Mechanism-based Screening for Cancer Therapeutics with Examples from the Discovery of Marine Natural Product-based HIF-1 Inhibitors

22

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Abstract

Recent advances in cancer genetics combined with an increasing number of new methods in molecular and cell biology provide exciting new antitumor drug targets and a wide array of means to design bioassay systems for the discovery of novel cancer chemotherapeutics. Marine natural products continue to play a vital role in molecular-targeted antitumor drug discovery. Although most recognize the critical and expanding role mechanism-based antitumor bioassays play in modern anticancer drug discovery, few natural products chemists have specific training in bioassay technology. Critical bioassay development factors are outlined and introduced at a level intended to provide a basic understanding to

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a general audience. These include molecular target identification, antitumor target validation, selection of assayable biochemical processes, data acquisition methods, experimental controls, bioassay validation and statistical methods, experimental artifacts, active compound identification, and the dereplication of nuisance compounds. Marine natural products have been identified that inhibit the activation of the anticancer drug target hypoxia-inducible factor-1 (HIF-1). Bioassay systems and recent results from marine HIF-1 inhibitor discovery programs are used to illustrate important factors that must be considered when using molecular-targeted antitumor bioassay methods.

22.1 Introduction

Marine natural products research is a dynamic and constantly changing field that has evolved and adapted new technologies with cutting-edge molecular and cell biology. Modern high-field multidimensional nuclear magnetic resonance (NMR) methods facilitate the rapid structure elucidation of minute quantities of pharmacologically active natural products. Advances in microbial sourcing have yielded exciting new biological diversity. This is especially true in the field of marine natural products, where marine bacteria and fungi have began to eclipse sponges and other invertebrates as important sources of novel chemistry (see ► Chap. 3) [1–5].

The activation of oncogenes and/or the inactivation of tumor suppressor genes plays an important role in the etiology and progression of cancer, an assemblage of diseases that result from accumulated mutations. Our knowledge of cancer genetics has expanded rapidly during the past few decades. Since the initial discovery of oncogenes, the field of cancer biology has grown to include the elucidation of the molecular mechanisms that underlie tumorigenesis, tumor growth, progression, metastasis, tumor cell death, and treatment resistance. As a result, mechanism-based drug discovery efforts have prospered and molecular-targeted agents (i.e., trastuzumab) are in clinical use. By integrating critical technological advancements with a growing number of important molecular mechanism–based antitumor targets, marine natural products research currently plays a vital role in anticancer drug discovery [6, 7]. In addition, newly developed strategies aim to alter the genetic regulation of secondary metabolite biosynthesis. Taken together, these advances show great promise to increase the structural diversity of natural products for drug discovery.

While the face of natural products chemistry has dramatically changed, the therapeutic potential of compounds identified in anticancer marine natural product programs remains inexorably tied to the quality of the bioassay methods used to direct the discovery efforts. In this respect, there is a fundamental need for cutting-edge molecular and biomedical research to support nearly all aspects of contemporary antitumor drug discovery. Most natural products chemists recognize the importance of excellent bioassays to support their research efforts. Yet, few chemists are specifically trained to critically evaluate the cell biology that supports the

potential significance of anticancer targets or the molecular biology involved in the design of molecular-targeted antitumor bioassay methods. To ensure the quality of these efforts, the most successful programs usually depend on strong collaborative research efforts with molecular and tumor cell biologists, pharmacologists, and experts in the design of high-throughput bioassay systems.

Important factors that must be considered when using modern molecular-targeted antitumor bioassay methods comprise a relatively short list of drug target–associated, bioassay method–dependent, and test sample source–specific factors. These may include, but are not limited to, the following list of points that require careful consideration: (a) identification of a molecular target; (b) validation of the antitumor target; (c) selection of a measurable biochemical, chemical, or biological process for the assay; (d) methods used to acquire or measure the data; (e) use of experimental controls; (f) bioassay validation and the application of appropriate statistical methods; (g) possible experimental artifacts; (h) active compound identification; and (i) nuisance compounds and methods for chemical dereplication. It may not be feasible to expect all natural products chemists to become experts in bioassay systems. Nonetheless, a general appreciation for these important assay components is critical to the success of every molecular-targeted antitumor drug discovery process.

The transcription factor hypoxia-inducible factor-1 (HIF-1) has emerged as an important target for anticancer drug discovery [8–12]. Hypoxic conditions (decreased oxygen tension) that are present within actively growing tumor masses activate HIF-1. The heterodimeric protein complex known collectively as HIF-1 then regulates the transcriptional response to tumor hypoxia [13]. Numerous natural products have been identified that regulate HIF-1 activation and suppress tumor-related HIF-1 target genes [14–16]. Because of its current distinction in the drug discovery process, HIF-1 has been selected as a representative example to demonstrate the importance of the previously outlined points that must be considered in the design and implementation of molecular-targeted bioassays used in the discovery of antitumor natural products. Screening efforts have shown the marine invertebrate and algal extracts in the U.S. National Cancer Institute's Open Repository to be a valuable source of new regulators of HIF-1 signaling [14]. Compounds isolated from marine organisms appear to either inhibit or promote HIF-1 activation. In addition to its coverage of basic concepts in molecular-targeted antitumor drug discovery, this chapter highlights the emerging role of marine natural products as potential regulators of HIF-mediated hypoxic signaling.

22.2 Bioassay Target Selection

Perhaps, nowhere in natural product–based drug discovery is bioassay target selection more important than in the field of molecular-targeted antitumor drug discovery. Traditional antitumor drug discovery has relied heavily on tumor cell viability assays for the identification of new natural product–based anticancer compounds [17–19]. Assay target selection consisted mainly of a choice in tumor cell lines and/or in the design of specific assays to discriminate between agents that

produce either a cytostatic or cytotoxic effect. In these cases, the bioassay target can be considered the tumor class, which is equated to the tumor cell line tissue source. Although this strategy may currently be considered less fashionable, it continues to produce some of the most promising anticancer marine natural products (salinosporamide [20, 21] and Yondelis/ET-743/trabectedin [22, 23]). Marine natural products researchers have taken advantage of modern high-throughput bioassays to identify new compounds that function through an assorted array of antitumor molecular targets [14].

The definition of an antitumor molecular target is somewhat subjective. One investigator may consider an enzyme/enzymatic activity, protein-protein interaction, or protein-DNA interaction as the molecular target. Superficially, enzymes such as receptor protein kinases, cyclin-dependent kinases, checkpoint kinases, topoisomerases, DNA polymerases, and other proteins that mediate important reactions can be thought of in this way. In this case, an antitumor molecular target is something as conceptually straightforward as a specific protein or protein-mediated biochemical reaction. However, another drug discovery group may consider the disruption of a genetically controlled process as the molecular target, even though many individual steps may be involved in regulating the control of the overall process. In this case, the molecular target is viewed more as a complex set of biochemical and genetic events that may involve many proteins or other macromolecules. Under this broader definition, antitumor molecular targets include such occurrences as apoptosis-related cellular signaling, mammalian target of rapamycin (mTOR)-regulated protein expression, proteasome-mediated protein degradation, heat shock protein–dependent protein stability, etc. This broader view also includes the targeting of specific genes and the transcriptional or translational events involved in protein-mediated processes. According to this broader definition, drug discovery groups may employ assays that specifically examine the effects of small molecules on the multistep processes that regulate the expression or function of genes that are considered to be important in tumorigenesis, tumor progression, or metastasis. In this light, oncogenic gene transcription and the proteins involved in regulating tumor-associated gene transcription have emerged as major targets for anticancer drug discovery programs that focus on the regulation of specific tumor-specific gene function [24].

The distinctions between various molecular target definitions quickly blur when one considers the interactions between the systems involved in each class of antitumor molecular target. Enzymes may be considered biochemical targets, but enzymes can regulate the synthesis of proteins that control gene expression. Similarly, oncogenes may code for the production of enzymes or other proteins that play an important role in biochemical processes that enhance the growth and spread of tumors. Oncogenes may also code for transcription factors that regulate the expression of other genes involved in tumorigenesis. In such cases, each specific step in the process of DNA replication, transcription, translation, and protein function may represent a potentially druggable step within a single molecular target.

Hypoxia is one of the signature features of the tumor microenvironment. The rapid growth of tumor cells outstrips the capacity of tumor blood vessels to supply oxygen. Newly formed tumor blood vessels often fail to mature, and the tumor blood flow is sluggish and irregular [25]. The combination of increased oxygen demand and insufficient oxygen delivery yields hypoxic regions that are commonly found in solid tumors. Clinical studies indicate that the extent of tumor hypoxia correlates with advanced disease stages and poor prognosis. Unlike normal cells from the same tissue, tumor cells are often chronically hypoxic [26]. Since hypoxia can activate both survival and death programs, it serves as a form of physiological pressure that selects for the oncogenically transformed cells with diminished apoptotic potential [27]. The tumor cells that have adapted to a hypoxic environment are more aggressive [25, 26, 28]. Because of hypoxia-associated resistance to radiation treatment and chemotherapy, hypoxic tumor cells are considered an important contributor to malignant progression and disease relapse [25, 26]. Approaches such as breathing carbogen (95% O_2, 5% CO_2) have been employed to overcome tumor hypoxia by increasing tumor oxygenation. Most of the initial drug discovery efforts target the direct effects of hypoxia – lack of cellular oxygen – by either discovering chemical sensitizers that improve the outcome of radiation or developing hypoxic cytotoxins that selectively kill hypoxic cells [25, 26]. No hypoxic cytotoxin is currently approved, and there is only one bioreductive drug (tirapazamine) in clinical trials [29]. Although mixed results have been reported from clinical trials with tirapazamine, it is undeniable that drugs that target tumor hypoxia have significant therapeutic potential as part of a combination therapy [30]. Clearly, tumor hypoxia represents an important unmet therapeutic need, and molecular-targeted drug discovery efforts should be directed at this target.

