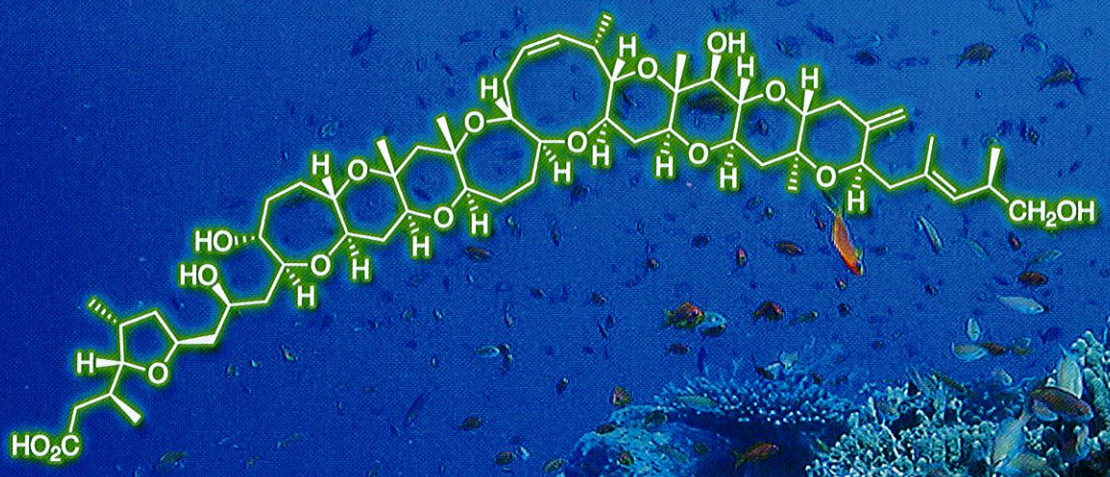
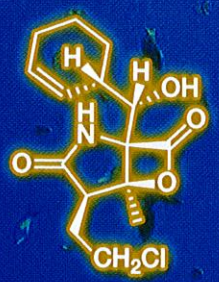
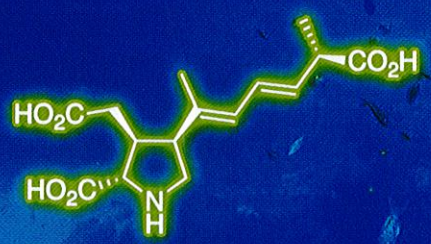
Outstanding Marine Molecules

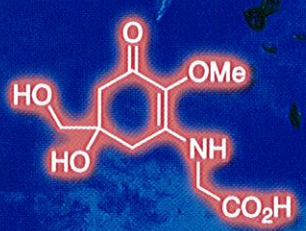
Chemistry, Biology, Analysis

Edited by Stéphane La Barre and Jean-Michel Kornprobst









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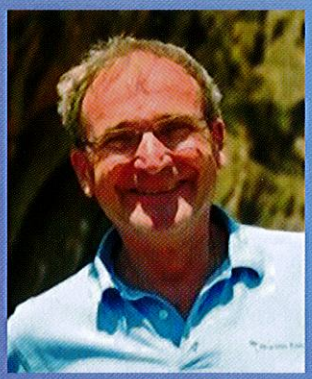
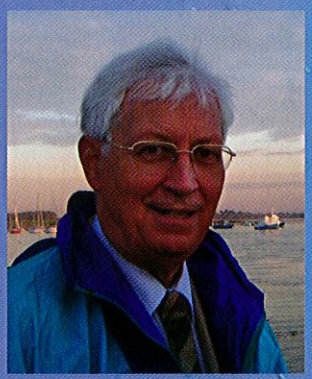
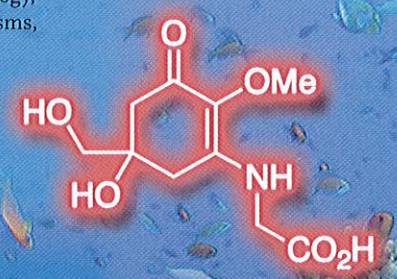
Using a number of outstanding examples, this text introduces readers to the immense variety of marine natural compounds, the methodologies to characterize them, and the approaches to explore their industrial potential. Care is also taken to discuss the function and ecological context of the compounds.
Meticulously produced and easy to read, this book serves students and professionals wishing to familiarize themselves with the field, and is ideally suited as a course book for both industry and academia.



Stéphane La Barre is a senior research scientist at the Centre National de la Recherche Scientifique in France. He gained his MSc from Auckland University, New Zealand, and his PhD from James Cook University, Townsville, Australia, before joining CNRS in 1984. His multi-disciplinary career includes marine chemical ecology, natural products chemistry of terrestrial and marine organisms, and polymer chemistry. Dr. La Barre is currently the coordinator of the research cluster BioChiMar (Marine Biodiversity and Chemodiversity), and is investigating novel analytical tools to evaluate and predict environmental change affecting coral reef diversity, both biological and chemical.



Emeritus professor at the University of Nantes, France, since 2003, **Jean-Michel Kornprobst** has a chemical engineering degree from Montpellier University and a PhD from the University of Lyon. After being assistant professor at the University of Paris 7 from 1970 to 1973, he became professor of organic chemistry at the University of Dakar, Senegal, where he worked on marine natural products before joining the University of Nantes in 1990. Professor Kornprobst has over 100 publications and three books to his name, and was responsible for two research programs on manapros in Doha, Qatar, and Jeddah, Saudi Arabia. He has recently been an invited professor at the universities of Louvain-la-Neuve, Belgium, Campinas, Brazil, and Blida, Algeria, and is currently an external member on the scientific advisory board of the Marine Biotechnology Research Center in Québec, Canada.



