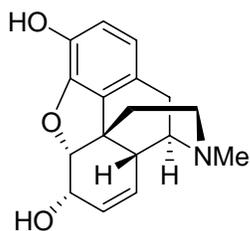


I. Natural products as medicines

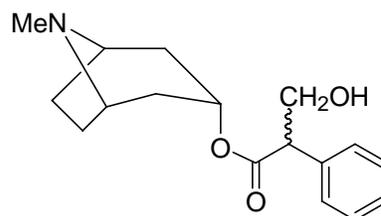
1.1 History and the earliest known medicines to man

For thousands of years natural products have played a very important role in health care and prevention of diseases. The ancient civilizations of the Chinese, Indians and North Africans provide written evidence for the use of natural sources for curing various diseases.¹ The earliest known written document is a 4000 year old Sumerian clay tablet that records remedies for various illnesses.² For instance, mandrake was prescribed for pain relief, turmeric possesses blood clotting properties, roots of the endive plant were used for treatment of gall bladder disorders, and raw garlic was prescribed for circulatory disorders. These are still being used in several countries as alternative medicines.

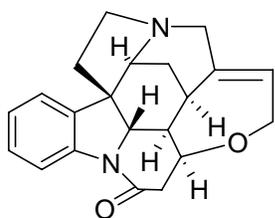
However, it was not until the nineteenth century that scientists isolated active components from various medicinal plants. Friedrich Sertürner isolated morphine (**1.1**) from *Papaver somniferum* in 1806, and since then natural products have been extensively screened for their medicinal purposes. Atropine (**1.2**) obtained from *Atropa belladonna*, strychnine (**1.3**), a CNS stimulant, ziconotide (**1.4**), identified from a cone snail, *Conus magus*, and Taxol® (**1.5**) obtained from the bark of the Pacific yew tree are a few examples of active components isolated from natural sources.



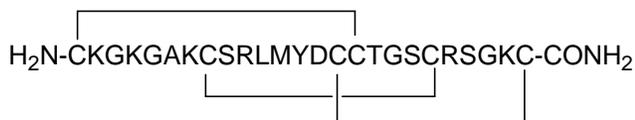
1.1 Morphine



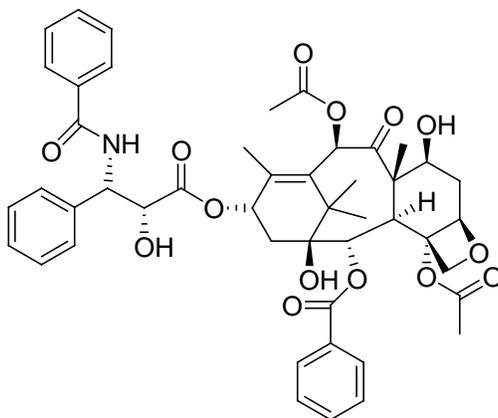
1.2 Atropine



1.3 Strychnine



1.4 Ziconotide



1.5 Paclitaxel (Taxol®)

According to recent studies conducted by the World Health Organization (WHO), about 80% of the world's population relies on traditional medicine.³ About 121 drugs prescribed in USA today come from natural sources, 90 of which come either directly or indirectly from plant sources.⁴ Forty-seven percent of the anticancer drugs in the market

come from natural products or natural product mimics.⁵ Figure 1.1 gives a graphical representation of the contribution of natural products to drug discovery.⁶

