

## HISTONE ACETYLATION AND THE CELL-CYCLE IN CANCER

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### TABLE OF CONTENTS

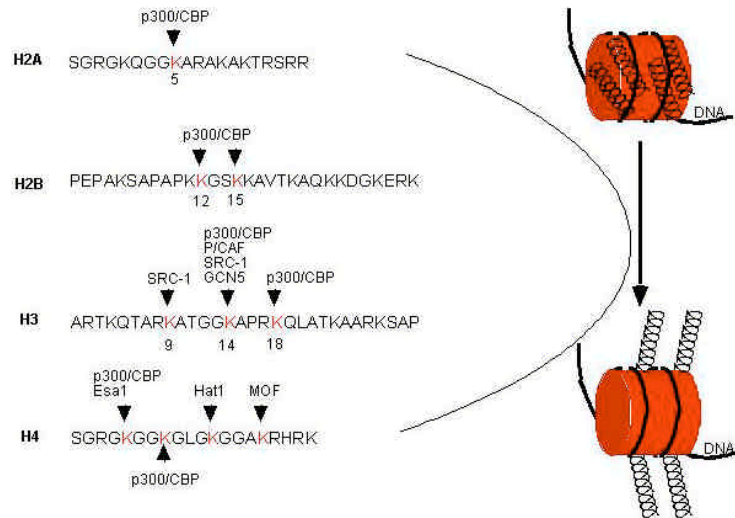
1. Abstract
2. Introduction
3. Chromatin modifying enzymes and mitogenic signaling.
4. Histone acetyltransferases and cancer.
5. Histone modification during the cell cycle, DNA damage and apoptosis
6. Histone deacetylases as cancer therapeutic targets.
7. Cyclin-dependent kinases and the regulation of histone acetylase activity.
8. Endogenous cyclin-dependent kinase inhibitors and histone acetylase activity.
9. Therapeutics targeting the cell cycle and histone deacetylation
  - 9.1. Histone deacetylase (HDAC) inhibitors
    - 9.1.1. Hybrid polar compounds
    - 9.1.2. Benzamide derivatives
    - 9.1.3. Trichostatins and cyclic tetrapeptide antibiotic
  - 9.2. Cdk modulators as Novel Cancer Therapy
    - 9.2.1. Flavopiridol.
    - 9.2.2. Staurosporine analogues and UCN-01.
    - 9.2.3. The Paullones
    - 9.2.4. Indirect Modulators of CDK function.
10. Conclusion
11. Acknowledgement
12. References

### 1. ABSTRACT

A number of distinct surveillance systems are found in mammalian cells that have the capacity to interrupt normal cell-cycle progression. These are referred to as cell cycle check points. Surveillance systems activated by DNA damage act at three stages, one at the G<sub>1</sub>/S phase boundary, one that monitors progression through S phase and one at the G<sub>2</sub>/M boundary. The initiation of DNA synthesis and irrevocable progression through G<sub>1</sub> phase represents an additional checkpoint when the cell commits to DNA synthesis. Transition through the cell cycle is regulated by a family of protein kinase holoenzymes, the cyclin-dependent kinases (Cdks), and their heterodimeric cyclin partner. Orderly progression through the cell-cycle checkpoints involves coordinated activation of the Cdks that, in the presence of an associated Cdk-activating kinase (CAK), phosphorylate target substrates including members of the "pocket protein" family. One of these, the product of the retinoblastoma susceptibility gene (the pRB protein), is phosphorylated sequentially by both cyclin D/Cdk4 complexes and cyclin E/Cdk2 kinases.

Recent studies have identified important cross talk between the cell-cycle regulatory apparatus and proteins regulating histone acetylation. pRB binds both E2F proteins and histone deacetylase (HDAC) complexes. HDAC plays an important role in pRB tumor suppression function and transcriptional repression. Histones are required for accurate assembly of chromatin and the induction of histone gene expression is tightly coordinated. Recent studies have identified an important alternate substrate of cyclin E/Cdk2, NPAT (nuclear protein mapped to the ATM locus) which plays a critical role in promoting cell-cycle progression in the absence of pRB, and contributes to cell-cycle regulated histone gene expression. The acetylation of histones by a number of histone acetyl transferases (HATs) also plays an important role in coordinating gene expression and cell-cycle progression. Components of the cell-cycle regulatory apparatus are both regulated by HATs and bind directly to HATs. Finally transcription factors have been identified as substrate for HATs. Mutations of these transcription factors at their sites of acetylation has been associated with constitutive activity

## Histone acetylation and the cell cycle



**Figure 1.** The core histone tail may function as a signaling platform. A schematic representation of a nucleosome core particle consisting of a histone octamer wrapped in DNA. Known phosphorylation sites and acetylation sites are indicated. Candidate HATs known to acetylate specific residues are shown. In response to mitogenic stimuli, the induction of ERK and p38 MAPK activity is associated with the induction of Rsk-2, which is capable of phosphorylating histone H3 (9) Adjacent residues are acetylated by recruited histone acetyl transferase (HAT) which facilitate transcriptional induction of immediate early genes.

and enhanced cellular proliferation, suggesting an important role for acetylation in transcriptional repression as well as activation. Together these studies provide a working model in which the cell-cycle regulatory kinases phosphorylate and inactivate HDACs, coordinate histone gene expression and bind to histone acetylases themselves. The recent evidence for cross-talk between the cyclin-dependent kinases and histone gene expression on the one hand and cyclin-dependent regulation of histone acetylases on the other, suggests chemotherapeutics targeting histone acetylation may have complex and possibly complementary effects with agents targeting Cdk.

## 2. INTRODUCTION

Cancer therapies target the activity of genes controlling cell-cycle progression, inducing differentiation or apoptosis. Although the precise mechanisms underlying the cell-cycle arrest induced by sodium n-butyrate is not known, this histone deacetylase inhibitor arrests the cell cycle or induces revertant phenotype in a variety of transformed cells. Compounds possessing HDAC inhibitory activity represent a new class of treatment for human cancers. The cyclin-dependent protein kinases are serine/threonine-specific holoenzyme protein kinases that drive cell-cycle progression. The activity of these kinases is inhibited by small molecular weight proteins of the p21<sup>WAF1</sup> and p16<sup>INK4</sup> family (1-3). Human cancers have abnormalities in one or more cell cycle component either hyperactivation of Cdk and/or a decrease in the inhibitors Cdk function. HDAC inhibitors induce CKI expression, which may contribute to their cell-cycle inhibitory effects. With the advent of newer biologic probes and techniques, rationally designed molecules that selectively inhibit Cdk function have entered the clinic. This review focuses on

the interface between the cell cycle and histone acetylation. The results of Cdk and HDAC inhibitors as anticancer agents in the laboratory and the clinic are described.

