IN VITRO SCREENING OF CRUDE EXTRACTS AND PURE METABOLITES
OBTAINED FROM MARINE INVERTEBRATES FOR THE TREATMENT OF
BREAST CANCER

by

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THESIS ABSTRACT

Fifteen samples of crude extracts and pure metabolites obtained from marine invertebrates collected from the offshore waters of British Columbia, Papua New Guinea and Sri Lanka had previously been shown to exert cytotoxic activity in the *in vitro* L1210 leukemic bioassay. For this thesis, these samples were screened for *in vitro* cytotoxic activity against the drug-sensitive breast tumor cell lines MCF-7, T-47D, ZR-75-1 and MDA-MB-231; the multidrug-resistant and P-glycoprotein (Pgp)-positive breast tumor cell lines MCF-7 Ad^r and MDA-A1^r; and normal and malignant human breast epithelial cells (HBEC) in primary culture. Eight samples exhibited significant [drug concentration resulting in a 50% decrease in cell growth as compared with controls (ED₅₀), < 25 µg/ml] dose-dependent cytotoxicity against the drug-sensitive cell lines; the ED₅₀ values were as low as 0.004 µg/ml. Five of the 8 samples exhibited significant cytotoxicity against the multidrug-resistant cell lines; the ED₅₀ values were as low as 0.0006 µg/ml. Incubation of the MCF-7 Ad^r cells with varying concentrations of compounds in the presence of Adriamycin demonstrated that none of the compounds tested appeared to interfere with Pgp function. Results obtained using HBEC in primary culture showed a wide range of chemosensitivities for a given drug against tissue taken from different patients, demonstrating the uniqueness of the response of different individuals to chemotherapy.

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LIST OF ABBREVIATIONS

A ₅₄₀	Absorbance at 540 nm
BSA	Bovine serum albumin
DMSO	Dimethyl sulfoxide
ED ₅₀	Effective dose which results in a 50% decrease in cell viability
FBS	Fetal bovine serum
HBEC	Human breast epithelial cells
HTCFA	Human tumor colony forming assay
ILS	Increase in life span
MDR	Multidrug resistance
MTT	3-[4,5-dimethyl(thiazol-2-yl)-3,5-depheryl] tetradium bromide
NCI	National Cancer Institute (USA)
PALA	N-(phosphonacetyl)-L-aspartate
Pgp	P-glycoprotein
SRB	Sulforhodamine B

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CHAPTER 1
INTRODUCTION

1. Treatment of Cancer with Chemotherapy

Over the last half century, approximately forty drugs suitable for the treatment of cancer have been developed (148). As a result, great strides have been made with the use of chemotherapy in the treatment of cancer. The cure rates of different types of disseminated malignancies with chemotherapy, alone or in combination with surgery and/or radiotherapy, is summarized in Table 1. Moderate to high cure rates are seen in the treatment of malignancies such as testicular carcinoma, Wilms tumor, childhood sarcomas and many of the leukemias and lymphomas. Unfortunately many common solid tumors, especially the epithelial neoplasms of adulthood still have a poor response rate to chemotherapy, thereby indicating that there is still a dire need for new and more effective chemotherapeutic agents and regimes (21, 62, 73, 138).

Table 1. Curability of disseminated cancer with treatment involving chemotherapy.*

<u>DISEASE</u>	<u>PROBABLE CURE RATE</u>
ADULTS	
Diffuse histiocytic lymphoma (III & IV)	50%
Hodgkin's disease (III & IV)	50%
Testicular cancer (III)	75%
Gestational choriocarcinoma	90%
Acute myelocytic leukemia	20%
CHILDREN	
Acute lymphocytic leukemia	50%
Non Hodgkin's lymphoma	50%
Burkitt lymphoma	50%
Wilm's tumor	50%
Childhood sarcomas	50%

*Adapted from: Chabner B.A. (1990) Clinical strategies for cancer treatment: The role of drugs. In: Cancer Chemotherapy, Principles and Practice. (Chabner B. and Collins J., eds.), JB Lippincot Co, New York. pp 3.

1a. Treatment of Breast Cancer with Chemotherapy

Breast cancer afflicts one in 9 women in North America. It arises from the epithelial lining of the ducts and lobules of the glandular portion of the breast. Usually at the time of clinical presentation, the cancer has metastasized to distant sites in the body, thereby making treatment of breast cancer a systemic problem. Currently the best form of treatment of advanced breast cancer is treatment of the primary tumor with either surgery or radiation followed by a short course of high-dose adjuvant chemotherapy. This chemotherapy is given to kill any residual cancer cells. Metastatic breast cancer is responsive to all major classes of chemotherapeutic agents, with response rates falling in the 20%-40% range (77, 155).

Response rates of approximately 55% are observed when combination chemotherapy is used instead of single-agent therapy (77). Currently the best chemotherapy regimes are the cyclophosphamide-methotrexate-5-fluorouracil-vincristine-prednisone (CMFVP) variants (18, 27, 71, 77, 79, 113) and Adriamycin (doxorubicin) based variants (ie: Adriamycin-cyclophosphamide and Adriamycin-cyclophosphamide-5-fluorouracil) (53, 77, 79, 119). The chances of survival of the patient with metastatic breast cancer treated with adjuvant chemotherapy is best illustrated in a report released in 1986 by the medical statistician Dr. R Peto (121, 155). This report analyzed the cumulative survival data of approximately 15,000 women who have participated in adjuvant chemotherapy trials over the years. It is very statistically powerful because of the high number of participants involved. The report states that adjuvant combination chemotherapy in premenopausal patients with metastatic breast cancer results in approximately a 30-35% decrease in risk of death as compared to patients not receiving therapy. This 30-35% decrease in risk of death translates into a benefit to approximately 10-20% of the patients. For example, instead of 55% rate of mortality after 10 years, combination chemotherapy will result in a 38% rate of mortality. Postmenopausal patients experienced little significant benefit ($12 \pm 5\%$) in risk of death (116). The long term survival of the first successful CMF trials is illustrated in Figure 1.

Figure 1. Total survival of premenopausal patients with advanced breast cancer after CMF chemotherapy.
(From: Bonadonna G. (1989) Conceptual and practical advances in the management of breast cancer. J. Clin. Oncol. 7:1380-1397).

2. The Basis of Chemotherapy

Chemotherapeutic drugs, for the most part, are cytostatic and/or cytotoxic agents. The exception to these are those agents which act as biological response modifiers. Examples of biological response modifiers are the interferons and the interleukins, which act to enhance the immune system (172). Most of the cytostatic/cytotoxic anticancer drugs can be categorized as agents that damage DNA, antimetabolites, mitotic spindle poisons, hormones and antihormones. Table 2 lists the mechanisms of action of many of the currently available anticancer drugs. The basis of the treatment of malignancy with cytostatic and cytotoxic drugs is that tumor cells are growing and dividing faster than nonmalignant cells and anticancer drugs tend to be more

effective against rapidly growing and dividing cells than quiescent cells (163). This accounts for the hair loss, myelosuppression and gastrointestinal problems associated with chemotherapy, since the epithelial cells of the hair follicle, the bone marrow stem cells and the epithelium lining the gut are all rapidly dividing cells. Unfortunately not all cancer cells are rapidly dividing (158); some cells have slow rates of division and some cells are quiescent, thereby making eradication of the tumor difficult. In addition, not all tumor cells are sensitive to chemotherapeutic drugs. These topics are discussed further in the *Kinetics of Tumor Growth* and the *Drug Resistance* sections.

Table 2. Known mechanisms of action of chemotherapeutic agents.*

-
1. Damage to the DNA template
 2. Interference with normal microtubule function
 3. Enzyme inhibition
 4. Enzyme stimulation
 5. Growth inhibition via a hormone receptor
 6. Modification of the immune system
-

*Adapted from: Yarbrow J.W. (1992) The scientific basis of cancer chemotherapy. In: The Chemotherapy Source Book (Perry M.C., ed). Williams and Wilkins, Baltimore. pp 3.

3. The Kinetics of Tumor Growth

3a. Skipper's Laws

In 1964, Skipper, Schabel and Wilcox using the *in vivo* L1210 mouse leukemia model made two historical observations (150). The first was that this tumor's growth rate is

logarithmic. This means that the doubling time of a population of cells from this tumor is constant, regardless of the number of cells in that population. For example, if it takes 2 cells 24 hours to divide and form 4 cells, then it will take 24 hours for 2000 cells to divide and form 4000 cells. This logarithmic growth implies that all the cells in the population are actively growing and dividing. A plot of tumor cell number vs time of this type of growth will yield a straight line on a semilog plot (Figure 2).

Figure 2. Skipper-Schabel curve for tumor growth.
 From: Skipper H.E., Schabel F.M., Jr. and Wilcox W.S. (1964)
 Experimental evaluation of potentail anticancer agents.
 XIII. On the criteria and kinetics associated with
 "curability" of experimental leukemia. Cancer Chemother.
 Rep. 35: 1-111.

The second observation Skipper and colleagues made was that treatment of this L1210 tumor with a cytotoxic agent resulted in a specific percentage of the tumor cells being

killed (depending on dose of drug administered), regardless of the original tumor cell number. For example, if the drug killed 90% of the cells in an original population of 1000 cells, after the first treatment 100 cells would survive. After the second treatment with the same dose, 10 cells would survive, and after the third treatment 1 cell would survive. Furth and Kahn in 1937 demonstrated that leukemia can be transmitted from one mouse to another with a single cell (56). Therefore the survival of a single malignant cell after treatment can result in relapse. In the example presented earlier, 4 treatments with our chemotherapeutic agent would be required to erradicate the tumor (ie: to get the surviving cell number < 1 , assuming that all cells are drug sensitive).

3b. The Goldie-Coldman Model

One of the greatest obstacles in the treatment of malignancy is the development of drug resistance. The mechanisms of drug resistance are varied and are discussed later under the *Drug Resistance* section. In 1952, Law demonstrated that resistance to a chemotherapeutic agent is a trait that can be acquired randomly through genetic mutation prior to exposure to that agent (88). A mathematical model relating drug sensitivity of tumors to this random acquisition of resistance has been constructed by Goldie and Coldman (58). According to their model, the proportion and the absolute number of drug-resistant cells

within a tumor will increase with time. The fraction of the tumor that is composed of drug-resistant cells will vary between tumors, depending on how early or late the mutation event occurred. Goldie and Coldman have calculated that the probability (P) of a tumor consisting of N cells not having a mutation that results in resistance to a given drug is:

$$P = \exp \{-x(N-1)\}$$

where x = spontaneous mutation rate

Studies have indicated that the probability of a genetic mutation occurring in a nonmalignant cell is in the order of magnitude of 10^{-6} (1, 32, 41, 143), whereas in malignant cells the probability is increased to 10^{-5} (31, 41, 143). The minimum size of early detection of a tumor is considered to be approximately 1 gram of tumor which corresponds to roughly 10^9 cells (174). Thus the probability of a tumor at the time of detection not having any cells resistant to a given drug is:

$$P = \exp \{-10^{-5}(10^9 - 1)\} \approx 0$$

The Goldie-Coldman model is based on the following assumptions:

1. The growth of the tumor is based on Skipper's model of exponential growth.
2. Drug-resistant cells are resistant to all concentrations of drug.
3. Treatment is based on single-agent chemotherapy.

Although the Goldie-Coldman model is a rather

simplistic representation of the evolution of drug-resistance within a tumor, one can still conclude with reasonable certainty that the appearance of drug-resistant cells is a major reason for treatment failure (58, 151). Several recommendations for improved chemotherapy can be put forth as a result of the Goldie-Coldman hypothesis:

1. The earlier chemotherapy is initiated, the increased probability of a cure being obtained.
2. Combination chemotherapy would be more effective in obtaining a cure than single-agent chemotherapy since the probability of a cell mutating to become resistant to several drugs is much less than it becoming resistant to a single agent.

The treatment of many different patients representing a variety of neoplasms has demonstrated that Skipper's model of exponential growth and cell kill is not a true reflection of tumor growth. Patients who have been treated with combination chemotherapy to induce remission are responsive to the same chemotherapeutic regime following relapse (78, 162). The achievement of a second remission is opposite of what one would expect if adhering to Skipper's model of exponential growth and the Goldie-Coldman hypothesis. It would be expected that a second round of chemotherapy would result in very little or no response since most or all the drug-sensitive cells should have been eradicated by the first drug treatment. Assuming that the drug-sensitive and drug-resistant cells have equal cycling times, the ratio of

drug-sensitive cells to drug-resistant cells should be less than one (152). Consequently no response should be observed since more drug-resistant cells are being added to the population than drug-sensitive cells being killed. This observation of a second remission indicates that drug-resistant cells are reverting back to a drug-sensitive phenotype after treatment and/or a substantial number of drug-sensitive cells are not being killed by the first treatment of chemotherapy. There is experimental evidence that the former can occur (36, 80), but a kinetic explanation, the Norton-Simon model, has been put forward to account for the treatment failure (110, 111).

3c. Gompertzian Growth and the Norton-Simon Model

The growth of experimental animal tumors (87) and clinically observed tumors (68, 109) when plotted (cell number vs time) yields a Gompertzian curve (Figure 3). This shape of growth curve indicates that this type of tumor growth is much more complex than that of Skipper's *in vivo* L1210 tumor model. In order to explain this observed Gompertzian growth, the concept of "growth fraction" must be introduced. Mendelsohn in 1960 suggested the notion that tumors are mixtures of proliferating and nonproliferating cells (97). He defined the growth fraction of a tumor as the ratio of proliferating cells to total cells. In 1968 Tannock demonstrated that vascularity of a tumor influenced the growth fraction (158). Using a unique mouse mammary tumor

model in which the tumor cells grew uniformly around straight blood vessels, he observed that tumor cells in the immediate vicinity of the oxygen and nutrient supply had the fastest growth rates and the highest growth fraction, whereas those cells further away from the blood supply had slower rates of division and lower growth fractions. The cells farthest from the blood vessels were observed to be necrotic.

Figure 3. Gompertzian Curve for Tumor Growth.

