MONOGRAPH

BioDiscovery

OPEN ACCESS

From Roscovitine to CYC202 to Seliciclib – from bench to bedside: discovery and development

Nikolai Zhelev[1*], Dimitar Trifonov[1], Shudong Wang[2], Moustapha Hassan[3,4], Ibrahim El Serafi[3],Vanio Mitev[5]

[1] School of Science, Engineering & Technology, University of Abertay Dundee, Bell Street, Dundee,UK, DD1 1HG
[2] Division of Health Sciences, School of Pharmacy and Medical Sciences, University of South Australia, Adelaide, Australia
[3] Experimental Cancer Medicine (ECM), Department of Laboratory Medicine, Karolinska Institutet, Stockholm, Sweden
[4] Clinical Research Centrum, Karolinska University Hospital - Huddinge, Stockholm, Sweden
[5] Department of Chemistry and Biochemistry, Medical University of Sofia, 1431 Sofia, Bulgaria

Abstract

This monograph reviews the discovery and development of the cyclin-dependent kinase inhibitor roscovitine (R-roscovitine, CYC202, Seliciclib). The authors summarise the in vitro and in vivo data that have formed the basis for clinical investigation of Seliciclib as an anti-cancer drug. Kinase selectivity, cellular effects and the pharmacological properties of the drug are discussed in addition to the clinical results of Seliciclib being reviewed. Novel results on the effect of the drug in cardiac hypertrophy are summarized and potential applications of Seliciclib in other therapeutic areas, including, inflammation, virology, glomerulonephritis and polycystic kidney disease, are discussed. Finally the authors argue that optimisation of the therapeutic effect of kinase inhibitors such as Seliciclib can be enhanced using a systems biology approach involving mathematical modelling of the molecular pathways regulating cell growth and division.

Citation: Zhelev N, Trifonov D, Wang S, Hassan M, El Serafi I, Mitev V. From Roscovitine to CYC202 to Seliciclib – from bench to bedside: discovery and development. *Biodiscovery* 2013; **10**: 1; DOI:10.7750/BioDiscovery.2013.10.1

Received: December 15, 2013**; Accepted:** December 29, 2013**;**
Available online /Published: December 30, 2013

Corresponding Author: N. Zhelev, e-mail: n.zhelev@abertay.ac.uk

1. Introduction

Dysregulation of the cell cycle often results in diseases that are leading cause of mortality and morbidity in the world. Experimental research into cell cycle biology has revealed that progression of the cell cycle is regulated by cyclin-dependent kinases (CDKs) which are activated by cyclins and inhibited by CDK inhibitors. The cell cycle checkpoints are regulated by the CDKs which combine both the mitogenic and growth inhibitory signals that are responsible for the cell cycle transitions [1]. Dysregulation of CDKs has been associated with several malignancies, neurodegenerative disorders, viral and protozoa infections, glomerulonephritis and inflammatory diseases.Cyclin-dependent kinase inhibitors have been recognized as a new class of compounds and introduced as treatment for several diseases, including cancer. This monograph discusses the biological properties and clinical applications of roscovitine – a synthetic tri-substituted purine that selectively inhibits CDK1, 2, 5, 7 and 9. In order to fully understand the mechanism of action of roscovitine we first describe the main CDKs, their functions, regulation and importance as drug targets for therapeutic intervention.

1.1. Cyclin-dependent kinases, functions, regulation and involvement in diseases

Cyclin-dependent kinases area family of serine or threonine protein kinases. Members of this family share the common characteristic of requiring the binding of a regulatory cyclin subunit for enzymatic activation, and may also require the phosphorylation of a threonine residue near the kinase active site for full enzymatic activity. They were originally identified as proteins that controlled cell cycle events but have also been discovered to be involved in other cellular processes. Animal cells contain at least thirteen CDKs, only four of which are directly involved in cell cycle control, namely CDK1, CDK2, CDK4 and CDK6. CDK7 and CDK9 are involved in cell growth and the others are involved in the control of CDK activity, nerve cell differentiation and the level of basal gene transcription (Table 1).

Table 1. Known cyclin-dependent kinases, their cyclin partners, and biological function(s).

CDK	Cyclin partner	Biological function
CDK1	Cyclin A, B	G2 and M phase,
CDK2	Cyclin A, B, E	G1,S and M phase, apoptosis, transcription
CDK3	Cyclin C, E	G0 and G1 phase
CDK4	Cyclins D1, D2, D3	G1 phase, cell differentiation
CDK5	Cyclins D1, D3, p35, p37	Transcription, DNA damage response
CDK6	Cyclins D1, D2, D3	G1 phase
CDK7	Cyclin H	CDK-activating kinase, transcription, DNA repair
CDK8	Cyclin C	Transcription
CDK9	Cyclins T1, T2a, T2b, K	Transcription, cell growth, cell differentiation, apoptosis
CDK10	?	?
CDK11	Cyclin L	?
CDK12	Cyclin L	?
CDK13	Cyclin L	?
?	Cyclin F	?
?	Cyclin G	?

Similar to other protein kinases, CDKs have a bi-lobed structure, and can undergo two conformational changes that inactivate the enzyme, should the partner cyclin be absent. These conformational changes were discovered through crystallographic studies on human CDK2. Firstly, a flexible loop present at the carboxyl- terminal lobe, called the T-loop or the activation loop, blocks the binding of protein substrates at the opening of the active site cleft. Secondly, in inactive CDKs, some catalytically important amino acid side chains are in conformations that do not allow efficient phosphate transfer. Upon cyclin binding two alpha helices induce conformational changes in the kinase that allow efficient catalysis. The

PSTAIRE helix reorients the residues that interact with the phosphates of ATP and the L12 helix reconfigures the residues of the T-loop and the active site. A brief description of the main CDKs is given below.

CDK1

CDK1 also known as the mitotic kinase [2] is the prototypic cyclin-dependent kinase. After its identification in fission yeast it was initially known as cdc2. It has a length of 297 amino acids and a corresponding molecular weight of 34 kDa. The human gene is located on chromosome 10 at 10q21. The designation cdc2 (cell division control molecule 2) is used for cell division control genes and proteins [3]. CDK1 activity is modulated by phosphorylation at both stimulatory and inhibitory sites. CDK1 kinase activity is stimulated by phosphorylation on Threonine 161 and is inhibited, should the enzyme be phorphorylated at Tyrosine 15 (Y15) and Threonine 14 (T14), at the active site of the kinase. These residues in CDK1 (Y15 and T14) are substrates for protein kinases, such as wee1, mik1 and myt1 and their phosphorylation inhibits the entry of the cell into the G2 phase of the cell cycle[4]. Overexpression of Cyclin B1 and hyper-activation of CDK1 have both been observed in several tumors, including breast-, colon- and prostate carcinoma [5].

CDK2

CDK consists of298 amino acids in length as has a molecular weight of 33.9 kDa. The human *CDK2* gene is located on Chromosome 12 at 12q13. The protein kinase is found in complex with its regulatory partners Cyclin E or Cyclin A. Cyclin E is necessary for the transition from phase G1 to S and the initiation of the S phase of the cell cycle while Cyclin A is required to progress through the S phase.CDK2 is regulated by its protein phosphorylation and by the inhibitors p21Cip1 and p27Kip1. Intriguingly, despite the key role of CDK2 in cell cycle regulation and the profound effects of CDK2 inhibitory compounds on cell fate, it has been reported also that *CDK2* knockout mice are viable [6]. However, studies with melanocytes demonstrate that they are uniquely dependent on CDK2 for proliferation [7]. In addition, CDK2 small-molecule inhibitors may have different cellular phenotypes compared with cells that have lost expression of CDK2.Inactivation of Cip/Kip inhibitors and overexpression of Cyclin

E and/or Cyclin A leads to deregulation of CDK2 in various malignancies, including melanoma, ovarian carcinoma, lung carcinoma and osteosarcoma [5].

CDK4

CDK4 is found complexed with Cyclin D and this complex is essential for the progression of cells through the G1 phase of the cell cycle. The protein consists of 303 amino acids and has a molecular weight of 34 kDa. The human *CGK4* gene is located on Chromosome 12 at position 12q14. CDK4/Cyclin D is one of the kinases that phosphorylate the retinoblastoma protein family (Rb), which leads to dissociation of the pRb-E2F complex and activation of the transcription of genes required for the entry into the S phase. Mutations in the *CDK4*gene were all found to be associated with tumorigenesis.

CDK5

CDK5 interacts with D1 and D3-type cyclins. The protein shows kinase activity only after interaction and activation by CDK5R1 (p35) or CDK5R2 (p39). Although p35 and p39 lack cyclin sequence homology, crystal structures show that p35 folds in a similar way as the cyclins.CDK5 has been found to modulate the metastatic potential of some malignancies, including breast and prostate carcinomas[8,9]. This kinase has been found to play an essential role in the central and peripheral nervous system [10].CDK5 has also been reported to regulate the cytoarchitecture of the developing brain [11] and to mediate the neuronal migration in post mitotic neurons. CDK5 also has several vital functions in neuronal cytoskeleton dynamics, synaptic plasticity [12], drug addiction [13], synaptic endocytosis [14] and neurotransmitter release [15]. Thus, CDK5 may be involved in the pathogenesis of several neurodegenerative disorders, such as Alzheimer's and Parkinson's diseases [10].

CDK6

CDK6 has a size of 326 amino acids, a molecular weight of 40 kDa and the human *CDK6* gene is located on chromosome 7 at position 7q21-22. Together with CDK4, CDK6 is known as G1-phase CDK and binds to Cyclin Ds, to form CDK6/Cyclin D complexes. The activity of thiskinase

plays a significant role in the progression of cells through the G1 phase of the cell cycle [16]. The two kinases, CDK4 and CDK6, share 71% identity at the amino acid level and both can bind *in vitro* with all 3 type-D cyclins to phosphorylate pRb [17–21]. *CDK4* and *CDK6* double knockout-mice die during development due to anemia, an observation which taken together with the mild phenotype exhibited by *CDK6* knockout-mice, whose only defects are also haematopoietic, points toward a role for the two kinases in haematopoiesis, and at least some redundancy in function between the two [22].

The two kinases have shown differing substrate specificities *in vitro*– with CDK4 phosphorylating Thr-826 of pRb, while CDK6 targets Thr-821 – a result which also suggests that the CDK alone can direct substrate recognition [23]. Expression studies in a variety of tumour and cell types have also shown differential expression and localisation of the two kinases. Mutations in the binding site of the CDK4 inhibitor $p16^{INK4A}$ have been discovered in cases of familial melanoma [24]; and in squamous cell carcinomas and neuroblastomas CDK6 has been found to be overexpressed, with CDK4 expression unchanged [25, 26]. In T cells CDK6 has been detected in both nuclear and cytoplasmic fractions; however, any pRB phosphorylating activity was limited to the nuclear fraction, indicating the possibility for differential kinase or inhibition activation across cellular compartments [27].

CDK6 activity has been implicated in the inhibition of differentiation across a number of cell types. In murine erythroid leukaemia cells inhibition of CDK6 has been shown to induce terminal differentiation while inhibition of CDK4 did not have this effect [28]. Osteoblast differentiation has also been shown to be inhibited by expression of CDK6, and this effect was said to be independent of any role in the control of the cell cycle [29]. Interestingly for a kinase, generally considered mitogenic, this role in differentiation may be due to CDK6 activity reducing rates of cellular proliferation.

CDK7

CDK7 exists as a part of a three protein complex consisting of CDK7, Cyclin H and MAT1 ("ménage à trois") that is also known as Cyclin-dependent kinase Activating Kinase (CAK) and is responsible for the phosphorylating activation of CDKs 1,2,4 and 6. This complex is also, along with another six proteins, part of the general transcription factor TFIIH, and so is involved in the early pre-initiation and initiation steps of transcription.

CAK either does not phosphorylate monomeric CDKs or does so only poorly, requiring the presence of the partner cyclin to cause a conformational change in the kinase, exposing the activation loop and allowing CAK to phosphorylate. Like other CDKs, CDK7 is phosphorylated on the activation loop, but this modification is not required for enzymatic activity, instead the presence of MAT1 in complex with CDK7 and Cyclin H can substitute for the activating phosphorylation.

As a part of TFIIH CAK hyperphosphorylates the C-terminal domain (CTD) of the large subunit of RNA polymerase II, thus promoting the initiation of transcription, an event that CAK alone is unable to catalyse. CAK, as a part of TFIIH, is phosphorylated by CDK1/Cyclin B during mitosis – an event that causes the loss of the TFIIH's CTD's phosphorylating activity, and is at least in part responsible for the silencing of transcription during mitosis.

Temperature sensitive CDK7 mutants in C. elegans exhibit a block in the cell cycle which can be attributed to loss of CAK activity rather than any CTD phosphorylating function [30]; and in Drosophila CDK7 mutations are lethal and cause death before or during pupation due to mitotic catastrophe, similar to mutations in the CDK1 gene suggesting that the gene is required for mitotic proliferation [31]. Similarly in mice deletion of the MAT1 causes embryonic lethality. In addition, RNAi knockdown of MAT1 in rat smooth muscle cells and abrogation of the protein in human osteosarcoma cell lines leads to arrest of the cell in the G1 phase of the cell cycle, apoptosis in the rat smooth muscle cells, and is associated with a reduction in pRb phosphorylation [32, 33].

CDK8

CDK8 is a 464 amino acid serine-threonine protein kinase with a molecular weight of 53.3 kDa whose gene *CDK4* is located on chromosome 13 at position 13q12. The protein is located in the nucleus and along with CDK7 and CDK9 makes up the transcriptional CDKs (rCDKs) as they have all been identified as having role(s) in transcriptional control. Similar to CDK7 – CDK8 can also phosphorylate the C-terminal domain of RNA Polymerase II *in vitro*;however, its specificity is for residues Ser-2 and Ser-5, as opposed to the Ser-5 and Ser-7 targeted by CDK7. The cyclin partner for CDK8 is Cyclin C, which does not show cell cycle-dependent fluctuations in concentration; control of CDK8 activity is modulated rather by other factors, such as association with Mediator Complex Subunit 12 (MED12) [34–38]. CDK8 is part of the large multi-subunit transcriptional regulatory complex Mediator that functions in eukaryotes as a transcriptional co-activator. This 25–30 member, 1.2MDa complex is required for successful transcription from almost every class II promoter, at least in yeast [39]. The complex binds to the CTD of RNA Polymerase II and acts as a co-activator of transcription. Many members of the complex provide a large and varied surface area for protein-protein interactions, thus acting as a scaffold between RNA Pol II and transcription factors [40, 41].

CDK8 has been attributed to oncogenic potential in colon cancer cells due to effects observed on the Wnt/β-catenin pathway in cultured cells [42]. CDK8 was identified in a shRNA screen as being required for colon cancer cell proliferation and Wnt/β-catenin driven signalling. CDK8's copy-number was also shown to be frequently increased in colon cancers, suppression of CDK8 expression reduced proliferation of colon cancer lines, overexpression of wildtype, but not kinase-dead, CDK8 led to transformation of NIH3T3 cells [42, 43].

