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Anticancer Agents from Unique Natural Products Sources

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Abstract

The National Cooperative Natural Products Drug Discovery Group (NCNPDDG) "Anticancer Agents from Unique Natural Products Sources, CA 67786" was first awarded in September 1995. The goal of the project is to discover and develop novel anticancer agents from a variety of natural products sources. The key accomplishments of this NCDDG which will be highlighted in this manuscript include:

Development of tools to probe fungi for the production of novel natural products by DNA-based probes. Discovery that the majority of these fungi can produce natural products via nonribosomal peptide synthetases, polyketide synthases, or both – a much larger percentage than current culturing techniques reveal.

Identification of the MDR-selective cytotoxic agent austocystin D, and use of a novel yeast deletion strain approach to help identify its molecular target(s).

Identification of hemiasterlin and other naturally occurring analogs as potent antimitotic agents with excellent in vivo activity against human solid tumors in mouse models.

Development of a total synthesis of hemiasterlin. The utilization of this methodology to provide the first SAR for the hemiasterlin family of antimitotic agents and to identify the synthetic analog HTI-286, which is being examined in clinical trials as an anticancer agent.

To provided technology transfer, educational opportunities and compensation to countries of origin for collection and study of their natural product resources. This NCNPDDG program has provided funding to research programs at the University of the Philippines, The University of the South Pacific in the Fiji Islands, Colombo University in Sri Lanka, the Instituto de Quimica de Sao Carlos, Universidade de Sao Paulo, Brazil, and the University of Papua New Guinea.

Keywords: Natural Products, antitumor agents, NCDDG, sponges, fungi, marine microorganisms, hemiasterlins, austocystins.

Introduction

Anticancer Agents from Unique Natural Products Sources is a consortium of researchers from academia and industry organized in 1995 in response to a request for proposals by the National Cancer Institute to establish National Cooperative Drug Discovery Groups (NCDDGs). The goal of the

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consortium is to discover and develop novel anticancer agents from a variety of natural product sources. The underlying theme of this program is to integrate the discovery of novel biologically active natural products from organisms that inhabit unique ecological niches with mechanism-directed cancer biology. The rationale behind this approach is that chemical diversity stems from biological diversity and environmental pressures that select for unique genotypes. Also, new mechanism-based assays that target receptors or pathways that are over- or selectively expressed in cancer cells, will further select for novel chemotypes with potential utility in the treatment of human cancers. Figure 1 illustrates the current structure of the consortium. The basic units within the consortium have remained constant although some investigators within each of the units have changed. Additionally, the actual name of the industrial partner has changed twice during the lifetime of the program and one of the academic units moved from Cornell to Harvard. Professor Chris M. Ireland, University of Utah, is the Principal Investigator of the consortium and responsible for overall administration and coordination of effort between the programs and liaison with the NCI representatives Drs. Gordon Cragg and Yali Hallock. Dr. Ireland also directs a natural products drug discovery program focused on investigating the chemical diversity of marine invertebrate animals. Professor Jon Clardy, Harvard Medical School, directs a program to investigate the chemical diversity of plant- and insect-associated fungi. Professor Raymond J. Andersen, University of British Columbia, directs a program to investigate the chemical diversity of marine sponges. Drs. Guy T. Carter, Valerie S. Bernan and Michael Greenstein, Wyeth Research, direct a program to investigate the chemistry of marine microorganisms. They also serve as the interface for entry and tracking of NCDDG samples in Wyeth's High Throughput Screening system. Dr. Semiramis Ayral-Kaloustian, Wyeth Research, directs the medicinal chemistry team that provides synthesis and SAR support for the program. The centerpiece of the program is Wyeth's Oncology Group, under the guidance of Drs. Philip Frost and Lee Greenberger. The Oncology Group is responsible for all initial drug screening, development and validation of screening paradigms, and where appropriate, preclinical and clinical development studies.

Sampling natural products chemical diversity

As stated earlier, a fundamental tenet of the consortium is to integrate the discovery of novel chemical entities with mechanism-directed cancer biology. Part of the strategy to generate chemical diversity has been to focus natural products chemistry efforts on taxonomic groups that are underrepresented in the chemical literature and also organisms from unique habitats. In addition, as will be illustrated in detail later, we have employed a variety of molecular approaches to probe the genomes of microorganisms within our collections for the presence of biosynthetic pathways that make nonribosomal peptides and polyketides. To access diversity, the discovery programs have established collaborations worldwide. These collaborations are outlined in Figure 2. Dr. Ireland's program involves collaborations with the University of the Philippines and the University of the South Pacific in the Fiji Islands to study marine organisms from those countries. Dr. Andersen's program has similar arrangements with universities in Brazil, Sri Lanka and Papua New Guinea. Dr. Clardy's program involves collaborations with Dr. Donna Gibson at the USDA-ARS to access the ARS Entomopathogenic Fungi (ARSEF) Culture Collection and Dr. Alice Churchill at the Boyce Thompson Institute to screen organisms in the ARSEF collection for specific secondary metabolic pathway genes.

Intellectual property

The penning of the Convention on Biological Diversity (CBD) (UN Convention on Biodiversity) at the 1992 United Nations convention in Rio de Janeiro ushered in a new era

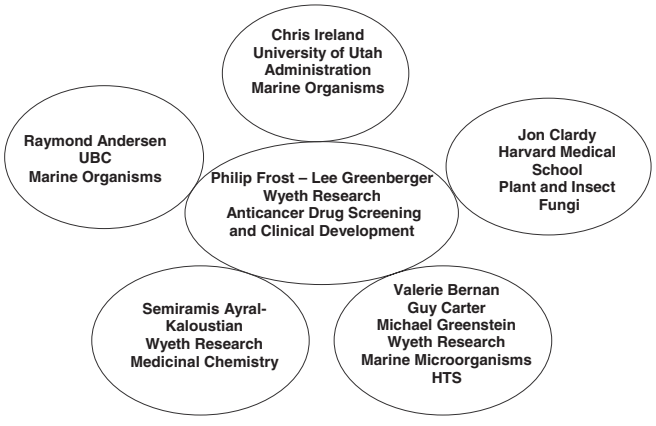


Figure 1. Organizational chart for the NCDDG "Anticancer Agents from Unique Natural Products Sources".

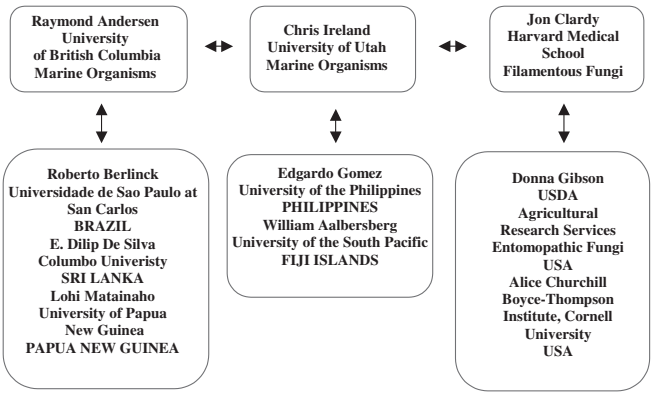


Figure 2. NCDDG collaborations established to access natural products diversity.

in natural products drug discovery and development. For the first time, the CBD called for recognition of the sovereign rights of countries to control utilization of their natural resources and genetic materials. The CBD also stipulated the importance of compensation to countries and indigenous populations for use of resources in the form of revenue sharing and technology transfer. Our consortium has actively embraced the principles of the CBD. The collaboration between the University of Utah (UU) and the University of the Philippines Marine Science Institute (UP-MSI) is presented here as an illustration of our approach to intellectual property (IP) and revenue sharing issues. At the outset of the program in 1995, an MOU was executed between UU and UP-MSI, which specified co-ownership of patents and equal sharing of revenues from IP. The foundation of this agreement, a requirement to validate co-authorship on patents, was that research had to be performed collaboratively at both institutions. The agreement also highlighted the importance of technology transfer and training opportunities for Filipino students and scientists. This agreement also recognized that funding was required to accomplish the goals of the MOU thus UP-MSI has been provided with an independent budget from NCDDG funds to support laboratory studies and scientific infrastructure. The agreement was renewed in 2003.

In 1997, the Philippines implemented new regulations regarding bio-prospecting, namely: 1) Executive Order EO 247: Prescribing Guidelines and Establishing a Regulatory Framework for the Prospecting of Biological and Genetic Resources, 2) Department Administrative Order DAO 96-20: Implementing Rules and Regulations of EO 247, and 3) DAO 97-27: Amendment of Section 15 (Transitory Provisions) of DAO 96-20 prohibiting issuance of gratuitous permits for bio-prospecting without a required academic/commercial research agreement. These new regulations mandated that a commercial research agreement (CRA) be obtained prior to any sample collection or bio-prospecting activity that involved a foreign collaborator. A CRA between the University of the Philippines-Marine Science Institute/University of Utah and a designated Philippine government agency, in this case, the Department of Agriculture-Bureau of Fisheries and Aquatic Resources (DA-BFAR) was signed in June 1998. This was the first CRA issued since the implementation of Executive Order 247 on bio-prospecting in the Philippines. To our knowledge this is one of only two CRAs that has been issued by the Philippine Government for marine bio-prospecting to a group outside of the Philippines. Our CRA was renewed in 2003.

Similar agreements and budgets have been established for all collaborators in the NCDDG consortium. The first fruits of these agreements has been a revenue stream to the University of Papua New Guinea and Papua New Guinea's Biodiversity and Conservation authority PNG-BioNET resulting from milestone payments under the licensing agreement between UBC and Wyeth to develop HTI-286 (**29**).

High throughput screening

The successful discovery of rare, novel therapeutic agents depends upon the rapid evaluation of large numbers of chemically diverse compounds in targeted, high-volume detection systems. All screens conducted at Wyeth employ state-of-the-art technologies that are compatible with high-throughput, automated strategies. Assays, conducted in either cell-free or whole-cell formats, monitor receptor-ligand interactions, enzymatic activities, or markers for cellular integrity. Receptor-binding, labeled ligands and enzyme substrates and their products are assayed in high throughput schemes using scintillation proximity assay (SPA) methodologies, luminescence, fluorescence, and spectrophotometric analyses. Promoter-luciferase reporter readouts or other types of reporter systems are commonly used in a number of Wyeth's cell-based assays. Yeast two-hybrid and other cellular assays are used to address protein-protein interactions. New approaches employing microfluidics and capillary electrophoresis are also being applied to screening efforts. Although automation is being utilized in many companies, Wyeth has the unique advantage of testing a wide variety of diverse compounds including natural products, as well as computerized data basing capabilities for the rapid correlative analysis of results from a large number of screening systems.

A centralized automation group develops robotic methods for the implementation of detection systems for new bioactive compounds useful in all therapeutic areas at Wyeth Research. This group operates in facilities at both the Pearl River, NY and Princeton, NJ sites. This venture has required extensive collaboration between the automation group and scientists throughout the company. Target selection, detection system development and screen validation are conducted within each therapeutic area. Robotic schemes for these screening systems are then developed by the automation group, and after further validation, they are implemented for the evaluation of our collections of compounds and natural products extracts. The emphasis is upon the performance of assays from beginning to end with minimal human intervention, and the continuing evolution of robots with increased capabilities will extend automation to many complex assay systems. The benefit of these robotic systems is that they permit the rapid evaluation of many samples in many assays simultaneously. The voluminous data generated from these assays are directly transferred to a NAPIS® Enterprise computerized database for analysis, storage, and retrieval. Further lead evaluation is conducted by the product area where the test system originated. However, semi-automated assays are always available to more fully evaluate samples, especially for dose titration and expanded profiling.

Screening paradigms

In 1995, the primary oncology screening model at Wyeth (then Cyanamid) was based on the use of a panel of human

solid tumor cell lines. The Cyanamid diverse 26 cell line screen was a refinement of the NCI Disease Oriented Tumor Cell Line Drug Screen (Monks, 1991). The NCI screen initially utilized a panel of 60 human tumor cell lines derived from seven cancer types (i.e., lung, colon, ovarian, leukemia, CNS, melanoma and renal), with their original impetus being to identify compounds with disease-specific activity (Boyd, 1989). Given the current view that resistance is a dominant factor responsible for the failure of existing cancer drugs (Gottesman, 2002), it may be more valid to screen compounds against cell lines with different resistance phenotypes rather than different tumor histotypes. This was one rationale employed by the Cyanamid screening program. A greater emphasis was placed on including human tumor cell lines that had been characterized for mechanisms of clinically-induced and/or intrinsic drug resistance (e.g., multidrug resistance, DNA repair, topoisomerase), in addition to including a diversity of tumor types. This diversity in resistance phenotypes is not reflected in screening models that rely on only one or a few cell lines or xenograft models. The rationale was that inclusion of clinically resistant cell lines would enable identification of compounds with activity against refractory cancers and would result in the selection of compounds with mechanisms of action different than previously identified using sensitive murine leukemia models.

Probably the most important result emanating from the NCI Disease Oriented Screen was the observation that compounds with similar mechanisms of action produced similar activity profiles against the 60 cell line screening panel (Boyd, 1989). The ability to predict structure and mode of action on the basis of pattern of activity was the essential feature of the NCI screen we wished to preserve. A criterion for selecting cell lines (outlined below) was established that enabled us to retain the prognostic capacity of the NCI screen while using far fewer cell lines.

Cell line criteria and selection

Diverse tumor types

Cancer is composed of more than 200 distinct diseases, each with its own etiology and pathology, which is most closely related to the tissue of origin. Therefore, 23 cell lines were selected that represent some of the more common solid tumor types (i.e., colon, lung, breast, prostate, ovarian, melanoma). Two human leukemia cell lines were included as a reference to help identify solid tumor selective compounds. A bFGF-dependent bovine endothelial cell line was also incorporated into the screen as a representative "normal" cell line, with the added potential benefit of facilitating the identification of compounds with selective anti-angiogenic properties.

Diverse drug-resistant phenotypes

The mechanisms of cancer drug resistance are numerous and multifactorial (Kramer, 1993; Kramer, 1988). Colon carcinomas are among the most resistant solid tumors, owing largely to the fact that these cancers are derived from the epithelial cells lining the intestine, a tissue exposed to high levels of ingested natural products and toxins. Thus colon carcinoma cells are a useful model for representing clinical cancer drug resistance (Kramer, 1993). We have characterized the intrinsic drug-resistant properties of more than 20 different human colon carcinoma cell lines (Kramer, 1993), and have included nine colon carcinoma cell lines that reflect a spectrum of clinically relevant resistant phenotypes. These include both P-glycoprotein dependent and independent mechanisms of resistance. P-glycoprotein independent lung and melanoma resistant cell lines were also included in the screen, as well as an ovarian carcinoma cell line established by selection with the clinically important DNA active agent, cisplatin. This cell line is resistant to most known DNA reactive compounds.

Cell kinetics

One explanation offered for the poor solid tumor performance of existing drugs is that the murine leukemia models used for identifying these agents have high growth fractions and short tumor doubling times compared to the majority of solid tumors. Thus, cell lines of varying doubling time were incorporated into the diverse cell screen. Methods of data analysis were developed that enable quantitation of the antiproliferative activity of test compounds against these slow growing cell lines.

Tumor progression/differentiation state

Cancer is a disease that progresses with time from a benign, relatively well-differentiated lesion to an aggressive poorly differentiated metastatic disease. These changes reflect cumulative perturbations/mutations/rearrangements of the cellular genome. Cell lines reflecting a spectrum of tumor progression were included in this screen.

