquartynoic acid (**25**), and two new analogs, (*S*)-17,18-dihydroxy-9,11,13,15- octadecatetraynoic acid (18-hydroxyminquartynoic acid, **26**) and (*S*)-17-hydroxy-15*E*-octadecen-9,11,13-triynoic acid (*E*-15,16-dihydrominqurtynoic acid, **27**). In our work, the previously unresolved absolute stereochemistry of **25** was determined at C-17 as *S*, using the Mosher ester method (Ito et al., 2001). At the same time as our work was in progress, Rasmussen et al. (2000) independently determined the C-17 stereochemistry of **25** by its chemical conversion to the known (*S*)-17-hydroxystearic acid methyl ester. Compound **25** showed broad but weak cytotoxic activity in the UIC tumor cell panel, with compound **26** exhibiting a general decrease in cytotoxic potency relative to **25**. Of the three compounds, **27** was the most potently cytotoxic for the KB (oral epidermoid carcinoma), LNCaP, and SW626 (human ovarian) cell lines (ED50 range 0.30–1.1 gg/ml). The supplies of compounds **25–27** were too small for the additional biological testing of these polyacetylenes from *O. amentacea* collected in Indonesia (Ito et al., 2001).

Phenols

Machaerium aristulatum (Spruce ex Benth.) Ducke is a member of the legume family, which grows widely as a vine in the Amazon basin. This plant was collected in Peru, and activity-guided fractionation led to the isolation and purification of a new cinnamylphenol (macharistol, **28**) and a known pterocarpan (+)-medicarpin (**29**) as cytotoxic principles in the KB cell line. When compound **28** was evaluated in the *in vivo* hollow fiber assay at

UIC, it was deemed to be inactive at the highest dose tested (25 mg/kg body weight), using the KB, Col-2 (human colon), and hTERT-RPE1 (human telomerase reverse-transcriptase-retinal pigment epithelial) cells (Seo et al., 2001).

Flavones

Vitex negundo L. (Verbenaceae) is a small aromatic plant with extensive use as a folk medicine in south and southeast Asia, particularly as an anti-inflammatory agent (Panthong et al., 1986). The known flavone, vitexicarpin (casticin; 5,3'- dihydroxy-3,6,7,4'-tetramethoxyflavone, **30**) was found to be the only potent cytotoxic agent in a chloroform-soluble extract of *V. negundo* leaves collected in Indonesia. When evaluated in an 11-cell line Oncology Diverse Cell Assay at Bristol-Myers Squibb, this compound was found to exhibit good potency and a high selectivity against the K562 human leukemia cell line. Accordingly, several semisynthetic derivatives of compound **30** were made, and the new compound **31** exhibited similar cytotoxic profiles to the parent compound at both UIC and B-MS. However, compound **31** was inactive in both the hollow fiber assay at UIC (evaluated with KB, LNCaP, and Lu1 cells) and the P388 *in vivo* assay at B- MS (135mg/kg/injection, injected i.p.) (Díaz et al., 2003). The mechanism of action of compounds **30** and **31** was not studied in our program. However, Ko et al. (2000) have shown that vitexicarpin (**30**) induces apoptosis in human myeloid leukemia cells. Beutler and colleagues (1998) have shown that for polymethoxylated flavones, the maximal binding to tubulin occurs in compounds having hydroxyl groups at C-5 and C-3' and methoxyl groups at C-3 and C4', so it is likely that vitexicarpin (**30**) inhibits tubulin polymerization, as already noted by Ono et al. (2002).

Stilbenes

A methanol-soluble extract of the root bark of *Ekebergia benguelensis* C. DC. (Meliaceae), collected in Zimbabwe, led to the isolation of three new stilbenoids (**32–34**). The first of these, 5-[(1*E*)-2-(4-hydroxyphenyl)ethenyl]-4,7- dimethoxy-3-methyl-2*H*-1-benzopyran-2-one (**32**), was characterized as an interesting stilbene-coumarin hybrid molecule based on a novel carbon skeleton, and the structure was proposed by single-crystal X-ray crystallography. In turn, compounds **33** and **34** are derivatives of *trans*-resveratrol having an unusual isobutyryl substituent, with compound **33** being considered the biogenetic precursor of compound **32**. Compounds **32–34** were only weakly cytotoxic for one or two cell lines each, when evaluated in the tumor panel at UIC (Chávez et al., 2001).

Xanthones

During the course of our investigation of various Indonesian plants, six new xanthones, called cratoxyarborenones A-F (**35–40**), were isolated from various plant parts of *Cratoxylum sumatranum* Blume (Guttiferae), which were individually examined. Four of these xanthones, which bear one or more geranyl and/or prenyl units (**35**, **36**, **38**, and **39**) were isolated and characterized from the leaves, while compound **37** was found in the stem bark, and compound **40** in the twigs (Seo et al., 2002).