## 3. CHROMATIN MODIFYING ENZYMES AND MITOGENIC SIGNALING

Chromatin structure is known to have profound effects on gene expression in eukaryotic cells. DNA in eukaryotes is typically packaged as repeating arrays of nucleosomes, in which 146 bp of DNA are wrapped around a histone octamer. Each histone octamer includes four histone proteins (H2A, H2B, H3 and H4). Nucleosomes are in turn organized into higher order complexes the structure of which is dynamically regulated during the cell cycle exemplified by the condensed chromatin structure found during metaphase. Chromatin structure is regulated by proteins, including the Swi-Snf and NURF complexes (4, 5) that remodel chromatin in an ATP-dependent manner. Other classes of histone modifying proteins alter chromatin proteins through phosphorylation, ADP-ribosylation, methylation (6), ubiquitination and acetylation. Histone acetylation involves the transfer of an acetyl group from acetyl Co-A to the  $\epsilon$ -amino group of lysine side chains within the substrate. Phosphorylation of histones occurs coincident with mitogenic stimulation through the ERK and the p38 MAPK pathway (7). Histone H3 phosphorylation is increased in mitogen or oncogene transformed cells (8) and requires Rsk-2 kinase (9). Epidermal growth factor synergistically enhances acetylation and phosphorylation of histone H3. The modification of the lysine groups of core histones by multiple post-translational events including phosphorylation and acetylation coincident with activation of mitogenic signaling (10, 11), has led to a model in which

## Histone acetylation and the cell cycle

the N-terminal tail of the core histone is considered to function as a signaling platform (Figure 1) (12). We had proposed that non-histone substrates of histone acetyl transferase FAT (factor acetyl transferases) might also function as signaling platforms in acetylation-phosphorylation cascades (13).

Specific lysine is acetylated by specific HATs suggesting both a complexity and specificity (Figure 1). How the post-translational modification of histones activates gene expression remains unclear. The modification of lysine groups or the addition of negatively charged phosphate groups to the N-terminal H3 tails may disrupt electrostatic interactions between histones and DNA and increase accessibility of nuclear factors to the DNA. Alternatively, these modifications may create a recognition site for recruiting other transcription factors or coactivator complexes. Precedents for histone modifying proteins in recruiting protein-protein interaction domains include the finding that the histone acetyl transferase P/CAF binds to acetyl lysines (14). Furthermore, TAF<sub>II</sub> 250, which conveys intrinsic HAT activity (15), binds acetylated lysines through its double bromodomain (16).

### 4. HISTONE ACETYL TRANSFERASES AND CANCER

HATs were historically classified as type A, located in the nucleus and known to acetylate nucleosomal histones within chromatin, and type B HATs, located in the cytoplasm with a housekeeping role consisting of acetylation free histones in the cytoplasm. A summary of known HATs and their substrates, divided into six groups, are shown in Table 1 (17). The GNAT (Gcn5-related N-acetyltransferase) super family includes the best-characterized member yeast Gcn5. Importantly; detailed mutagenesis of yeast Gcn5 demonstrated an important correlation between HAT activity and *in vivo* transcription and cellular growth (18). In mammals P/CAF was identified on the basis of homology to Gcn5 and was found to associate with the p300 coactivator protein (19). Intriguingly P/CAF inhibited cell-cycle progression and acetylated several substrates in addition to histones including HMG17, p53 and the androgen receptor (20). The first member of the human MYST protein family (MOZ, Ybf2/Sas3, Sas2 and Tip60) (21) to be discovered, Tip60, was identified as a Tat-interactive protein and was subsequently shown to confer intrinsic HAT activity. MOZ (monocytic leukemia zinc finger protein) was found as part of a chromosomal translocation in acute myeloid leukemia (MOZ-CBP) in which the N-terminus of MOZ was fused to the C-terminus of CBP, including its HAT domain. It was hypothesized that MOZ-CBP may contribute to oncogenesis through aberrant chromatin acetylation (21). Subsequent findings of MOZ-TIF2 translocations in certain leukemias (22) led credence to this model. Nuclear receptor coactivators of the p160 family acetylate either free histones or histones in mononucleosomes. AIB1/ACTR is amplified in human breast cancers and induced by mitogenic signaling (23, 24).

The HATs form multiprotein complexes, which contribute to their temporal, spatial, and substrate specificity (17). In yeast four complexes known as SAGA, ADA, NuA4, and NuA3 were identified. In humans the P/CAF containing complex resembles the yeast SAGA complex and contains hADA2, five TAF<sub>II</sub> or TAF<sub>II</sub>-related proteins and TRRAP (25, 26). The TRRAP protein is a member of the ATM family and regulates activity of E2F and c-Myc (27). In recent studies the G<sub>1</sub> regulatory cyclin, cyclin D1, was shown to associate with P/CAF suggesting components of the cell cycle may regulate function of the HAT complexes (28, 29). Dissection by site-directed mutagenesis indicated that cyclin D1 bound to the P/CAF HAT domain providing support for a model in which the abundance of a labile mitogen-inducible cell-cycle regulatory protein may interface with activity of histone modifying complexes (29). Whether cell-cycle components regulate other human HAT complexes (TIP60, TFIIC, HBO1 complexes and others), remains to be determined.

It was previously known that histone acetylation and deacetylation regulated chromatin structure and thereby indirectly regulated gene expression and that HATs regulated the activity of several components of the transcriptional regulatory apparatus. The coactivator proteins CREB-binding protein (CBP) and the related functional homologue p300 (30), regulate a broad array of genes and this activity has been linked to their histone acetylase activity (31). Acetylation facilitates binding of transcription factors to specific target DNA sequences by destabilizing nucleosomes bound to the promoter region of a target gene (32-35). Transcription factors also serve as direct targets of acetylation (36). p300/CBP regulated acetylation of the tumor suppressor p53 (37), the Kruppel-like factor (EKLf) (38), the erythroid cell differentiation factor GATA-1 (39), the androgen receptor (20) and histone acetylases themselves (40) (reviewed in: (41)). The identification of non-histone substrates of FATs (Factor acetyl transferase substrates) (Table 2) provided important insights into the mechanism by which acetylation may directly regulates gene expression.

The growing list of FATs including transcription factors, coactivators, general transcription factors, cytoplasmic microtubules ( $\alpha$ -tubulin), nuclear import proteins (Rch1, which regulates importin- $\beta$  nuclear translocation), suggests acetylation may function as a signaling mechanism which itself must be tightly regulated. The binding of HATs to target substrates *in vivo*, assessed by chromatin immunoprecipitation assays is quite transient (42). Coactivator-substrate interactions can lead to transcriptional attenuation (40). The attenuation of coactivator signaling by acetylation illustrates the importance of feedback loops in controlling acetylation-signaling pathways. These findings raise the possibility that cancers may evade these normal mechanisms of attenuation leading to sustained proliferative signaling. Although this remains to be formally established, recent studies have identified a mutant estrogen receptor in breast cancer (43), which escapes normal acetylation and functions in a constitutively active manner (44).