The initial portion of the Gompertzian curve represents a small number of rapidly dividing tumor cells (171). This portion of the Gompertzian curve resembles Skipper's model of exponential growth. Since cell number is so low, the overall slope of the curve is low, despite the fact that the growth fraction of the tumor is high. As the tumor expands and begins to outgrow its blood supply, the growth fraction of the neoplasm decreases (158, 111). At the other end of

the curve, where the tumor becomes lethal to the patient, tumor cell number is maximal and growth fraction is minimal, thereby resulting in a low slope of the curve. Maximal tumor growth (slope) is observed in between these two extremes where neither cell number nor growth fraction is maximal, but when their product is. The Norton-Simon model for Gompertzian tumor growth can be represented by the following equation (111):

$$dN(t)/dt = GF(N) \times N(t) - K \times L(t) \times GF(N) \times N(t)$$

where N = cell number

t = time

GF = growth fraction

K = constant

L = level of chemotherapy

or more simply stated:

change in tumor cell number = cell generation - cell death.

Cell death is dependent on the growth fraction of the tumor, since chemotherapeutic agents are more effective against mitotic cells than quiescent cells (163). The main implications of this equation is that the rate of tumor cell kill and the rate of regrowth increases as tumor size decreases and thus the level of therapy required to initiate a regression may not be adequate to maintain the regression and to produce a cure. A greater level of cell kill is counterbalanced by a greater fractional regrowth, so that

the only way a cure can be achieved is to use more *intensive* chemotherapy against very *small* tumors. To be curative, such intense chemotherapy will require the patient to have autologous bone marrow transplants and hematopoietic growth factor support to counteract the myelosuppressive action of the intense chemotherapy. In summary, it now appears that chemotherapy treatment failure is not entirely due to the development of drug resistance, but is also due in part to the kinetic parameters of Gompertzian tumor growth and the limitation in dosage of chemotherapy due to host toxicity.

4. Drug Resistance

One of the reasons for chemotherapy treatment failure is the acquisition of drug resistance by the tumor cells. This acquisition of drug resistance can occur randomly prior to treatment (88) or it can evolve during the course of chemotherapy (15, 90). The mechanisms by which cancer cells can become refractory to chemotherapy are diverse (35, 173) and some of the more important methods are summarized as follows:

1. Increased expression of the plasma membrane protein P-glycoprotein (Pgp).
2. Changes in expression of drug detoxifying enzymes (glutathione-S-transferase, dihydrofolate reductase)
3. Qualitative and/or quantitative changes in DNA repair enzymes (topoisomerase I and II, polymerase alpha and beta, thymidylate synthase).

4. Genomic alterations.

4a. P-Glycoprotein and Multidrug Resistance

One of the factors responsible for unsuccessful cancer chemotherapy is the acquisition of multidrug resistance (MDR). Multidrug resistance is a phenotype characterized by resistance to a drug which arises upon exposure of the cell to that specific agent (15, 90). Multidrug-resistant cells also exhibit a cross resistance to a number of compounds that are structurally and functionally unrelated to the drug that induced the *mdr* phenotype (6, 10, 92). Multidrug resistance has been observed to be associated with increased levels of a 170 kDa plasma membrane protein, P-glycoprotein (Pgp) (15, 164). Significant levels of Pgp have been identified in a wide variety of malignancies, including sarcomas, carcinomas, lymphomas and leukemias (59, 76). Structural analysis of Pgp predicts that it is an ATP-binding pore forming pump, and that it brings about drug resistance by actively pumping drugs out of the cell (28, 63, 76). The gene coding Pgp has been termed the *mdr1* gene. Pgp has been detected at low levels in a variety of normal tissues and is believed to have a role in normal physiology (54, 69). Amino acid sequencing confirms that Pgp shares extensive sequence homology with numerous bacterial and eukaryotic transport proteins (22, 76). This conservation of structure of Pgp throughout evolution indicates that this protein is a fundamental membrane transport protein. Pgp levels are regulated by gene copy number (81, 164), by rates

of transcription (15) and by post transcriptional modifications (15).

From a clinical point of view, the two most important questions are 1) does Pgp predict response to chemotherapy and 2) can the mdr phenotype be circumvented? The answer to the first question at this point in time is not entirely clear, since there is little data relating Pgp expression and patient response to chemotherapy (89). Two preliminary studies involving breast cancer have observed a correlation between Pgp expression (as determined by immunohistological methods) and response to chemotherapy (131, 166). A confounding problem associated with using Pgp to predict a response to chemotherapy is that many normal tissues express Pgp and it may be difficult to discriminate between normal and neoplastic tissue (30). Some *in vitro* studies using cancer cell lines (47, 140, 164) and malignant tissue in primary culture (5, 59, 81, 144) have correlated increased expression of Pgp with decreased sensitivity to chemotherapy, but other *in vitro* and *in vivo* experiments utilizing both cell lines and fresh tissue could not establish a strong correlation between Pgp expression and chemosensitivity (59, 65, 126). Although Pgp expression may be high, pump function may be zero.

In vitro experiments have established that inhibition of the energy-dependent Pgp-mediated drug efflux can result in circumvention of mdr. Calcium antagonists such as verapamil, diltiazem, nifedipine and others have enhanced

the cytotoxicity of the *Vinca* alkaloids (vincristine and vinblastine) and the anthracyclines (Adriamycin) against cell lines representing a variety of tumor types (98, 101, 159, 176). This enhanced cytotoxicity is a result of competition of binding to Pgp between the calcium antagonists and the antitumor agents (137, 176). The calcium channel blockers act to circumvent the mdr phenotype in a calcium-independent manner (19). A problem encountered in the use of calcium antagonists in circumvention of mdr is that the maximal clinically achievable dose of the antagonist is limited because calcium antagonists cause detrimental cardiovascular side effects *in vivo* (8). As a result, the maximal *in vivo* dose is below that required to reverse the mdr phenotype (98).

Calcium influx blockers are not the only agents that compete with binding of antitumor agents to increase intracellular drug concentration. Calmodulin inhibitors (85, 160), monoclonal antibodies (7, 16), dihydropyridines (175), several of the steroid hormones, especially progesterone (70, 108), and the antiestrogen tamoxifen (50) have all been shown to interfere with Pgp-mediated efflux of anticancer drugs. With respect to mdr and breast cancer, the use of the progestins and tamoxifen may prove to be interesting since both are relatively nontoxic (75, 77) and both are used for treatment of breast cancer (24, 125).

5. Historical Background of Anticancer Drug Development

The first nonhormonal antitumor agents to demonstrate activity in humans were the nitrogen mustards (60, 72, 128). These alkylating agents, which were used in the chemical warfare programs of World War I, demonstrated significant antitumor activity in patients with lymphomas. These observations signalled the beginning of the search for new anticancer agents. The pursuit for anticancer drugs prior to the 1950's was performed mainly by the pharmaceutical industry (38). Sixteen of our current armament of approximately forty drugs were developed during this era. In 1955, the Developmental Therapeutics Program at the National Cancer Institute (NCI), based in Bethesda Md, initiated its drug discovery and development program (38, 62). This program has been the main driving force behind the search for new anticancer drugs since its inception. Of the sixteen commercially available drugs introduced between 1955 and 1985, the NCI discovered eight of them. In addition, the NCI was involved in the clinical evaluation of all sixteen of these drugs (38).

5a. Preclinical Drug Discovery and Development

There were two methods by which the NCI approached new drug discovery: rational drug design and mass screening of synthetic and natural products. Anticancer drugs such as asparaginase, cisplatin, mitotane, levamisole and antimetabolites such as fluorouracil and N-(phosphonacetyl)-L-

aspartate (PALA) were discovered both by logical reasoning and serendipity (25, 38, 48, 66, 84, 132, 148). Although rational drug design had an important role in the discovery of anticancer drugs, it was more commonly used in improving leads discovered via mass screening. Biological screening of compounds so far has accounted for approximately half of the current repertoire of chemotherapeutic agents (Table 3). Screening of acquired compounds was performed using a series of *in vivo* tumor models. These models measured the increase in life span (ILS) of tumor-bearing mice receiving treatment with a potential anticancer drug vs tumor-bearing mice not receiving treatment. An ILS of at least 25% was required for the agent to be considered "active" so it can be forwarded to the next stage of testing (62). The evolution of these *in vivo* tumor models from 1955 to 1982 is presented in Table 4.

Table 3. Pathways to the discovery of current anticancer drugs.*

Targeted synthesis	Screening of natural products	Screening of chemicals	Analogue synthesis	Serendipity and rational application
Antimetabolites:	Dactinomycin	Busulfan	Cyclophosphamide	Mechlorethamine
Methotrexate	Vincristine	Dacarbazine	Chlorambucil	Asparaginase
Thioguanine	Vinblastine	Procarbazine	Melphalan	Mitotane
Mercaptopurine	Platamycin	Hydroxyurea	Ifosfamide	Cisplatin
Fluorouracil	Daunorubicin	Thiotepa	Etoposide	Levamisole
Cytarabine	Doxorubicin	Carmustine	Teniposide	Interferons
PALA	Mitomycin C	Lomustine	Carboplatin	
Hormones:	Bleomycin	Mitoxantrone		
Steroids	Streptozocin	Altretamine		
Tamoxifen	Taxol	Pentostatin		
Flutamide				
Leuprolide				
Octreotide				

*From: Sikic B.I. (1991) Anticancer Drug Discovery. J. Natl. Cancer Inst. (U.S.A.) 83: 738-742.

Table 4. Historical Evolution of *In Vivo* Screens at the NCI.*

<u>YEAR</u>	<u>IN VIVO SCREEN</u>
1955	sarcoma 180**, carcinoma 755, leukemia L1210
1960	L1210 + 2 models from a pool of 21
1965	L1210 + Walker carcinoma 256
1968	L1210 for synthetics, L1210 and P388 leukemia for natural products
1972	L1210 for synthetics, P388 for natural products, B16 melanoma and Lewis lung carcinoma for special testing
1975	P388 prescreen, followed by a panel of 8 models, including human xenografts

*From: Staquet M.J., Byar D.P., Green S.B. and Rozencweig M. (1983) Clinical predictivity of transplantable tumor systems in the selection of new drugs for solid tumors: Rationale for a three-stage strategy. Cancer Treat. Rep. 67: 753-765.

**All tumor models are murine except where noted.

The NCI's preclinical drug development protocol for the years 1975 to 1985 is illustrated in Figure 4. The first step (Stage I) of this protocol was a P-388 murine leukemia prescreen to eliminate the supposedly large number of clinically inactive compounds. Any agent considered "active" (ILS > 25%) in this prescreen was then forwarded to the *in vivo* tumor panel (Stage II) which consisted of a variety of human tumor xenographs and murine malignancies. If the agent demonstrated any activity (ILS > 50%) in Stage II, it was further investigated (formulation/toxicology stage) to determine structure, production, mechanism of action, dosage, toxic effects on animals and so on. The animal toxicology studies, which were usually performed on two

species of animals, were performed to characterize the drug's absorption (if oral), clearance, half-life, metabolism, excretion and either or both the maximum tolerated dose (MTD: dose which produces severe reversible toxicity) and the LD₁₀ (dose which is lethal to 10% of the population given that dose). These studies helped to determine if the agent was suitable for clinical trials and which dose to administer in clinical trials. The initial dose is usually a fraction of either the MTD or the LD₁₀ (26).

Figure 4. NCI preclinical drug development strategy (1975-1985).

5b. Clinical Evaluation of New Anticancer Drugs

Once the preclinical drug research phase is completed, the compound is introduced into clinical trials. Clinical trials are composed of three phases (I-III) (26, 94). The goal of Phase I is to assess the drug's qualitative and quantitative (maximum tolerated dose) toxicity upon the patient. The patients involved in Phase I are those who have

tumor types in which there is no suitable treatment available. In addition, patients are selected so that a broad spectrum of tumor types are represented by the patient panel. The assumption in cancer chemotherapy is that anticancer drugs are most effective when administered near to or at their MTD (55). For most clinically active drugs the bone marrow is usually the dose limiting factor, but the CNS, kidney, liver and other organs may also be dose limiting (94). The maximum tolerated dose for patients with a common tumor type is determined by using a "modified Fibonacci scheme" (26). The initial dose is extrapolated from the animal toxicology studies and is administered to three patients. If acceptable toxicity is observed with these patients, the dose is increased for the next three patients who enter the study. As long as the toxicity remains acceptable, the dosage will be escalated with decreasing increments for new patients entering the study.

The main goal of Phase II studies is to establish which tumor types the drug is effective in treating and to further define the drug's side effects. The patients in this phase comprise the Human Tumor Panel. This panel was created in 1975 and consists of patients with the same types of malignancies (lung, colon, breast, lymphoma, leukemia and melanoma) as those in the preclinical *in vivo* tumor panel. The purpose of selecting these two panels is to determine if results obtained from the *in vivo* preclinical tumor models correlate with those obtained using the Human Tumor Panel.

The goals of Phase III are to determine the response rate, response duration, survival, toxicity and relative activity (when compared to standard treatment) of the drug in patients with tumor types that were responsive in Phase II. In addition, Phase III studies explore the incorporation of the agent with other drug combinations, alternate routes of administration and high dose therapy in combination with rescue procedures.

