DOI: 10.1002/cmdc.200800185

ATP-Noncompetitive Inhibitors of CDK–Cyclin Complexes

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Progression through the cell division cycle is controlled by a family of cyclin-dependent kinases (CDKs), the activity of which depends on their binding to regulatory partners (cyclins A–H). Deregulation of the activity of CDKs has been associated with the development of infectious, neurodegenerative, and proliferative diseases such as Alzheimer's, Parkinson's, or cancer. Most cancer cells contain mutations in the pathways that control the activity of CDKs. This observation led this kinase family to become a central target for the development of new drugs for cancer therapy. A range of structurally diverse molecules has been shown to inhibit the activity of CDKs through their activity as ATP antagonists. Nevertheless, the ATP binding sites on CDKs are highly conserved, limiting the kinase specificity of these inhibitors. Various genetic and crystallographic approaches have provided essential information about the mechanism of formation and activation of CDK–cyclin complexes, providing new ways to implement novel research strategies toward the discovery of new, more effective and selective drugs. Herein we review the progress made in the development of ATP-noncompetitive CDK–cyclin inhibitors.

Introduction

The balance between proliferation and cell death plays a crucial role in tissue homeostasis. The cell cycle and cell death pathways (apoptosis) are the two main mechanisms that ensure the proper development of an organism. A deregulation of the cell cycle mechanism emerges on the origin of some degenerative diseases and tumor development. For instance, excessive proliferation is generally considered an indicator of cancer, whereas restrained proliferation causes hypotrophy.

The cell progression cycle is composed of two main phases, mitosis (M) and the interphase. The latter further divides into three stages: gap 1 (G_1) in which cells grow and prepare for DNA synthesis, S in which cells synthesize DNA, and gap 2 (G_2) in which cells prepare for M phase entry (Figure 1). Cells can remain in a nonproliferating state named G_0 or quiescence. The cyclin-dependent kinase (CDK) family of serine/threonine kinases has been identified as a key regulator of cell cycle progression and, as such, its members have become important targets for the development of therapeutic strategies. Cell cycle progression requires the sequential activation of CDKs through their association with regulatory subunits named cyclins. The CDKs needed for the G_1 and S phases are present in the cell at a constant level, unlike the levels of cyclins which fluctuate during cell cycle progression.[1] The accumulation of specific cyclins at precise moments of the cell cycle permits the formation of different CDK–cyclin complexes, which are activated and phosphorylate specific substrates. In this way, these complexes drive cell cycle progression. Cyclin C is expressed during the G_0–G_1 transition when it binds to and activates CDK3. Later, during early and mid-G_1 the different isoforms of cyclin D (D1, D2, and D3) are synthesized and associate with CDK4 and CDK6. The transition between the G_1 phase and the S phase implicates cyclin E and CDK2 complex formation, whereas progression through the S phase is controlled by the CDK2–cyclin A complex. Next, cyclin A binds to CDK1 in G_2 and, together with CDK1–cyclin B, lead to an entry into mitosis.

The activity of CDK–cyclin complexes is regulated by the phosphorylation of CDKs, and also by the association with the members of two inhibitor families (CKIs).[2–4] The INK4 (inhibitor of CDK4) family includes $p16^{Ink4a}$,[5,6] $p15^{Ink4b}$,[6] $p18^{Ink4c}$,[7] and $p19^{Ink4d}$,[8] which bind to and inhibit CDK4 and CDK6. The KIP (kinase inhibitor protein) family includes $p21^{Cip1}$,[9] $p27^{Kip1}$,[10] and $p57^{Kip2}$,[11] which bind to all the CDK–cyclin complexes and inhibit their activities.

Cell cycle progression is also under the surveillance of different mechanisms, known as checkpoints, which ensure the fidelity of cell proliferation. Checkpoints detect different types of anomalies and respond by blocking cell cycle progression and by inducing mechanisms to repair damage. Cell size, DNA damage, re-replication, premature mitosis entry, and metaphase–anaphase transition are all under the surveillance of major checkpoints. Thus, these checkpoints verify whether the progression through each step of the cell cycle has been accurately completed before allowing the transition to the next phase.

