2.20  Natural Products as Probes of Selected Targets in Tumor Cell Biology and Hypoxic Signaling

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2.20.1  Introduction

More than 50 years have past since the development of the first chemotherapeutic drugs. As clearly illustrated by several recent reviews, over 60% of the antitumor agents used in the clinic are natural products or small molecules based upon natural product leads.1–5 Natural products and natural product-derived agents continue to remain a substantial component of new chemical entities that are currently under development for cancer treatment.1–7 However, except for a small number of hormone-based treatment options, most of the small molecule-based chemotherapeutic drugs that were developed during the first 50 years have been relatively nonselective cytotoxic agents. These agents exhibit some level of efficacy in the treatment of certain forms of leukemia, lymphoma, and other malignancies that result from the proliferation of rapidly dividing cells. The relatively narrow therapeutic margins have limited their application toward the treatment of many solid tumors (see Chapters 2.19, 3.06–3.08).

Perhaps one reason why many plants, microbes, and marine invertebrates have been found to produce cytotoxic natural products is due to ecological pressures that select for the production of cytotoxic compounds as chemical defenses.8,9 We have previously proposed that it is also logical to conclude that the considerable number of cytotoxic natural products that have been discovered is an unintended consequence of the prevalent use of cytotoxicity-based screening methods by antitumor drug discovery programs.10 If this assumption is correct, then the selection of cytotoxicity as the primary anticancer drug target has been partially responsible for limiting the pool of tumor cell-selective chemotherapeutic agents that have been developed over the past decades. This premise should not be considered as a fatalistic indictment against the sometimes heroic attempts to discover and develop new drugs to treat cancer, but must be recognized as an exciting and potentially huge untapped opportunity for natural product research and discovery. Recently, dramatic progress has been made in understanding the unique physiological, biochemical, and molecular differences between normal and
malignant cells. Considerable knowledge of tumor cell biology has come from the study of natural products that modulate specific cellular processes within tumors. Small molecule-based pharmacological interference with vital genetic and biochemical processes within tumor cells has facilitated the discovery of important molecular targets for antitumor drug discovery. Antitumor chemotherapeutic agents have greatly contributed to this progress. However, some of the most important molecular probes of tumor cell biology have been found among the considerably larger number of biologically active natural products that may be deficient in pharmaceutically desirable properties and lack apparent druggability.

The fact that a large percentage of antitumor drugs continue to be natural products or derived from natural product leads indicates that these compounds remain an essential component of cancer chemotherapy. The study of biology has evolved to incorporate new methods such as transcriptome profiling, proteomics, and metabolomics to study various aspects of molecular and cell biology. In this same fashion, it is likely that bioactive natural products may find their most important potential as molecular probes of cell biology. This premise may seem biased, considering the well-recognized biomedical utility of natural products. However, while a single drug can play a significant role as a specific treatment for a disease, a biomolecular or pharmacological probe that facilitates the discovery of previously unknown biological processes can assist in the discovery of an entire array of new methods to treat disease and can streamline the development of a myriad of drugs that may have little or no resemblance to the original natural product-based molecular probe. To illustrate this point, consider the narcotic analgesic morphine. Morphine remains an important drug. Yet, as a probe of cell biology, morphine (and related compounds) has provided the foundation for opioid receptor pharmacology, endorphin research, and the development of numerous therapeutic options for the treatment of pain and other conditions.

Just as in the case of normal eukaryotic cells, tumor cells are complex 'machines' that tie together the function of numerous cellular organelles and a multitude of biochemical processes in order to survive and grow. The illustration in Figure 1 depicts a typical animal cell. Many aspects of tumor cell biology are both interconnected and interdependent in nature. In this light, the order of natural product molecular probe presentation in this chapter may seem somewhat subjective. This chapter aims to focus on the use of natural products as tools to investigate the functions of various cellular organelles and protein stability, with special relevance to tumor cell biology. This includes examples of molecular probes that are used to examine a few selected organelles (e.g., endoplasmic reticulum, Golgi apparatus, mitochondria) and natural products that serve as tools to investigate the molecular chaperone heat shock protein-90 (Hsp90) and the ubiquitin–proteasome system. Further, the interdependent nature of cellular processes is exemplified by the process of oxygen-regulated tumor cell signaling. Hypoxia-inducible factor-1 (HIF-1) is a transcription factor that promotes tumor cell adaptation and survival under hypoxic conditions. Within hypoxic regions of tumor masses, complex systems of biochemical processes and signal transduction networks simultaneously regulate HIF-1 activation, which in turn controls the expression of >100 hypoxia-responsive genes.
It is not within the scope of this chapter to comprehensively examine the hundreds of ways in which natural products have served as probes of tumor cell biology. This chapter also does not attempt to fully explore all of the numerous ways natural products have provided information regarding the interconnected biochemical and physiological networks required to coordinate vital cellular processes. Rather, the aim is to describe some of the interconnected cellular pathways involved in the regulation of hypoxic signaling and to highlight the benefits that can be derived, not from looking at natural products simply as drug candidates, but from tapping into the tremendous potential of these agents as pharmacological or biomolecular probes to specifically differentiate the unique properties that distinguish malignant cells from normal cells.

### 2.20.2 Organelle Function and Protein Stability

#### 2.20.2.1 Endoplasmic Reticulum

The endoplasmic reticulum (ER) is an interconnected network of branching tubules and flattened sacs that extend throughout the entire cytosol in eukaryotic cells. The ER lumen is a highly convoluted space enclosed by the largest set of internal membranes found within eukaryotic cells (Figure 2). The primary function of the ER is the synthesis and modification of proteins and the synthesis of various lipid molecules. The ER is composed of two structural components known as the smooth ER and the rough ER. The smooth ER is responsible for the biosynthesis of certain fatty acids and phospholipids and lacks membrane-bound ribosomes. The rough ER appears as a series of ‘rough’ interconnected vesicles because most of the surface of the rough ER is lined with bound ribosomes. These ribosomes within the ER produce many of the proteins required by membranes and certain organelles, but their most critical function is the production of nearly all secreted proteins. In certain specialized cells, the ER is also involved in functions that range from calcium sequestration to regulating detoxification reactions.

Most of the soluble and membrane-bound proteins synthesized in the rough ER are glycosylated through the covalent addition of N-linked oligosaccharides. These proteins are further modified in the Golgi apparatus. Tunicamycin (1–4) prevents N-linked glycosylation in the ER and has been widely used as a molecular probe.
to investigate the role of N-glycans in the maturation, secretion, and function of glycoprotein and to induce ER stress.\textsuperscript{20} As a family of homologous nucleoside antibiotics isolated from fermentation broth of \textit{Streptomyces lysosupericus},\textsuperscript{21–23} tunicamycin (1–4) contains mainly tunicamycins II, V, VII, and X (also referred to as A, B, C, and D, respectively). Tunicamycins are composed of a uracil unit, N-acetylglycosamine, tunicamycin (11 carbon aminodialdose), and a tunicamycin-linked fatty acid that varies between homologues in the chain length, degree of saturation, and branching. First discovered as an antibiotic with potent antiviral activity,\textsuperscript{22} tunicamycin was shown to interfere with glycoprotein synthesis in both yeast and mammalian systems.\textsuperscript{24,25} Subsequent studies revealed that tunicamycin inhibits eukaryotic GlcNAc-1-P transferase, blocking the transfer of GlcNAc from UDP-GlcNAc to dolichyl phosphate.\textsuperscript{26} Recent advances in the study and development of tunicamycin as an antimicrobial agent are summarized by Kimura and Bugg.\textsuperscript{27} Both the tumor microenvironment and genetic mutations contribute to the perturbation of ER homeostasis in tumor cells that activates the adaptive unfolded protein response (UPR) to modify protein entry, folding, and degradation in the ER.\textsuperscript{28} Tunicamycin has been used in hundreds of published research studies as a molecular probe to investigate the pathways that regulate the ER stress response.

Certain natural products indirectly induce ER stress. For example, agents that disrupt the functions of the Golgi apparatus can induce ER stress. Additionally, the plant-derived (\textit{Thapsia garganica}) sesquiterpene lactone thapsigargin (5) is an ATPase inhibitor, which inhibits intracellular Ca\textsuperscript{2+} transport and is also used as a probe to study ER stress.

