Understanding and controlling the cell cycle with natural products

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Small molecule natural products have aided in the discovery and characterization of many proteins critical to the progression and maintenance of the cell cycle. Identification of the direct target of a natural product gives scientists a tool to control a specific aspect of the cell cycle, thus facilitating the study of the cell-cycle machinery.

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Introduction

Nature has evolved an elegant solution to the complex problem of regulating cellular proliferation. With numerous, constitutive processes occurring in the background that contribute to the growth of cells, an impeccable clock controls specifically timed events in preparation for cell division. Malfunctioning of this clock wreaks cellular havoc and is the molecular basis for proliferative disorders such as cancer.

Many of the proteins that regulate critical events of the cell-division cycle have been identified. The classical genetic approach to understanding protein function in cells involves making mutations in genes that alter the function of the encoded protein. An alternative approach is to alter the function of the protein directly by using a cell-permeable ligand that binds to the protein in its intracellular environment. As with temperature-sensitive folding mutations, this chemical approach allows conditional alteration of function, causing either a loss [1] or gain [2] of function of the intracellular protein. The major challenge for this field is the development of methods for the identification of cell-permeable ligands for any given protein [3].

Currently, the most valuable collection of ligands for use in the study of protein function are natural products, or, in some instances, compounds that are closely related to natural products. Many small molecule natural products inhibit progression of the cell cycle by binding to a protein required for cell division, thus helping to determine the function of the protein. Conversely, an understanding of cell cycle events helps in understanding the mechanisms of action for many cell-cycle inhibitors. Thus, in the field of cell-cycle regulation, the marriage of cell biology and natural products chemistry has already proven fruitful, with continued promise for the future. In this review, we first outline the current state of knowledge of the cell cycle, before discussing the natural products and their derivatives that have illuminated cell-cycle events and ones with the potential for doing so.

The cell cycle

Two primary events in cell proliferation are DNA replication and cell division, the latter resulting in the formation of two cells from a single precursor (see [4] for a review of the cell cycle). Replication must necessarily precede chromosomal segregation and cell division to ensure fidelity in transmission of all genetic information. The cell cycle has been divided into four sequential phases (Fig. 1): G1 is the first gap phase in which the cell prepares for DNA replication; S phase is the period of DNA synthesis during

which a second copy of the entire genome is generated; G2 is a second gap phase in which the cell prepares for division; and, finally, M phase or mitosis is the period during which the two copies of DNA segregate and the cell divides into two genetically identical daughter cells. Entrance into and exit out of the cell cycle occurs as a cell passes between active proliferation and a quiescent or G0 state, in which the fundamental metabolism of the cell is depressed, including many of its usually active functions such as transcription and protein synthesis. Deprivation of growth factors can cause a cell to exit into G0, whereas stimulation with growth factors can signal a cell to re-enter the active cycle. A cell may also exit the cell cycle to undergo processes of differentiation or programmed cell death (apoptosis) [5,6]. Many complex signals interact to determine a cell's fate, specifying whether it should be quiescent, divide, differentiate, or undergo apoptosis.

The cell-cycle engine and brakes

The elements responsible for driving the cell cycle from one phase to the next are a series of protein kinases and phosphatases that activate and deactivate each other. The cyclin-dependent kinases (Cdks) are responsible for phosphorylating various substrates critical to cell-cycle progression. The levels of the Cdks are invariant throughout the cell cycle, but their activities are modulated by their interaction with another set of proteins called cyclins, whose levels fluctuate. The point in the cell cycle at which the various cyclins are expressed at the highest level is somewhat cell-line dependent and can vary dramatically between transformed and non-transformed cells. In general, however, the cyclin Ds are associated with G1, cyclin Es with the G1 to S transition, cyclin As with S phase as well as with the G2 to M transition, and cyclin Bs with the G2 to M transition (Fig. 1). The presence of a Cdk–cyclin complex does not ensure activity, however, as the Cdk–cyclin complexes can be inhibited by the Cdk inhibitors (CDIs; see below) [7].

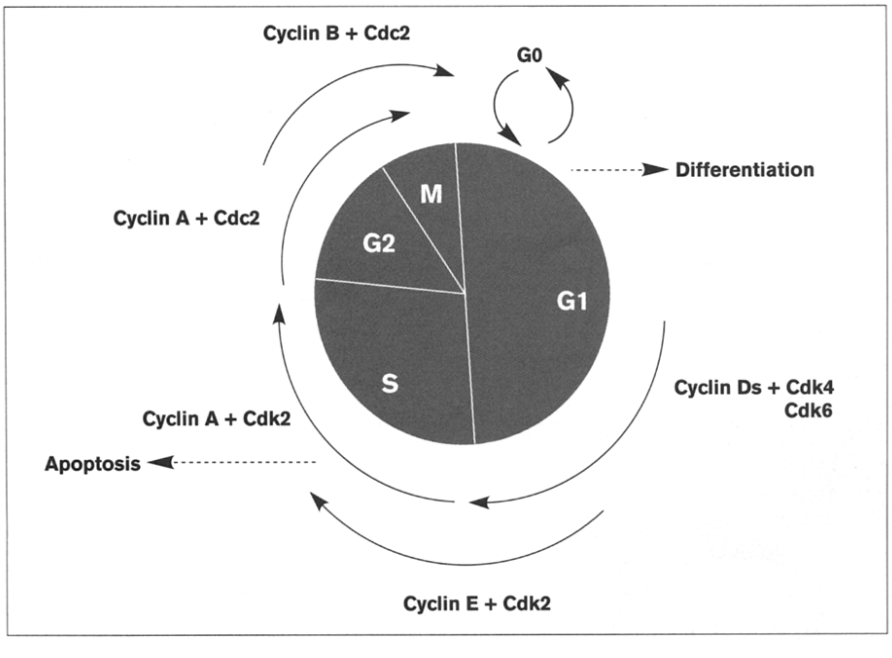
G1 phase and the G1/S transition

G1 is a critical point at which the cell assesses whether it should enter another full round of division. It is perhaps not surprising, then, that the proteins involved in G1 progression are frequently mutated in human cancers, and it is now recognized that these proteins are among the most attractive targets for the development of new therapeutic agents for the treatment of cancer. The progression from G0 to G1 is triggered by the binding of extracellular growth factors to specific cell-surface receptors. Signal transduction cascades downstream of these receptors then activate various intracellular processes required for proliferation, including the transcription and translation of many cell-cycle-dependent factors [8]. Among these factors are the G1 cyclins (predominantly the cyclin D family) that form active complexes with various Cdks. The Cdk–G1 cyclin complexes release a brake on the cell cycle, allowing it to progress forward into S phase.

S phase, G2 phase, and the G2/M transition

Driven by the the Cdk–G1 cyclin complexes, the cell progresses into S phase where a complicated assembly of DNA polymerases, primases, helicases, topoisomerases and accessory factors duplicate the genome. Independently, replication of the centrosome or microtubule-organizing center (MTOC) must also occur to ensure bipolar anchor

Figure 1



The cell cycle is driven by an ordered activation of various Cdk–cyclin complexes. The arrows show the portions of the cell cycle in which the particular Cdk–cyclin complex is active. Passage through these parts of the cell cycle require the activation of this complex, which is primarily driven by the accumulation of the specific cyclin.

sites for mitotic spindle formation. Upon completion of DNA synthesis, the cell progresses through G2, a period of active protein synthesis in preparation for cell division.

M phase and the exit from mitosis

Although a basic understanding of mitosis has existed for decades, the details of its regulation have emerged only recently. Following activation of a Cdk–cyclin complex (Cdc2–cyclin B) and condensation of the chromosomes, the paired sister chromatids must be pulled apart by the spindle into two distinct daughter cells. Formation of the spindle requires reorganization of the microtubule network via an increase in the rate of microtubule shrinking and a decrease in the rate of growth. The spindle grows from the two MTOCs, which contain γ-tubulin [9]. A subset of the microtubules also attach to the kinetochore of the chromosomes. The kinetochore is a complex of DNA and proteins that holds the two sister chromatids together prior to separation [10]. The microtubule motors dynein and kinesin align the chromosomes at the metaphase plate, and, following dissolution of a putative sister-chromatid 'glue', drive the separation of the sister chromatids.

Checkpoints

To ensure the integrity of the cell and its genetic material, the events of the cell cycle must occur in a well-defined sequence; completion of certain events must precede the initiation of others. For example, DNA replication must be completed before mitosis begins to ensure that both cells receive a complete copy of the genome. Checkpoint signaling pathways exist in the cell cycle to ensure the proper order and timing of events [11,12]. Checkpoints are thought to consist of a sensor that detects the problem, a signal transduction cascade that directs the sensor's output, and effectors that then stop transitions in the cell cycle by interacting with the cell-cycle machinery. In general, checkpoints function to allow time for a process (such as DNA replication or repair) to be completed, although after some types of DNA damage repair is also induced. Checkpoints provide the means by which many natural products arrest the cell cycle, as the damage inflicted by the natural product is detected and then the cell-cycle engine is halted.

