Small-molecule inhibitors of the cell cycle

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The cell cycle remains an attractive target for the development of small-molecule inhibitors for use as both novel chemotherapeutics and research probes. Given the importance of cytoskeletal dynamics and cyclin-dependent kinases for cell-cycle progression, much interest has focused on the identification of anti-mitotic agents and kinase inhibitors. However, recent advances in cell-based screening technologies and an increased interest in inhibitors with greater specificity are beginning to influence the search for novel cell-cycle inhibitors.

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Current Opinion in Chemical Biology 2000, **4**:47–53

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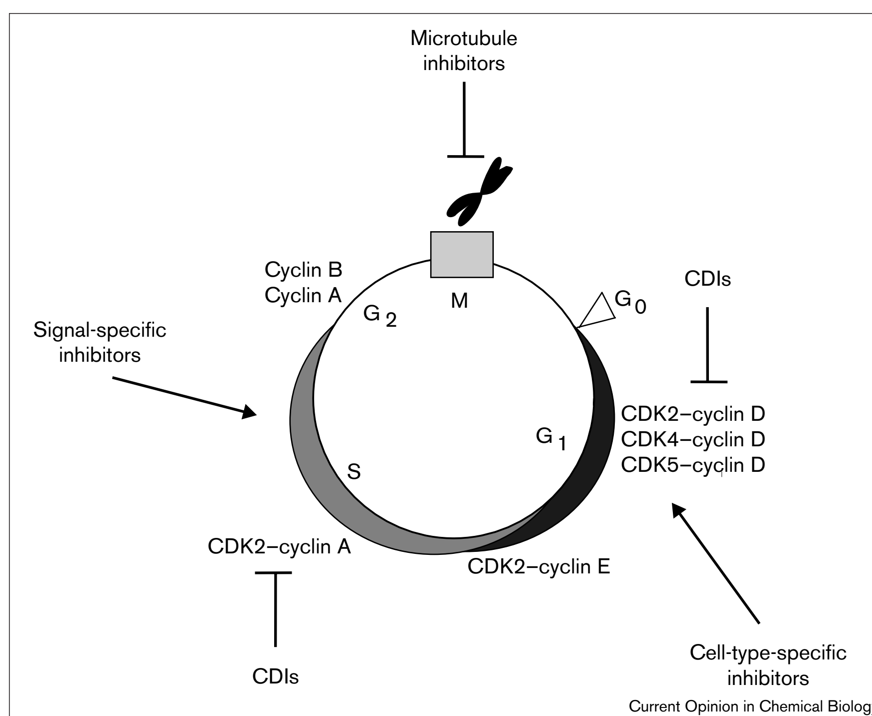
Abbreviations
CDI CDK inhibitor
CDK cyclin-dependent kinase
PFT-α pifithrin-α
TPS-A tryprostatin A

Introduction

Faithful duplication of the 3×10^9 nucleotide base pairs that encode the mammalian genome and successful segregation of the resulting genetic material between two cells poses many challenges to dividing cells. Because a single misstep at any point during the cell cycle can have catastrophic consequences, the regulatory processes governing chromosome duplication and cell division are tightly controlled. For cell-cycle researchers and the pharmaceutical industry these regulatory proteins represent key vulnerable targets for small molecule cell-cycle inhibitors.

The eukaryotic cell cycle is divided into four distinct phases (G_1, S, G_2, M), each of which is regulated by a series of proteins that are attractive targets for small molecule cell-cycle inhibitors (Figure 1). The critical point when a non-dividing quiescent cell in G_0 makes the commitment to enter another round of cell division is encountered at the $G_0 \rightarrow G_1$ transition. Once triggered to enter the G_1 phase, genes for cell-cycle proteins are transcribed and translated in preparation for the energetically demanding period of DNA synthesis. In the S phase the entire genome of the cell is duplicated. Upon completion of DNA synthesis, cells prepare for cell division by entering into the G_2 phase, a time when there is active protein synthesis. The cell then begins to divide its chromosomes between the two daughter cells, involving a number of molecular motors to drive this process. Chromosome segregation and cellular division occurs in the short span of the M phase, which is also known as mitosis.