CDK9

CDK9 is a 372 amino acid, 42.8 kDa serine-threonine kinase encoded by the *CDK9* gene on chromosome 9 at position 9q34.1. The kinase has been found complexed with two cyclins, Cyclin T and Cyclin K. The kinase subunit forms the catalytic core of positive transcription elongation factor b (p-TEFb) [44–47]. It plays a crucial role in increasing transcriptional elongation from RNA Pol II-dependent promoters, including many key developmental and response genes, as well as the vast majority of protein coding genes [48]. Activity of CDK9 is controlled by associations with other proteins, most notably its cyclin partners, Cyclin T and Cyclin K, but also other activatory cellular proteins, such as c-myc, NF-κB, androgen receptor; and inhibitory complexes, such as the 7SK small nuclear RNA containing complex [49–54].

Regulation of CDKs

Understanding the biological role and the regulation of cyclin-dependent kinases is important for selecting drug targets and developing strategies for modulating their activity for therapeutic purposes.

CDKs are regulated by several mechanisms, including transcription and translation of their subunits, heterodimerization with cyclins, post-translational modification by phosphorylation and dephosphorylation, and interactions with their natural inhibitors.The endogenous CDK inhibitors consist of two families, the INK4 family and the KIP (kinase inhibitor protein) family[55].

Members that fit into the INK4 family e.g.: $p15^{INK4B}$ and $p16^{INK4A}$, inhibit CKD4 and CDK6; while members of the KIP family, e.g.: $p21^{Cip1}$, $p27^{Kip1}$ and $p57^{Kip2}$, have diverse activities [55]. On one hand p21 and p27 activate formation of CDK/Cyclin D complexes trough increasing stability of Cyclin D's, facilitation of the assembly of these complexes, and increasing nuclear localization of CDK/Cyclin D complexes. On the other hand they bind to the assembled complex and inhibit its kinase activity. The above proteins and peptides derived from them have been widely used as tool compounds for studying the regulation of the cell cycle, and can have potential therapeutic application. Among the variety of cell cycle

inhibitors that are present, those important in the regulation and control of the cell cycle and used as tools for drug development are mentioned below.

p16

p16 is the main member of the INK4 family of inhibitors, which plays key role during the G1 and S phases of the cell cycle. The family includes p15, p16, p18 and p19 which tend to inactivate only the G1 CDK's – CDK4 and CDK6. Prior to their attachment with cyclins, they form stable complexes with the CDK's, and overexpression of cyclins will not separate them from the CDK. Due to the binding that occurs between the members of the p16 family and CDK4 or CDK6, it does not permit the phosphorylation and inactivation of pRb. [4].It is transcribed from the 9p21 locus and, rather than directing cells towards neoplasia, it may direct them towards senescence [56]. P16 is described as a tumour suppressor.

p21

The proteins of the p21 family are important in the regulation of G1 phase of the cell cycle and include waf1 (also called p21), p27 and p57. Except for CDK1, they can inhibit most of the CDKs. Due to its binding with cyclins, they prevent the CDK from phosphorylating the pRb and unlike p16, these induce dissociation from E2F. The role of p21 in oncogenesis is still unclear, but it might act as a tumour suppressor since it plays the role of an inhibitor during the cell cycle [4]. It is said by Gartel AL et al. [57], that p21 is regulated at the transcriptional level, it is an inhibitor of apoptosis, and its suppression may have anti-cancerous consequence. Its overexpression during the cell cycle may arrest the G1, G2 or the S phase. It is said to advance the development of active complexes in a concentration dependent manner by stabilising contact between CDK4or CDK6 and Cyclin D [57].

p27

p27 inhibits cyclin-dependent kinases that help in the regulation of the cell cycle through which cells progress. This inhibitor was initially discovered in cells that were arrested by transforming growth factor α (TGF-α) or by contact inhibition or after treatment with lovastatin. It is found to be in

conjugation with CDK/Cyclin D complexes in those cells that proliferate, while in those G1 arrested cells it binds itself to CDK2/Cyclin E complexes. Hence it is crucial to which complex p27 binds and there remains a competition between CDK/Cyclin D and CDK2/Cyclin E complexes. If the former can sequester p27 from the latter then it can push the cell into the S phase. In the experimental studies related to breast cancer, Chiarle et al compared the levels of p27 and the activities of CyclinE and Cyclin D1-dependent kinases and found that an inverse correlation exists between CyclinE-dependent kinase and p27 [55].

The activity of p27 seems to increase on loss of adhesion to the extracellular matrix, in response to differentiation signals and by the signals of TGF-α, a growth inhibitory factor, while mitogenic factors cause its loss. [55]. In inactive cells the level of p27 appears high and during the G1 phase the levels decrease reaching its lowest phase during the S phase. High levels of nuclear p27 protein are seen in most of the normal epithelial tissue, including prostate, ovary, lung and breast, especially in the terminally differentiated layers. On the contrary, in the proliferating cells, such as the basal layer of the epithelia or in the germinal centre lymphocytes, the level of p27 seems to be practically untraceable [55].

1.2. CDK inhibitors as potential therapeutics.

As already mentioned CDKs are key regulators of cell cycle and other fundamental biological processes, such as transcription. Consequently the main effects of CDK inhibitors are induced via modulation of the cell cycle progression and inhibition of transcription.

Notably dysregulation of the cell cycle is a common event in tumorigenesis and leads to uncontrolled cell proliferation and independence from extracellular mitogenic signals. In a normal cell, initiation of the cell cycle is achieved through stimulation with growth factors. Such mitogenic stimulation results in a cascade of serine-threonine kinase activities resulting with induction of Cyclin D1 transcription. Cyclin D1 then binds to CDK4 and CDK6 to give active

kinase complexes. The latter directly phosphorylates the retinoblastoma protein (Rb), causing dissociation of histone deacetylase 1, allowing histone acetylation, which is permissive for transcription. As a result key cell cycle regulators, such as Cyclin E are transcribed and translated. Cyclin E then forms a complex with CDK2, which further phosphorylates Rb with the release of the general transcription factor E2F. E2F stimulates the transcription of genes required for progression through to the synthesis phase of the cell cycle.

Dysregulation of the cell cycle occur through various mechanisms. They include: mutations leading to constitutive activation of the signal transduction pathways[58], which results in uncontrolled production of Cyclin D; loss, mutation or methylation of p16 – the endogenous inhibitor of CDK4/Cyclin D and CDK6/Cyclin D [59]; constitutive activation of CDK4 via mutations [60]; viral inactivation of p21 [61] and loss or mutations of Rb [62]. All of the above mechanisms override the anti-proliferative activity of Rb and confer a greater propensity for cells to progress through the cell cycle. That is why CDKs involved in cell cycle control have been considered as attractive targets to restrict uncontrolled cell proliferation. This includes CDKs involved in G1/S transition but also CDKs playing role in other phases of the cell cycle, such as CDK2/Cyclin A. The latter maintains pRb in its hyper-phosphorylated state allowing completion of the S phase of the cell cycle. As the cell exits S phase timely neutralisation of E2F transcriptional activity is required, otherwise the apoptotic mechanisms are triggered leading to cell death. The neutralisation occurs through CDK2/Cyclin A activity to E2F and leads to dissociation from the DNA. It is this pathway which is the most promising from a CDK inhibitory point of view, since inhibition of CDK activity would maintain E2F in its DNA bound state and trigger apoptotic cell death. In addition, as discussed above, E2F is commonly deregulated in many tumour cell lines, and this overexpression is a viable target for CDK inhibition – with the aim of keeping E2F DNA bound into G2, and hence, inducing apoptosis. The validity of CDK2/Cyclin A as a drug target in tumours with upregulated E2F activity has been proved experimentally – using cell-permeable peptides, which block the interaction between Cyclin

A and E2F [63–65]. Such peptides kill transformed cells, but not their normal counterparts [66].

In addition to control of the cell cycle, some CDKs are involved in regulation of RNA polymerase II-mediated transcription. There are three RNA polymerase II (RNAPII) directed cyclin-dependent protein kinases which play roles in RNAPII activation. These are CDK7, 8 and 9, and they all phosphorylate the serine-rich C-terminal domain (CTD) of RNAPII to allow the consecutive attachment of the elongational transcription factors. Soon after initiation, CDK7 (the kinase subunit of transcription factor TFIIH) phosphorylates Serine 5, causing RNAPII to become arrested in the promoter-region due to its interaction with negative elongation factors (NELF). Phosphorylated Serine 5 also mediates the recruitment of mRNA capping enzymes. After capping, CDK9 phosphorylates Serine 2 which counteracts the suppressive action by NELF and allows RNAPII to advance from the promoter and so allows transcriptional elongation [67]. Serine 2 is dephosphorylated by TFIIF-dependent CTD phosphatase FCP-1 during termination of transcription, and the unphosphorylated RNAPII is recycled.CDK8 is suppressive, in that it phosphorylates RNAPII preventing it from binding to the promoter, and has additional suppressive activity by phosphorylating Cyclin H, thus preventing the activation of CDK7. This suggests that CDK8 is a suppressor of transcription and CDK7 and in particular CDK9 appear to be more attractive as targets for drug development.

Importantly roscovitine inhibits RNAPII-mediated transcription, which downregulates the expression of anti-apoptotic proteins, such as Bcl-2, Mcl-1, survivin and others [68]. These proteins have short half-lives and their transient downregulation can trigger an irreversible activation of apoptosis in tumour cells, but not in normal cells, which do not depend on survival factors. Notably, myeloma cells treated with small molecule inhibitors of CDK9 have an increased level of apoptosis caused by the loss of short half-life proteins, such as the anti-apoptotic proteins XIAP and Mcl-1, and the proliferative D-type cyclins. These effects have been assigned to abrogation of transcription due to inhibition of CDK9 activity, although such an absolute assignment is difficult because the small

molecule inhibitors used in the studies, including roscovitine, inhibit multiple CDKs [69–73]. Various aspects of CDK 9 as a potential drug target in virology and inflammation have been reviewed by ourselves previously [74]. Here we give a brief overview of the latest insights regarding potential application of CDK9 inhibitors in cardiology and in particular in heart hypertrophy.

The amount of phosphorylated (active) RNAPII increases rapidly in response to hypertrophic stimuli, such as endothelin-1 (ET-1) and Angiotensin (Ang) II, due to increased phosphorylation of Ser-2 of the C-terminal domain–the serine rich region which is preferentially phosphorylated by CDK9. CDK9 is, therefore, the essential CTD kinase for hypertrophic growth – an observation further reinforced by much experimental data, such as that from Sano and Schneider(2004)[75], which showed that *in vitro* CDK9 activity was required for hypertrophy in a tissue culture model, and *in vivo*, in a mouse model,heart-specific activity of CDK9 by Cyclin T1 provoked hypertrophy in mice. In addition, they found that mutations in CDK9 blocked the effect of hypertrophic stimuli. Sano and Schneider(2004)[75] also showed that inhibition of CDK7 had a much smaller effect on the increase in size following growth stimuli, showing that CDK9 is the RNAPII kinase associated with abnormal protein synthesis in pathological cardiac hypertrophy.

Hypertrophic signalling pathways stimulate CDK9 activation by causing the dissociation of 7sk (a small nuclear RNA) from CDK9, unleashing CDK9 function. 7sk, when bound to CDK9, causes inhibition of the catalytic activity. Hypertrophic signals result in the liberation of functional P-TEFb (positive transcription elongation factor b) from its endogenous inhibitor, which is the CDK9/Cyclin T heterodimer. The following diagram (Fig. 1) helps to convey the activation of CDK9 by hypertrophic stimuli [75].

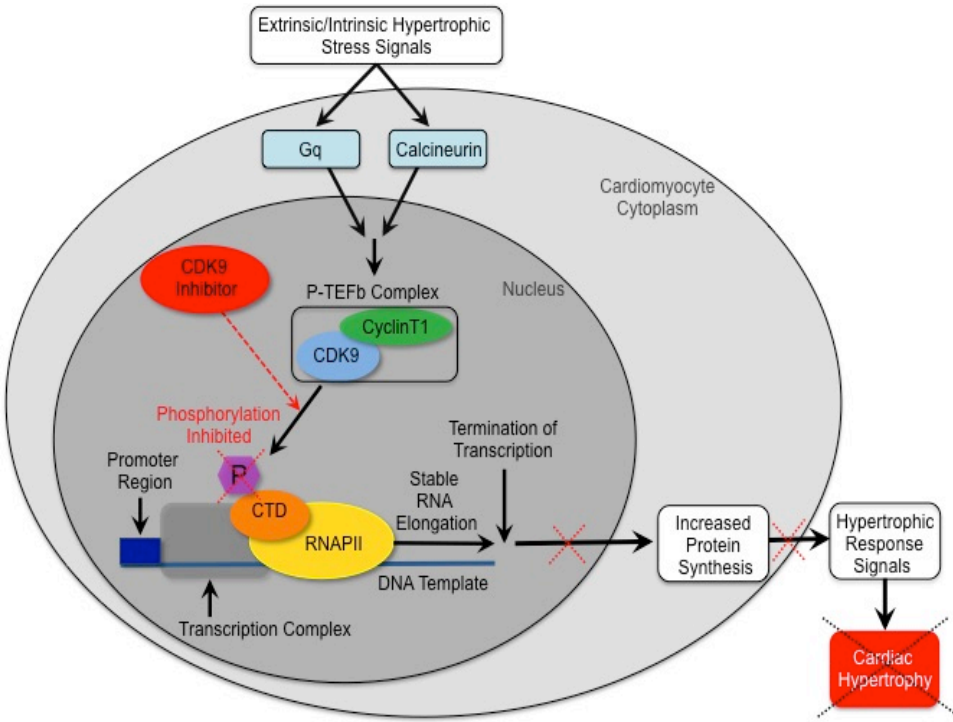

Figure 1. Role of CDK 9 in cardiac hypertrophy and as potential target for drug development.

CDK9 is also involved in mitochondrial dysfunction, observed as a result of cardiac hypertrophy. This dual transcription role of CDK9 in both hypertrophic growth and in mitochondrial dysfunction further highlights the potential utility of CDK9 as a drug target for the inhibition cardiac hypertrophy. Overall, the activity of CDK9 is upregulated in cardiac hypertrophy, and consequently, CDK9 inhibition is a potentially attractive molecular target for therapeutic intervention in cardiac hypertrophy [75].

2. Discovery of roscovitine

It was in the laboratory of Pierre Guerrier at the Roscoff Biological Station that Laurent Meijer, after spells in the labs of Maurice Durchon in Lille and David Epel at Stanford, realised that starfish oocytes were perhaps the ideal model to study the role of protein phosphorylation in the prophase/metaphase transition of the cell cycle [76–79]. Having studied the histone phosphorylation associated with this transition before, the starfish oocyte had advantages over the previous systems he had worked on, namely the ability to isolate the oocytes in large quantity in natural seawater. The oocytes are naturally arrested in prophase, but addition of the hormone 1-methyladenine induces a rapid and synchronous transition to metaphase [80, 81].