As already mentioned, alterations in CDK activity can be related to a variety of different diseases. Cyclin E is upregulated

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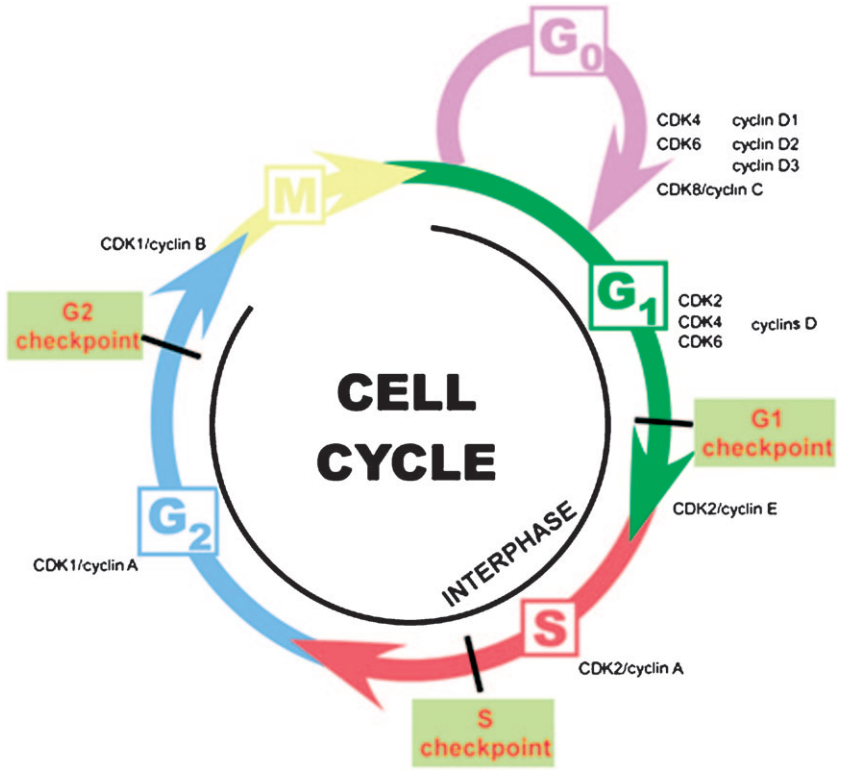

Figure 1. The cell cycle and its regulation. The eukaryotic cell cycle is divided into two phases: interphase and mitosis (M). Interphase is composed of three stages: gap 1 (G_1), DNA-synthesis (S), and gap 2 (G_2). The cells in the G_0 (quiescence) phase do not cycle.

in human malignant melanomes.[12] Decreased levels of CKI $p27^{Kip1}$ are associated with many cancer types,[13] and this fact not only correlates with a worse outcome in breast cancer, but also with many other tumors.[14] The concurrent amplification of cyclin E and CDK2 genes seems to play a role in colorectal carcinogenesis.[15] An elevated expression of CDK2 is a critical factor in oral cancer progression, and can be used as a negative predictive marker of the patients' prognosis.[16] Some studies report that the determination of CDK1- and CDK2-specific activities could be useful in the prediction of outcomes in breast cancer patients.[17] Moreover, abnormalities in CDK activity have also been associated with neurodegenerative disorders such as Alzheimer's[18] or Parkinson's disease.[19] CDK/GSK-3 inhibitors have demonstrated preclinical efficacy in mesangial proliferative glomerulonephritis, crescentic glomerulonephritis, collapsing glomerulopathy, proliferative lupus nephritis, polycystic kidney diseases, and diabetic nephropathy.[20]

With all these precedents, the need for precise CDK activity regulation in the cell clearly justifies the time and effort dedicated to design new strategies for finding novel generations of CDK inhibitors with improved activity and selectivity.