\subsection*{2.20.2.2 Golgi Apparatus}

Unlike prokaryotic cells, eukaryotic cells are elaborately compartmentalized into functionally distinct organelles enclosed by membrane. As depicted in Figure 2, the Golgi apparatus (aka Golgi complex, Golgi) is an intricate network of flattened membrane-enclosed sacs (the Golgi stack), bordered by the tubulovesicular \textit{cis}- and \textit{trans}-Golgi networks (CGN and TGN, respectively). Located in proximity to the nucleus and adjacent to the centrosome, the Golgi stack is composed of three functionally and morphologically distinct compartments – the \textit{cis}-, \textit{medial}-, and \textit{trans}-Golgi. As a major component of the secretory and endocytic pathways, the Golgi is not only involved in the transport of proteins, but also plays a crucial role in protein sorting and modification. For example, newly synthesized proteins that are transported within the secretory pathway (i.e., plasma membrane, secretory, organelle resident proteins, etc.) are produced by ribosomes within the rough ER and shuttled within the lumen of the ER to membrane-bound portions of the rough ER that are devoid of ribosomes. Those proteins that are properly folded and have undergone initial glycosylation are packaged into membrane-lined transfer vesicles and bud off from the ER. The coat protein complex II (COPII)-coated transport vesicles move toward the \textit{cis}-region of the Golgi that is directly adjacent to the rough ER, lose their coats, and fuse to become the ER–Golgi intermediate compartment (ERGIC). Numerous ERGICs subsequently merge to form the CGN and the proteins enter the
Golgi apparatus. Secretory proteins then move progressively through the various regions of the Golgi apparatus where the proteins are post-translationally modified by various enzymes and shuttled to the TGN, the Golgi exit site where the proteins are sorted for the final destination. The proteins are then packaged into specific coated vesicles or tubulovesicular carriers that transport the cargo proteins to the destined location. For example, secretory vesicles that bud off from the TGN transport the proteins to storage vesicles that are eventually discharged from the cell through the process known as exocytosis. In addition to function as the exit site for Golgi, the TGN also serves as one of the major sorting sites for endocytosed proteins. The traffic of proteins through the Golgi apparatus includes both anterograde and retrograde transport. One example of retrograde traffic is the vesicular transport of resident ER proteins in COPI-coated vesicles from the ERGIC back to the ER. Natural products have played an important role as molecular probes to investigate the structure and function of the Golgi apparatus. Representative natural product-based molecular probes used in Golgi-related studies are summarized in the following paragraphs.

Brefeldin A (6) is a *Penicillium brefeldianum* fungal metabolite that contains a 13-membered macrocyclic lactone ring. Following the initial discovery by Takatsuki and Tamura that brefeldin A inhibits protein secretion by blocking the secretory pathway upstream of the Golgi apparatus, brefeldin A has become one of the most used molecular probes to investigate cellular organelle structural integrity and the regulation of membrane traffic. Brefeldin A and its effects on biological systems have been extensively reviewed. Microscopic and biochemical studies revealed the existence of a Golgi to ER retrograde pathway that could be disrupted by brefeldin A. Treatment of cells with brefeldin A causes rapid redistribution of the cis/medial Golgi proteins back to the ER and this leads to the blockade of ER to Golgi protein transport and causes disassembly of the Golgi complex. The Golgi reorganizes into necklace-like structures composed of tubules of 90 nm in diameter that lack the apparent coats normally associated with the microtubules, while the TGN condenses close to the microtubule organizing center (MTOC). The effects of brefeldin A on both protein transport and Golgi structure are reversible. This retrograde transport requires energy and brefeldin A does not cause redistribution of proteins from the TGN to the ER. The initial observation that brefeldin A causes immediate dissociation of the nonclathrin-coated vesicle coatomer from Golgi membranes subsequently led to the discovery of brefeldin A’s mechanism of action. The ADP-riboyllation factors (ARFs) are a family of small G proteins that regulate vesicular traffic through mechanisms such as the recruitment of coat polymers. The ARFs cycle between the GDP-bound inactive and GTP-bound active forms and the GDP/GTP exchange reaction is catalyzed by guanine nucleotide exchange factors (GEFs) that contain the Sec7 domain. Structural studies revealed that brefeldin A inhibits ARF activation by binding to the interface of the transient, low-affinity complex of ARF–GDP–Sec7 and prevents the exchange reaction.
Ilimaquinone (7) was originally isolated from the marine sponge *Hippoponsea metachromia*. Takizawa and coworkers reported that ilimaquinone causes the vesiculation of Golgi membranes and inhibits the transport of proteins between successive Golgi cisternae in normal rat kidney (NRK) cells. In the presence of ilimaquinone, the Golgi membranes first became highly vesiculated but were retained in the pericentriolar region, then dispersed as clusters of tubulovesicular structures throughout the cytoplasm, and further fragmented into small vesicular structures (vesiculated Golgi membranes, or VGMs). The Golgi fragmentation effect of ilimaquinone is reversible, as indicated by the observation that the Golgi membranes reassemble within 2 h upon removal of ilimaquinone. Both the fragmentation and the recovery processes are energy-dependent and do not require protein synthesis. In the presence of ilimaquinone, protein transport from the ER to the cis-VGMs is not affected, while protein transport between successive Golgi compartments is inhibited. A later study revealed that ilimaquinone fragments Golgi membranes through the activation of heterotrimeric G proteins bound to the membrane. The Gβγ subunits activate protein kinase D, which then promotes the fission of transport carriers from the TGN. A recent study suggests that phospholipase D acts upstream of protein kinase D during ilimaquinone-induced Golgi fragmentation. The Golgi fragmentation effect of ilimaquinone is independent of its microtubule depolymerization activity. Ilimaquinone breaks down Golgi membranes regardless of whether microtubules are stabilized by taxol or depolymerized by microtubule disrupting agents. Upon removal of ilimaquinone, the reassembly of Golgi from the VGMs takes place in two steps: the VGMs first fuse together to form stacks of Golgi cisternae in a microtubule-independent process, and these Golgi stacks are subsequently transported to the perinuclear location via microtubules. Radeke and coworkers utilized an affinity chromatography-based approach and identified S-adenosylmethionine (SAM) synthetase, S-adenosylhomocysteinase, and methyl transferases as the cellular targets of ilimaquinone. Subsequently, it was found that the inhibition of methylation enzymes by ilimaquinone may cause Golgi vesiculation. Ilimaquinone has also been shown to inhibit gap-junctional communication independent of its effects on Golgi and protein transport.

Norrisolide (8) is a diterpene first isolated from the dorid nudibranch *Chromodoris norrisi*. It causes an irreversible fragmentation of the Golgi membranes in NRK cells. Unlike ilimaquinone (7), norrisolide did not affect the microtubule structure under conditions that disrupted Golgi membranes. Biochemical studies suggest that the perhydroindane core of norrisolide binds to a receptor on the Golgi membranes and triggers reversible fragmentation. When the side chain contains the uniquely reactive γ-lactone-γ-lactol moiety (i.e., as in norrisolide) or certain other electrophilic functionalities, the binding and fragmentation of Golgi membranes become irreversible. The new generation of synthetic trifunctional norrisolide probes may aid in the future identification of the precise molecular target of norrisolide and related Golgi inhibitors.

Monensin is a carboxylic ionophore isolated from the fermentation broth of a strain of *Streptomyces cinnamonensis*. Monensin can be readily incorporated into biological membranes due to its hydrophobicity.
and primarily mediates the transmembrane exchange of Na\(^+\) and H\(^+\) ions. The ion exchange promoting activity of monensin is highly dependent on the specific composition of the membrane. While monensin contains minor related compounds, the major constituent monensin A (9, especially the sodium salt form) is widely used as an antibiotic food additive in the poultry and cattle industry. The Golgi-disrupting effects of monensin were first reported by Tartakoff and Vassalli. Treatment of murine IgA myeloma MOPC-315 cells with monensin caused large smooth-surfaced vacuoles with an irregular perimeter to appear in the Golgi region in place of the normal flattened Golgi cisternae that were observed in control cells. Monensin treatment produces a ‘Golgi swollen’ response that has been observed in cells from a wide range of animal and plant species. The Golgi disrupting effects of monensin are most often observed in the \textit{trans}–Golgi cisternae, while organelles such as endosome and lysosome may also be affected. The systematic effects of monensin are both cell/tissue-specific and dependent on the route of administration.

Other natural products such as bafilomycin A (10), nocodazole (11), retinoic acid (12), okadaic acid (13), and nigericin (14) have been reported to disrupt the Golgi apparatus. In some cases, the Golgi-disrupting effects of these compounds are secondary to their effects on other primary targets (i.e., nocodazole disrupts cytoskeleton).

2.20.2.3 Heat Shock Proteins and Protein Stability

The process of folding denatured proteins into their correct form is generally an inefficient process \emph{in vitro}. This is not the case within living cells, where the overwhelming majority of cellular proteins remain in their native conformation. Perhaps, the single most important factor that facilitates the efficient folding of proteins in all living cells is the presence of chaperone proteins within all cellular compartments. Chaperone proteins are classified into two general classes – molecular chaperones and chaperonins. Molecular chaperones (e.g., Hsps) block the degradation of proteins by stabilizing unfolded proteins, while chaperonins bind to proteins and promote the folding of complex protein structures.

Hsps were first recognized as proteins that rapidly appear after cells are subjected to heat stress. They are named according to their molecular weights and include Hsp60, Hsp70, Hsp90, and Hsp100. When bound to ATP, Hsps adopt an ‘opened’ conformation with a hydrophobic binding pocket that is capable of recognizing and binding to hydrophobic regions of unfolded target proteins. Unfolded target proteins are subsequently released when the ATP is hydrolyzed. Typically, Hsps bind to nascent polypeptides that have been synthesized on the ribosome and prevent the degradation of the peptides while their folding is allowed to take place.

The eukaryotic chaperonin TRiC (T-complex polypeptide (TCP-1) ring complex or cytosolic chaperonin-containing TCP-1 (CCT)) is composed of an eight-subunit Hsp60 complex. The barrel-shaped TRiC complex binds to partially folded or misfolded target proteins and holds the protein to facilitate protein folding. The TRiC chaperonin is required for the folding of proteins such as tubulin, actin, and a number of other structurally complex proteins.