The use of protein phosphates and phosphatase inhibitors had led to the initial anecdotal identification of protein phosphorylation as a mechanism in the control of the cell cycle [78, 82]. While working with Steven Pelech in Edwin Krebs' laboratory during a sabbatical a histone kinase specific to the M-phase was identified that was strongly activated during the prophase metaphase transition [83, 84]. Further biochemical studies subsequently identified the kinase as a heterodimer of cdc2, the kinase subunit, and a regulatory protein–Cyclin B [85–87]. These observations taken together with many other studies [88–90] had finally solved the riddle of almost twenty years that CDK1/B was the "maturation promoting factor" present in all species, the factor that when injected into prophase-arrested oocytes induced the transition into metaphase.

The central role of CDK1 and its requirement for cell division made it an immediate target for scientists interested in identifying potential anti-cancer agents. A radioactive *in vitro* kinase assay [87, 90, 91] was established with the goal of identifying inhibitors of the kinase. Initial tests with then current chemotherapeutic agents were negative, only the non-specific kinase inhibitor Staurosporine (Fig. 2) was available as a control compound. It was only when the compound 6-dimethylaminopurine (DMAP) was tested that the first purine based kinase inhibitor was discovered (Fig. 2). The compound was initially

identified as a Puromycin analogue that, although it no longer inhibited protein synthesis, was a potent inhibitor of mitosis in the sea urchin embryo [92]. During work on the M-phase specific phosphorylation it had been observed that DMAP was a potent inhibitor of this event, raising the possibility, subsequently proven using *in vitro* kinase assays, that DMAP was an inhibitor of the maturation specific factor – CDK1/Cyclin B [91, 93, 94]. DMAP and a related purine, isopentenyladenine (Fig.2)were found to be inhibitors of CDK1/Cyclin B, but with IC_{50} values of 120µM and 55µM, respectively, were neither particularly potent nor selective.Isopentenyladenine was known to be key intermediate in the synthesis of cytokinins, plant hormones involved in many aspects of the control of plant cell growth; and this knowledge led to a contact of the Biological Station Roscoff scientists with JaroslavVesely and MiroslavStrnad at the Institute of Experimental Botany in Olomouc, who had a collection of isopentenyladenine analogues and other substituted purines. This collaboration led to the identification of 2-(2-hydroxyethylamino)-6-benzylamino-9-methylpurine, a compound subsequently named olomoucine (Fig. 2), as a kinase inhibitor with specificity for CDK and MAPK over some 30 other tested kinases [95]. This observed kinase specificity was surprising, given that the dogma of the time said that ATP-competitive molecules, such as substituted purines, would never show specificity given the similarity of these binding sites in kinases. Olomoucine was also approximately a lot more potent than isopentenyladenine with IC_{50} values of 7, 7, 7, 3 and 25µM against the kinases CDK1/Cyclin B, CDK2/Cyclin A and CDK2/Cyclin E kinases, the brain CDK5, and the map-kinase ERK1, respectively.

Further attempts to improve selectivity and potency of these substituted purines was carried out in the laboratories of MiroslavStrnad in Olomouc (Czech Republic) and Michel Legraverend at the Institute Marie Curie in Orsay(France) and resulted in the identification of the 6-(benzylamino)-2(R)-[[1-(hydroxymethyl)propyl]amino]-9-isopropylpurine (Fig.3), subsequently named roscovitine [96], a potent inhibitor of CDK1/Cyclin B with an IC_{50} value of 0.45µM. Subsequent crystallographic structures of roscovitine and olomoucine in complex with CDK2 provided the basis for a significant amount of structure-guided medicinal chemistry work [97].

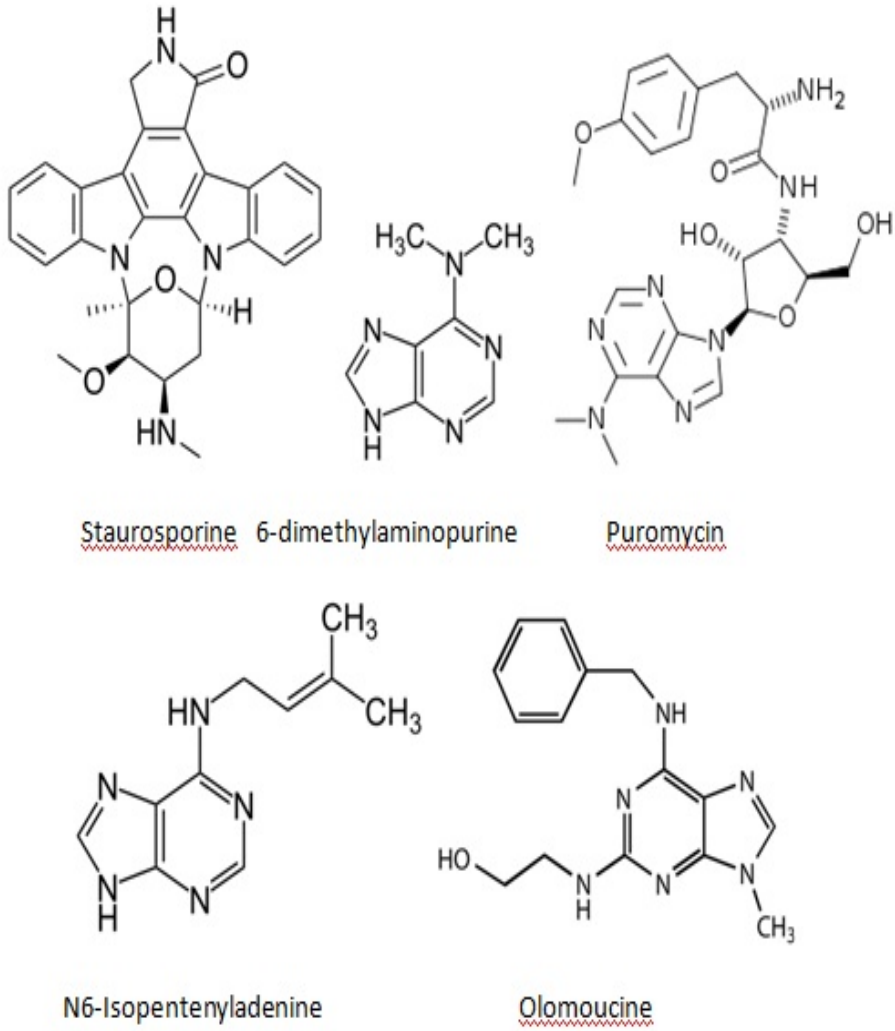

Figure 2. Chemical structures of some of the first CDK inhibitors.

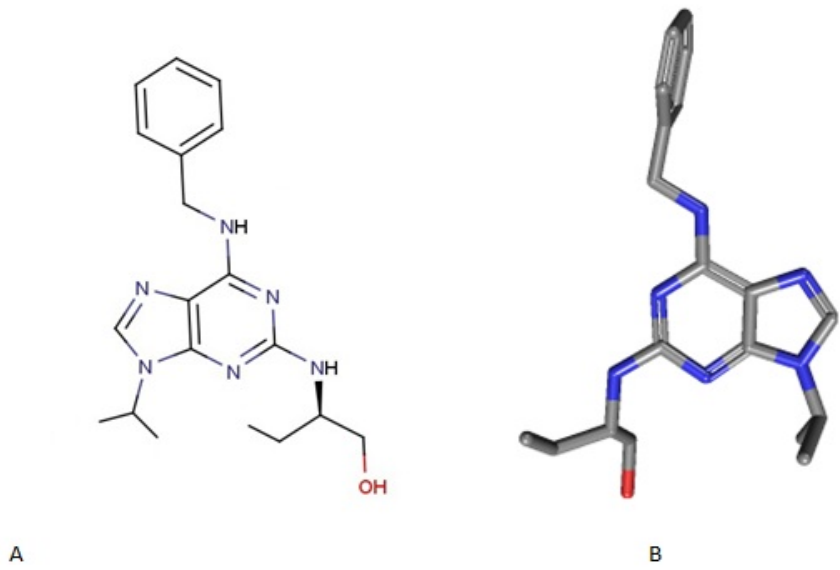

Figure 3. 2D (A) and 3D (B) structure of roscovitine.

3. Chemical synthesis and physicochemical properties of roscovitine

We have synthesised (R)-roscovitine (CYC202) and its (S)-enantiomer [98] and molecular structure of the produced CYC202 was determined unambiguously by X-ray diffraction. Briefly, compound 2,6-dichloropurine (1) was converted into *N*-benzyl-2-chloro-9*H*-purin-6-amine (2) up on heating with benzylamine in the presence of triethylamine in *n*-butanol for a period of 3 hours. Treatment of the intermediate 2 with isopropylbromide in the presence of potassium carbonate afforded *N*-benzyl-2-chloro-9-isopropyl-9*H*-purin-6-amine (3) in 85% yield. Amination of the latter with excess of (*R*)-2-aminobutan-1-ol (8 mol equiv.) at 160^{o}C for 8 hours, followed by re-crystallisation in ethyl acetate, yielded compound CYC202 as white crystalline (Fig. 4).

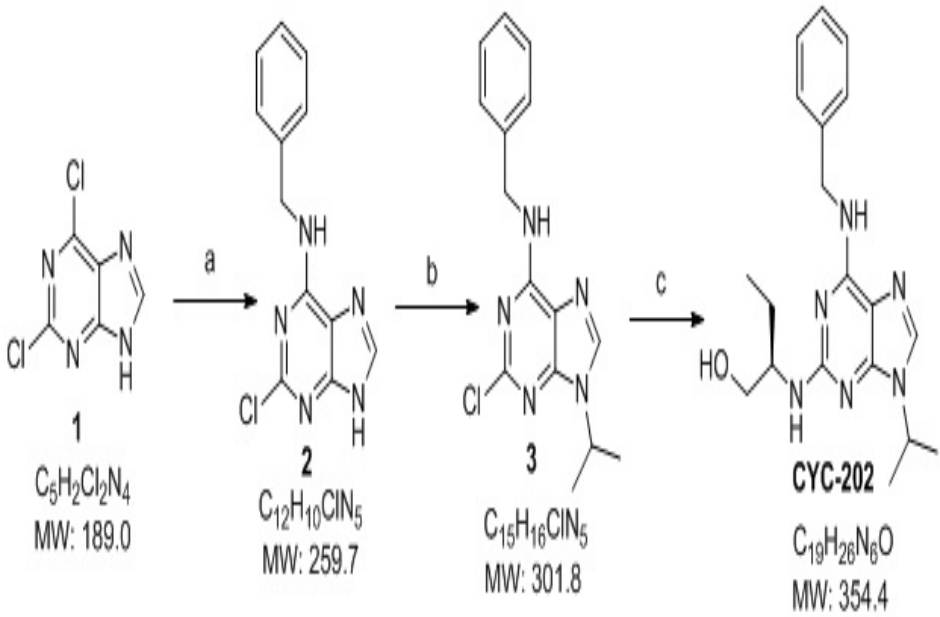

Figure 4. Synthesis of CYC-202 ((*R*)-Roscovitine). Reagents and conditions: (a) benzylamine, BuOH, NEt_3, 110°C, 3 h, 95%; (b) 2-bromopropane, K_2CO_3, DMSO, 18-20°C, 5 h, 85%; and (c) (*R*)-2-aminobutan-1-ol, 160°C, 8 h, 89%.

We have also determined some of the physiochemical properties of roscovitine [98], which are summarized in Table 2.

Table 2. Physicochemical properties of roscovitine.

IUPAC name	**2-(*R*)-(1-Ethyl-2-hydroxyethylamino)-6-benzylamino-9-isopropylpurine**
Chemical name	1-butanol, 2-[[9-(1-methylethyl)-6-[(phenylmethyl)amino]-9*H*-purin-2yl]amino], (2*R*)-(*R*)-2-(6-benzylamino-9-isopropyl-9*H*-purin-2-ylamino)-butan-1-ol
Other names	(R)–Roscovitine, (S)-Roscovitine, CYC202, Seliciclib
Molecular formula	$C_{19}H_{26}N_6O$
State	White powder
Solubility	Soluble in DMSO (up to 50 mM) and in 50 mM HCL with the pH adjusted to 2.5
Atomic composition	C= 64.38%; H=7.39%; N=23.71%; O=4.51%
Molecular weight	354.45
Rotation values	(R)-Roscovitine: $[\alpha]_D^{20}$+ 56.3, (S)-Roscovitine: $[\alpha]_D^{20}$-56.3
Melting point	106-108°C
pKa	4.4
Absorption λ max	230nm and 292nm

4. Inhibition of CDKs by R-roscovitine (CYC202)

De Azevedo et al. (1997) [97] investigated the kinetics of roscovitine inhibition of p34cdc2/Cyclin B kinase purified from starfish oocytes, and determined the crystal structure of the CDK2- roscovitine complex. Roscovitine was shown to be a competitive inhibitor of p34cdc2/Cyclin B kinase (Ki=1.2μM). X-ray crystallographic studies demonstrated that the purine portion of the roscovitine molecule binds to the adenine binding pocket of CDK2. The benzyl ring of the roscovitine molecule forms contacts with the CDK2 enzyme that are not observed in the ATP-CDK2 complex, which provides the basis for the selectivity of roscovitine. The interaction involves hydrophobic and van der Waals contacts and three hydrogen bonds. Two of them involve atoms N^7 and N^6 of roscovitine and backbone atoms of Leu83 of CDK2. The third hydrogen bond is formed between the hydroxyl group of roscovitine and a water molecule, which mediates the interaction with CDK2 (Fig. 5).

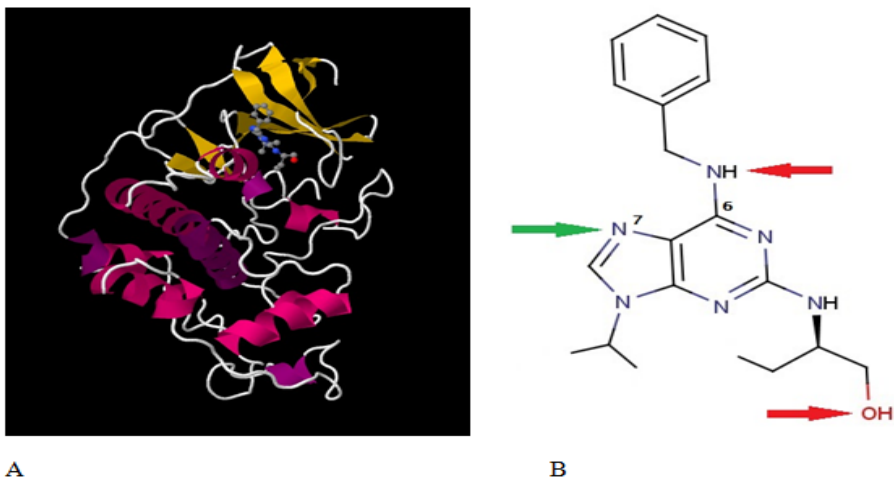

Figure 5. Interactions of (R)-Roscovitine with CDK2. A - 3D structure of human CDK2 with (R)-Roscovitine. B - (R)-Roscovitine's atoms involved in hydrogen bonds: red arrows -hydrogen donors, green arrow - hydrogen acceptor.