## Histone acetylation and the cell cycle

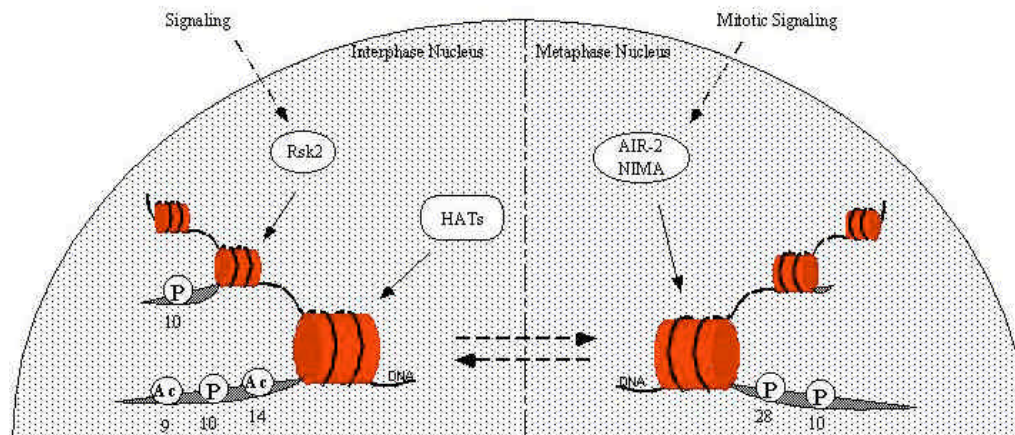
**Table 1.** Acetyltransferases and their histone substrates

HAT	Free Histone Substrate	Nucleosomal Histone Substrate
<b><u>Global coactivators</u></b>		
• p300/CBP	H2A, H2B, H3, H4	H2A, H2B, H3, H4
<b><u>Nuclear receptor coactivators</u></b>		
• SRC-1	H3, H4	H3, H4, H2A, H2B
• TIF2		
<b><u>Nucleosome complexes</u></b>		
• SAGA	H3	H3, H2B
• ADA	H3	H3, H2B
• NuA3		
• NuA4		H4, H2A
<b><u>NAT family</u></b>		
• Gcn5	H2A, H3, H4	H3
• P/CAF	H3, H4	
• TAF <sub>II</sub> 250	H3, H4	
• Tip60	H4, H3, H2A	

**Table 2.** FAT substrates

Substrates for FAT	FAT	Possible effects on transcription
<b><u>General transcriptional factors</u></b>		
• TFIIF	p300/CBP, P/CAF	Unknown
• TFIIE $\alpha$	p300/CBP, P/CAF, TAF <sub>II</sub> 250	Unknown
<b><u>Transcriptional effectors</u></b>		
• P53	p300/CBP, P/CAF	Up
• GATA-1, -3	p300/CBP, P/CAF	Up
• EKLF	p300/CBP	Up
• TCF	p300/CBP	Down
• c-Myb	p300/CBP, GCN5	Up
• HIV-1 tat	p300/CBP, P/CAF	Up
• E2F-1, -2	p300/CBP, P/CAF	Up
• TR-RXR	p300/CBP	Unknown
• MyoD	p300/CBP, P/CAF	Up
• TAL1/SCL	p300/CBP, P/CAF	Up
• AR	p300/CBP, P/CAF	Down
• ER $\alpha$	p300/CBP	Down
• Sp1	p300/CBP	N/A
• E1A	p300/CBP	Up
<b><u>Nuclear receptor coactivators</u></b>		
• p300/CBP	p300/CBP	Unknown
• P/CAF	P/CAF	Unknown
• ACTR	p300/CBP	Down
• SRC-1	p300/CBP	Unknown
• TIF2	p300/CBP	Unknown
<b><u>Nonhistone Chromatin proteins</u></b>		
• HMG1	p300/CBP	Unknown
• HMG2	-----	Unknown
• HMG14	p300/CBP	Unknown
• HMG17	P/CAF	Unknown
• HMG I(Y)	p300/CBP, P/CAF	Up(P/CAF), Down(p300/CBP)
• Sin1	Gcn5	
<b><u>Others</u></b>		
• $\alpha$ -Tubulin	-----	Unknown
• Importin- $\alpha$ 7	-----	Unknown

## Histone acetylation and the cell cycle



**Figure 2.** Cell-cycle regulation of histone phosphorylation. Cell-cycle signals activate mitotic H3 kinases, phosphorylating the N-terminus of H3 at Ser10 and Ser28. Apoptosis induced signals are associated with phosphorylation of histone H2B (50) and H2A.X (51).

### 5. HISTONE MODIFICATION DURING THE CELL CYCLE, DNA DAMAGE AND APOPTOSIS

During mitosis cell cycle signals activate mitotic H3 kinases, which phosphorylate the N-terminal tails of H3 at Ser10 (45) and Ser28 (46), together likely contributing to chromosome condensation. Mutation of H3 Ser10 in *Tetrahymena* induces abnormal chromosome segregation supporting the importance of histone H3 phosphorylation in mitosis and meiosis (47) (Figure 2). Candidate kinases responsible for H3 phosphorylation during mitosis include Ipl1/AIR-2 kinase in yeast (48) and the NIMA kinase in *Aspergillus nidulans* (49). Apoptosis induced signals through caspases are associated with phosphorylation of histone H2B (50) and H2A.X (51). The Survivin/BIR family was identified as anti-apoptotic proteins (52). Reduction in BIR-1 in *C. elegans* by RNA-mediated interference resulted in embryos lacking H3 phosphorylation, with abnormally segregated chromosomes implicating BIR proteins in H3 function (53).