Hsp90 has emerged as a major target for anticancer drug discovery. Excellent reviews by Len Neckers describe in great detail the connection between Hsp90 and cancer, and further delineate the critical role natural product inhibitors have played in validating Hsp90 as a molecular target for cancer. Hsp90 is overexpressed up to 10-fold in tumor cells, relative to normal cells. Under normal conditions, unchaperoned Hsp90 client proteins are typically ubiquitinated and proteolytically degraded by the 26S proteasome. Hsp90 is required for the stability of a wide array of client proteins that are involved in nearly every aspect of tumor formation, survival, growth, and metastatic spread. Six ‘hallmarks of cancer’ have been described by Hanahan and Weinberg. As reviewed by Neckers, Hsp90 client proteins are associated with all six ‘hallmarks of cancer’: (1) ‘self-sufficiency in growth signals’ (e.g., HER2/Neu (ErbB2), Met, Kit); (2) ‘insensitivity to growth-inhibitory (antigrowth) signals’ (e.g., cyclin-dependent kinase 2 (Cdk2), Cdk4, Cdk6, Cdk9, Cdk11, cyclin D, Wee1); (3) ‘evasion of programed cell death (apoptosis)’ (i.e., Akt kinase, survivin, insulin–like growth factor-1 receptor (IGF-1R), mutated p33); (4) ‘limitless replicative potential’ (e.g., telomerase); (5) ‘sustained angiogenesis’ (e.g., Met, SRC, HIF-1α, HIF-2α, vascular endothelial growth factor (VEGF), VEGFR2); and (6) ‘tissue invasion and metastasis’ (e.g., urokinase, matrix metalloproteinase-2 (MMP-2)).
Perhaps the most important natural product Hsp90 inhibitor is the *Streptomyces hygroscopicus* var. *geldanus* var. *nova* metabolite geldanamycin (GA, 14).74 Geldanamycin was the first benzoquinone ansamycin Hsp90 inhibitor to be isolated and, unlike other ansamycin antibiotics, GA was significantly cytotoxic to tumor cell lines. The development of GA as a therapeutic agent was precluded by its lack of adequate water solubility, insufficient tumor cell line selectivity, and the observation that it exhibited liver toxicity in dog models.75 However, GA was the first pharmacological tool available to examine the role of Hsp90 in tumors. Using GA, Neckers and coworkers were able to demonstrate the importance of Hsp90 in stabilizing the v-src protein complex for the oncogenic transformation of tumor cells.76 Because of the use of GA as a probe to disrupt Hsp90-mediated client protein stabilization, researchers have been able to identify many of the more than 200 known client proteins and to clearly establish the role of Hsp90 in tumorigenesis. Since GA interferes with the ability of nucleotides to bind to the Hsp90 binding pocket, it has served as a vital probe to elucidate the function of ADP/ATP hydrolysis on chaperone function and has served as a structural template from which to design new classes of Hsp90 inhibitors.70 While primarily developed as less toxic antitumor drug leads with improved pharmaceutical characteristic, semisynthetic analogues of GA (especially 17-allylaminogeldanamycin (17-AAG, 15) and 17-demethoxy-17-[[2-(dimethylamino)ethyl]amino]-geldanamycin (17-DMAG, 16)) have also proved to be valuable pharmacological probes of tumor cell biology. Numerous studies have used GA, 17-AAG, and 17-DMAG to show that highly mutated and chimeric proteins in tumors (i.e., mutated forms of BCR-ABL, KIT, B-Raf, and epidermal growth factor receptor (EGFR), androgen receptor in prostate tumors, and proteins involved in tumor angiogenesis (i.e., HIF-1) are Hsp90 client proteins that represent significant molecular targets that make tumor cells sensitive to Hsp90 inhibitors (reviewed by Neckers70).

The antitumor antibiotic radicicol (17) was isolated from *Monosporium bonorden*77 and *Nectria radicicola*.78 Radicicol has been shown to bind to the same amino terminal residue as geldanamycin and similarly block the
nucleotide pocket in Hsp90. Radicicol has also been used extensively as a probe of Hsp90 function and in vivo studies with an oxime derivative of radicicol have helped validate Hsp90 inhibition as an effective antitumor molecular target.

The antibiotic novobiocin (18), originally called ‘streptonivicin’, was isolated from Streptomyces niveus (aka Streptomyces spheroides). Novobiocin competitively interferes with the ATPase activity of bacterial DNA gyrase B. Similarly, it was shown that novobiocin weakly inhibited the Hsp90–ATP interaction and destabilizes the Hsp90 client proteins mutated p53, p60v-src, p185erbB2, and Raf-1. However, rather than binding to the same N-terminal site as GA and radicicol, novobiocin was shown to bind to a distinct carboxyl terminal ATP-binding site thought to be involved in Hsp90 dimerization and cochaperone binding. More potent truncated analogues of novobiocin have been prepared and found to selectively inhibit Hsp90 function and promote the degradation of androgen receptor, AKT, and HIF-1α proteins in prostate tumor cells.

The plant rotenoid deguelin (19) was originally isolated from Mundulea sericea (Leguminosae) and found to have potent antitumor activity. Recent studies have shown that deguelin binds to the Hsp90 ATP-binding pocket and exhibits in vivo antitumor and antiangiogenic activities that were attributed to deguelin’s ability to inhibit the stability of one of its important client proteins HIF-1.

2.2.2.4 Ubiquitin–Proteasome

The functional activity of cellular proteins is under tight regulation. This control is accomplished, in part, through specifically timed phosphorylation and dephosphorylation or other post-translational modifications that result in the activation or deactivation of proteins. Perhaps even more importantly, the activity of most functional cellular proteins is controlled by selective modulation of their stability. As noted in recent textbooks and reviews, the ubiquitin–proteasome complex is responsible for the controlled degradation of more than 80% of all cellular proteins. Ubiquitin is a small (76-residue) ‘ubiquitously’ conserved protein that can be covalently linked to target proteins and initiates a process that ‘tags’ the target proteins for degradation by proteolytic enzymes found within the ‘barrel-like’ structure of at least 44 distinct proteins that compose the massive 2.5 MDa eukaryotic 26S proteasome (Figure 3).

2.2.2.4.1 Ubiquitin and ubiquitin inhibitors

Three distinct classes of enzymes carry out the process of ubiquitination – ubiquitin-activating enzymes (E1), ubiquitin-conjugating enzymes (E2), and ubiquitin-protein ligases (E3). Protein ubiquitination (or ubiquitylation) is characterized by the ubiquitin ligase-catalyzed formation of a bond between the C-terminal glycine of ubiquitin to an ε-amino acid side chain of a lysine moiety of a target protein. The mono-ubiquitinated protein is then polyubiquitinated through a process of repeated ubiquitination of the lysine-48 residue of each successive ubiquitin unit. The polyubiquitinated target protein is then recognized by the eukaryotic 26S proteasome where it is de-ubiquitinated (often referred to as ‘de-ubiquitinylated’) and ‘chopped up’ into small peptide fragments that range from 3 to 25 amino acids in length (Figure 3).

Natural products have recently been shown to interfere with specific steps in both the ubiquitination and de-ubiquitination of proteins, effectively preventing their recognition and degradation by the 26S proteasome. Panepophenanthrin (20), isolated from the mushroom Panus radis, and himeic acid A (21), isolated from a marine strain of Aspergillus sp., have been shown to interfere with the ubiquitin-activating function of E1 enzymes. In this ATP-driven process, an adenylated ubiquitin–AMP intermediate is first formed. This ubiquitin intermediate then forms an activated ubiquitin–Cys thioester in the active site of E1. Both compounds have been shown to interfere with the formation of the adenylated ubiquitin–AMP intermediate that is required to form the activated ubiquitin thioester.
Figure 3 Probes of ubiquitin–proteasome-mediated protein degradation. Target proteins must first be polyubiquitinated by (E3) ubiquitin-protein ligases before they can be recognized by the 26S proteasome for degradation. Panepophenanthrin (20) and himeic acid A (21) disrupt the earliest steps in the process of E1-mediated ubiquitin activation. Chlorofusin (22) and certain plant-derived chalcones directly interfere with binding interactions required for protein recognition and E3 ubiquitin ligases to ubiquitinate target proteins. The antitumor sponge metabolite girolline (35) is believed to interfere with the function of adaptor proteins required for the recruitment of polyubiquitinated proteins to the proteasome. Before the proteasome can degrade polyubiquitinated target proteins, the proteins must be deubiquitinated by enzymes within the ‘lid’ structure of the 19S regulatory complex of the 26S proteasome. Prostaglandin $\Delta^{12}$-PGJ$_2$ (36) and chlorinated marine prostanoids (e.g., punaglandins (37 and 38)) inhibit the ubiquitin isopeptidases that release the client proteins for degradation by enzymatic activities within the 20S core ($\beta$-ring) of the proteasome. While each has uniquely different specificities for the ‘caspase-like’, ‘trypsin-like’, and ‘chymotrypsin-like’ peptidase catalytic sites, natural product proteasome inhibitors (e.g., lactacystin (23), salinosporamide A (26), epoxomicin (27), etc.) block the degradation of client proteins, including many that have been shown to be important in tumor cell biology (e.g., Bcl-2, XIAP, cyclin A, p21, p53, bax, c-Jun, $\beta$-catenin, and HIF-1$\alpha$, etc.). Reproduced from Pharmacognosy Course PHCG 422 (School of Pharmacy, University of Mississippi). Copyright 2008, Dale G. Nagle.
The E3 ubiquitin ligases appear to be involved in the binding recognition and subsequent ubiquitination of substrate proteins. Since the apoptotic protein p53 is recognized by an E3 protein known as MDM2 (transformed mouse 3T3 cell double minute 2 homolog), considerable efforts have been underway to identify inhibitors of this ubiquitin ligase that would facilitate the accumulation of p53 and subsequent induction of apoptosis in tumor cells. Both the *Fusarium* metabolite chlorofusin (22)\(^{95}\) and certain antitumor chalcone derivatives\(^{96}\) can disrupt the p53/MDM2 binding complex. Boronic-chalcone derivatives have been synthetically produced and found to be even more potent ubiquitin–proteasome inhibitors.\(^{97}\)

\[ \text{(22) chlorofusin} \]