These studies also revealed that it is the (R)-enantiomer of roscovitine which is bound by CDK2 (this enantiomer is about twice as potent as the (S)-enantiomer in inhibiting cdc2/Cyclin B). This provides the rationale for clinical development of the (R)-enantiomer of roscovitine.

The specificity of roscovitine was demonstrated by Meijer et al. (1997) [99] by screening for inhibition against 25 highly purified kinases. The above study confirmed that roscovitine has a narrow spectrum of activity showing specificity for cdc2/Cyclin B, CDK2/CyclinA, CDK2/CyclinE and CDK5/p35 (IC_{50} values for these enzymes were 0.65, 0.7, 0.7 and 0.2µM, respectively). The cyclin kinases CDK4/Cyclin D1 and CDK6/Cyclin D3 were not significantly inhibited by roscovitine(IC_{50}>100µM for both enzymes). It is worth noticing that the ATP concentration used in these experiments (15µM) was significantly lower than the concentration of ATP in cells (typically more than 1mM). We have confirmed that CYC202 (R-roscovitine) is a potent inhibitor of recombinant CDK1/CyclinB, CDK2/CyclinA, CDK2/CyclinE and CDK7/CyclinH, with IC_{50} values for these enzymes, respectively: 2.69, 0.71, 0.1, and 0.49 µM using *in vitro* kinase assays with 100mM ATP [100]. It has also been determined that roscovitine is a potent inhibitor of CDK9/CyclinH, although the IC_{50} values reported varied between 0.79 [74] and 3.2µM [101]. Notably a large number of kinases (including: AMPK, Aurora B, Aurora C, BRSK2, CAMK1, CAMKKα, CAMKKβ, CK1δ, CK2, EF2K, IKKβ, Lck, MAPKAP-K2, MAPKAP-K3, MARK3, MELK, MKK1, MSK1, MNK1, MNK2, ERK1, MAPK2/ERK2, MARK3, NEK2a, NEK6, NEK7, JNK1, JNK2, JNK3, JNK/SAPK1c, SAPK2a/p38, SAPK2b/p38, SAPK3/p38, SAPK4/p38, p38α/MAPK, P38β/MAPK, p38δ/MAPK, ERK8, MAPKAP-K1a, MAPKAP-K2, MSK1, MST2, HIPK2, HIPK3, PAK4, PAK5, PAK6, PIM1, PIM2, PIM3, PLK1, PRAK, PRK2, PKA, PKA1, PKCα,PKCζ, PDK1, PKBα, PKBβ,PRAK, RSK1, RSK2, SRPK1, SmMLCK, SGK, SGK1, S6K1, Src, GSK3β, ROCK-II, AMPK, CHK1, CHK2, CK2, PHK, LCK, CSK, GSK3β, CK1, DYRK1A,DYRK2, DYRK3) are poorly, if at all, sensitive to roscovitine [102, 103].

5. CYC202 inhibits cell growth *in vitro*

Due to the pivotal role of CDKs in a wide range of cellular functions, roscovitine has been suggested as a potential treatment for several diseases that involve the cell cycle. The effects of roscovitine have been studied *in vitro* in cell lines and *in vivo* in animal models. *In vitro* studies have been carried out in more than 100 cell lines, including the NCI-60 cell line panel of the national cancer institute (NCI).

Meijer et al. (1997) [99] demonstrated that roscovitine exhibited inhibitory activity against human tumour cell lines *in vitro*, in a study using 60 different tumour cell lines derived from nine different tumour types. Roscovitine was tested at concentrations ranging from 0.01µM to 100µM with a 48 hour exposure time. All cell lines displayed a similar sensitivity to roscovitine, and the average IC_{50} was 16µM. The nature of the inhibition was further characterised in experiments with the cell line L1210, which demonstrated that roscovitine arrested the cells in the G2/M phases of the cell cycle. Raynaud et al [104] reported 24 cell lines with an average IC_{50} of 14.6µM.

We have investigated the potential for CYC202 to inhibit the growth of cultured tumour cells *in vitro* using a panel of nineteen different human tumour cell lines and three non-proliferating human cell lines [100]. Cell growth was determined using an MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) assay. CYC202 exhibited growth inhibition for all cell lines tested, with IC_{50} values ranging from 7.9µM to 30.2µM. In addition, the data demonstrated an average of four-fold selectivity for actively dividing tumour cells over normal cells.

We have also studied the minimum length of exposure to CYC202 required to achieve the maximum growth inhibitory effect using the human ovarian cancer cell line A2780, and determined a required exposure time of at least 16 hours – a period of time equal to approximately one cell cycle for A2780 cell line. Given that these cells are asynchronous, it is, therefore, likely that the molecular target must be inhibited during the appropriate phase of the cell cycle in order to cause

growth arrest in the cells. In addition, we also evaluated the ability of CYC202 to induce cell cycle arrest and apoptosis in the human lung tumour cell line A549. Information on cell cycle distribution of the cells was obtained from DNA content analysis of ethanol-fixed, propidium iodide stained, A549 cells following treatment. The major effect observed following 24 and 48 hours exposure to 50 or 100μM CYC202 was the accumulation of a sub-G1 population of cells, indicative of induction of apoptosis. In addition, CYC202 caused accumulation of cells in the G2/M phases of the cell cycle at both concentrations investigated.In this study, the induction of apoptosis by CYC202 was quantified by DNA content analysis. Cells with a DNA content that is less than that of a cell in G1 (termed sub-G1 peak analysis) were scored as apoptotic. In a separate experiment the appearance of sub-G1 peaks was confirmed to be representative of apoptosis by TUNEL analysis of replicate samples. Apoptosis was detectable at 24 h in CYC202-treated cells. 48 hour exposure resulted in a small further increase in the proportion of apoptotic cells. TUNEL analysis also demonstrated that apoptotic cells (i.e. TUNEL positive) were produced from each of the cell cycle compartments. This indicates that the previously described G2 accumulation is not a result of selective induction of apoptosis from the other compartments (G1 and S) and is likely to be caused by inhibition of one or more cell cycle regulators.

Schutte et al. (1997) [105] studied the effect of roscovitine and its analogue olomoucine on cell cycle kinetics in the non-small cell lung cancer line MR65 and the neuroblastoma cell line CHP-212. The cells were pulse-labelled with bromodeoxyuridine, and were then incubated with roscovitine (10-40μM) or olomoucine (10-200μM). After being harvested at intervals the cells were subjected to irnmunocytochemical staining for bromodeoxyuridine and treatment with propidium iodide, followed by flow cytometric analysis. Dose-dependent inhibition of both the G1-S and G2/M-G1 phase transitions was observed, as well as slowed progression through S-Phase with both CDK inhibitors, although roscovitine was approximately 10-fold more potent than olomoucine. At the highest concentrations tested, both inhibitors induced a complete cell cycle block in both cell lines and causeddetachment of the cells from the

culture dish, membrane blebbing, chromatin aggregation/condensation/fragmentation, and the disintegration of the nucleus, suggesting that apoptosis had been induced. Levels of CDK1 were reduced by treatment with the CDK inhibitors as measured by western blotting.

Alessi et al. (1998) [106] investigated the effects of roscovitine, olomoucine and iso-olomoucine on the cell cycle of normal human fibroblasts in cell culture. Roscovitine and olomoucine (but not iso-olomoucine) induced a dose-dependent arrest in the G1-phase, which was reversible upon removal of the inhibitors. Western blot and immunohistochemical analyses of proliferating cell nuclear antigen (PCNA) and Cyclins D1 and E showed that levels of these were unaffected by treatment with either CYC202 orolomoucine. Treatment with the CDK inhibitors caused a reduction in the level of hyper-phosphorylated forms of the Rbprotein. Immunoprecipitated CDK2 from roscovitine or olomoucine treated cells had reduced activity compared with control; unlike CDK4, whose activity, when immunoprecipitated from treated cells, remained unchanged. Overall, the authors suggested that their experimental results pointed to CDK2 being the cellular target of the roscovitine and olomoucine.

Yakisichet al. (1999) [107] have reported the effects of roscovitine on DNA synthesis in tissue samplestaken from eight human malignant gliomas. Immediately, following resection, the tissue samples were cut into small fragments and incubated in culture medium in the presence of roscovitine (1-100µM). DNA synthesis was estimated by measuring the incorporation of tritiatedmethyl-thymidine. Roscovitine was found to inhibit DNA synthesis in a dose-dependent manner, an effect which was evident within 30 minutes from the start of the incubation. The highest concentration tested (100µM) inhibited DNA synthesis in the tissue fragments by 71-97% (mean 89%) in comparison with the control.

Iseki et al., (1997) [108] studied the ability of roscovitine and olomoucine (an analogue ofCYC202) to block CDK1 and CDK2 activity in the human gastric cancer cell line SUA, and inhibit the proliferation of this cell line

and three other human gastric cancer cell lines (AGS, MKN45-630 and SNU-I) in cell culture. Both roscovitine and olomoucine blocked inhibition of Histone H1, a proxy for CDK1 and CDK2 activity in SIIA cells, induced a large increase in the proportion of cells in the G2/M and S phases of the cell cycle, and also inhibited the proliferation of all four gastric cancer cell lines tested. The same group carried out a similar study using four human pancreatic cancer cell lines with a variety of genetic alterations (BxPC3, PANC-I, Capan-2 and CAV), and showed that both roscovitine and olomoucine blocked CDK2 activity in all four pancreatic cell lines and inhibited cell proliferation [109] in a dose dependent manner, independent of the K-Ras, p53 or p16 status of the cell lines, and thus suggested that CYC202 represented potential new therapeutics for pancreatic cancer.

Mgbonyebi et al, (1998) [110] investigated the effects of roscovitine on proliferation of immortal and neoplastic human breast epithelial cells in cell culture. The human breast epithelial cell lines studied were MCF-7 (oestrogen receptor-positive carcinoma), MD-MB-231 (oestrogen receptor-negative carcinoma) and MCF-10F (immortalised oestrogen receptor-positive). Treatment with roscovitine (1-40μg/mL) resulted in a dose and time-dependent inhibition of the proliferation of all three cell lines, suggesting a role for CYC202 in the treatment on both ER-ve and ER+ve breast tumours. Further studies by the same group on the nature of the inhibition in the MD-MB-231 cell line revealed that treatment with roscovitine (10μg/mL for 24–240 hours) induced cell death and morphological changes, such ascell shrinkage, chromatin condensation, changes in actin microfilament architecture, and extensive detachment of cells from the cell culture substratum, consistent with the induction of apoptosis [111].

In addition to its cytostatic and pro-apoptotic actions,roscovitine has also been reported to induce cell differentiation. In a study using the human non-small cell lung cancer cell line NCI-H348 [112] induction of mucinous differentiation was observed in NCI-H348 cells treated with 10μM roscovitine for 24 or 72 hours, as indicated by the development of positivity for periodic acid-schiff (PAS) staining.

The cellular effects of roscovitine have also been investigated in several non-mammalian models [99].Roscovitinehas been shown to arrest starfish oocytes and sea urchin embryos reversibly in late prophase, and showed *in vitro* inhibition of M-phase promoting factor activity and DNA synthesis in Xenopus egg extracts. The compound has also blocked progesterone-induced maturation of Xenopus oocytes, and *in vivo* phosphorylation of the elongation factor eEF-1.

Roscovitine was reported to induce apoptosis in several cell lines, regardless of p53 status; surprisingly, roscovitine was more effective in inducing apoptosis in wild type p53 cells, compared to p53 null cells. Cell death has been detected in all phases of the cell cycle, and different mechanisms may be involved, including inhibition of the cell cycle due to p53 activation and inhibition of CDK7/CDK9-dependent transcription inhibiting RNA polymerase II enzyme [113,114]. The effect of roscovitine on global transcription was shown to be limited, and only few proteins, including Mcl-1, XIAP, and survivin, were found to be reduced. Thus, cell death caused by roscovitine seems to be correlated rather well with inhibition of transcription of essential cell survival factors [115, 116]. It has been shown that downregulation of survivin and XIAP by roscovitine contributes to the activation of caspases in glioma cells. Alvi et al. [117] have reported that roscovitine induced apoptotic cell death in chronic lymphocytic leukemia B-lymphocytes at significantly higher levels, compared to that observed in normal blood mononuclear cells, purified B- or T-lymphocytes. The apoptosis process was shown to be caspase-dependent, but p53-independent, and was accompanied by downregulation of Mcl-1 and XIA [118].

The antitumor effects of roscovitine are mainly based on anti-proliferative and pro-apoptotic mechanisms; moreover, no resistance to roscovitine therapy has been reported so far [119].

Interestingly, tumor cells are more dependent on short-lived survival factors than normal cells. Thus, downregulation of these factors by roscovitine treatment has higher impact on tumor cells than on normal cells [120]. Roscovitine showed synergistic effects in combination with

other chemotherapeutic agents *in vitro*, e.g. camptothecin in MCF-7 breast tumor [121], histone deacetylase (LAQ824) in HL60 and Jurkat leukemic cells, doxorubicin in sarcoma cell lines [122] and irinotecan in p53-mutated colon cancer [123].

6. Anti-tumour activity of CYC202 *in vivo*

Roscovitine has been examined in a variety of cell lines from a number of different tissue types for anti-tumour activity in mouse xenograft models. Its anti-tumour activity is best described as mild, with reductions in tumour cell growth observed, rather than reductions in initial tumour cell volume. The compound has been delivered both orally and via intra-peritoneal injection, and a number of doses and dosing schedules have been employed. McClue and co-workers used the uterine cell line MES-SA/Dx5 and the colon cell line LOVO, which had been most sensitive to CYC202 in an *in vitro*, 19 member cell line panel. Mice bearing established tumours formed from LOVO were dosed with 100mg/kg CYC202 three times daily by intra-peritoneal injection for 5 days, and tumour growth was followed for 32 days post initiation of dosing, at which point CYC202 treated animals bore tumours that were approximately 55% of the volume of those of the control animals [100]. In another study mice bearing MES-SA/Dx5 tumours were dosed with either 200mg/kg or 500mg/kg of CYC202 by intra-peritoneal injection three times per day for either 10 days or 4 days, respectively. Again anti-tumour activity was mild with tumour growth inhibited by 35% at the 200mg/kg dose and by 62% at 500mg/kg [100].

Most other studies using roscovitine as a single agent have seen similar anti-tumour activities in xenografts using cell lines of colon, lung, brain, breast and nasopharangeal origin [124–128]. Interestingly, a study dosing mice bearing an Ewing's Sarcoma cell line A4573 with only 50mg/kg of roscovitine once per day has shown the most striking single-agent anti-tumour activity, inhibiting tumour growth by 85%, when compared to untreated animals [129]. Our results and data form other laboratories on anti-tumour activity of CYC202 *in vivo* are summarised in Table 3.