5c. Evaluation of the National Cancer Institute's Drug Discovery and Development Program

In 1984 it became evident that the NCI's criteria for identifying new chemotherapeutic drugs was unsatisfactory. This situation was illustrated in a report released in 1987 which evaluated the Phase II activity of all cytotoxic drugs introduced into clinical trials by the NCI between the years 1970 and 1985 (95). During this time period, 83 drugs were introduced into Phase I trials. Of these 83, 11 were terminated at the end of Phase I and 5 remained in Phase I at the time of the report. Of the 67 drugs that proceeded onto Phase II, 13 were still in Phase II and 7 were not evaluated properly. After Phase II testing, 24 of the remaining 47 agents demonstrated significant activity against at least one type of malignancy. Only 14 of 24 active agents were new chemical structures; the remaining 10 were analogues of already known chemotherapeutic drugs. With the NCI drug discovery program screening up to 40,000

compounds annually with the cost in the tens of millions of dollars per year (\$29 M in 1984)(21), only 14 novel compounds discovered after 15 years was highly unsatisfactory. Of a more serious concern was that of the active agents, 74% were rated active against lymphoma, 35% against leukemia and only marginal activity was observed against the solid tumors. It became apparent that the P-388 murine leukemia prescreen was not a valid representation of all types of malignancies. It is believed that the P-388 prescreen was filtering out agents that are potentially effective in treating solid tumors. The P-388 model was assumed to be a valid representation of all tumor types because of the P-388's and the L-1210's (P-388's predecessor) historical predominance in anticancer drug screening and of the fact that more agents discovered by the NCI program were effective against the *in vivo* L-1210 and P-388 leukemic bioassays than any other *in vivo* bioassay (156, 165), which of course is faulty logic. When the NCI's drug screening program was initiated, the L1210 and P-388 *in vivo* tumor models seemed to be the appropriate choices because of their technical feasibility. The NCI at the time was screening 7,000-12,000 compounds per year. At this level of screening, the use of a single *in vivo* preliminary screen required that the tumor model used have a doubling time of 10-15 hours so that the antitumor assay could be completed in 30 days (157). The NCI eventually dropped the colon and lung human tumor xenografts from their Stage II screen

because they were deemed too insensitive for drug screening (62, 114). This is somewhat ironic since lung and especially colon neoplasms are known to be clinically resistant to chemotherapy (59, 106). In fact, it would seem that the most important tumor models would be those that are nonresponsive to drugs currently in use. This is supported by the observation that out of 979 compounds rated not active in an *in vivo* L1210 leukemic prescreen, 119 were later found to have activity in at least one of the *in vivo* solid tumors from the Stage II secondary screen (165).

Another flaw in the NCI's use of murine tumor models was that for the P-388 Stage I screen and many of the murine tumor models in the Stage II screen, both the tumor and the drug were injected intraperitoneally (62). This form of tumor model, nicknamed "test tube in the mouse", does not represent the true *in vivo* environment since no physiological barriers have to be passed by the drug. As a result, drug activity is falsely magnified and the assay tends to isolate drugs that do not cross physiological barriers well (62).

In the mid 1980's the NCI reevaluated its drug discovery program. Several alternatives to the drug-screening system were proposed (21):

1. *Abandonment of all drug discovery contracted out by the NCI.* Approximately 50% of all compounds acquired for the NCI drug-screening program have been via a chemical synthesis

contractor and a collection contractor (38). Instead the NCI would redirect these funds towards drug discovery grants and clinical development of compounds submitted from outside the NCI. Active compound acquisition by the NCI and voluntary submission of agents to the NCI, which normally account for approximately 20% and 30% respectively of all compounds acquired, would then become the major sources of agents entering the greatly scaled down NCI drug-screening program. This proposal was rejected because it was felt that this would be too much of a negative signal to the few remaining private sector companies involved in drug-discovery programs.

2. *Development of new molecular screening targets.* With the science communities' ever-expanding knowledge of tumor biology and scientific technique, we are identifying more and more intracellular targets involved in induction and maintenance of neoplasia. These intracellular targets can be utilized in a screening protocol to identify agents that may inhibit the neoplastic process. Some examples of these targets are protein kinases, growth-factor inhibitors, oncogene products and proteins involved in multidrug resistance. The NCI rejected the development of this type of screen for two reasons. The first is that this type of screen utilizes a cell free system and thus is too far removed from the *in vivo* environment. Secondly, the importance of any one specific intracellular target involved

in the malignant process is still not clear (21). Many independent laboratories have adopted this screening process and, as our understanding of molecular biology increases, this format of drug discovery will become more prominent (73, 154). The NCI recognizing this, has established the National Cooperative Drug Discovery Groups, an organization that brings together molecular biologists, chemists and biochemists so as to expand the role of rational drug discovery (154, 157, 165).

3. *Creation of an in vitro human tumor cell line panel for initial testing of compounds.* To avoid the "filtering" effect observed with the use of the P-388 leukemia prescreen, the NCI decided to utilize an initial screening panel representing all the major categories of tumor types (14, 21, 103). An *in vitro* human tumor cell line panel was selected instead of an *in vivo* panel because it is economically and technically impractical to use *in vivo* models for mass screening.

6. Current Status of the National Cancer Institute's Preclinical Drug Discovery and Development Program

Figure 5 is a schematic representation of the preclinical antitumor drug screening protocol implemented at the NCI in 1985 (14, 62). In order to avoid the disease-specific Stage I screen and the "filtering" effect observed with the screening protocol used in earlier prescreens, the

NCI switched to a disease-oriented *in vitro* human tumor cell line panel. The ultimate goal is to have the human tumor cell line panel consisting of 120 cell lines representing 10-12 different tumor types (14, 103). Currently the pilot-scale cell line panel utilizes 60 human tumor cell lines organized into subpanels representing melanoma, leukemia and cancers of the lung, colon, kidney, ovary and central nervous system (103). Each tumor type is represented by multiple cell lines because the use of subpanels will allow for detection of agents that are specific for certain categories of malignancies (ie: histiospecificity) or, conversely, agents that are active but not tumor-specific (14, 103, 148). In addition, the use of just 1 or 2 cell lines originating from 1 type of cancer would not emulate the heterogeneity observed in naturally occurring tumors (51, 122). Cell lines are used instead of fresh tumor tissue in primary culture because the cells are plentiful, easy to grow and well characterized (ie: for mechanisms resulting in drug resistance). Previous attempts at using fresh tumor tissue from a variety of solid tumors (in conjunction with the human tumor colony-forming assay) was shown to be impractical for high volume screening (145, 146). The use of cell lines in the primary screen is also advantageous, because many human tumor cell lines can be propagated subcutaneously in athymic nude mice (14, 114), thereby providing a means of evaluating agents *in vivo* (Stage II of figure 5). This Stage II screening involves the testing of

agents in athymic nude mice with subcutaneous implants of the most chemosensitive cell lines from Stage I. In addition to the use of these subcutaneous human tumor xenografts, the NCI can further investigate promising compounds more thoroughly *in vivo* by using an orthotopic tumor model and the microencapsulated tumor assay (META) (14, 61, 157). In the orthotopic tumor model, the tumor cells are implanted *in vivo* in sites corresponding to the presumed origin of the malignancy rather than subcutaneously. For example, if an agent was observed to be active against renal carcinoma, the tumor cells would be implanted in the kidney. In the META, tumor cells are encapsulated in microcapsules, which in turn are implanted in the peritoneal cavity of nude mice. The capsules are designed to confine the tumor cells while still exposing them to an *in vivo* environment. Following drug exposure, the capsules are removed and the surviving cell number counted and compared to controls. Neither the META nor the orthotopic xenograft methods are to be used for first line *in vivo* screening since they are too technically impractical (14). Instead, these tumor models are to be used for more specialized preclinical investigation of promising anticancer compounds.

Figure 5. NCI preclinical drug development protocol (1985-present).

A drawback of using murine tumor models for chemosensitivity testing is that clinically achievable doses are 2- to 10- fold higher than that achievable in mice (148). In humans, we have ways we can support the myeloproliferative cells in the bone marrow (ie: growth factor and bone marrow transfusions and gene therapy) and thus we can increase the dose intensity (57, 82). But in mice, myelosuppression is the dose-limiting factor, therefore agents that are myelosuppressive in mice but not in humans could potentially be filtered out. A possible way to get around this problem is to use a transgenic mouse that has its bone marrow cells expressing the *mdr1* gene (102).

Inclusion of non-neoplastic myeloid colony forming cells as a control in the human tumor panel may give an indication of the therapeutic index (minimum toxic dose/minimum effective dose) of the test agent (21).

Once preclinical drug development is completed, the agent under study is introduced into disease-specific clinical trials. This strategy is based on the assumption that *in vitro* activity against specific tumor types will translate to *in vivo* activity in patients who have those tumor types. There is some evidence that histiospecific activity does exist for certain antitumor agents. Mitotane, asparaginase and ipomeanol demonstrate preferential activity for adrenocortical carcinomas, lymphoblastic leukemias and lung cancers respectively (23, 148). Several earlier studies investigating the feasibility of alternative drug screening methodologies have demonstrated that agents previously shown not to be active in a leukemic prescreen demonstrated activity in non-leukemic screens. An NCI pilot screen investigating the feasibility of the (*in vitro*) human tumor colony forming assay for large-scale drug screening detected activity for 14 of 79 compounds that were negative in the P388 leukemic prescreen (145). Of 1085 compounds tested against a panel of *in vivo* murine tumors representing a variety of murine and human tumor types, 106 were active in L1210 leukemia. Of the 979 compounds that were inactive in the L1210 tumor model, 78 were active against melanoma, 45 against human breast carcinomas, 18 against murine breast

carcinomas, 10 against murine lung cancers, 9 against murine colon cancers, 5 against human colon cancers and 1 against human lung cancer (156, 165).

To aid detection of potential histiospecific activity of anticancer agents, the NCI has developed the mean graph and the COMPARE computer program (118). The mean graph, which is a type of bar graph, displays cytotoxic activity of the test agent against each cell line in relation to the average activity observed against all cell lines of the tumor panel. The graph is constructed by projecting bars horizontally, to the right or left of the mean, depending on whether or not cell sensitivity to the test agent is greater or less than average. The length of the projection bars is directly proportional to the difference from the mean. Consequently, a "fingerprint" pattern is generated for each compound tested against the human tumor cell line panel. This COMPARE computer program can then compare this fingerprint pattern to patterns generated with other test agents, thereby identifying similarities in biological properties and/or chemical structure and properties (118).

7. Methods to Assess Chemosensitivity

One of the difficulties associated with *in vitro* drug screening is how to accurately measure tumor cell viability following exposure to a test agent. Numerous assays have been developed, but unfortunately each has its limitations. These assays include the human tumor colony forming

(clonogenic) assay (HTCFA) (64, 139, 145, 146), the sulforhodamine B (SRB) assay (149), tritiated thymidine uptake (74, 161), neutral red assay (12, 13, 49), the Coomassie blue protein assay (147), the Hoechst 33342 assay (39, 129), the fluorescein diacetate assay (133), the methylene blue assay (112), the XTT tetrazolium dye reduction assay (141), the MTT tetrazolium dye reduction assay (2, 20, 33, 67, 107, 134, 167) and many others.

The HTCFA, MTT, XTT, and SRB were all investigated as possible tools in the NCI's drug-discovery and development program. The HTCFA (Figure 6) was deemed not suitable for large-scale screening because it is labour intensive, is plagued by low plating efficiencies for certain tumor types and is difficult to obtaining single-cell suspensions (145, 146).

Figure 6. The human tumor colony forming assay.

The assay originally selected by the NCI to use in the *in vitro* Stage I screen was the colorimetric MTT (1-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl tetrazolium bromide) tetrazolium dye reduction assay (Figure 7)(2). The MTT assay was observed to be a rapid, sensitive, and reproducible method for *in vitro* anticancer drug screening (2, 20, 67, 134). MTT is a soluble yellow substrate that is cleaved by the dehydrogenase enzymes of mitochondria in living cells to form an insoluble purple formazan product (153). The amount of formazan produced by a population of cells is proportional to the number of viable cells in the population (107). Formazan levels are measured with a spectrophotometer designed to read 96-well microculture plates (ELIZA reader), which is interfaced with a computer to analyze results. The use of the 96-well microtiter plates in conjunction with the

ELIZA reader and the computer makes this procedure highly efficient.

Figure 7. NCI MTT assay protocol.

From: Rubenstein L.V., Shoemaker R.H., Paull K.D., Simon R.M., Tosini S., Skehan P., Scudiero D.A., Monks A. and Boyd M.R. (1990) Comparison of *in vitro* anticancer-drug-screening data generated with a tetrazolium assay versus a protein assay against a diverse panel of human tumor cell lines. *J. Natl Cancer Inst. (U.S.A.)* 82: 1113-1118.

A slight variation of this assay was developed to eliminate the aspiration/solubilization step (the insoluble formazan must be dissolved in solvent prior to colorimetry). The reduction of XTT (2,3-bis[2-methoxy-4-nitro-5-sulfophenyl]-5-[(phenylamino)carbonyl]-2H-tetrazolium hydroxide) results in a formazan product that is water soluble, thereby eliminating the need for additional solvents. Some serious drawbacks associated with the MTT and XTT assays are i) agents that reduce tetrazolium salts by direct chemical action may yield erroneous results (2), ii) agents that block cell-mediated MTT reduction indirectly by inhibiting cellular respiration may confound results (2), iii) MTT formazan production varies between cell lines and

culture age, iv) agents that alter the number of mitochondria per cell may confound results and v) the color intensity of the dissolved formazan is not stable, and has to be measured at a precise time point (136). The variation of formazan production between cell lines and culture age is related to the availability of glucose at the time of the assay. Low levels of available glucose is related to decreased mitochondrial activity, which in turn is related to decreased formazan production (167). With the NCI's Stage I screen operating at 1,000 microtiter plates/day, it became difficult to coordinate microculture plate reading. As a result the NCI was prompted to develop the SRB assay (Figure 8) (149). SRB is a aminoxanthene dye with two sulfonic groups that binds to basic amino acid residues of protein (91). The SRB assay was determined to be a sensitive and rapid method of measuring the cellular protein content of adherent and suspension cultures. Unlike the MTT and XTT assays, the SRB's colorimetric endpoint is indefinitely stable (136, 149). A study comparing the SRB assay and the MTT assay observed that the two perform similarly (136).

Figure 8. NCI SRB assay protocol.

From: Rubenstein L.V., Shoemaker R.H., Paull K.D., Simon R.M., Tosini S., Skehan P., Scudiero D.A., Monks A. and Boyd M.R. (1990) Comparison of *in vitro* anticancer-drug-screening data generated with a tetrazolium assay versus a protein assay against a diverse panel of human tumor cell lines. J. Natl. Cancer Inst. (U.S.A.) 82: 1113-1118.