Figure 1



The eukaryotic cell cycle. The cell cycle is driven by a sequential activation of CDK–cyclin complexes. This process is kept in check by the induced expression of CDIs. Quiescent cells in G_0 enter into G_1 through the activities of cyclin Ds in complexes with CDKs. The G_1 phase is a period of expression of cell-cycle regulatory genes in preparation for DNA synthesis. The G_1 to S transition is a critical point controlled mainly through activities of the cyclin E–CDK2 complex, and this point is also known as the restriction point. DNA synthesis occurs in S phase. Importantly, a cell may undergo programmed cell death or apoptosis when conflicting signals from G_1 are sensed. In G_2, cells pause in preparation for mitosis, or the M phase. Activation of cyclin Bs occurs at the G_2 to M transition. The requirement of numerous M-phase cell cycle proteins that regulate microtubule remodeling in preparation for cell division makes this cluster of molecules unique targets for small molecule inhibitors.

Arguably, the most important family of cell-cycle regulatory proteins is the family of kinases known as cyclin-dependent kinases (CDKs) (see [1]). Different CDKs are active at various times and are responsible for driving the cell cycle from one phase to the next. CDKs are themselves activated via phosphorylation by other kinases as well as through the interaction with a group of proteins known as cyclins, the levels of which fluctuate during the cell cycle. At different points throughout the cell cycle, different cyclin proteins are rapidly degraded resulting in loss of CDK activity. This loss of CDK activity, in turn, allows the transit from one phase of the cell-cycle to the next. Given the obvious importance of CDKs in facilitating cell cycle progression, CDK inhibition serves as a mechanism for cell cycle checkpoint control. A number of insults to a proliferating cell (e.g. lack of nutrients or damaged DNA) lead to CDK inhibition and cell-cycle arrest via the expression of CDK inhibitors (CDIs), a family of proteins that bind to and inhibit CDKs. The three major families of CDIs are the $p21^{CIP/WAF}$, $p27^{KIP}$ and $p16^{INK4a}$ families [2–4].

Two additional proteins that play important roles in cell-cycle checkpoints are p53 and the retinoblastoma gene product RB, both tumor suppressors. Because over 50% of human tumors contain a mutated p53 gene, much interest has focused on understanding the regulatory mechanisms by which cell proliferation is influenced by this tumor suppressor [5]. p53 is a transcription factor that is activated via phosphorylation in response to a variety of stress signals [6], leading either to cell-cycle arrest or apoptosis. Activation of p53 leads to a G_1 checkpoint through the transcriptional induction of the G_1 CDI ($p21^{CIP/WAF}$) gene expression [7]. RB, on the other hand, is a substrate for CDK2 [8]. By binding to and inhibiting the transcription factor E2F and cyclin A [9], unphosphorylated RB negatively regulates expression of genes required for S phase. Phosphorylated RB, however, cannot bind to and inhibit E2F, thereby permitting the transition from G_1 to S. As discussed below, both tumor suppressor pathways have been targeted in recent months by small molecule cell-cycle inhibitors.

Anticancer therapeutics have focused on the development of various classes of cell-cycle inhibitors [10••,11,12••]. Here, we review the development of small cell-permeable molecules that embrace different facets of cell-cycle inhibition; inhibitors that target microtubule assembly/disassembly, ones that target directly the activities of CDKs, and others that target regulatory pathways upstream and downstream of CDKs.

Small-molecule cyclin-dependent kinase inhibitors

Given the pivotal role of CDKs in cell-cycle regulation, much effort has focused on the development of small molecule CDIs. The purine analogs olomoucin, roscovitine, flavopiridol and purvalanol (Figure 2a) have proven useful both as probes for basic cell biology and as potential lead compounds for antiproliferative therapeutic development. Because these inhibitors have been the subject of reviews in recent years [10••,11,12••], we will highlight here two new CDIs.