Table 3. In vivo anti-tumour activities of Roscovitine in xenografts.

Cell Line	Tissue	Dosing Route	Dose (mg/kg)	Dose Frequency	Period of Dosing (Days)	Tumour Volume (T/C)%	Reference
LOVO	Colon	i.p.	100	t.i.d.	5	55	McClue et al 2002
MES-SA/Dx5	Uterus	oral	200	t.i.d.	4	65	McClue et al 2002
MES-SA/Dx5	Uterus	oral	500	t.i.d.	10	38	McClue et al 2002
MDA-MB-231	Breast	i.p.	100	Single	1	-	Maggiorella et al 2003
HCT-116	Colon	oral	500	b.i.d.	5	65	Raynaud et al 2005
A4573	Sarcoma	i.p.	50	q.d.	5	ca.15	Tirado et al 2005
H358	Lung	i.p.	50	b.i.d.	20 in 28	-	Fleming et al 2008
C666-1	Naso-Pharangeal	i.p.	50	b.i.d.	10 in 12	ca.75	Hui et al 2009
C15	Naso-Pharangeal	i.p.	50	b.i.d.	10 in 12	ca.50	Hui et al 2009
MCF7-HER2	Breast	oral	100	t.i.d.	10	ca.45	Nair et al 2011
MCF7-TamR	Breast	oral	100	t.i.d.	10	ca.60	Nair et al 2011
MCF7-LTLTca	Breast	oral	100	t.i.d.	10	ca.60	Nair et al 2011
GBM43	Brain	i.p.	50	q.d.	12	ca.60	Cheng et al 2012

Roscovitine has also been tested for anti-tumour activity in xenograft models in combination with ionising radiation and other small molecule kinase inhibitors, with sometimes impressive results (Table 4). Most striking is the anti-tumour activity seen with the combination of 50mg/kg roscovitine dosed twice per day for 5 days by intra-peritoneal injection, followed by a two day break, and a further 5 days of dosing in combination with a dose of 6Gy ionising radiation twice in the same dosing period. This treatment caused a reduction in tumour growth of

around 95% in two Epstein-Barr Virus (EBV) positive cell lines derived from nasopharyngeal carcinomas [126]. Maggiorella and co-workers also utilised ionising radiation in combination with roscovitine, and achieved an 80% reduction in tumour growth, compared to untreated animals over the 30 days following treatment [130].

In combination with other small molecule kinase inhibitors roscovitine has produced tumour growth inhibitions of around 75-93%, compared to untreated animals. Used together with erlotinib to treat mice carrying H358 (non-small cell lung cancer) tumours, Fleming and co-workers report that, when CYC202 was dosed at 50mg/kg twice per day for 5 days on / 2 days off for 28 days, and erlotinib was dosed orally, daily at 100mg/kg for all of the 28 days treatment period, tumour growth was reduced by 93% 7 weeks after the treatment was started. In the same study CYC202 alone, when dosed using the same schedule, failed to significantly inhibit tumour growth, and erlotinib inhibited only to 56% of untreated control, indicating some synergistic activity between the molecular targets of CYC202 and erlotinib.

Roscovitine has also been used successfully in combination with the small molecule PI3-Kinase inhibitor PIK-90 to inhibit growth of GBM43, glioma derived tumours, in immunodeficient mice. Dosedby intra-peritoneal injection at 50mg/kg, four times daily for 12 days together with PIK-90 four times per day at 40mg/kg, tumour volume was about 25% of that in untreated animals. Roscovitine when used as a single agent, inhibited growth by about 40% in the same model.

Table 4. In vivo anti-tumour activities of Roscovitine combinations in xenografts.

Cell Line	Tissue	Dosing Route	Dose (mg/kg)	Dose Frequency	Period of Dosing (Days)	Combinant	Dose	Dose Frequency	Period of Dosing (Days)	Volume (T/C)%	Reference
MDA-MB-231	Breast	i.p.	100	Single	1	Ionising Radiation	7.5 Gy	1	1	20	Maggiorella et al 2003
H358	Lung	i.p.	50	b.i.d.	20 in 28	Erlotinib	100 mg/kg	q.d.	28	7	Fleming et al 2008
C666-1	Naso-Pharangeal	i.p.	50	b.i.d.	10 in 12	Ionising Radiation	6 Gy	1	2 in 12	ca.5	Hui et al 2009
C15	Naso-Pharangeal	i.p.	50	b.i.d.	10 in 12	Ionising Radiation	6 Gy	1	2 in 12	ca.-5	Hui et al 2009
GBM43	Brain	i.p.	50	q.d.	12	PIK-90	40 mg/kg	q.d.	12	ca.25	Cheng et al 2012

7. Pharmacokinetics and pharmacodynamics of roscovitine

7.1. Pharmacokinetics and metabolism of roscovitine

The pharmacokinetics of roscovitine was studied in mice, rats and humans. Vita et al. [131] described roscovitine PK in rat as a two-compartment open model with a short elimination half-life of <30 min (Table 5).

Table 5. Pharmacokinetics of roscovitine in rats following i.v. administration at a dose of 25 mg/kg.

AUC: Area Under the Curve; T½-α: the distribution half-life; T½/ T½-β: the terminal elimination half-life; CL: Clearance; Vd: Distribution Volume

	Plasma	Brain	Muscle	Testis	Spleen	Kidney	Adipose	Lung	Liver
AUC (µg/ml x min)	169.3	45.7	120.8	133.9	163.6	466	673.4	747.8	830.7
T½-α (min)	5.8	1.71						1	
T½ / T½-β (min)	26	12.4	11.6	21.8	14.5	15.2	27.4	22.3	14.4
Cl (ml/min)	29.5	109.5	41.4	37.3	30.6	10.8	7.4	6.7	6
Vd (L/kg)	3.45	4.3	3.5	5.9	3.2	1.2	1.5	0.3	0.03

The distribution of roscovitine was highest in the lungs, followed by liver, fat, and kidney, while exposure to roscovitine in the brain was 30% of that observed in plasma (Figure 6).

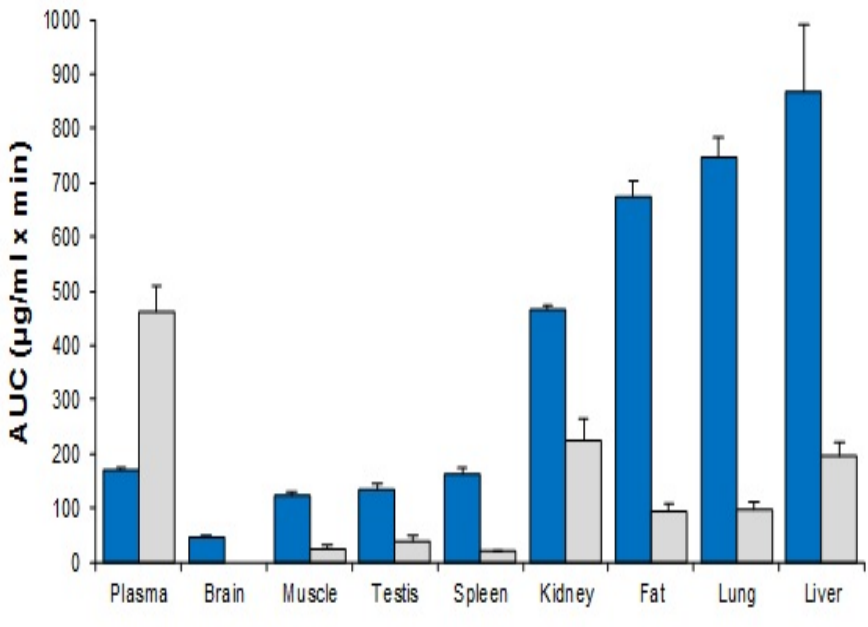

Figure 6. Organ exposure to roscovitine (■) and it major metabolite (■) in rats after bolus administration of 25 mg/kg body weight expressed as AUC ± SD.

Three major metabolites were detected in plasma (Figure 7).

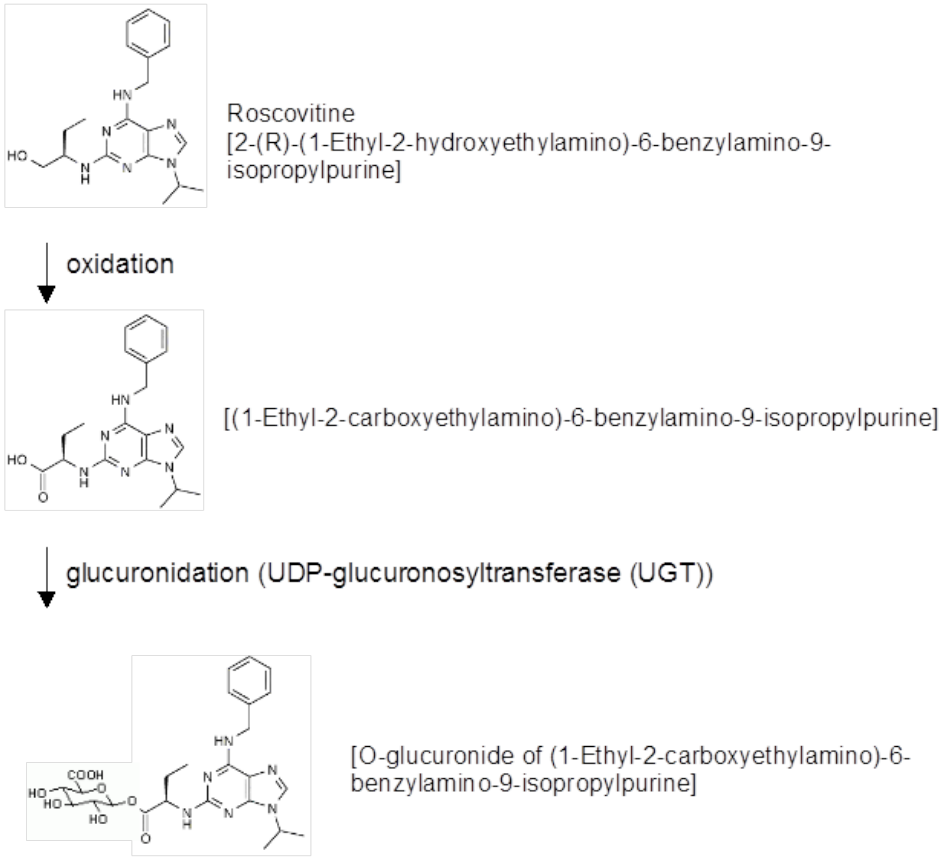

Figure 7. Roscovitine metabolites detected in rat plasma.

The first metabolite is the major metabolite. It is a carboxylated form of roscovitine, while the second metabolite is the glucuronide form of metabolite one. No metabolites were detected in the brain (Figure 8) [131, 132]. High protein binding of roscovitine (92% to 96%) was shown in human and mouse plasma [124, 133].

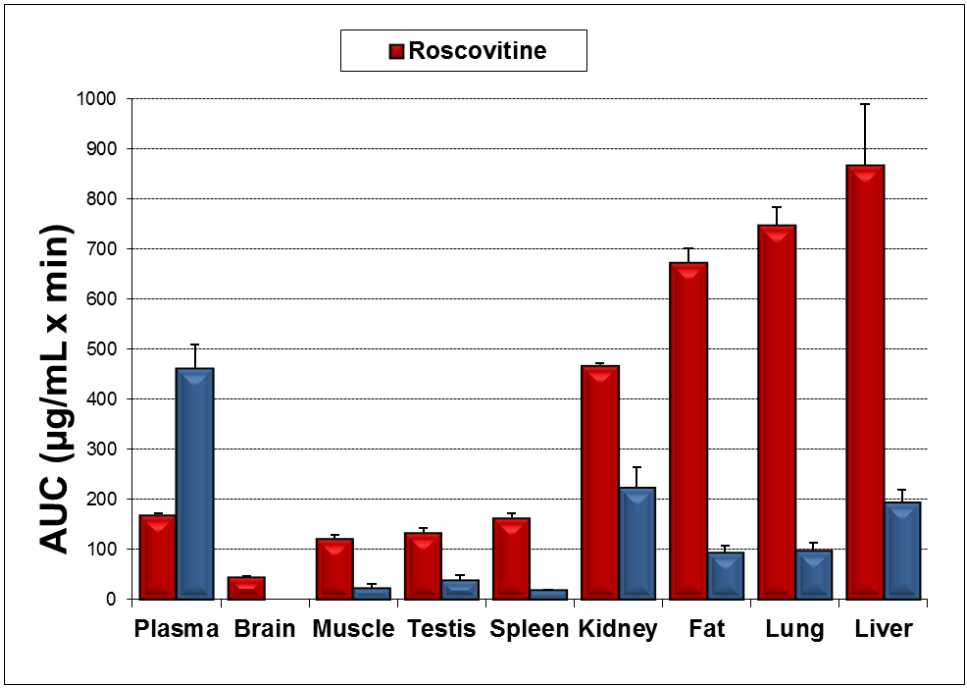

Figure 8. The distribution of roscovitine and it's major metabolite in different organs of the rat after the administration of a single dose of 25 mg/kg.

Pharmacokinetics of roscovitine was investigated in BALB/c and Tg26 mice. These studies showed rapid and biphasic clearance of roscovitine from plasma following i.v., i.p. or oral administration [124, 134, 135]. Roscovitine had rapid tissue distribution and rapid elimination with a half-life of 1.19hr. Plasma concentrations above 15µM (the average IC_{50}values obtained with various tumor cell lines) were observed for 4, 12, and 24hr following oral administration of 50, 500, and 2000mg/kg, respectively [124].

The pharmacokinetics of roscovitine in humans were reported in two phase-I trials. Roscovitine was administered orally as a single dose (50 to 800mg) to healthy volunteers and the concentrations of roscovitine and its carboxylated metabolite were followed in plasma and urine. Roscovitine

was found to undergo slow absorption from the gastrointestinal tract (GIT); however, the bioavailability of the drug was not affected by food intake. Roscovitine was found to have rapid metabolism and non-saturated high protein binding [136].

Twenty-one patients with a median age of 62 years (range: 39–73 years) were treated with roscovitine in doses of 100, 200 and 800mg twice daily for 7 days. The elimination half-life was found to be dose dependent and ranged between 2–5hr. Neither objective tumor responses nor inhibitions of retinoblastoma protein phosphorylation (PD endpoint) in mononuclear cells in peripheral blood were observed[137].

In vitro and *in vivo* metabolism of roscovitine was reported recently[131, 135, 138, 139]. Several metabolites were identified, including the carboxylate metabolite (oxidation of the alcohol group at C2 of the purine ring)[131]. Cytochrome P450, mainly CYP3A4 and CYP2B6 enzymes, has been shown to metabolize roscovitine. Roscovitine was found to undergo phase II metabolism through conjugation with glucoronic acid by the phase II UGT1A3, 1A9 and 2B7. Moreover, roscovitine was able to inhibit its own metabolism *in vitro* through inhibition of CYP3A4 with an IC_{50} of 3.2µM. Thus, possible drug-drug interactions should be considered in the clinic[139].