ATP-competitive versus noncompetitive inhibitors

The first generation of CDK–cyclin inhibitors is composed of ATP-competitive inhibitors. Among them flavopiridol and CY-202, which are involved in a large number of clinical trials (see Table 1), should be highlighted. Nevertheless, this type of inhibitor did not match initial expectations, and a second gener-

Table 1. Compounds in clinical trials: the current status of ATP-competitive and ATP-noncompetitive drugs.

Drug	Active substance	Clinical phase[a]
ATP-competitive drugs:		
Alvocidib	Flavopiridol	NCI, Phase II
Bryostatin-1	Macrocyclic lactone	DFCI, Phase II NCI, Phase II
CYC202 (Seliciclib)	(*R*)-roscovitine	CP, Phase II
P276-00	Derivative of rohitukine (flavone family)	NPI, Phase I
PD 0332991	Pyrido[2,3-*d*]pyrimidine-7-ones (paullone family)	Pfizer, Phase II
SNS-032 (former BMS-387032)	2-Aminothiazole derivative	SP, Phase I
SU9516	3-[1-(3*H*-Imidazol-4-yl)meth-(*Z*)-ylidene]-5-methoxy-1,3- dihydro-indol-2-one	Pre-clinical
UCN-01	7-Hydroxystaurosporine	NCI, Phase II
ATP-noncompetitive drugs:		
CIP	YFTLQIRGERFEMFRELNE	Pre-clinical
NBI1	rwimyf-NH_2[b]	Pre-clinical
Pipeline (CYC103)	Cyclin binding groove inhibitors	Pre-clinical
PSTAIRE	TYTKKQVLRMEHLVLKVLTFDL	Pre-clinical
Spa310	TSPTTLYDRYSSPPASTTR	Pre-clinical

[a] NCI: National Cancer Institute; DFCI: Dana Farber Cancer Institute; CP: Cyclacel Pharmaceuticals Inc.; NPI: Nicholas Piramal India Ltd.; SP: Sunesis Pharmaceuticals. [b] Lower-case letters denote the D stereoisomers of natural amino acids.

ation of compounds, designed with the lessons learned from the first generation in mind, is now undergoing preclinical and clinical studies (see reference [21]). One of the main problems associated with kinase ATP-competitive inhibitors is the lack of specificity toward the target kinase. In fact these off-target kinase interactions could explain the appearance of numerous side effects during treatment.[22] For example, flavopiridol provokes the appearance of the hyperacute tumor lysis syndrome which determines the dose limitation of the drug,[23] and the histopathological analysis of monkey-treated eyes showed specific cellular damage in the photoreceptor layer.[24] Additionally in combination with docetaxel, flavopiridol induces neutropenia in patients with metastatic breast cancer. Moreover, the antitumor activity of these compounds in clinical trials has been modest. One of the essential causes that could explain the limited antitumor activity is the low tolerable doses that could be achieved in patients. In that sense, improvement of specificity could help to solve the appearance of treatment side effects.

As there are thousands of different kinases in the cellular context, and it is well established that the ATP binding site is highly conserved among them,[25] development of specific inhibitors against this protein region has become a highly complicated goal. Herein we present the initial efforts that different groups have made to design ATP-noncompetitive inhibitors (Table 1). The establishment of new inhibition strategies could contribute to the development of novel inhibitors for CDK–cyclin complexes with improved activities.