### 2.20.2.4.2 26S Proteasome and proteasome inhibitors

At the center of the 26S proteasome is the 20S proteasome (Figure 3). The outer surface of 20S proteasome is composed of four layered rings – two innermost \(\beta\)-rings inserted between two outer \(\alpha\)-rings. Within the four stacked rings of the 20S proteasome are a series of channel-forming proteins and six proteolytically active sites that are completely shielded within its core. This complex structure restricts access to the proteolytic sites only to ubiquitin-tagged target proteins. At each end of the 26S proteasome structure are two 19S regulatory complexes. Each of these 19S regulatory complexes are made up of a set of proteins that form a ‘lid’ that recognizes and removes ubiquitin from polyubiquitinated target proteins and a ‘base’, which contains six ATPases that regulate the opening and closing of the channel proteins within the core of the 20S proteasome. In addition to opening the 20S proteasome channels to allow the entry of target proteins, the ATPases of the 19S regulatory proteins are also believed to facilitate the unfolding and movement of target proteins to the proteolytic sites in the 20S proteasome. Six specific sites within the 20S proteasome proteolytic core of the two interconnected \(\beta\)-rings are responsible for three different classes of peptidase activities. Two sites correspond to ‘caspase-like’ catalytic residues that cleave acidic residues, two ‘trypsin-like’ catalytic sites cleave basic residues, and two ‘chymotrypsin-like’ sites catalyze the degradation of hydrophobic amino acids residues.

Unlike other proteases, the proteasome utilizes threonine protease activity. The N-terminal threonines act as active nucleophiles that attack the amide carbonyl moiety of target proteins, cleave the amide bond to form an acyl–enzyme intermediate, and subsequently hydrolyze to release the free proteasome threonine residue and the remaining peptide residue.\(^{91}\) Small molecules, including natural product-based proteasome inhibitors have significantly contributed to our understanding of the specifics of proteasome protease function (reviewed by Tsukamoto and Yokosawa\(^{92}\)).

The importance of ubiquitin–proteasome-mediated protein degradation in mammalian systems cannot be overstated. Sequence analysis indicates that more than 2% of all genes in the human genome code for ubiquitin ligases. Degradation by the ubiquitin–proteasome regulates the functional stability of many critical proteins involved in controlling the growth, survival, and spread of tumors.\(^{91,98}\) As summarized by Kisselev and Goldberg,\(^{91}\) tumor cell-associated substrates for ubiquitin–proteasome pathways include proteins that regulate apoptosis (e.g., Bcl-2, cIAP, XIAP), proteins that regulate the cell cycle progression and oncogenesis (e.g., cyclin A, p21, p27\(^{kip1}\), p53, bax,
IκB-α (inhibitor of transcription factor NF-κB)), proteins that regulate cellular adaptation to hypoxic stress (i.e., HIF-1α), and proteins related to aberrant gene expression in tumors (e.g., c-Jun, β-catenin, IκB-α, E2F1). Proteasome inhibitors are under development as new antitumor chemotherapeutic agents and the proteasome inhibitor bortezomib (PS-341, Velcade) has been approved by the U.S. Food and Drug Administration (FDA).

Lactacystin (23), the first natural product reported to inhibit the proteasome, has become one of the most commonly used molecular probes for proteasome research. Lactacystin was originally isolated as a Streptomyces metabolite that induced differentiation in murine neuroblastoma cells and inhibited proteinases.99,100 Schreiber and coworkers subsequently demonstrated that lactacystin selectively inhibited all three classes (‘caspase-like’, ‘trypsin-like’, and ‘chymotrypsin-like’) of 20S proteasome peptidase catalytic functions without inhibiting the activity of other proteases.101 Further, evaluation with tritium-labeled lactacystin indicated that lactacystin inhibited proteasome activity by selectively binding to the β-subunit 5X of the 20S proteasome. The β-subunit X selectivity of lactacystin was later challenged when Rock and coworkers demonstrated that [3H]-lactacystin not only binds to the 20S proteasome β-subunit 5X but also lactacystin modifies all of the β-subunits.102 This study with lactacystin further indicated the critical role of the ubiquitin–proteasome in degrading intracellular proteins and in major histocompatibility complex (MHC) class I antigen presentation for immune recognition. Dick and coworkers showed that when in solution at pH 8, lactacystin undergoes hydrolysis to an inactive dihydroxy acid derivative through the formation of an active clasto-lactacystin β-lactone (24, also known as ‘omuralide’) intermediate. When evaluated under more acidic conditions (pH 6.3), which prevented hydrolysis of lactacystin, lactacystin showed no significant proteasome inhibitory activity. However, the clasto-lactacystin β-lactone intermediate remained active at lower pH.103 The same group also demonstrated that only the clasto-lactacystin β-lactone intermediate (not lactacystin) penetrates tumor cells where it reacts with the sulphydryl of glutathione (GSH) to form lactathione (25), which is believed to be an inactive intracellular repository form that releases the active β-lactone over time.104
The potently cytotoxic proteasome inhibitor salinosporamide A (26, NPI-0052) was isolated from a new genus of marine bacteria called *Salinispora*. The bicyclic (β-lactam-γ-lactam) core structure of salinosporamide A is also found in the activated form of lactacystin known as clasto-lactacystin β-lactone (24, omuralide). While structurally similar to omuralide, salinosporamide A (26) is pharmacologically and mechanistically distinct. Unlike omuralide, chloride displacement from the chlorinated side chain in salinosporamide A (26) results in the formation of an irreversible linkage to the catalytically active sites within the 20S proteasome. This irreversible binding appears to increase the duration of proteasome inhibition. Omuralide, bortezomib, and other proteasome inhibitors tend to selectively inhibit only one or, at most, two catalytic activities of the proteasome and only partially reduce the degradation of key proteins. Further, suppression of at least two catalytic activities is required to produce a robust reduction (i.e., >50%) of proteolytic activity. Salinosporamide A interferes with the catalytic activity at all three catalytic sites and is able to effectively suppress target protein degradation.

One of the most potent and selective classes of proteasome inhibitors are the ‘peptide epoxyketones’ such as epoxomicin (27) and dihydroeponemycin (28), which contain a reactive α,β-ketoepoxide moiety. Epoxomicin was isolated from a strain of *Actinomyces*, and eponemycin was isolated from a strain of *S. hygroscopicus*. Both compounds showed tumor cell line cytotoxicity that apparently resulted from proteasome inhibition. In spite of the fact that these natural products contain similar chemically reactive functional groups, the two compounds exhibit different levels of specificity. Epoxomicin is more ‘chymotrypsin-like’ site specific, while eponemycin also inhibits ‘caspase-like’ activity of the proteasome. Crystallographic studies suggest that the high level of specificity associated with these compounds may be due to their ability to react with both the amino and hydroxyl groups of the proteasome N-terminal threonine to produce a morpholino ring system. The similarly active semisynthetic eponemycin analogue dihydroeponemycin (28) was also shown to produce its cytotoxic effect on tumor cells by inducing apoptosis.

Studies by Dou and coworkers have shown that the green tea (dried fresh leaves of the plant *Camellia sinensis*) catechin (−)-epigallocatechin-3-gallate (EGCG, 29) specifically inhibits ‘chymotrypsin-like’ proteasome activity in Jurkat T cells at physiologically relevant concentrations. Inhibition of proteasome activity by EGCG caused cells to accumulate ubiquitinated p27Kip1 and IκB-α, two proteins that are associated with aberrant gene expression and oncogenesis. Much like lactacystin, the proteasome appears to react with the gallate ester bond in EGCG that results in acylation of the active site threonine. In a similar fashion, caffeoyl esters of quinic acid such as 3,5-dicaffeoylquinic acid (30) from mate tea (dried fresh leaves of the plant *Ilex*
paraguayensis) inhibit 'chymotrypsin-like' proteasome activity in Jurkat T cells and have served as models for disease-specific studies and analogue development. Dou's group also found that the dietary flavonoids apigenin (31) and quercetin (32) inhibit proteasome activity. Inhibition of the 'chymotrypsin-like' site (β5 subunit) by these compounds induced activation of caspase-3 and poly(ADP-ribose) polymerase cleavage in Jurkat T cells. Further, this study correlated the inhibition of the 20S proteasome with the same concentrations of these compounds that induce apoptosis in tumor cells.

Dou and coworkers have recently found that celastrol (33) (a triterpene from the Chinese 'Thunder of God Vive' (Tripterygium wilfordii)) and withaferin A (34) (a steroid lactone from 'Indian Winter Cherry' (Withania somnifera)) inhibit 'chymotrypsin-like' proteasome activity in PC-3 (androgen receptor negative) and LNCaP (androgen receptor positive) human prostate cells. Studies with these natural products indicated that both compounds cause the accumulation of tumor-related ubiquitinated substrate proteins (Bax, p27, and IκB-α), suppression of androgen receptor protein expression in LNCaP cells, and the induction of apoptosis.