7.2. Chronopharmacology of roscovitine

Treatment effect of roscovitine in non-nude BDF1 male mice bearing Glasgow osteosarcoma xenografts was investigated in relation to biological circadian rhythm. Roscovitine was administered orally (300 mg/kg ×1 daily) for 5 days at Zeitgeber time 3 (ZT3, 3 hours after light onset), or ZT11, or ZT19. Roscovitine reduced tumor growth by 35% when administered during the active time of the mice (ZT19), and 55% when administered during their rest span (ZT3 or ZT11) [140].

7.3. Pharmacokinetics and pharmacodynamics of roscovitine in mouse bone marrow

Myelosuppression is the dose-limiting factor for the majority of conventional chemotherapeutic anticancer agents and one of the most frequent complications. Chemotherapy may induce partial or complete myeloablation of the bone marrow, which in general is dose dependent. Studies on hematotoxicity *in vitro* and in animal models help to predict possible side effects before the start of clinical trials.

In a recent study, we investigated the myelosuppressive potential of roscovitine on bone marrow cells *in vitro* and *in vivo* in Balb/c mice. Bone marrow was incubated *in vitro* with roscovitine at concentrations of 25–250μM for 4hr and viability was studied using resazurin assay. Bone marrow cell viability was decreased in a concentration-dependent manner. At a concentration of 250μM, cell viability was significantly ($p=0.015$) reduced to 70%, compared to control mice, while significant effect was observed at lower concentrations. These results were in agreement with the findings that roscovitine induced apoptosis of mature neutrophils [141], eosinophils [142]. and proliferating T-cells [143] in a concentration and exposure-time dependent manner.

The myelosuppressive effect of roscovitine on hematopoietic progenitors was studied using clonogenic assay[144]. Bone marrow cells were incubated with roscovitine (25–100μM) for different time intervals up to 24hr. After washing, the capacity of hematopoietic progenitors to form colony-forming unit granulocyte/macrophage (CFU-GM), burst-forming unit erythroid (BFU-E), and colony-forming unit granulocyte/erythrocyte/macrophage/megakaryocyte (CFU-GEMM) colonies was studied using semisolid media. The clonogenic capacity of the bone marrow decreased in a dose- and time-dependent manner. BFU-E colonies were more sensitive compared to CFU-GM. BFU-E were completely blocked after 12 and 24hr incubation with both 50 and 100μM of roscovitine. Since the decrease in colony formation in controls after 12 and 24hr most probably is due to lack of growth factors in the suspension media, bone marrow cells were exposed to roscovitine (1–100μM) in semisolid MethoCult media containing growth factors. Suppression of

colony formation in a concentration- and cell type-dependent manner was observed. CFU-GEMM were most sensitive and were completely blocked at 25µM concentration, followed by BFU-E which were also significantly inhibited at 25µM, while CFU-GM were least sensitive and were inhibited at 100µM only.

We further studied the myelosuppressive effect of roscovitine *in vivo* in female Balb/c mice. Mice were treated with roscovitine, and bone marrow cells were cultured in the MethoCult media and assessed for clonogenic growth. After the administration of a single dose of roscovitine up to 250mg/kg, no myelosuppressive effect was detected. However, the administration of roscovitine at 175mg/kg twice daily for 4 days resulted in only transient inhibition of the BFU-E colonies, which was observed one day after the last dose of roscovitine. Colony formation capacity of bone marrow was recovered 5 days after the last dose of roscovitine (Figure 9).

The lack of activity of roscovitine on hematopoietic progenitors *in vivo* was not expected, since an inhibitory effect *in vitro* was observed, along with the reported activity on different xenografts *in vivo*[100,124, 129,140]. Therefore, we decided to study the distribution and PK of roscovitine in Balb/c mice. Roscovitine was administered as a single i.p. injection in a dose of 50 mg/kg. As presented in Table 5, roscovitine had a short half-life (less than 1hr), and only a small fraction of roscovitine (about 1.5%) reached the bone marrow compared to plasma. Thus, low distribution of roscovitine to bone marrow may explain the low hematotoxicity *in vivo*. This study is an excellent example illustrating the importance of PK/PD and biodistribution investigations in preclinical studies. This may also show that, despite the good cytotoxic effect of roscovitine in leukemic cell lines *in vitro*, its therapeutic potential in hematological malignancies may be rather limited.

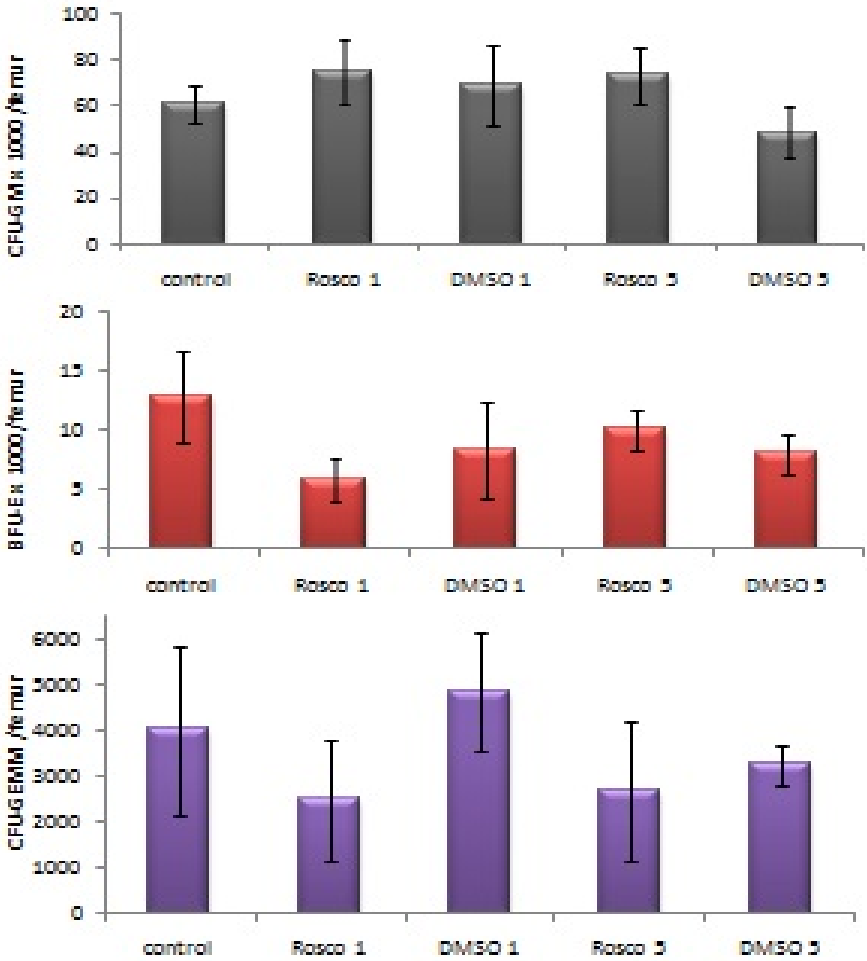

Figure 9. Effect of roscovitine on hematopoietic progenitors in vivo. Mice were treated with roscovitine (350 mg/kg/day) for 4 days divided into two daily doses. Mice treated with DMSO and untreated animals served as controls. Bone marrow was examined 1 and 5 days after the last dose of roscovitine. CFU-GM, BFU-E and CFU-GEMM were counted on day 12. Each group consisted of five mice, and the control group of eight mice. Results are expressed as mean ± SD.

7.4. Age-dependent kinetics and pharmacodynamics of roscovitine in rat brain

In the field of pediatric medicine, especially when the drug has a narrow therapeutic window, age-dependent pharmacokinetics becomes an important issue. Unfortunately, scaling down the PK data from adults to pediatrics has proved not to be predictive enough for many drugs. [145] Roscovitine was reported to inhibit different solid and hematological tumor cell lines, including ALL[146], which is frequent in children and is correlated with a high CNS relapse rate [147].

A recent study reported the effects of age on the pharmacokinetics of roscovitine and investigated the effect of roscovitine on two neuronal targets, CDK5 and Erk1/2, in different regions of the brain [148]. Fourteen-day-old pups and adult Sprague-Dawley rats were treated with a single i.p. injection of roscovitine at a dose of 25mg/kg. Plasma and brain were sampled at different time points. The pharmacokinetic parameters of roscovitine in plasma and in different brain regions in pups and adult rats are given in Table 6. The pharmacokinetics of roscovitine was fitted to a 2-compartment open model, with distribution half-lives of 0.6hr in pups and 0.06hr in adult rats. The elimination half-life (7hr) observed in plasma and brain of the rat pups was significantly longer compared to that observed in plasma and brain in adult rats (30 and 20 min, respectively).

Table 6. Pharmacokinetics of roscovitine in rat plasma and brain parts after single i.p. administration (25mg/kg). Abbreviations: AUC, area under the concentration-time curve; T_{α}, absorption half-life; T_{β}, elimination half-life; C_{max}, maximum reached concentration; V_{ss}, volume of distribution at steady state; CL, clearance.

PK parameters	Plasma	Frontal Cortex	Hippocampus	Cerebellum
AUC (h.µg/ml)	3.01 ± 0.21	0.71 ± 0.14	0.58 ± 0.03	0.62 ± 0.06
T_{α} (h)	0.081 ± 0.05	0.045 ± 0.02	0.062 ± 0.012	0.062 ± 0.018
T_{β} (h)	0.54 ± 0.26	0.35 ± 0.13	0.36 ± 0.15	0.42 ± 0.18
C_{max} (µg/ml)	17.71 ± 4.42	4.47 ± 0.70	4.64 ± 0.81	3.81 ± 1.22
V_{ss}(ml)	650 ± 223	1095 ± 167	2056 ± 219	1909 ± 484
CL (ml/h)	1637 ± 118	7262 ± 1612	8737 ± 452	8139 ± 727

The exposure to roscovitine expressed as AUC was 22-fold higher in pup plasma and 100-fold higher in pup brain tissue, compared to that seen in adult rats (Figure 10). However, no significant difference was observed between roscovitine AUC in plasma and AUCs in different brain regions in pups. On the contrary, in adult rats the AUC of roscovitine in the brain was about 25% of that found in plasma (Table 6). The maximum observed concentration (c_{max}) was significantly ($p<0.05$) higher (>22µg/g) in pup

brain tissue, compared to that found in plasma. However, 4-fold higher c_{max} was found in plasma, compared to that observed in the brain (17.7µg/ml and about 4µg/g, respectively) of adult rats. The high concentrations of roscovitine found in pup brain tissue indicate that roscovitine passes freely through the blood brain barrier (BBB).

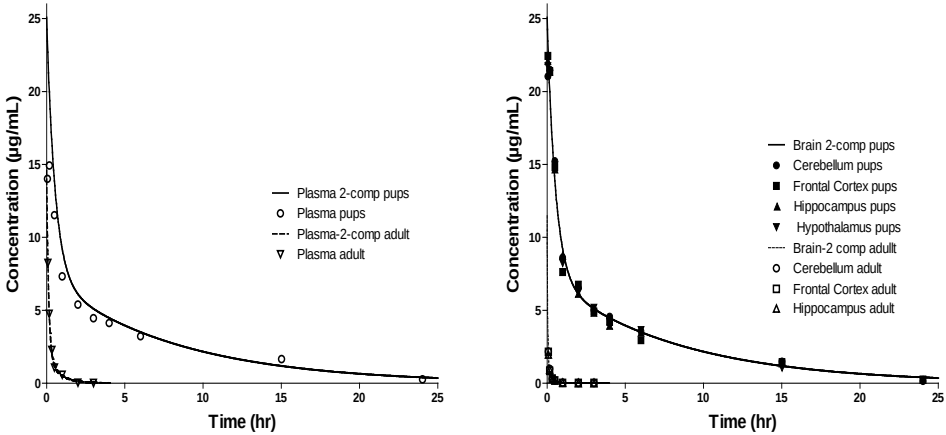

Figure 10.Concentration versus time curves of roscovitine after fitting to a two-compartment open model in the plasma (to the left) and different brain regions (to the right) in 14-day-old rat pups and adult rats following single i.p. administration of 25 mg/kg. The solid and dashed lines represent the theoretical model.

This difference in roscovitine kinetics might be due to the immaturity of the CYP450 enzymes responsible for roscovitine metabolism [149] or to the immaturity of the BBB. Roscovitine is metabolized mainly by the CYP3A4 and CYP2B6 enzymes in human [139]. Several CYP450 enzymes are not fully matured in rats at two weeks of age [150]. A similar situation was also reported in humans; CYP3A4, for example, only approaches full maturity after the first year of life [151, 152].

Most chemotherapeutic agents do not cross the blood brain barrier (BBB) and do not reach the CNS in high enough concentrations to eliminate tumor cells despite high systemic exposure. Roscovitine was highly presented in the brain of rat pups, and the exposure was observed in all studied regions (e.g. hippocampus, cerebral cortex and cerebellum). The brain exposure to roscovitine was 100% of that found in plasma, which can be compared to about 25% of that found in the brain of adult rats. The high distribution to the brain could be explained by an age-dependent variation in the maturity and function of the BBB. Butt et al. have reported that the BBB of the rat reaches full maturity 3–4 weeks after birth. [153] Roscovitine metabolites were not found in the brains of adult or young rats, probably due to their hydrophilic character.

Roscovitine concentrations in plasma and brain of rat pups were higher than the IC_{50}(10–15µM) reported for cancer cell lines for more than 8 hours. However, this level of exposure was achieved only for short time (<30 minutes) in plasma and brain of adult rats. These results may be utilized in the treatment of pediatric brain tumors.

Roscovitine has been reported to be a potent inhibitor of CDK5 that has an important function in the developing brain, such as neuronal migration [11]. Moreover, the negative feedback regulation of mitogen activated protein kinases (MAPK) signaling by CDK5 was suggested to be essential for neuronal survival [154].

The unexpected high concentrations of roscovitine in the brain of rat pups led us to assess the expression of p35 as an indicator of CDK5 activity. It is well known that the inhibition of p35 phosphorylation by CDK5 stabilizes it and delays its proteasomal degradation [155, 156]. We found that roscovitine induces a transient and significant accumulation of p35 protein in all brain regions in rat pups, which indicates inhibition of the CDK5 enzyme. Furthermore, increase in p35 was found in the frontal cortex about 1–2hr post-administration (140% in controls; Figure 7;p <0.05), in the hippocampus and in the cerebellum at 2hr post-administration (150% and 200%, respectively; Figure 11). The levels of p35 reached the normal level at 6–15hr post-administration. Surprisingly, no change in p35 levels was

observed in the brain of adult rats, which is most probably due to the low concentration and the short elimination half-life.