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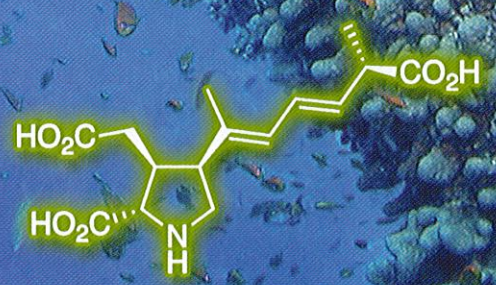

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23
High-Throughput Screening of Marine Resources

Arnaud Hochard, Luc Reininger, Sandrine Ruchaud, and Stéphane Bach

Abstract

The marine environment represents a vast resource to discover bioactive molecules with novel modes of action. The stagnation of the pharmaceutical market in terms of newly approved drugs, along with increased research and development costs, have created an urgent need to accelerate the discovery of new natural chemical scaffolds, and high-throughput screening (HTS) can be used for this purpose. The HTS process involves testing compounds in high numbers, to determine if they can modulate a given molecular pathway. Various HTS assays have been developed, depending on the therapeutic targets. In this chapter, an overview is provided of HTS procedures, and examples given that highlight the potential of marine products as pharmaceuticals, such as inhibitors of protein kinases.

23.1 Introduction

The oceans cover 70% of planet Earth, and contain the greatest diversity of living organisms. Considering the critical importance of natural products derived from living organisms in the discovery and development of bioactive agents of medical interest, the marine environment represents a vast resource to discover novel chemical structures with novel modes of action. For instance, 47% of all drugs used in anticancer treatments are natural products and/or derivatives (Skropeta, Pastro, and Zivanovic, 2011). During recent years, a growing number of new chemical entities with potent pharmacological properties obtained from marine organisms have been described (Vinothkumar and Parameswaran, 2013; Hu *et al.*, 2011; Molinski *et al.*, 2009) (Figure 23.1). Of particular note here are the bryostatin-like compounds, which are currently under investigation as anticancer therapies (see Section 23.3.4). At this point it is worth mentioning the growing interest in the development of marine natural products and chemical derivatives with applications in the agrochemical and cosmetics industries, all of which show great potential for an economic impact.

The recent stagnation of the pharmaceutical market in terms of newly approved drugs, along with increases in research and development costs –the so-called "big pharma crisis" – has created an urgent need to accelerate the discovery of new natural chemical scaffolds (Esteban, Lien, and Youn, 2008) (Figure 23.2). However, in order to be effective, it is important that the exploration of marine natural products is developed in parallel to high-rate analyses of bioactivity.

A critical step in the drug discovery process is to determine either a ***therapeutic target***, which can be defined as a cellular component (protein, DNA) or a *biological pathway* that is clearly involved in a specific pathology. Importantly, the therapeutic target must be "**druggable**" and compatible with the treatment of patients (e.g., small chemical compounds, antibodies) in order to inhibit/activate its cellular function. According to Hughes *et al.*, a "druggable" target is accessible to putative drug molecules and elicits a biological response which may be measured both *in vitro* and *in vivo* (Hughes *et al.*, 2011).

High-throughput screening (HTS) involves the testing of compounds in high numbers in order to determine if they can modulate a given molecular pathway. HTS relies on two major classes of assay:

- ***In-vitro* biochemical assays** which are developed to select compounds with activities on purified molecular targets (e.g., disease-related enzymes as protein kinases, ligand–receptor interactions).
- ***Ex-vivo* cell-based assays**, which are developed in mammalian cells, yeast or bacteria that have been engineered to overexpress the target protein (G-protein coupled receptors, ion-channels and others membrane receptors) or to express a reporter gene (for a review, see Michelini *et al.*, 2010).

In comparison with HTS, **high-content screening (HCS)** is based on the analysis of specific cellular phenotypes through automated image capture and analysis by heavy computing systems (Bickle, 2010; Brodin and Christophe, 2011). In this chapter, attention will be focused on HTS as applied to the identification and selection of marine natural products and derivatives with biological activities of medical interest.

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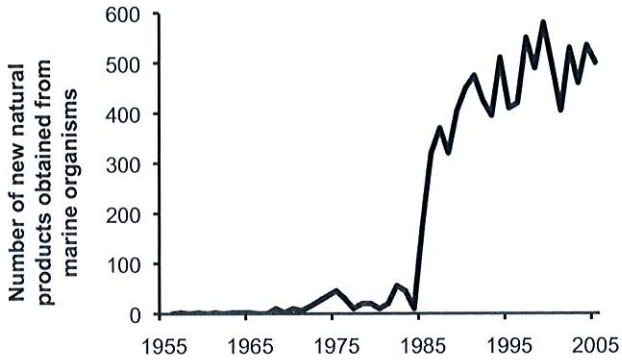

Figure 23.1 Temporal evolution in the number of novel chemical entities characterized from marine organisms. Adapted from Hu *et al.*, 2011.