8. Predictive Value of In Vitro Chemosensitivity Assays

The most paramount question to be answered with regard to *in vitro* assays is whether or not *in vitro* activity translates into *in vivo* activity. *In vitro* chemosensitivity assays can be categorized as patient-oriented or as drug-oriented. The former is used to predict the response of a patient to cytotoxic drugs. The latter is used for measuring the chemosensitivities of compounds. Although most of the studies addressing whether *in vitro* activity correlates with *in vivo* activity have been performed with respect to patient oriented *in vitro* assays, many of the conclusions are relevant to *in vitro* drug screening. The HTCFA (Figure 6), first described by Hamburger and Salmon (64) has been the most studied of the *in vitro* assays. In this assay, stem cells are exposed to various concentrations of drugs and the

proportion of colonies that develop relative to a control is plotted against the dose level of the agent. A review of 2,300 different *in vitro-in vivo* clinical correlations using clonogenic based assays was presented by Von Hoff in 1990 (170). The results of this review are as follows:

Sensitive <i>in vitro</i> and <i>in vivo</i>	512
Sensitive <i>in vitro</i> but resistant <i>in vivo</i>	226
Resistant <i>in vitro</i> but sensitive <i>in vivo</i>	135
Resistant <i>in vitro</i> and <i>in vivo</i>	<u>1,427</u>
Total	2,300

An analysis of these numbers reveals that if an agent is active *in vivo*, an *in vitro* assay will predict it 79% of the time. Conversely if the agent is not active *in vivo*, an *in vitro* assay will predict it 86% of the time. Since the number of correlations involved in generating these numbers is very large, these results are statistically powerful. Another study, in which patients with advanced metastatic cancer were randomized to receive single-agent chemotherapy as decided by an oncologist or by a clonogenic assay, demonstrated that an *in vitro* clonogenic assay could improve the response rates to chemotherapy (169). Patients who received chemotherapy as decided by the assay were observed to have a response rate of 21%, whereas those who were administered chemotherapy as selected by a clinician were observed to have a response rate of 3%. No difference in

survival was observed between the two arms of the study, but this can be attributed to the current stock of anticancer drugs and the single-agent chemotherapy regime administered. Although the results of these two reports confirm that *in vitro* activity can reasonably predict *in vivo* activity, several comments must be made in order to keep these results in perspective: 1) Only certain tumor types are suitable for the HTCFA (142, 145, 170). Von Hoff in his review of *in vitro-in vivo* correlations only examined solid tumors. Of the 13,932 tumors involved in the prospective randomized study mentioned above, approximately 30% of the tumors displayed adequate growth *in vitro*. Adequate growth *in vitro* has been considered to be ≥ 30 colonies per 5×10^5 cells plated (142). The tumor types which were found to be the most suitable for the HTCFA are mesothelioma, ovary, corpus uteri and kidney, whereas leukemias, lymphomas and myelomas were observed to be not suitable for the HTCFA. 2) Clonogenic assays have been plagued by quality control problems (ie: cell clumping) which have resulted in erroneous results (145, 146). Recent technical advances and the establishment of stringent assay parameters and controls have minimized these quality control problems (145).

There are several limitations associated with the predictive value of all *in vitro* assays. The most obvious is the inability of *in vitro* assays to detect agents that require metabolic activation, factors that could inhibit angiogenesis, invasion or metastases or agents that act via

effector cells of the immune system such as macrophages and natural killer cells. One method proposed to minimize the former of these is inclusion of liver microsomal preparations in the incubation mixture (3, 86).

Another problem associated with *in vitro* assays is that it is difficult to determine the appropriate drug concentrations and exposure times in culture that reflect those attainable *in vivo*. This problem is further compounded by the fact that drug penetration barriers exist within the tumor, thus different sites within the tumor may be exposed to different concentrations of drug (96). For patient-oriented predictive *in vitro* assays this is a serious problem. For drug-screening oriented *in vitro* assays, this problem is not as important since the purpose of these tests is to determine if a given agent has the potential for antitumor activity. Usually the drug concentrations used in preliminary drug-screening studies are five ten-fold dilutions starting with the highest soluble concentration or an arbitrarily selected concentration (usually 10^{-3} M for pure compounds or 250 $\mu\text{g/mL}$ for crude extracts) (14, 103). A knowledge of the potency of the test compound *in vitro* may provide a reference point on which to base *in vivo* drug dosages (122).

The basis of all *in vitro* chemosensitivity assays is the hypothesis that response *in vivo* is determined by the inherent chemosensitivity of the cells *in vitro*. Experimental evidence has demonstrated that this is not

always the case and that other factors determine tumor responses *in vivo* (4, 122). A study by Phillips *et al.* (122) demonstrated that a murine colon adenocarcinoma cell line grown *in vivo* exhibited varying responses to chemotherapy depending on the site of tumor growth (subcutaneous or intraperitoneal). The reasons for this site-dependent response to chemotherapy is believed to be due to the drug's ability to access different tumor sites, tumor hypoxia and cell kinetics (4, 96). Furthermore, factors affecting chemosensitivity such as the proliferative status of tumor cells, the 3-dimensional tumor geometry, the presence of an extracellular matrix and variations in pH, O₂ tension and osmolarity observed *in vivo* may not necessarily be reproduced *in vitro* (37, 122).

The validity of the HTCFA has been examined by testing the assay against a panel of standard agents (10 clinical agents, 5 highly toxic compounds with no therapeutic efficacy and 5 nontoxic clinically ineffective compounds) (145). Using a 20% *in vitro* response rate (decrease in colony number as compared to control) as activity criterion, 9 of the 10 clinical agents were observed to be active, as were all 5 of the highly toxic compounds. None of the nontoxic clinically ineffective compounds demonstrated significant activity. A study comparing the MTT and the HTCFA using a range of anticancer drugs on a variety of cell lines demonstrated that the two assays produce similar results (2, 20), as do both the MTT

and SRB assays with one another (149). Differences in relative chemosensitivities between cell lines were maintained when comparing the MTT and HTCFA assays (20). Other experiments comparing *in vitro* chemosensitivities of leukemia cell lines with carcinoma cell lines against a panel of cytotoxic agents has demonstrated that carcinoma cell lines tend to be more chemoresistant than leukemic cell lines, especially when exposed to DNA binding drugs (4, 52, 115). These results suggest that the refractoriness of carcinomas as compared with leukemias is due to inherent resistance of the tumor cells themselves. Ultimately, however, only results from clinical evaluations will tell whether or not an *in vitro* human tumor cell line panel is a valid means to detect clinically effective anticancer agents (14, 62, 122, 148).

9. Natural Products as a Source of New Anticancer Drugs

The NCI has not only changed the way they investigate new potential anticancer drugs, but also the way it selects compounds for screening. In addition to screening some acquired synthetic compounds, the NCI is putting a greater emphasis on screening natural products because they represent a vast untapped source of agents with novel modes of action and unique structures. Currently approximately 20-25% of U.S. prescriptions contain natural plant products, even though only a handful of a quarter of a million species

of tropical plants have been examined for pharmaceutical purposes (127). The yield of cytotoxic agents from natural sources is generally considered to be several orders of magnitude greater than randomly acquired synthetic agents (21, 83). Table 3 lists currently available anticancer agents that are derived from natural sources. In 1959, the NCI initiated its fermentation broth and plant screening programs. In 1972, the marine acquisition program was initiated. By 1980, approximately 200 000 fermentation broth extracts, 114 000 plant extracts and 17 000 marine extracts were tested (127, 157). Of these, samples from marine sponges and tunicates exhibited exceptional activity (157). These marine organisms produce toxins as a mechanism of self-defence (168). Many interesting and important compounds have been isolated from marine organisms. For example, the development of cytarabine, a widely used antileukemic chemotherapeutic agent, was based on leads provided by the isolation of 1- β -arabinofuranosyl derivatives of uracil from the Caribbean sponge *Cryptotethya crypta* (9, 89). Didemnin-B (Figure 9), a metabolite of the tunicate *Trididemnin solidium* (130), has entered Phase II human cancer trials (157, 168). The bryostatins, a family of metabolites isolated from the bryozoan *Bugula neritina* (120), is also undergoing clinical trials (157, 168). Palytoxin (Figure 10), whose structural analysis represents a considerable triumph in natural product research, is derived from the *Palythoa* species and is the most toxic non-proteinous agent

ever discovered (104). As the structures of these agents illustrate, the structural diversity of compounds isolated from marine organisms appears boundless.

Figure 9. Molecular structure of Didemnin B.
 From: Suffness M. and Thompson J.E. (1988) National Cancer Institute's role in the discovery of new antineoplastic agents. In: Biomedical Importance of Marine Organisms (Fautin D.G., ed). California Academy of Sciences, San Francisco. pp 155.

Figure 10. Molecular structure of palytoxin.
 From: Ireland C.M., Roll D.M., Molinski T.F., McKee T.,
 Zabriski T.M. and Swersey J.C. (1988) Uniqueness of the
 marine chemical environment: Categories of marine natural
 products from invertebrates. In: The Biomedical Importance
 of Marine Organisms (Fautin D.G., ed), California Academy of
 Sciences, San Francisco. pp 54.

The sources and number of samples collected by the NCI from 1986 to 1991 is summarized in Table 5. This acquisition program is devised so that the greatest possible diversity of plant and marine life is acquired.

Table 5. Sources and number of samples collected by the NCI (1986-1991).*

MARINE MICROORGANISMS

5 000 shallow water, Indo-Pacific
 5 000 deep water, Caribbean and Atlantic

TROPICAL RAINFOREST PLANTS

7 500 Southeast Asia
 7 500 Africa and Madagascar
 7 500 Central and South America

MICROORGANISMS

1 500 fungi, soil origin
 1 500 cyanobacteria, soil and other origins
 2 000 marine microorganisms

*From: Suffness M. and Thompson J.E. (1988) National Cancer Institute's role in the discovery of new antineoplastic agents. In: Biomedical Importance of Marine Organisms (Fautin D.G., ed). California Academy of Sciences, San Francisco. pp 155.

There are several problems associated with natural product screening:

1. Crude extracts may not be readily soluble.
2. The active ingredient in a crude extract may be present in exceedingly small amounts, so that even though it may potentially be extremely active, it may not be detected.
3. It may be difficult to isolate the active ingredient in the crude extract.
4. It may be difficult to acquire sufficient quantities of the active agent once it is identified.

This last problem is usually considered to be the most severe. Several options to resolve this obstacle have been proposed (11, 157):

1. Use tissue culture to grow the cell(s) responsible for the production of the agent of interest.
2. Identify a precursor to the agent in question that is easier to isolate.
3. Maintain rare marine invertebrates in tanks and plants in greenhouses.
4. Collect close relatives of the plant or animal in question.
5. Utilize genetic engineering techniques.

10. Thesis Objectives

The first objective of this thesis was to select and implement an *in vitro* assay suitable for evaluating the effects of various test agents on the cellular viability of human breast cancer cell lines and normal and malignant human breast epithelial cells (HBEC) in primary culture. This laboratory has, in the past, relied on the incorporation of radiolabelled thymidine as an indicator of cell growth. It was felt that this assay, although reproducible, was not suitable for larger scale screening. After a review of the literature, the MTT tetrazolium dye reduction assay was selected. Unfortunately, there was a great variation in the literature regarding the protocol for the assay, especially for assay parameters such as solvent, MTT incubation concentration and MTT incubation time. An MTT assay protocol had to be elucidated that would optimize the sensitivity and the accuracy of this assay for use on human breast cells.

The principal objective of this thesis was to determine the effects of 7 crude extracts and 8 pure metabolites derived from marine organisms on the cellular viability of 4 drug-sensitive and 2 drug-resistant cell lines. In addition, it was a prerogative to see if any agents that demonstrated significant cytotoxicity against the drug-resistant human breast cancer cell lines may circumvent the MDR phenotype.

This thesis also investigated the cytotoxicity of the more potent test agent on normal and malignant human breast

epithelial cells (HBEC) in primary culture, which more closely resemble counterpart cells *in vivo* than do cell lines. The normal HBEC were included in the screening protocol to detect tumor-specific agents.

CHAPTER 2

MATERIALS AND METHODS

1. Isolation and Purification of Compounds

The procedures used for the isolation and purification of the geodiamolides (34), bromotopsentin (105), xestospongins E (99), bastidin-4 (100), imbricatine (17), chromodorolide A (40), jaspamide (29), glaciasterol A (123) and the Garvea extract (46) have previously been described. The crude extracts of the other organisms were prepared by homogenizing the organisms in methanol, filtering and evaporating the methanol in vacuo. The isolation and purification of compounds used for cytotoxicity testing did not constitute part of this thesis work. They were provided by Dr. Raymond Andersen, Departments of Chemistry and Oceanography, U.B.C.

All samples were stored lyophilized in a refrigerator until ready for use. When ready for use, some of the compound was transferred to a 250 μ l microfuge tube, weighed and then dissolved in enough solvent so that all of the sample is dissolved but the volume is still workable (typically about 100 μ l). The solvent used for all samples except for imbricatine was ethanol. Sterile saline was used for dissolving imbricatine. The 250 μ l microfuge tubes containing dissolved samples were then placed within 1.5 ml screw cap microfuge tubes and these were then wrapped in parafilm (to minimize any evaporation of solvent) and stored in the freezer at -5°C.

2. Cell Line Culture

The MCF-7, T-47D, ZR-75-1 and MDA-MB-231 cell lines, which originated from effusions from women with metastatic breast cancer, were obtained from the American Type Culture Collection (ATCC). The MDA-A1^r cell line was furnished by Dr. W. McGuire at the University of Texas, and the MCF-7 Ad^r cell line was obtained from Dr. K. H. Cowan at the National Institute of Health (NIH). The cell lines were maintained in 25 cm² Corning tissue culture flasks containing 10 ml of growth medium consisting of Ham's F12/Dulbecco's modified Eagle medium [F12/DME; 1:1 (v/v); Terry Fox Media Preparation Services] supplemented with 10 mM HEPES (H) and either 10% fetal bovine serum (FBS; Gibco Laboratories; for MDA-MB-231), 5% FBS and 5 µg insulin/ml (Sigma; for MCF-7, T-47D and ZR-75-1), 10% FBS and 1 µg Adriamycin/ml (ADR; Adria Laboratories; for MDA-A1^r), or 5% FBS, 5 µg insulin/ml and 0.5 µg ADR/ml (for MCF-7 Ad^r) (Appendices 1-4). All of these cell lines grow adherent to the tissue culture plastic.