### 6. HISTONE DEACETYLASES AS CANCER THERAPEUTIC TARGETS

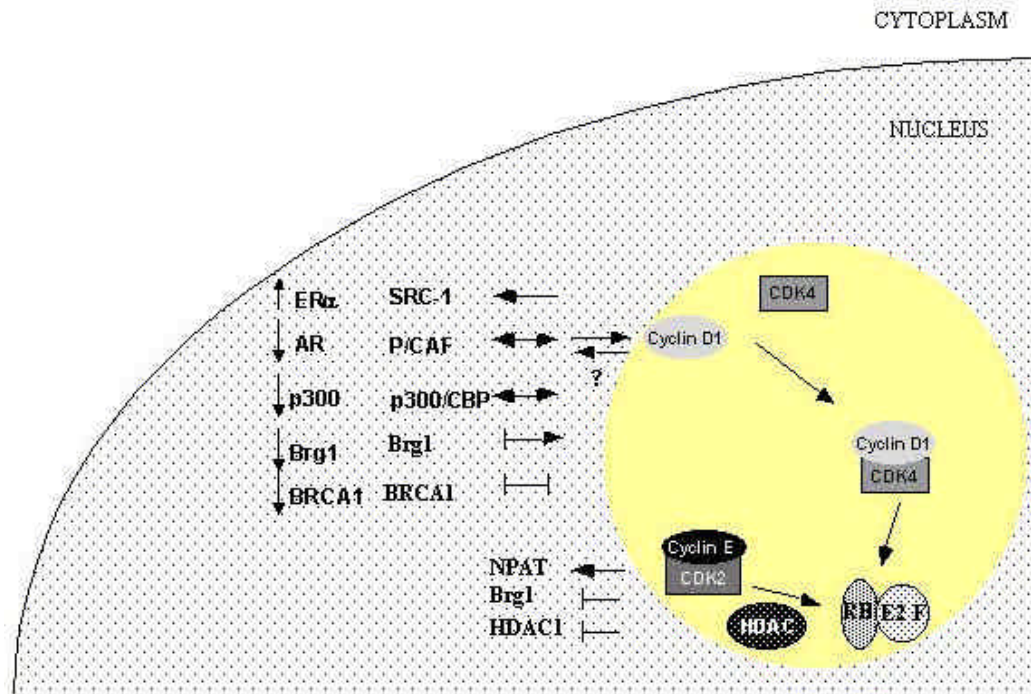
Histone acetylation is associated with transcriptional induction and the activity of HDACs has been frequently linked to transcriptional repressor activity. Histone acetylation is a dynamic process induced by histone acetyl transferases and reversed by histone deacetylases. HDACs were found as transcriptional repressors related to Rpd3 (54), Hda1 (55) and Sir2 (56). Transcriptional corepressors, including Sin3, N-CoR and SMRT were shown to recruit HDAC complexes to the promoter of target genes (57-61). HDAC1 also bound to the retinoblastoma protein, pRB and the related p130 and p107 proteins *in vitro* (62, 63) and *in vivo* (64) likely contributing to the transcriptional repression function of pRB. To date at least nine different mammalian HDACs have been described, and are divided into three classes; class I is related to Rpd3 (54, 57, 65, 66); class II which is related to Hda1 (67-70) and the Sir2 family in which the

activity depends upon nicotinamide-adenine dinucleotide (56). Inhibitors of HDACs function as potent anti-cancer therapeutics and are discussed below.

Several recent findings have shown important relationships between disruption of HAT or HDAC activity and cancer. Translocation of the coactivator p300/CBP is associated with acute myeloid leukemia, and CBP/p300 is associated with a subset of colorectal tumors (71). Patients with Rubinstein-Taybi syndrome are genetically predisposed to cancer and have a point mutation in the CBP gene (72). HDACs bind to pRB and various DNA tumor viruses (HPV16, SV40) thereby disrupting interactions between HDACs and the pRB tumor suppressor protein (19, 62). There is a correlation between the genesis of acute promyelocytic leukemia (PML) and the presence of chimeric proteins of RAR $\alpha$  and PML or PLZF. An RA-insensitive form of PML was characterized by the association of HDAC with both the RAR $\alpha$  and the PLZF component of the chimeric factors. The addition of retinoic acid dissociates HDAC from the RAR $\alpha$  but not from the PLZF component of the chimera, contributing to RA-resistance (73). Together these studies suggest mislocalization leading to enhanced HDAC activity or mutation of histone acetylases may contribute to the tumorigenic phenotype.

### 7. CYCLIN-DEPENDENT KINASES AND THE REGULATION OF HISTONE ACETYLASE ACTIVITY

Orderly cell cycle progression is required for the cell to replicate its DNA with high fidelity and separate into daughter cells. In eukaryotes the cell cycle is divided into four distinct phases: S-phase (synthetic phase), M (mitotic) phase, G<sub>1</sub> (Gap 1 phase) and G<sub>2</sub> (Gap 2 phase) (1, 74, 75). Transition through the cell cycle is orchestrated by an orderly cascade of holoenzymes, which are quite specific for each phase of the cell cycle. Passage through the restriction point in late G<sub>1</sub> (or START in yeast), is regulated by G<sub>1</sub> cyclins; in mid-G<sub>1</sub>, the D-type cyclins, and



**Figure 3.** Functional interactions between histone acetylation and the cell cycle. The cyclin D-Cdk4 complex is shown in relation to its substrate pRB. In addition cyclin D1 expression is regulated by the p300 HAT (78) Cyclin D1 binds to the HAT domain of P/CAF (29), to TAF<sub>II</sub> 250 (79) and to the p160 HAT SRC-1 (81) Cyclin D1 and cyclin E overcome the cell-cycle arrest function of Brg-1 (80) and the BRCA-1 mediated inhibition of liganded ER $\alpha$  (214) Cyclin E/Cdk2 phosphorylates NPAT contributing to the induction of histone gene expression, inhibits Brg-1 cell-cycle arrest (97).

in late G<sub>1</sub>, cyclin E. Phosphorylation of pRB is induced sequentially by first cyclin D-dependent kinases and then cyclin E-dependent kinases. Cyclin D1-induction of S-phase entry is thought to be pRB-dependent, whereas cyclin E is capable of inducing S-phase independently of pRB, suggesting important alternate substrates (reviewed in (1, 76, 77).

A series of recent observations has provided important evidence for an interface between the machinery regulating orderly cell-cycle progression and the processes regulating histone acetylation (Figure 3). Expression of the *cyclin D1* gene is induced by p300, and induction required the p300 HAT domain (78). As cyclin D1 abundance is rate-limiting in G<sub>1</sub> phase progression these studies suggest an important role for p300 HAT function in regulating S-phase progression. Cyclin D1 directly binds to P/CAF to regulate both ER $\alpha$  and AR activity in breast and prostate epithelial cells (29). Cyclin D1 binds to TAF<sub>II</sub> 250 to regulate Sp1 signaling (79). Cyclin D1 overcomes growth arrest induced by the chromatin remodeling protein Brg-1, although the role of Cdk-independent domains of cyclin D1 in this process remains to be determined (80). Intriguingly cyclin D1 conveys important Cdk-independent functions, which may contribute to aberrant proliferative signaling in hormonally responsive tissues, and may involve histone acetylases. In this context cyclin D1 binds to the estrogen receptor (ER $\alpha$ ) and the p160 HAT, SRC-1 (81-83). The abundance of cyclin D1 is

rate-limiting in serum- or growth factor-induced G<sub>1</sub> progression in a variety of cell types including MCF7 human breast cancer cells (84-89) and the cyclin D1 gene is induced by a broad array of oncogenic signals including activating mutation of Ras, Src, ErbB2, b-catenin, Rac and mitogen activated protein kinase kinases (90-95) demonstrating that cyclin D1 is a common target of oncogenic signals. The role of cyclin D1 binding of proteins with HAT regulatory function in the induction and progression of tumorigenesis remains to be determined.