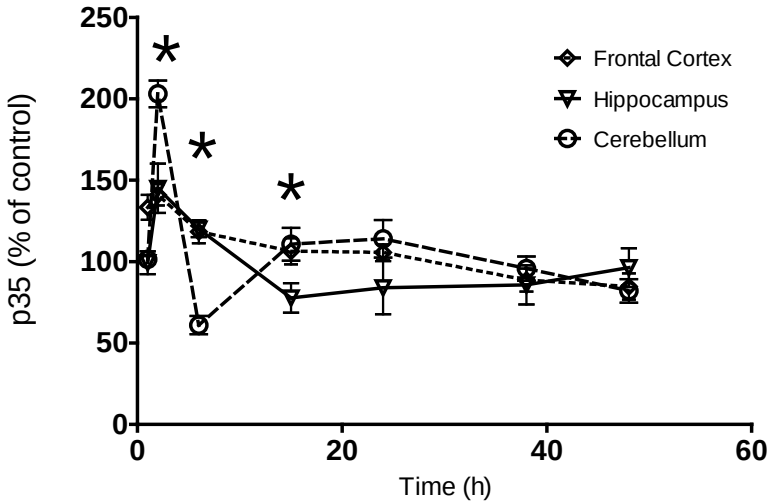

Figure 11.Effect of roscovitine on Cdk5-p35 in different brain parts of 14-day-old rat pups after single i.p. injection of 25 mg/kg. Pups were sacrificed at different time points after injection, and their brains were dissected, homogenized, and immunoblotted for active Cdk5-p35. The figure shows densitometric analysis of the Western blotting bands for p35 in the frontal cortex, hippocampus and cerebellum until 48 hr after single i.p. injection of roscovitine. Data are presented as mean± SD of values expressed as percentage of control animals (*, $p < 0.05$ for analysis of p35 data; ANOVA followed by Fisher's PLSD post-hoc test).

CDK5 was found to inhibit Erk1/2 phosphorylation by a MEK1 and RasGRF2 mediated mechanism. Moreover, it was reported that the inhibition of CDK5 by roscovitine increased the levels of phosphorylated Erk1/2 (active form) in neuronal cells *in vitro*[154, 157]. Soon after the administration of roscovitine, the accumulation of p35 protein was accompanied by increased levels of the phosphorylated (activated) form of

Erk1/2. A transient activation of Erk1/2 was observed at 1 and 2hr after administration in the frontal cortex and hippocampus (Figure 8). In the cerebellum, a significant increase of pErk1/2 levels at 2hr was followed by a significant decrease at 6hr after roscovitine administration (Figure 12). The levels of pErk1/2 returned to normal levels in all brain regions at later time points (Figure 12).

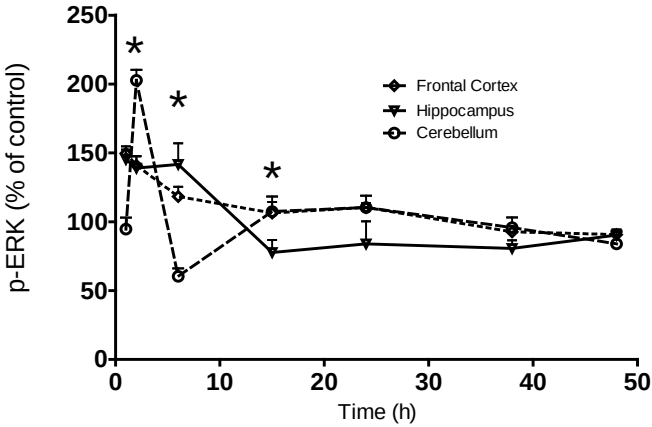

Figure 12. Effect of roscovitine on pErk in brain regions of 14-day-old rat pups after single i.p. injection of 25 mg/kg. Pups were sacrificed at different time points post injection. Their brains were dissected, homogenized, and immunoblotted for active phosphorylated Erk1/2. Control animals were injected with vehicle. The figure shows densitometric analysis of the Western blotting bands for pErk1/2 in the frontal cortex, hippocampus and cerebellum until 48 hr after single i.p. injection of roscovitine. Data are presented as mean± SD of values expressed as percentage of control animals (*, p < 0.05 is the significant level for analysis of pErk data; ANOVA followed by Fisher's PLSD post-hoc test).

These results show that roscovitine levels in the brain of rat pups were sufficient to inhibit CDK5, resulting in increased phosphorylation of Erk1/2.

7.5. Summary and future outlook

Cyclin-dependent kinases (CDKs) are essential kinases and play a key role in cell cycle progression and RNA transcription. The deregulation of CDKs has been described in several diseases, including cancer. The cyclin dependent kinase inhibitors (CDKIs) are synthetic small heterocyclic compounds that compete with ATP and inhibit the phosphorylation of the target substrates. Based on this knowledge, exposure of tumor cells to CDKIs will result in both cell cycle arrest and apoptosis.

2,6,9-trisubstituted purines are among the first described CDK inhibitors [158]. The (R)-stereoisomer of roscovitine is a member of this family that recently reached phase-II clinical trials for nasopharyngeal cancers and non-small cell lung cancer (NSCLC), as well as phase-I trials for glomerulonephritis. Several preclinical studies about the role of roscovitine in the treatment of neurodegenerative disorders, e.g. Alzheimer's disease, viral infections, protozoal infections, and inflammatory diseases, are ongoing. The poor pharmacokinetic profile (rapid metabolism and short elimination half-life in rodents and man) and the insufficient exposure to the drug in cancer patients may explain the modest success in clinical trials[124, 131, 135-137]. Several attempts to overcome pharmacokinetic barriers that limit the clinical use of roscovitine are ongoing. At the same time, second-generation analogues of roscovitine have been designed and are under development. Studies on the pan-CDK inhibitor, flavopiridol, confirmed the importance of optimizing the dosing schedule according to the PK/PD relationship. By changing the dosing schedule from 72hr infusion to 30 minutes i.v. bolus followed by 4-hr-infusion a significant difference in the clinical outcome and final response of refractory CLL patients was achieved[159].

The absence of myelosuppression, reported in preclinical and clinical studies of roscovitine [134, 137], is clinically beneficial. However, low hematotoxicity of roscovitine might in reality reflect poor distribution of roscovitine to the bone marrow. The hematopoietic progenitors were inhibited by roscovitine *in vitro* within the same exposure range as the tumor cells, when comparing the inhibitory AUC reported for tumor cell lines [100,124] with the inhibitory AUC of the hematopoietic progenitors

found in our study. The hematotoxicity of roscovitine may become more evident under the following conditions:

1. Changes of the administration form to increase the half-life of the drug may result in changes in biodistribution and higher exposure to roscovitine, and thus change its toxicity profile.
2. Radiation therapy increases the permeability of the blood-bone marrow barrier [160]. A combination of roscovitine with radiotherapy may increase the myelotoxicity of roscovitine.
3. Higher bone marrow exposure to roscovitine and thus a higher toxicity risk may occur in pediatric patients due to age-dependent pharmacokinetics [151].

Age-dependent pharmacokinetics is not a unique factor for roscovitine. Age-dependent pharmacokinetics has been reported for several drugs, including cisplatin, busulfan, thioguanine, etoposide, lamivudine, and mycophenolate mofetil [161-166]. Our studies showed that roscovitine elimination half-life was 14-fold higher in young rats compared to adults. Moreover, the exposure to roscovitine was 22-fold and 100-fold higher in plasma and brain, respectively. Age-dependent pharmacokinetics is an important issue in toxic drugs and drugs with a narrow therapeutic window, such as anticancer drugs. In these drugs, underdosing may lead to relapse, while overdosing can cause severe side effects.

8. Clinical results from investigation of Seliciclib in clinical trials

To date, CYC202 has been evaluated in several phase I and II studies sponsored by Cyclacel Pharmaceuticals Inc. as Seliciclib, and has shown signs of anti-cancer activity in approximately 240 out of 450 patients.

In a phase I trial Seliciclib was used to treat 21 pre-treated patients with refractory solid tumours. Dosing was started at 100mg twice per day for 7 days in a 3 week cycle and dose was escalated 100% after at least 3 patients had been treated at a given dose. Dose-limiting toxicities were seen with the 800mg dose schedule and included fatigue, rash, hyponatraemia, and hypokalaemia, with other reactions seen, including

increased creatinine, vomiting, and indications of abnormal liver function. Pharmacokinetic analyses showed that Seliciclib reached a maximum plasma concentration between 1 and 4 hours post dosing and that elimination half-life was between 2 and 5 hours. Stable disease was observed in 8 patients, including an ovarian cancer patient who was stable over 18 weeks of treatment. No tumour responses were observed [137].

In another phase I trial dosing Seliciclib orally as a single agent, 56 patients were dosed while examining 3 different dosing schedules. Schedule A dosed Seliciclib twice per day for 5 days every 3 weeks, schedule B dosed twice-daily for 10 days followed by a 2 week break, and schedule C dosed twice-daily for 3 days every 2 weeks. Dosing was initiated at 100mg Seliciclib twice per day with dose incremented up to 1600mg Seliciclib twice per day in schedule A, and 1800mg twice per day inschedule C. Schedule B was discontinued, due to the appearance of toxicities at lower doses than the alternative schedules. Pharmacokinetic analysis of plasma samples showed that exposure to Seliciclib was dose-dependent. The dose-limiting toxicities experienced by patients were hypokalaemia, asthaenia, nausea, and vomiting, with other reactions seen, including increases in serum creatinine and indications of liver toxicity. A single patient with a hepatocellular carcinoma had a partial response and other patients had periods of stable disease. The recommended dose for subsequent studies was concluded to be 1250mg twice per day for 5 days in a 21 day cycle, or 1600mg of Seliciclib twice per day for 3 days in a 14 day cycle [167].

CYC202 has also been tested in a phase I trial in combination with the current first line treatment for NSCLC Gemcitabine 1000mg/m^2+ Cisplatin 75mg/m^2. A total of 27 patients were enrolled and were dosed in a 21 day cycle with current first line NSCLC therapy (Gemcitabine 1000mg/m^2i.v. (days 5 and 12) and Cisplatin 75mg/m^2 i.v. (day 5) plus oral twice per day CYC202 at either 400mg, 800mg or 1200mg (days 1–4, 8–11, 15–18, respectively). No dose limiting toxicities (DLT) were seen at the lowest dose of CYC202 from 2 patients enrolled, 3 DLTs were seen in the 800mg CYC202 cohort from 19 patients dosed, and 3 DLTs were seen in the 1200mg CYC202 cohort from 6 patients dosed. The toxicities

observed were: elevated GGT, nausea, vomiting, hypokalaemia. Haematological toxicity was low. The maximum tolerated dose (MTD) was determined as 800mg CYC202 in combination with Gemcitabine (1000mg/m^2) and Cisplatin (75mg/m^2) within the dose schedule described above [168].

Interim results are available from an initial tolerability section of a phase I clinical trial in which 23 patients with nasopharyngeal cancer and other solid tumours were dosed orally with CYC202. Patients were divided into 2 groups, one was dosed with twice-daily 400mg of CYC202 for 4 days per week for 2 weeks and the other group was dosed with 800mg of CYC202 for 4 days per week for 2 weeks. 10 patients with NPC were enrolled in this section of the trial and 7 had stable disease while on trial. Thirteen patients with other advanced solid tumours were enrolled in the tolerability and 4 of these patients had stable disease while on trial. Seven patients had progressive disease and 5 patients could not be evaluated. Dose-limiting toxicities (grade 3 increase in ALT or AST (n=3) and treatment delay of >2 weeks due to grade 1 creatinine (n=1)) were observed in 4 patients, and so both dosing regimes were considered as tolerable and could be advanced to a randomised phase of the trial, uniquely with NPC patients [169].

In another trial in nasopharyngeal cancer patients with locally advanced disease 20 patients were treated twice-daily with either 800mg or 400mg of CYC202 on days 1 to 3 and 8 to 12. Three patients were treated at the 800mg twice-daily schedule and 2 of these had dose limiting toxicities (grade 3 liver toxicity and grade 2 vomiting), none of the 13 patients dosed at 400mg twice-daily showed any significant signs of toxicity. Tumour biopsies were taken at day 1 (just prior to dosing) and on day 13 (after dosing had ceased). Fifty percent of the 14 evaluable patients showed signs of a reduction in tumour volume and were associated with increases in levels of apoptosis, necrosis, and reductions in plasma EBV DNA levels following dosing [170]. It was promising that this short treatment with CYC202 had such apparently positive outcome at the lower dosing schedule and no limiting toxicities.

Seliciclib was tested in a randomised phase II clinical trial in which 187 Non-Small Cell Lung Cancer **(**NSCLC) patients, with at least two prior treatments, were dosed with 1200mg of the drug twice per day for 3 days in a 2 week cycle. Although there was no statistically significant difference in the primary endpoint of progression free survival relative to placebo, patients treated with Seliciclib had a longer median survival (388 days compared to 218 days) and the study showed that Seliciclib was safe at the dose level and regime used in the study (Cyclacel website – www.cyclacel.com).

Phase I clinical trial with dose expansion to determine the maximum tolerated dose of liposomal Doxorubicin in combination with Seliciclib for the treatment of patients with metastatic triple negative breast cancer was initiated in 2011 (Cyclacel website – www.cyclacel.com).

Seliciclib has been entered into novel clinical trials in combination with other drugs. Phase 1 escalation trial started in 2013 of a nucleoside analogue Sapacitabine (CYC682), and Seliciclib, as an orally-administered sequential treatment regimen in patients with advanced solid tumours (Cyclacel website – www.cyclacel.com).

Investigators at Vall d'Hebron University Hospital and Cyclacel collaborated to study the combination of Seliciclib and the Epidermal Growth Factor Receptor (EGFR) inhibitor Erlotininb (Tarceva®) in patients with advanced solid tumours. The study aimed to identify the recommended dose of Seliciclib and Erlotinib, and investigated the pharmacokinetics of the combination and some potential pharmacodynamic markers (Cyclacel website – www.cyclacel.com).

In addition to cancer, Seliciclib has been evaluated in clinical trials for other disorders. In 2013 scientists at the University of Newcastle, together with colleagues at the University of Birmingham and Glasgow, initiated a clinical trial aiming to investigate the utility of Seliciclib in the treatment of patients with rheumatoid arthritis, who have not responded to current conventional treatments.