Hypoxia-inducible factor-1 is an important molecular target for anticancer drug discovery that targets tumor hypoxia. First discovered as the transcriptional activator that binds to the promoter of human erythropoietin (*EPO*) gene and activates transcription under hypoxic conditions [31], HIF-1 was later shown to regulate the expression of genes that promote cellular adaptation and survival under hypoxic conditions [32, 33]. For example, Semenza and coworkers [32, 33] have generally classified the HIF-1 target genes that enhance the survival of hypoxic tumor cells into the following major functional groups: (1) those that increase oxygen delivery by promoting erythropoiesis [erythropoietin (EPO), transferrin, etc.], angiogenesis [vascular endothelial growth factor (VEGF), etc.], and vasodilatation (heme oxygenase, nitric oxide synthase II, etc.); (2) those that decrease oxygen consumption by switching to anaerobic metabolism (glucose transporter 1, glycolytic enzymes that include hexokinase 1, hexokinase 2, aldolase A, enolase 1, lactate dehydrogenase A, etc.); and (3) those that promote autocrine growth/survival (insulin-like growth factor II, etc.). Recent studies indicate that HIF-1 also regulates the expression of genes that are involved in processes ranging from tumor cell immortalization, genetic instability, dedifferentiation, invasion, metastasis, to treatment resistance [32–34]. These observations suggest that HIF-1 plays an important role in the etiology and malignant progression of cancers.

22.3 Molecular Target Validation

Molecular target validation is the process of establishing that a potential molecular target is essential for a disease process and that inhibition (or dysregulation) of that specific target can produce a potential therapeutic benefit. Screening efforts that use poorly validated antitumor molecular targets can be extremely expensive and time consuming in identifing active compounds that may fail to produce a significant antitumor effect in living cells. Demonstrating that a drug or other representative inhibitor of the molecular target produces the desired therapeutic outcome in patients may be the ultimate form of target validation. For example, the validation of antitumor targets such as tubulin/microtubules or topoisomerases may be considered solidly validated as druggable molecular targets by the therapeutic efficacy of the microtubule-targeted anticancer drugs vincristine and paclitaxel and by the topoisomerase inhibitors topotecan and etoposide, respectively. However, most innovative molecular-targeted marine natural product discovery programs aim to identify potential new agents that function through nontraditional mechanisms, rather than simply seek to isolate compounds that act in the same manner as clinically approved drugs. Under these conditions, molecular target validation can be considered the construction of an evidence-based case to support the essential nature and causative role of the target to a specific disease. Evidence to validate the target can come from clinical studies, but is most often derived from a combination of in vitro and preclinical animal-based experimental results.

Drugs often fail in clinical studies because they either do not produce the desired therapeutic effect or because they exhibit severe side effects, or toxicities, that render them unsafe for patient use [35]. Both reasons for the clinical failure of new drugs have been attributed to a lack of thorough target validation [35]. Therefore, it is essential to design a strategy for target validation that not only evaluates the therapeutic relevance of the target but that will also examine the specificity of the molecular target for the biochemical or physiological processes that contribute to the disease process. This is crucial in order to discriminate between disease-specific activity and off-target-related effects. While many in vitro cell-based methods (e.g., DNA-microarray or proteomic-based systems) are widely used in target validation, the most convincing data usually come from those models that more closely represent the disease condition in humans [36]. Various forms of gene/target knockout models are used to examine the physiological relationship between a specific gene, or gene product protein, and a particular disease phenotype. Traditional gene knockout methods (e.g., homologous recombination–based methods) and alternative gene knockdown or silencing methods (e.g., RNA interference) have proven invaluable as in vivo means to validate the critical role of a selected molecular target for a specific disease process [37]. Data from animal-based models that include physiological, genomic, or proteomic results from in vivo models are generally considered important for reliable target validation [35].

As a model for a validated antitumor drug target, multiple lines of evidence support the critical involvement of HIF-1 in the growth, progression, and metastatic spread of various forms of cancer [8–12]. In line with the laboratory findings, clinical studies indicate that overexpression of the oxygen-regulated HIF-1α protein correlates with advanced disease stages, treatment resistance, and poor prognosis among patients with tumors derived from tissues that range from brain to breast [25, 26]. In general, the availability and activity of the oxygen-regulated HIF-1α subunit determines the activity of HIF-1, a heterodimer of the bHLH-PAS (basic helix-loop-helix–PER-ARNT-SIMM) proteins HIF-1α and HIF-1β/ARNT (aryl hydrocarbon receptor nuclear translocator). Under normoxic conditions, HIF-1α protein is degraded rapidly (protein half-life <5 min) while HIF-1β protein is constitutively expressed. In many of the clinical specimens examined, the expression of HIF-1α protein is directly linked to the extent of tumor hypoxia. The decrease in oxygen tension inhibits the hydroxylases that tag HIF-1α protein for degradation and inactivation. The stabilized HIF-1α protein then translocates into the nucleus, heterodimerizes with HIF-1β protein, binds to the hypoxia response elements (HREs), and activates transcription of HIF-1 target genes (Fig. 22.1). In other cases such as renal clear cell carcinoma, HIF-1α protein is expressed at high levels even under well-oxygenated conditions [38]. One of the molecular mechanisms for hypoxia-independent induction of HIF-1α protein is the loss of function of the tumor suppressor von Hippel-Lindau gene (VHL). The VHL gene product pVHL mediates the degradation of HIF-1α protein by proteasome. In the presence of oxygen and iron, HIF-1α proteins are modified posttranslationally by prolyl hydroxylases [39–41]. The prolyl hydroxylated HIF-1α proteins are then recognized by pVHL, polyubiquitinized by the pVHL-associated E3 ubiquitin ligase complex, and degraded by the proteasome. Inactivation of pVHL due to loss of function prevents the degradation of HIF-1α protein. As a result, HIF-1 is activated under normoxic conditions and the HIF-1 regulated pathways such as angiogenic processes are highly active. In addition to the loss of function of VHL, other tumor-specific mechanisms that induce HIF-1α protein and activate HIF-1 in a hypoxia-independent manner include the activation of oncogenes such as ras (rat sarcoma viral oncogene homolog), src (avian sarcoma viral oncogene), myc (avian myelocytomatosis viral oncogene homolog), and the loss of tumor suppressor genes such as PTEN (phosphatase and tensin homolog) [42, 43].

Results from numerous animal-based studies further support the notion that HIF-1 is an important molecular target for anticancer drug discovery [44, 45]. In general, overexpression of a gene product in tumor cells does not necessarily support the role of that particular gene as a molecular target for cancer therapeutics. It is anticipated that the inhibition of a true molecular target will suppress tumor progression, while the activation of a molecular target will promote tumor progression in animal-based models. Most of the animal-based studies that substantiate HIF-1 as a target for molecular-based therapeutics focus on the HIF-1A gene.

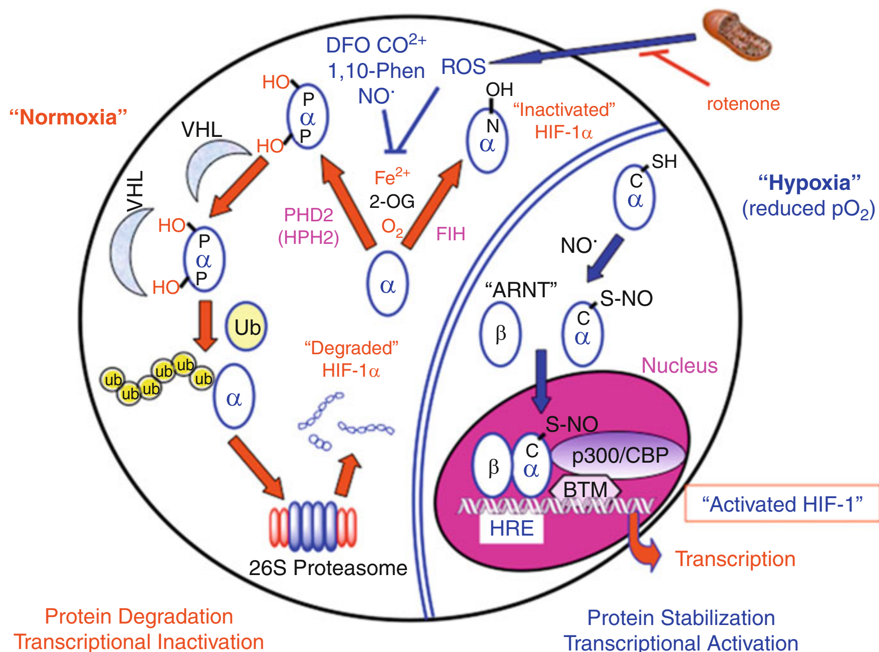


Fig. 22.1 Hypoxic regulation of hypoxia-inducible factor-1 (HIF-1). The transcription factor HIF-1 is a heterodimer composed of an HIF-1α subunit that is regulated by cellular oxygen levels and an HIF-1β subunit (also known as ARNT) that is constitutively expressed. Under normoxic conditions, HIF-1α protein is hydroxylated at specific proline residues by Fe^{2+}/2-oxoglutarate/O_2-dependent prolyl hydroxylase enzymes (e.g., PHD2). This prolyl-hydroxylation "tags" HIF-1α protein for von Hippel-Lindau tumor suppressor protein (pVHL)-E3 ubiquitin ligase-mediated polyubiquitination. The "ubiquitn-tagged" HIF-1α protein is then rapidly degraded by the 26S proteasome. Hydroxylation of an asparagine residue in the C-terminal transcriptional activation domain (CTD) contributes another level of oxygen-dependent regulation by inactivating HIF-1α protein. Like PHD2, this asparaginyl hydroxylase ["factor inhibiting HIF" (FIH)] is also an Fe^{2+}/2-oxoglutarate/O_2-dependent hydroxylase that modifies the asparagine residue in the CTD region of HIF-1α protein. Once hydroxylated, the interaction between HIF-1 and the coactivator CBP/p300 is disrupted and transcriptional activation is blocked. Besides hypoxic conditions, HIF-1α protein can be stabilized by addition of iron chelators, transition metals, nitric oxide radical (NO·), or inhibitors of PHDs [e.g., dimethyloxaloylglycine (DMOG)]. Such inducing conditions inactivate the prolyl hydroxylases that tag HIF-1α protein for ubiquitination and proteasomal degradation, and suppress the asparaginyl hydroxylase that normally inactivates the transcriptional activity of HIF-1. In addition, binding between HIF-1 and CBP/p300 can be enhanced by direct nitrosylation of a sulfhydryl moiety in HIF-1α. When the level of O_2 decreases to a level below a certain threshold, reactive oxygen species (ROS) generated by hypoxic mitochondria inhibit PHD2 and FIH by oxidizing the Fe^{2+} in their catalytic sites. Natural products that inhibit the mitochondrial electron transport chain (e.g., rotenone) block HIF-1 activation by suppressing the hypoxia-induced increase in ROS production by mitochondria. This promotes the PHD2-mediated degradation and FIH-facilitated inactivation of the HIF-1α subunit (Figure and caption reproduced with the permission of D.G. Nagle [© 2010] at the University of Mississippi)