Cultures were maintained in a humidified atmosphere consisting of 5% CO₂ and 95% air at 37°C. Media changes were every second to third day (ie: M-W-F). Multiple flasks of each cell line were maintained. The rationale for this was that the drug samples were dissolved in very small amounts of ethanol (— 100 µl). As a result of this small working volume, it was felt that repeated opening and closing of the drug-containing microfuge tubes would result in excessive

evaporation of the solvent, thus altering the concentration of the drug. To minimize the number of opening and closing of the tube, multiple flasks of cells were used to independently set up (ie: no cell pooling) separate drug-testing cultures on microtiter plates at one time. If at any time the stock drug concentrations became suspicious, either the vehicle was evaporated in a N₂ atmosphere and the sample was reweighed and redissolved, or a fresh stock solution was prepared from previously undissolved drug.

Cells were subcultured when approaching confluence by removing the growth medium, washing the culture with 5 ml of F12/DME/H (to remove any residual FBS), and adding 7 ml of either warmed saline-trypsin-versene (MCF-7, T-47D, ZR-75-1, MDA-MB-231 and MCF-7 Ad^R) (Appendix 5) or warmed calcium and magnesium-free phosphate-buffered saline (PBS) supplemented with 1 mM ethylenediaminetetraacetic acid (EDTA; MDA-A1^R) (Appendix 6). Gentle agitation of the tissue culture flasks for 3-5 min resulted in a single-cell suspension. Too much or too little agitation resulted in cell clumping, which was found to be irreversible. The cell suspension was then centrifuged in a clinical centrifuge at 100 x g for 3 min. The supernatant was then removed and 2 ml of the appropriate growth medium (Appendices 1-4) were then added to resuspend the cells. A small fraction (0.4 ml) of this suspension was then used to seed a new tissue culture plate. At the time of the experiment the cells were resuspended and centrifuged at 100 x g for 3 min. The supernatant was then

removed and the pellet was resuspended in 2 ml of F12/DME/H. An aliquot of the cell suspension (0.1 ml) was placed in a small glass test tube and a drop of trypan blue dye was added. Viable cells are able to prevent the trypan blue dye from penetrating the plasma membrane, whereas dead cells take up the dye and thus stain blue. The viable cell number was then determined by counting on a hemacytometer. After cell counting, the remaining 1.9 ml of cells suspended in F12/DME/H was centrifuged at 100 x g for 3 min, the supernatant removed and the cell pellet resuspended in F12/DME/H supplemented either 5% FBS and 5 μ g insulin/ml (MCF-7, T-47D, ZR-75-1 and MCF-7 Ad^r) or 10% FBS (MDA-MB-231 and MDA-A1^r) to result in a final viable cell concentration of 4 x 10⁴ cells/ml. Cells were then seeded at 4 x 10³ cells/well onto 96-well microtiter plates in 100 μ l volume of the appropriate medium (Appendices 7 and 8) and then reincubated. Cells were only seeded in the inner 10 x 6 matrix of the 12 x 8 microtiter plate, whereas cell-free F12/DME/H was placed in the wells bordering the edge of the microtiter plate. This was done to minimize any discrepancies in the microenvironments between wells bordering the plate edge and those that do not. In addition, only the 8 x 6 matrix towards the left side of the microtiter plate was used for the T-47D cell line since it was observed in early experiments that alterations in pH of the growth medium stimulated the growth of this cell line. Since I work right to left when removing and adding media,

the cells to the right side of the microtiter plate were exposed to the air of the laminar flow hood longer than those towards the left side of the plate, and thus had the pH of their growth media altered to a greater extent.

3. Human Breast Epithelial Cells in Primary Culture

Normal tissue cells from reduction mammoplasties and malignant tissue from biopsies and mastectomies were obtained from collaborating surgeons at local hospitals. Malignant breast tissue was used only from patients who had no previous chemotherapy. During surgery, the tissue was aseptically placed into plastic sterile cups containing transport media (Appendix 9). Immediately following surgery, the cups were placed on crushed ice in a cooler and transported to the tissue culture room. Under sterile conditions, excess fat was removed from the tissue samples and the samples were finely minced using 2 scalpels. The minced tissue was then transferred to 1.7 ml cryotubes so that the tubes were half filled. Freezing medium (Appendix 10) was then added to bring the volume to 1 ml. The tissue was then gradually frozen in liquid nitrogen to be used at a later date. At the time when the tissue was needed, the vial was removed from liquid nitrogen and quick-thawed by agitating in a 37°C waterbath. The vial was wiped with a Kim-wipe saturated with 70% ethanol prior to opening. Once opened, the minced tissue was transferred to a 15 ml centrifuge tube. The vial was then rinsed with a few ml of

F12/DME/H which was also placed into the centrifuge tube. The tube was then centrifuged at 100 x g for 3 min and the supernatant discarded. Following this, the pellet was washed with 5 ml of F12/DME/H, recentrifuged at 100 x g for 3 min and then resuspended in 5 ml warmed dissociation medium (Appendix 11). This mixture was then transferred to a 125 ml Bellco dissociation flask. The 15 ml centrifuge tube was rinsed several times with warmed dissociation medium that was also transferred to the dissociation flask. The total volume of dissociation medium in the flask was then brought up to 50 ml. The flask was covered first with sterile tin foil followed by parafilm, then placed in a gyrating shaker inside a 37°C incubator for 16-18 h (until no large tissue fragments remained). After dissociation, the solution had a cloudy appearance with a layer of liquid fat floating on top. The dissociation mixture was transferred to a 50 ml centrifuge tube. The flask was rinsed twice with 5 ml of F12/DME/H, with each rinsing being added to the 50 ml centrifuge tube. The tube was then centrifuged at 40 x g for 30 sec to separate undigested tissue fragments from the cell suspension. The supernatant was transferred to a second 50 ml centrifuge tube. The pellet in the first tube was washed with 5 ml of F12/DME/H, spun at 40 x g for 30 sec, with the supernatant being added to the second 50 ml centrifuge tube. This washing and centrifuging of the pellet was repeated, with the supernatant being added to the second centrifuge tube and the pellet being discarded. The cell suspension of

the second tube was centrifuged at 80 x g for 4 min to separate the epithelial fraction (pellet) from the stromal cells, undigested stroma and cellular debris (supernatant). After discarding of the supernatant, the pellet was resuspended in 1 ml of F12/DME/H and viable cells determined by trypan blue exclusion and counted on a hemacytometer. Following cell counting, the suspension was centrifuged at 100 x g for 5 min, the supernatant discarded and the epithelial cell pellet resuspended in growth medium (Appendix 12) such that the final cell concentration was 10^5 cell/ml. These cells were plated into as many wells as possible in the inner 10 x 6 matrix of collagen coated 96-well microtiter plates. The wells were collagen coated by adding one drop of rat tail collagen (Appendix 14) to each well, swirling the plates to ensure complete coverage of the well and then allowing the plate to dry for 1 h in a laminar flow hood under UV light. Since the cell yield from each tissue sample was very small, usually only a portion of each microtiter plate was utilized. Tissue samples which had insufficient cell yields were discarded. All wells not receiving cells contained F12/DME/H. The cell concentration used was 10^4 cells/well in 0.1 ml of growth medium. The cultures were incubated at 37°C in 5% CO₂ and 95% air. The growth medium of these cultures contained the patient's own serum (Appendix 13). Culturing cells in the serum of the patient from whom the tissue was received is the routine protocol for chemosensitivity testing in our laboratory (42,

43). It has been observed that fibroblasts in serum-containing media are stimulated to grow (44). As a result of this situation, the tissue culture plates during their 5 day culturing period were monitored using a phase contrast microscope to ensure that the dominant cell population (>80%) had an epithelial-like morphology (ie: polygonal shaped).

4. Compound-Testing Protocol

On day 0, the cells (cell lines and HBEC in primary culture) were seeded onto the microtiter plates in the appropriate growth medium (Appendices 7, 8 and 13) as described earlier. After 24 h, the medium was removed and 100 μ l of fresh medium containing either 5 serial dilutions (typically 100, 10, 0.1, 0.01 and 0.001 μ g/ml) of the agent to be tested or 5 serial dilutions of the corresponding drug vehicle (ethanol or saline) were added to the wells. Drug control cultures received the appropriate growth medium with no drug or vehicle. Each drug concentration, drug vehicle concentration and control condition (no drug or vehicle) was represented by 4 to 6 wells within each experiment. Each experiment was repeated 2-4 times, using independent cultures each time. Cultures were incubated with the drugs or drug vehicles for 48 h. The cells were then washed once with F12/DME/H to remove any residual drug and cultured in the appropriate drug-free medium (Appendices 7 and 8) for 48 h. A 48 h drug incubation period was selected to ensure that

all cells passed through at least one cell cycle, thus allowing phase-specific and cell-cycle-specific agents to exert their effects. The cell-cycle times of MCF-7, T-47D, ZR-75-1, MDA-MB-231, MCF-7 Ad^r and MDA-A1^r cells used in this laboratory are 30, 22, 44, 21, 23 and 18 h, respectively. A 48 h drug recovery period was selected to ensure enough time had been allowed for the cytotoxic effects of the drug to be realized. Inhibitors of protein synthesis usually require a drug recovery period of approximately 24 h, whereas inhibitors of DNA synthesis require a recovery period of 48-96 h (135). After the recovery period, viable cells were quantified by the MTT assay.

In some experiments performed to detect agents that might circumvent the MDR phenotype, MCF-7 Ad^r cells were incubated in medium containing the concentration of Adriamycin that results in a 50% decrease in cell growth for this line (ED₅₀, 5 µg/ml) as well as serial dilutions of the drug to be tested.

5. Preparation of Standard Curves for Each Cell Line

Since the relationship between absorbance at 540 nm and cell number is not necessarily linear when using the MTT assay (124), a standard curve for each cell line was prepared. Serial dilutions (2×10^3 to 65×10^3 cells/well) of each cell line were seeded onto 96-well microtiter plates in the appropriate growth medium (Appendices 7 and 8).

Fifteen hours after cell seeding, the cells were quantified using the MTT assay.

6. MTT Assay

a. Preparation of MTT

MTT (Sigma) powder was weighed, placed in a 17 x 100 mm polypropylene test tube and dissolved in 8 ml of warmed serum-free and phenol red-free F12/DME/H (Sigma). With the use of a small spatula, the aggregates of MTT were broken up and the solution stirred until all the MTT was dissolved. The solution was filter sterilized through a Millex GV 0.22 μ m syringe filter to remove any pathogens, undissolved MTT and any spontaneously formed formazan crystals. Warmed serum-free and phenol red-free F12/DME/H was then added to bring the final concentration to 1 mg MTT/ml.

b. MTT Assay Protocol

The procedure for the MTT assay used in all drug chemosensitivity experiments is summarized in Figure 11. The medium was removed and 100 μ l of MTT (1 mg/ml) dissolved in serum-free and phenol red-free F12/DME/H were added to each well. Serum-free medium is used so that there is no precipitation of any residual serum protein upon addition of the solvent, thereby decreasing background absorbance levels. Similarly, phenol red-free medium is used to prevent interference of the red pH indicator during the absorbance readings. Following a 5 h incubation period at

37°C in 5% CO₂ and 95% air, 100 µl of 20% formol in saline were added to each well. Following 30 min at room temperature, the medium was removed using a multichannel pipette (Titertek) and 100 µl of anhydrous isopropanol were added to each well to dissolve the formazan crystals. After 1 h, the plates were gently agitated, and absorbance values of each well were determined at 540 nm using a 96-well microtiter plate reader (model EL 311, Biotek Instruments) interfaced with a MacIntosh Apple computer.

Figure 11. MTT assay protocol.

To take into account any absorbance due to residual MTT, MTT control wells (which do not contain cells) were incorporated into the experiment. Absorbance values obtained for these MTT control wells were subtracted from absorbance

values obtained for cell-containing wells. The corrected absorbance values were then converted to cell numbers via standard curves specific for each cell line. Cytotoxicity was determined by comparing drug-treated cultures with control cultures (which are assigned a value of 100%). The ED₅₀ value was then determined from the dose response curve.

A control to monitor reduction of MTT to formazan by drugs that have reducing properties was not included in the experiments. Previous studies have demonstrated that reducing agents do not influence results generated by the assay (20).

