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Review Article

Cell cycle inhibitors for the treatment of cancer

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CONTENTS

Abstract

The loss of cell cycle control leading to deregulated cell proliferation is one of the hallmarks of cancer. The cell cycle progression is regulated by the activities of cyclin-dependent kinases (CDKs) and their subunits known as cyclins. Cell cycle regulators are often mutated in human neoplasia, resulting in outcomes such as overexpression of CDKs and cyclins, as well as loss of natural inhibitors of CDKs, and consequently hyperactivation of CDKs. Inhibition of CDKs thus provides an attractive therapeutic strategy for the treatment of cancer. Over the past few years, many small-molecule CDK inhibitors have been discovered and at least 4 have entered into clinical trials. These include both pan CDK inhibitors such as flavopiridol and UCN-01, along with the more selective CDK2 inhibitors, (*R*)-roscovitine and BMS-387042. On the other hand, the transition from one phase of the cell cycle to the next is controlled by cell cycle checkpoints and may represent a viable alternative target for cell cycle modulation. The recent understanding of the critical role of checkpoint kinase 1 (Chk1) in the G_2 checkpoint has generated great interest in the discovery of Chk1 inhibitors. This review discusses the progress of preclinical research and clinical developments of CDK inhibitors and the recently disclosed Chk1 inhibitors.

Introduction

One of the characteristics of cancer is uncontrolled cell growth and proliferation. Drugs targeting the cell cycle have proven clinically useful in the treatment of cancer. The antimitotics – drugs that inhibit mitosis – such as taxanes have been very successfully used as chemotherapy agents. The clinical and commercial success of paclitaxel (Taxol®) and docetaxel (Taxotere®) have stimulated efforts in the discovery of new antimitotic agents with improved efficacy, particularly in multidrug-resistant (MDR) tumors (1). Among antimitotics with a similar mechanism of action as paclitaxel, the epothilones and their analogs appear to be the most promising. Four epothilone derivatives (EPO-906, KOS-862, BMS-247550 and BMS-310705) are currently in various stages of clinical trials (2, 3). Epothilones were shown to be active in MDR cell lines *in vitro* and paclitaxel-resistant tumor models *in vivo*. A new direction in the discovery of antimitotic agents is the search for compounds with novel mechanisms. Ro 31-7453, a cell cycle inhibitor that arrests cells in the G_2/M phase, showed *in vivo* efficacy in 15 tumor models including 3 paclitaxel-resistant tumor models (4). SB-715992, a potent inhibitor of mitotic kinesin KSP, caused mitotic arrest in all tumors tested and showed *in vivo* activity in a number of human xenograft models (5).

With the development in the understanding of the genetic basis of carcinogenesis, and the intricate cell cycle machinery, a number of new cell cycle targets have been identified. Many, if not all of these, are being aggressively pursued by pharmaceutical companies as well as academic researchers in an effort to identify more specific molecular targeted therapies in cancer.

The cell cycle

The cell cycle consists of a series of events that a cell undergoes in order to grow and divide into two cells (6). It is divided into five distinct phases, G_0, G_1, S, G_2 and M phases. G_0 is the quiescent state in which a cell remains metabolically active (7). In response to the extracellular stimuli, a cell leaves G_0 and enters the first gap phase G_1,

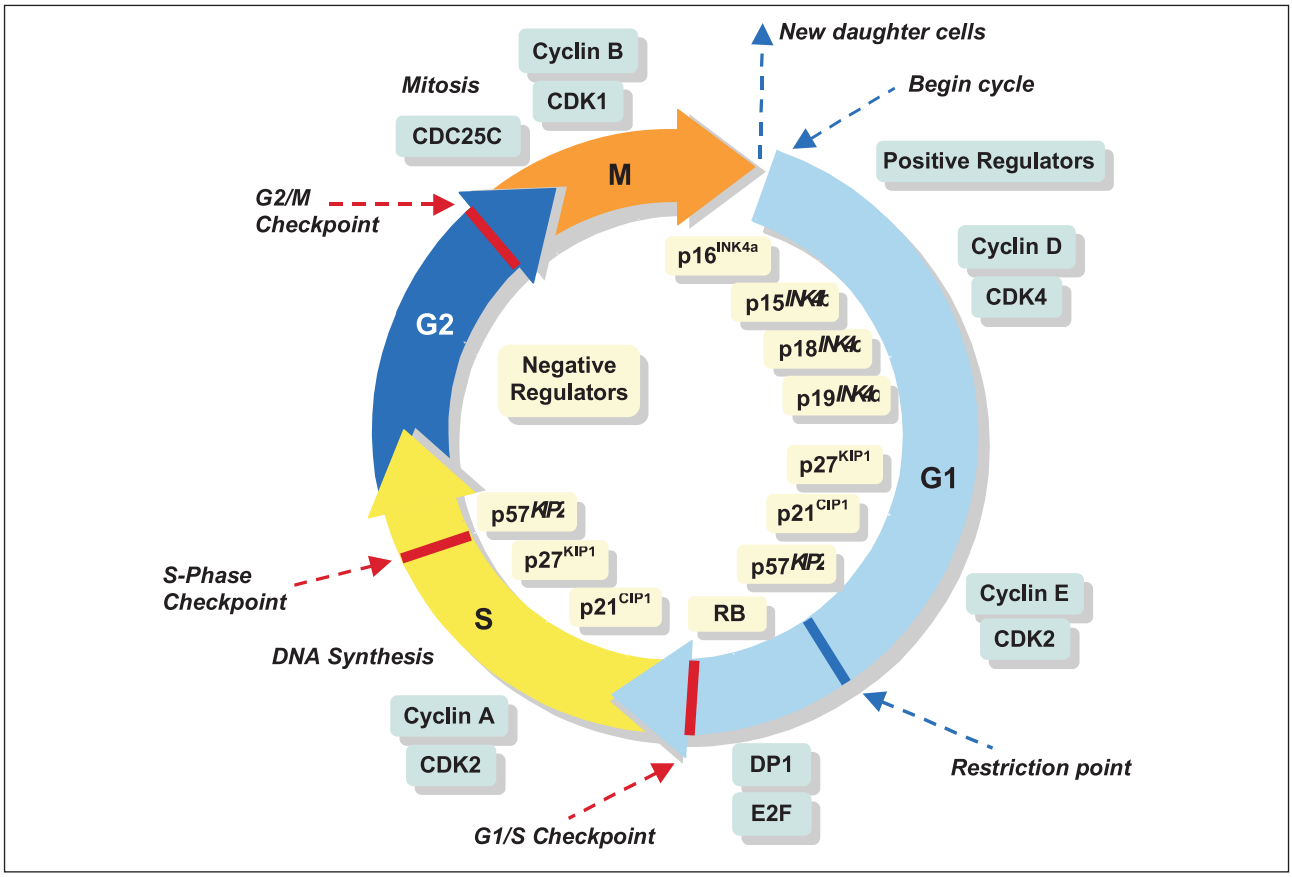

Fig. 1. Cell cycle.

preparing its DNA for replication. This is followed by the S phase, during which a cell replicates its DNA. Following DNA replication, a cell enters the second gap phase G_2 and prepares for mitosis. The M phase is the period of cell division for the generation of two daughter cells (Fig. 1).

Cyclin-dependent kinases

Cyclin dependent kinases (CDKs) are key regulators of the cell cycle progression. In order to be active, CDKs need to form complexes with proteins known as cyclins. While the levels of CDKs remain relatively constant during the cell cycle, the levels of their cyclin counterpart fluctuate, thus allowing a regulated control of activity (8). Cyclins contain destruction boxes (cyclins A and B), or PEST domains (Pro-, Glu-, Ser- and Thr-rich domains), that allow their targeting for efficient ubiquitination and degradation as a means to control their levels (9, 10). The activity of the resulting complex or holoenzyme is further regulated through phosphorylation of key amino acids. Phosphorylation of threonine residues in the CDK catalytic subunit (Thr161 in CDK1, Thr172 in CDK4 and Thr160 in CDK2) is essential for catalytic activity. This phosphorylation is carried out by CDK7/cyclin H, which is also known as CDK-activating kinase (CAK) (11). On the other hand, phosphorylation of the nearby Threonine and Tyrosine residues (Thr14 and Tyr15 in CDK1) by dual specifity kinases such as MYT1 and WEE1 keeps the CDKs in an inhibited state. Dephosphorylation of Thr14 and Tyr15 by the CDC25 family of protein phosphatases results in reactivation (12).