Paullones

Given the success of flavopiridol to arrest cells in the G_1 phase through the nanomolar inhibition of CDC2 and CDK2 activity [13,14,15•], the National Cancer Institute's computer-based algorithm COMPARE was employed to find compounds with flavopiridol-like activities. This analysis identified a novel class of inhibitors, the paullones (Figure 2b) [16••]. Kenpaullone (9-bromopaullone) inhibits CDK1/cyclin B (IC_{50}, 0.4 μM), CDK2/cyclin A (IC_{50}, 0.68 μM) and CDK2/cyclin E (IC_{50}, 7.5 μM), and the activity of CDK4/cyclin D1 at much higher concentrations (IC_{50} 100 μM); IC_{50} is the concentration of the drug at which 50% inhibition is achieved. In addition, molecular modeling studies using the crystal structure of CDK2 suggested that the paullones bind competitively to the ATP-binding site, making contacts with residues outside the ATP-binding domain. Interestingly, the studies predict that kenpaullone occupies little of the ATP-binding pocket where the sugar and phosphate groups of ATP reside. Therefore, it should be possible to design kenpaullone analogs that extend into these vacant sites while still retaining CDK-specific interactions.

Indirubin

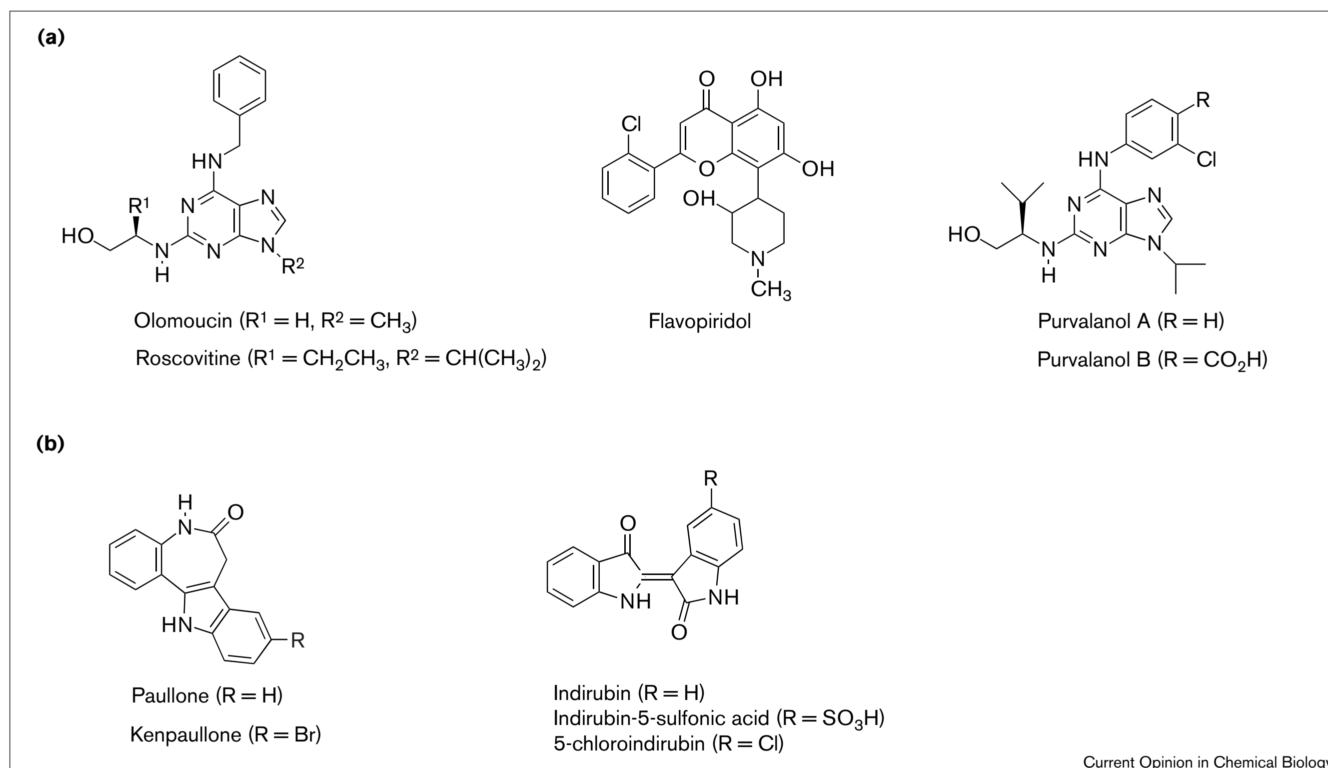
Interest in ethnopharmacology has increased in recent years and traditional Chinese medicine, in particular, has been a rich source for the identification of novel biologically active natural products [17,18]. Some years ago, one of the principal active agents of Danggui Longhui Wan — a complex mixture of herbs used to treat myelocytic leukemia — was identified as indirubin (Figure 2b), an isomer of indigo [19]. Recently, indirubin and its analogs were found to inhibit cell proliferation in late G_1 and G_2/M phases of the cell cycle via CDK inhibition and RB phosphorylation [20••]. Indirubin inhibits all CDKs equally well (IC_{50} ~10 μM), whereas the 5-chloro- and 5-sulfonic-acid substituents reveal greater specificity for CDK1, CDK2, and CDK5 over CDK4. In what is emerging as a common theme for CDK specificity determination among small molecule CDIs, X-ray crystallography of indirubin-5-sulfonate with CDK2 revealed that the inhibitor targets the ATP-binding site in a manner analogous to roscovitine and olomoucin [20••].