pRB/HDAC complexes regulate the expression of the cyclin E gene and cyclin E/Cdk2 in turn plays a role in regulation of histone gene expression (96, 97). Histone biosynthesis is induced during G<sub>1</sub>/S phase transition, contributing to the increase in mRNA synthesis that occurs as cells progress into S-phase. The coordinate induction of histone gene expression is important in maintaining the integrity of genomic replication. In yeast, overexpression of histone H2A and H2B or H3 and H4 increase chromosome loss (98). One substrate for cyclin E/Cdk2, NPAT (99), was found within nuclear Cajal bodies. These structures are thought to contribute to assembly of transcriptional complexes (100). NPAT overexpression can induce both histone gene expression and G<sub>1</sub>/S phase transition (96, 97), suggesting these two properties may be functionally linked. Additional levels of interaction between cyclin E/Cdk2 and chromatin include findings that cyclin E can directly associate with the HAT p300 (101) and phosphorylation of

## Histone acetylation and the cell cycle

CBP at the G<sub>1</sub>/S boundary by cyclin E/cdk2 correlated with an increase in CBP HAT activity (102). Cyclin E can also bind to the chromatin remodeling protein Brg1 and inhibit Brg1-mediated growth arrest (80).

### 8. ENDOGENOUS CYCLIN-DEPENDENT KINASE INHIBITORS AND THE REGULATION OF HISTONE ACETYLASE ACTIVITY.

Activity of the Cdks is regulated at several levels. Activation of the cyclin/Cdk complex requires CAK. Full induction of Cdk2 requires dephosphorylation by the cdc25A phosphatase (103). Two broad categories of endogenous Cdk inhibitors (CKIs) are found in most cells and are frequently inactivated during tumorigenesis. The Ink4s (p16<sup>INK4a</sup>, p15<sup>INK4b</sup>, p18<sup>INK4c</sup>, p19<sup>INK4d</sup>), which inhibit Cdk4 or 6, contain conserved ankyrin motifs, and the Cip/KIP family (p21<sup>Cip1</sup>, p27<sup>Kip1</sup>, and p57<sup>Kip2</sup>). The Cip/KIP family share partial structural homology, inhibit cyclin Cdks in a concentration-dependent manner, and play a dual function as they are also required for assembly of the cyclin/Cdk complexes (2). CKIs typically cause G<sub>1</sub> growth arrest when overexpressed in transfected cells (1). p21<sup>Cip1</sup> inhibits Cdk4 and 6 with a Ki of 0.5-0.15 nM, while p21<sup>Cip1</sup> weakly inhibits Cdc2/cyclin B (Ki = 400 nM) (104). p21<sup>-/-</sup> embryonic fibroblasts are deficient in G<sub>1</sub> arrest after DNA damage and exhibit significant growth alteration with saturation densities as high as that seen with p53<sup>-/-</sup> cells (105). Evidence is mounting to suggest the CKIs regulate function of HATs (101). Furthermore the frequent induction of p21<sup>Cip1</sup> by HDAC inhibitors implicates p21<sup>Cip1</sup> in mediating the cell-cycle arrest (106, 107).

The role of p27<sup>Kip1</sup> as a target of HAT function is less well established. The abundance of p27<sup>Kip1</sup> remains low during cell cycle transition; however, mitogens reduce p27<sup>Kip1</sup> protein levels via ubiquitin-mediated proteolysis (108-110). During quiescence, cyclin E/Cdk2 associates with p27<sup>Kip1</sup> and the latter inhibits cyclin kinase activity; however, when p27<sup>Kip1</sup> is bound to cyclin D1/Cdk4 the kinase activity may not be inhibited. It has been proposed that the removal of p27<sup>Kip1</sup> from the cyclin E/Cdk2 complex is essential for S-phase entry and that this in part is achieved through binding of cyclin D1/Cdk4, thus titrating p27<sup>Kip1</sup> from binding and inhibiting cyclin E/Cdk2 (1, 111-113). p27<sup>Kip1</sup> interacts with cyclin E/Cdk2 in two very different ways. p27<sup>Kip1</sup> binds directly with cyclin E/Cdk2 holoenzyme and inhibits cyclin-dependent kinase activity resulting in reduced phosphorylation of target substrates. Secondly, p27<sup>Kip1</sup> is phosphorylated by cyclin E/Cdk2, releasing p27<sup>Kip1</sup> leading to ubiquitin-mediated proteolysis (1). Recent studies show reduced p57<sup>Kip2</sup> protein levels in cancer (114). p57<sup>Kip2</sup> causes growth arrest in G<sub>1</sub> when overexpressed in cells. Like p21<sup>WAF1/CIP1</sup>, both the amino terminal cyclin/Cdk binding domain and carboxy terminus PCNA binding domains of p57<sup>Kip2</sup> are required for anti-mitogenic activity. However, unlike p21<sup>Cip1</sup>, p57<sup>Kip2</sup> is not regulated by p53.

## 9. THERAPEUTICS TARGETING THE CELL CYCLE AND HISTONE DEACETYLATION

The vast majority of human cancer has abnormalities in a component of the pRB-p16 or ARF-p53 pathway, making components of these pathway logical targets for chemotherapeutics. Empirical observations in which inhibitors of HDAC activity proved cytostatic in tumor therapy, implicated histone acetylation in the mechanisms maintaining the tumor phenotype. Only relatively recently have important molecular interactions been identified that provide the basis of cross talk between the cell cycle and control of histone acetylation. Deletions of the CKI p16 have been reported in approximately 50% of gliomas and mesotheliomas, 40% of pancreatic tumors, and 20-30% of acute lymphoblastic leukemia and bladder tumors. Nearly 40-60% of pancreatic and biliary tract neoplasms have p16 mutations (115-121). There seems to be a reciprocal relationship between cyclin D1 or p16 expression and pRB such that it is rare to find alterations in both families of proteins in the same tumor cell, suggesting Cdk hyperactivation is important in maintaining neoplastic transformation. Designing drugs to either modulate aberrant Cdk activity or inhibit histone deacetylases, may prove useful as cancer therapy (122, 123).