The different phases of the cell cycle are controlled by the activation of various CDK-cyclin complexes (13). In early to mid G_1, activation of CDK4/cyclin D1 and CDK6/cyclin D3 by the extracellular signal induces the phosphorylation of the retinoblastoma protein (Rb). Phosphorylation of Rb releases the transcription factor E2F, which can then enter the nucleus to turn on the genes for the expression of cyclin E and cyclin A. CDK2/cyclin E promotes the G_1/S transition along with additional phosporylation of Rb, resulting in further E2F release. After entering into the S phase, CDK2/cyclin A phosphorylates a number of substrates, resulting in DNA replication and the inactivation of G_1 transcription factor. At the late S/G_2 stage, CDK1 is activated in a complex with cyclin A and B. CDK1/cyclin B mediates the G_2/M transition by phosphorylating the anaphase complex APC, which in turn leads to the completion of mitosis.

CDKs are also negatively regulated by endogenous CDK inhibitors. There are two categories of CDK inhibitors, the INK4 family and the Cip/Kip family. The INK4 family consists of at least four members, $p16^{INK4a}$, $p15^{INK4b}$, $p18^{INK4c}$ and $p19^{INK4d}$, which can only inhibit CDK4 and CDK6. The Cip/Kip family of CDK inhibitors comprises at least three proteins, $p21^{cip1}$, $p27^{kip1}$ and $p57^{kip2}$, which negatively modulate the kinase activities of CDK2/cyclin E and CDK2/cyclin A. The function of these inhibitor proteins is also subject to regulation, mainly through their expression and posttranslational modifications (*e.g.*, phosphorylations). Disruption of these pathways is also observed in human cancers.

CDK inhibitors

Dysregulation of cell cycle progression is a universal characteristic of cancer cell proliferation (6, 11, 13). The majority of human cancers have abnormalities in some components of the Rb pathway. The loss of Rb function is mainly a result of hyperactivation of CDKs due to overexpression of cyclins, downregulation of endogenous CDK inhibitors or mutation of Rb. The direct consequence of this Rb inactivation is the activation of CDK2/cyclin, which in turn can participate in maintaining inactivation of Rb in tumor cells (14). Abnormal expression of CDK2/cyclin E has been characterized in ovarian, breast and lung cancers (15). CDK2/cyclin E was shown to phosphorylate p27, which leads to its degradation. CDK2/cyclin E also appears to have other important functions in cell cycle progression: centrosome duplication and histone protein expression. These findings point to CDK2 as a critical regulator of cell cycle progression. For the above stated reasons, regulation of CDK activity is an attractive anticancer strategy. This has prompted considerable interest in the design and synthesis of small-molecule CDK inhibitors, and in particular more selective CDK2 inhibitors as novel cancer therapeutic agents. There are at least four CDK inhibitors in clinical trials. Flavopiridol and UCN-01 are nonspecific pan CDK inhibitors which are in phase II clinical trials against a variety of tumors. Other compounds in the clinic include (*R*)-roscovitine and BMS-3870342, which are modestly selective CDK2 inhibitors. Over the past few years, a large number of CDK inhibitors have been reported in the literature, and much of the earlier work has been reviewed extensively (13, 16-29). This review is mainly focused on the work published over the last 2 years.

Flavopiridol and related compounds

Flavopiridol, **1** (HMR-1275, L86-8275, NSC-649890) (Fig. 2), a synthetic flavone derived from rohitukine, has been shown to be an inhibitor of CDK1, CDK2 and CDK4 with IC_{50}s in the range of 40-200 nM, and more recently CDK9 (30). The crystal structure of flavopiridol with CDK2 (31) demonstrated that the 4-keto and 5-hydroxy groups of the compound form two hydrogen bonds with the α amino group of Leu83 and the carbonyl of Glu81 in the CDK2 backbone, respectively.

Flavopiridol causes cell cycle arrest at both G_1 and G_2 phases, consistent with its inhibition of CDK1, CDK2 and CDK4. In the National Cancer Institute (NCI) anticancer screen, flavopiridol exhibited significant *in vitro* activity against all 60 human tumor cell lines with an average IC_{50} of 66 nM (11). Further investigations have revealed the ability of this compound to decrease cyclin D1 concentrations (32, 33), induce apoptosis in a variety of cell lines (33-36), inhibit angiogenesis (37-39) and enhance the radiosensitivity of ovarian carcinoma cells (40). In multiple myeloma, induction of apoptosis has been linked to transcription repression and downregulation of Mcl-1 (41).

Flavopiridol was evaluated in a number of xenograft models (42). Fourteen of 21 tumor models responded to flavopiridol treatment with an average tumor growth delay of 35-45%. Treatment with flavopiridol of head and neck (HN12) xenografts in nude mice, at 5 mg/kg/day i.p. for 5 consecutive days, resulted in a 60-70% tumor reduction which was sustained for 10 weeks (33). In another study, upon treatment with flavopiridol as a 7.5 mg/kg/day i.v. bolus in nude mice for 5 consecutive days, 11/12 advanced-stage s.c. human HL-60 xenografts underwent complete regression and animals remained disease free for several months. However, flavopiridol administrated as a 72-h continuous infusion only achieved tumor growth delay (43).

Flavopiridol is still the most widely studied CDK inhibitor in the clinic. Several phase I and phase II clinical trials with different dosing regimens (72-h and 1-h infusions) have been completed (44-47). Recent reviews (23-25, 44, 45) have been published covering the clinical trials of the compound. In a phase I trial which included 21 patients having advanced cancer where flavopiridol was administrated as a 72-h continuous infusion regimen every 2 weeks, the maximum tolerated dose was defined at 40 mg/m^2/day x 3. Dose-limiting toxicities included diarrhea and orthostatic hypertension (45). A complete response was observed in 1 patient with metastatic gastric cancer. With this encouraging result, a number of phase II clinical trials were initiated in renal, gastric, non-small cell lung and colorectal cancers using 72-h and 1-h infusions (24, 44-48). However, there has been no major response reported at this time. Since flavopiridol has been shown to have synergistic effects with other standard chemotherapeutic agents, several combination studies with paclitaxel (49), docetaxel (50, 51), cisplatin (52), irinotecan (53) and paclitaxel/carboplatin (54) have been initiated (25). At the NCI, a phase I combination study with paclitaxel has been completed (49). A recommended phase II dose has been determined to be a 3-h infusion of paclitaxel at 175 mg/m^2 on day 1 followed by a 24-h infusion of flavopiridol at 70 mg/m^2 on day 2. One complete and one partial response in patients with adenocarcinoma of the esophagus were observed among 54 patients with a variety of different tumors.