Inhibitors directed to the cyclin binding groove

In 1996, Kaelin and co-workers identified an eight-residue peptide (PVKRRLDL) derived from the sequence of the CDK2 substrate, E2F1, which inhibits the CDK2–cyclin A/E complexes.[26] Similar CDK–cyclin recognition motifs were found not only in other CDK substrates, but also in p21Cip1^{Cip1}-like CKIs, (p21Cip1^{Cip1}, p27^{Kip1}, and p57). These studies gave rise to the definition of a ZRXL binding motif on the sequence of some CDK substrates (in which Z is a basic residue or cysteine, and X is frequently a basic residue).[27] The peptides derived from these regions were able to promote the formation of stable CDK2–cyclin A complexes, but inhibited the phosphorylation of the retinoblastoma tumor suppressor protein (pRb) substrate. These results, together with advances in the understanding of the molecular mechanism behind the action of the natural CDK inhibitors, led Fischer's research group to account for CDK inhibition. The determination of the CDK2–cyclin A–p27^{Kip1} complex structure provided clues to design a new class of inhibitors.[28] p21^{Cip1} interacts with the CDK–cyclin complex on a binding groove located on the surface of cyclin A. This site, known as the cyclin substrate recruitment site (CRS), directs the appropriate orientation of the amino acid to be phosphorylated toward the ATP active site. Thus, the inhibitory activity of p21^{Cip1} relies on the competitive occupancy of this site that prevents the recruitment and phosphorylation of defined CDK substrates. In fact, the synthesis of an octapeptide derived from the p21^{Cip1} C-terminal sequence has been described, which binds to the CRS and inhibits kinase activity[29–32]

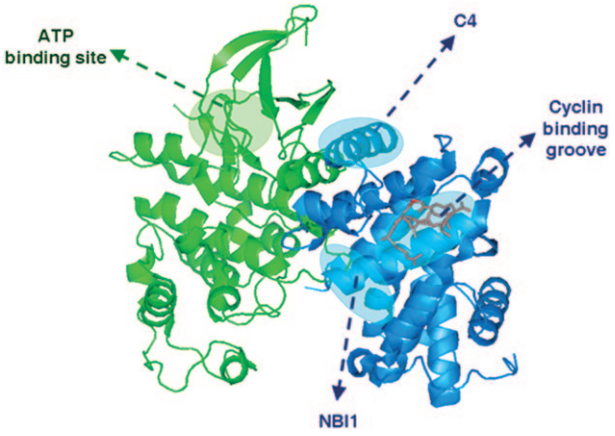

Figure 2. Potential points of intervention for drug discovery. Crystal structure of the CDK2–cyclin A complex (PDB accession code 1JSU). The green circle represents the ATP binding region of intervention; the blue circles represent the ATP-independent regions of intervention: cyclin binding groove, α5 helix on cyclin A that interacts with the PSTAIRE motif of CDK2 (the strategy adapted during the development of the C4 peptide), and a cleft on the surface of cyclin A, which is important for the binding of cyclin A to CDK2 (the predicted NBI1 region of interaction).

(Figure 2). Based on these approaches, Cyclacel Pharmaceuticals opened a targeted drug pipeline (CYC103) to develop CDK inhibitors directed to the CRS and have developed a broad family of inhibitors ranging from peptides to peptidomimetics (for more detailed information, see references [33–38]) together with the development of a second generation of ATP competitors, such as 4-arylazo-3,5-diamino-1*H*-pyrazoles or 2-anilino-4-(1*H*-pyrrol-3-yl) pyrimidines.

Interestingly in this context, a computational strategy named REPLACE (replacement with partial ligand alternatives through computational enrichment) has been developed. This strategy was specially designed to improve drug discovery by the inhibition of protein–protein interactions. REPLACE is based on the replacement of amino acids using peptide inhibitors as a scaffold to generate pharmaceutically acceptable lead molecules. This innovative approach will certainly improve and accelerate the generation of new leads for the development of CDK inhibitors.