V= Vaccine

B= Biological

NP= Natural product

NPD= Natural product derivative

SNP= Synthetic derived from NP

S= Synthetic

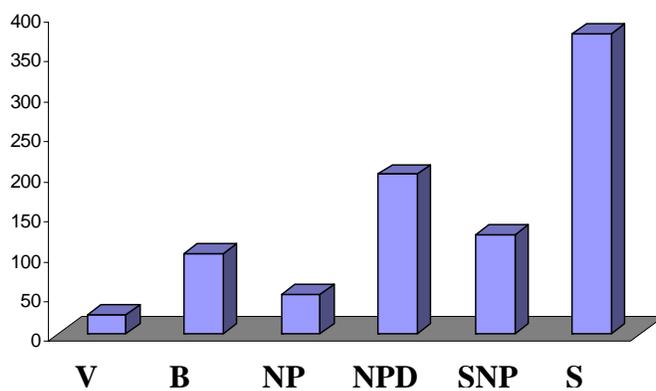
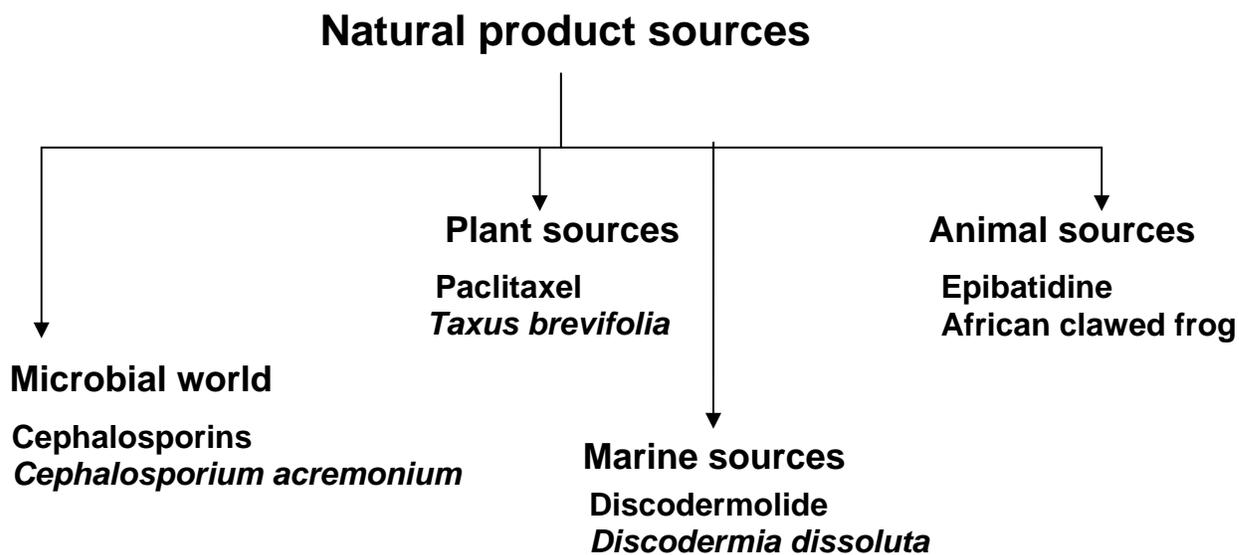


Figure 1.1 Distribution of natural products as drugs
Source: *J. Nat. Prod.* **2003**, 66, 1022–1037.

Between the years 1981-2006, about a hundred anticancer agents have been developed, of which, twenty five are natural product derivatives, eighteen are natural product mimics, eleven candidates are derived from a natural product pharmacophore, and nine are pure natural products.⁵ Thus natural sources make a very significant contribution to the health care system.

1.2 Types of Natural products

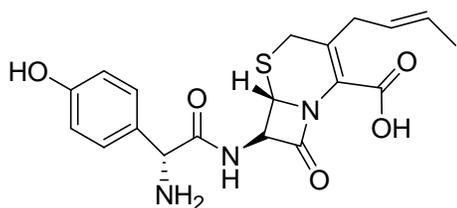
As noted above, several drug candidates are derived from various naturally occurring medicinal sources. These can be broadly divided into four categories:



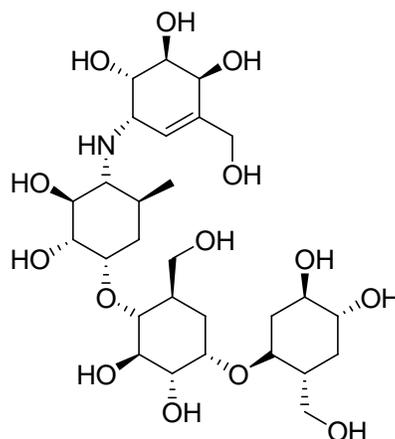
Scheme 1.1 Natural product sources

1.2.1 Natural products from microorganisms

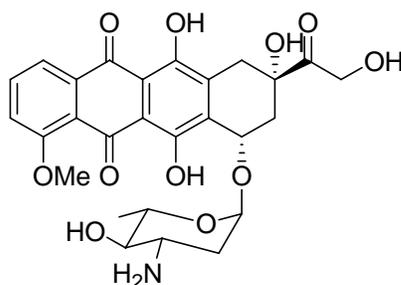
Microorganisms as a source of potential drug candidates were not explored until the discovery of penicillin in 1929. Since then, a large number of terrestrial and marine microorganisms have been screened for drug discovery. Microorganisms have a wide variety of potentially active substances and have led to the discovery of antibacterial agents like cephalosporins (1.6), antidiabetic agents like acarbose (1.7), and anticancer agents like epirubicin (1.8).⁷



1.6 Cefprozil (cephalosporin)



1.7 Acarbose

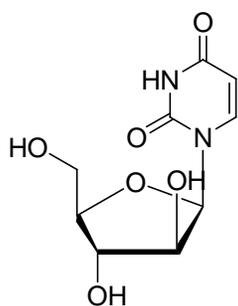


1.8 Epirubicin

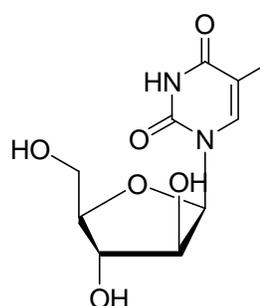
1.2.2 Natural products from marine organisms

The first active compounds to be isolated from marine species were spongouridine (1.9) and spongothymidine (1.10) from the Caribbean sponge *Cryptotheca crypta* in the 1950s. These compounds are nucleotides and show great potential as anticancer and antiviral agents. Their discovery led to an extensive research to identify novel drug candidates from marine sources. About 70% of the earth's surface is covered by the oceans, providing significant biodiversity for exploration for drug sources. Many marine organisms have a sedentary lifestyle, and thereby synthesize many complex and extremely potent chemicals as their means of defense from predators.⁸ These chemicals can serve as possible remedies for

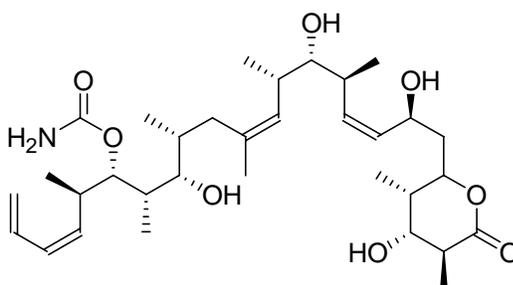
various ailments, especially cancer. One such example is discodermolide (**1.11**), isolated from the marine sponge, *Discodermia dissoluta*, which has a similar mode of action to that of paclitaxol® and possesses a strong antitumor activity. It also exhibits better water solubility as compared to paclitaxol®. A combination therapy of the two drugs has led to reduced tumor growth in certain cancers.⁹



1.9 Spongouridine



1.10 Spongothymidine

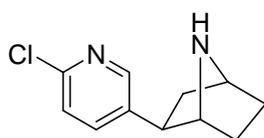


1.11 (+)- Discodermolide

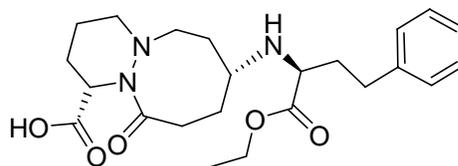
1.2.3 Natural products from animal sources

Animals have also been a source of some interesting compounds that can be used as drugs. Epibatidine (**1.12**), obtained from the skin of an Ecuadorian poison frog, is ten times more potent than morphine.¹⁰ Venoms and toxins from animals have played a significant role

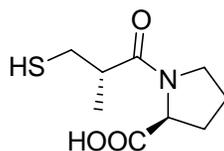
in designing a multitude of cures for several diseases. Teprotide (pry-trp-pro-arg-glu-ile-pro-pro), for example, extracted from a Brazilian viper, has led to the development of cilazapril (1.13) and captopril, which are effective against hypertension (1.14).¹¹