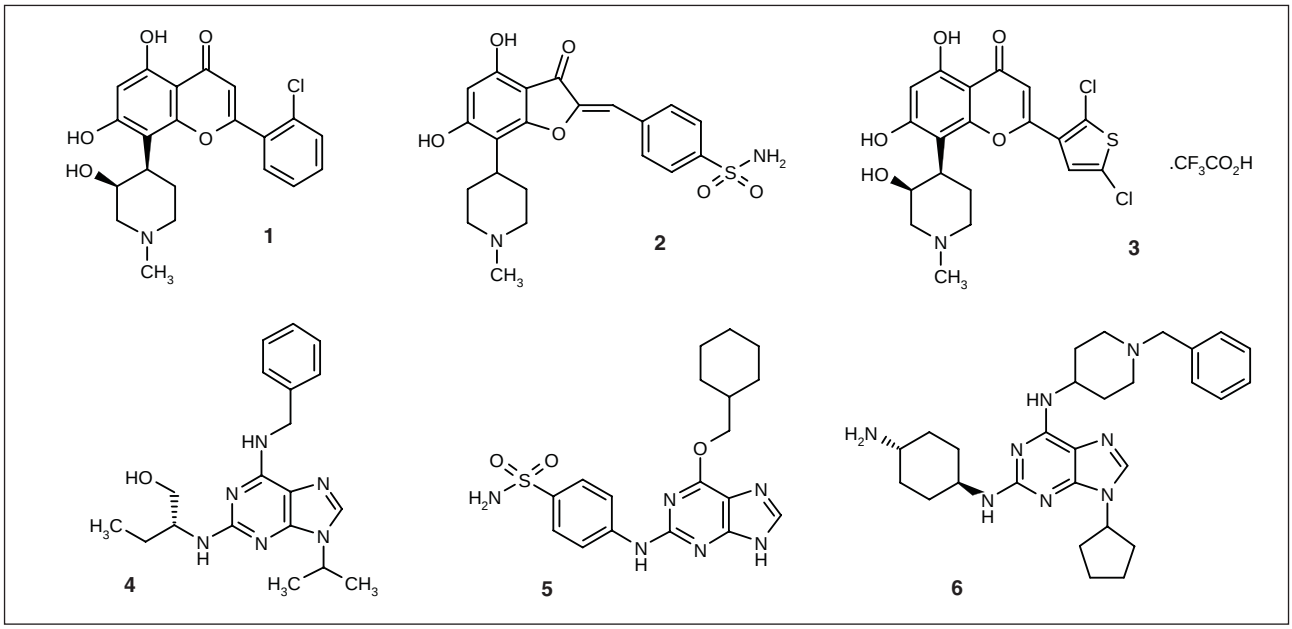

Fig. 2. Flavones and purines.

The continued interest in flavopiridol in the clinic has promoted a search for alternative scaffolds based on the flavone core. A novel 2-benzylidene-benzofuran-3-one scaffold mimicking the flavonoid structure (55) was designed based on the crystal structure of flavopiridol complexed to CDK2 (31). Compound **2** was a more potent and selective CDK1 and CDK2 inhibitor compared with flavopiridol, with CDK1, CDK2 and CDK4 inhibitory activities of 9 nM, 30 nM and 1.87 μM, respectively. However, this class of compounds showed very limited activity in cellular assays.

In a recently published patent, researchers at Aventis used a bioisosteric approach to modify flavopiridol at C2. For example, replacement of chlorophenyl with dichlorothiophenyl afforded **3**, which was claimed to act as a CDK inhibitor (Fig 2). No biological data was disclosed (56).

Purine derivatives

The purine ring system, which is found in ATP itself, has been used in a large number of CDK inhibitors (17-19). (*R*)-Roscovitine **4** (CYC-202) (Fig. 2) is a substituted purine analog conceptually derived from 6-dimethylaminopurine and isopentenyladenine. It is a potent and selective CDK inhibitor over other kinases, with the greatest potency against CDK2/cyclin E (IC_{50} = 100 nM) (57) and only modest activity against CDK1/cyclin B (IC_{50} = 2.69 μM) and CDK4/cyclin D1 (IC_{50} = 14.2 μM). Against a panel of 19 human tumor cell lines, (*R*)-roscovitine exhibited an average cytotoxic IC_{50} of 15 μM. In the human uterine xenograft model, MESSA-DX5, treatment with (*R*)-roscovitine at 500 mg/kg p.o. 3 times daily for 4 days afforded a 62% inhibition of tumor growth (57).

A phase I clinical trial of (*R*)-roscovitine has been completed. Nineteen patients with a variety of advanced malignancies, received the drug at doses of 100-1250 mg b.i.d. for 5 days every 3 weeks. No dose-limiting toxicity was observed up to 800 mg b.i.d., while at 1000 mg b.i.d. grade 3 nausea/vomiting and asthenia were observed (58). The maximum tolerated dose was not established. Two phase IIa trials were initiated. One trial will explore the use of (*R*)-roscovitine in the treatment of stage IIIb/IV non-small cell lung cancer in combination with gemcitabine and cisplatin. The other trial will evaluate the use of (*R*)-roscovitine in the treatment of advanced breast cancer in combination with capecitabine.

The crystal structure of (*R*)-roscovitine bound to CDK2 has been determined (59, 60). The purine ring of (*R*)-roscovitine occupies roughly the same plane in the inhibitor-enzyme complex as ATP in the ATP-enzyme complex, but in a completely different orientation, with N7 of (*R*)-roscovitine close to N1 of ATP. There are two hydrogen bonds between N6 of (*R*)-roscovitine and the carbonyl oxygen of Leu83, and between N7 and the backbone NH of Leu83.

Using structure-based drug design, scientists at the University of Oxford, AstraZeneca and the University of Newcastle-upon-Tyne discovered a highly potent CDK inhibitor **5** (NU-6102) (Fig. 2) with a K_i of 9 nM against CDK1 and 6 nM against CDK2 (61). In addition, NU-6102 is more than 1000 times selective against CDK4 and 27 other kinases. NU-6102 inhibited tumor cell growth in MCF-7 breast carcinoma cells (GI_{50} = 8 μM) for over 48 h and induced cell cycle arrest at both G_1 and G_2 phases, consistent with CDK1 and CDK2 inhibition. The crystal structure of NU-6102) bound to CDK2 (60) indicated that the donor-acceptor-donor motif of the purine ring forms

hydrogen bonds to Glu81 and Leu83 (NH at C2 and N3 with Leu83 and NH9 with Glu81). The cyclohexyl methyl group occupies the same space as the ribose ring of ATP. In addition, there are interactions between the phenyl ring and the region of hydrophobic residues, and two additional hydrogen bonds between the sulfonamide and Asp86. These interactions might contribute to the high potency of this compound. This binding mode is completely different from the binding mode of ATP and (*R*)-roscovitine to CDK2 (60, 61).

A series of piperidine-substituted purine analogs based on the olomoucine structure were recently reported (62). These compounds demonstrated potent antiproliferative activity against breast, lung, colon and prostate tumor cell lines. Although the authors have reported that the antiproliferative activity of this series of compounds correlates well with its CDK2 activity, they neglected to give other convincing data such as protein phosphorylation (*e.g.*, Rb) and cell cycle analysis. The lead compound in this series, MDL-108522 (**6**, Fig. 2) is a CDK2/cyclin E, CDK4/cyclin D inhibitor with IC_{50}s of 190 nM and 410 nM, respectively, and has excellent activity in HT 29 cells (IC_{50} = 0.2 μM). When given orally at 3 mg/kg in nude mice, MDL-108522 significantly inhibited tumor growth in a PC-3 human prostate tumor xenograft model. The similarity of IC_{50} values against CDK2 and in the cell-based antiproliferative assay points to the conclusion that CDK2 is not the only target of this series of compounds, and most likely other kinases such as CDK1 contribute to the observed cellular potency.

Oxindole derivatives

The oxindole scaffold has provided the starting point for many small-molecule kinase inhibitor programs. Numerous examples have been published with oxindoles as serine/threonine or tyrosine kinase ligands. The substitution pattern on the oxindole core (most notably on the aromatic ring) can dictate the selectivity towards the different classes of kinases. It is not surprising to find multiple oxindole hits in high-throughput screens of kinases. Whereas some may label oxindole as a frequent hitter to be avoided, others would refer to it as a priviledged scaffold. The cyclin-dependent kinases have not escaped the oxindole realm. A series of substituted oxindoles as CDK4 and CDK2 inhibitors have been claimed by GlaxoSmithKline (63). As an example, oxindole **7** (Fig. 3) shows greater selectivity towards CDK4 (IC_{50} < 0.1 μM) than CDK2 (IC_{50} < 1 μM). When tested in Rb^+ cells (U2OS, MDA-MB231), the compound exhibits submicromolar activity. GW-491619 (a substituted oxindole with a presently undisclosed structure) is a very potent CDK4 inhibitor (IC_{50} = 25 nM) under development at GlaxoSmithKline (64). GW-491619 inhibits HCT-116 human colon tumor cells with an IC_{50} of 0.7 μM and inhibits normal human fibroblast cells with an IC_{50} of 7 μM, showing a selectivity of 10-fold. The compound is also 4-fold selective for Rb^+ human breast tumor cells (MDA-MB-231) relative to Rb^- cells (MDA-MB-468) in the cell survival assay (IC_{50} = 0.7 μM *vs.* 2.9 μM). When GW-491619 was administrated to tumor-bearing mice (50 mg/kg b.i.d. x 16 days), 49% inhibition of tumor growth was observed in HCT-116 human colon xenografts.