### 9.1. Histone deacetylase (HDAC) inhibitors

Inhibition of Histone Acetyl Transferase (HAT) activity or disruption of HDAC function by oncogenes and tumorigenic viruses is associated with the development of cancer. Sodium n-butyrate (NaBu), an inhibitor of HDAC, is known to induce cell cycle arrest or differentiation in a large number of tumor cell types including leukemias (124, 125), colorectal cancers (126, 127), hepatic cancer (128), breast cancer (129, 130) and fibroblasts transformed by oncogenes (131). The low antiproliferative activity and short half-life of NaBu in the blood resulted in poor efficacy in clinical trials. Correlative observations that inhibitors of HDAC activity were cytostatic for tumorigenic cell growth lead to the identification of a number of HDAC inhibitors now in clinical trials. These agents include 1) hybrid polar compounds (suberoylanilide hydroxamic acid) (132), 2) synthetic benzamide derivatives MS-27-275 (107), 3) butyrate and its analogues (e.g. phenylbutyrate), 4) trichostatins (isolated from *Streptomyces hygroscopicus*) (133), and cyclic tetrapeptide antibiotics including trapoxins (133), 5) apicidin (134) and FR901228 (135).

#### 9.1.1 Hybrid polar compounds

Hybrid polar compounds (HPCs) were developed as HDAC inhibitors. The prototype HPC hexamethylene bisacetamide (HMBA) induced remissions in patients with myelodysplastic syndrome and acute myelogenous leukemia. However, it is not currently used as a clinical agent because of the high dosage required (millimolar blood levels) and toxic side effects (thrombocytopenia) (136). Second-generation HPCs, suberoylanilide hydroxamic acid (SAHA) and m-carboxycin-namic acid bishydroxamide (CBHA) are 2,000-fold more potent. CBHA and SAHA inhibit histone deacetylase 1 (HDAC1) and histone deacetylase 3 (HDAC3) activity *in vitro* (132).

## Histone acetylation and the cell cycle

**Table 3.** Cdk Modulators

### **Direct Cdk Inhibitors (designer small molecules)**

1. Natural Products or Synthetics
  - Flavopiridol (and deschloroflavopiridol)
  - Staurosporine and UCN-01
  - 9-Hydroxyellipticine
  - Toyocamycin
  - Suramin
  - Butyrolactone I
  - Paullones
  - 9-hydroxyellipticine
2. Purines and Purine-like Analogs
  - 6-dimethylamino purine
  - Isopentenyladenine
  - Olomucine
  - Roscovitine
  - Purvalanol
3. Peptides
  - p16<sup>INK4a</sup> and p21<sup>Cip1</sup> mimics

### **Indirect Cdk Inhibitors**

1. Decreasing Cyclin D Levels (?)
  - Rapamycin
  - Tyrphostins
  - Benzoquinoid ansamycins
  - Cyclin D1 antisense approaches
  - Flavopiridol
2. Increase of endogenous Cdk inhibitors
  - Butyrates
  - Retinoids
  - Differentiation agents
  - Inhibition of proteasomal degradation (e.g., 20S proteasome inhibition PS-341, increases (p27<sup>Kip1</sup>) p16<sup>INK4a</sup>, p21<sup>CIP1/waf1</sup> and p27<sup>Kip1</sup> overexpression)
3. Inhibitors of Histone deacetylase (142)
  - Butyrate
  - trichostatin A,
  - hybrid polar compounds
  - FR901228
  - phenyl butyrate
  - Oxamflatin
  - MS-27-275
  - apicidin A
4. Altering Checkpoint Control
  - Phosphatase inhibitors (e.g. okadaic acid)
  - UCN-01
  - Caffeine
  - Pentoxifylline

From: (159)

### **9.1.2. Benzamide derivatives**

Benzamide derivatives with HDAC-inhibitory activity were identified during a search for compounds to treat refractory malignancies including multidrug resistance (137-139). The most active derivative, MS-27-275, inhibited S-phase entry in K562 tumor cell lines associated with increased histone acetylation and the induction of p21<sup>Cip1</sup> abundance (107). The induction of p21<sup>Cip1</sup> occurred independently of the cells p53 status (107).

### **9.1.3. Trichostatins and cyclic tetrapeptide antibiotics**

Trichostatins (isolated from *Streptomyces hygroscopicus*) (133), and cyclic tetrapeptide antibiotics,

including trapoxins (133), are potent HDAC inhibitors. The recent crystallographic analysis of a bacterial enzyme related to HDAC (histone deacetylase like protein) (140) and subsequent co-crystallization with TSA showed that the inhibitors mimic the substrate. The chelation of zinc in the catalytic pocket by the hydroxaminic acid group is the main mechanism of inhibition (132). The active site pocket is conserved between the bacterial enzyme and class I (HDAC1,2,3,8) and class II HDACs (HDAC 4,5,6,7). Derivatives of trapoxin (CHAP1) strongly and reversibly inhibited HDAC1, however HDAC6 was relatively resistant (106). As specific HDACs regulate subsets of target genes,



## Histone acetylation and the cell cycle

these finding suggests further development of HDAC inhibitors may result in more selective therapeutics.

Several HDAC inhibitors can cause a G<sub>1</sub> arrest and G<sub>2</sub>/M block perhaps in part through the induction of p21<sup>Cip/WAF1</sup> and the transcription factors Sp1 which binds HDAC, and the inhibition of cyclin D1 which is induced by oncogenic signals through Sp1 (90). In clinical trials, the short half-life of butyrate (~6 mins) limited its applicability; however, the hybrid polar compounds are effective in mice (141) as are MS-27-275 and oxamflatin (for review see: (142)). There is observed synergy between DNA methyltransferase inhibitors (e.g., 5-Aza-2deoxycytidine) and HDAC inhibitors (e.g., Trichostatin A) in terms of re-expression of genes silenced in cancer (e.g., p16) (143, 144). The clinical implications of this strategy are currently under investigation.

### 9.2. Cdk modulators as Novel Cancer Therapy

Cdks are small 30-40 kDa Ser/Thr kinases that display common subdomains shared by all protein kinases. The ATP-binding site located in a deep cleft between the two lobes of Cdk2, for example, contains the catalytic residues, which are conserved across eukaryotic protein kinases. Many novel Cdk inhibitors target the ATP-binding site thereby preventing cyclin-Cdk interactions necessary for phosphorylation events. The therapeutic strategies aimed at modulating Cdks may be subdivided into several categories (122, 123) (Table 2). These compounds have diverse specificities for CDK inhibition. The staurosporines, suramin and 6-dimethylaminopurine are relatively nonspecific protein kinase inhibitors; however, olomucine, butyrolactone 1 and the paullones are much more selective for Cdk inhibition (145-147). Flavopiridol inhibits all Cdks tested; however, butyrolactone 1, olomucine, purvalanol and paullone derivatives are relatively selective for Cdk 1 and Cdk 2 but spare Cdk4 and Cdk6 (145-149). The direct Cdk inhibitors have been piloted in the clinic. The first two direct Cdk modulators tested in human clinical trials were flavopiridol (Table 3) and UCN-01. Flavopiridol is a synthetic flavone, developed by Aventis and the National Cancer Institute (NCI) as a novel antineoplastic agent.