Substrate-derived peptides

p53-derived peptides

Transcription factor p53 is one of the key factors in cell cycle control (reviewed in [39,40]). The activity of this tumor suppressor is widely regulated by different modifications, including ubiquitination, proline isomerization, acetylation, and phosphorylation. CDK1 and CDK2 are among kinases which control the activity of p53. As it is well established that the catalytic subunits of protein kinases have affinity binding sites for protein motifs different from the phosphorylation sites, the docking site of the p53 tetramerization domain on the CDK2 surface has inspired the design of p53 derived peptides (Figure 3 A). The inhibition of CDK2-mediated p53 phosphorylation was achieved using a 20-mer peptide named CIP (YFTLQIRGER-

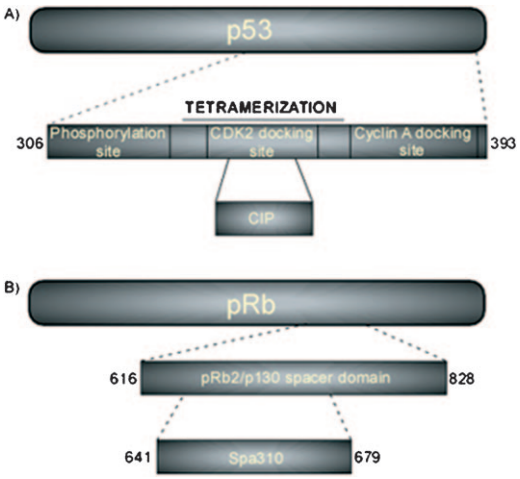

Figure 3. A) CDK2 docking site as a target for a p53 peptide inhibitor. The domain structure of p53 represents localization of the CDK2 docking site in comparison with the phosphorylation and cyclin A binding sites. Peptides derived from the tetramerization domain of p53 block CDK2 phosphorylation (see text for details). B) pRb2/p130 spacer domain as a target of inhibitory peptide development. The Spa310 construct, which spans the region between amino acids 641 and 679 (39 residues), maintains the specific inhibitory ability of the pRb2/p130 spacer domain on CDK2 activity.

FEMFRELNE). Furthermore, a GFP-CIP fusion protein attenuates p53 activity, suppresses $p21^{Cip1}$, and induces cell death in the A375 melanoma cell line. These results suggest that this docking site on the surface of CDK2 could be exploited as a pharmacological target. Moreover, the authors of this study compare the action of CIP with well established ATP-competitive inhibitors, such as roscovitine A[41] and NU2058.[42] Treatment with these inhibitors produces different cellular consequences in A375 cells. Whereas roscovitine treatment increases p53 and $p21^{Cip1}$ levels and arrests cells in G_1/S, NU2058 treatment does not change p53 levels but decreases the levels of $p21^{Cip1}$ and arrests cell cycle in G_2/M. The CIP-induced cell death mechanism is similar to that described for NU2058, and clearly differs from that induced by roscovitine. The different cellular consequences derived from roscovitine treatment could be explained if a different kinase-based mechanism was compromised by this drug, probably through the interaction with the ATP binding pocket. Once again, these differences reflect the specificity problems associated with ATP-competitive drugs. Further progress on this p53-based strategy has been made with the identification of a cyclin A docking site that directs p53 to the cyclin A surface. This new interaction site could provide results of pharmaceutical interest in the near future.[43]

pRb-derived peptides

Members of the pRb family are among the targets that CDKs phosphorylate. The binding of pRb to the E2Fs transcription factors prevent them from interacting with the cell transcription machinery. When pRb is phosphorylated, E2Fs are liberated and, in turn, activate the expression of the genes implicated in the G_1/S transition.[44–48] The characterization of these proteins led to the development of a 39-residue peptide (spacer 310), derived from the spacer domain of pRb2/p130, which is able to inhibit CDK2 activity[49,50] (Figure 3B). Treatment with the spacer 310 peptide arrests cells in the G_0/G_1 phase of the cell cycle. Moreover, when such a peptide was fused to a cell-penetrating peptide derived from the HIV-1 Tat protein (TAT) to improve its cellular uptake,[51] a significant decrease in tumor size in nude mice was also observed