Arylideneoxindole **8** (Fig. 3) exhibits an IC_{50} of 0.3 nM against CDK1 and demonstrates *in vitro* antiproliferative activity against a panel of tumor cell lines, with IC_{50} values below 1.5 μM in most cases (65). This compound also exerts antimitotic and proapoptotic effects in HCT-8 tumor cells, and in human colon carcinoma HCT-116 cells induces accumulation in the G_2/M phase.

Hoffmann-La Roche has reported a class of tricyclic oxindoles as CDK2 inhibitors (66). Compound **9** (Fig. 3) inhibits CDK2/cyclin E with an IC_{50} of 5 nM. It also shows very potent antiproliferative activity against MDA-MB-435 breast carcinoma (IC_{50} = 120 nM) and RKO colon carcinoma (IC_{50} = 70 nM) cell lines.

Based on the crystal structure of an SU-9516-CDK2 complex, Sugen scientists (67) designed a novel series of pyrrolyllactone and pyrrolyllactam oxindoles in an attempt to create a hydrogen bond with Lys89 in CDK2. Both 4-substituted oxindole **10** and 5-substituted oxindole **11** (Fig. 3) have an IC_{50} of 9 nM against CDK2. In general, the pyrrolyllactone oxindoles are more potent than their pyrrolyllactam counterpart. While SU-9561 is not a selective molecule, these new derivatives are selective against CDK2 relative to FLK and VEGFR.

Substituted pyrazoles

Recently, a number of companies have disclosed several substituted pyrazoles and bicyclic pyrazoles as CDK inhibitors (68). Pharmacia (now Pfizer) identified 2-phenyl-*N*-(1*H*-pyrazol-3-yl)-acetamide derivatives as CDK inhibitors (68). Pyrazole **12** (Fig. 3) is a very potent CDK2/cyclin A inhibitor with an IC_{50} of 8 nM. Agouron (now Pfizer) described aminopyrazole **13** (69) (Fig. 3) as a selective CDK2/cyclin A inhibitor with a K_i of 16 nM, 56 times more selective relative to CDK4 (K_i = 900 nM). Bicyclic aminopyrazole **14** (Fig. 3) was also claimed as a CDK2/cyclin A inhibitor (K_i = 34 nM) with modest selectivity against CDK4/cyclin D3 (K_i = 390 nM) (70). BMS-265246 **15** (Fig. 3) was reported as a potent CDK1/cyclin B (IC_{50} = 6 nM) and CDK2/cyclin E (IC_{50} = 9 nM) inhibitor, with some selectivity against CDK4/cyclin D (IC_{50} = 230 nM) (71). Pyrazole derivative **16** (Fig. 3) is a very potent CDK2 (K_i = 21 nM) inhibitor with good selectivity against CDK4 (K_i = 3.2 μM) and exhibits potent antiproliferative activity against HCT-116 cells (IC_{50} = 370 nM) (72). LG Chem reported indazole **17** (Fig. 3) as a CDK2/cyclin A (IC_{50} < 50 nM) and CDK4/cyclin D1 (IC_{50} < 10 μM) inhibitor (73). Hoffmann-La Roche claimed a series of pyrazolobenzodiazepines (*e.g.*, **18**) as CDK2 inhibitors with IC_{50} values ranging from 0.01-1 μM (74).

High-throughput screening, followed by medicinal chemistry efforts at DuPont (now Bristol-Meyers Squibb) led to the identification of indenopyrazoles as novel CDK

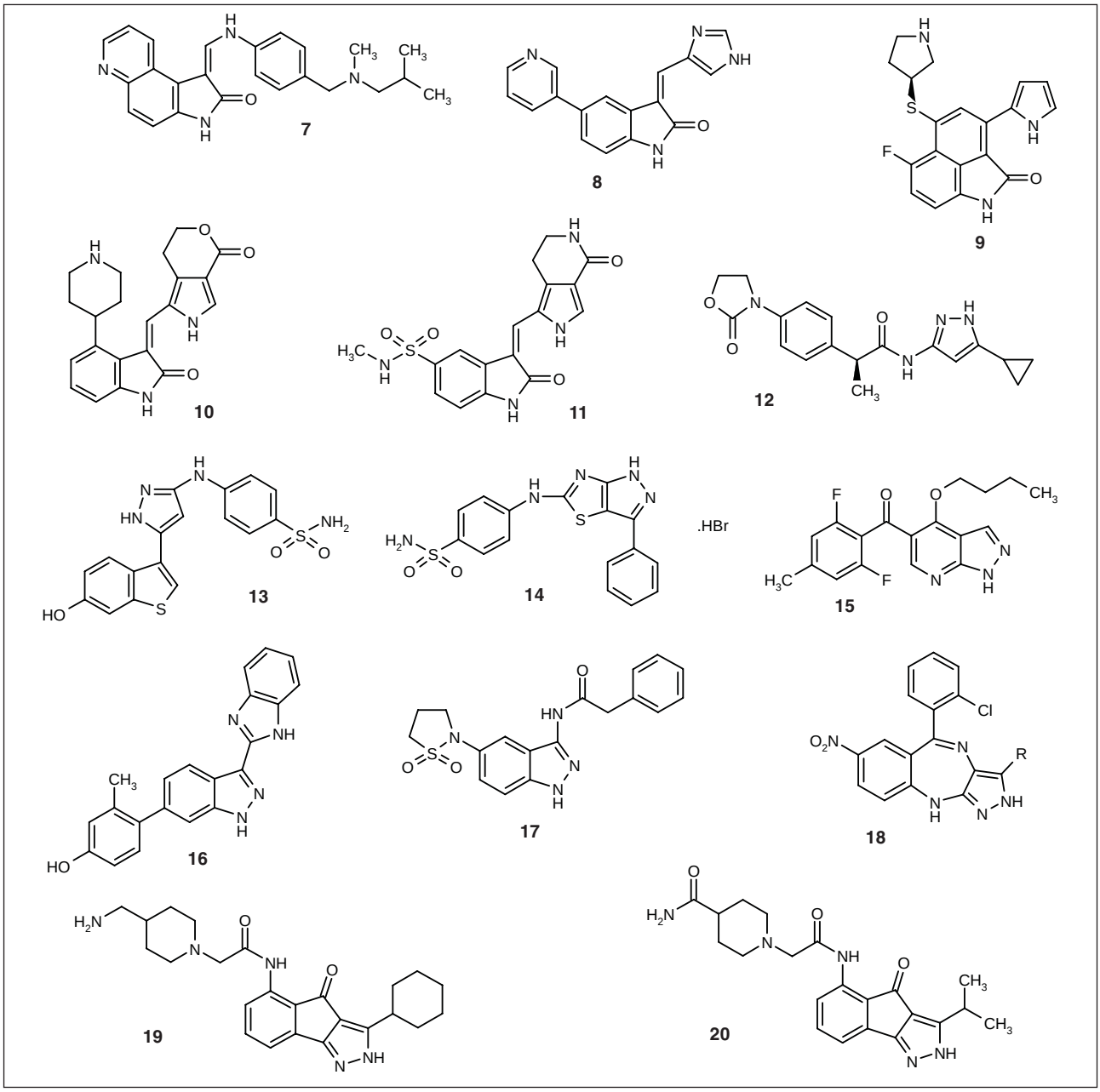

Fig. 3. Oxindole and pyrazole derivatives.

inhibitors (75-77). This class of compounds is selective for the CDK related kinase family and active in cell culture against a transformed colon cell line (HCT-116). Furthermore, compounds from this class demonstrated *in vivo* activity in a human xenograft model in a dose-dependent manner. The crystal structure of indenopyrazole **19** (Fig. 3) with CDK2 indicated that the inhibitor binds into the ATP binding pocket of CDK2 with the indenopyrazole core occupying the same region as the adenine ring of ATP. Both nitrogens of the indenopyrazole core of **19** form hydrogen bonds with Leu83 in the hinge region. The other compound in this series, **20** (Fig. 3) is a potent (IC_{50} = 21 nM) and selective CDK2/cyclin E inhibitor (62 times selective) against CDK4/cyclin D1 with excellent cell-based activity against HCT-116 cells (IC_{50} = 290 nM) (77).