23.2 High-Throughput Screening and Drug Development

HTS is based on the development of automated and reproducible assays that are sufficiently robust to be miniaturized and fit different microtiter plate formats (96- to 3456-well plates). Typically, the HTS approach is fully integrated into a drug development strategy (Figure 23.3), its central role being to permit the pharmaceutical companies to save both the time and money spent on basic research. The main purpose of the HTS assay is to select optimal chemical structures that will subsequently be analyzed during preclinical and clinical studies.

23.2.1 Screening Assay Development and Validation

As noted above (and also shown in Figure 23.3), the identification of a therapeutic target is an essential step prior to any screening development. Several targets can be identified and validated for a single disease (Westby *et al.*, 2005), and tools available to identify therapeutic targets include mRNA/protein levels analyses, genetic associations, bioinformatics (data mining) (Hughes *et al.*, 2011), or RNA interference (RNAi)

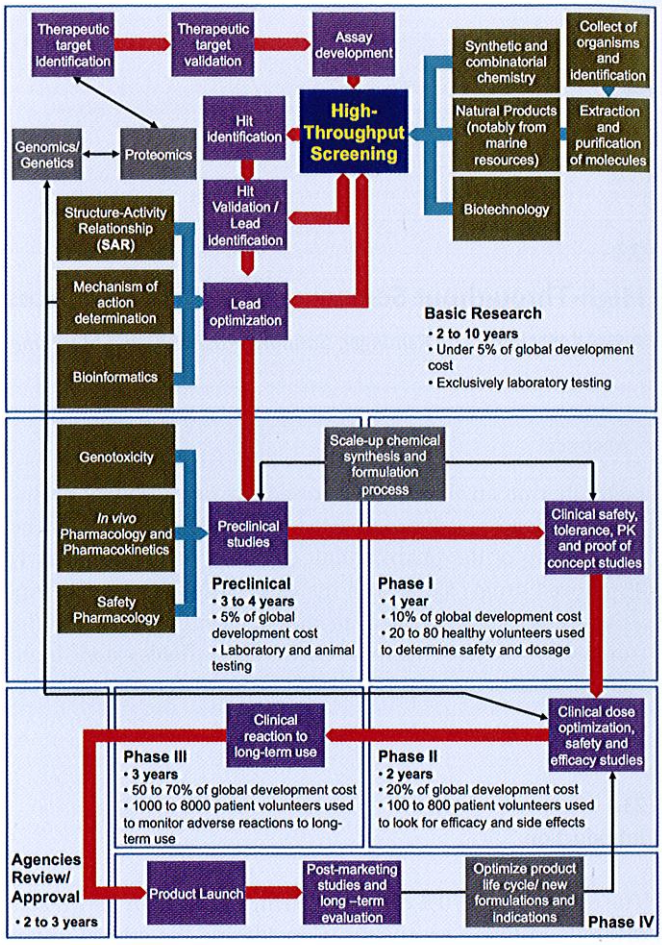

Figure 23.3 Drug development: From bench to the market, a long adventure. Drug development is used to define the process of bringing a new drug to the market. Screening is one of the major initial steps.

technology (Tyner *et al.*, 2009). The next step is to determine whether the molecular pathway needs to be inhibited or activated in order to produce significant beneficial effects. Strategies for *in-vivo* and *ex-vivo* drug target validation include notably

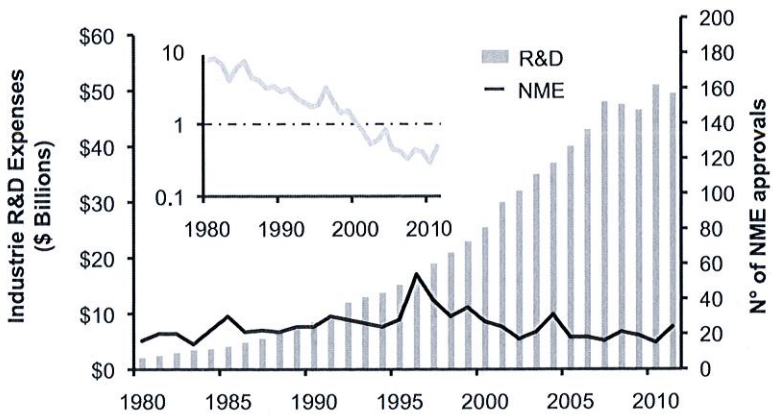

Figure 23.2 The "R&D productivity crisis" in the pharmaceutical industry (Esteban, Lien, and Youn, 2008). The curve in light gray (top left) represents the ratio of NME approved by FDA to R&D expenses per year. NME, New Molecular Entities; FDA, US Food and Drug Administration.

gene knockout/knockin, dominant negative mutants, or RNAi (Hughes *et al.*, 2011).