Targeting microtubules and the mitotic spindle apparatus with small molecules

Tryprostatin A

The discovery of paclitaxel (Taxol®) as a specific stabilizer of microtubules that arrests cells in mitosis [21] stimulated the search for novel agents with a similar mode of action. Several years ago, tryprostatin A (TPS-A; Figure 3), an anti-mitotic natural product from *Aspergillus fumigatus* was discovered using the temperature-sensitive $p34^{cdc2}$ mutant cell line, tsFT210 [22,23]. At the restrictive temperature, cells arrest in G_2 and form large colonies. Upon release from G_2 arrest, cells that enter

Figure 2



Small-molecule inhibitors of CDKs. **(a)** Olomoucin, roscovitine, flavopiridol and purvalanol (A and B) form the class of trisubstituted purine-based CDIs. **(b)** Examples of the paullones and the indigoids, which form two new classes of CDK1–cyclin-B inhibitors.

Figure 3

Small molecules that target the microtubule machinery. Monastrol induces a monoastral conformation of microtubules by inhibiting the mitotic kinesin, Eg5. DHP2 is an inactive analog of monastrol. Trypostatin A and B are natural products, which prevent the interaction between microtubule-associated proteins with the carboxy-terminal domain of tubulin. Pironetin is a tubulin-binding natural product.