22.4 Selection of Measurable Biochemical Processes Suitable for Bioassay Development

Since HIF-1 is a transcription factor, a cell-based reporter assay should be selected as the method to monitor the activity of HIF-1. Upon induction and activation, HIF-1 binds to the HRE present in the promoter region and activates transcription of the reporter gene [45, 46]. In general, the reporter gene may encode an enzyme such as luciferase, whose activity can be easily measured in a high-throughput format. The activity of the reporter (e.g., luciferase) correlates with the expression of the reporter gene that serves as an indicator of HIF-1 activity. It is anticipated that compounds/extracts that activate HIF-1 will increase the activity of the reporter, while HIF-1 inhibitory compounds/extracts will decrease the reporter activity. The advantage of such a cell-based reporter assay is that it will detect a wide range of chemicals with dissimilar mechanisms of action. The challenge is that it may take considerable effort to resolve the mechanism(s) of action once the active compounds are identified. In the event that the active compound(s) has been well characterized, the compound may serve as a molecular probe to further investigate the crosstalk between signaling pathways.

In contrast, assays that monitor one specific step within a signaling pathway will facilitate the discovery of active leads with a defined mechanism of action. For example, an assay measuring the activity of a particular kinase can be used to identify inhibitors of that particular kinase. Among the approaches used to discover HIF-1 inhibitors, one is to identify compounds that can disrupt the interaction between HIF-1 and the coactivator CBP/p300 [(cAMP-response element-binding protein)-binding protein/E1A-binding protein, 300 kD] [47]. In general, the activity of HIF-1 is determined by the availability and activity of the oxygen-regulated HIF-1α subunit (Fig. 22.1). In the presence of oxygen and iron(II), the HIF-1α subunits are hydroxylated by prolyl hydroxylases, and this posttranslational modification triggers pVHL-mediated proteasome degradation of HIF-1α proteins [39–41]. Upon a reduction in cellular oxygen levels or in the presence of iron chelators, the hydroxylation reaction is inhibited and the stabilized HIF-1α proteins translocate into the nucleus, heterodimerize with HIF-1β subunits, and activate gene transcription. The interaction between HIF-1 and CBP/p300 enhances the formation of the transcriptional initiation complex and increases the transcription of target genes. Oxygen-dependent asparaginyl hydroxylation of HIF-1α protein (Asn803) by factor inhibiting HIF (FIH) abrogates the interaction between HIF-1 and CBP/p300. To discover disruptors of HIF-1α/p300 interaction, Kung and colleagues employed a time-resolved fluorescence high-throughput screening assay to detect the binding between a 41-amino-acid HIF-1α-derived polypeptide representing the minimal p300/CBP binding domain and a glutathione-*S*-transferase (GST) fusion protein that contains the 122-amino-acid minimal HIF-1α-binding domain from p300 (GST-CH1) [47]. The biotinylated HIF-1α-derived polypeptide was immobilized onto streptavidin-coated plates. The interactions between the HIF-1α polypeptide and the GST-CH1 fusion protein were examined using time-resolved fluorescence to monitor a europium-conjugated anti-GST

antibody probe. A library of >600,000 natural products and synthetic compounds was evaluated, and the dithiodiketopiperazine metabolite chetomin was identified as a submicromolar inhibitor of HIF-1α/p300 interaction. The advantage of such a biochemical process-based assay is that it can be used to discover active compounds that selectively target a specific predetermined mechanism. However, some of the active compounds identified in this type of in vitro assays may not be further pursued if they fail to demonstrate efficacy in cell-based systems. In the case of chetomin, it exhibited anticancer efficacy in vivo. Since the CH1 domain is also required for CBP/p300 to serve as a coactivator to other transcriptional activators, the relative "nonspecificity" and subsequent toxicity prevented the further development of chetomin as a chemotherapeutic drug.

Small-molecule HIF-1 inhibitors have also been identified using other approaches such as an ELISA (enzyme-linked immunosorbent assay)-based assay that detects the interaction between the HIF-1α and HIF-1β PAS A domains [48]. As previously discussed, biochemical process-based in vitro assays may facilitate the discovery of HIF inhibitors that target one specific mechanism or process. For enzyme-based assays, the recombinant protein should retain the native and active conformation. Active compounds identified from the screening effort may have a higher possibility of being active in cell-based assays, if they can penetrate the host cells. In the case of in vitro assays that monitor protein interactions, the proteins used are often only polypeptides or otherwise truncated proteins that contain the domain(s) of interest. These "protein" reagents may not retain the native conformation that is required for proper binding. As such, a large percentage of the actives from the primary screening efforts with such biochemical assays may fall out of the "hit list" when evaluated in cell-based systems. In addition, further studies are still required to discern the specificity and the bioavailability of active compounds before the agent can be advanced to animal-based studies.

22.5 Techniques Used to Acquire or Measure the Bioassay Data

Following rapid advances in molecular techniques, most of the assay-related reagents are available from commercial sources. For screening purposes, it is important to select the method(s) that is suitable for a high-throughput format as well as cost effective. In the case of discovering small-molecule HIF-1 inhibitors, we have chosen a cell-based luciferase assay as the format for primary screening [49]. In this cell-based assay, the activity of HIF-1 correlates with the enzymatic activity of the luciferase reporter. The luciferase assay itself is straightforward, and the reagents are readily available from commercial sources. In addition, other accessories such as cell culture plates and plate readers are available in both 96- and 384-well format for the purpose of screening. Cell-based reporter assays require cellular uptake of the reporter constructs by either transient or stable transfection. The transient transfection method is relatively fast, but the cells need to be transfected every time the assay is performed. The stable transfection approach is relatively time consuming in regard to the initial effort to establish

a genetically modified cell line that has incorporated the reporter construct into its genome. However, once a stable cell line is established, the cells can be directly assayed without transfection.

Upon selecting a transient transfection approach for our HIF-1 inhibitor discovery efforts, the initial studies were directed at identifying the optimal model system for the cell-based reporter assay. A panel of human breast tumor cell lines that represent different disease stages and malignant progression were selected as in vitro models (early stage estrogen dependent: T47D and MCF-7; highly metastatic estrogen independent: MDA-MB-231). First, the conditions for transient transfection were optimized in each cell line using a control construct (pGL3-control, Promega) that expresses luciferase under the control of a constitutively active promoter. Second, a cell-based reporter assay for HIF-1 activity was performed in each cell line. The objective was to identify the cell line with the highest level of HIF activation. In order to improve screening efficiency, a robust assay with a high level of induction was sought to provide a low rate of background noise–associated false-positive experimental artifacts. The cells were transiently transfected with the pHRE3-TK-luc reporter to monitor HIF-1 activity and exposed to inducing conditions that activate HIF-1. The conditions that activate HIF-1 range from hypoxia (decreased oxygen tension), iron chelators, transition metals (Co^{2+}, Ni^{2+}, etc.), to oncogenic mutations (activation of oncogenes and/or inactivation of tumor suppressor genes). In solid tumors, hypoxia is a common pathophysiological condition and the extent of tumor hypoxia correlates with advanced disease stages and poor prognosis. Since HIF-1 is a key regulator of hypoxia-regulated gene expression that promotes tumor cell adaptation to hypoxia and overall treatment resistance, compounds that inhibit hypoxic activation of HIF-1 represent potential drug leads that selectively target tumor hypoxia. The focus of our discovery project was the identification of small molecules that inhibit HIF-1 activation by hypoxia. Among the cell lines examined (Fig. 22.2), T47D breast tumor cells exhibited the highest level of HIF-1 induction upon hypoxic exposure (1% O_2/5% CO_2/94% N_2, 16 h). This T47D cell-based HIF-1 reporter assay produced the greatest signal to noise (background) ratio. The effectiveness of the cell-based reporter assay for detecting HIF-1 inhibitors was examined with a known HIF-1 inhibitor, MEK1 (meiosis-specific serine/threonine protein kinase) inhibitor PD98059. In MCF-7, MDA-MB-231, and T47D cells, PD98059 inhibited HIF-1 activation by hypoxia with comparable potencies (Fig. 22.3). Further cell viability studies in T47D cells excluded the possibility of false positives due to cytotoxicity.