Substituted pyrimidines

Based on some initial findings with substituted guanines, groups at University of Newcastle and

AstraZeneca designed a series of pyrimidine analogs to mimic the donor-acceptor-donor motif in the purine type of CDK inhibitors (78-80). The 5-nitrosopyrimidine **21** (NU-6027) (Fig. 4) was thus designed with the concept that the nitroso group would form an intramolecular hydrogen bond with the 6-NH_2 group allowing the other hydrogen on 6-NH_2 group to have the correct orientation to form a hydrogen bond with Glu81. NU-6027 is an inhibitor of CDK1 and CDK2 with IC_{50} values of 2.9 μM and 2.2 μM, respectively. The crystal structure of NU-6027 complexed with CDK2 demonstrated that the binding mode is different from that of 6-aminopurine-based inhibitors, such as (*R*)-roscovitine, but is nearly identical to that of alkoxyguanine derivatives, such as NU-6102 (60). The key interactions in the ATP binding pocket are indeed comprised of hydrogen bonds formed through the donor-acceptor-donor motif (2-NH_2 and N1 to Leu83, 6-NH_2 to Glu81). Additionally, the co-crystal structure of NU-6027 and CDK2 revealed the presence of an intramolecular hydrogen bond between the 5-nitroso group and the 6-NH. Interestingly, replacement of the nitroso group with the formyl isostere does not result in a similar intramolecular hydrogen bond as demonstrated by X-ray crystallography. This lack of intramolecular hydrogen bond may be the reason for the reduced inhibitory activity of the corresponding formyl analog (79).

When the C-2 amino group was substituted with a phenylsulfonamide group to provide additional interactions with Asp86 of CDK backbone, the resulting derivatives are potent CDK2 inhibitors, as exemplified by compound **22** (81) (Fig. 4) with an IC_{50} value of 17 nM.

In an elegant piece of virtual screening based on the CDK2-ATP binding pocket, researchers at Cyclacel and University of Edinburgh applied their database mining program, LIDAEUS™, for the docking of small ligands to the active site of CDK2 (82, 83). Using this approach, screening of about 200 compounds from a virtual set of approximately 50,000 drug-like molecules resulted in the identification of a new pyrimidine-based phamacophore. The results were further supported through co-crystallization of the prototypical compounds with CDK2. Structure-guided lead optimization afforded **23** (Fig. 4), which inhibits CDK2/cyclin E and CDK4/cyclin D with IC_{50} values of 0.9 μM and 5.5 μM, respectively. Further optimization generated an extremely potent and selective CDK2 inhibitor **24** (Fig. 4) with an IC_{50} value of 0.2 nM against CDK2/cyclin E and IC_{50} of 410 nM against CDK4/cyclin D1 (84, 85). Compound **24** also shows potent antiproliferative activity in the human tumor cell lines A549, HT29 and Saos-2 with a median IC_{50} of 0.3 μM. Furthermore, compound **24** inhibits Rb phosphorylation, blocks cells in the G_1/S phase and induces apoptosis in A549 cells, consistent with its CDK2/CDK4 inhibition. The crystal structure of **24** with CDK2 indicates the presence of two hydrogen bonds between the NH and carbonyl of Leu83 and the pyrimidine N1 and the NH attached to the pyrimidine. Additional hydrogen bonds between Asp86-NO_2 and Asn132-NH_2 may account for the further increase in potency. Compound **24** also shows significant *in vivo* activity in the MES-SA mouse xenograft model. Pyrimidine derivative **25** (Fig. 4), from AstraZeneca, was reported to be an extremely potent CDK2 inhibitor (IC_{50} < 3 nM) and was selective relative to CDK4 (IC_{50} = 2.5 μM) (86). Very recently, GlaxoSmithKline disclosed pyrimidine **26** (Fig. 4) as a CDK4 inhibitor (IC_{50} < 0.1 μM) (87). Another series of diamino pyrimidines were also identified as CDK4 inhibitors (88). The combination of 5-substitution and N-alkylation on the aminopyrimidine core generated the CDK4 inhibitor **27** (Fig. 4) (IC_{50} = 10 nM), with 20-fold selectivity against CDK2.

Aminothiazole derivatives

Aminothiazole **28** (BMS-387032) (Fig. 4) is a potent CDK2/cyclin E inhibitor (IC_{50} = 48 nM) with significant selectivity against other protein kinases (IC_{50} > 25 μM against 15 other kinases) as well as CDK1/cyclin B (IC_{50} = 480 nM) and CDK4 (IC_{50} = 925 nM (89-93). This compound shows broad cytotoxicity against a panel of 40 tumor cell lines including A2780 cells (IC_{50} = 95 nM). It also induces cell cycle arrest and apoptosis, and inhibits phosphorylation of the CDK2 substrate, the retinoblastoma protein (Rb). BMS-387032 demonstrated *in vivo* efficacy in five tumor models including the P388 mouse leukemia and A2780 human ovarian carcinoma, with superior efficacy to flavopiridol. Treatment with BMS-387032 (qd x 8, i.p. or i.v.) in A2780 ovarian tumor bearing mice produced a marked antitumor activity of > 4.0 log cell kill (LCK) and >50% complete regression and cures. This compound is currently in phase I clinical trials. A more potent compound in this series, **29** (BMS-419437) (Fig. 4), has an IC_{50} of 3 nM against CDK2/cyclin E and is at least 10-fold more selective than CDK1/cyclin B and CDK4/cyclin D and over 1000-fold more selective against a panel of kinases (94). This compound also shows *in vivo* antitumor activity in the human ovarian carcinoma A2780 xenograft model in mice.

PNU-252808, another thiazole derivative (structure not disclosed), is a potent CDK2/cyclin A (IC_{50} = 48 nM) inhibitor which is selective against a panel of 30 kinases, including CDK4, MAPK, PKA, EGFR, *etc.*, while being moderately selective against CDK1/cyclin B (IC_{50} = 470 nM) (95). In human colon adenocarcinoma HT-29 cells, PNU-252808 causes G_1 block and inhibits Rb phosphorylation upon exposure to 0.3 μM for 24 h. It selectively induced apotosis in proliferating A2780 human ovarian carcinoma cells, but not in proliferating normal cells. PNU-252808 demonstrated *in vivo* activity in three tumor models, including A2780 ovarian carcinoma, DU145 prostate carcinoma and HCT-116 colon carcinoma after oral or i.p. administration. In addition, PNU-252808 is synergistic with other chemotherapeutic agents, such as gemcitabine (96). In A549 cells, these effects require the administration of chemotherapy prior to PNU-252808, which is similar to the schedule dependency previously observed with flavopiridol.