Hormone/growth factor dependent

Cancer of the prostate and breast comprises between 25–33% of all adult cancers, and anti-hormonal therapies (e.g., tamoxifen) represent an important therapeutic niche. Hormone unresponsive variants of these diseases are more aggressive and have a poor prognosis. Prostate and breast carcinoma cell lines were characterized for androgen and estrogen growth dependency, and cell lines expressing either hormone dependent or independent phenotypes were included in the diverse cell screen. Similarly, growth factor antagonists are viewed as potential new therapeutic targets for cancer (e.g., Her2/neu, EGF, PDGF, bFGF). Cell lines were characterized for the: a) expression of growth factor receptors, and; b) the effect on cell proliferation of growth factors and/or antibodies directed against these growth

factors. These studies served as the basis for selecting growth factor-responsive cell lines.

Oncogene/suppressor gene (OSG)

The identification of oncogenes/suppressor genes (Stehelin, 1976) and the studies that have led to an understanding of their role in tumor pathogenesis (Weinber, 1991) are among the most important developments in cancer biology. OSG expression in the diverse tumor cell line screening panel was initially investigated by literature search, and this was supplemented by Cyanamid's own ongoing research effort. Functional assessment of wild-type p53 function was established using radiation induced G1 arrest.

Mechanism/structure vs. Meangraph (MG) pattern

The potential value of this screening program was in its ability to relate a given mechanism or structural class to a unique profile of activity, i.e., MeanGraph pattern. In this way, unknown test compounds could be tentatively assigned to a given mechanistic or structural class on the basis of their pattern. Following this logic, novel compounds should generate novel patterns. In order to test this hypothesis, initial studies were conducted using a diverse spectrum of compounds with known mechanisms of action, including different classes of anticancer agents (e.g., intercalation, alkylation, antimetabolites, topoisomerase and microtubule inhibitors). Representative MG patterns generated by different classes of compounds are shown in Figure 3. For example, the estrogen receptor antagonist 257 was 840-fold more active against an estrogen dependent breast carcinoma cell line (designated Breast 4), compared to the average cell response (i.e., the Mean IC_{50} value of all 26 cell lines). Other compounds (e.g., paclitaxel) are known substrates for the drug efflux pump P-glycoprotein, and this property is clearly reflected in the pattern of resistance shown in Figure 3, where the degree of paclitaxel resistance in each cell line is proportional to the level of P-glycoprotein expression. Other compounds that effect microtubule polymerization/depolymerization (i.e., vincristine, colchicine) produce similar MG patterns. Compounds that react with DNA topoisomerases generate a clearly recognizable MG pattern, as shown for VP-16 in Figure 3. A cisplatin resistant cell line designated OvCa 1 in Figure 3 is used for predicting DNA reactivity. The ratio of IC_{50} values (>10) in this cisplatin resistant cell line/parental sensitive cell line is prognostic of DNA reactivity. We have established that most clinically useful DNA active agents (e.g., cisplatin, adriamycin, etoposide) have cisplatin resistant/sensitive ratios between 10–100. An unanticipated cellular response was observed when testing the protein kinase C (PKC) agonist, TPA (phorbol ester). Several cell lines were found to be >1000-fold more sensitive to the antiproliferative effects of the TPA treatment. This observation prompted biochemical studies that established that the sensitive cell lines all expressed >10-fold higher levels of PKC activity. A series of TPA analogs (e.g., teleocidin, lyngbyatoxin) were all found to produce "TPA-like" MG patterns that differed only with respect to potency. These results demonstrate that the Cyanamid diverse 26-cell line screen was able to predict structure and mechanism.

Molecular targets

Since 1995, the Oncology Department at Wyeth moved to molecular targeting and implemented screens for inhibitors of protein kinases, cell cycle, transcription, and translation. Combined with advances in robotics, these screens are capable of identifying active molecules from a library of greater than 500,000 compounds in three months. The utility of the mechanism-based approach is based on its rationale, speed, and its proven track record to yield novel drugs with potential clinical utility. Since it is impossible to investigate all known molecular mechanisms that govern cancer growth, the Oncology Department selected molecular targets based on:

1. An association of the overexpression, mutation, deletion and/or change in function of the target with the development of cancer in humans. In many cases, the correlation with cancer has been made between the alteration in the target and patient prognosis or treatment outcome.
2. Demonstrable changes of the target with increased proliferation or increased chemosensitivity of human tumors in tissue culture or animals. For example, overexpression of the proto-oncogene, HER2, in tumor cells stimulates the growth of transfected cells in culture and confers sensitivity to EGFR kinase inhibitors.
3. A clear association of inhibition of molecular pathway(s) with inhibitory effects on tumor growth in animals. For example, we have recently shown that an analog of rapamycin inhibits tumor growth (Gibbons, 1999). Rapamycin inhibits protein translation. Therefore, we have directed our efforts toward inhibiting molecular targets involved in protein translation.

Using these guidelines, the Wyeth Oncology Department implemented 11 screens for kinase inhibitors, and screens for 1) the inhibition of the activity and/or expression of metalloproteinases, 2) compounds that overcome unregulated growth due to loss of cell cycle control, 3) proteins that act downstream of the mutated APC gene in colon cancer, 4) inhibitors of a protease essential for signaling through receptor tyrosine kinases, and 5) inhibitors of the target of rapamycin (TOR) pathway.

Inhibitors of kinases

Protein kinases compose one of the largest superfamilies of eukaryotic proteins. Approximately 400 proteins have been identified and another 600 genes are likely to exist in the genome (Hanks, 1995). These enzymes transfer a phosphate

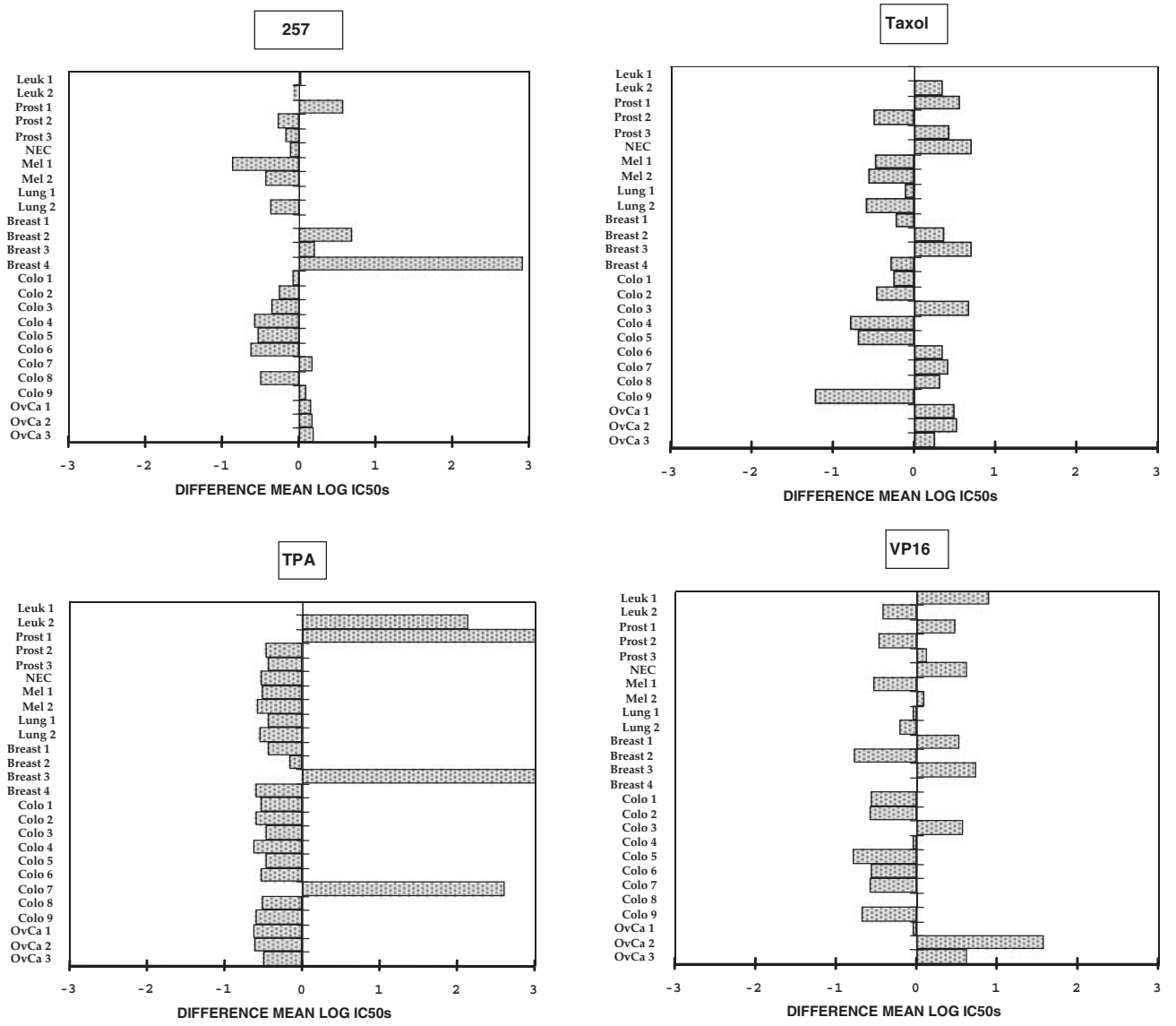


Figure 3. MeanGraph (MG). This graphic representation of data compares the individual IC_{50} values of a particular compound to the Mean IC_{50} value for all 26 cell lines. Specifically, the logarithm of each individual cell line IC_{50} value is subtracted from the geometric mean of the logarithm of the IC_{50} values for all 26 cell lines (Log Geometric Mean IC_{50} – Log Individual IC_{50}). The center line in this figure represents the Mean IC_{50} value. Bars projecting to the right of the mean represent cell lines that are more sensitive to the test drug, and bars to the left indicate cell lines that are more resistant. The length of the bar is proportional to the difference between the logarithm of the cell line IC_{50} and the mean. A bar projecting 1 log unit to the right of the mean, for example, reflects a cellular response that is 10 times more sensitive than the average of all cellular responses.

from ATP to serine, threonine, or tyrosine residues. A similar catalytic domain consisting of 250–300 amino acids relates the proteins. While broad-spectrum inhibitors of protein kinases may have utility, our working hypothesis is that selective inhibitors of kinases relevant to cancer would be the most useful. Therefore, we have attempted to identify potent and selective inhibitors of certain kinases that are effective in our animal models. Tyrosine kinases are enzymes that regulate signal transduction in cells leading to mitogenesis, cell movement, apoptosis and other cellular functions (Strawn, 1998). Both membrane-bound kinases that contain extracellular ligand-binding domains (so-called receptor tyrosine kinases or RTKs) and cytoplasmic kinases are in the screening panel. RTKs are of particular interest since aberrant activity in many of these proteins is linked to oncogenesis and other proliferative diseases including psoriasis, atherosclerosis, and restenosis (Gibbs, 2000). In the non-diseased state, ligand binding to RTKs induces receptor dimerization. The dimerized receptor then undergoes a conformation change leading to activation of the catalytic domain in the receptor. Upon enzymatic activation, tyrosine phosphorylation of the receptor (autophosphorylation) or phosphorylation of

another protein occurs. A phosphorylated recognition peptide recruits the binding of enzymes, adapter proteins that link the receptor to other enzymes, scaffold proteins, or negative regulators (Pawson, 1999) that ultimately lead to cell growth and motility. In cancer, mutation and/or overexpression of some RTKs lead to constitutive activity (i.e., independent of ligand activation). Some of the kinases linked to disease states include platelet-derived growth factor receptor, fibroblast growth factor receptor, vascular-endothelial growth factor receptor, epidermal growth factor receptor (EGF-R), HER-2, and src (Strawn, 1998).

Although a complete list of tyrosine kinase programs at Wyeth is proprietary, the EGF-R program, which is a prototype for the kinase programs at Wyeth, is discussed below. EGF-R is an RTK in which EGF, TGF-α, and other dimer-inducing ligands leads to autophosphorylation of tyrosine residues at the C-terminus of the molecule. The phosphorylated receptor recruits adapter molecules and/or directly binds and activates enzymes in the Ras-raf-MEK, PI3K, and/or PLC pathways. Activation of EGF-R can lead to cell proliferation as well as a number of other important processes for tumor progression, including cell motility, invasion, cell survival and angiogenesis (Woodburn, 1999). Overexpression and/or mutation(s) of EGF-R have been associated with a variety of solid human tumors and are believed to induce elevation and/or constitutive activation of EGF-R (Salomon, 1995).

There is considerable precedent to justify the exploration of natural products as inhibitors of tyrosine kinases. Many natural products including genistein, lavendustin A, and erbstatin (all fungal products) are tyrosine kinase inhibitors known to inhibit multiple tyrosine kinases (Levitzki, 1995). Since these compounds can be competitive and non-competitive with respect to ATP and/or peptide substrates, their lack of specificity may be understandable. Consistent with this, synthetic efforts based on erbstatin led to the development of tyrphostins that have improved potency and selectivity (Levitski, 1995). It is likely that compounds that target the interaction of ATP with the catalytic domain of tyrosine kinases will be most interesting in the future. Despite the fact that a lack of specificity of such inhibitors might be expected, a high level of specificity among kinase inhibitors directed to the catalytic domain has been demonstrated (Woodburn, 1999). As an example, Wyeth's irreversible inhibitor CL 387,785 binds to the catalytic domain of EGF-R alone, and modeling supports the hypothesis that specific modifications in the pharmacophore are essential for mediating specific interaction with an amino acid, cys^{773}, that is uniquely found in the catalytic domain of EGF-R and not in other RTKs (Discafani, 1999). Beyond this, six of the seven tyrosine kinase inhibitors in clinical trials are ATP competitive inhibitors. One of these clinical leads was derived from the natural product staurosporine a pan-kinase inhibitor, (Angeles, 1996).

Like tyrosine kinases, protein serine-threonine kinases participate in critical regulatory functions in the cell including signal transduction (MAP kinases), cell proliferation (cyclin-dependent kinases) and apoptosis (Akt). One or more of these pathways is often deregulated in cancers, resulting in uncontrolled proliferation. Inhibitors of these kinases are, therefore, predicted to be useful in the treatment of a variety of malignancies.

One serine-threonine kinase program in the Wyeth Oncology Department is directed at identifying inhibitors of cyclin-dependent kinases (cdks). Cdks and their activating partners, the cyclins, regulate progression through the cell cycle. Different combinations of cyclins and cdks perform distinct functions at each stage of the cycle. In the gap 1 (G_1) phase, extracellular growth signals induce the synthesis and activation of the G_1 cyclin/cdk complexes (primarily cyclin D1/cdk4) (Hunter, 1997; Morgan, 1995). These complexes cooperate to phosphorylate and inactivate the retinoblastoma gene product (pRb), which is required for the onset of DNA synthesis (S phase) (Angeles, 1996). The G_1 cdks themselves are negatively regulated by a family of small protein inhibitors, the cyclin-dependent kinase inhibitors (cdkis) in response to growth-inhibitory environmental signals (Hunter, 1997). Altered regulation of the G_1 kinases (by amplification of cyclins and cdks and/or inactivation of the cdkis) is observed in a variety of tumors (Kamb, 1994; Nobori, 1994; Hirami, 1995; Hall, 1996; Foulkes, 1997). Potent and selective antagonists of these kinases will be useful in the treatment of tumors that have aberrantly regulated G_1 kinase activity. Natural products have provided a rich source of leads for the development of cdk inhibitors (e.g., butyrolactone, flavopiridol, and paullone). These compounds are competitive inhibitors of ATP, have broad activity against several cdks, and preferentially inhibit cyclin B/cdc2 (Meijer, 1997; Zaharevitz, 1999). Structural studies with flavopiridol and other synthetic inhibitor complexes with cdks have revealed the mechanism underlying the specificity of these molecules for cdks (Meijer, 1997). This information and the identification of new pharmacophores by high throughput screening will allow us to identify and develop novel cell cycle inhibitors with improved selectivity, potency, and pharmacological properties.