Biochemical assays are well adapted to assess any effects on enzymatic reactions or binding interactions, whereas cell-based assays are preferred for molecular pathways, multiple targets or cell membrane compartment analyses. The currently available methods are:

- ***Colorimetric*** (Lavery, Brown, and Pope, 2001), such as enzyme-linked immunosorbent assay (ELISA; Hochard *et al.*, 2013).
- ***Fluorescent***, such as fluorescence resonance energy transfer, (FRET; Mere *et al.*, 1999; Zhu, Fu, and Luo, 2012), dissociation-enhanced lanthanide fluorescent immunoassay (DELFIA; Newbatt *et al.*, 2013), fluorescence polarization (Owicki, 2000), time resolved-FRET (TR-FRET), homogeneous time-resolved fluorescence (HTRF; Degorce *et al.*, 2009; von Ahsen *et al.*, 2006), fluorogenic substrates (Mitnaul *et al.*, 2007), calcium flux (Luo *et al.*, 2011), and membrane translocation (Vijayakumar, Ajay, and Bhat, 2010).
- ***Luminescent***, such as bioluminescence resonance energy transfer (BRET; Boute, Jockers, and Issad, 2002), bioluminescent substrates (Yasgar *et al.*, 2010), or luciferase reporter gene (Siebring-van Olst *et al.*, 2013).
- ***Based on the use of radioactive elements***, such as scintillation proximity assay (SPA; Khawaja, Dunlop, and Kowal, 2008).

Screening assays are considered to be **heterogeneous** (e.g., ELISA) or **homogeneous** (e.g., HTRF). Heterogeneous assays (multistep) require stages that go beyond simple reagent additions, incubations, and readings (e.g., filtration, centrifugation, and plate-washing steps), whereas homogeneous assays require only additions and incubations, followed by reading. Multistep assays prevent the tested compounds from interfering with the readout, and usually produce a higher signal-to-noise ratio (SNR), whereas homogeneous assays are more amenable to high-throughput analysis. Sensitivity, cost, automation, speed, robustness, reliability are important criteria to be taken into consideration. It should also be noted that the downscaling of assays, thus reducing the volumes of materials used, can lead to significant reductions in the total cost of HTS screens (Table 23.1).

23.2.2 Statistical Tools for Quality Assessment of HTS Assays

Generally, screening evaluation is based on the percentage of inhibition/activation obtained by the tested molecule on a specific target. Experimental data analysis requires their normalization in order to remove systematic plate-to-plate variation, and to standardize measurements across plates. Quality indicators such as reproducibility and accuracy are essential to assess the suitability of the assay for HTS. Many factors can affect the reproducibility of microtiter plate assays, such as "edge-effect" in cell-based assays. One widely used method of determining the suitability of an assay, prior to launching a full-scale HTS, is to calculate the Z′-factor coefficient, a statistical parameter which is defined as follows (as originally described in Zhang, Chung, and Oldenburg, 1999):

$$\text{Z′-factor} = 1 - \frac{3(\sigma_p + \sigma_n)}{|\mu_p - \mu_n|}$$

where μ and σ are the mean and standard deviation of the positive (p) and negative (n) sets of data obtained with dedicated controls, respectively. An acceptable Z′-factor should be greater than 0.5, as described in Table 23.2. In the context of an enzyme inhibition-based assay, the positive control is defined as the signal obtained when the enzyme is incubated with its substrate and a known inhibitor that exerts 100% of the desired effect or in the absence of the enzyme (i.e. background), whereas the negative control is defined as the enzyme incubated with its substrate in the absence of any interfering compound. As most chemical compounds are dissolved in dimethyl sulfoxide (DMSO), controls should contain the same amount of DMSO, and its potential effect evaluated. The use of more than 1% DMSO (final concentration) is not recommended for cell-based assays. As the Z′-factor is totally dependent on the HTS-assay conditions, a new Z′-factor must be determined whenever changes are implemented in the assay (including reagent variability). Statistical tools are available to achieve quality assessment, including percentage of control, median absolute deviation (MAD), SNR and signal window (SW) (for reviews, see Malo *et al.*, 2006; Goktug, Chai, and Chen, 2013).

Table 23.2 Interpretation of the Z′-factor value, a crucial step in the preparatory phase of a HTS campaign (Zhang, Chung, and Oldenburg, 1999).

Z′-factor	Interpretation
1.0	Ideal. Z′-factors can never exceed 1.
$0.5 > Z' < 1.0$	An excellent assay.
$0 > Z' < 0.5$	A marginal assay.
Under 0	There is too much overlap between the positive and negative controls for the assay to be useful.

Table 23.1 Impact of miniaturization on the cost of HTS assays. A library containing 200 000 compounds is evaluated in this example.

Plate Format	No. of tested compounds per plate	No. of plates	Average volume of target added/well (µl)	Assay volume (µl)	Total volume of target added (ml)	Cost (US$)
96	88	2273	10	100	2182	436 400
384	352	568	2	20	436	87 200
1536	1408	142	0,4	4	87	17 400
3456	3168	36	0,08	0,8	17,4	3480

23.2.3 Choice of Screening Strategy

In a typical HTS assay, a ***primary screen*** is performed at a single dose for all compounds, after which the results obtained are validated in a second round of screening. Other strategies that can be used include ***molecular pool*** and ***replicate screening***.

The molecular pool involves testing a pool of compounds in one reaction, the main advantage of the technique being to increase the screening output. However, sorting steps are required for every positive reaction in order to extract and identify the interesting molecule. This strategy is somewhat reminiscent of natural extract screening, where several rounds of fractionation/screening are necessary before approaching the positive compound.

A replicate screening strategy (where the number of independent experiments is more than three) allows not only the direct elimination of false-positives but also the probability of reproducing experimental error being reduced in line with the number of replicates (see Section 23.2.4.1 for definition of false-positive). Inevitably, the chosen strategy will have direct effects on the screening cost and speed, and should be carefully evaluated.

Whichever strategy is chosen, it is necessary to determine an activity threshold to identify "hits." Such a threshold can be set using a variety of methods, including percentage inhibition cut-off or Bayesian methods (Goktug, Chai, and Chen, 2013).