22.6 Design and Use of Proper Experimental Controls

In an ideal situation, all molecular-targeted screening efforts should be conducted in a statistically significant way ($N \geq 3$) to achieve accuracy and reliability. However, it is time consuming and not feasible for many small academic laboratories with limited resources to examine tens of thousands of samples in triplicate. To meet this challenge, one approach is to include some element of replication and

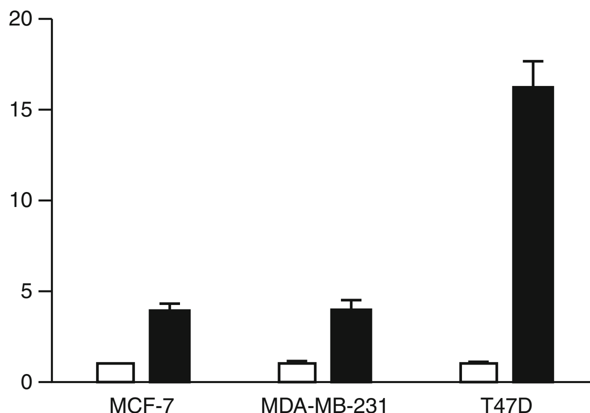


Fig. 22.2 Cell line–dependent hypoxic activation of HIF-1 determined in a cell-based reporter assay. Three human breast tumor cell lines (MCF-7, MDA-MB-231, and T47D) were transiently transfected with a pHRE-TK-Luc construct and exposed to hypoxic conditions (1% O_2/5% CO_2/ 94% N_2, 16 h). Control cells were incubated under normoxic conditions (95% air/5% CO_2, 16 h). The cells were lysed, and luciferase activity was determined and presented as relative luciferase activity to the control. Data shown are averages from one representative experiment performed in triplicate, and the error bars indicate one standard deviation

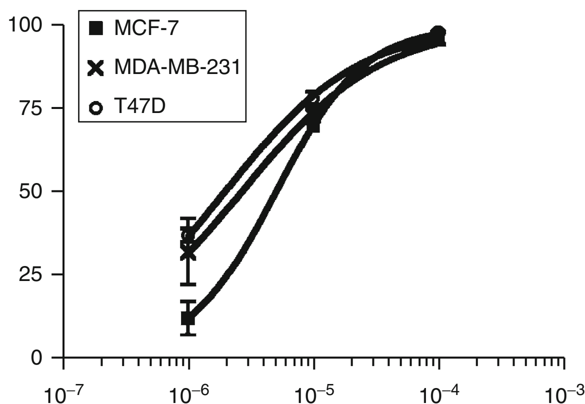
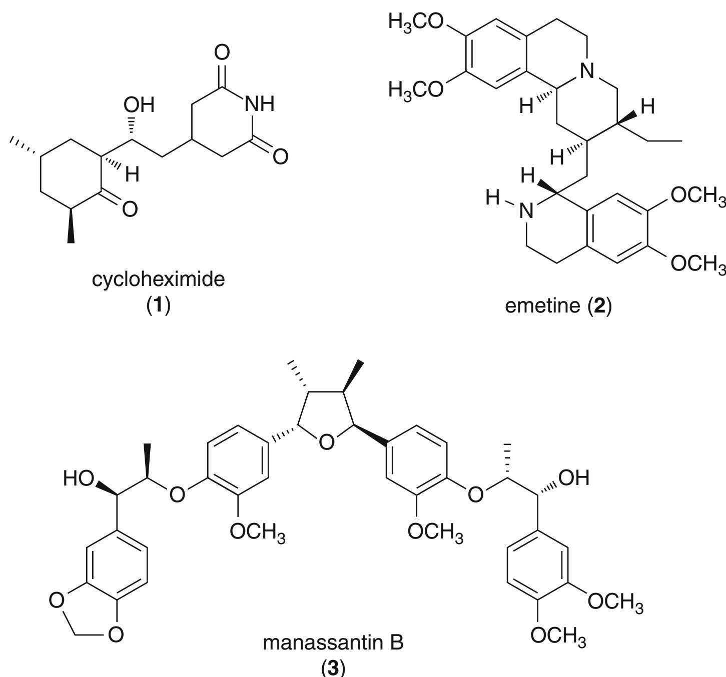


Fig. 22.3 PD98059 inhibits hypoxic activation of HIF-1 in a concentration-dependent manner. Human breast tumor cells (MCF-7, MDA-MB-231, and T47D) were transiently transfected with a pHRE-TK-Luc construct and exposed to hypoxic conditions (1% O_2/5% CO_2/94% N_2, 16 h) in the presence of PD98059 at the final concentrations of 1, 10, and 100 μM. The cells were lysed, and luciferase activity was determined and presented as percent inhibition of the solvent-treated control. Data shown are averages from one representative experiment performed in triplicate, and the error bars indicate one standard deviation

proper experimental controls to ensure the effectiveness of the bioassays. If an assay is deemed effective, then we can assume that the results obtained with the samples will have a higher probability of accuracy. In the T47D cell-based reporter assay for HIF-1 activity, the ratio of luciferase activity under hypoxic conditions

versus that under normoxic conditions serves as an indicator for the extent of HIF-1 activation. The inhibition of HIF-1 activity observed in the presence of an HIF-1 inhibitor (e.g., cycloheximide) (**1**) indicates the effectiveness of the assay for detecting HIF-1 inhibitors [50]. Other HIF-1 inhibitors have also been used as positive controls in HIF-1 bioassays. These positive controls include the nonselective alkaloid-based protein synthesis inhibitor emetine (**2**) [51–53] and *Saururus cernuus* dineolignan HIF-1 inhibitor manassantin B (**3**) [54].



Upon identification of the proper controls, another factor that impacts the screening outcome is the final concentration of the test sample. When the sample concentration is too high, nonspecific and/or less potent inhibitors will appear as positives in the assay, and this will lead to an unmanageably high hit rate. As shown in Fig. 22.4a, approximately 78% of the extracts on a 96-well sample plate inhibited HIF-1 activation by >50% when tested at the concentration of 50 μg/mL. In contrast, the hit rate in the primary assay was reduced to 2% when the same samples were examined at 5 μg/mL (Fig. 22.4b).

22.7 Bioassay Validation and the Application of Appropriate Statistical Methods

The results of molecular-targeted antitumor assays can only be considered reliable if the experiments are properly controlled, the bioassay methods have been

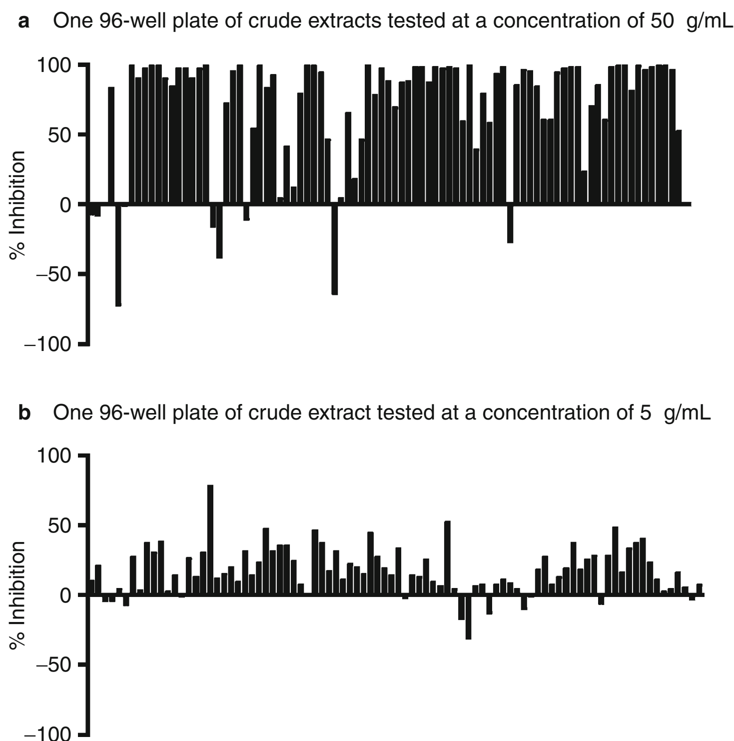


Fig. 22.4 Results for hypoxia-induced HIF-1 inhibitory effects observed for a representative 96-well plate of extracts evaluated at two different final concentrations. T47D human breast tumor cells were transiently transfected with a pHRE-TK-Luc construct and exposed to hypoxic conditions (1% O_2/5% CO_2/94% N_2, 16 h) in the presence and absence of natural product–rich extracts. The cells were lysed, and luciferase activity was determined and presented as percent inhibition relative to a nontreated hypoxic control. (**a**) Approximately 78% of the extracts on a 96-well sample plate of chemically diverse plant extracts inhibited HIF-1 activation by >50% when tested at the concentration of 50 μg/mL. (**b**) When the identical 96-well plate of extracts was evaluated at a final concentration of 5 μg/mL, the corresponding hit rate in the primary assay was reduced to only 2%

rigorously validated, and the results are analyzed using suitable statistical methods for the particular bioassay design. Despite the fact that these criteria are necessary for the publication of manuscripts that specifically describe new bioassay methods, such standards are seldom given sufficient attention in publications that describe the biological activities associated with either marine or terrestrial natural products. It is relatively common to see publications that combine the isolation and structure elucidation, total synthesis, and other aspects of medicinal chemistry with biological testing results. All too often these reports focus on the natural products chemistry but fall short in respect to their standards for the bioassay acquisition methods and statistical data analysis. Experimental results are commonly reported

without including the data for positive or negative experimental controls, IC_{50} values are frequently reported without any indication of experimental replication, and structure-activity relationship studies are sometimes supported by tables of results that do not specify the magnitude of the observed experimental error or if the differences in bioactivity between structurally related compounds have statistical significance.

It is beyond the scope of this chapter to describe the statistical methods by which various molecular-targeted bioassays must be evaluated. However, recent articles, reviews, and texts provide a detailed perspective of statistical methods used to analyze and evaluate bioassay data [55–59].