2.20.2.4.3 Inhibitors of ubiquitinated protein recruitment and ubiquitin isopeptidases

The antitumor marine natural product girolline (35) has been isolated from the marine sponges Pseudaxinyssa cantharella and Axinella brevisystyla. Treatment with girolline causes tumor cell lines to accumulate polyubiquitinated p53 but does not directly inhibit proteasome activity. Girolline may represent an interesting molecular probe of the ubiquitin–proteasome in tumor cells, since it is believed to prevent the recruitment of ubiquitinated proteins and may interfere with the function of adaptor proteins required for recruitment of polyubiquitinated proteins.

![Girolline](image)

In order for polyubiquitinated proteins to be degraded by the proteolytic enzymes within the proteasome, the ubiquitin proteins must be removed. Ubiquitin isopeptidases function to cleave the isopeptide ubiquitin linker and release the client proteins for proteasomal degradation (Figure 3). The prostaglandin Δ12-PGJ2 (36) was shown to inhibit ubiquitin isopeptidase. Subsequently, the unusual chlorinated prostanoids, punaglandins 4 (37) and 6 (38), from the soft coral Telesto risseti were found to inhibit ubiquitin isopeptidase. Studies in several tumor cell lines demonstrated that punaglandins 4 and 6 cause the accumulation of proteasome client proteins (i.e., p21 and p53), enhance caspase activity, and exhibit antitumor activities that appear to be associated with disrupting ubiquitin–proteasome function.

The use of different classes of natural product-based proteasome inhibitors as molecular probes has revealed that just as each structural class reacts with the proteolytic sites within the 20S proteasome through a distinct chemical mechanism, each class of inhibitor also exhibits a unique pattern of active site selectivity and protein specificity. Therefore, natural products and other small molecule proteasome inhibitors have not only proved to be invaluable probes to study proteasome function, but have also been valuable tools to examine the diversity of pharmacological/cellular responses produced by selective (or in some cases relatively nonselective) suppression of distinctly different catalytic sites within the eukaryotic 26S proteasome.


2.20.2.5 Mitochondria and Oxidative Phosphorylation

Mitochondria are among the most common cellular organelles and can occupy as much as one quarter of the cytoplasmic volume in eukaryotic cells. Mitochondria play a critical role in the initiation and control of programmed cell death or apoptosis. Numerous studies have explored the relationship between natural products and this particular aspect of mitochondrial function. Most publications have highlighted the importance of natural products in regulating the various apoptotic pathways. Rather than cover apoptosis in this section, attention is given to the often overlooked impact of mitochondrial energy production on tumor cell biology.

The mitochondrial respiratory chain drives the production of ATP in cells under aerobic conditions and is responsible for the production of superoxide and other reactive oxygen species (ROS). While often overshadowed by their importance in apoptotic cell death, tumor cells have specific differences in energy requirements and in other mitochondria-mediated signaling processes that are only recently becoming recognized as potential nonapoptotic antitumor drug targets.

The mitochondrial respiratory chain is composed of more than 80 proteins grouped into 5 distinct complexes that form an integrated electron transfer chain (ETC). Initiation of electron transport takes either from complex I (reduced nicotinamide adenine diphosphate (NADH)–ubiquinone oxidoreductase) or from complex II (succinate–ubiquinone oxidoreductase) to complex III (ubiquinol–cytochrome c oxidoreductase) by ubiquinone (UQ, coenzyme Q).

![Diagram of the mitochondrial electron transport chain](image)

Figure 4 Probes of mitochondrial electron transport chain (ETC) and oxidative phosphorylation. Natural products such as rotenone, piericidin A, annonaceous acetogenins, capsaicin, myxothiazol, stigmatellin, and antimycin A inhibit mitochondrial electron transport at various points along the ETC. Oligomycins, apoptoloidin, and other natural products bind to the oligomycin sensitivity-conferring protein (OSCP) within the F0 subunit located in the stalk of the F1F0-ATPase and inhibit the phosphorylation of ADP to ATP. Certain antibiotic natural products (e.g., valinomycin, gramicidin, nigericin, etc.) facilitate the ion flux through membranes and can interfere with the ability of mitochondria to establish proper ionic balance or maintain the proton gradient required to drive the F1F0-ATPase. Superoxide anion leakage can occur during each of the early steps of the ETC. However, release of superoxide from the Qp site of complex III (ubiquinol–cytochrome c oxidoreductase) is believed to play a critical role in cellular signaling for the hypoxic regulation of hypoxia-inducible factor-1 (HIF-1).

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ubiquinol (UQH₂, 40) by a two-electron process that involves the production of the intermediate ubisemiquinone radical (UQ⁺, 41) that can readily lose a proton to form the reactive ubisemiquinone anion radical (UQ⁻, 42). The transfer of electrons then continues from complex III to complex IV (cytochrome c oxidase) by the protein cytochrome c (cyt c). Although the precise process is exceedingly complex, complex III recycles the UQ from UQH₂, also producing ubisemiquinone anion radicals (UQ⁻). Driven by the electrons it receives from cytochrome c (that rapidly diffuses from complex III), the cytochrome c oxidase (complex IV) reduces available O₂ to water. This process is primarily responsible for mitochondrial oxygen consumption. Powered by the resulting electrochemical proton gradient (protonmotive force (Δρ)), the F₁F₀-ATPase complex (also known simply as ATP synthase, F₀F₁-ATPase, or sometimes referred to as mitochondrial complex V) catalyzes the subsequent formation of ATP from ADP.

The process of UQ redox cycling within the mitochondrial ETC is potentially dangerous to the cell because the oxidation of the ubisemiquinone anion radical intermediate (UQ⁻, 42) by O₂ can produce superoxide anion radical (·O₂⁻), which is dismutated to H₂O₂ by the enzyme superoxide dismutase (SOD). Hydrogen peroxide can then form highly reactive hydroxy radicals with the oxidation of heme Fe(II) to Fe(III) in the Fenton reaction. In order to diminish the potential oxidative stress created by this process, mitochondria contain significantly high levels of SOD to remove superoxide and GSH peroxidase to remove hydrogen peroxide.

### 2.20.2.5.1 Complex I electron transport inhibitors

Perhaps the most important small molecule probe of cellular respiration is the insecticidal natural product rotenone (43), isolated from roots of legumes Lonchocarpus nicou, Derris spp., etc. Several hundred studies have used rotenone as a general inhibitor of mitochondrial function. As such, rotenone has served as the primary pharmacological probe to examine the role of mitochondrial complex I in the ETC, the generation of ROS, and in the generation of mitochondrial signals that regulate apoptosis. Rotenone has also been shown to induce an ROS-associated cytotoxicity in dopaminergic neurons. Through the use of dopaminergic neuroblastoma tumor cells, rotenone has been used to investigate the potential of complex I inhibition to induce the progressive degeneration of dopaminergic neurons associated with Parkinson’s disease. As such, drug discovery programs have developed tumor cell line-based models to screen for small molecule inhibitors of
rotenone-induced SH-SY5Y cytotoxicity as a means of identifying new agents for the chemoprevention and
treatment of Parkinson’s disease.\textsuperscript{133–137} Suppression of mitochondria function depletes ATP production and can
slow replication of tumor cells and other mammalian cells.\textsuperscript{138,139} However, unlike other mitochondrial ETC
inhibitors, rotenone (43) has been shown to inhibit mammalian cell division by directly interfering with
microtubule polymerization.\textsuperscript{140–142} Most studies that employ rotenone as a molecular probe of mitochondrial
complex I function in tumor cell growth and apoptosis do not address this aspect of the pharmacological activity
of rotenone. This would appear to significantly limit the potential conclusions obtained from rotenone-based
studies of tumor cell biology. However, as judged by the widespread use of rotenone as a pharmacological tool
to inhibit mitochondrial function, the antimiticotic activity of rotenone has not seemed to affect its continued
application as a major probe of complex I function. This is of particular concern since numerous groups
interpret pharmacological and toxicological results obtained from \textit{in vitro} and \textit{in vivo} studies with rotenone as a
direct indication of the consequences of selectively blocking mitochondrial complex I-mediated electron
transport.

Next to rotenone, the \textit{Streptomyces mobaraensis} metabolite piericidin (also known as shaoguanmycin B),
specifically piericidin A\textsubscript{1} (44), is perhaps the most studied NADH–UQ oxidoreductase inhibitor and is
commonly used as a pharmacological probe of the role of complex I in the mitochondrial ETC. For
example, glucose deprivation causes upregulation of ER chaperone protein GRP78 and induces etoposide
resistance in human cancer cells. GRP78 is also known to protect tumor cells from apoptosis induced by
topoisomerase inhibitors.\textsuperscript{143} Studies have shown that glucose-deprived etoposide-resistant HT-29 human
colon carcinoma cells are uniquely sensitive to piericidin A.\textsuperscript{144} These studies further indicate that
piericidin prevents the upregulation of GRP78 in tumor cells. As in the case of rotenone (43), the structure of piericidin A1 (44) and many other inhibitors of the mitochondrial ETC owe their potent biological activities to structural similarities between these natural products and the lipophilic electron carrier molecules UQ (39), UQH2 (40), and their reactive intermediates ubisemiquinone radical (41) and ubisemiquinone anion radical (42). Depending on their specific structures, this allows these compounds to inhibit the various steps in the ETC by selectively interfering with any number of various UQ-mediated electron transfer reactions.