23.2.4 Data Analysis: From Hits to Leads

23.2.4.1 Hits

Those products tested during screening and having a positive action on the therapeutic target are called "**Hits**" (Figure 23.3). Valuable hits are potent and specific; they have a strong effect on the target of interest, and minor off-target effects. However, even if the screening is based on a reliable technique and all quality controls validate the assay, the possibility of having identified a **false-positive or false-negative compound** remains. False-positives can be identified by a preliminary analysis of the compounds to be screened; for example, it is well known that some dyes can interfere with activity readout, or that alkylating agents can alter the target irreversibly and have no specific inhibitory activity. Reproducible hits without activity on the therapeutic target can be safely regarded as false-positives and eliminated. When required (e.g., targeted pathology), the cell mortality can be determined in parallel using a cell-based assay to discard cytotoxic compounds.

Finally, any remaining false-positive compounds can be identified in subsequent screening rounds by performing a dose–effect assay, using serial dilutions of the compound. In the case of a false-positive, no effect will be observed whereas, for a "confirmed" hit the **IC_{50}-value** (the concentration needed for a 50% inhibition of the maximum biological; Figure 23.4) or the **EC_{50}-value** (the compound concentration where 50% of its maximal effect is observed, as is commonly used for agonist/stimulator assays) can each be determined. It is important to indicate here that various factors may influence IC_{50}-values. For example, there is a recognized interdependency between the IC_{50}-value and adenosine triphosphate (ATP) concentration for ATP-dependent enzymes such as kinases. The IC_{50}-values can be used to compare the efficiencies of the various antagonists, and the most potent hits will be identified and selected. It should be noted that it is important to select molecules with chemical structures that are representative of each family identified, in order to maintain the ***highest degree of chemical diversity*** during the screening process.

To continue on the subject of hit validation, selected structures can be analyzed by using various other bioactivity assays to evaluate the relative ***selectivity*** of the molecules to their primary targets. In the case of protein kinase inhibitors (PKIs), large panels of kinases (>89% of the whole kinome) can be tested to

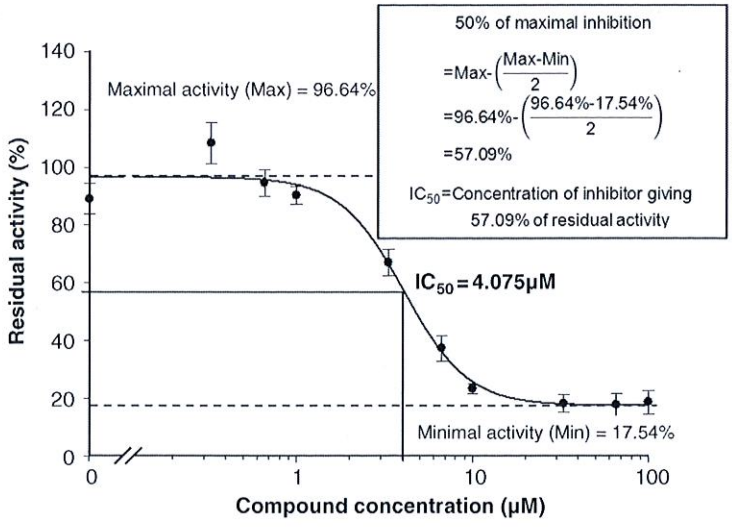

Figure 23.4 Determination of a relative IC_{50}-value on a dose–response curve.

obtain a comprehensive view of the molecule's selectivity (KINOMEscan® program; DISCOVERx, Fremont, USA).

A chemical scaffold can be analyzed according to the ***Lipinski's Rules*** **or the** ***Rule of 5*** **(RO5**, where all numbers are multiples of five) before further investigating its mechanism of action. These rules are used to evaluate whether a chemical compound has properties that would cause it to be an orally active drug in humans. These rules were formulated by Christopher A. Lipinski in 1997 (Lipinski *et al.*, 1997), and are based on the observation that most drugs administered as medication have relatively small and lipophilic molecules. In fact, these characteristics are associated with 90% of all orally active drugs that have achieved Phase II clinical status. The rules state that, in general, an orally active drug has no more than one violation of the following criteria: (i) molecular weight <500 Da; (ii) less than five hydrogen bond donor groups (i.e., nitrogen or oxygen atoms with one or more hydrogen atoms); (iii) less than 10 hydrogen bond acceptor groups (i.e., nitrogen or oxygen atoms); and (iv) the log of the octanol–water partition coefficient (P) is less than 5.

These rules are not always applicable to every drug, however. Indeed, the orally administered Navitoclax®, which is produced by Abbott/Genentech and currently in Phase II studies, violates three of Lipinski's rules for oral-drug-likeness; the molecular mass is 975 Da, the log $P = 12$, and it has 11 hydrogen-bond acceptors) (Mullard, 2012). Therefore, it is clearly very important to keep an open mind when screening complex and highly diverse marine natural chemical scaffolds (Colas, 2008).

The next step in hit validation involves the evaluation of molecular properties such as solubility and stability, and the use of a panel of *in-vitro* assays to assess intestinal and hematoencephalic permeabilities, metabolic stability, activity against cytochrome P450 (CytP450) enzymes, or cell permeability (Hughes *et al.*, 2011).