Molecular screening of the ARS Collection of Entomopathogenic Fungi (ARSEF) for secondary metabolite genes

The goal of this project is to identify fungi with the genetic potential to produce novel metabolites by using a molecular pre-screen to look for the presence of key genes predicted to be involved in natural product biosynthesis. Since polyketides, nonribosomal peptides, and polyketide/peptide hybrids are some of the most common biologically active natural products produced by microbes and other organisms, we focused on the identification of fungi that encode polyketide synthase (PKS) and nonribosomal peptide synthetase (PS) genes. Cloned PKS and PS genes from such fungi can

then be used to assess gene expression and metabolite biosynthesis under a variety of nutritional and environmental conditions.

The ARSEF culture collection in Ithaca, NY, which was screened in this study, is the largest and most diverse repository of insect-associated fungi in the world. It contains more than 6100 accessions, 425 fungal taxa, and 100 genera from more than 900 hosts (including insects, mites, spiders, nematodes, other invertebrates, and cold-blooded vertebrates) from more than 1200 locations worldwide. As such, the collection represents a taxonomically characterized set of fungi with significant metabolic potential. Previously, a subset of this collection (157 isolates), which represented the overall diversity of the collection, was screened for the presence of polyketide synthase (PKS) genes (Lee, 2001), and 42% of the fungi screened were shown to contain one or more PKS genes. The Churchill and Gibson labs extended the results of this study by screening many of the same subset of isolates for the presence of nonribosomal peptide synthetase genes. Degenerate primer pairs that hybridize to conserved domains within peptide synthetase (PS) genes were used against genomic DNA of 161 fungi to PCR-amplify fragments of PS genes. These fragments were cloned, sequenced, and analyzed by BLAST analyses (Altschul, 1990) to determine sequence similarities with genes in the NCBI/GenBank databases. These studies gave us the opportunity to identify taxonomically defined fungi that have the genetic potential to make nonribosomal peptides and/or polyketides, as well as hybrid molecules, and to target such isolates for further chemical analyses.

Molecular screen for peptide synthetase genes in fungi

In the Churchill lab, we have cloned and sequenced one or more peptide synthetase (PS) or PS-like gene fragments (ca. 150–1200 bp in size) from more than half of the fungi screened (161 isolates). We classified gene fragments into one of three PS or PS-like groups. Most PS gene fragments cloned are of unknown function and are from fungi not reported to produce nonribosomal peptides. These "unknown PS genes" have the potential to synthesize enzymes that make novel chemistries. A second class of genes was those most closely related to cyclosporin synthetase genes, which may participate in the synthesis of known chemistries (i.e., cyclosporin), variants of cyclosporin, or novel chemistries. The smallest number of fragments fell within the class of genes most closely related to the PS-like α-aminoadipate reductase genes, which may function solely in lysine biosynthesis or also participate in the synthesis of known secondary metabolites (e.g., β-lactams), variants thereof, or novel chemistries. Although we have grouped the PS gene fragments into these three classes, some of which are better defined than others, we emphasize that sequence similarity does not necessarily infer the function of any of these genes.

More than 70% of the PS-like gene fragments cloned from the ARSEF isolates have low similarity to fungal or bacterial PS genes with known or unknown functions, i.e., sequence similarity to already cloned genes with or without known functions is not sufficiently high for most sequences to suggest functionality. We propose that these genes have the greatest potential to synthesize novel chemistries. Twenty percent of the PS-like sequences were more similar to bacterial PS genes than to fungal genes. Of the approx. 30% of gene fragments having small E-values (i.e., defined here as <e-60), the great majority were highly similar to several different amino acid-activation domains of the *simA* cyclosporin synthetase (CS) gene from *Tolypocladium niveum* (Weber 1994). The sequence similarities between many of the CS gene fragments and the known *simA* gene are high enough to suggest, but not prove, that the domains represented could be part of a functional cyclosporin synthetase gene. However, it is also possible that these CS-like fragments represent highly conserved amino acid-activation domains within functionally unique genes. Most of the fungi from which the CS-like fragments were cloned have not previously been reported to synthesize cyclosporin. Some CS-positive isolates also contain additional PS gene fragments of unknown function.

The remaining gene fragments with small E-values are most similar to fungal α-aminoadipate reductase (α-AAR) genes. α-AARs are fungal-specific proteins and a key enzyme in the evolution of fungal lysine biosynthesis. They are most closely related to bacterial peptide synthetases involved in antibiotic production (An, 2003). α-Aminoadipate, which originates from the lysine biosynthetic pathway, is one of three amino acids to form the tripeptide ACV involved in the biosynthesis of penicillin and other β-lactam antibiotics (Martin, 1998). Interestingly, more than half of the α-AAR fragments cloned from the ARSEF isolates had relatively low similarities to known α-AAR genes, suggesting the possibility that these genes encode enzymes that make novel chemistries.

Many of the fungi that encode PS sequences also contain PKS genes of unknown function, suggesting that these fungi make polyketides and nonribosomal peptides, and/or polyketide/peptide hybrid molecules. Additionally, many fungi from which PKS fragments were *not* detected in a previous screen (Lee, 2001) contained PS-type genes. The PS and PKS sequences cloned in this study will be of value in assessing gene expression in correlation with the production of novel metabolites. Fungi containing uncharacterized PS and/or PKS genes have the genetic potential to synthesize new chemistries if cultured under conditions that support metabolite gene expression. Such fungi have been targeted for further chemical prospecting, with extracts being tested in cell- and receptor-based anticancer assays by the Wyeth Research group. A manuscript describing the methodologies and detailed results of this molecular screen for PS genes in insect-associated fungi is in preparation. In conclusion, our genetic data suggest that a greater degree of metabolic diversity is present in fungi than is evident in chemical screening programs alone. Accessing this metabolic potential will

likely prove to be a challenge but one that is worthy of serious effort since the chemical diversity of fungi has been just barely tapped to date.

Recent developments in the isolation of bioactive lead compounds

Austocystin D (1)

Aspergillus isolate UGM218 was selected as a lead culture based on the activity of its fermentation extracts in the Wyeth 26-cell line panel. The 100–300 fold greater activities of these extracts against a cell line (MIP101) overexpressing the MDR1 p-glycoprotein multi-drug efflux pump (MIP) compared with a cell line (SW620) lacking the pump were of particular interest. In addition to this demonstrated selectivity, extracts from UGM218 were potent, as exemplified by an IC_{50} for one of the crude extracts of <0.01 μg/mL against MIP. In order to identify the active components and to prepare pure compound for further testing, efforts were initiated early in 1998 in the Natural Products group at Wyeth to develop fermentation scale-up conditions. Extracted fermentation material from initial scale-up efforts was provided to Dr. Clardy (then at Cornell University) for compound isolation and chemical structure elucidation. Dr. Clardy and his team identified austocystin D (**1**) as the major component responsible for the differential MIP/SW620 activities using the material from these early fermentation efforts. The isolation of the active principle from the ethyl acetate extract of UGM218 involved a bioassay-guided fractionation using the closely related human tumor cell lines MIP101, which expresses multidrug resistance (mdr) by MDR1, and SW620, which does not. The assay followed the MDR-ratio, the IC_{50} value of MIP101 divided by the IC_{50} value of SW620. The crude extract typically had an MDR ratio of 0.01. A solvent separation scheme (hexane extraction of 90% aqueous methanol, carbon tetrachloride extraction of 80% aqueous methanol, and methylene chloride extraction of 60% aqueous methanol) was used initially and the best MDR ratio was in the carbon tetrachloride fraction. Further chromatography on a C18 flash column eluted with a step-gradient of methanol water gave fractions with MDR ratios from 0.0016 to 0.0047. From those fractions, austocystins D, H, and B were isolated, and austocystins D and H, but not B, were responsible for the activity. Structure elucidation was straightforward, and the spectral data and interpretations were in accord with those given by the Steyn group in their original isolation and structure determination of the austocystins (Steyn, 1974; Steyn, 1975). In view of the close relationship of the austocystins with known Ames test mutagens, and suspected human carcinogens, it's worth noting that in the Ames test, austocystin D was not mutagenic (50 μg/plate) while austocystin H was mutagenic at the same dose. Metabolic activation enhanced the mutagenic effect (Kfir, 1986). Metabolic activation undoubtedly involved epoxidation of the dihydrofuran ring on the right-hand side of austocystin D (**1**).

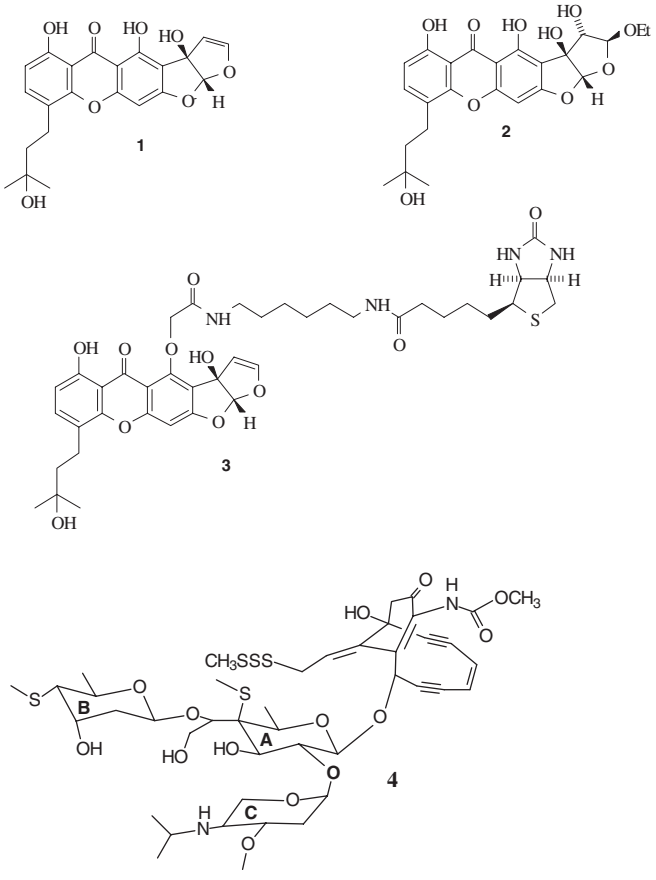
With this information available, austocystin titers in subsequent fermentations were monitored by HPLC rather than cytotoxicity assays. Scale-up yields observed for UGM218 were variable, and 1 L fermentations in Potato Dextrose Broth in 2.8 L Fernbach flasks were initially emphasized. Extracts of this isolate obtained from 10 L fermentors often yielded little activity, and conditions (air flow and agitation rates were varied) supporting consistent production of cytotoxic activity were not identified. With time, it was decided to evaluate another culture, *Aspergillus ustus* ATCC 36063, which is known to produce the austocystins, and fermentation on solid media, such as corn meal, as a means of promoting higher, consistent yields. Variables such as incubation temperature and water activity of the solid media were studied and optimized. Our data indicated that a combination of an improved seed medium, the use of cornmeal as a solid substrate, along with carefully controlled moisture conditions and an extended incubation time, resulted in consistent yields that were 3- to 6-fold higher than previously obtained. Roughly 65% of the total of 3923 mg of austocystin D generated in Wyeth fermentations by mid-2001 was accomplished in the last 1.5 years of the project employing solid medium conditions. In further testing at Wyeth, the IC_{50} of austocystin D against MIP was determined to be 0.001 μg/mL, while its IC_{50} against SW620 was 0.9 μg/mL. It was further demonstrated that austocystin D had approximately 20-fold more potency in cells lines where P-glycoprotein overexpression was either induced by drug-selection or by transfection of the gene into a melanoma cell line. In addition, austocystin D was found to inhibit the growth of an MDR1-over-expressing tumor cell line placed into hollow fibers and implanted into nude mice. Further *in vivo* testing demonstrated that austocystin D (3–5 mg/kg administered daily on days 1–5 by intraperitoneal injection) profoundly inhibited the growth of a human colon carcinoma derived from LS174T, when the tumor was implanted into nude mice subcutaneously. However, upon further experimentation, it was concluded that the compound had a low safety window. Therefore, further development of austocystin D was terminated.

While the therapeutic window of austocystin D was too narrow for further development, it seemed possible that it could be a useful reagent to identify a sensitive target for cancer therapy. As described above, the effect of austocystin seems to be coupled to P-glycoprotein overexpression, but its effect is not the usual one seen for P-glycoprotein 'pump blockers'. Known pump blockers are by themselves not very toxic, and their utility, if any, is to restore sensitivity to other cytotoxic agents. Austocystin D's ability to selectively kill MDR cancer cells by itself suggests a different target. As is invariably the case, attempts to identify targets reflect the technology available at the time, and one of the most pressing needs in natural products research is generally applicable methods to identify targets.

The first attempt was to produce affinity reagents that could be used to identify austocystin D's target. The initial

set of affinity reagents involved epoxidation of the 2,3-double bond and subsequent chemistry. These manipulations were all difficult as they proceeded in variable but typically low yield. While this approach was eventually abandoned for the approach described below, it did yield the austocystin D derivative shown as (**2**). Since this derivative had most of the potency and selectivity of the starting compound, it indicated that the presence of the 2,3-double bond was not absolutely essential for activity. We then turned our attentions to derivatizing the phenolic hydroxyls and were able to prepare the biotin-linked affinity reagent shown as **3**. This reagent was used to pull down a few candidate proteins, but they all appeared to be common housekeeping proteins that were known to give false positives in affinity assays. It seemed likely that austocystin D's target might be an integral membrane protein or a low abundance cytoplasmic protein, either of which would be difficult to identify through classical affinity reagent methods.

The second attempt was to use microarray-based genome profiling of austocystin D. In this approach, cells are treated with the test compound, austocystin D, while companion cells are left untreated. Messenger RNA (mRNA) transcripts are then measured to eventually give the ratio of mRNA transcripts in treated vs. untreated cells. We used yeast cells as the yeast genome was well characterized and microarrays covering essentially the entire genome were available to us.



This approach showed that a total of 59 mRNA transcripts were upregulated, approximately 1% of the genome, but the overall response was rather "flat". This result was not totally unexpected as austocystin D is not very toxic to yeast. However two transcripts that were upregulated are worth noting. One, YKR104W is a member of the ATP-binding cassette (ABC) superfamily. Its closest human homolog is ABCC4 (44% identity). The yeast protein interacts with the RIB4 gene product, a riboflavin biosynthesis pathway enzyme. The second, YPR156C, encodes a member of the drug : proton antiporter DHA12 family of multidrug efflux proteins in the major facilitator superfamily. Homologs cannot be found in human by sequence homology, but they do exist in rats. The yeast protein interacts with three proteins, two with unknown function and the third is a homolog of Erv1, which is a flavin-linked sulfhydryl oxidase essential for mitochondrial biogenesis and cell viability. The search for austocystin D's target continues, and experiments with human gene microarrays at Harvard Medical School are beginning.