Extracts from the seeds and other parts of the custard apple family (also known as ‘pawpaw’) and related members of the Annonaceae family contain long alkyl chain acetogenins with one or two tetrahydrofuran rings. Numerous reviews have described the chemistry and biological activities associated with the acetogenins.145,146 Extracts of pawpaw and other related acetogenin–rich species are commonly used as herbal therapies for the treatment of various forms of cancer. More than 400 of these compounds, collectively known as annonaceous acetogenins have been isolated and many have been shown to be extremely potent and selective inhibitors of mitochondrial complex I electron transfer.145,147 Acetogenins such as rolliniastatin-1 (45) and bullatacin (46) have been well studied for their effects on NADH–UQ oxidoreductase and used to examine the potential of selective complex I ETC inhibitors to suppress tumor growth and regulate apoptosis.

Perhaps best known as an inhibitor of vanilloid receptors, the Capsicum spp. hot chili metabolite capsaicin (47) is also an inhibitor of mitochondrial complex I.148,149 Capsaicin has been used to examine the effects of vanilloid receptor modulation and mitochondrial disruption on tumor cell proliferation and apoptosis.149

2.20.2.5.2 Complex II and III electron transport inhibitors

The stolonoxide oxylipins (e.g., stolonoxides A (48) and C (49)) were isolated from the Mediterranean tunicate Stolonica socialis. These cyclic peroxides possess a unique structure with a 1,2-dioxane ring directly linked to a tetrahydrofuranyl moiety.150,151 Such an arrangement is closely related to the bis-tetrahydrofuranyl groups of annonaceous acetogenins.152 The ability of these compounds to inhibit mitochondrial respiration was evaluated in an assay system that used beef heart submitochondrial particles. Stolonoxide A methyl ester (50) and stolonoxide C methyl ester (51) completely inhibited cytochrome c reductase activity with either NADH or succinate as the electron donor. When assayed using deacylubiquinone,153 both compounds strongly reduced the specific activity of succinate-sustained UQ reduction in complex II, and only slightly reduced complex I UQ-sustained NADH oxidation.154 These data and the cytochrome c reductase activity suggest that both stolonoxides behave as specific inhibitors of complex II and complex III (at submicromolar concentrations). While this dual functional inhibition of the mitochondrial electron chain is potentially compelling, these compounds have not since found significant use as molecular probes in mitochondrial research or tumor cell biology.

\[ RO\]
\[ H-O-O=H\]
\[ \text{stolonoxide A} \]
\[ R = H \]
\[ \text{methyl stolonoxide A} \]
\[ R = CH_3 \]

\[ RO\]
\[ H-O-O=H\]
\[ \text{stolonoxide C} \]
\[ R = H \]
\[ \text{methyl stolonoxide C} \]
\[ R = CH_3 \]

\[ \text{(52) myxothiazol} \]
The antibiotic myxothiazol (52) was originally isolated from the myxobacterium *Myxococcus fulvus* and found to inhibit fungal cell respiration.\(^{155,156}\) Myxothiazol was established to inhibit events at the Q\(_p\) site (UQ/UQH\(_2\) binding site toward the cytoplasmic P-side of the inner mitochondrial membrane) of complex III and has since emerged as a major natural product probe of mitochondrial structure and function (Figure 4). Myxothiazol (52) has been used as a pharmacological tool to investigate the role that mitochondrial-independent cell surface oxygen consumption plays in the total cellular oxygen consumption in glycolytic cancer cell lines.\(^{157}\)

The antifungal antibiotic strobilurin A (53) was first isolated from the basidiomycete mushroom *Strobilurus tenacellus*. Strobilurins have been shown to target the Q\(_p\) site of complex III and function in a similar manner to that of myxothiazol.\(^{158}\) The critical pharmacophore in both myxothiazol and strobilurins is the \(\beta\)-methoxyacrylate moiety.\(^{159}\) This pharmacophore is strikingly similar to the \(\beta\)-methoxyacrylate moiety in myxothiazol (52). Azoxystrobilurin (54, azoxystrobin) and other strobilurins have found great utility as agricultural fungicides. Perhaps owing to their lack of potency in mammalian cells, relative to myxothiazol, natural and synthetic strobilurin derivatives have not emerged as major probes of mitochondrial function in tumor cell biology.

Another major probe of mitochondrial function within tumors and other eukaryotic cells is the *Streptomyces* metabolite antimycin A (55) that was first isolated as a potent antimicrobial agent.\(^{160}\) The unique feature of antimycin A that has earned it its role as a probe of mitochondrial function is that, in contrast to myxothiazol (52), antimycin A was found to act at the Q\(_p\) site (UQ/UQH\(_2\) binding site toward the matrix N-side) of mitochondrial complex III and block the formation of ubisemiquinone radical anions \(42\) (Figure 4).

Using bacterial genetics, Darrouzet and Dupuis\(^{161}\) isolated *Rhodobacter capsulatus* mutants with rotenone- and piericidin-resistant mitochondrial complex I proteins. The effects of the natural product complex I inhibitors rotenone (43) and piericidin (44) and complex III inhibitors myxothiazol (52) and stigmatellin A (56) were evaluated on these complex I mutated strains.\(^{162}\) The unique selectivity of these inhibitors for the mutated mitochondrial proteins were used to construct a model for the binding of piericidin, quinine, and other mitochondrial complex I inhibitors composed of a polar ‘quinone-ring’ binding site and an ‘isoprenyl’ lipid-binding site within the mitochondrial membrane. Dupuis further postulated that differences in the affinity of various complex I inhibitors for either the hydrophilic or the hydrophobic binding domains was responsible for the broad structural diversity (and biological activity) of complex I inhibitors.

### 2.20.2.5.3 Ionophores

Various lipophilic natural product antibiotics that have a hydrophobic exterior and a shielded hydrophilic interior capable of binding and transporting ions are known as ionophores. The three ionophores that are most commonly used as probes of ion transport across mitochondrial membranes are the *Streptomyces* cyclic dodecadepsipeptide valinomycin (57), *Bacillus brevis* gramicidin pentadecapeptides (e.g., valine-gramicidin A, 58), and *S. hygroscopicus* polyether metabolite nigericin (59).
(57) valinomycin

(59) nigericin

(60) monactin $R^1 = R^2 = R^3 = R^4 = \text{CH}_3$

(61) nonactin $R^1 = \text{CH}_2\text{CH}_3; R^2 = R^3 = R^4 = \text{CH}_3$

(62) monensin A

(63) dianemycin

OHC-Val-Gly-Ala-d-Leu-Ala-d-Val-Val-d-Val-Trp-d-Leu-Trp-d-Leu-Trp-d-Leu-Trp-NH(CH$_2$)$_2$OH

(58) Val-gramicidin A
Valinomycin (57) is a charge-neutral potassium-selective mobile ion carrier. Since valinomycin has no net charge it accepts the charge of the K⁺ but remains lipophilic and freely diffuses through mitochondrial membranes in either the ion-free or ion-complexed form. By rapidly shuttling through the lipid membranes, even low nanometer concentrations of valinomycin are capable of catalyzing the transfer of large numbers of K⁺ ions through mitochondrial membranes. Valinomycin has been used to examine the role of mitochondrial ion transport and membrane potentials in prokaryotic and various eukaryotic systems including tumor cells. These macrotritile antibiotics, sometimes referred to as ‘nactins’ have been used to examine ion flux, apoptosis, glycoprotein-mediated multidrug efflux, and other cellular processes in tumor cell-based systems.

The mixture of peptides referred to as gramicidins or gramicidin D is composed of a mixture of gramicidin A (e.g., valine-gramicidin A (58)), B, and C-type linear pentapeptides and other cyclic peptides. Gramicidins form dimeric complexes that create channels that allow protons, K⁺, and other monovalent cations to rapidly transverse mitochondrial membranes and other lipid bilayers.

The S. hygroscopicus natural product nigericin (59) and several other antibiotics (e.g., monensin (62) and dianemycin (63)) belong to a third class of ionophores. Because of their carboxylic acid moieties, these linear lipophilic polyether acids form a neutral deprotonated complex when binding K⁺ and other metal ions and remain neutral in their protonated forms. This allows members of this class of ionophore to remain lipophilic and rapidly diffuse through mitochondrial membranes in either form. This property of nigericin and related polyethers facilitates the rapid exchange of K⁺ and Na⁺ with H⁺ and thus disrupts the mitochondrial proton gradient without altering the net charge across the membrane.

Highly lipophilic weak acids and bases that have the capacity to remain lipophilic in both their protonated and deprotonated forms can act as protonophores. Such compounds belong to another class of ionophores that are often referred to as mitochondrial uncouplers because of their unique ability to translocate protons across mitochondrial membranes, resulting in the subsequent loss of the mitochondrial proton gradient that is required to drive oxidative phosphorylation. While certain natural products act as mitochondrial uncouplers, most of the protonophores used as pharmacological probes are not natural products but are low-molecular-weight synthetic compounds (e.g., carbonyl cyanide p-trifluoromethoxyphenylhydrazone (FCCP)).