1.12 Epibatidine



1.13 Cilazapril



1.14 Captopril

1.2.4 Natural products from plant sources

The use of plants as medicines has a long history in the treatment of various diseases. The earliest known records for the use of plants as drugs are from Mesopotamia in 2600 B.C., and these still are a significant part of traditional medicine and herbal remedies.¹² To date, 35,000-70,000 plant species have been screened for their medicinal use.¹³ Their

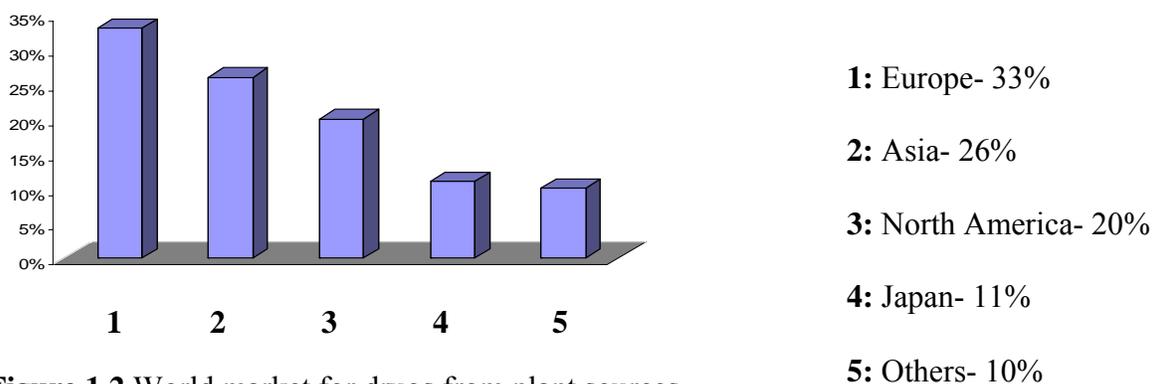


Figure 1.2 World market for drugs from plant sources

Source: *Environmental Health Perspectives* 1999, 107, 783–789

contribution to the world market for herbal remedies is as shown in Figure 1.2.¹⁴

Several important drugs such as Taxol®, camptothecin, morphine and quinine have been isolated from plant sources. The first two are widely used as anticancer drugs, while the remaining are analgesic and antimalarial agents, respectively.

1.3 Plant based anticancer drugs

Cancer is the second leading cause of death among children between the ages of one and fourteen and it is also responsible for 25% of all deaths today.¹⁵ There were 10.9 million new cancer cases diagnosed in USA and 6.7 million deaths in 2002.¹⁶ Seventy-seven percent of all the cancers diagnosed are observed in people aged 55 years or older.¹⁷ These figures indicate that the death toll from cancer is going to rise with the aging of US population.

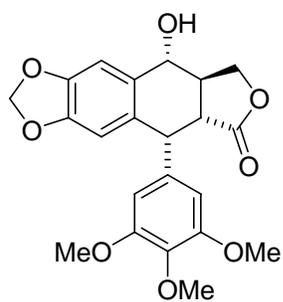
In spite of the availability of a large number of anticancer drugs and various chemotherapy options, there is still an acute need for less toxic and more potent cancer drugs and continues be the concern. Most of the drugs available are not selective to cancer cells and affect the normal cells as well leading to severe side effects. However, these drugs are currently the most effective means to combat cancer. The aim of research in cancer drug development is to find new drugs that are specific to cancer cells, or to develop a method that alters the nature of the drug administered such that it acts only on the target cells and not the regular normal functioning cells, thereby reducing the side effects.



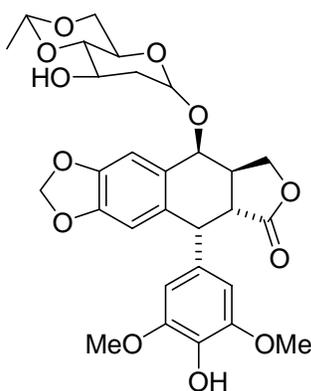
Figure 1.3 *Podophyllum peltatum*
Source: University of Georgia Herbarium

Several anticancer agents available in the market today derive their origin from natural sources. One of the early compounds isolated as an anticancer agent was podophyllotoxin (**1.15**), a compound obtained from *Podophyllum peltatum* (Fig. **1.3**),¹⁸ in 1944.¹⁹ It was initially used therapeutically as a purgative and in the treatment of venereal warts.²⁰ Later, in 1974, it