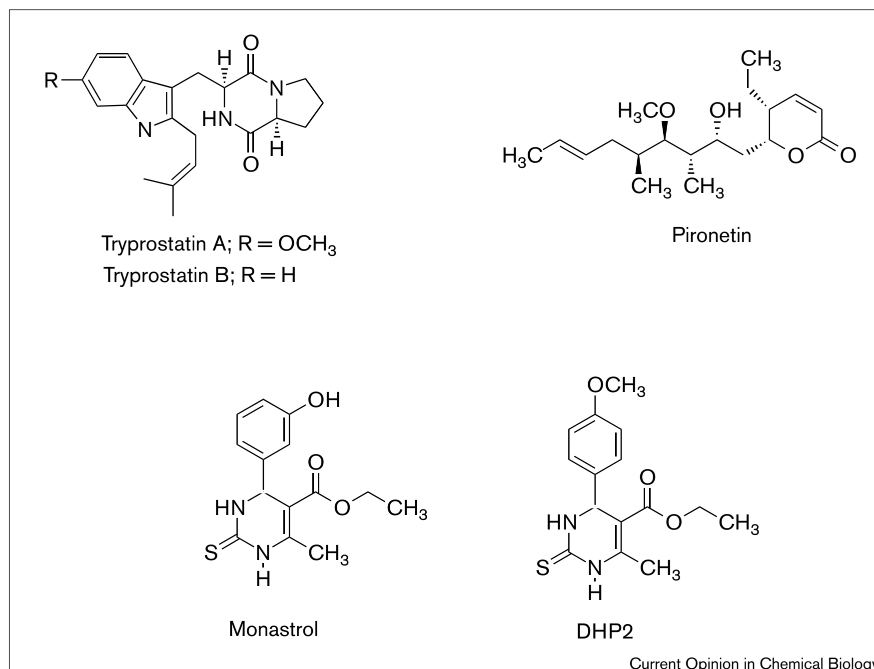
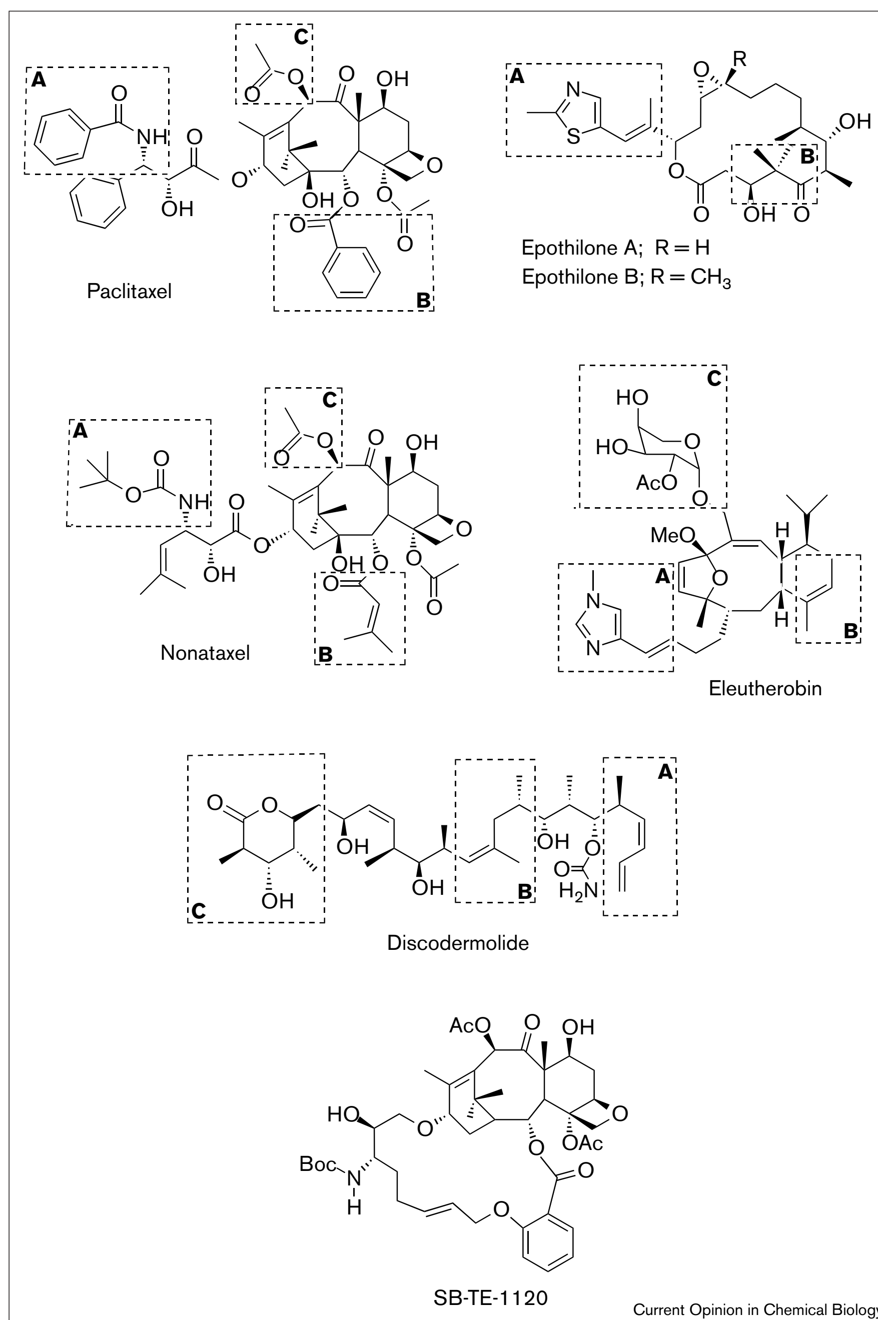


Figure 4



Pharmacophore profiling of microtubule inhibitors using NMR conformational analysis. Common areas of conformational overlap between the compounds (indicated by the dashed-line boxes) were incorporated in the design of the hybrid anti-mitotic compound SB-TE-1120 shown at the bottom of the figure.