2.20.2.5.4 ATPase inhibitors

The mitochondrial ETC is used to establish a proton motive force (Δp) that drives the production of ATP from ADP by the mitochondrial ATP synthase known as F₁F₀-ATPase (or F₀F₁-ATPase). The polyketide macroide antibiotics known as oligomycins have been the best-studied pharmacological probes of the mitochondrial F₁F₀-ATPase. Oligomycins A (64), B (65), and C (66) were originally isolated and identified from cultures of Streptomyces diastatochromogenes over 50 years ago. Purified oligomycin A and oligomycin mixtures are commonly used to study oxidative phosphorylation in numerous model systems such as bacteria, fungi, and higher animals. Oligomycins have been used extensively to examine various mechanistic aspects of the ATP formation and energy requirements in tumor cell biology and apoptosis. Oligomycin has been shown to block the flow of protons required to drive the F₁F₀-ATPase by binding to the oligomycin sensitivity-conferring protein (OSCP) within the F₀ subunit found in the stalk of the ATP synthase structure (Figure 4). Oligomycin has been used to probe tumor cell dependence on glycolytic metabolism and the role of oxidative phosphorylation on the activation of BAX and Bak tumor suppressors. Other polyketide F₁F₀-ATPase inhibitors that have been used as probes of tumor cell energetics and cell growth and survival include the Norcardiopsis sp. metabolite apoptolidin (67), S. diastatochromogenes compound cytovaricin (68), and S. hygroscopicus var. ossamyceticus natural product ossamycin (69). Along with oligomycin these F₁F₀-ATPase inhibitors produced very unusual and somewhat characteristic patterns of growth inhibition in the U.S. National Institutes of Health – National Cancer Institute (NIH–NCI) anticancer screening program. These compounds are among the most cell line-specific inhibitors of tumor cell growth (as exemplified by their relative GI₅₀ values) ever evaluated at the NCI. Taken together, these F₁F₀-ATPase inhibitors have been instrumental in formulating a hypothesis that highlights specific differences in cell line sensitivity to specific
differences that result from the Warburg effect, \textsuperscript{181,182} whereby tumor cells retain the propensity to maintain a high level of anaerobic carbon metabolism even under normoxic conditions.\textsuperscript{183}
Watanbe and Nakaki\textsuperscript{132} have recently used a panel of natural mitochondrial inhibitors to probe the role of ATP depletion and ROS generation in the induction of apoptosis in human dopaminergic cells. Inhibition of ATP production with the F\textsubscript{1}F\textsubscript{0}-ATPase inhibitor oligomycin (64), ionophore valinomycin (57), and uncoupler 2,4-dinitrophenol did not induce apoptosis. However, inhibition of ETC-generated ROS production with the complex I inhibitor rotenone (43), complex III inhibitor antimycin A (55), and other ETC inhibitors all induced apoptosis. While this investigation did not use tumor cells, these studies with natural product mitochondrial inhibitors suggest that generation of mitochondrial ROS is responsible for the induction of apoptosis, rather than ATP depletion.

2.2.3 Oxygen-Regulated Cell Signaling – Interactions between Cellular Processes

2.2.3.1 Hypoxia-Inducible Factor-1

One of the best examples of how cellular organelles, a diverse set of signal transduction pathways, and numerous biochemical reactions all interact in a concerted manner is in the process of oxygen-regulated cell signaling within tumor cells. As solid tumors grow, they rapidly outstrip the oxygen-carrying capacity of the existing capillary bed and blood vessels in the surrounding vasculature, thus creating hypoxic regions within the solid tumor mass. Tissue hypoxia stimulates tumor angiogenesis. However, the newly developing capillaries and blood vessels that formed in response to this rapid tumor growth typically fail to fully mature. This creates chronically hypoxic conditions within the growing tumor mass.\textsuperscript{184–187} At the organism level, when we physically exert ourselves and our bodies become deprived of oxygen, we respond by increasing our respiration rate and other physiological changes occur that enhance the blood flow to our tissues. In contrast to acute hypoxic adaptation, tumor masses gradually become hypoxic as they grow and tumor cells adapt to this potentially lethal physiological stress by altering the expression of genes that promote hypoxic adaptation and survival.

As illustrated in reviews by Semenza and others,\textsuperscript{17,188,189} the principle genetic regulator of oxygen homeostasis in eukaryotic cells is the transcription factor HIF-1. HIF-1 is a heterodimer of the basic helix-loop-helix (bHLH) PER-ARNT-SIM (PAS) proteins HIF-1\textalpha and HIF-1\beta (also known as arylhydrocarbon receptor nuclear translocator or ARNT). Once HIF-1 is translocated into the nucleus, it binds to hypoxia-response elements (HREs, minimal core sequence 5\textprime-RCGTG-3\textprime) in the promoter regions of hypoxia-responsive genes and activates transcription. Over one hundred identified HIF-1 target genes encode proteins that regulate numerous aspects of cell physiology that include: (1) cell growth and survival (e.g., cyclins, insulin–like growth factors (IGFs), platelet-derived growth factors (PDGFs)); (2) energy metabolism (e.g., glucose transporter 1 (GLUT1), glyceraldehyde 3-phosphate dehydrogenase (GAPDH)); (3) cytoskeletal structure, cell adhesion, and motility (e.g., chemokine (C-X-C motif) receptor 4 (CXCR4), MMPs); (4) apoptosis (e.g., B-cell leukemia/lymphoma 2/adenovirus E1B 19kDa interacting protein 3 (NIP3)); and (5) angiogenesis, erythropoiesis, and other aspects of oxygen transport and delivery (e.g., erythropoietin (EPO), angiopoietin-2 (Ang-2), vascular endothelial growth factor-A (VEGF-A)) (Figure 5).

The availability and activity of the oxygen-regulated HIF-1\textalpha subunit serves to mediate the bioactivity of HIF-1 in response to variations in cellular oxygen concentrations.\textsuperscript{17,188} In contrast to the constitutively expressed HIF-1\beta subunit, HIF-1\textalpha is rapidly degraded (t\textsubscript{1/2} 5 min) in well-oxygenated normal cells and stabilized under the hypoxic conditions that exist within poorly vascularized tumor cells. As depicted in Figure 5, cellular oxygen concentrations regulate the stability and accumulation of HIF-1\textalpha protein because O\textsubscript{2} is required by the oxygen-dependent prolyl hydroxylases that modify HIF-1\textalpha protein (also known as prolyl hydroxylase domain-containing proteins, PHDs). Under normoxic conditions, oxygen-dependent PHDs (i.e., PHD2) hydroxylate prolyl residues P402 and P564 in the oxygen-dependent degradation domain (ODDD) of HIF-1\textalpha protein. The prolyl hydroxylated HIF-1\textalpha subunit is then recognized by an E3 ubiquitin ligase complex that contains the von Hippel–Lindau tumor suppressor protein (pVHL). The HIF-1\textalpha protein is subsequently polyubiquitinated and shuttled to the 26S proteasome where it is rapidly degraded (Figure 5).

As a second means of hypoxic HIF-1 regulation, HIF-1 activity is mediated through the action of an oxygen-dependent asparaginyl hydroxylase, sometimes referred to as factor inhibiting HIF-1 (FIH). The FIH
hydroxylates an asparagine residue (N803) in the C-terminal transcriptional activation domain (C-TAD) of HIF-1 protein. Oxygen-dependent asparaginyl hydroxylation inactivates HIF-1 protein by preventing the interaction between HIF-1 protein and the transcriptional coactivator CBP/p300 (cAMP-response element-binding protein (CREB)-binding protein/E1A-binding protein, 300 kD). In addition, the synthesis of HIF-1 protein is regulated by a series of different signaling pathways (e.g., PI3K/AKT, MEK/ERK, AMPK, etc.) that constitute the mammalian target of rapamycin (mTOR) signaling cascade. Hypoxic conditions suppress mitochondrial ATP synthesis, resulting in an increase in the ratio of AMP to ATP in the cell. This induces the phosphorylation of LKB1 kinase, which activates AMP-activated protein kinase (AMPK). Phosphorylation by AMPK activates the tumor suppressor complex TSC2–TSC1 (tuberous sclerosis complex or TSC) that suppresses the activation of mTOR. Suppression of mTOR blocks protein translation by inhibiting eukaryotic initiation factor 4E-binding protein-1 (4EBP-1) and ribosomal p70 S6 kinase (S6K) (**Figure 6**). The cumulative combined is general reduction of protein synthesis, while the production of certain specific proteins, such as HIF-1α protein, is increased through some of the following mechanisms.

As summarized in several reviews and other publications, numerous studies have contributed to the understanding of how the phosphoinositoll 3-kinase/protein kinase B (PI3K/AKT) signaling pathway regulates the accumulation of HIF-1α protein. Growth factors, hormones, and other stimuli activate the PI3K/AKT and Ras–ERK pathways (**Figure 6**). The tumor suppressor complex TSC1–TSC2 processes and integrates signals...
from each of these pathways. In an opposing fashion to the hypoxia-induced, AMPK/TSC2–TSC1/mTOR-mediated suppression of protein synthesis, AKT and ERKs enhance mTOR-mediated protein synthesis by phosphorylating and inhibiting TSC2 in the TSC1–TSC2 integration complex. The GTPase activity of the activated TSC1–TSC2 complex suppresses the ability of the small G protein known as Rheb–GTP to activate mTOR.