Namenamicin (4)

An extract of the orange sheet ascidian *Polysyncraton lithostrotum* showed very potent cytotoxicity. Crude extracts obtained from multiple collections of this organism generated reproducible MeanGraph profiles, which showed very high correlation (rho = 0.914) with those of calicheamicin, Figure 4. All of these data were indicative of a DNA cleavage agent; however structure determination studies were hampered by the very low yield of the compound in the extracts, 10^{-4}%. Ultimately, a collection of 7 kg of tissue from Namenalala reef in the Fiji Islands yielded 3 mg of the active metabolite that we named namenamicin (**4**). Namenamicin contains the same "enediyne warhead" as the calicheamicins, however, the attached carbohydrate moiety differs in replacement of the N-O sugar linkage between the A and B sugars with a C-O, an S-methyl substituent at A4, and the absence of a benzoate ring appended to the B sugar. Namenamicin exhibited potent *in vitro* cytotoxicity with a mean IC_{50} of 3.5 ng/ml in Wyeth's cell panel and *in vivo* antitumor activity in a P388 leukemia model in mice (ILS 40 @ 3 g/kg). The sequence specific DNA interactions of namenamicin were mapped on a 142 base pair pBR322 restriction fragment (Hind III-NciI) and compared to calicheamicin 8_1^I. Namenamicin produced fewer high specificity cleavage sites than calicheamicin and cleaved DNA less efficiently. There were some similarities in the sequence specific recognition patterns between the two compounds and several distinct differences. For example, TCCT, the primary recognition site for calicheamicin was cleaved with greatly diminished cleavage intensity. The primary recognition site for namenamicin in this restriction fragment was 5′ATCTA3′ with cleavage occurring at C. This was also a strong recognition site for calicheamicin but cleavage occurred at the 5′T residue. Interestingly, TTGT, a strong cleavage site in the case of

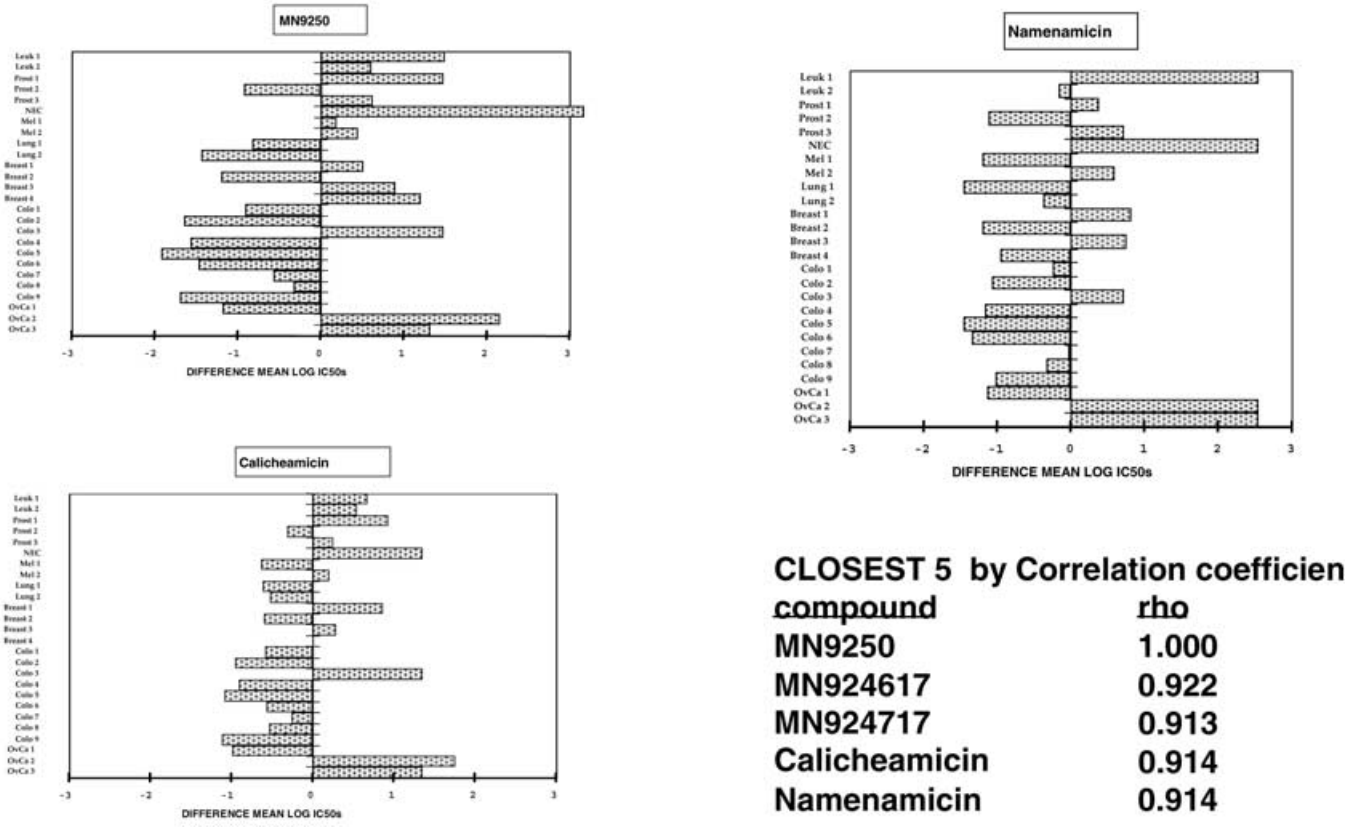


Figure 4. MeanGraph profiles comparing crude extracts derived from *P. lithostrotum* (MN9250, MN924617 and MN924717) to the isolated metabolites namenamicin, and calicheamicin.

calicheamicin was not cleaved by namenamicin (McDonald, 1996). The observations that namenamicin produced fewer high affinity cleavage sites and a slightly altered recognition pattern are consistent with its truncated structure. The lowered cleavage efficiency and the altered selectivity are possibly due to the absence of the rhamnose sugar and thiobenzoate ring and more importantly due to the change in glycosidic linkage between the A and B ring sugars. The C-O linkage could conceivably enhance flexibility, thereby lowering the DNA binding energy and consequently decreasing sequence selectivity.

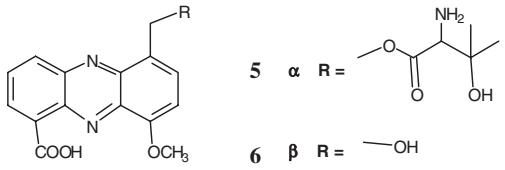
Namenamicin represents the first enediyne isolated from a marine organism and also from a non-microbial source. Because of Wyeth's extensive experience with this class of compounds, most notably the calicheamicins, a program was initiated to isolate and characterize the microbial population associated with *Polysyncraton lithostrotum* to see if a microbial symbiont might be involved with the production of namenamicin. To isolate the microorganisms associated with the tunicate, 10 grams of ascidian were macerated and plated onto eight different selective agars. After a one month incubation, 41 eubacteria, 17 actinomycetes and two fungi were isolated and taxonomically characterized (Table 1). All of these isolates were fermented in 8 different media and tested in the BIA for DNA-damaging activity. Four isolates, three different *Micromonospora* species and one halophilic Gram-negative eubacterium, were active in this assay. The halophilic Gram-negative isolate, designated strain LL-14I352, was shown to be a halophilic, nonmotile, pleomorphic, eubacterium with a morphologically complex life cycle. Large-scale fermentations were carried out to furnish material for isolation and structure elucidation. Two BIA-active compounds were isolated and were designated LL-14I352 α and β (**5–6**). The α compound contained a phenazine moiety linked to an uncommon β-hydroxy valine residue through an ester bond. The β component structure was established based on its molecular weight and the fact that hydrolysis of α yields β. The α component demonstrated antitumor activity with a mean IC_{50} of 0.48 μg/ml in cell culture based cytotoxicity assays, while β had a mean IC_{50} of 1.8 μg/ml in this system. However, in animal experiments, both LL-14I352 α and β were inactive in a murine P388 leukemia model. This lack of *in vivo* activity may be due to the fact that LL-14I352 α is chemically unstable and converts to the less active β. At the same time our work was in progress, Imamura et. al (1997) described these same compounds and called them pelagiomicins. The microorganism that produced their compounds was described as a new marine bacterium and classified as a new genus and given the name *Pelagiobacter variabilis*.

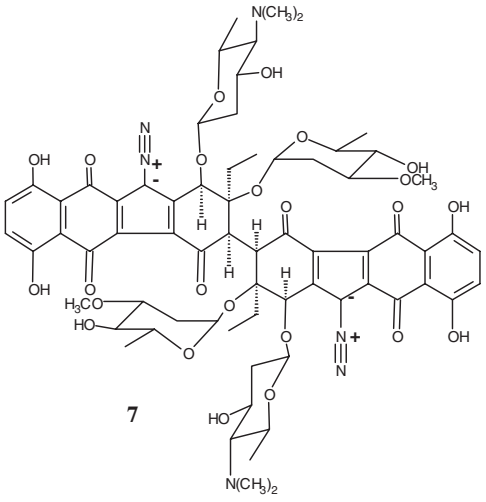
Extensive work on the *Micromonospora* spp. both in liquid fermentation and on agar surface growth has demon-

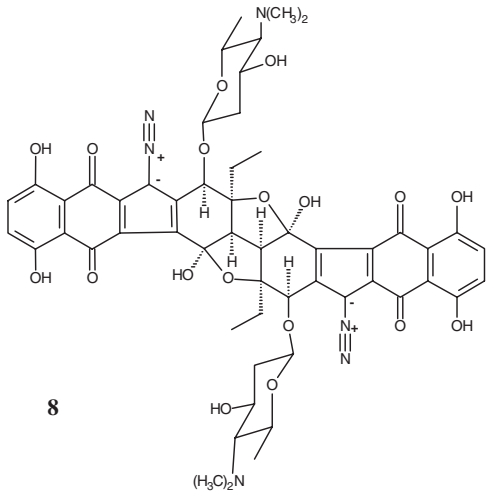
Table 1. Types of Organisms Isolated from *Polysyncraton lithostrotum.*

Organism Type	Total Number of Isolates	Number of Unique Isolates
Fungi	2	2 genera
Mycobacterium	2	1 species
Oceanospirillium	5	2 species
Bacillus	4	3 species
Pseudomonas	3	2 species
Agrobacterium	5	1 species
Rhodococcus	1	1 species
Micromonospora	17	3 species
Unidentified	24	6 genera; 10 species

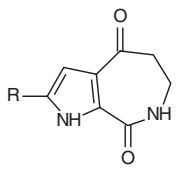
strated BIA activity from these cultures. Among these actinomycetes, a halophilic strain was identified as a new species of the genus *Micromonospora* on the basis of its morphological properties and 16S rDNA sequence and was named "*Micromonospora lomaivitiensis*". The fermentation broth of this organism exhibited potent BIA activity and was extremely cytotoxic against a panel of cancer cell lines. Using BIA-guided fractionation, two novel dimeric diazobenzofluorene glycosides, responsible for the bioactivity, were isolated and designated lomaiviticins A and B (He, 2001). Lomaiviticins A (**7**) and B (**8**) were produced by fermentation of the organism in a seawater-based liquid medium. The active compounds were recovered by incorporating HP-20 resin in the fermentor. The active material was eluted from the resin and purified by chromatography to afford lomaiviticins A (70 mg) and B (12 mg) from 70 liters of broth. The molecular formula of **7** was determined by high resolution Fourier transform ion cyclotron resonance (FTICR) mass spectrometry to be $C_{68}H_{80}N_6O_{24}$. Initial analysis of the ^1H and ^{13}C NMR spectral data in CD_3OD indicated the presence of 34 carbons and 36 unexchangeable protons, which only accounted for half of the carbons and less then half of the protons in the molecular formula. Therefore, **7** was most likely a symmetric dimer. Detailed analysis of NMR data, including 2-D ^1H-^1H COSY, TOCSY, ^1H-^{13}C HMBC, and HMQC spectra, revealed several substructures which were assembled with the aid of ROESY data. Lomaiviticin A (**7**) was determined to be a dimeric benzofluorene glycoside attached to two diazo functional groups at C-5, and -5′. The chemical shifts for C-5 and -5′ at δ 78.8 were consistent with the literature data for the corresponding carbons in the monomeric benzofluorene antibiotics, kinamycins (Mithani, 1994). The molecular formula of lomaiviticin B was determined to be $C_{54}H_{56}N_6O_{18}$ by high resolution FTICR mass spectrometry. Similar to lomaiviticin A, this compound was also considered to be a symmetric dimer. By comparison of the NMR data with **7**, **8** lacked proton and carbon signals for the sugar moieties B and B′, and the ketone signal at δ 198.4 in **7** was replaced by a hemiketal resonance at 96.5 in **8**. Therefore, the center region of compound **8** was elucidated to be a fused furanol system that was supported







by the ^{1}H-^{13}C correlations in an HMBC spectrum. The remaining portions of the molecule, for example, the two units of diazobenzofluorene and the sugar moieties A and A′, were determined to be identical to lomaiviticin A. Lomaiviticin B may be derived from lomaiviticin A by formation of furanol rings between 3-hydroxyl and 1′-ketone and between 3′-hydroxyl and 1-ketone following hydrolysis of sugars B and B′. The nature of the lomaiviticins interaction with DNA is still under investigation.