G_1-phase cells can be easily discriminated from the G_2/M-arrested cells by their smaller size. Using this cell-morphology-based assay, TPS-A was shown to arrest cells in the G_2/M interphase at a concentration of 125 μM. Although tryprostatin B is more potent that TPS-A, its inhibitory activity on the cell cycle was not specific to the M phase. Recently, it was shown that the G_2/M arrest is dependent on microtubule-associated protein-2 and tau, two endogenous stimulators of tubulin assembly [24•,25]. Interestingly, TSP-A does not inhibit glutamate-induced or Taxol®-induced microtubule assembly, and, thus, it represents a new class of cell-cycle inhibitor lead for drug development.

Pironetin

Another novel antitumor compound that targets the cytoskeleton is pironetin, a pyran-based molecule containing a simple alkyl chain (Figure 3) [26]. Pironetin is a potent anti-mitotic agent that disrupts the mitotic spindle at nanomolar concentrations via inhibition of microtubule assembly [27•]. Pironetin also induces the disassembly of microtubules in a dose-dependent and reversible manner, while sparing actin

filaments. In addition, pironetin inhibits the binding of vinblastine to tubulin while promoting the binding of colchicine to tubulin. These results suggest that pironetin prevents microtubule assembly through direct tubulin binding.

Cytoblot analysis: a novel chemical genetic approach to anti-mitotic small molecule discovery

A 'chemical genetic' approach [28••] to the identification of novel cytostatic/cytotoxic agents is also proving fruitful. This strategy entails screening for small molecule inhibitors by looking for 'phenotypic' changes in a manner analogous to a conventional genetic screen [29•]. Using an innovative whole-cell immunodetection ('cytoblot') assay, Mitchison and co-workers [30••] screened a library of compounds for anti-mitotic activity by investigating the phosphorylation status of nucleolin, a nucleolar protein that is phosphorylated as cells enter mitosis [31]. This screen identified one compound that causes bipolar mitotic spindles to form a monoastral microtubule array surrounded by a ring of chromosomes, and thus it was named monastrol (Figure 3). Focusing on factors important for bipolar spindle formation, they found that a member of the mitotic motor kinesin family, Eg5, was inhibited by monastrol in *in vitro* motility assays. It is noteworthy that microtubule motility driven by Eg5, but not by conventional kinesins, was inhibited by monastrol (IC_{50} = 14 μM). DHP2, a structural congener, does not exert the biological activities of monastrol. In addition, monastrol does not influence motor protein localization, an activity that distinguishes it from other general inhibitors of motor proteins. Thus, monastrol constitutes a powerful new tool to probe the signaling pathway connecting a novel microtubule target to the cell-cycle machinery.

Predicting a common microtubule-binding pharmacophore from antitumor natural products

Small molecule inhibitor conformational analysis is also beginning to play an important role in drug design. On the basis of the finding that structurally dissimilar natural products, epothilones A and B, elutherobin, and discodermolide bind competitively with [^{3}H]-paclitaxel to microtubules to induce mitotic arrest, Danishefsky and co-workers [32••] compared the NMR-defined conformations of each compound with that of paclitaxel and its related analog, nonataxel. This novel approach revealed that all six compounds contained a putatively shared pharmacophore (Figure 4). In an elegant proof of this approach, a synthetic hybrid compound was subsequently generated that demonstrated striking tubulin-binding characteristics. This hybrid compound (SB-TE-1120) potently inhibits the growth of the human breast cancer cell line MDA-435/LCC6-WT with an IC_{50} value of 0.39 μM. The predictive power of this approach has great potential for the design of improved taxoid mimetics.