At a critical point at the G1/S interface (called the restriction point in multicellular organisms), the cell's ability to complete a full cell cycle, including DNA synthesis, is confirmed prior to committing to S phase. In yeast, cell size is monitored at this point to determine whether sufficient nutrients are available to complete the cell cycle [13]. The cell is delayed in G1 until it is large enough to pass through the checkpoint.

Genetic analysis has identified several genes whose products monitor the integrity and replication of DNA [14,15]. Studies in both budding and fission yeast have identified genes (including, for budding yeast, the RAD and MEC genes) whose products are necessary for cell-cycle arrest either in G1 or G2 in response to DNA damage and also in response to incomplete DNA replication in G2. In mammals there is a prominent G1 DNA-damage-dependent arrest that seems to be mediated by the tumor suppressor p53 [16,17]. Although it is not required for normal growth, p53 is essential for sensing DNA damage after X-ray irradiation and causing cell-cycle arrest to permit DNA repair.

Clearly, checkpoints must be intimately tied to the regulatory events that drive or halt the cycle. For example, p53 arrest seems to be mediated by a recently discovered family of endogenous CDIs [18] that negatively regulate or halt the cell cycle. p53 induces the transcription and subsequent expression of a 21 kD protein, p21, in response to DNA damage [19]. It has been shown that p21 interacts with Cdks in complex with different cyclins [20], preventing their ability to phosphorylate and activate substrates necessary for G1 progression. The related CDI, p27, is also capable of causing G1 arrest [21,22]. Mammalian cells also arrest in G2 in response either to DNA damage sustained during S phase, or incomplete DNA replication [23].

A distinct G2 checkpoint may be activated by the inhibition of topoisomerase II [24]. Topoisomerases regulate the topology of DNA, unwinding DNA during transcription or replication, and condensing it during cell division. Whereas topoisomerase I is active constitutively, topoisomerase II expression and activity is highest during G2 and M phases [25]. Selective inhibition of topoisomerase II, in the absence of DNA damage, results in G2 arrest at a checkpoint that most probably monitors the ability to condense chromosomes.

A checkpoint also exists in mitosis to monitor mitotic spindle formation. A signal arising from incorrectly attached kinetochores [26], and possibly also an incomplete spindle structure, prevents chromosome separation and mitotic exit. Once the cell passes this last checkpoint, it divides and the two daughters reenter G1.

Natural product inhibitors of the cell cycle

Small molecule natural products have aided in disentangling the complex web of cell-cycle events in several ways. Irrespective of their targets, natural products have been invaluable as agents to achieve arrests at specific points in the cell cycle, allowing synchronization of a population of cells. Identification of the direct targets of natural product inhibitors of the cells cycle implicates specific proteins or activities as being essential to cell-cycle-related functions. Components downstream of these direct targets can then be identified on the basis of their indirect inhibition by the same agent. Once a specific binding interaction is

established, a cell-permeable natural product can be used to understand the function of its protein target in living cells. Exposure of cells to the natural product causes a conditional loss of function in the target protein, a result similar to that achieved by the use of temperature-sensitive mutations in the gene encoding a protein of interest. Commonly, such mutations result in the unfolding of the mutant protein at the non-permissive temperature, resulting in loss of the protein's function.

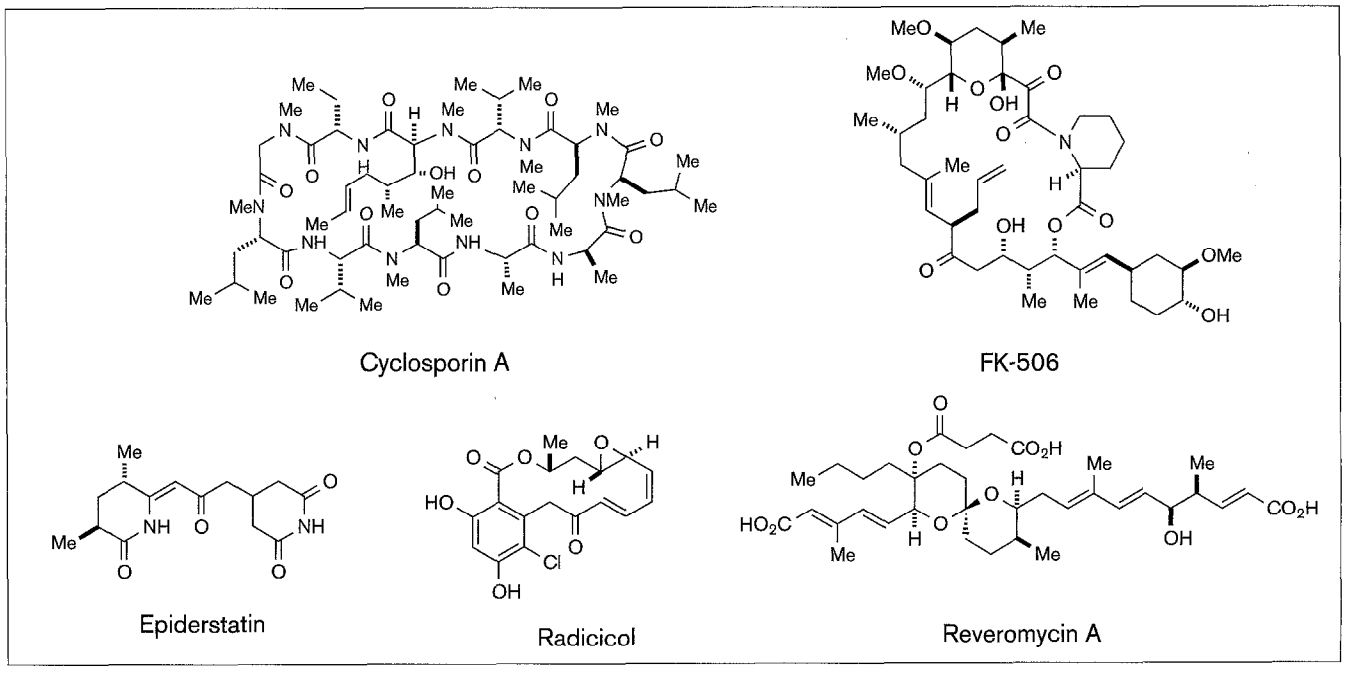
Entry into the cell cycle – the G0 to G1 transition

One class of natural products (Fig. 2) warrants brief mention prior to discussion of agents that arrest the active cell cycle. In response to certain external signals, a cell will enter the active cell cycle from a resting G0 state. Various exogenous growth factors act to stimulate the cell through activation of their respective receptors, located on the plasma membrane of the cell [27,28]. Many of these receptors are tyrosine kinases which, upon activation, initiate an intracellular signal transduction pathway whose ultimate end point is cell proliferation. Some natural products inhibit the signal-transduction pathways required for the G0 to G1 transition. Perhaps the most thoroughly studied examples are FK506 and cyclosporin A, which inhibit the T-cell receptor (TCR) pathway through inhibition of calcineurin [29,30]. The TCR pathway prepares a resting (G0) T cell for the cell cycle in part by inducing the synthesis of interleukin-2 (IL-2) and the IL-2 receptor, which, when combined, activate a second signaling pathway required for T cells to progress through the G1 phase of the cell cycle. Other natural products that inhibit the G0 to G1 transition include radicicol, which inhibits the platelet-derived growth factor (PGDF) receptor pathway [31], and epiderstatin [32] and reveromycin [33], which inhibit some element of the epidermal growth factor (EGF) receptor pathway. The PDGF receptor pathway is generally viewed as one used by some cells to exit quiescence and enter the cell cycle (G0 to G1), whereas the mechanistically related EGF receptor pathway has been viewed as facilitating further progression through G1.