The elucidation of the various assay parameters (solvent, MTT incubation concentration and MTT incubation time) presented in this assay protocol were determined as described below (c-e).

c. Selection of Solvent for the MTT Assay

i) With No Removal of Supernatant Prior to Solvent Addition

MCF-7 cells were seeded at 1.3×10^5 cells/ml in 430 μ l of 1 mg MTT/ml serum-free and phenol red-free F12/DME/H in 3 12 x 75 mm polypropylene test tubes. Following a 4 h incubation at 37°C in 5% CO₂ and 95% air, 870 μ l of either anhydrous isopropanol, ethanol or spectrophotometric grade dimethyl sulfoxide (DMSO) were added to each tube. The cell generated formazan was allowed to dissolve in the solvent for 2 h at room temperature. The formazan solvent mixture was then transferred to a glass cuvette and a wavelength

scan from 400 to 640 nm was generated for each solvent mixture using a UV/visible scanning spectrophotometer (Beckman Instruments). The reference cell for each of the 3 wavelength scans was the solvent containing 1.3×10^5 dead (lysed) MCF-7 cells and 430 μ l of 1 mg MTT/ml in F12/DME/H.

ii) With Removal of MTT Supernatant Prior to Solvent

Addition

MCF-7 cells were seeded at 2×10^5 cells/ml in 1.3 ml of 1 mg MTT/ml serum-free and phenol red-free F12/DME/H in 3 12 x 75 mm polypropylene test tubes. Following a 4 h incubation at 37°C in 5% CO₂ and 95% air, the tubes were centrifuged at 100 x g for 3 min, the supernatant was removed and 1.3 ml of either isopropanol, ethanol or DMSO were added to each tube. The cell generated formazan was then allowed to dissolve in the solvent for 1 h at room temperature. The formazan-solvent mixture was then transferred to a glass cuvette and a wavelength scan from 400 to 640 nm was generated for each solvent mixture using a UV/visible scanning spectrophotometer. The reference cells used for each of the 3 wavelength scans was the solvent containing 2.6×10^5 dead (lysed) MCF-7 cells (cells that were not exposed to MTT).

d. Selection of Optimal MTT Concentration

Multiple wells of serial dilutions of MCF-7 cells (312 to 5×10^5) were seeded onto 96-well microtiter plates in $100 \mu\text{l}$ of growth medium (Appendix 1). Following a 24 h incubation at 37°C in 5% CO_2 and 95% air, the growth medium was removed and serial concentrations (0.125 - 5 mg/ml) of MTT dissolved in $100 \mu\text{l}$ serum-free and phenol red-free F12/DME/H were added to the wells. MTT addition was such that every concentration of MTT tested was exposed to the entire spectrum of cell densities. Following a 4 h incubation period at 37°C , the medium was removed and $100 \mu\text{l}$ of isopropanol were added to each well. The formazan was allowed to dissolve for 1 h at room temperature and then the plates were read on a 96-well microplate reader at 540 nm.

e. Selection of Optimal MTT Incubation Time

MCF-7 cells were seeded at 4×10^3 cells/well in growth medium (Appendix 1) on 7 different 96-well microtiter plates. Following a 24 h incubation at 37°C in 5% CO_2 and 95% air, the growth medium was removed and $100 \mu\text{l}$ of 1 mg MTT/ml serum-free and phenol red-free F12DME/H were added to all the wells. The cells were reincubated at 37°C and at 1 h intervals for 7 h, a single microtiter plate was taken out of the incubator and the mean absorbance of each plate was measured at 540 nm.

f. Removal of MTT Supernatant

In the initial experiments outlined above (c-e), it was observed that removal of the MTT-F12/DME/H supernatant with a multichannel pipettor (Titertek) resulted in cell loss and therefore increased assay error. It became apparent that MCF-7 cells, once they have formed intracellular formazan, become easily detached from the tissue culture plastic. To correct for this problem, a variety of alternative methods were tested for removing the MTT supernatant. Briefly, MCF-7 cells were seeded at 4×10^3 cells/well in 42 wells on 5 different 96-well microtiter plates. Following a 24 h incubation period at 37°C in 5% CO₂ and 95% air, the growth medium (Appendix 1) was removed and 100 µl of 1 mg MTT/ml serum-free and phenol red-free F12/DME/H were added to each well. Following a 5 h MTT incubation period, the MTT supernatant was removed by a variety of methods: 1) The cells, prior to MTT supernatant removal, were fixed to the tissue culture plate by the addition of 100 µl of 20% formol in saline (to result in a final concentration of 10% formol). After 30 min at room temperature the MTT supernatant was gently removed using a multichannel pipette. 2) The MTT supernatant was removed by submersing the plate in saline and then blotting the plate on paper towels. 3) The MTT supernatant was removed by first inverting the plate and "flicking" it with a sudden wrist motion, then dipping the plate in saline and finally by blotting on paper towels. 4) The MTT supernatant was removed by pipetting with a

multichannel pipette.

Following removal of the MTT supernatant, 100 μ l of isopropanol were added to each well and the absorbance value at 540 nm of each cell-containing well was measured. From these values, the coefficient of variation (the ratio of the standard deviation (S) relative to the mean (X) expressed as a percentage; $S - X \cdot 100\%$) was determined for each plate.

g. Absorbance Limit of the MTT Assay

To determine the upper limit of absorbance at 540 nm in which the assay becomes insensitive to differences in formazan concentration (ie: cell number), the absorbances of serial dilutions (1-800 μ g/ml) of pure MTT formazan (Sigma) dissolved in isopropanol were measured using a spectrophotometer. The absorbance values were used to construct a graph relating absorbance to formazan concentration.

To relate the absorbance spectrum of pure formazan to cell-generated formazan, a wavelength scan (400-640 nm) of pure formazan dissolved in isopropanol at 30 μ g/ml was generated using a UV/visible scanning spectrophotometer.

CHAPTER 3

RESULTS

1. Optimization of MTT Assay Parameters

a. Selection of Solvent for the MTT Assay

The MTT tetrazolium dye reduction assay utilizes formazan production as an index of cell population viability. Figure 12 shows the wavelength scan from 400 to 640 nm of cell-generated formazan dissolved in isopropanol, ethanol and DMSO in the presence of serum-free and phenol red-free F12/DME/H. A peak in absorbance was observed for isopropanol and ethanol at approximately 560 nm. The height of the isopropanol peak (0.71) was slightly higher than that for the ethanol peak (0.68). A peak in absorbance for the DMSO curve was observed at a wavelength of 520 nm. The height of this DMSO peak (0.53) was substantially lower than that for isopropanol and ethanol. It was observed in this experiment that it was difficult to get the formazan crystals to dissolve in DMSO in the presence of F12/DME/H, even when the formazan dissolving period was extended to 2 h. As a consequence, the experiment was repeated with the modification that the MTT supernatant was removed prior to solvent addition.

Figure 13 shows the wavelength scan from 400 to 640 nm of cell-generated formazan dissolved in isopropanol, ethanol and DMSO. A peak in absorbance was observed for isopropanol at 540 nm, for ethanol at 580 nm and for DMSO at 500 nm. The height of the isopropanol peak (2.67) was slightly higher

than that for the ethanol peak (2.39) and the DMSO peak (2.35). Since the ELIZA reader was equipped with a 540 nm filter, these results indicated that isopropanol was the most suitable solvent for the MTT assay. No difficulty in dissolving the formazan crystals was observed when using DMSO as the solvent in this second experiment. The heights of all the peaks for the 3 different solvents in the second experiment (removal of MTT supernatant) were several magnitudes higher than those generated in the first experiment (no removal of MTT supernatant).

Figure 12. Wavelength scans of cell-generated formazan dissolved in DMSO, isopropanol and ethanol in the presence of the MTT supernatant.

MCF-7 cells were incubated in 1 mg MTT/ml serum-free and phenol red-free F12/DME/H for 4 h. Following the incubation period, 100 μ l of either DMSO, isopropanol or ethanol were added to the cells to dissolve the cell-generated formazan. Two hours later, a wavelength scan from 400 - 640 nm was generated for each formazan-solvent mixture. The results indicated that isopropanol was the most suitable solvent for reading A_{540} since it generated the highest peak (0.71) at this wavelength.

FIGURE 12

68A

Figure 13. Wavelength scans of cell-generated formazan dissolved in DMSO, isopropanol and ethanol.

MCF-7 cells were incubated in 1 mg MTT/ml serum-free and phenol red-free F12/DME/H for 4 h. Following the incubation period, the supernatant was removed and 100 μ l of either DMSO, isopropanol or ethanol were added to the cells to dissolve the cell-generated formazan. One hour later, a wavelength scan from 400 - 640 nm was generated for each formazan-solvent mixture. The results indicated that isopropanol was the most suitable solvent for reading A_{540} , since it generated the highest peak (2.67) at this wavelength.

FIGURE 13

69A

b. Selection of Optimal MTT Concentration

Figure 14 illustrates the relationship between MTT concentration and absorbance over a variety of cell densities. At lower cell densities, slight differences in absorbance were observed between all the concentrations of MTT, with 1 mg MTT/ml being optimal. As cell number increases, the difference in absorbance between the different concentrations of MTT also increases. At the highest cell density (5×10^5 cells/well), 5 mg MTT/ ml resulted in the greatest absorbance (0.871), whereas 0.125 mg MTT/ml resulted in the lowest absorbance (0.239). It was expected that cell density at the end of the drug testing experiments would generally be $\sim 2 \times 10^4$ cells/well. Consequently, 1 mg MTT/ml was selected as the most suitable MTT concentration, because it resulted in the highest A_{540} below this cell concentration.

Figure 14. Formazan production as a function of MTT incubation concentration

Serial dilutions of MCF-7 cells ($312 - 5 \times 10^5$) were exposed to serial concentrations of MTT (0.125 - 5 mg/ml) such that every cell density was exposed to every MTT concentration. Following the MTT incubation period, the supernatant was removed, the formazan crystals were dissolved in isopropanol and the A_{540} was measured. At cell densities below 10^4 cells/well, 1 mg MTT/ml resulted in slightly higher absorbance than the other MTT concentrations. Above 10^4 cells/well the higher MTT concentrations resulted in greater formazan production, with 5 mg MTT/ml being the optimal at the very high cell densities. Since it was expected that the final cell density following the drug incubation period would generally be 2×10^4 cells/well, 1 mg MTT/ml was selected as the optimal MTT concentration.

FIGURE 14

71A

c. Selection of Optimal MTT Incubation Time

Figure 15 illustrates the relationship between length of MTT incubation and absorbance at 540 nm. Absorbance (0.302) was highest when the MCF-7 cells were incubated with the MTT solution for 5 h, and consequently a 5 h MTT incubation time was selected for all further experiments. It is interesting to note that the longer the MCF-7 cells were exposed to the MTT solution after 5 h MTT incubation, the greater the value was for the coefficient of variation (which is directly proportional to error). This relationship is illustrated in Figure 16.

Figure 15. Formazan production as a function of MTT incubation time.

MCF-7 cells were seeded at 4×10^4 cells/well on 7 different microtiter plates. Twenty-four hours later, the medium (Appendix 1) was removed and 1 mg MTT/ml was added to all the cells. The cells were reincubated at 37°C and at 1 h intervals, a single microtiter plate was removed from the incubator, and the mean A_{540} of its' cell containing wells was determined. A peak in absorbance (0.302) is observed for cells that were incubated for 5 h, thereby indicating that a 5 h MTT incubation time results in optimal formazan production. The error bars represent the standard error of the mean (standard deviation/ $\sqrt{n}$) of 2 experiments.

FIGURE 15

73A

Figure 16. Coefficient of variation as a function of MTT incubation time.

The error associated with Figure 15 is represented here as the coefficient of variation (standard deviation/mean x 100%). The results demonstrate that the error associated with the assay increases significantly after 5 h MTT incubation time.

FIGURE 16

74A

d. Removal of MTT Supernatant

Table 6 lists the coefficients of variation for different methods of removal of the MTT supernatant. Formol-fixing the cells to the microtiter plate and then removing the MTT supernatant with a multichannel pipettor resulted in the lowest coefficient of variation (8.8%), and consequently formol-fixing the cells followed by multichannel pipetting was used for all subsequent experiments. The control studies for this experiment demonstrated that formalin did not interfere with absorbance at 540 nm.

Table 6. Coefficient of variation associated with different methods of MTT supernatant removal.

Method of MTT supernatant removal	Coefficient of variation (%)
Formol fixing and multichannel pipetting	8.8
Saline dipping with no flicking	15.8
Saline dipping with flicking	11.0
Multichannel pipetting	16.5

e. Absorbance Limit of the MTT Assay

Figure 17 illustrates the relationship between the concentration of pure formazan and absorbance at 540 nm. The upper limit of absorbance was approximately 3, which corresponded to a concentration of roughly 200 ug formazan/ml. Absorbance readings from the drug-screening

experiments very rarely exceeded 1.0.

Figure 18 is the wavelength scan of pure formazan from 400 to 640 nm. Included in the Figure is the corresponding wavelength scan of cell-generated formazan (from Figure 13). The peaks of the pure formazan and the culture-generated formazan were both in the 540 to 560 nm range, thereby indicating it is valid to use pure formazan in the determination of the upper absorbance limit of the MTT assay.

Figure 17. Absorbance limit of the MTT assay.

Serial dilutions (1 - 800 $\mu\text{g/ml}$) of pure formazan were dissolved in isopropanol and the A_{540} of each dilution was measured. The upper limit of absorbance is observed to be approximately 3.

FIGURE 17

77A

Figure 18. Wavelength scans of pure formazan and cell-generated formazan dissolved in isopropanol.

Pure MTT formazan dissolved in isopropanol was used to generate a wavelength scan from 400 - 640 nm. The wavelength scan of cell-generated formazan dissolved in isopropanol was also included in Figure 18. Both absorption spectrums had a peak in the 540 - 560 nm range.

FIGURE 18

78A

2. Compounds Screened Against Drug-Sensitive Cell Lines

The MTT assay protocol determined from the experiments described above is briefly described as follows. Following the 48 h drug recovery period, the medium was removed and 100 μ l of MTT (1 mg/ml) were added to each well. Following a 5 h incubation period at 37°C, 100 μ l of 20% formol in saline was added to each well to fix the cells. Following 30 minutes at room temperature, the supernatant was removed and 100 μ l of isopropanol was added to each well. One hour later, the plates were gently agitated and the absorbance values of each well was determined using a 96-well microtiter plate reader.

Figure 19 illustrates the wide range of chemical structures of all the pure samples tested in our *in vitro* screening protocol. Table 7 summarizes the ED₅₀ values obtained for compounds screened against the MCF-7, T-47D, ZR-75-1 and MDA-MB-231 cell lines. Of the 15 samples screened, 8 demonstrated significant cytotoxicity (ED₅₀, $\geq$ 25 μ g/ml) against either the MCF-7 or the T-47D cell line. Only these 8 samples were screened against ZR-75-1 and MDA-MB-231 cells; all 8 compounds were significantly cytotoxic against the former cell line, whereas 6 were significantly cytotoxic against the latter. The ED₅₀ values for Adriamycin were determined in all of these cell lines to provide a standard with which to compare the samples. The ED₅₀ values recorded for the two pure samples, the geodiamolides and jaspamide, were comparable with or lower than those noted

Figure 19.

Molecular structures of pure metabolites isolated from marine organisms.

FIGURE 19

TABLE 7. ED₅₀ values obtained for compounds screened against the drug-sensitive breast cancer cell lines MCF-7, T-47D, ZR-75-1 and MDA-MB-231.