These compounds were evaluated against the KB cell line, and all of them were found to exhibit EC50 values in the range 1.0–4.3 mg/ml. Due to the availability of a sufficient amount of one of these compounds, cratoxyarborenone C (**37**) was evaluated in the 25 cell-line Oncology Diverse Cell Assay and then in the *in vivo* P388 murine leukemia system at B- MS. However, when tested at 72mg/kg/injection (i.p.), this compound was deemed inactive (Seo et al., 2002). Also isolated as a cytotoxic principle (EC50 1.3 mg/ml) in this investigation on *C. sumatranum* was the previously known anthracene derivative, vismione B (**41**). Vismione A, an acetylated derivative of compound **41**, was found by earlier investigators to exhibit *in vivo* activity against an ovarian carcinoma and the B16 melanoma models, but not the P388 system (Cassinelli et al., 1986). Two other compounds from *C. sumatranum*, representing a new structural class of geranylated anthraquinobenzophenones were also isolated and characterized, but these were not found to be cytotoxic for KB cells in our investigation (Seo et al., 2002).

Summary and conclusions

Although some have questioned the need to prospect the full diversity of nature in order to find leads for new drugs (Tulp & Bohlin, 2002), others have challenged this (Cragg & Newman, 2002). In the previous work in our multidisciplinary NCDDG project, we have attempted to maximize the plant diversity sampled, through collections in both tropical and temperate countries (Cordell et al., 1993; Kinghorn et al., 1995, 1998, 1999). It is our intention to develop plant collection agreements with as many new countries as reasonable, and new agreements have recently been signed with the Congo and Papua New Guinea. The plot collection approach described earlier in this review is worthy of additional investigation, and is particularly advantageous when it is necessary to organize plant recollections.

It has been apparent for some time that natural products can serve as excellent biological probes for cellular targets that are associated with cancer chemotherapy (Newman et al., 2002). It is to be noted that even previously well-known classes of plant-derived secondary metabolites with antineoplastic and/or cytotoxic activity, such as cucurbitacins (Blaskovich et al., 2003), *ent*-kaurene diterpenoids (Lee et al., 2002), naphthopyrones (Li et al., 2003), rocaglamide derivatives (Baumann et al., 2001), and simple bisindole alkaloids (Marko et al., 2001), have been demonstrated to act on specific cancer-related targets. Accordingly, there is value in the isolation and biological and mechanistic evaluation of known compounds along with the discovery of new potential antineoplastic agents from plants, since potent and specific biological probes may be elucidated.

Previously, betulinic acid was selected for preclinical development through the RAID (Rapid Access to Intervention Development) initiative of the National Cancer Institute (Cragg & Newman, 2000; Cragg et al., 2000). Pervilleines A–C (**1–3**) and F (**6**) are also being evaluated in the NCI RAID program, for which the work performed so far has involved the recollection of 50 kg each of the roots and stem bark of *Erythroxylum pervillei* from the original collection site in southern Madagascar. These materials have been sent to the National Cancer Institute at Frederick, Maryland, where these four pervilleines are being isolated in gram quantities each, and tested in a HT-15 colon carcinoma *in vivo* hollow fiber assay. In this manner, it is planned to select one of the pervilleines currently being investigated (i.e., either pervilleine A, B, C, or F) for further preclinical development.

Acknowledgements

This review is dedicated to our preeminent colleague and friend, Dr. Monroe E. Wall (1916–2002), who served as a powerful driving force in this project from its inception to his passing. We are particularly grateful to Dr. John M. Pezzuto, currently Dean, Schools of Pharmacy, Nursing, and Health Sciences at Purdue University for his outstanding contribution to this project while at the University of Illinois at Chicago. Dr. Qi Gao, Bristol-Myers Squibb Pharmaceutical Research Institute, Wallingford, Connecticut, is acknowledged for the X-ray crystallographic structure determination of 5-[(1*E*)-2-(4-hydroxyphenyl)ethenyl]-4,7-dimethoxy-3- methyl-2*H*-1-benzopyran-2-one from *Ekebergia benguelensis* and (16*R*)-*ent*-16,17-dihydroxy-19-nor-kaur-4-en-3-one from *Exostema acuminata*. We are very grateful to our botanist collaborators in several countries for their cooperation with the plant collections made. We also thank the many outstanding staff and faculty colleagues, postdoctoral associates, and graduate students who have participated in this collaborative research project, and whose names are indicated in the reference section below. The authors of this review wish to acknowledge, with many thanks, grant U19- CA52956 from the National Cancer Institute (NCI), National Institutes of Health, Bethesda, Maryland, as well as NCI contract support for the compound development through the RAID program.

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