G1-phase-specific arrest agents

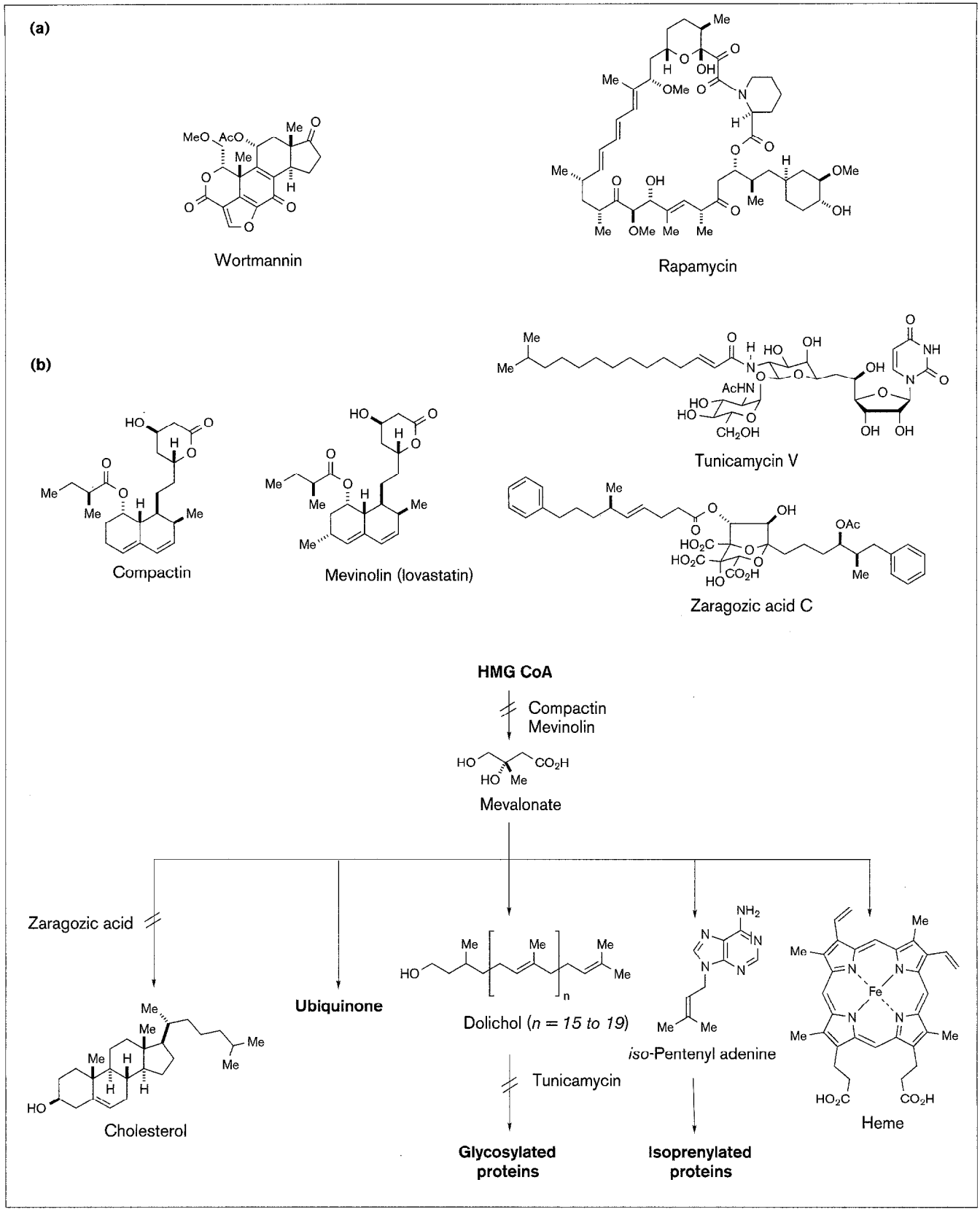
A molecule that inhibits a process that affects a cell-cycle protein, such as its synthesis, post-translational modification, or degradation, tends to be less useful in cell cycle studies than an inhibitor that binds to the actual protein itself, because other, non-related proteins can be regulated by the same upstream mechanism. Thus, the degree of specificity of an arrest agent may be proportional to its functional proximity to actual cell-cycle regulatory events. Within the class of G1 arrest agents, at least two inhibitors, rapamycin and wortmannin (Fig. 3a), act directly on cell-cycle events themselves. Rapamycin, in complex with its intracellular binding protein FKBP12, inhibits FRAP (FKBP–rapamycin binding protein) [34–36], a signaling molecule required for the activation of the downstream

Figure 2



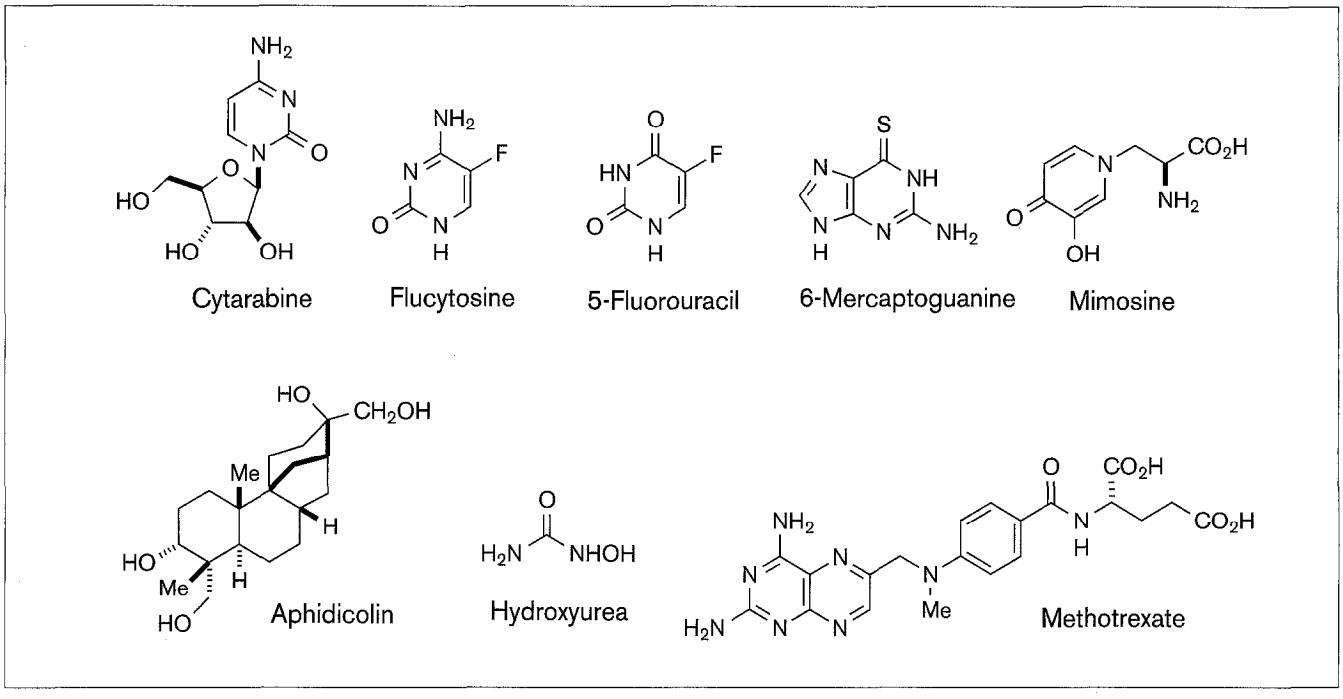
G0 to G1 inhibitors. Cyclosporin A and FK506 inhibit the T-cell receptor pathway, which in turn prevents the induction of interleukin-2 (IL-2) and the IL-2 receptor; these molecules activate a pathway that drives T cells from G0 into G1. Epiderstatin, radicicol and reveromycin A all inhibit pathways from G0 to G1 that are activated by extracellular growth factors.

Figure 3



G1-specific arrest agents. **(a)** Rapamycin and wortmannin inhibit the activation of p70 S6 kinase, thought to be required for progression from G1

Figure 4



G1/S- and S-phase-arrest agents that inhibit DNA synthesis.

effector molecule p70 S6 kinase and for the inactivation of the inhibitor of translation initiation, 4E-BP1 [37]. Further downstream in the FRAP pathway are the cyclin-dependent kinase inhibitors p21 and p27. Neither the direct action of FRAP nor the precise mechanism by which it regulates the levels of p21 and p27 are currently known, although it appears to involve the regulation of translation of specific mRNA transcripts. Wortmannin also prevents p70 S6 kinase activation, in this case by inhibiting phosphatidylinositol 3-kinase (PI3K) [38–40]. The functions of PI3K's phosphorylated substrates are at present incompletely defined. Nevertheless, FRAP and PI3K's common downstream target, p70 S6 kinase, is thought to be required for G1 progression into S phase [41–43]. Both of the natural products have been used widely to identify components of the FRAP- and PI3K-dependent signaling pathways. Indeed, these pathways were discovered by their response to the natural products.