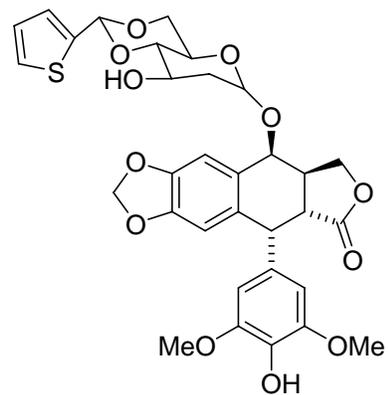
was shown that it acts as an anticancer agent by binding irreversibly to tubulin.²¹ Etoposide (**1.16**) and teniposide (**1.17**), the modified analogs of podophyllotoxin, however, cause cell death by inhibition of topoisomerase II, thus preventing the cleavage of the enzyme- DNA complex and arresting the cell growth.²² Both these analogs are used in the treatment of various cancers.²³



1.15 Podophyllotoxin



1.16 Etoposide



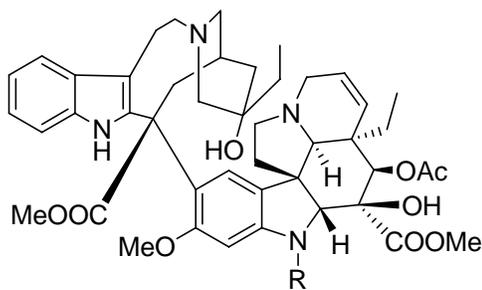
1.17 Teniposide



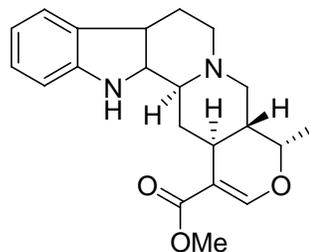
Figure 1.4 *Catharanthus roseus*
Source: New York Botanical Garden

The Madagascar periwinkle, *Catharanthus roseus* (Fig. 1.4),²⁴ a member of the Apocynaceae family, is important because of its diverse medicinal properties. It is a rich source of indole alkaloids which include the anticancer alkaloids vincristine (**1.18**) and vinblastine (**1.19**), and also the antihypertensive alkaloid, ajmalicine (**1.20**). For centuries, this plant was used as remedy for diabetes, as it was believed to enhance the production of insulin by the body. Both

vinblastine and vincristine are now known to prevent cell division by inhibiting mitosis in the cell cycle. They irreversibly bind to tubulin, thereby blocking cell multiplication and eventually causing cell death.²⁵



1.18 Vincristine R= Me
1.19 Vinblastine R= CHO



1.20 Ajmalicine

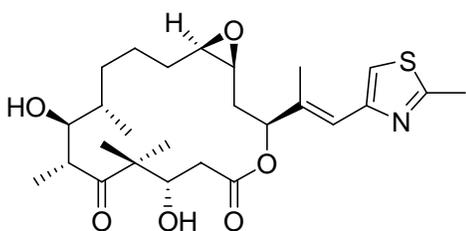


Figure 1.5 *Taxus brevifolia*
Photo: © Dave Ingram

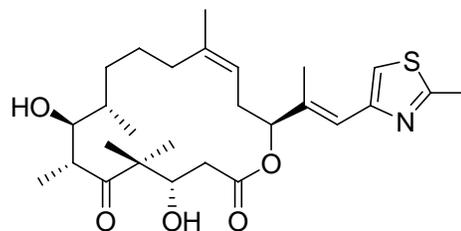
An extract of the Pacific yew tree, *Taxus brevifolia* (Fig. 1.5),²⁶ was discovered to possess excellent anticancer properties in 1963, and its active component was isolated only a few years later in 1967 by Monroe Wall and his co-worker, Mansukh Wani.²⁷ They published their findings as well as the structure of the active component, paclitaxel (Taxol®), in 1971 (1.4).²⁸ Susan B.