AGENT	ED ₅₀ values (μg/ml)			
	MCF-7	T-47D	ZR-75-1	MDA-MB-231
GEODIAMOLIDES	0.0068±.0003	0.044±.003	0.033±.024	0.004
XESTOSPONGIN E	>>50	>50	ND	ND
BROMOTOPSENTIN	>>100	32±5	ND	ND
PNG-31A ^a	5.4±.5	0.6	7	24±1
BASTIDIN-4	6.0±.6	0.58±.05	2.9±.1	2.6±.1
IMBRICATINE	>50	23±9	ND	ND
CHROMODOROLIDE A	>>100	>>50	ND	ND
GARVEA ^a	33±9	19±3	21±1	23±1
QCI-32 ^a	1.2±.4	0.09±.02	0.09±.01	0.018±.001
QCI 4-16-89 ^a	>100	50±22	ND	ND
PNG-137A ^a	30±3	13±3	16±3	>50
PNG-137B ^a	98±2	35±12	ND	ND
QCI-117 ^a	>>100	>>100	ND	ND
GLACIASTEROL A	19±1	21±3	26±1	>>50
JASPAMIDE	0.027±.004	0.019±.009	0.042±.002	0.024±.001
ADRIAMYCIN	0.10±.01	0.065±.009	0.005±.001	0.038±.006

Data represent mean values ± standard error of the mean (standard deviation/√n). Only the compounds that exhibited significant cytotoxicity towards the MCF-7 and T-47D cell lines were screened against the ZR-75-1 and MDA-MB-231 cell lines. ND, not done.

^a Crude extract

for Adriamycin. The values obtained for another sample, QCI-32, which is a crude extract, were 12-, 1.4-, 18- and 0.47-fold higher than those found for Adriamycin in screening against the MCF-7, T-47D, ZR-75-1 and MDA-MB-231 cell lines, respectively. ED₅₀ values as low as 0.6 µg/ml were noted for the crude extract PNG-31A in the T-47D cell line; this value is 10-fold higher than that determined for Adriamycin.

Values recorded for the 5 remaining compounds that exhibited significant cytotoxicity ranged from 0.58 to 33 µg/ml and were at least 9-fold higher than those found for Adriamycin.

Figures 20-23 represent the standard curves relating absorbance at 540 nm to cell number for the MCF-7, T-47D, ZR-75-1 and the MDA-MB-231 cell lines. Neither ethanol nor saline had any effect on drug-sensitive cell line viability in the concentrations used in the compound-testing experiments.

3. Compounds Screened Against Drug-Resistant Cell Lines

Table 8 summarizes the ED₅₀ values obtained for the samples and for Adriamycin during screening against the Pgp-positive cell lines MCF-7 Ad^r and MDA-A1^r. Of the 8 samples screened, 6 demonstrated significant cytotoxicity against either the MCF-7 Ad^r or the MDA-A1^r cell line. The geodiamolides were 54- and 11 600- times more cytotoxic than Adriamycin when screened against the MCF-7 Ad^r and MDA-A1^r cell lines, respectively. Jaspamide was 6- and 318- times more cytotoxic than Adrimycin when screened against the

TABLE 8. ED₅₀ values obtained for compounds screened against the multidrug-resistant breast cancer cell lines MCF-7 Ad^r and MDA-A1^r.

AGENT	ED ₅₀ values (μg/ml)	
	MCF-7 Ad ^r	MDA-A1 ^r
GEODIAMOLIDES	0.092 ± .015 (14)	0.0006 ± .0001 (0.15)
PNG-31A ^a	51 ± 2 (9.4)	41 ± 4 (1.7)
BASTIDIN-4	>>20 (>3.3)	>100 (>38)
GARVEA ^a	27 ± 4 (0.82)	25 ± 3 (1.1)
QCI-32 ^a	10 ± 4 (8.3)	1.3 ± .4 (72)
PNG-137A ^a	>50 (>1.7)	25 ± 2 (<0.5)
GLACIASTEROL A	18 ± 1 (0.95)	19 ± 2 (<0.38)
JASPAMIDE	0.77 ± .02 (29)	0.022 ± .005 (0.92)
ADRIAMYCIN	5 ± 2 (50)	7 ± 4 (184)

Data represent mean values ± standard error of the mean (standard deviation/√n) for 2 - 4 experiments. Values in parenthesis indicate the magnitude of increase in resistance noted for the MDR daughter cell lines as compared with the corresponding drug-sensitive parent lines (ED₅₀ of the resistant daughter cell line/ED₅₀ of the sensitive parent line).

^aCrude extract

MCF-7 Ad^r and MDA-A1^r cell lines, respectively. MCF-7 cells were 50-times more resistant to Adriamycin than was the parent MCF-7 cell line, whereas MDA-A1^r cells were 184-times more resistant than was the parent line MDA-MB-231. Table 8 also compares the ED₅₀ values found for compounds screened against the MDR cell lines MCF-7 Ad^r and MDA-A1^r with those obtained using the drug-sensitive parent cell lines (ED₅₀ drug-resistant/ED₅₀ drug-sensitive). The geodiamolides Garvea, PNG-137-A, glaciasterol A and jaspamide were observed to have ED₅₀ ratios μ 1 for one or both cell types.

Figures 24 and 25 represent the standard curves relating absorbance at 540 nm to cell number for the MCF-7 Ad^r and MDA-A1^r cell lines. Neither ethanol nor saline had any effect on cell viability of the drug-resistant cell lines in the concentrations used in the compound-testing experiments.

Figures 20 - 25. Standard absorbance curves for the MCF-7, T-47D, ZR-75-1, MDA-MB-231, MCF-7 Ad^r and MDA-A1^r cell lines.

Serial dilutions of the cell lines were seeded in the appropriate growth medium (Appendices 7 and 8). Following attachment of the cells to the tissue culture plate (15 h), the A₅₄₀ value of each cell density was determined (Figure 11). An A₅₄₀ versus cell number graph was plotted for each cell line (Figure 20: MCF-7, Figure 21: T-47D, Figure 22: ZR-75-1, Figure 23: MDA-MB-231, Figure 24: MCF-7 Ad^r and Figure 25: MDA-A1^r) and a computer generated equation for the best-fit line was determined (MCF-7: $y = 8.060e-4 * x^{0.7038}$, T-47D: $y = 0.0048 * x^{0.4852}$, ZR-75-1: $y = 8.924e-4 * x^{0.6849}$, MDA-MB-231: $y = 4.944e-4 * x^{0.6968}$, MCF-7 Ad^r: $y = 1.343e-4 * x^{0.7953}$ and MDA-A1^r: $y = 0.001 * x^{0.6477}$). Error bars represent standard error of the mean (standard deviation/√n) of 2 - 4 experiments.

FIGURE 20

85A

FIGURE 21

85B

FIGURE 22

85C

FIGURE 23

85D

FIGURE 24

85E

FIGURE 25

85F

4. Compounds Screened Against MCF-7 Ad^r Cells in the Presence of Adriamycin

To determine whether compounds whose ED₅₀ ratios were μ 1 might circumvent the MDR phenotype by interfering with Pgp, MCF-7 Ad^r cells were incubated in the presence of the ED₅₀ concentration (5 μ g/ml) of Adriamycin and in varying concentrations of the geodiamolides, Garvea, PNG-137A, glaciasterol A and jaspamide. No synergy was observed between any of the samples and Adriamycin (Figures 26-30), suggesting that these compounds do not interfere with Pgp function. The ED₅₀ values of the various test agents in the presence of Adriamycin are 0.95, 11, >50, 13 and 1 μ g/ml for the geodiamolides, Garvea, PNG-137A, glaciasterol A and jaspamide, respectively. Additive cytotoxicity was observed between some agents (Garvea, PNG-137A, glaciasterol A and Jaspamide) and Adriamycin (Figures 27 - 30). A 10-fold reduction in the cytotoxic activity of the geodiamolides was observed when MCF-7 Ad^r cells were exposed to the geodiamolides in the presence of Adriamycin (Figure 26).

Figures 26 - 30. Dose response curves of MCF-7 Ad^r cells to varying concentrations of the geodiamolides, Garvea, PNG-137A, glaciasterol A and jaspamide in the presence of the ED₅₀ concentration of Adriamycin.

MCF-7 Ad^r cells were exposed to serial dilutions of either the geodiamolides, Garvea, PNG-137A, glaciasterol A or jaspamide in the presence of 5 µg/ml Adriamycin. Following the drug recovery period the surviving cells were quantified (Figure 11) and a dose response curve for each compound was constructed (Figure 26: geodiamolides, Figure 27: Garvea, Figure 28: PNG-137A, Figure 29: glaciasterol A and Figure 30: jaspamide). Dose response to 5 µg/ml Adriamycin in the presence of no test compound is plotted as 50%. The dose response of the cells to the various test agents is plotted relative to this 50% level. No synergy was observed between any of the samples and Adriamycin. The ED₅₀ values of the various test agents in the presence of Adriamycin were 0.95, 11, >50, 13 and 1 µg/ml for the geodiamolides, Garvea, PNG-137A, glaciasterol A and jaspamide, respectively. A 10-fold decrease in the cytotoxicity of the geodiamolides is observed. Error bars represent the standard error of the mean (standard deviation/√n) of 2 experiments.

FIGURE 26

87A

FIGURE 27

87B

FIGURE 28

87C

FIGURE 29

87D

FIGURE 30

87E

5. Compounds Screened Against Normal and Malignant Human Breast Epithelial Cells in Primary Culture

Table 9 summarizes the cytotoxicities of the geodiamolides and jaspamide as tested against malignant and normal HBEC in primary culture. No significant trend indicating tumor specificity was observed for the geodiamolides. Minimal data is available for the jaspamide sample due to limited quantity of drug. Large variation was seen in the cytotoxicity for a given agent for different tissue samples: the ED₅₀ values recorded for the geodiamolides ranged from 0.018 to 5.5 µg/ml and from 0.21 to > 10 µg/ml in malignant and normal tissue, respectively, whereas the corresponding values determined for jaspamide ranged from 0.0005 to 3.8 µg/ml and from < 0.05 to 0.5 µg/ml respectively. Adriamycin ED₅₀ values were included for reference in cases in which sufficient tissue was available.

Table 9. ED₅₀ values obtained for the geodiamolides and jaspamide tested against normal and malignant HBEC in primary culture.

Agent	ED ₅₀ values (µg/ml)							
	N1	N2	N3	Ca1	Ca2	Ca3	Ca4	Ca5
Geodiamolides	0.21	>10	>6	0.1	0.018	5.5	0.5	ND
Jaspamide	<0.05	0.08	0.5	ND	ND	ND	0.0005	3.8
Adriamycin	ND	0.25	0.29	ND	ND	ND	ND	ND

N, Normal cells; Ca, malignant cells; ND, not done.

CHAPTER 4

DISCUSSION

Past drug-screening programs have illustrated that the incorporation of solid tumors into a drug-screening protocol is essential, since agents that are effective in treating leukemias are not necessarily effective against solid tumors (21, 62, 95). The crude extracts and pure metabolites evaluated in this thesis had previously been screened and found to be cytotoxic *in vitro* in the leukemic L1210 bioassay (34, 100, 105, 117 and personal communication with Dr. R Andersen). In the present study, mammary carcinoma was used as a solid-tumor model. Breast cancer cell lines were selected for initial screening because the cells are plentiful and easy to grow. However, cell lines result from cell selection and the populations are very homogenous in their characteristics. Therefore, four drug-sensitive and two drug-resistant cell lines were used to represent the heterogeneity seen in naturally-occurring tumors. Primary cultures of epithelial cells from malignant breast tissue were incorporated into the screening protocol, because they more closely resemble counterpart cells *in vivo*. Epithelial cells from normal tissue were also included to determine the tumor specificity of the geodiamolides. As the results illustrate, there was broad variation in the biological activity of cytotoxic compounds tested on different cell lines and on different primary cultures.

Several assays were considered to measure the cytotoxic effects of the test compounds on the cell lines and the HBEC in primary culture. Incorporation of radiolabelled precursors, protein binding assays, the neutral red assay and the MTT assay were all reviewed. This laboratory has traditionally used incorporation of radioactive thymidine as a measure of cell viability, but this method was rejected because it is impractical for large-scale screening. Also, a potential problem that may arise when using this method to screen drugs that have unknown mechanisms of action is that the drug under study and the radiolabelled precursor may share the same biochemical pathways and/or transport mechanisms, thus there may be competition between the label and the drug resulting in irregularities in the results (Ruben 1988). Protein binding assays measure the total protein content of a population of cells. Consequently, a potential problem with protein binding assays is that they could incorporate the protein content of nonviable cells in the final results (136). This is especially so if the time interval between drug addition and the assay is short. The neutral red assay was deemed inadequate, because it relies on the accumulation of neutral red dye into lysosomes and it was this investigator's opinion that lysosome number would be more susceptible to change when exposing cells to unknown potentially cytotoxic agents. The MTT assay was selected, because it is a measure of cell viability and it has correlated well with the SRB assay (136), a ^{51}Cr release

assay (67), the radiolabelled thymidine incorporation assay (unpublished results from our laboratory), the HTCFA and a dye exclusion assay (20).

Unfortunately, there is a large amount of variation in the literature regarding the exact procedure of the MTT assay. The cause of this variation has to do with the fact that in order for the MTT assay to be a reproducible measure of cell survival, the parameters of the assay have to be optimized for the type of cells used and the culture conditions used to grow these cells. The parameters that had to be optimized for the drug screening experiments outlined in this thesis were the type of solvent, the concentration of the MTT solution and the length of the MTT incubation time. The original MTT protocol as described by Mosmann (107) called for the MTT-containing medium not to be removed before addition of the solvent. MTT assays performed for this thesis had the MTT supernatant removed prior to solvent addition, because the results of the two wavelength scans of cell-generated formazan dissolved in different solvents (Figures 12 and 13) indicate that greater assay sensitivity can be achieved when the MTT supernatant is removed. These wavelength scans also demonstrate that isopropanol is the most suitable solvent for determining formazan absorbance at 540 nm. The NCI, when investigating the feasibility of the MTT assay for its large scale drug-screening program, observed that DMSO is the most suitable solvent. This discrepancy can be explained by the fact that the NCI

cultures most of their cell lines in media supplemented with 10% FBS (2) and, at the time of assay, they add their MTT directly to the existing drug-containing growth medium (Figure 7). Thus, when the MTT supernatant is removed, there is a relatively high concentration of residual serum clinging to the cell monolayers and the walls of the wells. High concentrations of residual serum have been shown to enhance the absorption of formazan dissolved in DMSO (2). The MTT assay protocol outlined in this thesis (Figure 11) requires that the growth medium first be removed prior to the addition of MTT dissolved in serum-free and phenol red-free F12/DME/H. Since there is relatively little residual serum present, formazan dissolved in isopropanol illicit the highest peak in the wavelength scan.