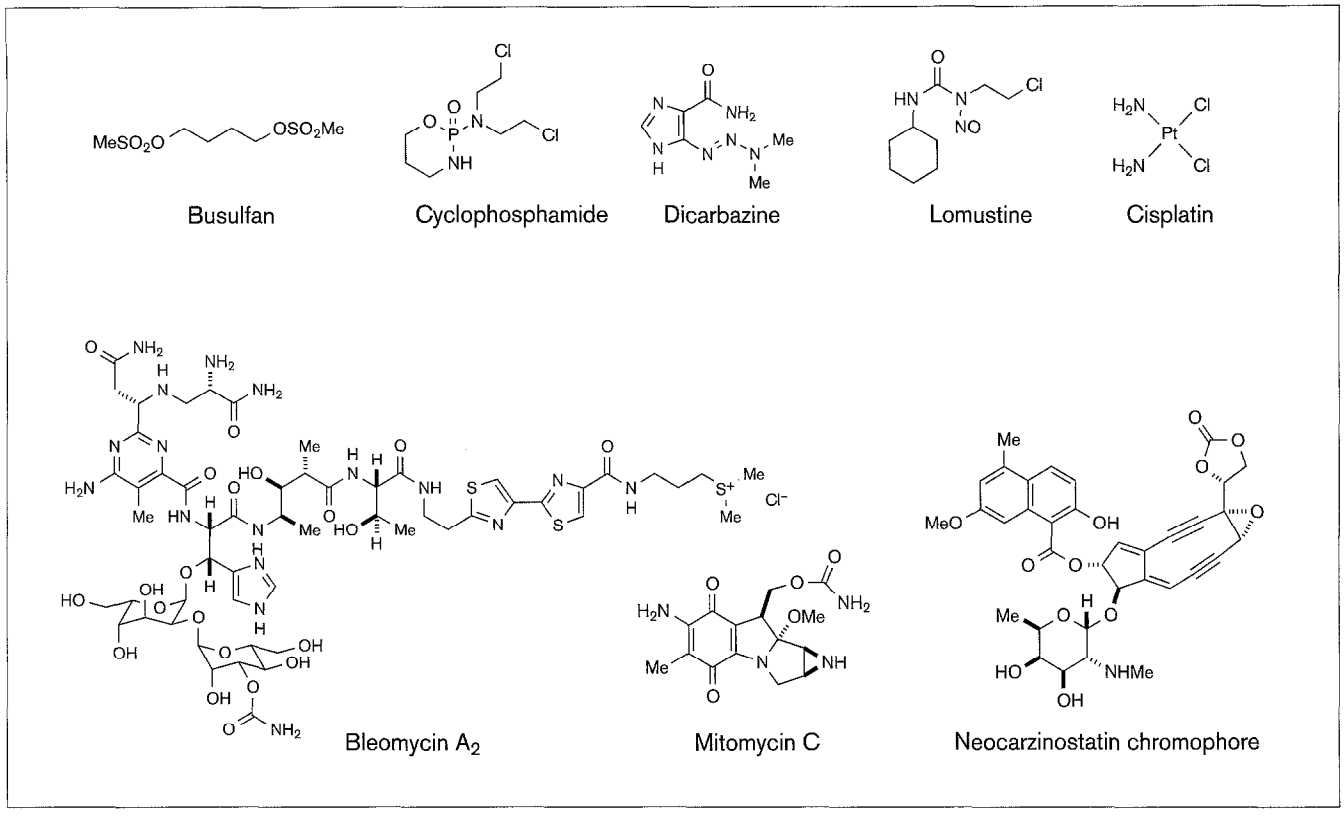
Another major subset of G1 arrest agents are compounds that interfere with the mevalonate metabolic pathway (Fig. 3b). Mevalonate is the isoprenoid precursor to many essential cellular components including cholesterol, dolichol, ubiquinone, heme, tRNA, and isoprenylated proteins. Inhibitors of HMG-CoA reductase (an enzyme required for mevalonate synthesis) such as compactin and lovastatin, result in G1 arrest [44]. Several different mevalonate requirements exist for cell-cycle progression in G1. Compactin causes a very early G1 arrest, most likely related to a cholesterol requirement, since addition of cholesterol allows the cell to recover and progress [45]. Zaragozic acid, an inhibitor of squalene synthase, also causes this early G1 block by inhibiting cholesterol synthesis (Fig. 3) [46].

Products of mevalonate metabolism other than cholesterol are also required for G1 progression, since a later G1 block is insensitive to the addition of cholesterol [47]. A late G1 arrest can also be induced by tunicamycin (Fig. 3) [48,49]. Tunicamycin inhibits the first step in the glycosylation of proteins, namely, the transfer of N-acetylglucosaminyl-1-phosphate to dolichol, an isoprenoid lipid derived from mevalonate [50]. This dolichol requirement strongly suggests that glycoproteins are essential for progression through late stages of G1 [51]. A third requirement derived from the mevalonate pathway was revealed by the inability of cholesterol and dolichol addition to overcome another late compactin block [52]. Instead, the addition of isopentenyl adenine was more effective at restoring cell-cycle progression [53]. Isopentenyl adenine is an essential element in the isoprenylation of proteins. This finding suggests that isoprenylated proteins have an important role in proliferation (Fig. 3), as in the case of the protein Ras, now a target for drug development [54,55]. Surprisingly, inhibition of mevalonate biosynthesis results in a G1 arrest only, implying that none of these same components or functions are required in later cell-cycle phases.

G1/S-transition and S-phase arrest agents

Small molecules that cause cell-cycle arrest by affecting DNA fall into two classes (Fig. 4). One class of compounds

Figure 5



DNA-damaging agents that cause G1 and G2 arrest.

directly inhibits DNA synthesis by affecting either the replication machinery or the nucleotide pool, resulting in arrest at the G1/S interface, or in S phase if the compound is added after passage into S phase. The second class causes damage to DNA, leading to a G1 or G2 checkpoint-mediated arrest, and will be discussed in a later section.

A majority of the established chemotherapeutic and antineoplastic agents are synthetic antimetabolites that inhibit the synthesis of deoxyribonucleotides, thus preventing elongation of nascent polynucleotide chains. Although they are not strictly cell-cycle-specific, cells are more sensitive to their actions in late G1 and S. Representative members include methotrexate (an inhibitor of dihydrofolate reductase); 5-fluorouracil, cytarabine, and flucytosine (pyrimidine analogs); and 6-mercaptoguanine (a purine analog) (Fig. 4) [56]. Hydroxyurea (HU) also arrests cells at the G1/S interface if they have not yet entered S phase, or in S phase if they have [57]. HU inhibits ribonucleotide diphosphate reductase, an enzyme that converts ribonucleotides to deoxyribonucleotides. The fairly immediate arrest observed upon addition of these inhibitory compounds suggests tight coordination between DNA synthesis and cell-cycle progression. Either there is only a very small pool of excess deoxyribonucleotides so that constant nucleotide synthesis is required, or there is a monitoring system (analogous to cell-cycle checkpoints) that is tightly coupled to nucleotide synthesis and quickly senses its inhibition. HU is also one of the most effective reagents in cell-cycle studies, as it can be used to synchronize cells at the G1/S interface. Removal of HU by simple washing allows a population of cells to continue through the cell cycle in a synchronized manner.