The PI3K inhibitors wortmannin (isolated from Penicillium funiculosum) and a synthetic chromone derivative LY294002 (isolated from S. hygroscopicus) were shown to inhibit HIF-1 activation by inactivating the downstream targets that promote HIF-1α mRNA translation (Figure 6), while some studies suggest that the PI3K/AKT pathway is not involved in mediating the hypoxic induction of HIF-1α protein. However, it appears that the regulatory effects exerted by the PI3K/AKT pathway on HIF-1 may be cell line-specific and may also be related to the nature of the stimulus (e.g., growth factor stimulation). The critical importance of the PI3K/AKT pathway in HIF-1 regulation is based on the ability of AKT to modulate the activity of the protein kinase mTOR. As a pharmacological probe, the mTOR inhibitor rapamycin (isolated from S. hygroscopicus), was shown to inhibit HIF-1α protein accumulation by at least two different mechanisms: inhibition of HIF-1α protein synthesis and promotion of HIF-1α protein degradation (Figure 6).
Natural product-based inhibitors can act at early points in HIF-1 signaling to regulate HIF-1α synthesis and HIF-1 activation under both hypoxic and normoxic conditions. By inhibiting receptor tyrosine kinases (RTKs), the plant isoflavone natural product genistein (73) blocks HIF-1α and HIF-1β synthesis and interferes with HIF-1 DNA-binding activity (Figure 6). Similarly, the flavone-based synthetic mitogen-activated protein kinase (MAPK) inhibitor PD98059 (74) inhibits the activation of MAPK that enhances the interaction between the HIF-1α C-TAD and the transcriptional coactivator CBP/p300 without affecting the level of HIF-1α protein or the binding between HIF-1 and HRE.

Studies with natural product-based pharmacological probes have highlighted the importance of the ER, Golgi apparatus, and other cellular organelles in the regulation of HIF-mediated hypoxic adaptation of tumor cells. One crucial study demonstrated that the Fenton reaction and ROS formation is localized to the ER. Tunicamycin (1–4) was used to examine the effect of ER stress on prosurvival and prodeath effects of HIF-1 in neuroblast HT22 cell lines. Decreasing concentrations of cytosolic calcium appear to increase HIF-1α
protein accumulation. However, a recent study has demonstrated that ER stress induced by either tunicamycin (1–4) or brefeldin A (6) appears to increase the transcription of HIF-1α mRNA and may play a more important role in promoting HIF-1α accumulation than alterations in intracellular calcium concentrations.216

Numerous studies have used natural product-based biochemical probes to investigate the interaction between HIF-1α protein and Hsp90.217–222 The molecular chaperone Hsp90 binds to the HIF-1α PAS domains and stabilizes HIF-1α protein, and the HIF-1β/ARNT subunit competes for the same binding sites with Hsp90 to stabilize HIF-1α protein.223,224 GA (14) has been shown to inhibit the activation of HIF-1 by promoting pVHL-independent proteasomal degradation of HIF-1α protein under both normoxic and hypoxic conditions.225,226 By contrast, the Hsp90 inhibitor radicicol (17) also binds to the amino-terminal ATP/ADP-binding pocket,227 but inhibits HIF-1 in Hep3B cells by a different mechanism. Radicicol appears to block the binding of HIF-1 to the HREs in the promoters of HIF-1 target genes.221 Other Hsp90 inhibitors, for example, 17-DMAG (16) and novobiocin (18), have also been shown to inhibit HIF-1α protein accumulation.219,220

Since the proteasome mediates the oxygen-dependent degradation of HIF-1α protein, natural product-based proteasome inhibitors have become important tools to probe HIF-1 regulation. Salceda and Caro found that inhibition of the ubiquitin–proteasome system with lactacystin (23) caused HIF-1α accumulation, indicating that HIF-1α protein is rapidly degraded by the ubiquitin–proteasome under normoxic conditions and that redox-induced biochemical modification mediates the changes in HIF-1α protein stability.228

Mitochondria ETC inhibitors constitute one group of recently recognized small molecule HIF-1 inhibitors.17,229 Opposing theories have emerged to explain the role of mitochondria in HIF-1 regulation.230–232 Several studies have used small molecule ETC complex I inhibitors such as rotenone (43), complex III inhibitors such as stigmatellin (56) and myxothiazole (52), and F1F0-ATPase inhibitors such as oligomycin A (64) as probes to investigate the involvement of hypoxia-induced mitochondrial ROS in cellular oxygen signaling.233–237 Under hypoxic conditions, superoxide anion, hydrogen peroxide, and perhaps other ROS produced by the Qp site of mitochondrial complex III (Figure 4) appear to serve as ‘signal molecules’ that oxidize the catalytic Fe(II) in Fe(II)-dependent HIF-prolyl hydroxylases that are required to initiate the ubiquitin-mediated proteasomal HIF-1α protein degradation process (Figure 5).233–238 Similarly, mitochondrial ROS may also block the Fe(II)-dependent asparaginyl hydroxylases (aka FIH) that normally interfere with the transcriptional activation of HIF-1 by hydroxylation of the N803 asparagine in the C-TAD of HIF-1α. In this respect, inhibitors of the mitochondrial ETC may prevent the production of essential ROS signaling molecules required to (1) stabilize HIF-1α under hypoxic conditions and (2) block the ability of oxygen-dependent FIH asparaginyl hydroxylases to inhibit HIF-1 activation. Studies with natural product-based inhibitors of the mitochondrial ETC that regulate HIF-mediated hypoxic tumor cell survival and adaptation have spurred a revitalization of interest in tumor mitochondria as a target for anticancer drug discovery.239

Redox proteins such as redox factor-1 (REF-1) and thioredoxin upregulate HIF-1 activity by enhancing the interaction between HIF-1α and transcriptional coactivators CBP/p300 and SRC-1. In MCF-7 human breast tumor cells, the overexpression of thioredoxin-1 induced the accumulation of HIF-1α protein, enhanced HIF-1 activation, and increased the expression of HIF-1 target genes without affecting the level of HIF-1α mRNA. Pleurotin (75), a toxic antibiotic obtained from the mushroom Pleurotus griseus, inhibits thioredoxin-1 reductase. Inhibition of thioredoxin reductase by pleurotin inhibits HIF-1α protein accumulation under both normoxic and hypoxic conditions (Figure 6).240 The fungal natural product chetomin (76, isolated from Chaetomium coelbioides) was found to disrupt the HIF-1α/p300 interaction from a library of >600 000 natural and synthetic compounds.241 Chetomin binds to the CH1 domain of p300, disrupts the tertiary structure, and inhibits both hypoxia- and iron chelator-induced HIF-1 activation. While toxicity limited its therapeutic potential, chetomin has since found use as a probe in the study of HIF-1 regulation and cell biology.242,243

2.20.4 Conclusions and Future Directions

Before the advent of gene overexpression methods, small interfering RNA (siRNA) technologies, and other procedures to produce genetic knockout systems, natural products, and other biologically active small molecule-based compounds provided the first efficient means to probe cell biology. Natural toxins and
clinically important natural product-based drugs have long-served as pharmacological means to either disrupt (or activate) specific enzymes, receptors, genes, or functionally important structural components of cells. While modern molecular methods have found great utility in the study of cellular processes, low-molecular-weight small molecules continue to play a critical role as pharmacological/molecular probes of cell biology. This is particularly true in regard to the study of enzyme mechanisms, drug receptor structure and specificity, ligand-activated transcription factors, and the investigation of other biochemical and biophysical processes that involve the interaction of small molecule chemical entities with crucial biological macromolecules. One reason for this is that natural products and other small molecule pharmacological inhibitors can often be used to probe a particular cellular target even when knockouts of the specific gene(s) are lethal or otherwise fail to produce a useful model system. Natural products often interact directly with specific binding sites within enzymes, drug receptors and other vital proteins, lipid structures, and genetically important macromolecules. This property of natural products facilitates their use as direct probes of the precise structural features required for essential biochemical reactions critical to the function of enzymes, receptors, and other biomolecule-mediated cellular processes.

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References

Biographical Sketches

Dr. Dale G. Nagle earned his B.S. in pharmacy and his Ph.D. in pharmacy – marine natural products chemistry with Professor William H. Gerwick at the Oregon State University. His graduate research detailed the chemistry and biosynthesis of marine eicosanoids and anti-tumor agents. Dr. Nagle worked as a postdoctoral fellow in marine chemical ecology with Professor Valerie J. Paul at the University of Guam Marine Laboratory. He took a second postdoctoral fellowship in small molecule regulators of mammalian gene expression with Professor Steven L. McKnight at the University of Texas – Southwestern Medical Center at Dallas. Dr. Nagle joined the faculty of the Department of Pharmacognosy at the University of Texas – Southwestern Medical Center at Dallas.
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Dr. Yu-Dong Zhou received her B.S. in biochemistry at Fudan University, Shanghai, China, and her M.S. and Ph.D. in biochemistry and molecular biology at Emory University. Her graduate research with Professor Kenneth E. Bernstein involved the study of tissue-specific gene expression in vitro and in vivo. Dr. Zhou then pursued postdoctoral training with Professor Steven L. McKnight in the Department of Biochemistry at the University of Texas Southwestern Medical Center at Dallas. Her research at UT Southwestern focused on the discovery and molecular characterization of mammalian neuronal PAS domain protein 1 (NPAS1) and the elucidation of the biological function of NPAS1. Dr. Zhou received the 2004 Jack L. Beal Award (jointly with Dr. Dale G. Nagle) from the American Society of Pharmacognosy. Dr. Zhou was appointed as a Research Assistant Professor of Pharmacognosy in 2007. Dr. Zhou's research focuses on the regulation of hypoxic signaling in tumor cells and the discovery of molecular-targeted small molecule natural products.