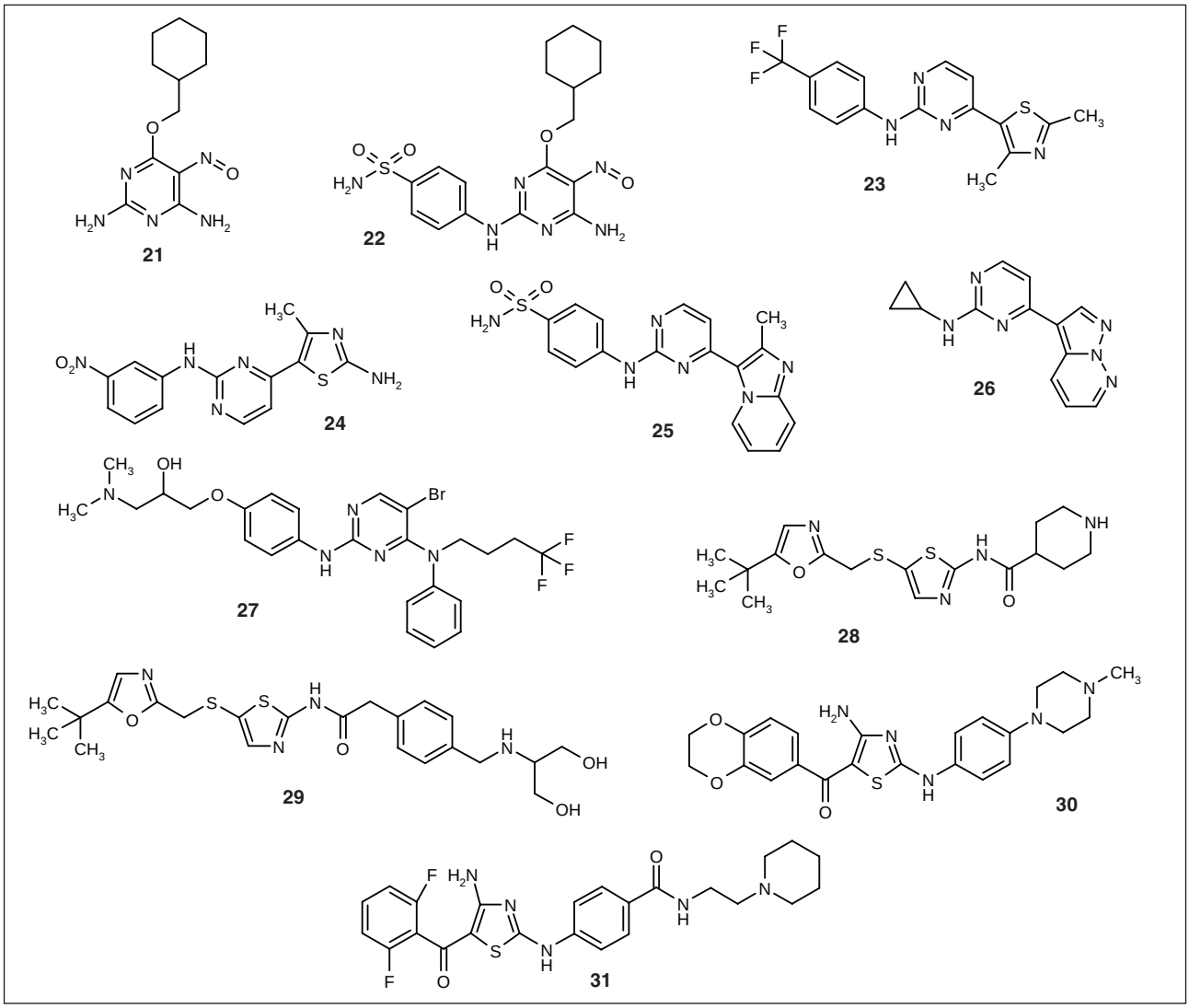

Fig. 4. Pyrimidine and aminothiazole CDK inhibitors.

Aminothiazole **30** (Fig. 4) is a potent CDK4/cyclin D1 (IC_{50} = 20 nM) inhibitor and is more than 100 times selective against CDK2 and CDK1 (97). Treatment of HCT-116 cells with compound **28** caused a time- and dose-dependent decrease in Rb phosphorylation, which corresponds to CDK4 inhibition. Compound **30** also blocks the G_1 phase and induces apoptosis in HCT-116 cells. When dosed intraperitoneally, twice a day for 10 days, **28** inhibited the growth of HCT-116 human colon tumor xenografts in nude mice by 74%. Pfizer recently claimed a series of diaminothiazoles as potent and selective CDK4 inhibitors (98). Compound **31** (Fig. 4) inhibits CDK4 and CDK2 with IC_{50} values of 7 nM and 150 nM, respectively.

Miscellaneous

Banyu and Merck scientists identified a new class of CDK inhibitors through docking experiments directed at a model of CDK4 meticulously constructed from the known crystal structures of CDK2 and CDK6 (99, 100). Diaryl urea **32** (Fig. 5), a potent CDK4/cyclin D (IC_{50} = 2 nM) inhibitor, is 190 times more selective against CDK2/cyclin E, 760 times more selective against CDK1/cyclin B and more than 430 times selective against many other kinases. In the T98 human glioblastoma cell line *in vitro*, 32 inhibited Rb phosphorylation, blocked cells in the G_1 phase and inhibited E2F transcriptional activity (101).

Parke-Davis (now Pfizer) claimed a pteridinone **33** (Fig. 5) as a selective CDK4/cyclin D inhibitor with IC_{50} values of 7, 180, 750, 3300 nM against CDK4/cyclin D, CDK2/cyclin A, CDK1/cyclin B and c-Src, respectively (102).

Eli Lilly reported two series of closely related indolocarbazoles as CDK4/cyclin D inhibitors (103-106). These ATP competitive inhibitors block the cell cycle at G_1 and inhibit human colon cell line HCT-116 with micromolar

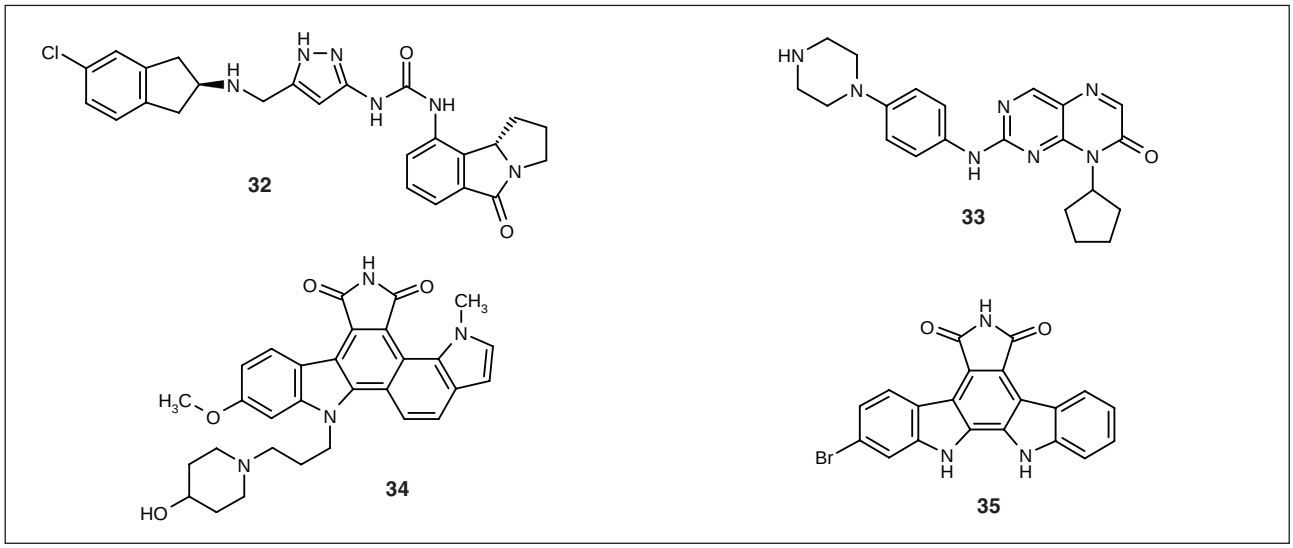

Fig. 5. Miscellaneous CDK inhibitors.

activity. Compound **34** (Fig. 5) has an IC_{50} of 4 nM against CDK4 using Rb[21] as the substrate and is more than 500 times selective against CDK2/cyclin E (105). Compound **35** (Fig. 5) is a less selective CDK4 and CDK2 inhibitor with IC_{50}s of 76 nM and 520 nM, respectively (106).