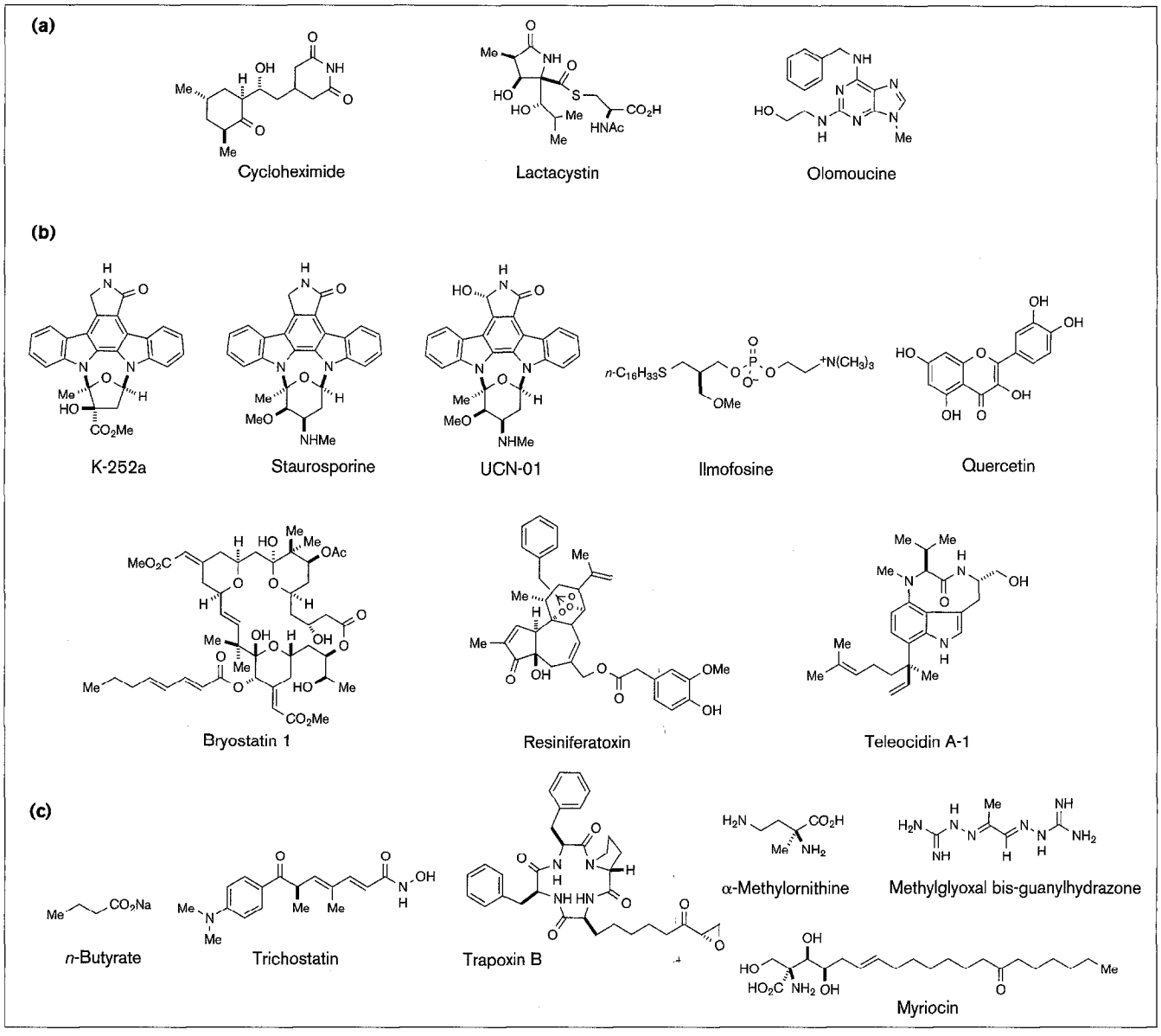
Aphidicolin causes S-phase arrest by inhibiting DNA polymerase α (Fig. 4) [58]. It inhibits expansion and elongation of the replication bubble, but not replication fork formation. Mimosine, on the other hand, has been traditionally thought to inhibit the formation of replication forks, formally causing a late G1 arrest. Recent work now suggests that mimosine may be affecting the deoxyribonucleotide pool in a manner similar to HU, causing S-phase arrest [59].

G1- and G2-phase arrest agents

Several classes of compounds cause both G1 and G2 arrest. Either they cause damage to the cell that can be detected by either a G1 or G2 checkpoint, or they inhibit targets that are essential to both phases.

One major class of compounds consists of the DNA damaging agents (Fig. 5). Surprisingly, at low concentrations, they do not cause S-phase arrest. Instead, cells in S phase

Figure 6



G1- and G2-arrest agents. **(a)** Agents affecting the cell-cycle engine by inhibition of either synthesis (cycloheximide) degradation (lactacystin) or activitation (olomoucine) of cell-cycle components. **(b)** PKC modulators. **(c)** Agents inhibiting other processes.

treated with DNA-damaging agents progress to a G2 arrest. Clinically used synthetic DNA alkylating agents include the nitrogen mustards (e.g., cyclophosphamide), alkyl sulfonates (e.g., busulfan), nitrosoureas (e.g., lomustine), and triazenes (e.g., dicarbazine) [56]. Recently, natural products that damage DNA have been the focus of great interest to chemists, both synthetically, because of their complex architecture and functionality, and mechanistically. These compounds include neocarzinostatin [60,61] mitomycin C, and bleomycin [62]. The mechanisms of cell-cycle arrest by DNA-damaging agents can be complicated. The DNA-damaging agent cisplatin illustrates this point. Cisplatin was originally thought to act simply by crosslinking DNA via nucleophilic displacement of its chlorides by the N7 of guanines. Recent work however, demonstrates that this cisplatin–DNA adduct binds certain nuclear proteins, such as SRY (the human testis-determining protein) and hMSH2. This protein–ligand complex may shield the DNA damage from repair mechanisms [63–65].

These compounds have facilitated an understanding of the cellular response to DNA damage. It now appears that it is not the DNA damage inflicted by these compounds that directly causes arrest. Instead, in mammalian cells, the DNA damage causes arrest at a checkpoint by activating

p53 by an unknown mechanism, leading to the transcriptional activation of the gene encoding p21 (a Cdk inhibitor, see above) [66,67]. In budding yeast, two *rad* mutants (see above) that are defective at this checkpoint are insensitive to the DNA-damaging agent bleomycin [68]. These results demonstrate that DNA-damaging agents cause arrest by activating a DNA-damage checkpoint in the cell cycle, resulting in inhibition of cell-cycle progression (through p21 in mammalian cells) and activation of the DNA-repair machinery. Only if the damage is not repaired will apoptosis be induced. Thus, DNA-damaged cells are either repaired or eliminated.

A second class of G1- and G2-arrest agents includes inhibitors of protein synthesis or degradation, which arrest the cell cycle in different phases, depending on the time of treatment (Fig. 6a); these arrests may be a result of an inhibition of the synthesis or degradation of many proteins. For example, cycloheximide [69] blocks the translation of cyclins [70,71], whereas lactacystin inhibits protein degradation by the 26S proteasome by binding to its X/MB1 subunit [72], a close homolog of the LMP7 subunit encoded by the major histocompatibility complex. This binding blocks the degradation of proteins that normally prevent neurite outgrowth [73], and presumably of cyclin, as it is degraded by the proteasome.

The aminopurines are also thought to mediate their effects by modulating Cdk–cyclin activity, but in a more direct manner than affecting protein levels (Fig. 6a). Because the aminopurines are ATP analogs, they can serve as nonselective protein-kinase inhibitors. The analog olomoucine, however, is selective for the Cdks. It competitively inhibits ATP binding to the kinase domain of the Cdc2–cyclin B, Cdk2–cyclin A, and Cdk2–cyclin E complexes [74]. G1 and G2 arrest probably occur by the inhibition of Cdk2–cyclin E and Cdc2–cyclin B, respectively.

Inhibitors of other protein kinases also cause arrest in G1 and G2 (Fig. 6b). Their mechanisms of action are much less clear, however, due in part to a poorer understanding of the role of these kinases in the cell cycle. Staurosporine and K252a are examples of protein kinase C (PKC) inhibitors that cause G1 arrest at low concentrations (20 nM) and G2 arrest at higher concentrations (200 nM) [75–77]. These compounds are not, however, selective for PKC, as they also inhibit Cdc2–cyclin B, Cdc2–cyclin A, and Cdk2–cyclin A complexes *in vitro* [78]. In contrast, the structurally related UCN-01 [79] and quercetin [80] are much more selective PKC inhibitors, which cause predominantly G1 arrest. Finally, a third type of PKC inhibitor, characterized by the structurally unrelated ilmofosine, a thioether phospholipid that specifically inhibits PKC by competitive binding to the phospholipid site, causes G2 arrest, indirectly suppressing Cdc2–cyclin B activity by affecting levels of cyclin B [81]. Clearly, the more specific inhibitors UCN-1, quercetin, and ilmofosine suggest that PKC has a role in both G1 and G2 progression. They also demonstrate the diversity of PKC and its many isozymes. In yeast, it has been shown that a conventional form of the kinase, cPKC, is essential in the G2 to M progression [82], and in mouse fibroblasts a novel form, nPKC, is essential in the G1 to S progression [83]. Thus, the differences in the phase of cell-cycle arrest observed with the various PKC inhibitors may reflect their specificities for the various PKC isoforms. The complexity of PKC is demonstrated by the varying effects of numerous other PKC modulators such as bryostatin [84], teleocidin [85], and perhaps resinferitoxin [86] on cellular proliferation. A promising area of research combines the use of natural products as templates for combinatorial chemistry and screens capable of identifying variants that inhibit specific kinases.