In order to ensure the reliability of bioassays used to screen extracts, active chromatographic fractions, and purified natural products from marine organisms, these assays must be validated with respect to an array of assay parameters (robustness, linearity, accuracy, precision, sensitivity, and specificity). For our screening purposes, we define these bioassay validation terms in a similar manner to the "Guidelines for Industry" text on Validation of Analytical Procedures of the International Conference on Harmonisation (ICH) [60–62]:

- Assay "robustness" is a measure of an assay's tendency to be susceptible to minor background variation in responses and is reflected by the magnitude of response (or signal) relative to background variation (or noise). Robust bioassays are generally more sensitive and require fewer replicates to observe statistical significance.
- Assay "linearity" is the ability of the assay to obtain test results that are uniformly proportional (within a specific range) to either the molecular/biochemical process evaluated or, in the case of analytical procedures, directly proportional to the concentration of sample.
- Assay "accuracy" is a measure of how close the results of the assay are relative to accepted reference or control values.
- Assay "precision" is the variation between multiple replicates and is generally expressed in terms of standard deviation, coefficient of variation, etc. Bioassay precision is a measure of experiment to experiment repeatability (intra-assay precision within a given laboratory), intermediate variation (between various equipment, personnel, etc.), and assay reproducibility between different laboratories. The sensitivity is a measure of the minimum detection limits of a bioassay method.
- Assay "specificity" is determined by the ability of the assay method to specifically respond to the desired molecular or biochemical process, relative to the bioassay's susceptibility to false positives due to off-target effects produced by test substances.

Shen and colleagues took an siRNA (small interfering RNA)-based loss-of-function screening approach to identify potential druggable targets that control the HIF-1 pathway [63]. A non-small-cell lung carcinoma H1299 cell–derived stable HIF-1 reporter cell line (H1299_HRE) was established. Expression of this luciferase reporter was under the control of the HRE from the enolase promoter and hypoxic exposure increased luciferase activity by three- to fivefold. For assay

validation, H1299_HRE cells were transfected with an HIF-1A siRNA as a positive control and a scramble siRNA as a negative control. The transfected cells were exposed to hypoxic conditions and the luciferase activities determined. The assay used in this initial screening had Z-factor values that were greater than 0.5. This indicated that the H1299_HRE-based reporter assay was suitable for high-throughput screening. The Z-factor is a simple statistical parameter used to assess assay quality, defined as $\text{Z-factor} = 1 - [3 \times (\sigma_p + \sigma_n)]/|\mu_p - \mu_n|$ [64]. The four parameters are the means (μ) and the standard deviations (σ) of the controls [positive (p) and negative (n)]. An ideal assay will yield a Z-factor of 1, an excellent assay Z-factor between 0.5 and 1.0, a marginal assay Z-factor between 0 and 0.5, and a highly variable assay will produce a Z-factor less than 0.

Based on the Z-factor analysis, the H1299_HRE-based reporter assay was considered suitable for high-throughput screening (HTS) and was used to examine an siRNA library against approximately 4,000 druggable targets. However, confirmation studies revealed that the hits identified in the primary assay were caused by siRNA-mediated off-target gene silencing that nonspecifically inhibited HIF-1. The H1299_HRE cells were used to establish a 384-well-based reporter assay for HIF-1 activity. A library of 691,200 small molecules was examined in this assay (Z-factor 0.18), and this chemical genomics approach led to the identification of alkyliminophenylacetate compounds as potent HIF-1 inhibitors. The low Z-factor indicates that this latter HTS assay would have only marginal reliability.

22.8 Bioassay Method–Specific Experimental Artifacts

Every assay format has method-specific advantages and disadvantages. For the purpose of HTS screening, most bioassays employ colorimetric, fluorescent, or luminescent methods to measure the outcomes. In the case of marine organism extracts, many substances in the crude extracts can potentially interfere with fluorescence- or luminescence-based methods due to autofluorescence or quenching of the fluorescent and/or luminescent signals. To reduce the rate of false positives associated with method-related experimental artifacts, the active samples should be first evaluated in a secondary screening system that acts as a control for substances that particularly interfere with the selected bioassay method. For example, active extracts that inhibit HIF-1 activation in our previously described T47D cell-based reporter assay are then examined in T47D cells that are transfected with a control construct (pGL3-control) [49, 65]. The pGL3-control construct contains a simian virus 40 (SV40) promoter and enhancer sequences that strongly express a modified firefly luciferase reporter gene (luc+) in many mammalian cell lines. A false positive that inhibits the luciferase reaction and/or expression will also suppress luciferase activity in the tumor cells that have been transfected with the control construct. Similarly, active samples identified using a fluorescence-based method can be evaluated in a separate fluorescence-based assay for an unrelated target. In general, false positives associated with

a specific method will inhibit all similar assays that examine unrelated targets using the same assay method. In this respect, it is critical to establish a screening protocol that incorporates procedures that remove method-related experimental artifacts.

22.9 Identification of Active Marine Natural Products

Just as demonstrating clinical efficacy results is the most definitive form of target validation, the identification of therapeutically effective compounds that regulate the selected molecular target can be considered strong practical proof of bioassay validation. The subject of molecular-targeted anticancer marine natural product discovery has been reviewed with respect to antitumor compounds that were specifically identified through the use of molecular-targeted bioassays [6].

To date, only a relatively small number of marine natural products have been found to inhibit HIF-1 activation in various tumor cell lines (reviewed in 14). The University of Mississippi HIF discovery program identified most of these marine-derived HIF-1 inhibitors [66]. Over 10,560 lipid extracts of marine invertebrates and algae crude extracts have been evaluated in the primary T47D human breast tumor cell–based reporter assay for the ability to inhibit hypoxia-induced HIF-1 activation [49]. As previously described, the extracts were examined in the T47D cell-based reporter assay (5 μg/mL = 5 ppm) and the threshold for actives was set at ≥70% inhibition. Actives from the primary screen were subjected to a panel of additional bioassays designed to aid in the confirmation, prioritization, deselection, and dereplication process (Fig. 22.5).

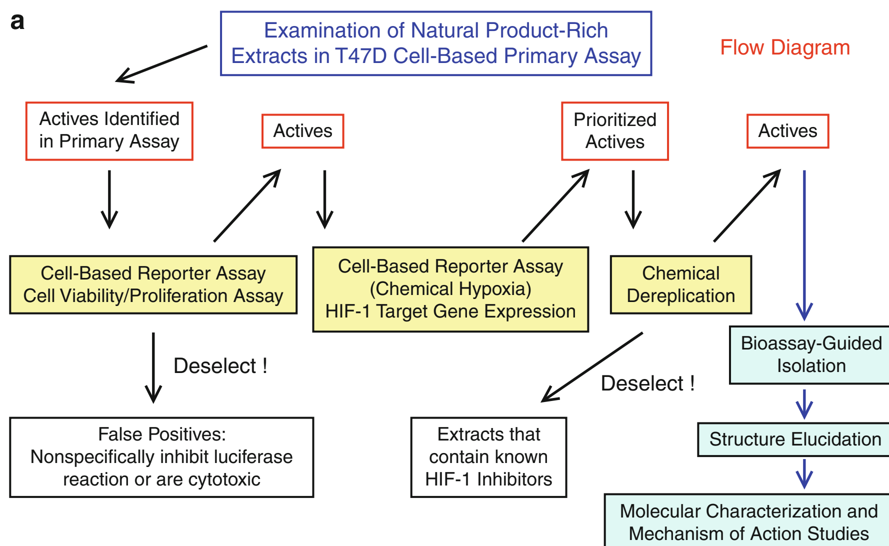


Fig. 22.5 (continued)

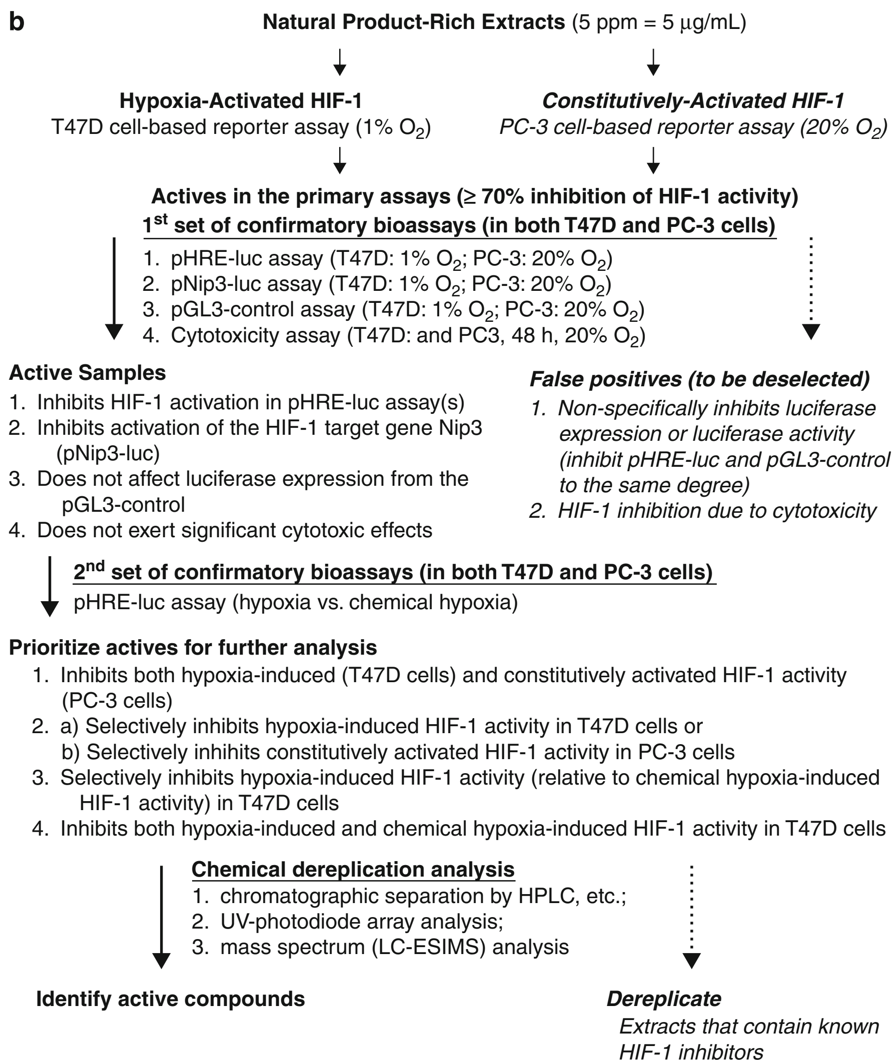


Fig. 22.5 Flow diagrams depicting representative bioassays used in HIF-1 inhibitor confirmation, prioritization, deselection, and dereplication process. (**a**) General flow diagram outlining HIF-1 bioassay evaluation system; **(b)** specific experimental protocols used to confirm, prioritize, deselect, and dereplicate extracts, fractions, and purified compounds detected in primary HIF-1 luciferase reporter assay system