23.2.4.2 Leads

Following the screening campaign, and the evaluation of selectivity and pharmacokinetics properties of the selected hits, the more potent molecules will achieve **"lead"** status (Figure 23.3). Medicinal chemistry is then applied in order to obtain derivative structures that exhibit optimized bioactivities; thus, the lead structure serves as a starting point for improvements by chemical modification. At this point, dose–response curves are generated using primary screen assays for each pharmacomodulation to determine activity through IC_{50} or EC_{50} evaluation to achieve the structure–activity relationship (SAR; this is the relationship between the chemical or three-dimensional structure of a molecule and its bioactivity). Additional screens can be performed to monitor selectivity and pharmacokinetics properties. Overall, the ***drug candidate*** will be a compromise between all of these parameters, as it will modulate the target efficiently and specifically, have a good metabolic stability and bioavailability, and be soluble and slightly lipophilic (Hefti, 2008).

At this stage, further experiments might help to characterize the lead compounds, and especially their mechanism(s) of action. Different affinity-based analyses (Guiffant *et al.*, 2007) or other "omics"-based methods can be used to carefully and comprehensively determine the cellular targets of the new chemical scaffold. Unexpected cellular targets may explain potential side effects that could appear during preclinical or clinical phases.

23.2.5 From HTS Assay to Market: The Drug Development Process

After selection, drug candidates are submitted for clinical trials (Figure 23.3). The first step of these trials is the ***pre-clinical*** **stage**, which involves studies with animals to check parameters such as genotoxicity, drug absorption, metabolism and metabolite elimination, as well as the toxicity of the drug and its metabolites (ADME-tox). The clinical stage is divided into four phases:

- **Phase I** is carried out on a small number of healthy human volunteers. During this phase, the metabolic and pharmacological effects of drugs are determined, along with any associated side effect(s). The main aim of Phase I studies is to determine safety profiles.
- **Phase II** consists of a further evaluation of safety together with efficiency in patients, and in the selection of a dose regimen.
- **Phase III** is intended to gather additional information about efficiency and safety, in order to evaluate the overall benefit–risk relationship of the drug.
- **Phase IV** is the long-term monitoring for adverse reactions, as reported by pharmacists and doctors.[1)]

Overall, drug development is a very expensive and lengthy process; typically, it may cost up to US$ 1.7 billion and take between 12 and 15 years for a new drug to become commercially available. The efficiency of drug development from lead compounds selected by screening is estimated at 1 in 5000 (Collier, 2009).

23.3 Examples of High-Throughput Screening

Many HTS assays have been developed depending on the therapeutic targets, some of which have helped to highlight the potential of marine products for pharmaceutical use.

23.3.1 Chemical Libraries: The Fuel of HTS

Along with HTS development, many molecule libraries have been compiled: for example, the ICSN-CNRS' (Institut de Chimie des Substances Naturelles, Gif-sur-Yvette, France) library constituted of 4000+ compounds from synthetic and natural origin, while the Greenpharma Natural Compound

1) http://www.fda.gov/drugs/resourcesforyou/consumers/ucm143534.htm (04/28/2013)

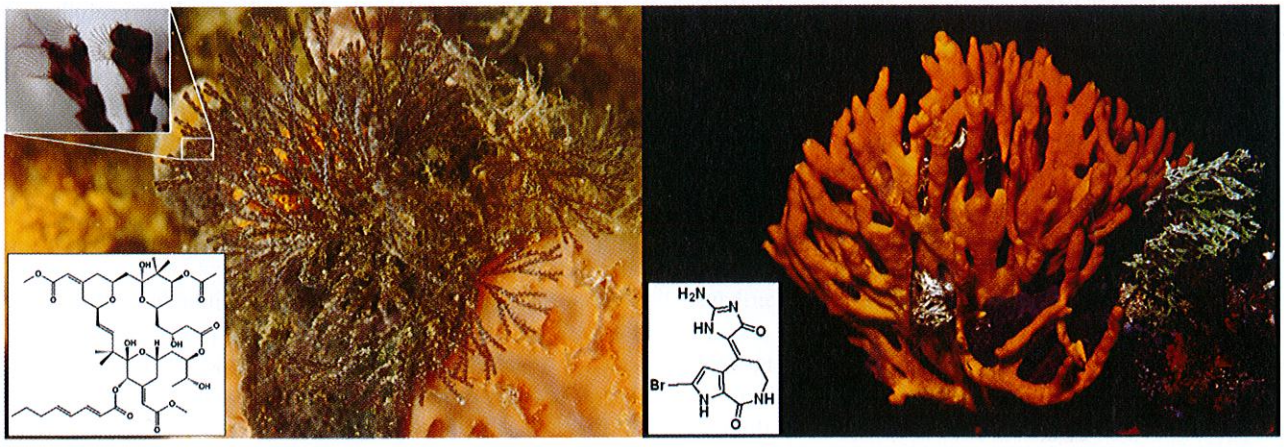

Figure 23.5 Images of marine producers of bioactive molecules. Left: *Bugula neritina* (© V. Lamare for the whole organism; © T.H. Ermak for the inset) for Bryostatin-1. Right: *Axinella verrucosa* (©, Oceanopolis, Brest, France) for Hymenialdisine. The corresponding chemical structures are depicted on the pictures.

library constituted 240 known phytochemicals (Greenpharma SAS, Orléans, France). The development of these libraries is a long and tedious process, as each compound must be controlled (mass spectrometry and/or NMR), diluted to specific concentration, archived, and stored. Thus, screening requires organization, a large storage capacity and, occasionally, a laboratory information management system (LIMS). In addition, "working" microtiter-plates (also known as "daughter" plates) will be generated to limit the freeze–thaw cycles and manipulation of the "mother" plates. A LIMS solution will allow inventory tracking, especially if multiple users are involved on the screening platform.