Small-molecule inhibitors that target specific regulatory pathways

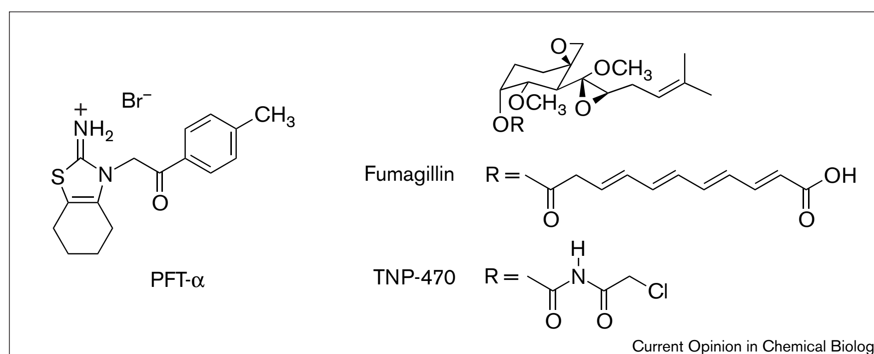
Pifithrin-α, a novel inhibitor of p53 activity

In cancer therapy, one of the major challenges has been how to abate the sequelae of inflammatory responses that arise in normal tissues because of radiation or chemotherapeutics. It is thought that much of the pain derived from these potent therapies is a result of p53-induced gene expression of inflammatory mediators. Gudkov and co-workers [33••] have recently addressed this issue by looking for small molecules that are capable of suppressing the transcriptional activation of p53-responsive genes. They discovered a small molecule, pifithrin-α (PFT-α; Figure 5), by screening a chemical library for agents that would block the transcriptional activation of a reporter gene driven by the p53 promoter. PFT-α arrests γ-irradiated cells in G_2 and prevents them from undergoing apoptosis. Interestingly, the effects of PFT-α are reversible and require the continued presence of the drug. Because PFT-α does not exert any activities on the p53/apoptosis pathway in the absence of irradiation, a stress-induced factor is thought to be targeted by this compound.

Fumagillin induces p53 activation specifically in endothelial cells

The surprising endothelial cell specificity of the antiangiogenic compound fumagillin has recently been explained by its differential activation of the p53 pathway. Fumagillin, a metabolite secreted by *A. fumigatus* [34], inhibits endothelial cells late in the G_1 phase of the cell cycle [35] through the inhibition of RB phosphorylation

Figure 5



Cell-cycle arrest is induced by small molecules that target p53-mediated regulatory pathways. PTF-α is a small-molecule cell-cycle inhibitor that targets p53-induced transcription; however, its target has not yet been identified. Fumagillin and its synthetic derivative TNP-470 are endothelial cell-type-specific inhibitors of the cell cycle. Both these compounds bind to and inhibit the enzyme methionine aminopeptidase-2 [36,38].

and CDK2 and CDK4 activities. In our studies of its antiangiogenic mode of action [36], we have recently discovered that fumagillin and its analog TNP-470 (Figure 5), exert endothelial cell-specific nanomolar growth arrest by engaging a pathway leading to p53 activation causing increased $p21^{CIP/WAF}$ expression [37]. The ability of fumagillin to induce p53 and $p21^{CIP/WAF}$ is critical for its antiproliferative activity, because fibroblasts, which are resistant to fumagillin at 1000-times higher concentrations, do not induce p53 or $p21^{CIP/WAF}$ in response to fumagillin. In addition, endothelial cells from $p53^{-/-}$ and $p21^{CIP/WAF-/-}$ deficient mice were shown to be resistant to TNP-470, strongly suggesting that p53 and $p21^{CIP/WAF}$ are required for TNP-470's biological activity. Moreover, new blood vessel growth (angiogenesis) stimulated *in vivo* by surgically implanting growth factor pellets in the avascular cornea is inhibited by TNP-470 treatment in wild-type mice, but not in $p21^{-/-}$ mice. Current efforts are focused on the endothelial cell-specific signaling pathway that mediates the TNP-470 induced p53 activation.

Conclusions

Although advances in synthetic methods promise powerful cell-cycle inhibitors for cell biology and pharmaceutical development, natural products continue to be useful structural platforms in inhibitor design. The application of chemical genetic approaches holds much potential for the identification of both new cell-cycle inhibitors as well as novel regulatory targets that signal to cell-cycle checkpoints. Cell-based screening strategies will probably play an increasingly important role in the discovery of small molecule cell-cycle inhibitors that effect cell growth inhibition in a cell-type-specific manner or in response to a specific stimulus.

Acknowledgements

Craig M Crews is a Burroughs Welcome Fund and Donaghue Biomedical Foundation New Investigator. The National Institutes of Health are also gratefully acknowledged for their support (CA74967).

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