MTT was added to the cell monolayer dissolved in fresh serum-free and phenol red-free medium rather than added directly to the existing growth medium for 2 additional reasons. The first is that a positive correlation has been established between the D-glucose concentration of the culture medium at the time of assay and the production of formazan (167). Thus, adding MTT to nutrient-depleted growth medium runs the risk of decreasing the sensitivity of the assay. The relationship between D-glucose availability and formazan production may also account for the non-linearity of the cell number vs A_{540} curves, since one would expect D-glucose availability to be decreased when cell density is high. The second reason is that the pH indicator present in

the normal growth medium increases absorbance background (33), and thus phenol red-free medium is used instead.

A possible explanation for the observation that the coefficient of variation increases proportionally with the MTT incubation time (Figure 16) is that, as the cells generate more and more intracellular formazan crystals, they begin to lose their attachment to the microtiter plates. When the MTT supernatant is removed, these cells are lost. To minimize cell loss in all subsequent experiments, formalin was used to fix the cells to the tissue culture plastic.

Of the the 15 compounds tested against the drug-sensitive cell lines MCF-7 and T-47D, 8 demonstrate significant activity. Agents with an ED₅₀ value of up to 25 $\mu\text{g/ml}$, except for imbricatine, were selected for further testing against the ZR-75-1 and MDA-MB-231 cell lines and the drug-resistant cell lines. There was an insufficient amount of imbricatine available for further testing in these cell lines. An ED₅₀ of 25 $\mu\text{g/ml}$ was selected as a cutoff value because many of the compounds tested were crude extracts, and a lower cutoff point might have resulted in the exclusion of potentially toxic compounds from further testing. The cutoff value used by other researchers in the drug-screening field is typically 1 $\mu\text{g/ml}$. In addition, glaciasterol, which exhibited ED₅₀ values in the 25 $\mu\text{g/ml}$ range, also exhibited low *in vivo* host toxicity (personal communication with Dr. R Andersen).

The geodiamolides (a mixture of two cyclodepsipeptides that are identical except that one is brominated and the other iodinated) and jaspamide are very promising compounds, since they exhibit cytotoxicity comparable with that of Adriamycin when tested against the drug-sensitive cell lines and are several orders of magnitude more cytotoxic than Adriamycin when screened against the MDR cell lines. Jaspamide, when screened against the NCI's human tumor cell line panel, also exhibits selective cytotoxicity against renal and rectal cancer cell lines (personal communication with Dr. R Andersen). This selective activity suggests that jaspamide may have a novel mode of action. Adriamycin was selected as a standard by which the test samples could be compared because it is commonly used, in conjunction with cyclophosphamide and 5-fluorouracil, in the treatment of breast cancer.

The observation that compounds such as the geodiamolides are much less cytotoxic against MCF-7 Ad^r cells than the parent line MCF-7 whereas they were more cytotoxic against MDA-A1^r cells than the parent line MDA-MB-231 (Tables 7 and 8) suggests either that cellular mechanisms in the MDA-A1^r cells may enable the drugs to overcome the effects of Pgp overexpression or that these drugs are simply not affected by this efflux pump. The presence of Pgp in these cell lines is known since other researchers in this laboratory have demonstrated using immunohistochemical techniques that the MCF-7 Ad^r and MDA-

A1^r cell lines are Pgp-positive, whereas the MCF-7 cell line is Pgp-negative. The Pgp staining status of the MDA-MB-231 is currently being determined in this laboratory.

It is observed for the Garvea extract, the geodiamolides, PNG-137A, glaciasterol and jaspamide that the ED₅₀ values generated for either the MCF-7 or MDA-MB-231 cells were not much different than those obtained using the drug-resistant daughter lines. This suggests that either these agents, in addition to being cytotoxic, may circumvent the MDR phenotype, or they may not be affected by the presence of the Pgp pump. Glaciasterol is particularly interesting since its' molecular structure is analogous to that of steroid hormones. Steroid hormones, particularly progesterone and the antiestrogen tamoxifen, have been shown to interfere with Pgp-mediated efflux of anticancer drugs (50, 70, 108). To gain an understanding of the relationship between these 5 samples and the Pgp pump, serial dilutions of these agents were exposed to the MCF-7 Ad^r cells in the presence of Adriamycin. Adriamycin is a known substrate for the Pgp pump. The failure to observe cytotoxic synergy between the samples and Adriamycin suggests that these drugs do not interfere with Pgp function.

In retrospect, with regard to these particular experiments, it may have been a more suitable experimental design if varying concentrations of Adriamycin and the ED₅₀ concentration of our test agent were incubated with the MDR cells rather than varying concentrations of our test agent

and the ED₅₀ concentration of Adriamycin. The reason for this is that one wants to see if the test agent can modulate the activity of Adriamycin (ie: decrease ED₅₀), rather than vice-versa. However, the experiments actually performed are not invalid, since ultimately the ED₅₀ concentrations of both Adriamycin and the test agent are exposed to the same MDR cells. The *in vitro* tumor model used in these experiments resembles a Skipper-Schabel model of tumor growth much more than a Gompertzian model, since the cells are growing at a low cell density (subconfluent) and there is easy access to O₂ and nutrients. Consequently one would expect Skipper's constant-cell-kill law to prevail. If the 2 agents in the test system worked independently from one another, then exposure of the MDR cells to the ED₅₀ concentration of both agents should result in 25% of the cells surviving treatment. This observation appears to hold true for all the samples (Figures 27 - 30) tested except the geodiamolides (Figure 26). If the agents work synergistically with one another (ie: the test agent interferes with Pgp function), then one would expect >75% cell kill. Conversely, if the 2 agents interfere with one another then decreased cell kill (<75%) would be observed. The geodiamolides appear to fit into this category (Figure 26).

It should be stressed that although the MTT assay has been shown to be a reproducible and accurate assay for generating dose response curves, there was great difficulty

in accurately weighing the compounds and maintaining the appropriate drug concentrations. For some of the pure samples, only a very small amount (~ 1 mg) of drug was available for testing. To keep drug vehicle (ethanol and saline) concentrations to a minimum, the drugs had to be dissolved in a minimal volume (~ 100 μ l) of vehicle. Consequently it was difficult to maintain a constant drug concentration when working with such a small volume of volatile organic solvent, especially when repeated opening of the drug-containing vials was required.

Previous experiments in this laboratory have demonstrated that cells from the same tumor cultured in different media vary in their responses to chemotherapeutic agents (43). These previous experiments also demonstrated that medium supplemented with the patient's serum promotes the attachment and growth of HBEC. Since medium containing the patient's serum more closely simulates the *in vivo* environment than defined medium and medium supplemented with FBS, the most appropriate nutrient environment for determining the effects of therapeutic agents should include serum of the patient from whom the tumor is removed (43). A wide range of chemosensitivities for the geodiamolides is observed when tested against normal and malignant breast tissue taken from different patients, although there appears to be no significant trend indicating tumor specificity. Several notes of caution should be mentioned regarding the interpretation of these results obtained from HBEC in

primary culture. Naturally occurring breast tumors *in vivo* follow Gompertzian growth kinetics (109). This is likely due to the fact that they are very heterogenous, being composed of both drug-resistant and drug-responsive cells. All the malignant tissue samples used in these experiments were from biopsies and mastectomies. Furthermore, the tissue sample received from the hospital is likely composed of normal as well as malignant cells. Another confounding factor in the interpretation of these results is that the growth media used to culture these cells also promotes the growth of fibroblasts (44). The presence of fibroblasts in the culture probably helps mirror the *in vivo* environment since at least some epithelial stromal interactions are maintained. However, in the *in vivo* environment the malignant epithelial cells are much more mitotically active than the stromal cells, whereas in the *in vitro* condition both populations are highly mitotic. Chemotherapeutic drugs are generally more effective on mitotic cells than on quiescent cells (163) and thus the presence of mitotically active fibroblasts could influence the interpretation of an *in vitro* assay. However, previous studies in this laboratory have demonstrated that the HBEC culturing methods outlined in this thesis select for ~ 85% epithelial cells at the time of cell seeding (44). It was also observed in these experiments that fibroblast overgrowth occurs 7 - 10 days after seeding. The drug screening protocol outlined in this thesis requires cultures to grow for only 5 days. A defined

medium which inhibits the growth of stromal cells could be used instead of the patient's serum for the drug sensitivity testing, but this has previously been shown to alter the response of HBEC in primary culture to cytotoxic agents (43).

The geodiamolides, jaspamide and the five crude extracts PNG-31A, Garvea, BC-3, QCI-32 and PNG-137-A warrant further investigation, since they demonstrate significant cytotoxicity in the preliminary cell-line screens. The full potential of the crude extracts will not be realized until the pure active metabolites have been isolated. The next step in the identification of these agents as cytotoxic would be additional testing against other *in vitro* human tumor models representing a variety of tumor types, followed by *in vivo* testing of the tumor types that demonstrate activity in the *in vitro* screens. This laboratory has a mouse colony for maintaining the transplantable Shionogi mammary carcinoma (45). In conjunction with these *in vitro* and *in vivo* screens, future experiments will also include mechanism based testing to determine modes of action of these agents.

Drug resistance and host toxicity, as outlined by the Goldie-Coldman model and the Norton-Simon hypothesis, are the reasons for chemotherapy failure. As the structures of the pure metabolites illustrate, marine organisms represent a vast reservoir of new and interesting chemical families that potentially provide novel modes of action, better

therapeutic indices and fewer side effects than
chemotherapeutic agents in clinical use against currently
uncurable solid tumors.

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APPENDICES**APPENDIX 1: Growth Medium for MCF-7, T-47D and ZR-75-1 Cell Lines for Routine Passaging**

F12/DME	(1:1)
Hepes buffer	10 mM
FBS	5%
Insulin	5 µg/ml

APPENDIX 2: Growth Medium for the MDA-MB-231 Cell Line for Routine Passaging

F12/DME	(1:1)
Hepes buffer	10 mM
FBS	10%

APPENDIX 3: Growth Medium for the MCF-7 Ad^r Cell Line for Routine Passaging

F12/DME	(1:1)
Hepes buffer	10 mM
FBS	5%
Insulin	5 µg/ml
Adriamycin	0.5 µg/ml

APPENDIX 4: Growth Medium for the MDA-A1^r Cell Line for Routine Passaging

F12/DME	(1:1)
Hepes buffer	10 mM
FBS	10%
Adriamycin	1 µg/ml

APPENDIX 5: Preparation of Saline-Trypsin-Versene

NaHCO ₃	0.35 g
NaCl	8.0 g
KCl	0.4 g
Glucose	0.35 g
EDTA	0.25 g
Trypsin (Gibco)	0.5 g
Distilled water	1000 ml

The first 5 ingredients were weighed out and placed in a large Erlenmeyer flask. Nine hundred and fifty ml of

distilled water was added and the solution was stirred until the ingredients were dissolved. Following this, the trypsin was added and stirred until dissolved (_ 1h). The pH was adjusted to 7.4 by the addition of either NaOH or HCl, and then the remaining distilled water was added. Under aseptic conditions, the solution was filter sterilized using a Millipore peristaltic pump. The sterilized solution was then stored as 10 ml aliquots in 17 x 100 mm propylene plastic test tubes in a -20°C freezer.

APPENDIX 6: Preparation of Calcium and Magnesium-Free PBS

NaCl	0.8 g
KCl	0.03 g
Na ₂ HPO ₄ · 7H ₂ O	0.0138 g
KH ₂ PO ₄	0.02 g
Glucose	0.2 g
EDTA	0.038 g
Deionized	
distilled water	100 ml

All the above ingredients are mixed into the deionized distilled water until dissolved. The solution is then filter sterilized using a Millipore peristaltic pump. The final solution is stored in the refrigerator at 5°C.

APPENDIX 7: Growth Medium for the MCF-7, MCF-7 Ad^F, T-47D and ZR-75-1 Cell Lines for Drug Testing

F12/DME	(1:1)
Hepes buffer	10 mM
FBS	5%
Insulin	5 µg/ml

APPENDIX 8: Growth Medium for the MDA-MB-231 and MDA-A1^F Cell Lines for Drug Testing

F12/DME	(1:1)
Hepes buffer	10 mM
FBS	10%

APPENDIX 9: Transport Medium

F12/DME	(1:1)
Hepes buffer	10 mM
Bovine serum	5%
Insulin	5 µg/ml

APPENDIX 10: Freezing Medium

DME	50%
Bovine serum	44%
Dimethylsulfoxide	6%

APPENDIX 11: Dissociation Medium

F12/DME	(1:1), 18 ml/sample
Hepes buffer	10 mM
BSA	0.4 g/sample
Insulin	5 μ g/ml
Collagenase	300 U/ml
Hyaluronidase	100 U/ml

APPENDIX 12: Growth Medium for Human Breast Epithelial Cells (Normal and Malignant) in Primary Culture

F12/DME	(1:1)
Hepes buffer	10 mM
Insulin	5 μ g/ml
Patients' serum	5%

APPENDIX 13: Preparation of Human Serum Samples

Serum samples were collected in the mornings from patients who had fasted over the previous 8-12 h. Blood was received in nonheparinized tubes, incubated for 30 min at 37°C, centrifuged at 100 x g and the serum was collected. If not used immediately, the serum was stored at -20°C.

APPENDIX 14: Preparation of Rat Tail Collagen

The collagen solution was prepared by placing rat tails in 95% ethanol for 15 min. The tendons were dissected out, weighed and then submersed in sterile deionized water while under UV radiation in a laminar flow hood. The fibers were then submersed in a 0.01N acetic acid solution and stirred for 48 h at 4°C. Subsequently they were left in the acid solution at 4°C for 24 h with no stirring. The solution was then transferred into 50 ml centrifuge tubes and centrifuged in a Sorvall ultracentrifuge for 30 min at 10 000 x g. The supernatant consisted of the collagen solution and was bottled and stored at 4°C.