JNJ-7706621 (structure not disclosed) (107) was identified as a CDK inhibitor (structure not released) with good selectivities over other unrelated kinases. It arrests the cell cycle at the G_2/M phase and shows cytotoxicity *in vitro* against a wide range of tumor cell types with 10-fold less toxicity against normal cells. *In vivo* studies show that JNJ-7706621 inhibits the growth of human prostate carcinoma and melanoma cancer cells in nude mice when dosed alone.

Cell cycle checkpoints

While cell cycle progression is regulated by cyclin-dependent kinases, the cell cycle transitions are controlled by cell cycle checkpoints. Checkpoints are signaling cascades that monitor the integrity and replication status of the genetic material before cells commit to replicate (in the S phase) or segregate (in mitosis) their DNA (108). In response to DNA damage, checkpoints are activated to arrest the cell cycle so that the cells can repair their DNA. There are at least three cell cycle checkpoints throughout the cell cycle, the G_1/S, G_2/M and S checkpoints. The G_1/S checkpoint ensures that cells do not start DNA synthesis until the molecular machinery for DNA replication is ready and DNA is intact. The G_2/M checkpoint prevents cells from entering mitosis if there are any replication errors. The S phase checkpoint arrests the cell cycle in the S phase due to either depletion of nucleotides or DNA damage.

The G_1/S checkpoint is regulated by Rb and p53 pathways (108). The Rb pathway plays a key role in the G_1/S transition and controls the activity of CDK2/cyclin E, which is essential for S phase entry. The G_1/S checkpoint is also p53 dependent. DNA damage leads to the rapid induction of tumor suppressor gene p53, which stimulates transcription of different genes including the CDK inhibitor p21. The upregulation of p21 results in CDK2 and CDK4 inhibition, and cell cycle arrest at G_1.

The S phase checkpoint is relatively poorly understood. Recent studies (109) have shown that Cdc25A phosphatase is important for the initiation and progression of the S phase. Cdc25A dephosphorylates and activates CDK2/cyclin E, a key kinase for the S phase progression. In response to DNA damage, protein kinases ATM and ATR activate the cell cycle checkpoint kinases Chk1 and Chk2, resulting in the rapid degradation of Cdc25A and consequently S phase arrest (110-113). The ATM-Chk2-Cdc25A pathway requires both ATM and the chk2-mediated phosphorylation of Cdc25A on Ser123 (111). Another study indicates that the DNA damage dependent S phase checkpoint is regulated by two parallel pathways: the ATM-Nbs1-Mre11 and the ATM-chk2-Cdc25A pathways (114).

The G_2/M transition is controlled by the activity of CDK1/cyclin B. CDK1 is activated through the dephosphorylation at Thr14 and Tyr15 by Cdc25C phosphatase. In eukaryotic cells, DNA damage activates the ATM and ATR kinases, which in turn phosphorylate the downstream protein kinases Chk1 and Chk2. Activated Chk1 and Chk2 phosphorylate Cdc25C on Ser216, preventing Cdc25 from activating CDK1 by binding to 14-3-3 protein, and leading to cell cycle arrest at G_2/M (115, 116). Cdc25A is also important during the G_2 and M phases of the cell cycle (109, 117). Cdc25A binds to and activates the mitotic inducer CDK1/cyclin B and its presence delays the entry of cells into mitosis. Furthermore, recent studies demonstrated that phosphorylation of Cdc25A by Chk1

was required for cells to arrest at the S and G_2 checkpoints in response to ionizing radiation (112). The DNA damaging agents camptothecin and doxorubicin, which induce S and G_2 arrest, can activate Chk1 and cause the rapid proteolysis of Cdc25A (113).

Chk1 inhibitors

To date, some of the most effective anticancer agents used in the clinic are still DNA targeting agents, such as bleomycins and cisplatin. These drugs have produced significant increases in the survival of cancer patients when combined with other drugs with different mechanisms of action (118). The resistance to these drugs after initial treatments is a major limitation of these cancer therapies. One major mechanism of drug resistance comes from the cell cycle checkpoints. Tumor cells can take advantage of the cell cycle arrests at checkpoints to repair DNA. Therefore, abrogation of the DNA damage checkpoints could enhance the cytotoxcity of DNA damaging agents.

Mutations in the p53 tumor suppressor gene occur in 50% of tumors. p53 is required for DNA damage induced G_1 arrest, but has little effect on S or G_2 arrest. Whereas normal cells arrest at G_1, p53 mutated tumor cells can only arrest in the S and G_2 phases. Therefore, by specifically abrogating the G_2 checkpoint, normal cells can still arrest at G_1 and repair the DNA damage, while tumor cells that lack the G_1 checkpoint will undergo mitotic catastrophe and eventually cell death.

As stated above, Chk1 is a serine/threonine kinase that serves as the effector of the DNA damage signal to block the cell cycle at the G_2/M checkpoint. Although there are two checkpoint kinases, Chk1 and Chk2, Chk1 was shown to be the essential gene for the cell cycle G_2 checkpoint (119). Liu (120) demonstrated that Chk1 is required for the G_2 DNA damage checkpoint in mammals, since mice lacking Chk1 die in early embryogenesis. Thus, Chk1 inhibitors would abrogate the DNA damaging agent induced G_2/M checkpoint and enhance the cytotoxicity of the DNA damaging agent (121, 122).

Staurosporine analogs

UCN-01 (**36**) (Fig. 6) is regarded as the standard for G_2 checkpoint modulators. Originally isolated as a PKC inhibitor, UCN-01 (123) abrogated the G_2 checkpoint in p53 mutated cancer cells. Recent works indicate that UCN-01 is a potent inhibitor of Chk1 and interferes with the degradation of Cdc25C phosphatase (124). This induces the activation of $p34^{cdc2}$ and subsequently causes the G_2 checkpoint abrogation.

The crystal structure of UCN-01 in complex with the Chk1 kinase domain was determined at GlaxoSmithKline (125). UCN-01 binds to the ATP binding pocket of Chk1 in a manner similar to many other known ATP-competitive protein kinase inhibitors: the 6-amino group on the lactam moiety of UCN-01 forms a hydrogen bond with the backbone carbonyl oxygen of Glu85 and the 5-keto of the inhibitor accepts a hydrogen bond from the amide nitrogen of Cys87, while the terahydropyran ring sits in the ribose-binding pocket. The selectivity of UCN-01 towards Chk1 over CDK2 can be explained by the presence of a hydroxyl group in the lactam moiety interacting with the ATP binding pocket (125).

In addition to its Chk1 activity, UCN-01 is also an inhibitor of CDK1 and CDK2 (IC_{50} ~ 600 nM) and arrests cell cycle at G_1/S (126). Most recent studies show that UCN-01 inhibits the Akt pathway (127) and the cell cycle effects of UCN-01 are mediated by upregulation of p21 (128). The multiple mechanisms of action enable UCN-01 to interact synergistically with diverse chemotherapeutic agents. As a G_2 checkpoint abrogator, UCN-01 can sensitize the action of DNA damaging agents such as cisplatin, mitomycin C (123) and ionizing radiation (129). UCN-01 not only abrogates the G_2 checkpoint but also abrogates the S phase checkpoint when combined with SN-38, an active metabolite of the topoisomerase I inhibitor CPT-11 (130).

UCN-01 entered clinical trials as a CDK inhibitor. The unusually long half-life observed in phase I is attributed to its binding to human plasma protein. The recommended phase II dose is a 72-h infusion at 42.5 mg/m^2/day followed by monthly 36-h infusions at the same dose. Dose-limiting toxicities included hyperglycemia with metabolic acidosis, dyspnea and/or hypoxemia, nausea, vomiting and hypotension. One partial response in a melanoma patient was observed (131). In a phase II trial conducted at the University of California at San Francisco, 15 patients with advanced renal cell carcinoma were treated with UCN-01. Although stable disease was observed in several patients, there were no objective responses (132). Several combination trials with standard cytotoxic agents have recently begun (133-135). Early results of a phase I study of UCN-01 in combination with topotecan have been reported (135). This combination was well tolerated with some preliminary evidence of efficacy.