#### 9.2.1 Flavopiridol

Flavopiridol is derived from the flavanoid rohitukine, Flavopiridol has antitumor effects that are associated with its 1) direct competitive inhibition of Cdks for ATP; 2) inhibition of Cdk7/cyclin H preventing threonine 160 phosphorylation on Cdk; 3) repression of cyclin D1 transcriptional activity; 4) inhibition of angiogenesis through decreased VEGF mRNA stability; 5) DNA binding activity; 6) decreased intracellular drug accumulation; 7) interaction with MRP1. Other mechanisms of cellular enzyme inhibition involve binding to the inhibitor site of glycogen phosphorylase resulting in the inhibition of glycogenolysis (150). Flavopiridol inhibits epidermal growth factor receptor tyrosine kinase (IC<sub>50</sub>=25 mM), serine/threonine kinases, protein kinase C (IC<sub>50</sub>=65 mM), and protein kinase A (IC<sub>50</sub>=145 mM) and inhibited CDKs at very low concentrations (IC<sub>50</sub>'s ~ 50 to 100 nM) (151-153). Flavopiridol arrests cells *in vitro* at the G<sub>1</sub>-S phase transition as well as the G<sub>2</sub>-M phase transition, suggesting that it may effect distinct populations of cell cycle proteins (154). Co-

crystallization studies with Cdk2 and des-chloroflavopiridol confirmed tight binding to the ATP site. In contrast, like the benzylamino and anilino substituents of roscovitine and purvalanol B, flavopiridol has a phenyl ring at the C2 position, which may confer specificity (155, 156). *In vitro* studies revealed that unlike other direct CDK inhibitors, flavopiridol inhibited Cdk1, 2 and 4 with similar potency associated with functional loss of regulatory tyrosine and threonine phosphorylations of these Cdks (151, 152). Several more potent analogues of flavopiridol have been synthesized and include the thio (IC<sub>50</sub> 110 nM) - and oxoflavopiridols (IC<sub>50</sub> 130 nM), which contain a sulfur (16) or oxygen (18) atom linker between a chromone ring and the hydrophobic side chain. These are selective cyclin-dependent kinase 1 (Cdk1) inhibitors (157). Flavopiridol inhibited the growth of several human tumor cell lines *in vitro* (123, 158-160). In most cell types the antiproliferative effect of flavopiridol was accompanied by apoptosis (161, 162) with the exception that post mitotic PC12 cells are protected from apoptosis (163).

Flavopiridol induces apoptosis of SUDHL-4 (B cell lymphoma) and Jurkat (T-cell leukemia), which is p53-independent and is associated with a reduction of cyclin D1 (164). p53-independent apoptosis has also been observed in squamous head and neck cell lines that are insensitive to irradiation. Neither Bcl-2/BAX nor p53 appears to be involved; however, in other hematopoietic cells Bcl-2 may be downregulated. Because cyclin D1 conveys an anti-apoptotic function in several cell types (78, 165), the reduction in cyclin D1 may play an important role in the apoptotic properties of flavopiridol function. There is selective early depletion of cyclin D1 in MCF7 cells exposed to flavopiridol within 3 hours. Subsequently, cyclin D3 levels decrease without alteration in cyclin D2, G or cyclin E (166). Flavopiridol binds directly to DNA, however the role of DNA-binding in flavopiridol cellular cytotoxicity is unknown (167). In addition to cytostatic and apoptotic activities, flavopiridol is antiangiogenic decreasing blood vessel formation in a mouse Matrigel model of angiogenesis (168) and inhibiting hypoxia-induced VEGF from human monocytes (169).

In animal models flavopiridol administration demonstrated cytostatic effects in Colo205 (colorectal) and LNCap/DU-145 (prostate) xenografts (158). The National Cancer Institute has sponsored two phase I studies (170) and a second phase I trial was conducted at the University of Wisconsin, Madison. The dose-limiting toxicity was diarrhea. Phase II efficacy studies have been completed in colon and kidney cancer where the activity is limited (171). In one published study, there has been a higher than previously reported incidence of severe asthenia (9%) and serious vascular thrombotic events (29%)(171). Combination therapies using flavopiridol are based on *in vitro* observations. *In vitro* studies with flavopiridol and cytotoxic chemotherapy show synergy in A549 lung carcinoma cells with several agents including paclitaxel, topotecan, doxorubicin and etoposide (172, 173).

#### 9.2.2. Staurosporine analogues and UCN-01

Activation of the PKC signaling pathway can either stimulate (174-176) and transform cells (177, 178) or inhibit cellular proliferation (176, 179-181). Specific cell cycle

**Table 4.** IC<sub>50</sub>'s towards Cdk and selectivity of Cdk Inhibitors

Inhibitor	IC <sub>50</sub> (mM)	Selectivity	Cdk2-Inhibitor	Crystal Structure (ref)
6-Dimethylaminopurine	120.000	Poor	-	
Isopentyladenine	56.000	Poor	Yes	(202)
Staurosporine	0.004	Poor	Yes	(203)
Olomucine	7.000	Good	Yes	(202)
Flavopiridol	0.400	Good	-	
9-hydroxyellipticine	1.000	Poor	-	
Roscovitrine	0.450	Good	Yes	(148)
CVT-313	4.200	?	-	
Purvalanol A	0.004	Good	Yes	(205)
CGP60474	0.020	?	-	
Kenpaullone	0.400	Poor	-	
Alsterpaullone	0.035	?	-	
Indirubin-3f-monoxime	0.180	Good	Yes	

transitions are governed in specific cell types by specific isozymes. The G<sub>1</sub>-S transition is regulated by the PKC $\eta$  isozyme in NIH3T3 cells (182) while in vascular smooth muscle cells G<sub>1</sub>-S is regulated by PKC $\alpha/\epsilon$  (183). Overexpression of PKC $\alpha$  promotes cellular proliferation in several cell types including human breast cancer cells (184, 185) whereas PKC $\delta$  isoform in the presence of phorbol ester, induces G<sub>2</sub>/M arrest in Chinese hamster ovary fibroblasts (186). Staurosporine, a potent PKC inhibitor, was isolated from a culture broth of streptomycetes (187). Staurosporine arrested cell cycle progression of normal and transformed cells in either G<sub>1</sub> or G<sub>2</sub> phase in a concentration-dependent manner induced a G<sub>1</sub> phase arrest at low concentrations (low nM range), staurosporine typically and a G<sub>2</sub> arrest at high concentrations. UCN-01 was developed as the 7-hydroxy analog of staurosporine and was found to be a more potent and selective inhibitor of PKC (188, 189). Recent studies have found it to be a potent and selective antagonist of PKC isozymes without inhibition of the atypical PKCs (190). UCN-01 irreversibly inhibited cell growth in G<sub>1</sub> after 24 hr in several cell types (191-194) associated with apoptosis. GF10923X inhibited PKC activity but did not induce apoptosis (195). Human epidermoid carcinoma cells exposed to UCN-01 displayed clear evidence of G<sub>1</sub> arrest accompanied by pRB hypophosphorylation and accumulation of p21<sup>Cip1</sup> and p27<sup>Kip1</sup> independent of the p53 and pRB status (191). UCN-01 inhibited Cdc2 and Cdk2 *in vitro* (IC<sub>50</sub> 300-600 nM), however, there is a paradoxical activation of the same kinases in intact cells exposed to UCN-01.