23.3.2 Biochemical Assay: The Example of Protein Kinases

A protein kinase is an enzyme that transfers a phosphate group from ATP to a specific substrate, in a process called "phosphorylation." The 518 human protein kinases play an essential role in regulating cellular growth and survival, differentiation or membrane transport. The deregulation of kinases has been notably described in cancer (since 1978 and the discovery of the first oncogene, Src, a tyrosine kinase), inflammatory or Alzheimer's disease and related neuronal disorders. This led to a growing interest in protein kinases and the development of specific and potent small-molecule inhibitors of kinase activity, historically targeting the ATP-binding pocket. Today, these enzymes have emerged as the most important targets in oncology drug discovery, and more than 20 compounds are already available commercially, such as Imatinib (Gleevec®, Glivec®), which targets Bcr-Abl (Chahrour, Cairns, and Omran, 2012). The market for kinase inhibitors is expected to reach US$11.6 billion in the US, and US$40.3 billion globally by 2016,[2] yet to date only a small part of the kinome has been targeted by already approved drugs. Clearly, kinase inhibitors have great potential. HTS assays have been developed using multiple technologies that include radioactivity-based assays, luminescent technology, TR-FRET, fluorescent polarization and scintillation proximity assays (SPAs).

Screening campaigns have identified several marine compounds as protein kinase inhibitors (Leclerc *et al.*, 2001; Bettayeb *et al.*, 2007; Bharate *et al.*, 2012; Debdab *et al.*, 2011). One of these, **Hymenialdisine** (HMD) (Figure 23.5), was originally identified based on its antiproliferative effects on cultured lymphocytic leukemia cells, and characterized using an SPA. The latter is a homogeneous method that monitors kinase activity based on the amount of ATP left when a kinase reaction has been determined. In this case, the luminescent signal is proportional to the amount of ATP, and inversely proportional to the amount of kinase activity (Meijer *et al.*, 2000).

HMD is a bromopyrrole alkaloid isolated from a variety of marine sponges, including *Hymeniacidon aldis* (or *Stylissa massa*), *Axinella verrucosa* (Figure 23.5), *Acanthella aurantiaca* during the early 1980s. It is a potent inhibitor of several closely related cyclin-dependent kinases (CDKs), such as CDK1/cyclin B ($IC_{50} = 22$ nM), CDK2/cyclin A ($IC_{50} = 70$ nM), CDK2/cyclin E ($IC_{50} = 40$ nM), and CDK5/p25 ($IC_{50} = 28$ nM), as well as against more distantly related kinases such as $GSK3_{\beta}$ ($IC_{50} = 10$ nM), casein kinase 1 ($IC_{50} = 35$ nM) or Mek1 ($IC_{50} = 6$ nM) (White *et al.*, 2012). Kinetic analyses also showed that HMD acts in an ATP-competitive fashion, and the cocrystal structure with CDK2 revealed that HMD occupies the ATP site and shows many of the hydrogen-bonding interactions seen in other CDK–inhibitor complexes (Wan *et al.*, 2004).

23.3.3 Protein–Protein Interactions (PPIs)

Interactions between proteins are at the center of the interactomic of any living cell, which makes ***PPI modulation*** one of the

2) http://www.prnewswire.com/news-releases-test/kinase-inhibitors-market-to-reach-116-billion-in-us-403-billion-globally-by-2016-161937715.html (04/28/2013)

most challenging tasks in drug discovery. Despite their structural diversity, protein–protein interfaces are in many cases large and compact hydrophobic and relatively flat surfaces, and thus can be considered as poorly druggable (Ofran and Rost, 2007; Nooren and Thornton, 2003). Nevertheless, only a few residues on protein–protein interfaces show a dominant contribution to the binding free energy. These residues are known as **"hot spots,"** and their discovery by crystallographic analysis and directed mutagenesis has been a major breakthrough in the development of small-molecule inhibitors for PPIs (Mullard, 2012).

The extreme complementarities of protein–protein interfaces allows the development of specific inhibitors, and also offers some advantages such as the emergence of a lower drug resistance due to the inhibitor binding outside the active site of the targeted proteins. This should be compared, for example, to the ATP-mimetic inhibitors of protein kinases (as described above).

Despite these advantages, the identification of small molecules that modulate PPIs remains largely untapped by pharmacological research (Thiel, Kaiser, and Ottmann, 2012). Few natural compounds were, nonetheless, identified such as microtubule-stabilizing agents (e.g., taxanes, and the marine compounds discodermolide, eleutherobin or laulimalide) (Bergstralh and Ting, 2006) and microtubule-destabilizing molecules (e.g., vinca alkaloids, and the marine compounds halichondrin B, dolastatin 10 or hemiasterlin), all of which have important applications notably in cancer treatment (Kingston, 2009).