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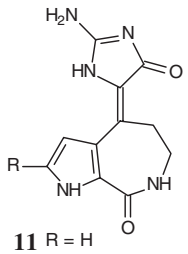
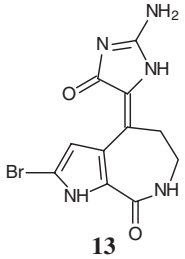
10 R = Br



11 R = H
12 R = Br



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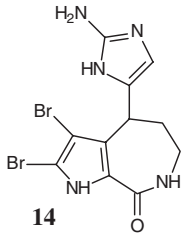

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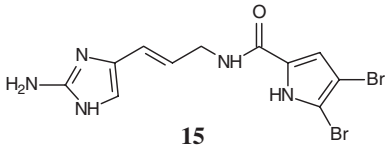

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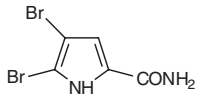


16

Stylissa massa

Raf/MEK/MAPK proteins play crucial roles in cellular signaling processes downstream of the RTK growth factors such as EGF-R. The Ras-MAPK signaling cascade (MAPK module) is found in all eukaryotic organisms and is involved in transmitting signals from the extracellular compartment into the cytosol and nucleus (Robinson, 1997). This cascade is activated by GTP- loaded Ras which recruits Raf-1 (c-Raf) to the inner cell membrane where it is activated by phosphorylation. Activated Raf-1 phosphorylates and activates the dual specificity kinase MEK (MAP kinase kinase) on two different serine residues (Lewis, 1998; Kolch, 2000). Activated MEK-1 phosphorylates and activates MAPKs (Mitogen-Activated Protein Kinases), that can translocate to the nucleus and modulate cytoplasmic events such as cell proliferation and differentiation, through the phosphorylation of a variety of substrates (Lavoie, 1996; Barbacid, 1987). Since the oncogenic form of Ras is associated with 30% of all cancers, Ras and the downstream kinase effectors of Ras, represent attractive targets for pharmacological intervention. Both *in vitro* and *in vivo* studies have shown that selective Raf and/or MEK-1 inhibitors are important pharmacological targets (O'Dwyer, 1999; Sebolt-Leopold, 1999; Lackey, 2000). Raf/MEK-1/MAPK cascade assays, as well as individual Raf, MEK-1, or MAPK activity assays, have been described (Alessi, 1995; McDonald, 1999). A sensitive, non-radioactive, high-throughput Raf/MEK-1/MAPK cascade ELISA was recently developed by Wyeth (Mallon, 2001). Using this assay, we have screened several thousand marine sponge extracts to identify inhibitors of this signaling cascade. The extract of the sponge *Stylissa massa* collected from the Philippines showed significant activity in this assay. Bioassay-guided fractionation yielded a family of known pyrrole alkaloids **9–16** (Tasdemir, 2002). In secondary assays, 10*E*-hymenialdisine (**12**) and 10*Z*-hymenialdisine (**13**) were shown to be potent inhibitors of MEK-1 with IC_{50}'s of 3 and 6 nM, respectively. Hymenialdisine has recently been reported to display significant inhibitory potential against a number of cytoplasmic kinases (Meijer, 2000; Curman, 2001), however, this represents the first report of activity for this class of compounds specifically against MEK-1. None of the compounds displayed activity in the Raf to MEK-1 assay, whereas all compounds showed essentially identical IC_{50} values in the MEK-1 to MAPK assays (Table 2) as in the Raf/MEK-1/MAPK cascade assay. For comparison, the IC_{50} values of staurosporine and PD98059 (Pang, 1995), in both Raf/MEK-1/MAPK ELISA and secondary assays are shown in Table 2. This activity profile prompted us to test compounds **9–16** for their abilities to inhibit the growth of two human colon tumor cell lines, LoVo and Caco-2. LoVo cells have been shown to be sensitive to growth inhibition by farnesyl protein transferase inhibitors (FTIs) at low nM levels (Lerner, 1997). Sensitivity of LoVo cells to FTIs is attributed to the presence of mutant activated K-Ras. Mutant K-Ras causes activation of the Raf/MEK-1/MAPK signaling cascade that emanates from Ras, thus inhibitors of Raf or MEK-1 should inhibit LoVo growth. Table 2 shows that the most potent MEK-1 inhibitors, 10*E*-hymenialdisine (**12**) and 10*Z*-hymenialdisine (**13**), also inhibited growth of LoVo cells (IC_{50}'s 586 and 710 nM, respectively). Caco-2 contains wild-type K-Ras, which correlates with resistance to the growth inhibitory effects of FTIs (Lerner, 1997). 10*E*-hymenialdisine (**12**) and 10*Z*-hymenialdisine (**13**) were significantly less active against Caco-2 cells (Table 2). Hamilton FTI-276, a known Ras inhibitor is also included in Table 2 for reference. No further development studies with the hymenialdisines are planned at this time.

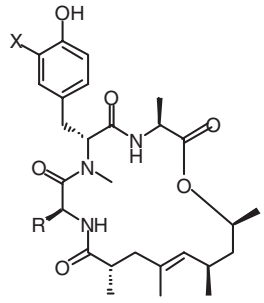
The hemiasterlins: HTI-286 (29)

The development of the anticancer clinical candidate HTI-286 started with a series of collecting expeditions to Papua New Guinea in the late 1980s. The goal of the trips was to collect marine invertebrates, mainly sponges, to provide a wide diversity of natural product extracts for a murine P388

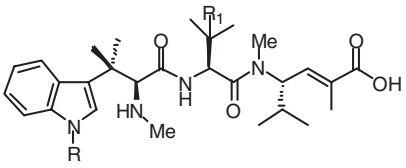
Table 2. Kinase Enzyme Inhibitory Activity and *in vitro* Antitumor Activity of Compounds **9–16** (IC_{50} nM).

Compound (nM)	Raf/MEK1/ MAPK ELISA	Raf to MEK-1 Assay	MEK-1 to MAPK Assay	Caco-2	LoVo
(**9**)	>2,500	2,500	>2,500	>10,000	>10,000
(**10**)	539	2,500	539	>10,000	>10,000
(**11**)	881	2,500	824	>10,000	>10,000
(**12**)	3	2,500	6	>3,867	586
(**13**)	6	2,500	9	>7,799	710
(**14**)	1,288	2,500	1,288	>10,000	>10,000
(**15**)	>2,500	2,500	>2,500	>10,000	>10,000
(**16**)	>2,500	2,500	>2,500	>10,000	>10,000
Staurosporine	2.5	2.5	2.5	NT	NT
PD98059	2,800	>10,000	2,800	NT	NT
Hamilton FTI	NT	NT	NT	>10,000	50

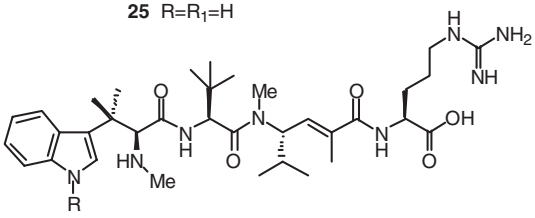
NT = not tested.



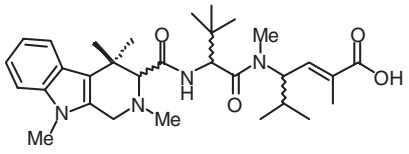
17 X=I, R=Me
18 X=Br, R=Me
19 X=Cl, R=Me
20 X=I, R=H
21 X=Br, R=H
22 X=Cl, R=H



23 R=R$_1$= Me
24 R=H, R$_1$=Me
25 R=R$_1$=H



26 R=H
27 R=Me



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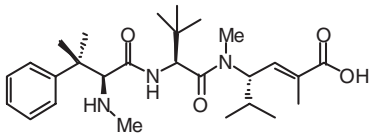


29

in vitro cytotoxicity screening program. This effort was being conducted as a collaboration between Theresa Allen in the Department of Pharmacology at the University of Alberta and Raymond Andersen at the University of British Columbia. The crude extract of one of the sponge specimens, a *Cymbastela* sp., showed extremely potent activity in the assay and consequently it was selected for chemical study. Bioassay-guided fractionation of the extract initially resulted in the isolation and structure elucidation of the known cyclic depsipeptides geodiamolides A (**17**) and B (**18**) along with the new analogs C (**19**) to F (**22**) (de Silva, 1990). Publication of the structures of **19** to **22** along with the first report of the potent *in vitro* cytotoxicity of pure compounds and promising *in vivo* activity against P388 exhibited by the crude *Cymbastela* sp. extract attracted the attention of scientists at NCI. They inquired about the possibility of getting sufficient quantities of the various geodiamolides for testing in their *in vivo* hollow fiber assay. In order to satisfy the NCI requirements for mg quantities of the geodiamolides, the source sponge was recollected in 1993. Bioassay-guided fractionation of the extract from the new specimens again yielded the desired geodiamolides, but also led to the isolation of the cytotoxic peptides **23** to **27**, which were initially named criamides A to E after the Christiansen Research Institute, where some of the sponges had been collected. The constitutions of peptides **23** to **27** were elucidated by analy-

sis of NMR and MS data and the absolute configurations of the N-methyl homo vinylogous valine (MHVV) and *tert*-leucine residues were shown to be L by chemical degradation followed by Marfey's analysis (Coleman, 1995). The configuration of the tetramethyltryptophan residue was shown to be L by single crystal x-ray diffraction analysis of the methyl ester of hemiasterlin (Coleman, 1996). While Theresa Allen was in the process of defining the biological activity of peptides **25** to **29**, two papers appeared in the literature at essentially the same time describing in one case the structure of peptide **23** and in the second case the structure of the closely related tripeptide **28**. Crews et al. reported isolating milnamide A (**28**) and jaspamide from specimens of *Auletta* cf. *constricta* also collected in Papua New Guinea (Crews, 1994). The configurations of the amino acids in **28** were not determined. Milnamide A (**28**) was reported to have *in vitro* cytotoxicity against several cell lines with IC_{50}'s in μg/mL of 4.1 (A549), 2.8 (HT-29), 3.8 (B16/F10), and 0.74 (P388). Kashman and co-workers isolated tripeptide **23** along with jaspamide and geodiamolide TA from the sponge *Hemiasterella minor* collected in South Africa (Talpir, 1994). They named peptide **23** hemiasterlin after the genus name of the source sponge. Very small amounts of hemiasterlin (**23**) were obtained by Kashman's group, precluding a determination of its absolute configuration. However, the optical rotation reported for the *H. minor* derived sample of **23** was similar to that measured for the sample isolated in the Andersen lab from *Cymbastela* sp. indicating that both tripeptides had all L amino acids. Kashman reported that hemiasterlin was cytotoxic against P388 with an IC_{50} of ca. 0.01 μg/mL. However, he cautioned that ". . . as hemiasterlin . . . might have contained impurities of jaspamide, the assays will have to be repeated when additional amounts of hemiasterlin . . . will be available" (Talpir, 1994). The PNG specimens of *Cymbastela* sp. provided sufficient quantities of the hemiasterlins **23** to **25** and criamide A (**26**) to allow Allen to determine more accurate IC_{50}'s for their *in vitro* cytotoxic activities (Table 3). The most striking result was the measured IC_{50} of 0.05 ng/mL for hemiasterlin (**23**) against P388, which indicated it was approximately a thousand-fold more potent than reported by Kashman. Based on these encouraging *in vitro* activities, Allen evaluated hemiasterlin in an *in vivo* P388 murine leukemia model and she found that it gave a %T/C 308 (5 doses @ 0.45 μg/mouse). It was also tested in an *in vivo* Gzhi (metastatic murine breast cancer) model (5 doses @ 1.0 μg/mouse) with the result that 60% of the mice were long-term survivors.

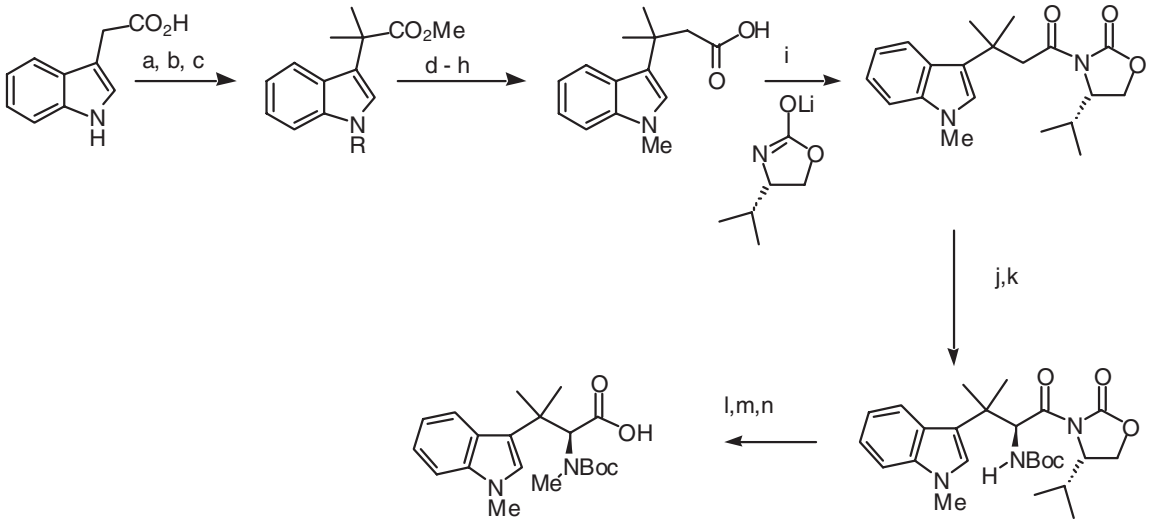
Based on the promising *in vitro* and *in vivo* activities documented for hemiasterlin by Allen, the Andersen lab in collaboration with E. Piers at UBC initiated a chemical synthesis of the compound. In addition, a small sample of hemiasterlin was sent to Lederle Laboratories of American Cyanamid as part of a pre-NCDDG collaboration between Bill Maise at Lederle and Andersen's group at UBC. Rob Kramer at Lederle ran hemiasterlin through his cell-line panel and determined a mean bar graph profile for the compound which confirmed its broad spectrum *in vitro* cytotoxicity and generated an 'interesting' differential cytotoxicity (mean bar graph) profile. This was the state of affairs when the first Ireland NCDDG proposal was submitted in September 1994 and further progress on the compound awaited the completion of the total synthesis.

The objectives of the total synthesis of hemiasterlin were several-fold. Primary among these was to solve the supply problem. It was clear that no matter how promising the activity of the compound, it was never going to generate serious interest at Cyanamid unless there was an efficient method to produce sufficient material for further testing and to demonstrate that in the long-term there was the possibility of an industrial method of production. Secondary, but equally important goals, were to demonstrate that the biological activity of the natural material actually came from the defined structure and not some very potent impurity, and to explore the SAR for this family of tripeptide cytotoxins with an eye towards making more potent analogs and analogs that were easier to synthesize.

The synthetic route to hemiasterlin and related analogs followed a convergent approach that involved synthesis of each of the appropriately protected N-terminal and C-terminal amino acids and subsequent coupling via standard methods to protected central aliphatic amino acids that were commercially available. Scheme 1 outlines the synthesis of Boc-protected tetramethyltryptophan, the N-terminal residue in hemiasterlin, and Scheme 2 outlines the synthesis of the ethyl ester of MHVV, the C-terminal residue. The steps involved in coupling the three amino acid residues into the natural product hemiasterlin (**23**) are shown in Scheme 3. Completion of the synthesis of hemiasterlin in mid 1996 provided sufficient material for additional biological evaluation of the compound (Andersen, 1997).

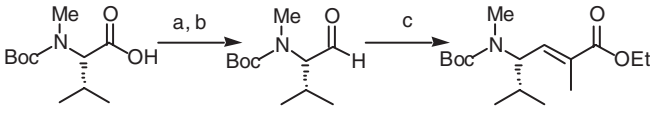
Table 3. *In vitro* cytotoxicities for natural hemiasterlin. IC_{50}s are listed in μg/mL.

Cell line	Hemiasterlin (**23**)	Hemiasterlin A (**24**)	Hemiasterlin B (**25**)	Criamide A (**26**)
Murine leukemia P388	0.000046	nd	0.007	0.0073
Human breast cancer MCF7	0.089	nd	0.066	6.8
Human glioblastoma/astrocytoma U373	0.012	0.0015	nd	0.27
Human ovarian carcinoma HEY	0.0014	0.0076	0.016	0.19



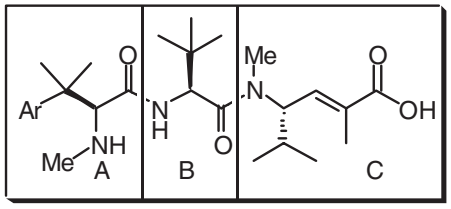
a) CH_2N_2, Et_2O; b) KHMDS, MeI, THF; c) KHMDS, MeI, THF;
d) DIBAL-H,THF, -78°C to 0°C; e) TPAP, NMO, CH_2Cl_2;
f) Ph_3PCH_2OMeCl, KO*t*-Bu, THF; g) TsOH,Dioxane, H_2O;
h) $NaClO_2$, NaH_2PO_4, *t*-BuOH, H_2O; i) i) pivaloyl chloride, THF; ii) **25**, THF, -78°C;
j) KHMDS, trisylN_3, THF, -78°C; k) H_2/Pd(C), Boc_2O, EtOAc or i) $SnCl_2$, dioxane, H_2O; ii) Boc_2O, $NaHCO_3$, dioxane, H_2O; l) LiOH, H_2O_2, MeOH; m) NaH, MeI, DMF; n) LiOH, MeOH, H_2O.