Since the number of samples in the NCI Open Repository of marine invertebrate and algae extracts [67] was relatively large (10,560 lipid extracts), screening results from this large group of samples were analyzed and summarized in the following synopsis. A total of 109 active extracts (1% hit rate) were identified in the primary screen (Fig. 22.6a). Over one-half of the active samples were lipid extracts of marine sponges (57 out of 109). Although, a broad range of organisms were shown to have HIF-1 inhibitory activity (sea hares, algae, echinoderms, bryozoans, nudibranchs, and cnidarians). Active extracts were subjected to additional bio-assays that include the following: (1) a pNip3-luc reporter assay for detecting substances that inhibit HIF-1 activation [68]; (2) a pGL3-control (Promega) reporter assay to deselect false positives that inhibit luciferase expression/activity [49, 65]; (3) a cell proliferation/viability assay to exclude cytotoxic extracts; and (4) a pHRE-TK-Luc reporter assay to confirm the initial results and discern the specificity toward the inhibition of low-oxygen (1% O_2) hypoxia-induced HIF-1 activation relative to the ability of chemical hypoxia [1,10-phenanthroline (10 μM)] to induce HIF-1 activation. Between 80% and 90% of the active extracts were confirmed upon retesting. Less than one-half of the samples suppressed tumor cell proliferation/viability by more than 50%. Over 50% of the active extracts showed selectivity for inhibiting hypoxia-induced HIF-1 activation over chemical hypoxia-induced HIF-1 activation. From the original 109 extracts that were active in the primary T47D cell-based reporter assay, 40 extracts (0.38% of the 10,560 extracts examined) withstood confirmatory secondary testing and were prioritized for further study (Fig. 22.6b). Samples from the NCI Open Repository are available on a first-come-first-serve basis, and the availability of many extracts is extremely limited. Supply-based prioritization suggested that only 33 extracts (out of the original 109 active extracts) were available from the NCI-Developmental Therapeutics Program (NCI-DTP) in sufficient quantity (typically 2–4 g) to guarantee any probability of successful bioassay-guided isolation and structure elucidation efforts, to ensure that the quantities of active pure compounds isolated would be adequate for in vitro mechanistic studies, and/or to evaluate for efficacy in vivo. Based on results from the biological confirmatory studies and the quantity of samples available, about one-third of the samples were assigned as "high-priority" for isolation efforts.

This synopsis exemplified several of the advantages of sourcing the NCI Open Repository – the large number of samples available for HTS assays, low-cost access to chemical diversity, logistical ease relative to investigator-initiated collection efforts and intellectual property negotiations, and the ability to identify new pharmacological activities even for "known" natural products. Efforts to source NCI samples are also associated with a number of disadvantages. These include limited sample availability due to previous use by other investigators and a lack of samples from microbial sources. Despite these possible disadvantages, a variety

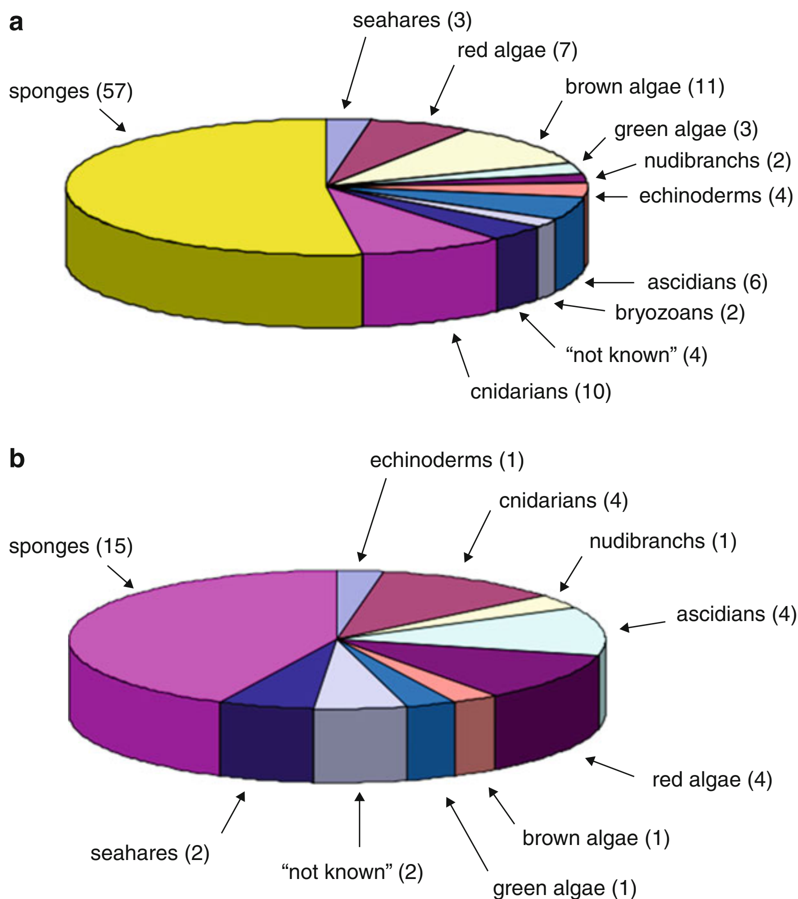


Fig. 22.6 Charts illustrating numbers and distribution of marine organism extracts found to inhibit HIF-1 activation in a cell-based reporter assay. Lipid extracts (10,560) from the NCI Open Repository of marine invertebrate and algae extracts were examined for the ability to inhibit hypoxia-induced HIF-1 activation in a T47D breast tumor cell line–based luciferase reporter assay. (**a**) Primary screening revealed 109 extracts strongly inhibited HIF-1 activation in the primary assay (a $\geq$70% decrease in pTK-HRE3-Luc). (**b**) Only 40 extracts remained following secondary evaluation in assays designed to confirm and prioritize the samples that were shown to be active in the primary reporter assay. Extracts from a variety of marine organisms were found to contain substances that potently inhibited hypoxic-induced HIF-1 activation (Figures and caption reproduced with the permission of D.G. Nagle [© 2010] at the University of Mississippi)

Table 22.1 Examples of marine natural products that inhibit HIF-1 activation

Compound name (no.)	Source	IC_{50} (μM)[a]	References
Laurenditerpenol (**4**)	*Laurencia intricata*	0.4	[70]
7-Hydroxyneolamellarin (**5**)	*Dendrilla nigra*	1.9	[53]
Furospongolide (**6**)	*Lendenfeldia* sp.	2.9	[72]
Sodwanone V (**7**)	*Axinella* sp.	15	[73]
9,9′-Oxybis-neocomantherin (**8**)	*Comantheria rotula*	0.8	[65]
Neocomantherin (**9**)	*C. rotula*	1.9	[65]
Comantherin (**10**)	*C. rotula*	2.7	[65]
5,8-Dihydroxy-6-methoxy-2-propyl-4*H*-benzo[*g*]chromen-4-one (**11**)	*C. rotula*	0.6	[65]
8-*O*-Methylneocomantherin (**12**)	*C. rotula*	2.0	[65]
TMC-256A1 (**13**)	*C. rotula*	0.9	[65]
Comaparvin (**14**)	*C. rotula*	3.0	[65]
Mycalenitrile-6 (**15**)	*Mycale* sp.	7.8	[74]
Mycalenitrile-7 (**16**)	*Mycale* sp.	8.6	[74]
Caulerpin (**17**)	*Caulerpa* spp.	10	[75]
Strongylophorine 2 (**18**)	*Petrosia strongylata*	8[b]	[76]
Strongylophorine 3 (**19**)	*P. strongylata*	13[b]	[76]
Strongylophorine 8 (**20**)	*P. strongylata*	6[b]	[76]
Latrunculin A (**21**)	*Negombata magnifica*	6.7	[52]

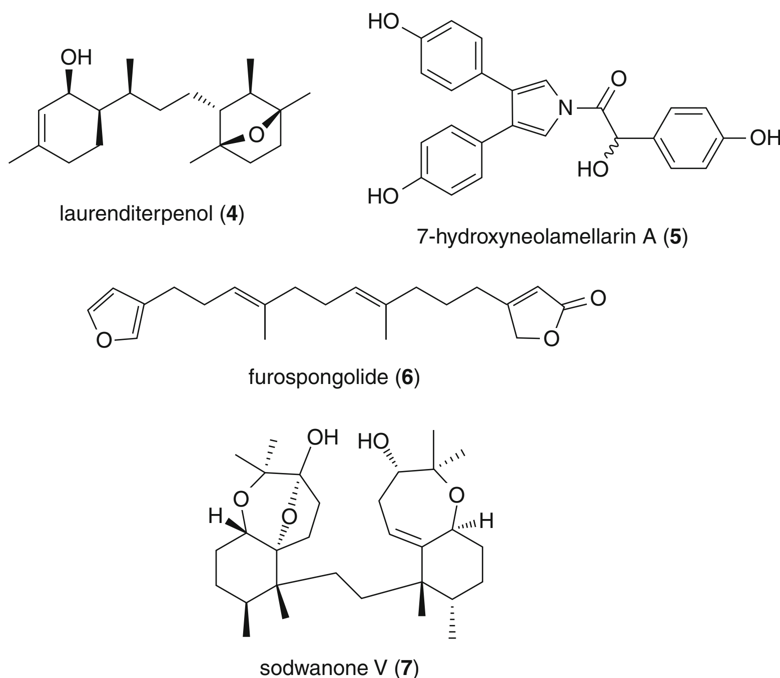
[a] IC_{50} values for hypoxia (1% O_2)-induced HIF-1 activation in a T47D cell-based reporter assay, unless otherwise noted

[b] EC_{50} values for hypoxia (1% O_2)-induced HIF-1 activation in a U251 cell-based reporter assay

of novel HIF-1 inhibitors were discovered from the available samples, and some of these were described in two recent reviews on natural product–derived inhibitors of HIF-1 (Table 22.1) [14, 69].