Horwitz, a molecular pharmacologist, established the novel mechanism of action of paclitaxel in 1979. Paclitaxel irreversibly binds to β -tubulin, thus promoting microtubule stabilization.²⁹ This tubulin- microtubule equilibrium is essential for cell multiplication, and its stabilization causes programmed cell death.³⁰ Previously reported anticancer drugs, vinblastine, vincristine and podophyllotoxin also bind to tubulin, but prevent rather than promote microtubule formation. Paclitaxel was the first compound to be discovered to promote microtubule formation. It has been used in the treatment of several types of cancer, but most commonly for ovarian and breast cancers as well as non-small cell lung tumors.³¹ It had sales of \$750 million in 2002 and \$1.0 billion in 2003.³² Shortly after the discovery of paclitaxel and its unique mechanism, several compounds having the same mode of action were discovered. The epothilones, discovered from the myxobacterium *Sorangium cellulosum*, possess potential anticancer properties (1.21, 1.22) and show high in vivo activity, including activity against taxane-resistant cell lines. However, they exhibit moderate

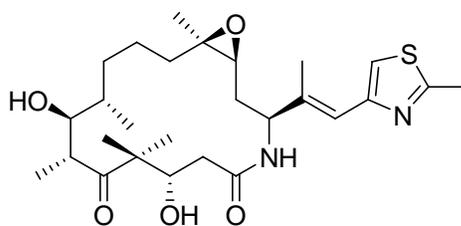
in vitro cytotoxicity.³³ Several semisynthetic analogs of epothilones such as ixabepilone (**1.23**) have been developed which are currently in Phase II clinical trials for treatment of breast cancer.⁷



1.21 Epothilone A

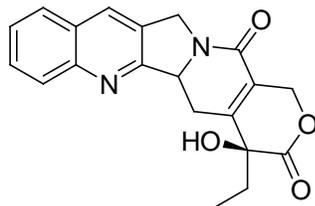


1.22 Epothilone D

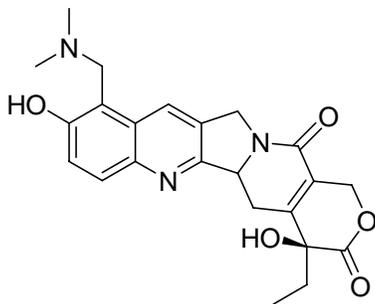


1.23 Ixabepilone

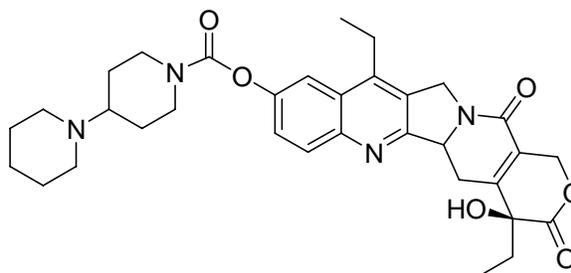
Camptothecin (**1.24**), discovered from the deciduous tree *Camptotheca acuminata*, is also an anticancer agent which has a unique mechanism of action. Camptothecin and its derivatives are topoisomerase-I inhibitors, and cause cell death by DNA damage.³⁴ However, camptothecin itself is too insoluble to be used as a drug but its several water-soluble analogs, namely, topotecan (**1.25**) and irinotecan (**1.26**) have been developed as effective drugs.³²



1.24 Camptothecin



1.25 Topotecan



1.26 Irinotecan

1.4 International Cooperative Biodiversity Group (ICBG)

As the awareness and the importance of natural resources as a source of medicines is increasing, the biodiversity of the planet is disappearing rapidly. Many plant extracts that are needed to be investigated for the isolation of promising drug candidates are obtained from the tropical rainforests of developing countries. In addition, many people in these countries mainly depend on plants as their source of medicine. The continuous loss of tropical rainforests causes potentially important plant species to be lost forever without being explored. It also deprives people of these countries of the sources of their natural medicines.

The ICBG program was initiated in 1992 by the joint efforts of the National Institutes of Health (NIH), the National Science Foundation (NSF) and the U.S. Agency for International Development (USAID). This program is focused on three main aspects: drug

discovery, biodiversity conservation, and economic development of underdeveloped countries. The ultimate aim is the discovery of natural products which would eventually benefit both developed as well as developing countries. The Kingston group was awarded an ICBG grant in 1993 for work in Suriname and the program is currently based in Madagascar. The main focus of the work at Virginia Polytechnic Institute and State University is the isolation of anticancer agents from plant sources.