UCN-01 inhibits breast carcinoma cells lines (MCF7) (194), human epidermoid carcinoma A431 cells, human lung cancer A549 and Ras-transformed rat fibroblast cells (192). In human leukemia cells (Jurkat, Molt-3, Molt-4, and Huy-78), UCN-01 induced loss of G<sub>2</sub>-M DNA content and apoptosis at concentrations above 300 nM coincident with the activation of Cdk1 and 2 (195). UCN-01 has been shown to inhibit hchk1 kinase activity (196). The disruption of DNA-damage G<sub>2</sub> checkpoint arrest by UCN-01 was shown to be mediated through inhibition of the cdc25C kinases, hchk1 and cTAK1. Notably, hchk2 is not able to override the pharmacological inhibition of hChk1 kinase activity by UCN-01 (196). Intravenous UCN-01 inhibited growth of the A498 renal tumor cell line and MCF7 cells when implanted in animals (123). Results from early clinical trials in humans have

shown unique pharmacologic features that were not predicted by the preclinical animal toxicology studies. There was an unexpected very long half-life (~ 600 hours), over 100 times the predicted value from preclinical studies and this attributed to specifically high binding to  $\alpha$ 1-acid glycoprotein (197, A. Senderowicz, Proc ASCO 1999).

### 9.2.3. The Paullones

The paullones exhibited *in vitro* antiproliferative agent in the NCI's anticancer drug screen panel (198). Several novel Cdk inhibitors were identified in this manner using analysis of compounds that inhibited proliferation of the NCI tumor cell line including kenpaullone (NSC 664704) (Table 4), a potent inhibitor of Cdk1/cyclin B (IC<sub>50</sub>=0.4 mM), Cdk2/cyclin A (IC<sub>50</sub>=0.68 mM), cdk2/cyclin E (IC<sub>50</sub>=7.5 mM) and cdk5/p25 (IC<sub>50</sub>=0.85 mM). Kenpaullone acts through competitive inhibition of ATP binding. Molecular modeling studies have shown that kenpaullone binds to the ATP binding site of Cdk2 with residue contacts similar to other Cdk2-bound inhibitors (199). The analogs of kenpaullone, for example 10-bromopaullone (NSC 672234), inhibit various protein kinases including Cdk2. Proliferation of MCF 10A breast epithelial cell line was inhibited at the G<sub>1</sub>/S boundary by kenpaullone (IC<sub>50</sub>=30 mM) (199).

Relatively selective Cdk inhibitors included 6-dimethylaminopurine (IC<sub>50</sub> = 120 mM) (200, 201) and substituted purine derivatives: - isophenyl adenine (IC<sub>50</sub>=55 mM) and olomucine (IC<sub>50</sub>=7 mM). Olomucine inhibits Cdk1, 2, 5 and Erk1. Since its discovery, several Cdk1 and 2 inhibitors have been crystallized in complex with Cdk2 (202, 203). Crystal structures of isopentyladenine and olomucine with Cdk2 show that both purines bind in the deep groove located between the N and C-terminal domains of the kinase usually occupied by the adenine ring of ATP. Newer CKIs have been discovered and include roscovitrine (148, 204), purvalanol A/B (205) and CVT-313 (206). The crystal structures of roscovitrine and purvalanol B in complex with Cdk2 show that both inhibitors also bind with the same orientation as olomucine, but that the increased affinity results from improved hydrophobic contact sites between the active site and the inhibitor. The selectivity and relative IC<sub>50</sub>'s of these novel agents towards Cdk1 are demonstrated in Table 4 (155).

### 9.2.4. Indirect Modulators of Cdk function

Rapamycin inhibits the efficient translation of a variety of mRNAs by inhibiting the phosphorylation of the PHAS2 family of translational repressors. This results in a G<sub>1</sub> arrest possibly through transcriptional repression of cyclin D1 mRNA and increased destruction of cyclin D1 protein. Herbimycin also decreases cyclin D1 protein levels by reducing the efficiency of cyclin D1 mRNA translation. A molecule structurally related to herbimycin, 17-allylamino-17-dimethoxygeldanamycin (17-AAG), is in early clinical trials. Geldanamycins bind to Hsp90 and thereby affects the stability of Hsp90 targeted proteins such as receptor tyrosine kinases, steroid receptors, raf and Cdk4 (207-210).

Several differentiation agents including phenylacetate, butyrate, retinoids and vitamin D analogs have been shown to increase expression of p21<sup>Cip1/Waf1</sup> (211-213). Whether this effect is a direct consequence of altered p21<sup>Cip1</sup> gene expression or protein stabilization is unknown. In a number of instances p21 induction occurs independently of p53 activation suggesting that these agents may bypass loss of normal p53 function through regulation of p21<sup>Cip1</sup>. Another approach is to increase the expression of the endogenous Cdk inhibitor p16<sup>INK4a</sup>. In selective tumor types reduced p16<sup>INK4a</sup> expression occurs through methylation. Demethylating agents or agents that inhibit DNA methyltransferase (e.g., 5-deoxyazacytidine) result in hypomethylated DNA and increased p16<sup>INK4a</sup> expression.

## 10. CONCLUSION

Recent studies have provided substantial evidence for important cross talk between the cyclin-dependent kinases and factors regulating histone acetylation and chromatin structure. The cyclin D1 gene is induced by the p300 HAT domain (78) and cyclin D1 binds directly to the P/CAF HAT domain (29) and TAF<sub>II</sub> 250 (79). The identification of non-histone HATs suggest diverse mechanisms by which P/CAF and related HATs control cellular function. The cyclin E gene is regulated by pRB/HDAC and the activity of cyclin E/Cdk2 can induce histone gene expression (97). Cyclin E and cyclin D1 inhibit the cytostatic effect of chromatin remodelling proteins in the Brg1 family (80). The successful development of selective HDAC inhibitors with cytostatic effects has provided important new approaches to cancer therapeutics. The observations that NaBu inhibited cellular proliferation and HDAC activity, the subsequent cloning of mammalian HDACs, and the crystalization of a bacterial HDAC, have lead to substantial progress in the synthesis of selective HDAC inhibitors. Since subsets of HDACs bind and regulate select subsets of genes, it is likely that the identification of select HDAC inhibitors will provide better therapies for human cancer.

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