Scheme 1.



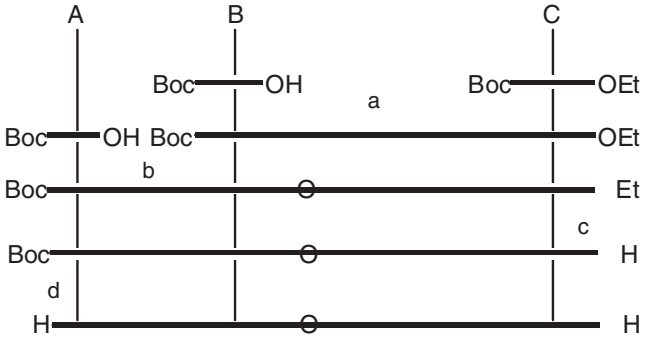
a) PyBOP®, $[H_2N(Me)OMe]Cl$, DIEA, CH_2Cl_2; b) LAH, THF;
c) $Ph_3PC(Me)CO_2Et$, CH_2Cl_2

Scheme 2.



Hemiasterlin (**7**): Ar= 1-methylindol-3-yl
SPA110/HTI 286 (**13**): Ar= phenyl

The *in vitro* cytotoxic IC_{50}s for synthetic hemiasterlin (**23**) were found to be identical with the IC_{50}'s for the natural product isolated from *Cymbastela* sp. Michel Roberge in the Biochemistry Department at UBC used a sample of the synthetic material to examine its mechanism of action. Roberge and co-workers found that hemiasterlin induced cell cycle arrest in mitotic metaphase (Andersen, 1997b). At low concentrations, it produced abnormal mitotic spindles like other microtubule inhibitors such as the Vinca alkaloids, and at higher concentrations, it caused microtubule depolymerization. Roberge concluded that hemiasterlin exerted its potent cytotoxic effects by inhibiting microtubule dynamics. Peter Lassota at Cyanamid carried out further investigations of hemiasterlin's interaction with tubulin. Although the results were never published, Lassota showed that hemiasterlin inhibited tubulin polymerization and had data to suggest that it was a noncompetitive inhibitor of vincristine binding to tubulin. Re-examination of hemiasterlin in Cyanamid's 26 cell-line panel showed that its mean bar graph profile had



a) i) TFA, CH_2Cl_2; ii) HATU, HOAt, DIEA, CH_2Cl_2 or pivaloyl chloride, DIEA, THF, -78°C to rt;
b) i) TFA, CH_2Cl_2; ii) PyBOP®, DIEA, CH_2Cl_2;
c) LiOH, MeOH, H_2O; d) TFA, CH_2Cl_2

Scheme 3.

strong similarities to that of the antimitoic agent taxol, as expected. Subsequently, Hamel and co-workers have provided more details of the interaction of hemiasterlin with tubulin (Gamble, 1999; Bai, 1999). They found that it was a competitive inhibitor of dolastatin 10 binding to tubulin and that it interfered with nucleotide exchange on β-tubulin. As part of the ongoing examination of hemiasterlins in the Andersen lab, more than 25 analogs of hemiasterlin were synthesized and evaluated for *in vitro* cytotoxicity and antimitotic activity against human breast cancer MCF7 cells (Andersen, 1999; Nieman, 2003). A new cell-based assay developed by Roberge was used to determine the antimitoic activities (Roberge, 2000). Figure 5 summarizes the first SAR information obtained for this family that came from evaluating these analogs. One of the synthetic analogs, a compound initially designated SPA 110 (**29**) (SPA: synthetic peptide analog) (Loganzo, 2003), was found to be approximately three-fold more potent than the natural product hemiasterlin (**23**). All of the other synthetic analogs were less potent. In addition, synthesis of the N-terminal amino acid in SPA 110 (**29**) (Scheme 4) was shorter (seven fewer steps) and higher yielding than the synthesis of the tetramethyltryptophan residue in hemiasterlin. Based on its ease of synthesis and its favorable cytotoxicity profile, tens of mg of SPA110 (**29**) were provided to Cyanamid (now Wyeth) for more in-depth biological evaluation. As a result of extensive *in vivo* tests (vide infra) conducted at Wyeth on SPA110, this compound was selected as a candidate for preclinical development and given the Wyeth code number HTI-286 (HTI-hemiasterlin tubulin inhibitor) (Loganzo, 2003).

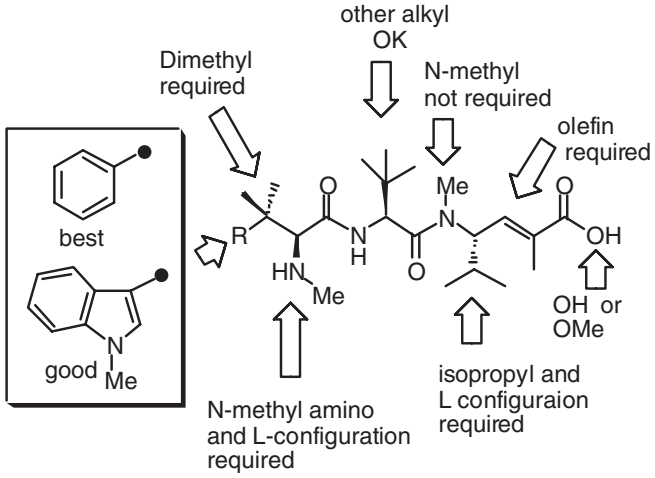
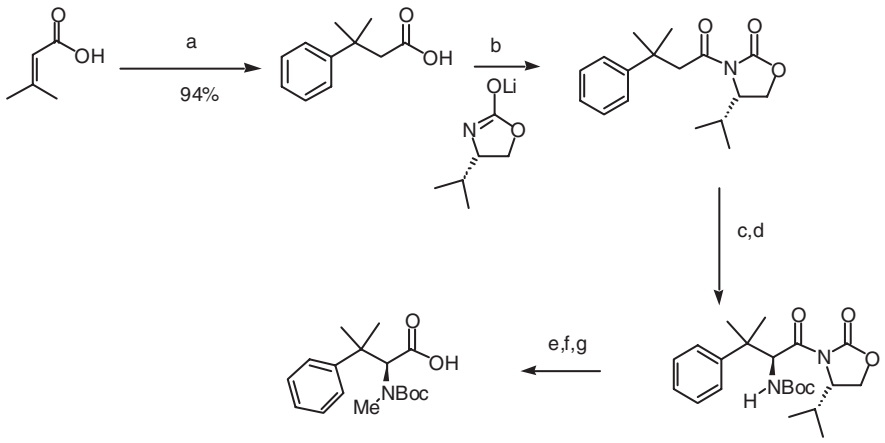


Figure 5. Preliminary SAR information for the hemiasterlin tripeptides generated in the Andersen/Roberge labs.

Wyeth's interest in hemiasterlins was multi-fold. First, Wyeth wanted to further explore the SAR beyond what Andersen's laboratory had provided. The second goal was to evaluate the potency and efficacy of hemiasterlin analogs in paclitaxel-sensitive and paclitaxel-resistant cell lines and tumor models. This would help justify clinical development of the agent. The third goal was to determine if resistance to HTI-286 might be encountered and if so, what could be the mechanistic basis of the phenotype. Finally, Wyeth hoped to understand the drug binding sites of hemiasterlin analogs within tubulin. The Wyeth Medicinal Chemistry Group dedicated a large synthetic effort to systematically explore each position of the tripeptide to achieve a thorough understanding of the SAR of HTI-286 (Fig. 6). Functionality required for activity, as well as positions tolerant of functionalization, were further elucidated. A key feature crucial for activity was branching at the beta position of the B-piece amino acid with the correct S configuration. The CD-piece olefin (a further subdivision of Andersen's "C" region) was not required for activity as reduction gave a diastereomer that retained substantial potency. The A-piece amine was necessary and activ-



a) $AlCl_3$, PhH, 65 °C; b) i) pivaloyl chloride, THF; ii) **25**, THF, -78 °C; c) KHMDS, trisylN_3, THF, -78 °C; d) H_2/Pd(C), Boc_2O, EtOAc; e) LiOH, H_2O_2, MeOH; f) NaH, MeI, DMF; g) LiOH, MeOH, H_2O.

Scheme 4.

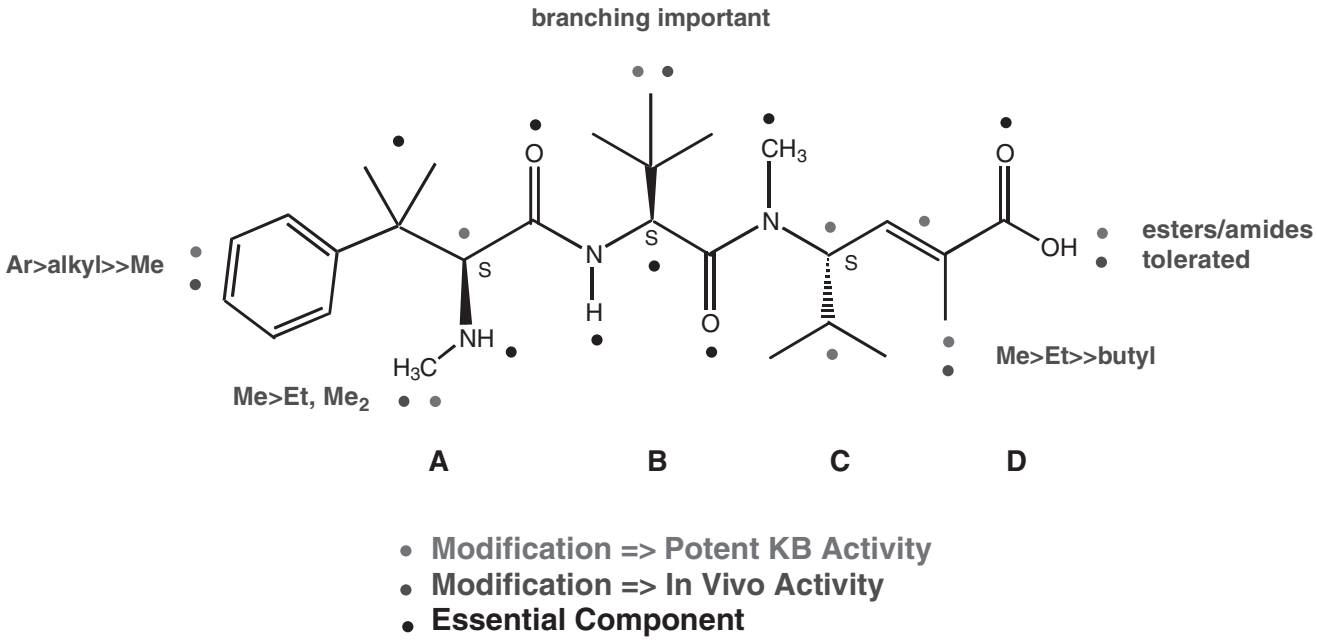


Figure 6. SAR information for the hemiasterlin tripeptides generated at Wyeth.

Table 4. Resistance profile of HTI-286 compared to other cytotoxic agents in cell lines that overexpress P-glycoprotein.

Compound	KB-3-1 IC_{50}, *nM*	KB-8-5 (P-glycoprotein +++) IC_{50}, *nM*	RR	KB-V1 (P-glycoprotein +++++) IC_{50}, *nM*	RR
HTI-286	0.96 ± 0.5	2.3 ± 1.2	2.4	77.4 ± 44	81
Hemiasterlin	0.319 ± 0.095	1.0 ± 0.5	3.2	76.1 ± 13.8	239
Paclitaxel	3.9 ± 1.8	63.3 ± 29	19	5,484 ± 2,780	1406
Docetaxel	0.55 ± 0.45	9.7 ± 6.6	18	368 ± 257	669
Vinblastine	0.79 ± 0.5	29.2 ± 22	37	1,464 ± 1,022	1848
Vinorelbine	2.4 ± 1.7	125.1 ± 50.3	52	>3,000	>1250
Colchicine	6.4 ± 1.3	62.7 ± 9.2	9.8	2,442 ± 2,176	382
Dolastatin-10	0.037 ± 0.02	0.263 ± 0.05	7.1	21.2 ± 1.4	573
Doxorubicin	43.3 ± 32	658 ± 483	15	11,489 ± 9,672	265

Data are mean IC_{50} (nM) ± standard deviation for the indicated agents based on two or more independent experiments. RR, relative resistance = ratio of IC_{50} of the resistant cell line to IC_{50} of the corresponding sensitive cell line. Lower relative resistance values indicate greater sensitivity of cells to the drug. P-glycoprotein +++ = moderate level expression; P-glycoprotein +++++ = very high level of expression as determined by immunoblot analysis.

ity was lost if it was acylated or replaced with carbon or a heteroatom. Disubstitution on nitrogen was tolerated but groups larger than ethyl led to significant loss of activity. The A-piece geminal dimethyl group was critical with removal of either diastereotopic methyl group leading to a loss of potency. The A-piece phenyl ring could be replaced with other lipophilic groups (consistent with the activity observed for hemiasterlin (**23**)) and was tolerant of a wide variety of substituents. The D-piece carboxylic acid could be esterified with retention of potent in vivo activity. D-piece amides could be quite potent with selection of the proper substituents. Proline gave a low nM analog and use of segments from the peptides dolastatin-10 or cemadotin gave sub nM compounds**.**

Based on the initial cell-based profiling of hemiasterlin, and subsequently more extensive analyses with HTI-286 in cells that had known mechanisms of resistance to paclitaxel, Wyeth established that HTI-286 had activity in tumor cell lines and human tumor xenograft models that were resistant to paclitaxel and Vinca alkaloids (Zask, 2002). For example, it was found that only 2- and 80-fold resistance to HTI-286 was found in cells that have approximately 20- and 1400-fold resistance to paclitaxel, respectively; the amount of resistance depended upon the level of expression of P-glycoprotein (Table 4). This was a critical distinction since resistance, either at the onset of therapy or during multiple rounds of treatment, occurs to all anti-microtubule agents

currently used to control the growth of solid tumors in patients (Rowinsky, 2001). Furthermore, taxanes are the principle anti-microtubule agents used in therapy for lung, breast, and ovarian cancers (Rowinsky, 2001). While the basis for resistance to agents such as the Vinca alkaloids or taxanes is complex in patients, in tissue culture systems, the ABC transporter known as P-glycoprotein mediates profound resistance to these agents in tissue culture systems (Dumontet, 2000). In addition, point mutations in tubulin mediate selective resistance to paclitaxel and epothilones (another microtubule stabilizing class of natural products) (Altmann, 2003). HTI-286 also overcame this mechanism of resistance (Loganzo, 2003). Beyond the *in vitro* data, HTI-286 inhibited the growth of tumors derived from cells that did not overexpress P-glycoprotein (and were sensitive to taxanes) as well as those that overexpressed P-glycoprotein and failed to respond to paclitaxel given on an optimal schedule and dose (Table 5). After extensive testing for tolerability and metabolism in rats and dogs, it was deemed that HTI-286 had a safety profile that would allow testing in humans. A Phase I trial in humans for the treatment of cancer has been completed (Ratain, 2003). Tolerated doses have been established and Phase II testing is in progress.