The use of other natural products that cause cell-cycle arrest has led to the identification of additional conditions required for cell-cycle progression (Fig. 6c). Histone deacetylation seems to be critical to both G1 and G2, since its inhibition by *n*-butyrate, trichostatin, or trapoxin [87] can results in arrest in either of these phases. The relationship between hyperacetylation of core histones and cell-cycle regulation however, remains to be elucidated. The recent discovery and cloning of the target of trapoxin and trichostatin, human histone deacetylase-1 (HD1), promises to expand this exciting area of research [88]. Further, inhibition of polyamine synthesis by α-methyl ornithine (an inhibitor of L-ornithine decarboxylase in the biosynthesis of putrescine) or methylglyoxal bis-guanylhydrazone (an inhibitor of S-adenosyl methionine decarboxylase in the biosynthesis of spermidine and spermine) blocks DNA synthesis and cell division [89]. Polyamines may stabilize DNA folding [90] or activate DNA polymerase [91]. Lastly, growing interest in sphingolipids as signaling molecules for cellular proliferation has resulted in the identification of a glucosylceramide synthase inhibitor *threo*-1-phenyl-2-decanoylamino-3-morpholino-1-propanol (PDMP) [92]. PDMP blocks the G1/S and G2/M transitions by effecting decreases in Cdc2 and Cdk2 activity. Although the mechanism of this decrease remains a mystery, sphingolipids have been implicated in cellular proliferation and differentiation, DNA synthesis [93], transcription [94], and apoptosis [95]. The natural product myriocin may help in understanding how these sphingolipid-dependent signaling pathways tie in with cell-cycle progression. Myriocin has been reported to inhibit a serine palmitoyltransferase activity that is required for the first step of sphingolipid biosynthesis [96,97]. Thus the cell cycle, particularly the critical G1/S and G2/M transitions, requires a multitude of protein mediators, and disruption of just a single one of these activities is sufficient to interrupt its progression.

G2-phase-specific arrest agents

Two major classes of G2-specific, cell-cycle arrest agents are the inhibitors of topoisomerase II (Fig. 7a) and

serine/threonine phosphatases (Fig. 7b). The tyrosine kinase inhibitors genistein [98] and herbimycin [99] (Fig. 7c) comprise a recently discovered third class, but their mechanisms of action are not yet understood.

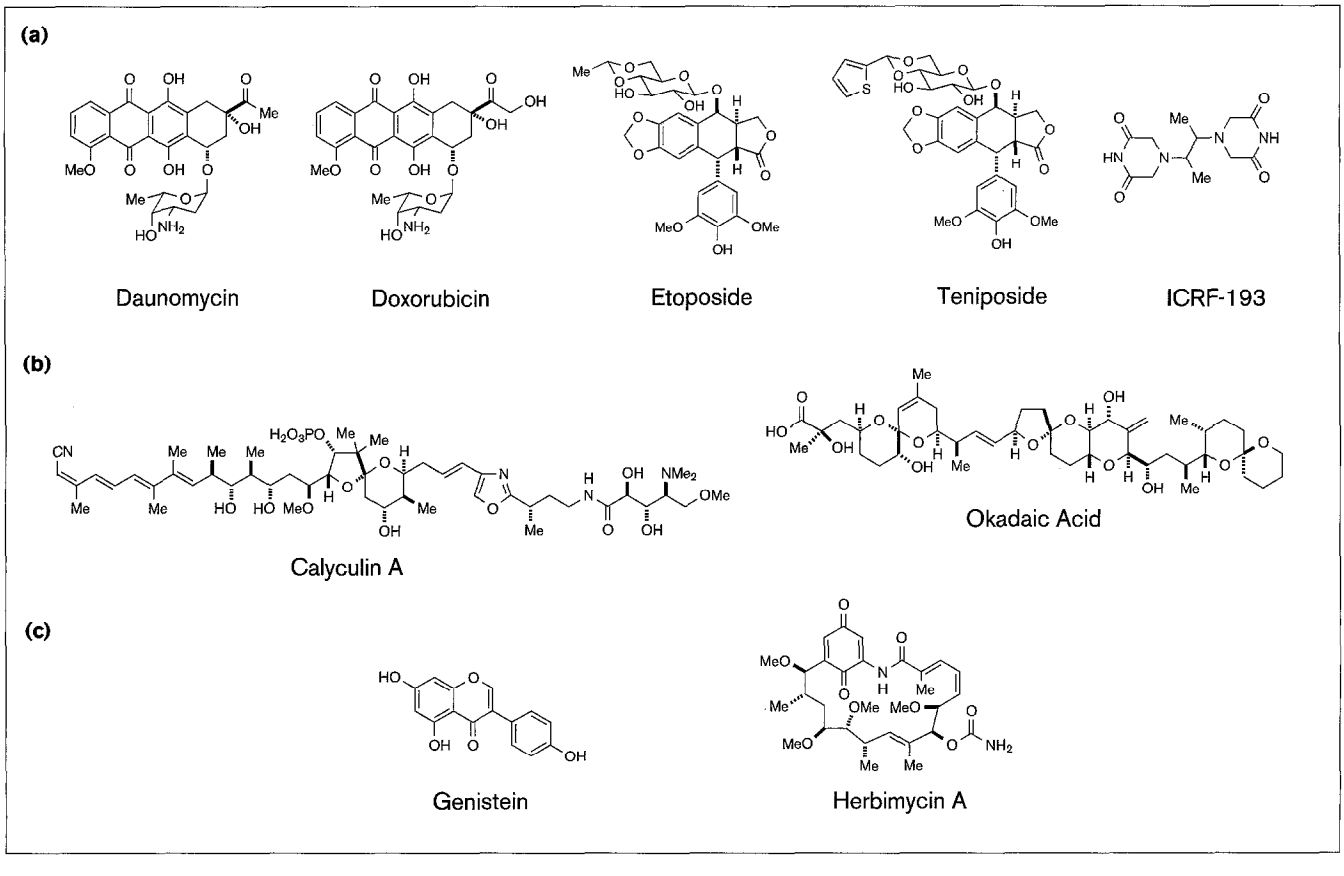
The anthracyclines doxorubicin and daunomycin, and the podophyllotoxins etoposide and tenoposide, were originally thought to act simply by cleaving DNA via semiquinone radicals (Fig. 7). The observation that some doxorubicin-resistant tumors contain undetectable or very low topoisomerase II activity [100], however, suggests that these molecules may require topoisomerase II [101] to arrest cells. Some evidence suggests that arrest occurs at concentrations sufficiently low that no DNA cleavage is observed, but that topoisomerase II activity is inhibited. Alternatively, topoisomerase II may potentiate the ability of the anthracyclines to cleave DNA, thus lowering the necessary effective concentration. Confirmation that inhibition of topoisomerase II may be sufficient to effect arrest came from studies done with a different topoisomerase II inhibitor, ICRF-193 [102]. ICRF-193 inhibits topoisomerase II in a DNA-independent manner, without direct damage to DNA. This compound, like doxorubicin and daunomycin, also causes G2 arrest in mammalian cells, indicating that prior to entry into mitosis, a checkpoint monitors successful chromosomal condensation and proper topology resulting from topoisomerase II activity, independent of DNA-damage and -repair pathways [24].

Okadaic acid and calyculin A are potent protein phosphatase (PP) inhibitors, selective for the serine/threonine phosphatases PP1 and PP2A. At low concentrations (20 nM), they cause a G2/M block, prior to activation of Cdc2–cyclin B complexes [103]. The mechanism of this arrest may involve the PP2A-like activities that regulate Cdc25 phosphatase and wee1 kinase, which in turn regulate the phosphorylation status of Cdc2. Sodium orthovanadate is an inhibitor of tyrosine phosphatases. It also causes a G2 block, perhaps by inhibiting Cdc25 dephosphorylation and activation of Cdc2–cyclin B complexes [104].

M-phase-specific arrest agents

Most of the mitotic arrest agents (Fig. 8) seem to act by interfering with cytoskeletal organization, predominantly affecting the microtubules required for spindle formation and chromosome segregation, although some agents interfere with actin, which is required for cytokinesis. (A recent

Figure 7



G2-specific arrest agents. **(a)** Topoisomerase II inhibitors. **(b)** Phosphatase 1 and 2A inhibitors. **(c)** Tyrosine kinase inhibitors.

exception is ilimaquinone, which causes a complete breakdown of the Golgi and thus inhibition of secretion, as well as mitotic arrest by an unknown mechanism [105].) The mechanism of action of cytochalasins is seemingly simple — at the end of mitosis they inhibit cytokinesis by binding to actin and preventing its polymerization [106] — but the end effect on the cell cycle is actually quite complicated. While the cytochalasins act during mitosis to prevent actin polymerization, they do not cause M-phase arrest but allow the cell to progress into the next G1 phase. Release from the block within a certain time frame allows cytokinesis to occur in G1 [107]. Thus, it appears that mitosis and cytokinesis are two distinct and somewhat independent events.