About 90% of the land in Suriname is covered by tropical rainforests and is estimated to contain 5000 different species of plant.³⁵ It was thus selected initially for drug discovery and conservation work. The Zahamena forest in Madagascar was the second center for the ICBG project, during the period 1998-2003, until a major part of the project was shifted to northern Madagascar in 2003. The Madagascar ICBG program has six collaborating groups. The Missouri Botanical Garden is responsible for plant collection and Centre National d'Application et des Reserches Pharmaceutiques (CNARP) prepares extracts of collected plants and also collaborates in other ways. VPI&SU, Eisai Research Institute and Dow Agrosiences are involved in the isolation and characterization of natural products isolated from the plant extracts obtained through this project. The Centre National de Reserches Sur l'Environnement is responsible for collection of marine samples and their identification.

The plant samples that are collected from the rainforests of Madagascar are dried, ground and extracted with ethanol at CNARP. The extracts are then evaporated and placed in voucher vials. The dried extracts are shipped to VPI&SU for bioassay, isolation and characterization of anticancer compounds.³⁶

1.5 Bioassays

Bioassays are crucial for the successful isolation of active compounds from various natural sources. The usual method for isolation of active components is the bioassay guided fractionation. Several bioassays are available to evaluate different types of bioactivities of different types of compounds. The assays can be chosen based on the nature and the type of activity that is desired to isolate. An ideal bioassay would be highly sensitive to small amounts of active material, selective to the specific bioactivity, cost effective and easy to run and maintain.³⁷

In general, bioassays are broadly classified into two categories; mechanism-based assays and cell-based assays.

1.5.1 Mechanism-based assays

Mechanism-based assays involve measurement of the specific activity of the drug towards a specific enzyme, DNA, receptor etc. Targeting these isolated systems involved in various metabolic pathways is an effective method for drug discovery. However, these assays are conducted in an artificial environment which is very different from the physiological environment. Hence they must be properly configured for accuracy and effectiveness. A properly designed assay is robust and provides the ability to accurately determine the activity of the compound at very low concentrations.³⁸

Though mechanism-based assays are highly sensitive and useful in determining the specific activity of the compound or extract, these assays have several disadvantages. They only approximate the *in vivo* environment, and it is likely that certain pathways or

mechanisms are missing and the system is incomplete. Also, certain compounds could be effective agents, but could act by a different pathway, inhibiting the activity of a different enzyme or receptor. This would lead to a ‘miss’ in the drug discovery process as the drug would not show any activity in an assay that is not associated with its mechanism. Also, compounds active in the mechanism-based assay may be inactive *in vivo* due to their incapability of permeating through the cell membrane.

1.5.2 Cell-based assays

Cell-based assays involve drug-cell interactions with the whole intact cell rather than just isolated systems. Though it is usually not possible to determine the exact mode of action of the compound with these assays, a wide range of active compounds can be detected. This method also proves useful in screening out compounds that cannot pass through the cell membrane. In spite of a few disadvantages like high maintenance and being tedious to perform, these assays have many advantages in screening a broad class of compounds and a much higher ‘hit rate’ than mechanism-based assays.

1.6 A2780 Bioassay

The cell line for assay used in the Kingston group is the A2780 human ovarian cancer cell line. The assay performed using this cell line is called an *in vitro* antiproliferative assay, to distinguish it from an *in vivo* assay in animals. Although it is not possible to determine the mode of action of the active compound, a wide range of compounds functioning with different mechanisms can be detected. The assay is usually carried out in a 96 well microtiter plate (Fig. 1.26). The cells, suspended in RPMI 1640 media with L-glutamine (Gibco) and

10% Fetal Bovine Serum, are transferred to each well of columns 1 to 11 at a density of 2.7×10^5 cells/mL. Column 12 is the positive control which contains only the media without any cells in it.