Since resistance to all anticancer agents has been encountered in the clinic, it is anticipated that resistance to HTI-286 would also be encountered. To help understand which patients might benefit the most from therapy with HTI-286, the laboratories of Lee Greenberger and Frank Loganzo (Wyeth) and Tito Fojo (NCI) selected tumor cell lines for resistance to HTI-286 in tissue culture and then attempted to determine the mechanistic basis for the resistance. To do this, two distinct cell lines, 1A9 ovarian (Poruchynsky, 2003) and KB-3-1 epidermoid carcinoma cells (Loganzo, 2003), were selected for resistance to HTI-286 in a step-wise fashion. The cell lines had similar but distinct phenotypes. Both cell types were about 10–15 fold resistant to HTI-286 and analogs. In addition, cross-resistance was observed to some agents that bind to the vinca site (i.e., vinblastine, rhizoxin) or vinca-peptide site (i.e., dolastatin-10) within tubulin (Hamel, 1996; Hamel, 2002), but little or no resistance was observed to taxanes or colchicine. In fact, enhanced sensitivity to tubulin polymerizing agents was seen in the 1A9 cells selected for HTI-286 resistance. These data are consistent with the hypothesis that hemiasterlin binds to the Vinca-peptide binding site in tubulin (Hamel, 2002) that is distinct from colchicine or taxane binding domains. Mechanistically, no increase in P-glycoprotein or *mdr*1 RNA was found based on immunoblotting and RT-PCR methodology, respectively. However, point mutations, mostly in alpha tubulin, were found in both series of resistant cell lines, and they may be useful in identifying HTI-286 – tubulin interaction sites. Beyond this, the HTI-selected KB cells had energy-dependent low drug accumulation that would be consistent with the expression of a novel ABC transporter (Gottesman, 2002).

While the data on the mechanism of resistance to HTI-286 suggested possible binding sites for the agent, Wyeth pursued

Table 5. The response of human xenograft tumors to HTI-286 and other anti-microtubule agents.

Tumors with little or no detectable levels of P-glycoprotein expression				
Tumor Type		%T/C[1]		
LOX melanoma	N	Day 7	Day 14	Day 21
HTI-286	5	17 × 1	3 × 1	–
Paclitaxel	2	12 × 4	3 × 2	–
Vincristine	4	17 × 6	6 × 2	–
KB-3-1 epidermoid ca.				
HTI-286*	2	6 × 5	3 × 1	20 × 3
Paclitaxel	1	12	5	5
Vincristine	1	11	9	9
LOVO colon ca.				
HTI-286	1	88	35	20
Paclitaxel	1	17	6	8
Vincristine	3	34 × 8	37 × 15	45 × 15
MCF-7 breast ca.				
HTI-286	1	15	6	27
Paclitaxel	1	41	3	0
Vincristine	2	33	16	14
Tumors with little to high detectable levels of P-glycoprotein expression				
KB-8-5 epidermoid ca.[3]				
HTI-286	3	21 × 4	9 × 4	47[2]
Paclitaxel	2	72 × 9	77 × 10	109[2]
Vincristine	2	88 × 16	84 × 1	81[2]
SW-620-W colon ca.[3]				
HTI-286	5	19 × 4	9 × 4	1[2]
Paclitaxel	3	60 × 10	73 × 5	55[2]
Vincristine	3	81 × 6	63 × 3	59 × 3
DLD-1 colon ca.[3]				
HTI-286	4	37 × 8	37 × 8	54 × 12
Paclitaxel	2	71 × 1.5	73 × 21	101 × 4
Vincristine	2	123 × 10	125 × 6	153 × 24
HCT-15 colon ca.[3]				
HTI-286	4	39 × 12	35 × 9	38 × 7
Paclitaxel	3	103 × 8	93 × 11	109 × 7
Vincristine	3	61 × 26	55 × 14	59[2]

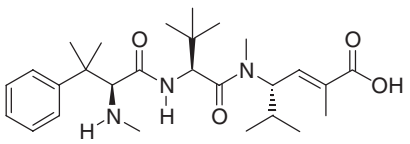
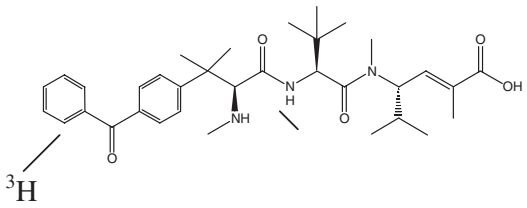
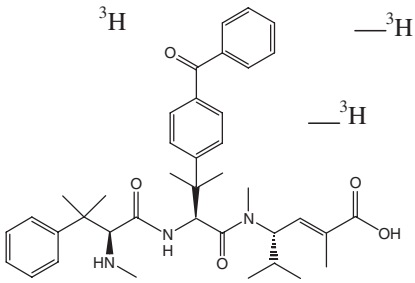
[1] Tumor cells were implanted into the flanks of nude mice. %T/C = percent tumor size in treatment group vs. control on days 7, 14, and 21 after drug dosing. Values shown are mean × standard errors (where available). N = number of independent experiments. HTI-286 was given at 1.5 mg/kg IV or 2.0 mg/kg IV (indicated by *), except LOX given at 1.0 mg/kg. Paclitaxel was given at 60 mg/kg IV. Vincristine was given at 0.8–1.0 mg/kg IP. All drugs were given on day 1, 5, and 9 to tumors that had an established size of approximately 100 mg.

[2] Control tumor grew too large at this time point in one experiment in this group and no standard error could be computed.

[3] The level of P-glycoprotein expression in SW-620-W (previously reported as MX1W), KB-8-5, DLD-1, and HCT-15 were approximately +, +++, +++, and ++++, respectively.

an independent path to identify such sites. Since the SAR of HTI-286 was well defined, Arie Zask and Joshua Kaplan were able to synthesize potent radioactive photoaffinity analogues of HTI-286 that might mimic the parent molecule (Kaplan, 2002). Researchers at Wyeth were able to show that two of these molecules, designated probe 1 and 2, retain high affinity for tubulin, were potent inhibitors of tumor cell growth, and were potent inhibitors of tubulin polymerization in a cell-free system (Nunes, 2002) (Table 6). Maria Nunes demonstrated that both probes bound exclusively to alpha tubulin, even though both alpha- and beta-tubulin were present in a 1 : 1 ratio (Fig. 7). The binding of both probes to tubulin was competed by non-radiolabeled probe. To date, probe 1 has been studied in detail, while further studies with probe 2 are in progress. It was found that the binding of probe 1 to tubulin was competed by dolastatin-10, and Vinca alkaloids, but not by colchicine or paclitaxel. In fact, the latter two agents enhanced the binding under certain conditions. The labeling site for probe 1 was localized to residues 314–334 of alpha-tubulin that, based on electron crystallographic analysis of zinc-induced tubulin sheets (Nogales, 1999), corresponded to the sheet 8 – helix 10 region of tubulin (Fig. 8). This region has longitudinal interactions with beta-tubulin and lateral interactions with adjacent protofilaments (Bai, 1999). It may explain why hemiasterlin induces abortive attempts to form microtubules (Hamel, 2002). Although mapping drug-binding sites within tubulin using photoaffinity labeling has limited resolution (Downing, 2000) and certain technical limitations, these data are the first to suggest that the peptide-binding site resides in alpha tubulin. The rest of the binding site either straddles the *inter* dimer alpha-beta tubulin interface – between dimers – or resides solely within alpha-tubulin. This contrasts with the colchicine binding site that straddles the *intra* dimer alpha-beta tubulin interface, and the paclitaxel binding site that resides solely in beta-tubulin (Downing, 2000). Ultimately, HTI-286 co-crystallized with tubulin will be needed to better understand the interaction of hemiasterlin analogs with tubulin.

Table 6. Structure and Activity of Photoaffinity Probes for HTI-286.

Compound	Structure	(μM)	IC_{50} KB cells[d] (nM)	% inhibition of tubulin polymerization[e]
HTI-286		0.4–0.8	0.96 ± 0.5	87.5 ± 12.2
Probe 1[a]		0.2–0.8	1.8 ± 0.1 (n = 2)	88
Probe 2[b]		1.1–6	22.4 ± 0.7 (n = 2)	69

[a] The chemical name for Probe 1 is 4-benzoyl-N,β,β-trimethyl-L-phenylalanyl-N1-[(1S,2E)-3-carboxy-1-isopropylbut-2-enyl]-N1,3-dimethyl-L-valinamide. It has been tritiated within the benzene rings: specific activity = 70.6 Ci/mmol, purity >99%.

[b] The chemical name for Probe 2 is N,β,β-trimethyl-L-phenylalanyl-4-benzoyl-N-[(1S,2E)-3-carboxy-1-isopropyl-2-butenyl]-N,β,β-trimethyl-L-phenylalaninamide. It has been tritiated within the benzene rings: specific activity = 82.1 Ci/mmol, purity > 98.8%.

[c] Affinity constant determined binding of non-radiolabeled probe to tubulin by fluorescence analysis.

[d] KB cells were grown in the presence of the test agent for 72 hours. Cell survival was determined by the SRB method. IC_{50} = the amount of drug needed to inhibit cell growth by 50%.

[e] Bovine brain tubulin was allowed to polymerize in the absence or presence of 0.3 μM test agent according to previously described methods.

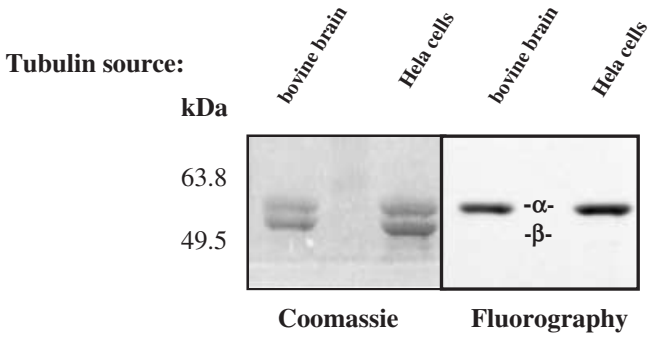


Figure 7. Photoaffinity labeling of tubulin derived from bovine brain or Hela cells by probe 1.

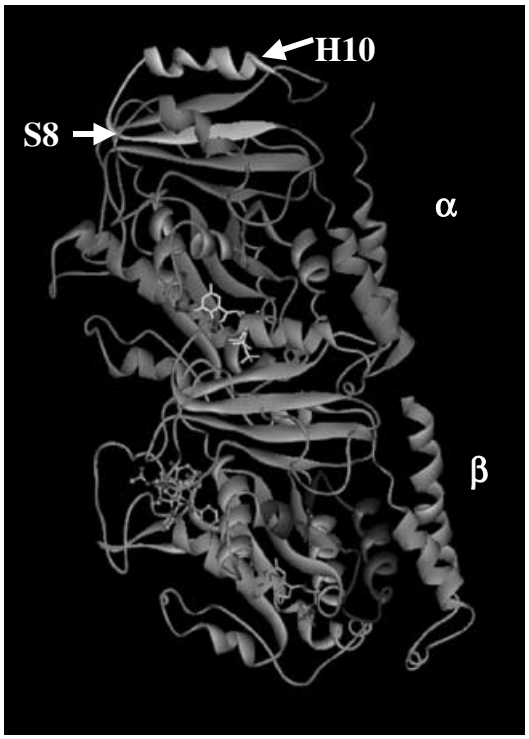


Figure 8. Site of major photoaffinity labeling domain by probe 1 within the model structure of tubulin as determined by Nogales, 1999.

References

Alessi DR, Cuenda A, Cohen P, Dudley DT, Saltiel R (1995): PD-098059 is a specific inhibitor of the activation of mitogen-activated protein kinase kinase *in vitro* and *in vivo. J Biol Chem 270*: 27489–27494.

Alessi DR, Cohen P, Ashworth A, Cowley S, Leevers SJ, Marshall CJ (1995): Assay and expression of mitogen-activated protein kinase, MAP kinase kinase, and Raf. *Methods Enzymol 255*: 279–290.

Altmann KH (2003): Epothilone B and its analogs – a new family of anticancer agents. *Mini Rev Med Chem 3*: 149–158.

Altschul SF, Gish W, Miller W, Myers EW, Lipman DJ (1990): Basic local alignment search tool. *J Mol Bio 215*: 403–410.

An K-D, Nishida H, Miura Y, Yokota A (2003): Molecular evolution of adenylating domain of aminoadipate reductase. *BMC Evol Biol 3*: 9–15 (http://www.biomedcentral.com/1471-2148/3/9).

Anderson HJ, Coleman JE, Andersen RJ, Roberge M (1997): Cytotoxic peptides hemiasterlin, hemiasterlin A, and hemiasterlin B induce mitotic arrest and abnormal spindle formation. *Cancer Chemother Pharmacol 39*: 223–226.

Andersen RJ, Coleman JE, Piers E, Wallace DJ (1997): Total synthesis of (−)-hemiasterlin, a structurally novel tripeptide that exhibits potent cytotoxic activity. *Tetrahedron Lett 38*: 317–320.

Andersen RJ, Piers E, Nieman J, Coleman J, Roberge M (1999): WO Patent 99/3250.

Angeles TS, Steffler C, Bartlett BA, Hudkins RL, Stephens RM, Kaplan DR, Dionne CA (1996): Enzyme-linked immunosorbent assay for trkA tyrosine kinase activity. *Anal Biochem 236*: 49–55.

Bai R, Durso NA, Sackett DL, Hamel, E (1999): Interactions of the sponge-derived antimitotic tripeptide hemiasterlin with tubulin: comparison with dolastatin 10 and cryptophycin 1. *Biochemistry 38*: 14302–14310.

Barbacid M (1987): RAS Genes. *Ann Rev Biochem 56*: 779–822.

Boyd, MR (1989): Status of the NCI preclinical drug discovery screen. In: *Principles and Practices of Oncology 3*, pp. 1–10, J.B. Lippincott Publish.

Coleman JE, de Silva ED, Kong F, Andersen RJ, Allen TM (1995): Cytotoxic peptides from the marine sponge *Cymbastela* sp. *Tetrahedron 51*: 10653–10662.

Coleman JE, Patrick BO, Andersen RJ, Rettig SJ (1996): Hemiasterlin methyl ester. *Acta Cryst C52*: 1525–1527.

Crews P, Farias JJ, Emrich R, Keifer PA (1994): Milnamide A, an unusual cytotoxic tripeptide from the marine sponge *Auletta* cf. *constricta J Org Chem 59*: 2932–2934.

Curman D, Cinel B, Williams DE, Rundle N, Block WD, Goodarzi AA, Hutchins JR, Clarke PR, Zhou B-B, Lees-Miller SP, Andersen RJ, Roberge M (2001): Inhibition of the G_2 DNA damage checkpoint and of protein kinases Chk1 and Chk2 by the marine sponge alkaloid debromohymenialdisine. *J Biol Chem 276*: 17914–17919.

de Silva ED, Andersen RJ, Allen TM (1990): Geodiamolide C to Geodiamolide F, new cytotoxic cyclodepsipeptides from the marine sponge *Pseudaxinyssa* sp. *Tetrahedron Lett 31*: 489–492.

Discafani CM, Carroll ML, Floyd Jr MBF, Hollander IJ, Husain Z, Johnson BD, Kitchen D, May MK, Malo MS, Minnick Jr AA, Nilakantan R, Shen R, Wang Y-F, Wissner A, Greenberger LM (1999): Irreversible inhibition of epidermal growth factor receptor tyrosine kinase with *in vivo* activity by N-[4-[3-bromophenyl)amino]-6-quinaxolinyl]-2-butynamide (CL-387,785). *Biochem Pharmacol 57*: 917–925.