During the past decade, following spectacular developments in genomics and proteomics, a growing number of potentially druggable PPIs have been identified, and this has pressured the development of HTS assays in this area. As a consequence, several technologies have been developed, including yeast two-hybrid (Y2H), reverse yeast two-hybrid (Rezwan and Auerbach, 2012), bacteria two-hybrid (Stynen *et al.*, 2012), Mammalian Protein–Protein Interaction Trap (MAPPIT) (Lievens *et al.*, 2009), reverse MAPPIT (Lemmens *et al.*, 2006), bimolecular fluorescence complementation (BiFC), and FRET- and BRET-based methods (Ciruela, 2008; Corbel *et al.*, 2011).

One of the most promising areas of research in the design and development of new PPI-based therapeutics is related to human immunodeficiency virus (HIV) propagation. Indeed, the viral infection relies heavily on PPIs, especially between the virus and host proteins, in almost every step of the virus' lifecycle. A Y2H screening assay led to the discovery of a hexapeptide from the marine worm, *Eunicidae* gen. sp., which showed potent inhibitory properties on the 3′-processing activity of HIV-1 integrase through an unusual homoserine residue (Maes, Loyter, and Friedler, 2012).

Finally, as an emerging and promising concept, studies of PPIs are still in their infancy and have not yet allowed the discovery of many interesting drugs. However, the development of HTS in this field of application will most likely allow the identification of potentially interesting new molecules, and thus offer innovative treatment opportunities. In this respect, and due to their high chemical diversity, marine molecules will certainly play a major role.

23.3.4
Cell-Based Assay: The Example of Bryostatins

In 1965–1966, **Georges Pettit** and his collaborators began the first systematic study of marine invertebrates and vertebrates as potential sources of new and potentially useful cancer chemotherapeutic drugs. For this, they used the NCI P388 *ex-vivo* lymphocytic leukemia screening system to measure antineoplastic activity (Pettit, Herald, and Hogan, 2002). In 1981, the fractionation and purification of bryozoan *Bugula neritina* extracts led to the characterization of a macrocyclic lactone, Bryostatin-1 (Figure 23.5), which showed cell growth-inhibiting properties at subnanomolar concentrations (Pettit, Herald, and Hogan, 2002). Subsequently, additional studies led to the isolation of 20 new bryostatins from *B. neritina* collections from the Gulf of Mexico, Gulf and coast of California and Japan (Gulf of Sagami) (Pettit, Herald, and Hogan, 2002). Interestingly, in a recent study it was reported that bryostatins are produced by symbiotic bacteria from *B. neritina* (Wender *et al.*, 2011). Bryostatin-1 is one of the most abundant and best-studied compounds of this structural family, and was found to target protein kinase C (PKC) through high-affinity binding to the diacylglycerol-binding site of the C-1 regulatory domain of the enzyme.

Whilst originally described as inhibiting cell growth (Pettit, Herald, and Hogan, 2002), Bryostatin 1 has subsequently shown a wide range of properties that include anticancer activity, a synergetic effect with other anticancer agents, a reversal of multidrug resistance, stimulation of the immune system, improvement of learning and memory, neuroprotection after stroke, reduction of amyloid plaque formation, and activity against HIV (Trindade-Silva *et al.*, 2010). Yet, after undergoing more than 30 Phase I and Phase II clinical trials[3] in a variety of cancers, both alone and in combination with other chemotherapy agents, bryostatin 1 failed to reach Phase III due to a lack of efficacy in patients. Nonetheless, Bryostatin-1 is still in clinical trial Phase II for the treatment of Alzheimer's disease.[4]

23.4
Conclusions and Perspectives

HTS assay development has supported an increased rate of discovery of marine bioactive compounds with various medical applications (Singh *et al.*, 2008). Recent methodological screening developments, which allow the identification of PPI modulators or the development of cell-based HCS assays will certainly lead to the discovery of new marine-derived medicines. One of the current challenges in pharmaceutical research is to increase the access to new chemical scaffolds in order to widen the

3) clinicaltrials.gov/(04/28/2013).
4) http://www.brni.org/scientific_research/clinical_trials.aspx (04/28/2013).

diversity of chemical libraries, this being seen as the "key to success" in drug discovery. Mother Nature is an amazing medicinal chemist, providing surprisingly innovative chemical scaffolds of which marine biodiversity is a vast, as-yet under-explored supplier. Yet, undoubtedly, there are many great discoveries to come, bearing hope for new and highly potent human therapies.

List of Abbreviations

BRET	bioluminescence resonance energy transfer
CDKs	cyclin-dependent kinases
CK1	casein kinase 1
CytP450	cytochrome P450
Da	daltons
DMSO	dimethylsulfoxide
EC_{50}	half-maximal effective concentration
ELISA	enzyme-linked immunosorbent assay
FRET	fluorescence resonance energy transfer
GPCR	G-protein coupled receptor
$GSK3_{\beta}$	glycogen synthase kinase 3_{β}
HCS	high-content screening
HMD	Hymenialdisine
HTRF	homogeneous time-resolved fluorescence
HTS	high-throughput screening
IC_{50}	half-maximal inhibitory concentration
NMR	nuclear magnetic resonance
PK	pharmacokinetics
PKC	protein kinase C
PKIs	inhibitors of protein kinase
PPIs	protein–protein interactions
RNAi	ribonucleic acid interference
SAR	structure activity relationship
SNR	signal-to-noise ratio
SPA	scintillation proximity assay
TR-FRET	time-resolved-FRET
SW	signal window
Y2H	yeast two-hybrid

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