Inhibitors of the CDK–cyclin interaction

C4: a peptide derived from cyclin A

Another strategy for the specific inhibition of kinase activity is the development of molecules that interfere with the protein–protein interface between CDK and cyclin. Crystal structures of free CDK2 and cyclin A, in their active and inactive forms, as well as in complex with each other, provide a rationale for this kind of inhibitor design.[52–54] The cyclin subunit binds to one side of the catalytic cleft and forms a continuous protein–protein interface by interacting with both the N- and C-terminal lobes of CDK2. Consequently, CDK2 undergoes a large structural rearrangement on the binding of cyclin A, which involves an association between the PSTAIRE helix of CDK2, and helices α3 and α5 of cyclin A in the first rapid step of the complex formation. However, it results in an inactive intermediate complex. The active CDK2–cyclin A complex is formed by a further slow structural reorganization that involves the exposure of the T-loop of CDK2 for phosphorylation by the CDK-activating kinase (CAK), and the formation of the substrate binding site.[55] The characterization of the CDK2–cyclin A complex formation led to the development of a 22 residue-long peptide (named C1) that targets the CDK2–cyclin A protein–protein interface.[56] This peptide corresponds to the sequence of the α5 helix of cyclin A (285-TYTKKQVLRMEHLVLKVLTFDL-306) (Figure 1), and abolishes the in vitro CDK2–cyclin A kinase activity toward histone H1 and a GST fusion of pRb (GST–pRb). A derivative peptide of C1 (named C4; bearing a point mutation E295 A which increases the solubility of the peptide) was shown to inhibit the kinase activity of CDK2 associated with either cyclin A ($IC_{50} = 1.8$ μM) or cyclin E ($IC_{50} = 1.5$ μM), although it did not affect the activity of CDK1–cyclin B ($IC_{50} > 200$). Its specificity toward the CDK–cyclin complex was shown by the lack of inhibition toward either protein kinase A or protein kinase C. Circular dichroism spectroscopy analysis of C4 showed that the peptide adopts an α-helical structure and may therefore inhibit the CDK2–cyclin A kinase activity by mimicking the hydrophobic interactions between the two components of the complex. As the inhibitory effect of C4 remained insensitive to the pre-existence of the CDK2–cyclin A complex (the same results were obtained when the peptide was added before or after the reconstitution of CDK2–cyclin A), and as C4 did not disrupt the CDK2–cyclin A interactions, it was concluded that the peptide affected protein–protein conformational changes, and "arrested" the CDK2–cyclin A complex in its inactive conformation. In accordance with these data, surface plasmon resonance studies showed that C4 did not bind to free CDK2. In contrast, it strongly interacted with

the CDK2–cyclin A complex ($K_d = 2.5 \pm 0.4$ nM) and showed a lowered affinity for free cyclin A. Thus, C4 appeared to be a selective inhibitor of CDK2–cyclin A, and was able to inhibit the proliferation of estrogen-independent MDA-MB-231 breast cancer cells. This inhibition was particularly efficient when C4 was fused to the cell-penetrating peptide TAT.[57]