Downing, KH (2000): Structural basis for the interaction of tubulin with proteins and drugs that affect microtubule dynamics. *Annu Rev Cell Dev Biol 16*: 89–111.

Dumontet C (2000): Mechanism of action and resistance to tubulin-binding agents. *Exp Opin Invest Drugs 9*: 779–788.

Foulkes WD, Flanders TY, Pollock PM, Hayward NK, (1997): The CDKN2A (p16) gene and human cancer. *Mol Med 3*: 5–20.

Gamble WR, Durso NA, Fuller RW, Westergaard CK, Johnson TR, Sackett DL, Hamel E, Cardellina JH, Boyd MR (1999): Cytotoxic and tubulin-interactive hemiasterlins from *Auletta* sp. and *Siphonochalina* sponges. *Bioorg Med Chem 7*: 1611–1615.

Gibbons JJ, Discafani C, Peterson R, Hernandez R, Skotnicki J, Frost P (1999): The effect of CCI-779, a novel macrolide anti-tumor agent, on the growth of human tumor cells *in vitro* and in nude mouse xenografts *in vivo. Proc Am Assoc Cancer Res 40*: 301.

Gibbs (2000): Anticancer drug targets: growth factors and growth factor signaling. *J Clin Invest 105*: 9–13.

Gottesman MM (2002): Mechanisms of cancer drug resistance. *Annu Rev Med 53*: 615–627.

Gottesman MM, Fojo T, Bates SE (2002): Multidrug resistance in cancer: role of ATP-dependent transporters. *Nat Rev Cancer 2*: 48–58.

Hall M, Peters G (1996): Genetic alterations of cyclins, cyclin-dependent kinases, and Cdk inhibitors in human cancer. *Adv Cancer Res 68*: 67–108.

Hamel E (1996): Antimitotic natural products and their interactions with tubulin. *Med Res Rev 16*: 207–231.

Hamel E, Covell DG (2002): Antimitotic peptides and depsipeptides. *Curr Med Chem Anti-Canc Agents 2*: 19–53.

Hanks SK, Hunter T (1995): The eukaryotic protein kinase superfamily: kinase (catalytic) domain structure and classification. *FASEB 9*: 576–596.

He H, Ding W-D, Bernan VS, Richardson AD, Ireland CM, Greenstein M, Ellestad GA, Carter GT (2001): Lomaiviticins A and B, potent antitumor antibiotics from *Micromonospora lomaivitiensis. J Am Chem Soc 123*: 5362–5363.

Hirama T, Koeffler HP (1995): Role of the cyclin-dependent kinase inhibitors in the development of cancer. *Blood 86*: 841–854.

Hunter T (1997): Oncoprotein networks. *Cell 88*: 333–346.

Imamura N, Nishijima M, Takadera T, Adachi K, Sakai M, Sano H (1997): New anticancer antibiotics pelagiomicins, produced by a new marine bacterium *Pelagiobacter variabilis*. *J Antibiot 50*: 8–12.

Kamb A, Gruis NA, Weaver-Feldhaus J, Liu Q, Harshman K, Tavtigian SV, Stockert E, Day III RS, Johnson BE, Skolnick MH (1994): A cell cycle regulator potentially involved in genesis of many tumor types. *Science 264*: 436–440.

Kaplan JA, Nunes M, Ayral-Kaloustian S, Krishnamurthy G, Loganzo F, Greenberger LM, Minnick A, May M, Zask A (2002): Hemiasterlin photoaffinity ligands: [^{3}H] benzophenone analogs of HTI-286. *National Medicinal Chemistry Symposium*: abstract 49.

Kfir R, Johannsen E, Vleggaar R (1986): Mutagenic activity of austocystins-secondary metabolities of *Aspergillus ustus. Bull Environ Contam Toxicol 37*: 643–650.

Kolch W (2000): Meaningful relationships: the regulation of the Ras/Raf/MEK/ERK pathway by protein interactions. *Biochem J 351*: 289–305.

Kramer RA, Zakher J, Kim G (1988): Role of glutathione redox cycle in acquired and *de novo* multidrug resistance. *Science 241*: 694–698.

Kramer RA, Weber TK, Morse B (1993): Constitutive expression of multidrug resistance in human colorectal tumors and cell lines. *Br J Cancer 67*: 959–968.

Lackey K, Cory M, Davis R, Frye SV, Harris PA, Hunter RN, Jung DK, McDonald OB, McNutt RW, Peel MR, Rutkowske RD, Veal JM, Wood ER (2000): The discovery of potent cRaf-1 kinase inhibitors. *Bioorg Med Chem Lett 10*: 223–226.

Lavoie JN, L'Allemain G, Brunet A, Muller R, Pouyssegur J (1996): Cyclin D1 expression is regulated positively by the p42/p44 MAPK and negatively by the p38/HOGMAPK pathway. *J Biol Chem 271*: 20608–20616.

Lee T, Yun S-H, Hodge KT, Humber RA, Krasnoff SB, Turgeon GB, Yoder OC, Gibson DM (2001): Polyketide synthase genes in insect- and nematode-associated fungi. *Appl Microbiol Biotechnol 56*: 181–187.

Lerner EC, Zhang TT, Knowles DB, Qian Y, Hamilton AD, Sebti M (1997): Inhibition of the prenylation of K-Ras, but not H- or N-Ras, is highly resistant to CAAX peptidomimetics and requires both a farnesyltransferase and a geranylgeranyltransferase I inhibitor in human tumor cell lines. *Oncogene 15*: 1283–1288.

Levitzki A, Gazit A (1995): Tyrosine kinase inhibition: an approach to drug development. *Science 267*: 1782–1788.

Lewis TS, Shapiro PS, Ahn NG (1998): Signal transduction through MAP kinase cascades. *Adv Cancer Res 74*: 49–139.

Loganzo F, Annable T, Xingzhi T, Morilla DB, Hari M, Musto S, Zask A, Kaplan J, Poruchynsky M, Fojo TMGL (2003): Cells resistant to HTI-286 do not over-express P-glycoprotein but have low drug accumulation and a point mutation in α-tubulin. *Proc Amer Assoc Cancer Res 44*: 1307 (2nd ed.).

Loganzo F, Discafani C, Annable T, Beyer C, Musto S, Tan X, Hardy C, Hernandez R, Baxter M, Singanallore T, Khafizova G, Poruchynsky MS, Fojo T, Nieman JA, Ayral-Kaloustian S, Zask A, Andersen RJ, Greenberger LM (2003): HTI-286, a synthetic analogue of the tripeptide hemiasterlin, is a potent antimicrotubule agent that circumvents P-glycoprotein-mediated resistance *in vitro* and *in vivo. Cancer Res 63*: 1838–1845.

Mallon R, Feldberg LR, Kim SC, Collins K, Wojciechowicz D, Hollander I, Kovacs ED, Kohler C (2001): An enzyme-linked immunosorbent assay for the Raf/MEK-1/MAPK signaling cascade. *Anal Biochem 294*: 48–54.

Martin JF (1998): New aspects of genes and enzymes for β-lactam antibiotic biosynthesis *Appl. Microbiol Biotechnol 50*: 1–15.

McDonald LA, Capson TL, Krishnamurthy G, Ding W, Ellestad GA, Bernan VS, Maiese WM, Lassota P, Discafani C, Kramer RA, Ireland CM (1996): Namenamicin, a new

enediyne antitumor antibiotic from the marine ascidian *Polysyncraton lithostrotum. J Amer Chem Soc 118*: 10898–10899.

McDonald OB, Chen WJ, Ellis B, Hoffman C, Overton L, Rink M, Smith A, Marshall CJ, Wood ER (1999): A scintillation proximity assay for the Raf/MEK/ERK kinase cascade: high-throughput screening and identification of selective enzyme inhibitors. *Anal Biochem 268*: 318–329.

Meijer L, Kim S-H (1997): Chemical inhibitors of cyclin-dependent kinases. *Methods Enzymol 283*: 113–128.

Meijer L, Thunnissen A-MWH**,** White AW, Garnier M, Nikolic M, Tsai L-H, Walter J, Cleverley KE, Salinas PC, Wu Y-Z, Biernat J, Mandelkow E-M, Kim S-H, Pettit GR (2000): Inhibition of cyclin-dependent kinases, GSK-3 and CK1, by hymenialdisine, a marine sponge constituent. *Chem Biol 7*: 51–63.

Mithani S, Weeratunga G, Taylor NJ, Dmitrienko GI (1994): The kinamycins are diazofluorenes and not cyanocarbazoles. *J Amer Chem Soc 116*: 2209–2210.

Morgan DO (1995): Principles of CDK regulation. *Nature 374*: 131–134.

Monks A, Scudiero D, Skehan P (1991): Feasibility of a high-flux anticancer drug screen using a diverse panel of cultured human tumor cell lines. *J Natl Cancer Inst 83*: 757—766.

Nieman J, Coleman J, Wallace D, Piers E, Lim LY, Roberge M, Andersen RJ (2003): synthesis and antimitotic/cytotoxic activity of hemiasterlin analogs. *J Nat Prod 66*: 183–199.

Nobori T, Miura K, Wu DJ, Lois A, Takabayashi K, Carson DA (1994): Deletions of the cyclin-dependent kinase-4 inhibitor gene in multiple human cancers. *Nature 368*: 753–756.

Nogales E, Whittaker M, Milligan RA, Downing KH (1999): High-resolution model of the microtubule. *Cell 96*: 79–88.

Nunes M, Kaplan J, Loganzo F, Zask A, Ayral-Kaloustian S, Greenberger L (2002): Two photoaffinity analogs of HTI-286, a synthetic analog of hemiasterlin, interact with alpha-tubulin. *Eur J Cancer 38*: S119.

O'Dwyer PJ, Stevenson JP, Gallagher M, Cassella A, Vasilevskaya I, Monia BP, Holmlund J, Dorr FA, Yao KS (1999): c-Raf-1 depletion and tumor responses in patients treated with the c-Raf-1 antisense oligodeoxynucleotide ISIS 5132 (CGP 69846A). *Clin Cancer Res 5*: 3977–3982.

Pang L, Sawada T, Decker SJ, Saltiel AR (1995): Inhibition of MAP kinase kinase blocks the differentiation of PC-12 cells induced by nerve growth factor. *J Biol Chem 23*: 13585–13588.

Pawson T, Saxton TM (1999): Signaling networks – do all roads lead to the same genes? *Cell* 97: 675–678.

Poruchynsky MS, Kim JH, Nogales E, Loganzo F, Greenberger LM (2003): Tumor cells resistant to a microtubule-depolymerizing hemiasterlin analog, HTI-286, have mutations in α- or β-tubulin and increased microtubule stability. *Proc Am Assoc Cancer Res 44*: 535 (2nd ed.).

Ratain MJ, Undevia S, Janisch L, Roman S, Mayer P, Buckwalter M, Foss D, Hamilton BL, Fischer J, Bukowski RM (2003): Phase I and pharmacological study of HTI-286, a novel antimicrotubule agent: correlation of neutropenia with time above threshold plasma concentration. *Proc Am Soc Clin Oncol*: abstract 516.

Roberge M, Cinel B, Anderson HJ, Lim L, Jiang X, Xu L, Kelly MT, Andersen RJ (2000): Cell-based screen for antimitotic agents and identification of analogues of rhizoxin, eleutherobin, and paclitaxel in natural extracts. *Cancer Res 60*: 5052–5058.

Robinson MJ, Cobb MH (1997): Mitogen-activated protein kinase pathways. *Curr Opin Cell Biol 9*: 180–186.

Rowinsky EK, Tolcher AW (2001): Antimicrotubule Agents. In: Devita Jr VT, Hellman S, Rosenberg SA, eds., *Cancer Principles and Practice*. 6th ed., Philadelphia, Lippincott Williams and Wilkins, pp. 431–452.

Salomon DS, Brandt R, Ciadiello F, Normanno N (1995): Epidermal growth factor-related peptides and their receptors in human malignancies. *Crit Rev Oncol Haematol 19*: 183–232.

Sebolt-Leopold JS, Dudley DT, Herrera R, Van Becelaere K, WilandA, Gowan RC, Tecle H, Barrett SD, Bridges A, Przybranowski S, Leopold WR, Saltiel AR (1999): Blockade of the MAP kinase pathway supresses growth of colon tumors *in vivo*. *Nat Med 5*: 810–816.

Stehelin D; Varmus HE, Bishop JM, Vogt PK (1976): DNA related to the transforming gene of avian sarcoma virus is present in normal avian DNA. *Nature 260*: 170–173.

Steyn PS, Vleggaar R (1974): Austocystins. Six novel dihydrofurol[3′,2′:4,5]furo[3,2-b]xanthenones from *Aspergillus ustus*. *J Chem Soc Perkin I 19*: 2250–2256.

Steyn PS, Vleggaar R (1975): Dihydrofuro[3′,2′:4,5]furo[3,2-b]xanthenones: the structures of austocystins G, H, and I. *J South African Chemical Institute 28*: 375–377.

Strawn, LM, Shawver LK (1998): Tyrosine kinases in disease: overview of kinase inhibitors as therapeutic agents and current drugs in clinical trials. *Exp Opin Invest Drugs 7*: 553–573.

Talpir R, Benayahu Y, Kashman Y, Pannell L, Schleyer M (1994): Hemiasterlin and geodiamolide-TA – 2 new cytotoxic peptides from the marine sponge *Hemiasterella minor* (Kirkpatrick). *Tetrahedron Lett 35*: 4453–4456.

Tasdemir D, Mallon R, Greenstein M, Feldberg LR, Kim SC, Collins K, Wojciechowicz W, Mangalindan GC, Concepción GP, Harper MK, Ireland CM (2002): Aldisine alkaloids from the Philippine sponge *Stylissa massa* are potent inhibitors of mitogen-activated protein kinase kinase-1 (MEK-1) *J Med Chem 45*: 529–532.

U.N. Convention on Biological Diversity, June 2, 1992, U.N. Doc. DPI/130/7.

Weber G, Schorgendorfer K, Schneider-Scherzer E, Leitner E (1994): The peptide synthetase catalyzing cyclosporine production in *Tolypocladium niveum* is encoded by a giant 45.8-kilobase open reading frame. *Curr Genet 26*: 120–125.

Weinber RA (1991): Molecular Biology of Carcinogenesis. In: Broder S, ed., *Molecular Foundations of Oncology*. Williams & Wilkins, pp. 27–39.

Woodburn JR (1999): The epidermal growth factor receptor and its inhibition in cancer therapy. *Pharmacol Ther 82*: 241–250.

Zaharevitz DW, Gussio R, Leost M, Senderowicz AM, Lahusen T, Kunick C, Meijer L, Sausville EA (1999): Discovery and initial characterization of the paullones, a novel class of small-molecule inhibitors of cyclin-dependent kinases. *Cancer Res 59*: 2566–2569.

Zask A, Birnberg G, Cheung K, Cole D, Kaplan J, Niu C, Norton E, Sandanayaka V, Suayan R, Tang Z, Yamashita A, Loganzo F, Beyer C, Discafani C, Greenberger LM, Ayral-Kaloustian S (2002): Synthesis and biological activity of analogs of the antimicrotubule agent HTI-286. *Proc Amer Assoc Cancer Res 43*: 737.