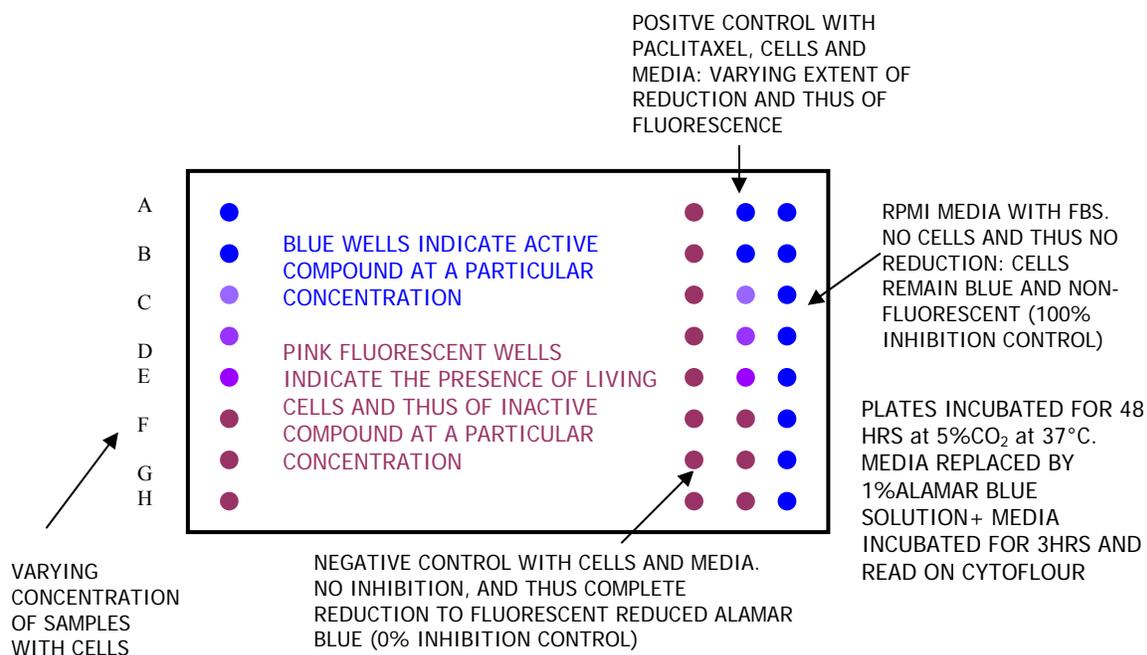


Figure 1.6 96 well microtiter plate for A2780 bioassay

The plates are then incubated at 37°C and 5% CO₂ to allow the cells to adhere to the bottom of each well. The samples are dissolved in DMSO to get a concentration of 50 µg/mL and 20 µL of this solution is transferred to the first and the fifth row of each column from 1 to 10 of the microtiter plate. Three dilutions are carried out so that the final concentration of the compound in each well is 20, 4, 0.8, and 0.16 µg/mL. The eleventh column has a series of four dilutions of paclitaxel, which is used as the positive control. The last four wells of this column, E-H, are used as the negative control, and contain only the cells and the media,

Various metabolic pathways that take place within the cell involve oxidation-reduction reactions. The redox potential of Alamar Blue is $E_0 = +380$ mV. The redox potential of various cellular components such as cytochromes, FADH, NADPH, etc. involved in cellular respiration is lower than that of Alamar Blue. Thus Alamar BlueTM can be used to determine cell viability and cell proliferation, as it can be reduced by the metabolic processes taking place within the living cell.³⁹ The percentage reduction of the dye is related to the percentage of growing cells and in turn to the percentage inhibition caused by the drug.

1.7 Methods for Structure determination

Natural product chemists mainly use mass spectrometry (MS) and nuclear magnetic resonance spectroscopy (NMR) for structure elucidation of the compounds isolated from various natural sources. A few other analytical methods, for instance, infrared spectroscopy, UV-Vis spectroscopy, and X-ray crystallography, are used to provide supplementary information to confirm the proposed chemical structure for the compound. Several compounds are not UV active, while others like glycosides are hard to crystallize to give good quality crystals for X-ray analysis. MS and NMR methods, however, are usually sufficient to elucidate the structure of the compound.

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