By far the largest class of mitotic arrest agents affect microtubule formation (Fig. 8). Microtubules and their attachment to the kinetochore have a critical role in cell division, since unattached kinetochores can prevent exit from mitosis by generating a checkpoint signal that inhibits the cell-cycle machinery [26]. The spindle is composed of microtubules, and the mechanics of microtubule elongation and shortening control mitotic spindle formation. Because the dynamic behavior of microtubules is critical for proper chromosome alignment and segregation, it is not surprising that compounds that exaggerate either the stability or instability of microtubules will disrupt their mechanical function. The majority of these compounds are destabilizing agents.

Tubulin exists as a dimer consisting of α and β subunits. Microtubule-destabilizing agents tend to bind the free dimer, preventing polymerization. Two major binding sites have been identified for these compounds. Colchicine binds to a single site on the dimer with pseudo-irreversible kinetics, suggesting that conformational changes occur in both the ligand and tubulin dimer. By photoaffinity labeling, the colchicine binding site has been localized to two small amino-terminal and carboxy-terminal regions on β-tubulin that lie near β-tubulin's point of interaction with α-tubulin [108]. Other microtubule-destabilizing agents, including podophyllotoxin [109] and nocodazole [110], bind at sites that overlap with the colchicine site, but in a reversible manner. The colchicine site is distinct from the vinca alkaloid binding site. Vinblastine and vincristine [111] bind at two high affinity sites on each tubulin dimer resulting in stabilization of the αβ heterodimer but destabilization of microtubule polymers. Whereas the vinca alkaloids bind in a reversible manner, maytansine binds covalently at the vinca alkaloid site, alkylating tubulin with its epoxide group [112]. Halichondrin [113], ustiloxin A [114], and spongistatin [115] also bind tubulin at the vinca alkaloid site. The binding site on tubulin of estramustine has not been determined [116]. Interestingly, substoichiometric amounts of each of these compounds are sufficient to inhibit completely microtubule assembly [117]. This phenomenon has led to the proposal that further polymerization is inhibited by the 'capping' of the growing 'plus' end of a microtubule by a tubulin–drug complex; meanwhile, the dynamic nature of microtubules and the difference in polymerization rates at the two ends permits continued depolymerization at the 'minus' end, resulting in net disassembly.

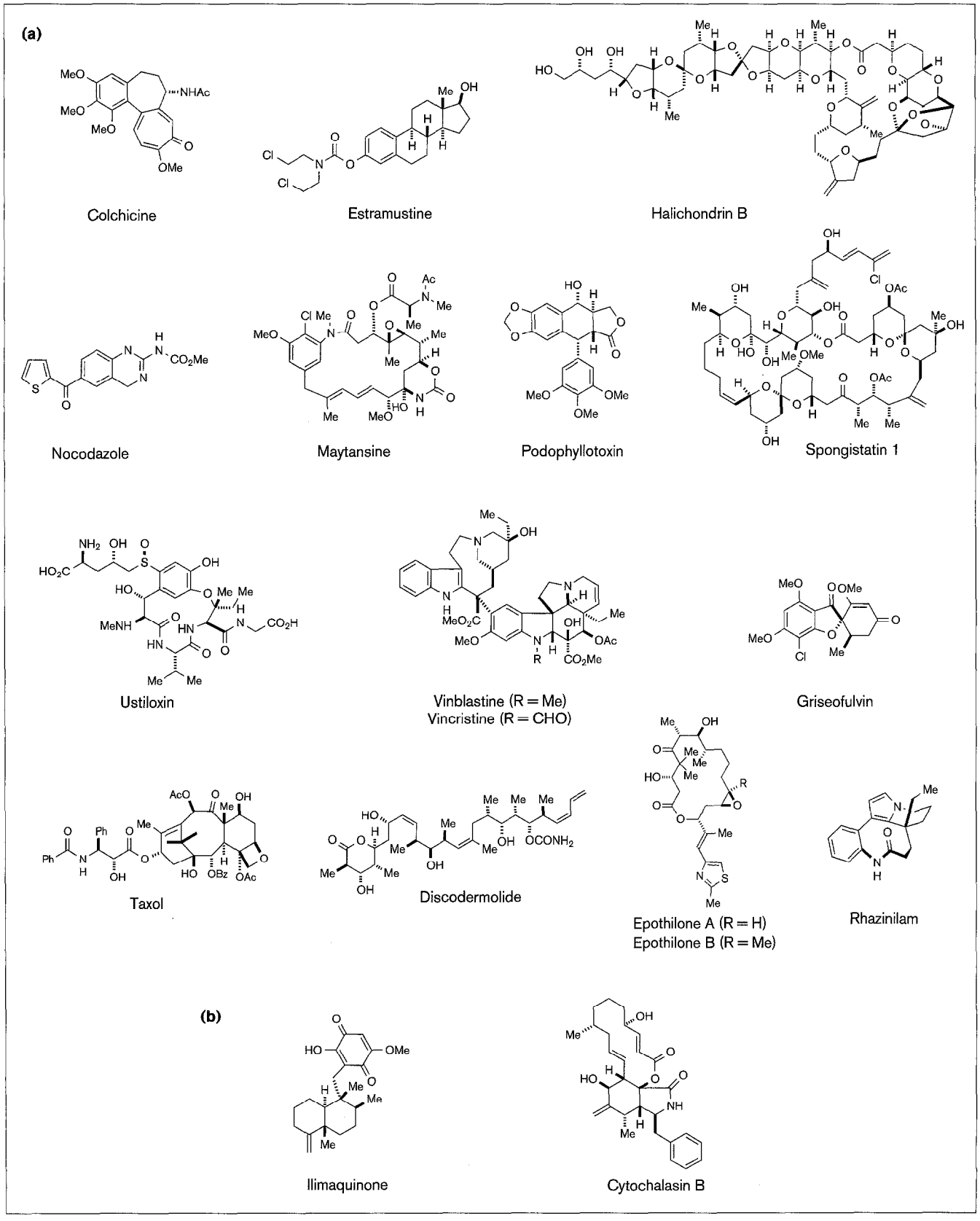
In contrast to the destabilizing agents, taxol [118] promotes the stabilization of microtubules, both *in vitro* and *in vivo*, presumably causing mitotic arrest by preventing proper assembly of the mitotic spindle [119] or by reducing chromosome oscillations [120]. Structures of taxol bound to microtubules have been reported using electron diffraction [121] X-ray scattering [122] and electron microscopy [123]. Photoaffinity labeling of tubulin by a taxol analog localizes the taxol-binding site to the amino terminus of β-tubulin [124]. The marine natural product discodermolide also stabilizes microtubules [125] by binding directly to them with a higher affinity than is found for taxol binding [126]. As with taxol, binding was found to be stoichiometric with tubulin dimers. Using tritiated taxol and discodermolide, it was found that the binding of these two natural products is mutually exclusive, suggesting a possible common binding site despite the lack of any obvious structural similarities. Epothilone is a competitive inhibitor of taxol, which also stabilizes microtubules and shows no obvious structural similarities to either taxol or discodermolide [127]. Rhazanilam has been reported both as a stabilizing and destabilizing agent towards microtubules [128].

Whereas the agents discussed above interfere with microtubule stability by direct interactions with tubulin, one natural product, griseofulvin, may have an indirect effect. Griseofulvin was originally reported as a mitotic arrest agent that did not alter microtubule stability [129]. Later work, however, revealed that the compound interferes with microtubule polymerization without binding to purified microtubules [130]. This finding raises the possibility that griseofulvin may have an indirect effect on microtubules, possibly mediated by microtubule-associated proteins (MAPs), which regulate microtubule polymerization. Thus, whether they function directly through association or indirectly through MAPs, it is clear that compounds that disrupt the normal balance of polymerization and depolymerization of microtubules cause mitotic arrest.

Natural products that activate programmed cell death

Prolonged disruption of the cell cycle results ultimately in death that has been proposed to occur via programmed cell death (apoptosis). In other words, if the cell is not viable, it commits suicide. Many small-molecule, cell-cycle arrest agents such as aphidicolin [131], vincristine [132], taxol [133], mitomycin C and staurosporine [134] cause apoptosis by either p53-dependent or -independent pathways

Figure 8



Mitotic arrest agents. **(a)** Agents that disrupt microtubule dynamics or structure. **(b)** Other M-phase arrest agents.

[135]. In addition to activating a checkpoint arrest in DNA-damaged cells, p53 also seems to be responsible for inducing death if repair fails [136]. Clearly, studying the mechanisms of many of these natural product inhibitors will help to elucidate the regulation of the suicidal process. Understanding their relationships to apoptosis may result in the development of more efficacious chemotherapeutic approaches to inducing apoptosis in cancer cells.