Once the active compounds were isolated, a combination of spectroscopic and spectrometric methods including multidimensional NMR spectroscopy and high-resolution mass spectrometry (HRMS) were employed to elucidate the structures of active compounds. This unique HIF-1 inhibitor discovery effort that combined the chemical diversity offered by natural products with effective and reliable bioassays has resulted in the identification of some of the most potent HIF-1 inhibitors known [16]. The first marine natural product found to inhibit hypoxia-induced HIF-1 activation in tumor cells was from the lipid extract of a Jamaican collection of the red alga *Laurencia intricata* Lamouroux (Rhodomelaceae). Laurenditerpenol (**4**), a novel bicyclic diterpene, was found to be the active constituent of the alga. Compound **4** inhibited hypoxia (1% O_2)-induced HIF-1 activation in T47D cells at submicromolar concentrations (Table 22.1) [70]. The absolute configuration of **4**

has recently been defined by total synthesis [71]. Total synthesis may also afford sufficient quantities of **4** and various related isomers for further biological evaluation and a study of structure-activity relationships (SARs).



laurenditerpenol (**4**)

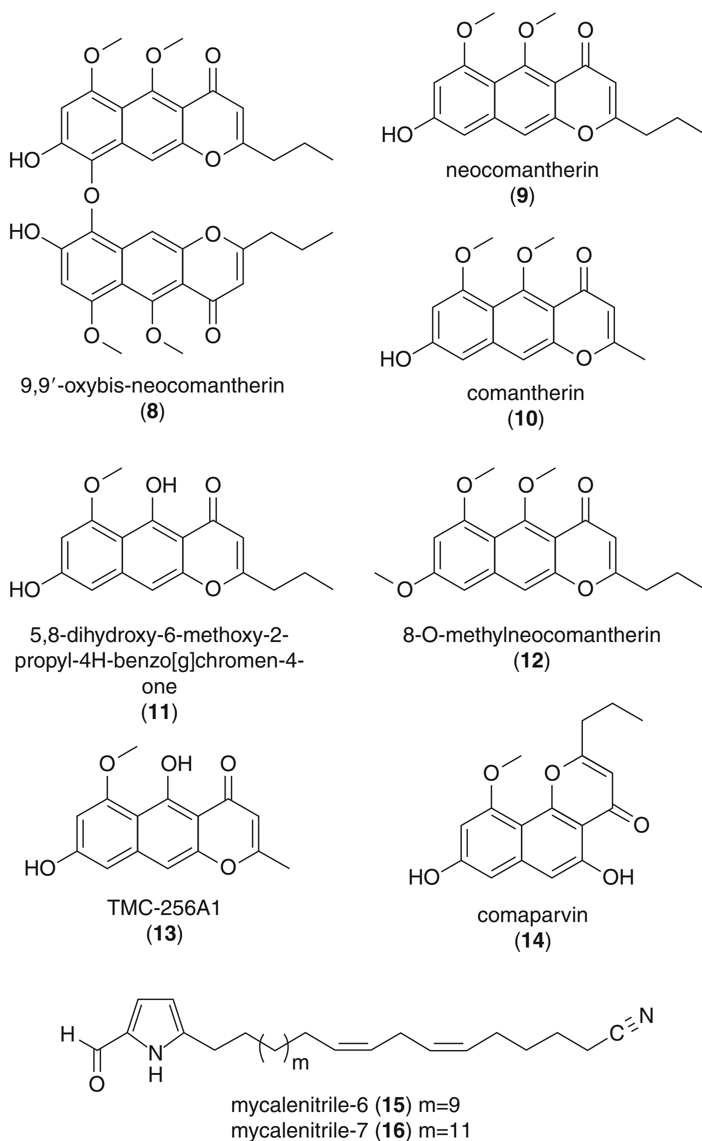
7-hydroxyneolamellarin A (**5**)

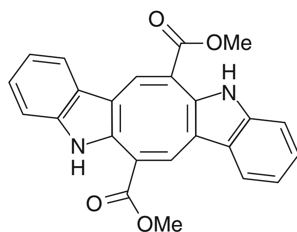
furospongolide (**6**)

sodwanone V (**7**)

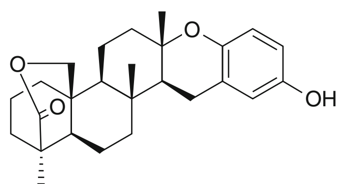
The NCI Open Repository of marine invertebrate and algae extracts has proven to be a valuable source of small-molecule inhibitors of HIF-1 activation. These marine natural product–based HIF-1 inhibitors include the sponge metabolites 7-hydroxyneolamellarin (**5**) [53], furospongolide (**6**) [72], and a series of sodwanone and yardenone triterpenoids [i.e., sodwanone V (**7**)] [73]. Similarly, benzo[*g*]chromen-4-one and benzo[*h*]chromen-4-one pigments (**8**–**14**) from a tropical marine crinoid (Comasteridae) were also found to inhibit hypoxia-induced activation of HIF-1 [65]. However, these benzochromenones were not further pursued because their ability to inhibit HIF-1 activation did not translate into a significant effect on the HIF-1 target genes examined (i.e., secreted VEGF) and, for all practical purposes, these crinoid pigments are now considered possible nuisance compounds. Recently, bioassay-guided fractionation of an active extract of a *Mycale* sp. sponge yielded 18 new and 8 previously reported lipophilic 2,5-disubstituted pyrroles, collectively known as mycalenitriles and mycalazals [e.g., mycalenitrile-6 (**15**) and mycalenitrile-7 (**16**)] [74]. The red pigment caulerpin (**17**) was first isolated from green algae of the genus *Caulerpa* [75].

Caulerpin (**17**) inhibited hypoxia-induced and 1,10-phenanthroline-induced HIF-1 activation [51]. The angiogenic factor VEGF is regulated by HIF-1. Caulerpin (10 μM) suppressed hypoxic induction of secreted VEGF protein and the ability of hypoxic T47D cell-conditioned media to promote tumor angiogenesis in vitro.

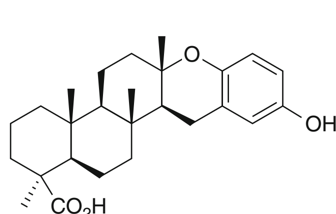




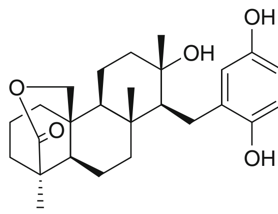
caulerpin (**17**)



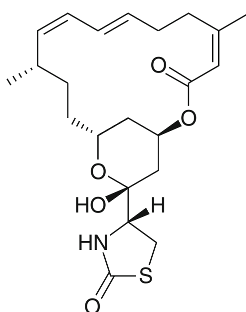
strongylophorine 2 (**18**)



strongylophorine 3 (**19**)



strongylophorine 8 (**20**)



latrunculin A (**21**)

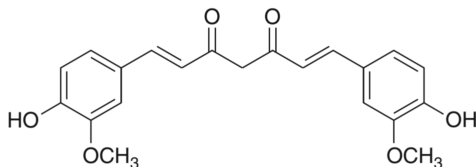
In addition to sourcing from the NCI Open Repository, several laboratories have reported the isolation and identification of HIF-1 inhibitors from their field collections of marine organism extracts. Ireland and coworkers recently found that an extract from a Papua New Guinea collection of the sponge *Petrosia* (*Strongylophora*) *strongylata* significantly inhibited HIF-1 activation at 1 μg/mL [76]. Through a process of bioassay-guided isolation, three previously reported strongylophorine meroditerpenoids were identified to be responsible for the

observed HIF-1 inhibition. Strongylophorines 2 (**18**), 3 (**19**), and 8 (**20**) inhibited hypoxia-induced HIF-1 activation in a genetically engineered U251 human glioma cell–based luciferase reporter assay [76]. The Red Sea sponge macrolide latrunculin A (**21**) disrupts actin polymerization and inhibits microfilament formation by reversibly binding to actin monomers [77–79]. This sponge-derived actin inhibitor has recently been shown to inhibit hypoxia-induced HIF-1 activation in T47D cells [52]. Not only are these marine natural products inhibitors of HIF-1 activation in tumor cells, but many appear to function through mechanisms that have not yet been recognized to regulate HIF-1 activity. A summary of the marine-derived HIF-1 inhibitors discovered in this program is provided in Table 22.1.