NBI1

In some instances, the screening of combinatorial libraries has paved the road to the discovery of molecules that interfere with protein–protein interactions.[58–60] In our research group, we used this approach for the discovery of the ATP-noncompetitive inhibitors of CDK2–cyclin A complexes. To this end, we envisioned a biological assay under restrictive conditions which limited hits to molecules that could not compete with ATP. The output of the study was the identification of a CDK2–cyclin A inhibitory hexapeptide (rwimyf-NH_2; named NBI1), which is either ATP noncompetitive or targets the cyclin recruitment motif (CRM).[61] The inhibitory activity of NBI1 appears to depend on the cyclin subunit associated with CDK rather than on CDK itself, as shown by a fluorescence polarization-based assay and as confirmed by surface plasmon resonance. NBI1 binds to a minimal structural domain within the cyclin A sequence (amino acids 257–345), which is highly conserved (Figure 1). This region compromises helices α3, α4, and α5, and represents a structural domain implicated in the interaction with CDKs, thus suggesting that NBI1 might affect the stability of the complex. Indeed NBI1 was shown to disrupt CDK2–cyclin A interactions in a way that implicates competition for cyclin A. Besides targeting the CDK2–cyclin A complex ($IC_{50} = 1.1$ μM), NBI1 was also observed to inhibit CDK1–cyclin B1 ($IC_{50} = 2.9$ μM) and CDK6–cyclin D3 ($IC_{50} = 6.4$ μM), and to lead to a very weak inhibition of CDK2–cyclin E ($IC_{50} = 51.4$ μM). NBI1 showed no inhibitory effect toward a representative set of other serine/threonine protein kinases, such as glycogen synthase kinase 3 ($IC_{50} = 10.4$ μM), mitogen-activated protein kinase II ($IC_{50} = 18.8$ μM), or CK2 ($IC_{50} > 200$ μM).[61] Altogether, these data suggest that the action of NBI1 is highly selective toward CDK–cyclin complexes. To confirm these results in vivo, a derivative of NBI1 (TAT–NBI1) was tested on a number of tumor cell lines (HCT116, HT29, T98G, and A2780). Cells were treated with increasing concentrations of the compound (0–100 μM), and the measurement of their viability showed a net dose-dependent decrease. The kinase activity assay on immunoprecipitates obtained from cells treated with TAT–NBI1 was also in agreement with the in vitro results. Both CDK2 and CDK1 activities decreased whereas CDK4 activity was only slightly inhibited. Additionally when using nonsynchronized cells, treatment with TAT–NBI1 was observed to provoke cell cycle arrest, mainly at the S phase. This was further confirmed using HCT116 cells synchronized in G_1, G_1/S, and in the M cell cycle phase. The most sensitive cells to the peptide were those in the S or G_2M phase, which is in agreement with the specificity of NBI1 toward CDK2–cyclin A and CDK1–cyclin A/B, that is, the complexes that control S and G_2M progression.

Recent results from our research group show that TAT–NBI1 induces cell death in the p53-deficient leukemia cell line HL60 (unpublished results), revealing an apoptosis induction mechanism that is p53-independent. Structure-based studies in our laboratory (to be published elsewhere) suggest that NBI1 binds to a cleft on the surface of cyclin A, which is important for its binding to CDK2. If further results confirm these cumulative evidences, NBI1 will represent a peptide ligand directed to a new CDK2 docking site on cyclin A, which could be potentially important for the design of a new generation of ATP-noncompetitive drugs directed to regulate the activity of the CDK–cyclin A complexes.

Outlook

When the cell cycle is out of control, a direct repercussion is noted on the appearance of human malignancies. The altered expression of CDKs and their modulators, including the overexpression of cyclins or a loss of expression of CDK inhibitors, results in a deregulated CDK activity and provides selective growth advantages for abnormal cells. The development of ATP-competitive inhibitors for CDKs has been the conventional method to deal with these unbalances, and nowadays a considerable number of clinical trials with different anticancer proposals are in progress (see Table 1). Nevertheless, the propensity of classic ATP-competitive inhibitors to share multiple targets determined the need for new strategies. Increasing knowledge of the both structure and the mechanism of action and modulation of CDK–cyclin complexes provided clues for the development of the new inhibition strategies presented in this review. Despite being in the first steps of drug development, these new families of CDK inhibitors are expected to reveal promising improvements in selectivity. Thus, the development of ATP-noncompetitive inhibitors of CDK–cyclin complexes as modulators of the unregulated cell cycle have stirred the interest of those pharmaceutical companies involved in oncology programs. In the near future, after having concluded the still needed intensive studies into these new in-class inhibitors, we will probably witness new discussion forums on their selectivity, potency, and toxicity and, hopefully, on human anticancer activity without, or with less, side effects.

Acknowledgements

The work done in the authors' laboratory has been supported by grants from the Spanish Ministry of Education and Science (BIO2004-00998, BIO2007-60066). We especially thank Dr. Nuria Canela for her participation in setting the foundation of the NBI1 project.

Keywords: cell cycle · cyclin · inhibitors · kinases · peptides

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Received: June 19, 2008
Revised: September 30, 2008
Published online on November 27, 2008