Natural products to probe checkpoint-signaling pathways

Checkpoint signaling is an important yet poorly understood area of signaling research. Rapamycin (see above) is one important tool that is helping to illuminate these fascinating processes. Human FRAP and the yeast TOR proteins, the targets of FKBP12–rapamycin, appear to act as G1 sensors. One hypothesis is that these proteins monitor cell size and/or the existence of external nutrients by monitoring the state of messenger RNA molecules. These proteins are homologs of the yeast Mec1 protein (see above), which is a sensor of the status of DNA molecules, and are members of a newly identified family of proteins, the phosphatidylinositol kinase (PIK)-related kinases, that appear to be critical to several checkpoint-signaling pathways that monitor the status of polynucleotides [137]. The mutation of one member of this gene family is responsible for the disease ATM (ataxia telangiectasia), which seems to be caused by an error in a signal to the tumor suppressor and checkpoint protein p53 [17,138].

Natural products to control the cell cycle

The ability to control the timing of arrest facilitates efforts to understand cell-cycle regulation, and the extensive collection of specific arrest agents provides the tools necessary for this control. The definition of the arrest point within the cycle is a necessary prerequisite before a natural product can be used in this manner. Analysis of cyclin expression has proven to be more precise than the determination of the DNA content of cells by flow cytometry alone (2N for G1 diploid cells, 4N for G2 or M diploid cells). For example, in synchronized cells that have been treated with a G1 arrest agent, the appearance of cyclin D, but not cyclin E, indicates that the block is in early to mid G1. In contrast, appearance of both cyclins indicates a late G1 block. Given the caveat of cell-line dependence, this type of analysis can be used in G1 by examining cyclin D and E expression [76,139], and in G2 by examining cyclin A and B expression [140]. Although the majority of compounds have not been analyzed at this level of detail, Figure 9 summarizes the results obtained for some compounds. These molecules can be used to synchronize an entire population of cells at many different and now characterized stages of the cell cycle, as the blocks are reversible. Washing the cells with media free of the blocking agent allows the cells to continue through the cycle, beginning precisely at the stage of the reversible block.

Orphan ligands – new probes of the cell cycle

A cornucopia of small molecules exist that have potential as tools for studying the cell cycle. Some of them show cell-cycle-specific effects, yet their mechanisms of inhibition are unknown. Even more of them have gone undetected within the pool of known natural products as they have not yet been examined for this activity. We have depicted some interesting candidates in Figure 10, chosen primarily based on the criterion of provocative or unknown biological activity. These compounds include deoxyspergualin [141], depudecin [142], didemnins (for which two protein receptors have been identified, yet for which a clear mechanism of cell-cycle arrest has not emerged) [143–145], leptolstatin [146], palau'amine [147], and diazonamide A [148]. These molecules are but a few representatives of an important resource from which invaluable tools for dissecting the cell cycle may be found.

Natural products have been described as 'genetically-encoded libraries of peptidomimetic, small molecule

Figure 9

Relative timing of arrest by different cell-cycle arrest agents.

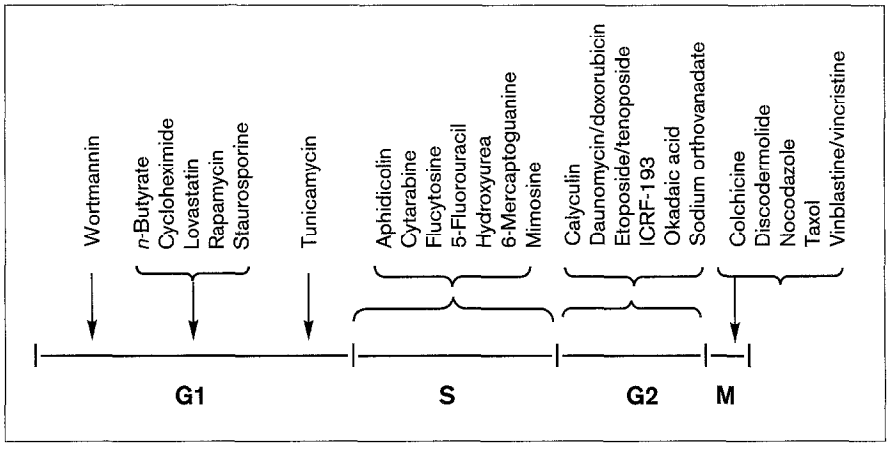
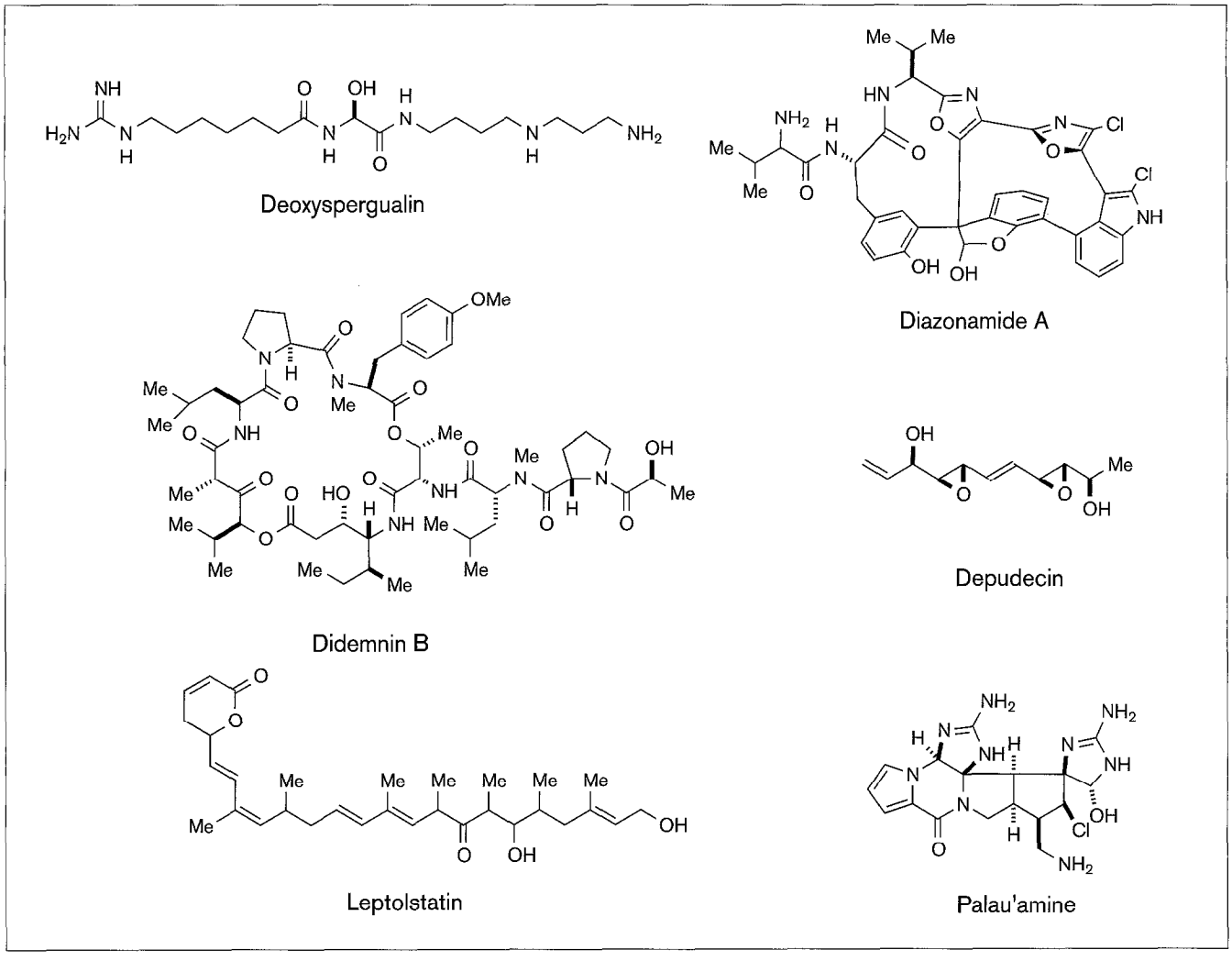


Figure 10



Natural products with unknown mechanisms of action that could potentially be useful in cell-cycle studies.

ligands' (J. Clardy, personal communication), and these naturally-occurring compounds have been central to the illumination of many molecular details of the cell cycle. The recent emergence of combinatorial chemistry may be the harbinger of yet another exciting merging of the fields of organic chemistry and cell biology. Combinatorial synthesis is a laboratory emulation of nature's generation of diversity, and this ability to generate tremendous numbers of diverse molecules gives this method the potential to be a very powerful tool for the next generation of investigators of the cell cycle.

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