Gö-6976 (**37**) (Fig. 6) is another PKC inhibitor that can abrogate the S and G2 checkpoints in response to DNA damage (136). Analysis of proteins that regulate cell cycle arrest indicated that Gö 6976 inhibits Chk1 and/or Chk2. In contrast to UCN-01, Gö-6976 can abrogate S and G_2 arrest in human serum, which suggests that it has little protein binding. In addition, Gö-6976 abrogates S and G_2 arrest at a much lower concentration than that required to inhibit PKC, suggesting that it may have fewer side effects associated with PKC inhibition (136).

Novel Chk1 inhibitors

In a recent patent application, Agouron (now Pfizer) disclosed a series of potent chk1 inhibitors (72). Indazole **38** (Fig. 6) inhibits Chk1 with an IC_{50} of 5.2 nM. However, this class of compounds also inhibits VEGF-R2.

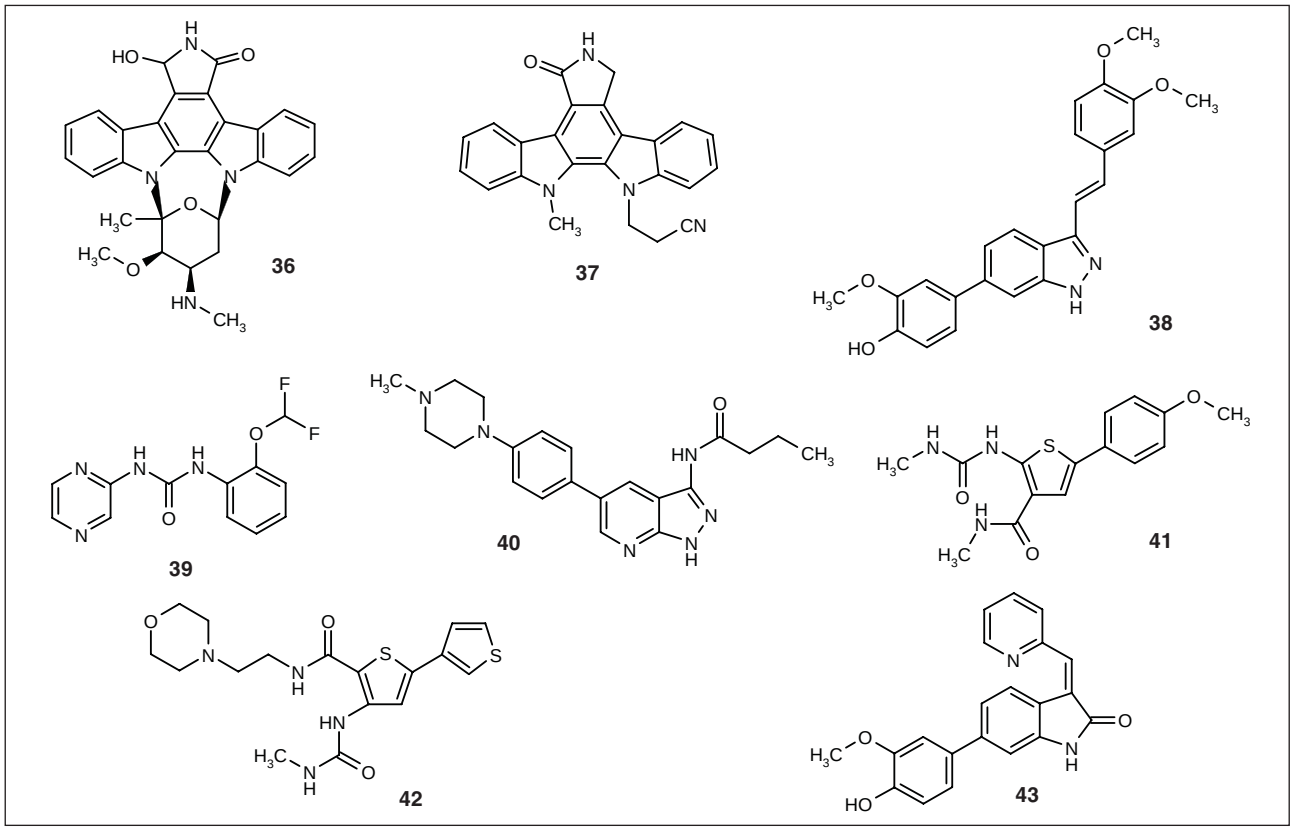

Fig. 6. Chk1 inhibitors.

Several other classes of Chk1 inhibitors such as **39** (137), **40** (138), **41** (139), **42** (140) and **43** (141) (Fig. 6) were also disclosed in patent literature without any associated biological data.

Conclusions

One of the greatest challenges in the discovery of molecular targeted therapies for cancer continues to be the identification of the appropriate targets to modulate. The success of antimitotic agents and the discovery of key mutations in the various regulators of the cell cycle in human tumors have prompted a great deal of interest in and research on cell cycle targets over the past few years. The further elucidation of the role that CDK2/cyclin E has in cell cycle progression and the prevalence of the aberrant expression of the complex in a variety of cancers has refined the focus of research on more selective inhibitors of CDK2. (*R*)-Roscovitine and BMS-387042, currently in clinical trials, are the outcome of such endeavors. It is expected that these two agents will offer a better efficacy and safety profile than the less selective agents. Recent *in vitro* work from McCormick (142, 143), however, showed that tumor cells deficient in CDK2 kinase activity can sustain cell proliferation, thereby suggesting that, because of redundant or compensating pathways, targeting CDK2 selectively may not be a viable therapeutic approach. Although tremendous strides have been made in the field during the last few years, many of the key elements of the cell cycle machinery and their interplay in the development of tumors are still missing. The complexity is further amplified by the inherent heterogeneity of human cancer. Emerging technologies, such as RNAi, offer the hope of providing a greater understanding of the biological processes and eventually new therapeutic approaches.

Checkpoints have attracted the attention of both the academic communities and the pharmaceutical industry, because these targets provide unique opportunities to develop drugs that have the potential to selectively target cancer cells over normal cells. The recently discovered Chk1 inhibitors provide the tools for the validation of this concept in *in vivo* models, and ultimately in the clinical setting.

Understanding the target is only part of the challenge. Chemical tools are needed to test biological hypotheses. The more selective the molecules are, the more relevant is the correlation between observed effect and the target in question. Selective molecules also allow the possibility of targeting multiple kinases cleanly or in concert in order to get a better insight into redundancy and cooperativity of various regulatory proteins in the cell cycle. The discovery and design of ATP mimics described in this review,

and in many others, has offered a significant starting point towards the discovery of these selective tools. In light of the fact that the ATP binding site of kinases is to a great extent conserved, it has been impressive to realize that a great deal of selectivity can be achieved by simply making modifications of the functional groups around these scaffolds. Some scaffolds offer a greater range of diversity in functionalization than others and allow for greater selectivity. It should be realized, however, that selectivity is a relative term and reflects the specific targets evaluated. Indeed, all of the kinase programs discussed in this review are only concerned about the activity of their inhibitors against a small handful of kinases without regard to the many others in the proteome that may be targeted. In fact, it is possible that a multitude of other kinases, purine binding proteins and many other unrelated proteins are targeted by these "selective" molecules and result in undesired effects. Moreover, these effects may significantly limit the effective dosing of these compounds in the clinical setting and therefore hinder the evaluation of the full potential of such agents. It is clear that the ultimate goal should still remain the discovery of selective molecules, or at the very least molecules that exclusively target a set of desired proteins. With the current ATP mimics, and the technologies available to us, this goal remains a challenge.

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