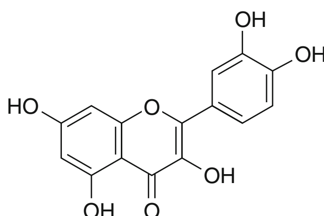
22.10 Dereplication of Nuisance Compounds

The concept of "nuisance" compounds is highly subjective and depends on the focus of the individual antitumor discovery group. In high-throughput screening, nonselective compounds that exert a variety of effects on various assay systems are typically considered nuisance compounds. Similarly, if the research program is solely interested in the discovery of novel chemical entities with a particular pharmacological activity, the researchers may consider all previously identified known compounds that show activity in other systems to be nuisance compounds. Each particular type of bioassay method is associated with assay-specific nuisance compounds. Chemically reactive compounds, including certain phenolic compounds, may nonselectively bind to proteins and inhibit enzyme-based assays [80, 81]. Pigments and other colored natural products can interfere with colorimetric assays. It is widely known that fluorescence-based assays may be susceptible to interference by fluorescent natural products [e.g., curcumin (**22**)] or compounds such as the flavonoid quercetin (**23**) that acts to quench the fluorescence [82]. Poorly controlled fluorescence-based bioassays have been used to support the premise that these natural products regulate a plethora of diverse molecular targets. Although less appreciated, pigmented and fluorescent natural products may also interfere with luciferase reporter gene assays that rely on fluorescent reagent formulations to enhance their light readouts. Cytotoxic natural products may be considered nuisance compounds when observed in cell-based bioassays that screen for inhibitors of a tumor cell selective molecular target. Similarly, compounds that do not penetrate cell membranes may be regarded as nuisance compounds in solution-based enzyme/protein-based in vitro assays. In such cases, whether or

not certain compounds are regarded as nuisance compounds depends on the particular objectives of the screening program.



curcumin (**22**)



quercetin (**23**)

Just as in other forms of natural product screening, molecular-targeted drug discovery programs that aim to identify marine natural product–derived anticancer agents must avoid the unnecessary replication of previously identified inhibitors (or activators) of the selected molecular target and common substances that exert nonselective effects on biological systems. One of the most powerful and inexpensive means to reduce the replication of chemical isolation and structure elucidation efforts in marine natural products is the use of chemotaxonomic literature related to the particular marine organism. Databases of marine natural products literature are readily accessible to most molecular-targeted drug discovery groups. These include general chemical (Chemical Abstracts Service (American Chemical Society)-SciFinder/SciFinder Scholar [83]) and biological (U.S. National Library of Medicine/National Institutes of Health-MEDLINE database accessible through PubMed [84]) databases. Highly specialized marine natural product databases (Marinlit [85]) that include searchable NMR and other spectroscopic data are also available and can greatly enhance chemical dereplication efforts. These databases can be used to identify known compounds and to distinguish previously reported redundant biological activities that may be associated with a particular marine natural product. Dereplication with chemotaxonomic data from chemical and biological literature databases is most efficient when examining well-characterized species of marine invertebrates and algae, but may be only of limited utility in screening programs that focus on poorly characterized new species of cultured marine microbes. One potential limitation of the use of chemotaxonomic literature for chemical dereplication efforts is that many species of marine organisms are

poorly characterized chemically as well as taxonomically [2, 86]. Since most marine natural products chemists are not in the habit of regularly reporting known compounds when they are found to occur in new species, estimates of the potential chemical diversity of many readily collectible species may be significantly underestimated. Chemical and biological dereplication for many of these species is often achievable by analysis of the chemotaxonomic and pharmacological literature related to other members of the same genera or taxonomic family. While practical limitations exist, the importance of chemical and biological databases in reducing the need for unnecessary marine natural product isolation and structure elucidation efforts cannot be overstated.

The subject of natural product dereplication has been the subject of recent reviews [87]. Compound dereplication strategies also commonly include various libraries of compound data sets that are used with a "hyphenated" technique that combines a purification method with a spectroscopic or spectrometric detection system. Sarker and Nahar have recently reviewed the field of hyphenated separation and spectroscopic/spectrometric techniques with respect to various classes of natural products [88]. Typical examples may include traditional methods such as high-performance liquid chromatography (HPLC) coupled with ultraviolet (UV) photodiode detection. Dereplication efforts have more recently come to rely on methods that combine liquid chromatography (LC) and mass spectrometry (MS) or nuclear magnetic resonance (NMR) spectroscopy. Tandem LC-MS and LC-MS-MS methods are among the most sensitive means to dereplicate known compounds. While inherently less sensitive, coupled LC-NMR methods have the advantage that they can be simultaneously used for dereplication and the structure elucidation of new natural products. Hyphenated techniques and their use in the dereplication of natural products are further described in recent reviews [87].

The expenditure of unnecessary efforts due to the occurrence of natural products that act by relatively general means to produce numerous nonspecific effects in bioassay systems must also be reduced. The previously described methods of compound dereplication can be used to identify known compounds that exert nonselective effects on assay systems. Alternatively, experimental procedures with appropriate controls can be designed directly into the assay systems to dramatically cut the number of bioassay "hits" that result from active compounds with no selectivity for the molecular target. This strategy can readily deselect nuisance compounds without requiring any sample purification, chemical analysis, or other dereplication procedure. Examples of this type of bioassay design–based nuisance compound dereplication include the use of alternative isoforms of a particular enzyme in a parallel screening platform [89] and the concurrent measurement of cell viability with cell-based bioassays for a molecular target that should not produce a cytotoxic effect on certain cell types.

The need to dereplicate known marine natural product HIF-1 inhibitors has only recently emerged as several groups have begun to identify marine invertebrate and

algae compounds that inhibit HIF-1 activation (reviewed in 14). The marine natural product–based HIF-1 inhibitors described in Table 22.1 include algal metabolites, crinoid benzochromenone pigments, latrunculin actin inhibitors, terpenes, and various lipids from marine sponges. In addition, various HIF-1 discovery groups have examined terrestrial natural products [15, 16] and synthetic pure compound libraries [63] and have reported that compounds that regulate certain central cellular biochemical processes may also suppress HIF-1 activation. Both academic and industrial HIF-1 screening efforts have found that mitochondrial electron transport chain (ETC) inhibitors suppress HIF-1 activation by hypoxia [63, 70], presumably by interfering with mitochondrial reactive oxygen species (ROS)-mediated signaling and destabilizing HIF-1α protein under hypoxic conditions. The emerging role of mitochondrial reactive oxygen species (ROS) on the regulation of HIF-mediated hypoxic signaling is highlighted by a recent review by Hamanaka and Chandel [90]. New classes of unique ETC inhibitors have been identified that may prove to be pharmacological leads or valuable probes of HIF-1 signaling and mitochondrial function. However, at some point, these terrestrial and marine mitochondrial ETC inhibitors may be deemed biochemically active nuisance compounds, for the purpose of HIF-1 inhibitor drug discovery. Similarly, the expression of HIF-1α protein and subsequent activation of HIF-1 can be strongly suppressed by pharmacologically active compounds that inhibit eukaryotic protein translation [15, 50, 91, 92]. While translation inhibitors have been found to inhibit HIF-1 signaling in tumor cells, the off-target effects associated with the generalized inhibition of protein synthesis may limit the therapeutic potential of these compounds and render such agents to be essentially nuisance compounds, at least for the purpose of bioassay dereplication.

22.11 Concluding Remarks

The field of antitumor marine natural products research has evolved over the years to incorporate an emphasis on molecular-targeted drug discovery. These changes have dramatically increased the appreciation among most natural products chemists for molecular and cell biology in natural product–based drug discovery. This shift in focus has also spurred a similar need for a general understanding of the factors that must be considered when using modern molecular-targeted antitumor bioassays. Researchers working in natural products must be acutely aware of the nature and validity of their selected molecular target, consider what particular methods their assays will use to measure the target processes, make sure appropriate controls are used, appreciate how the data will be analyzed, understand how the assay method will be validated, and establish suitable means for chemical dereplication. The scientific validity and clinical potential of any newly discovered antitumor natural product are only as solid as the reliability of the biological data that support its potential activity. This is true not only for the researchers involved in mechanism-based drug discovery; it is also true for those involved in the peer

review process. If manuscripts and grant proposals that involve molecular-targeted natural products research are not held to acceptable standards, the value of this discipline to the broader drug discovery community will be diminished. Therefore, an appreciation for all of these factors is essential for modern molecular-targeted antitumor drug discovery. Through mechanism-based drug discovery efforts, marine natural products have been identified that potently inhibit the hypoxia-induced activation of HIF-1 in tumor cells. As an important antitumor molecular target, the bioassay systems used in the identification of new HIF-1 inhibitors provide compelling examples to illustrate the various components of molecular-targeted bioassay design and analysis that are essential for the discovery of antitumor marine natural products.

22.12 Study Questions

1. Why is it important for natural products chemists (even those that do not perform their own bioassays) to have an understanding of the biological principles involved in bioassays and the important considerations used to evaluate assay quality?
2. Why are thoroughly validated and established molecular targets often less-exciting targets for novel drug discovery than newer, less fully validated, targets?
3. Why is the mere overexpression of a particular gene (or protein) in a disease condition insufficient to validate the gene (or protein) as a disease-specific molecular target?
4. What are the potential consequences of poor or inadequate molecular target validation in the drug discovery and development process?
5. What features of the transcription factor hypoxia-inducible factor-1 (HIF-1) make it a suitable representative molecular target to illustrate each of the principles involved in quality bioassay design and analysis?
6. What is the difference between bioassay validation and target validation?
7. Why is it critical for bioassay method validation to include both positive and negative control compounds?
8. Why is the definition of a bioassay “nuisance” compound in any particular bioassay considered to be subjective?
9. Why is compound dereplication so vital to a successful high-throughput screening assay–based drug discovery effort?
10. Chemical and biological literature databases are among the most economical sources of compound information regarding the production of specific natural products by any given species of organism. What are the practical limitations of literature databases in regard to the marine natural product dereplication efforts?

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