9. Potential therapeutic applications of Roscovitine

Polcystic Kidney Disease

Polycystic kidney disease (PKD) is one of the most common life-threatening genetic diseases, affecting some 12.5 million people across the globe. The condition is normally inherited in a dominant Mendelian manner, but can also be passed on recessively, and causes the formation of multiple, fluid-filled cysts in the kidneys, causing massive enlargement and leading to a reduction kidney function. Three genes have been linked to the condition all of which manifest similar phenotypes. Polycycstic Kidney Disease 1 (PKD1) is the most common genetic mutation and accounts for about 85% of dominantly inherited cases. It is located on chromosome 16 and encodes polycystin-1 (PC-1), a 4303 amino acid transmembrane glycoprotein involved in Wnt and GPCR-coupled signalling and in mediating calcium channel activity [171–174). The protein has been found localised to the desmosome and to focal adhesion complexes suggesting a role in the maintenance of cell-cell adhesion. Indeed in PKD cells PC-1 together with other normal desmosomal proteins are absent from intercellular junctions, remaining in the cytoplasm, despite unchanged levels of expression, thus suggesting that expression of normal PC-1 is required for correct assembly of desmosomal junctions [175]. There is also evidence that the expression of mutated PC-1 leads to a disruption in the polarity of renal epithelial cells [176] causing mislocalisation of ion channels and growth factor's receptors that in turn can reverse fluid flow and drive cellular proliferation in the kidney [177].

This increased renal cell proliferation has been targeted using roscovitine, an approach that has met with some success. Mouse models of both indolent (jck) and aggressive(cpk) forms of the disease show inhibition of disease progress and improvements of renal function when the mice are treated with roscovitine [178]. In a 2006 study Bukanov and co-workers showed that renal cells from roscovitine treated mice suffering with indolent PKD showed decreased phosphorylation of Rb and Cyclin D levels consistent with a block at the G1/S phase of the cell cycle. Interestingly these effects did not require daily roscovitine dosing and the

benefits persisted long after dosing of the mice had been stopped. This short term dosing would be a highly beneficial in the treatment of such a chronic condition.

Glomerulornephritis

Roscovitine has also been tested in a number of animal models of the kidney disease glomerulonephritis. Pippin and co-workers showed that the CDK inhibitor caused a reduction in mesangial cell proliferation and improved renal function in rats with experimental mesangialglomerulonephritis (Thy1 model) [179]. Podocytes or visceral epithelial cells are cells in the Bowman's capsule of the kidney and loss of these cells is a contributing factor in glomerulosclerosis in the kidney and subsequent proteinuria. Rats with passive Heymann nephritis, a model system for membranous glomerulonephritis, showed no signs of podocyte damage when treated with CYC202 (R-roscovitine) for 4 weeks, supporting the idea that this compound could provide a safe therapeutic approach in renal diseases characterized by podocyte injury [180]. Recently in a study in Wistar rats CYC202 was dosed orally to examine its effect on both established glomerulonephritis and its ability to prevent glomerulonephritis. The compound was shown to be effective at both prevention of crescentic glomerulonephritis and treatment of the pre-existing condition. The number of glomerular macrophages was reduced, as was a range of other disease markers, adding further weight to the argument that roscovitine may provide an effective therapeutic option for patients with, or at risk of developing, glomerulonephritis [181].

Systemic lupus erythrematosus (SLE or Lupus) is an autoimmune disease that can affect many parts of the body. It is characterised by the activation and proliferation of autoreactive T and B cells and the production of autoantibodies targeting endogenous antigens or nuclear antigens. This results in inflammation and tissue damage, and, in addition, antibody-immune complexes can precipitate and invoke a further immune response [182]. These immune complexes can also be retained in the kidney, where they can lead to complement activation and recruitment of T cells and macrophages. Cytokines secreted by these recruited cells can drive cellular proliferation in the kidney and exacerbate any renal disease [183,

184]. CYC202 has been tested in the NZBxNZW model of autoimmune lupus nephritis [185, 186] and, when dosed early, disease progression successfully delayed the appearance of proteinuria and slowed the decline in renal function. Importantly CYC202 treatment prolonged the survival of the treated mice compared to untreated control animals [187].

Anti Virals

Viruses, such as papilloma- or adeno-virus that can replicate only in dividing cells, have a requirement for cellular CDK activity to drive the cell into and through the phase of the cell cycle in which they are replication competent. Viruses, such as HIV and HSV, which can replicate in non-dividing cells, have a less obvious requirement for CDK activity, although some studies have revealed that, at least *in vitro*, replication of many of these viruses can be inhibited by small molecule inhibitors of the CDKs, such as roscovitine.

Human Cytomegalovirus (HCMV) is a double stranded DNA virus member of the β-Herpes family of viruses. Exposure rates across the general population are high but infection in healthy individuals rarely has any serious effects, however, immune-compromised individuals can develop retinitis, pneumonitis or gastroenteritis, increasing morbidity and mortality. These complications are more commonly seen in HIV infected patients [188] and in the newborn, where HCMV is a relatively common congenital infection and treatment options are limited [189]. Efficient HCMV replication requires CDKs 1,2,7and 9 [190–193]. Infection induces a cell cycle arrest in a G1-like state with high levels of Cyclin E and CyclinE-associated kinase that supports productive viral replication [194]. HCMV infection acts to create a cellular milieu favourable to viral transcription, protein modification and viral-genome replication that is disrupted by the inhibition of CDKs by small molecule ATP antagonists, such as roscovitine [195, 196]. Treatment of HCMV infected cells with roscovitine has been shown to reduce viral DNA synthesis, production of viral late proteins and ultimately the production of infectious viral particles. CDKs have been shown to phosphorylate and modulate the localisation and activity of the key viral multifunctional regulator protein pUL69 [197] causing its co-localistion with CDK9 and Cyclin T1 in

intranuclear speckles. This observation, when taken together with the demonstration that CDK9/Cyclin T1 phosphorylates pUL69 *in vitro*, raises the possibility that CDK activity is important in regulating pUL69 function and that small molecule inhibitors of CDKs have a future as HCMV therapeutics.

HIV-1 gene expression is controlled by the viral Tat transactivator as well as host cell transcription factors (reviewed in 198–200). Tat promotes efficient transcriptional elongation from the HIV-LTR, increasing the proportion of full length transcripts transcribed [201, 202]. Tat binds to a bulge in the stem-loop of the transactivation response element (TAR) at the 5' end of the viral transcript [203–206]. A number of host cell proteins interact with TAR and/or Tat to promote full transactivational activity, among them a protein kinase, originally designated as Tat-associated kinase (TAK) which phosphorylates the c-terminal domain of RNA Pol II [207]. It has been shown that the heptapeptide repeats of the C-terminal domain (CTD) of RNA Pol II are phosphorylated on Ser-2, Ser-5 and Ser-7 during transcription and that CDK7 is responsible for the phosphorylation of Ser-7 [208–211]. TAK has been identified as CDK9/Cyclin T1 and shown to interact with the TAR loop structure [212–214]. Ser-5 of the RNA Pol IICTD in the pre-initiation complex may be phosphorylated by TFIIH-associated CDK7 when induced by Tat causing its dissociation and allowing CDK9 autophosphorylation and efficient binding of CDK9/Cyclin T1 to the TAR stemloop [215–218]. In addition cells latently infected with HIV-1 have been shown to have increased CDK2/Cyclin E kinase activity due to loss of the CDKI – p21/waf1 [219]. These observations clearly pointed to the fact that inhibitors of CDKs 2,7 and 9 could be of potential benefit in inhibiting HIV infection. Roscovitine, which exhibits CDK inhibitory activity against CDKs 2,5,7 and 9, has been tested *in vitro* for its ability to inhibit HIV replication. The compound reduced viral titre and induced apoptosis in T-cells, monocytes and PBMCs, and was active against wildtype and drug resistant strains of the virus. Interestingly it was noted that anti-HIV activity was independent of the phase of the cell cycle [220]. It seems that a number of CDKs are involved in the efficient replication of HIV-1 and that inhibition of these

kinases has the real potential to significantly reduce viral load and offer complementary strategies inthe treatment of HIV infections.

Herpes viruses are one of the groups of viruses that carry genes for protein kinases in their genome [221]. However, many viral functions rely on and are regulated by host cell proteins; and so inhibitors of these host cell functions may inhibit viral replication. Rosovitine has been shown to inhibit viral transcription leading to a reduction in the number of viral transcripts [222, 223]. CDK8 has been reported as inhibiting HSV-1 transcription by phosphorylating the CDT of RNA Pol II prior to it being recruited to viral promoter sequences and by phosphorylating promoter-bound transcription factors [224, 225]. In contrast the inhibitory effects of CDK8, both CDK7 and CDK9 have been shown to enhance viral transcription and processivity by phosphorylating sites in the CTD of RNA Pol II [226–231]. CDK1 and CDK2 are also capable of phosphorylating the CTD of RNA Pol II so have potential roles in transcriptional control [232–235]. Roscovitine may also inhibit viral transcription via its effects on chromatin structure [236].

Inflammation

Roscovitine has been tested in a variety of animal models of inflammatory disease, including pleurisy, arthritis, and lung injury [141]. In a carrageenan-induced pleurisy model treatment of the animals with roscovitine caused a reduction in oedema and reduced concentrations of pro-inflammatory cytokines, and caused a reduction in the populations of neutrophils, macrophages, and monocytes. Concomitant treatment with the apoptosis inhibitor zVAD-fmk ablated these beneficial effects, suggesting that the benefits of roscovitine were due to enhanced apoptosis of inflammatory cells.

In a bleomycin-induced lung injury model mice treated with roscovitine saw a reduction in the number of neutrophils present in fluid from bronchial lavage and a reduction in inflammation. Importantly, roscovitine led to an increase in survival of the treated animals compared to untreated [141]. *In vitro* roscovitine led to rapid onset of apoptosis in neutrophils, effects that were observed even in the presence of potent survival factors, such as GM-CSF and LPS [141]. These observations are potentially very

interesting, given that efficacy is maintained in spite of the redundancy and diversity in the signalling pathways activated by these factors, implying perhaps that the anti-inflammatory action is due to an effect of roscovitine on a more fundamental cellular function. It has been shown recently that inhibition of CDK7 and CDK9 by roscovitine causes loss of phosphorylation of the RNA Pol II CTD and affects neutrophil transcriptional activity, and is responsible for the induction of apoptosis in neutrophils [237].

Pneumonia remains one of the most common infections in the world with around 450 million cases per year, resulting in about 4 million deaths. Typically caused by viral or bacterial infections, pneumonia is an inflammatory condition causing the alveoli to fill with fluid, reducing blood oxygenation levels [238–240].One of the most common causative agents is the gram-positive bacteria Streptococcus pneumonia, a component of whose cell wall, lipoteichoic acid (LTA), is a major pro-inflammatory molecule that can induce release of reactive oxygen and nitrogen, hydrolases, proteases, growth factors and cytotoxic cytokines from neutrophils and macrophages. Roscovitine has been shown *in vitro* to increase apoptosis in polymorphonuclear (PMN) cells and reduce secretion of TNF-α and keratinocyte chemoattractant (KC) from alveolar macrophage and respiratory epithelial cell lines after exposure to LTA [241]. Similarly positive results were observed with roscovitine in an *in vivo* mouse LTS-induced lung inflammation model, where numbers of PMN cells in fluid from broncheolavage were reduced, an effect which was reversed when apotosis was inhibited [241].

Fibrosis

Scleroderma is a chronic autoimmune condition that affects approximately 240 million individuals worldwide and is characterised by the formation of an excess of fibrous connective tissue (fibrosis), either cutaneously or systemically, resulting in changes in the vasculature. In a tissue culture model roscovitine inhibited the expression of collagen, fibronectin and connective tissue growth factor (CTGF) in growth-arrested normal and systemic sclerosis (SSc) fibroblasts. This reduction in expression of sclerotic proteins was the result of a decrease in transcription of the genes

rather than any effect on cell cycle, and was not reversed by treatment with the pro-fibrotic cytokines IL-6 or TGF-β [242]. Indeed, in the same study the authors also report that roscovitine treatment causes a reduction in the secretion of the pro-fibrotic cytokine IL-6. Given the current lack of clinical options for treating scleroderma, these observation strengthen any case for the testing of roscovitine in a more clinically relevant model.

Stroke

Ischaemic strokes arise when blood flow to a part of the brain is restricted, leading to tissue damage and loss of function in the area affected. Neuroprotective therapies are being sought to counter ischaemic injury and CDKs (being involved in apoptosis, they are one of the groups of drug targets being examined)[243–246]. CDK5 plays a pivotal role in the regulation of neuronal cell death and survival; and in pathological situation its activity is dysregulated by proteolytic cleavage of its partner protein p35 to p25. A CDK5/p25 complex is translocated to the cytoplasm and is promiscuous in its activity, compared to CDK5/p35, hyperphosphorylating substrates leading to neuronal cell death [11, 247]. Cyclin D1, CDK4 and CDK2 upregulation have also been observed during neuronal apoptosis and during proliferation of glial cells following cerebral ischaemia [248–251]. S-roscovitine has shown neuroprotective effects when used in models of ischaemia. The compound crossed the blood-brain barrier and showed protective effects (and reduced CDK5/p25 activity) when dosed after an ischaemic episode, suggesting that the compound functions by a mechanism involving CDK5 [252].

Glaucoma

Glaucoma is a term used to describe a number of eye disorders caused by changes in intraocular pressure. Most commonly associated with increased intraocular pressure it can, if left untreated, lead to irreversible retinal damage and blindness. Roscovitine has been shown to induce a relaxation of the pig trabecular meshwork, a zone around the cornea responsible for draining aqueous humour from the eye, suggesting a possible role in the control of intraocular pressure (IOP). CDKs have been reported to be involved in cellular responses to increased IOP, the cause of retinal cell damage. Both R- and S-roscovitine have been tested *in vivo* in a model of

rabbit glaucoma and both have shown beneficial effects in reducing the IOP. S-roscovitine was thought by the authors to be of greater potential benefit, because *in vitro* R-roscovitine, in contrast to S-roscovitine, amplified the effects of the Endosplasmic Reticular Stress, inducing compound tunicamycin, and increased oxygen-glucose deprivation induced cell death [253].

Seizure

Paroxysmal attacks are brief spasms or seizures often associated with other disorders, including multiple sclerosis, encephalitis, head trauma, stroke, and epilepsy, amongst others [254]. Attacks can be brought on by a wide variety of stimuli, including touch, heat, movement, and exercise. They are painful and can cause speech difficulty, lack of coordination, and loss of feeling. They resemble epileptic seizures, but lack the abnormal, rhythmic discharging of cortical neurons, and can vary enormously in frequency, but generally respond well to treatment with drugs, typically the anticonvulsant carbamzepine [255].

γ-aminobutyric acid (GABA) is a mammalian neurotransmitter and is a key molecule in controlling neuronal excitability throughout the nervous system. It functions primarily at inhibitory synapses in the brain by binding to two classes of transmembrane receptors in the plasma membrane, leading to hyperpolarisation of the neuron, and causing an inhibitory effect on neurotransmission by reducing the possibility of a successful action potential. Small molecules with the capacity to alter GABA receptor activity have the potential to be of use in the treatment of epilepsy and similar neurological conditions. Roscovitine has been shown to increase GABA mediated current in rat hippocampal neurons, without modifying $GABA_A$ receptors. The compound leads to an increase in neuronal GABA concentration and suppresses spiking in hippocampal pyramidal cells, a characteristic that may ultimately be beneficial in the prevention of paroxysmal activity [256], an observation that is worthy of further research.

Ischaemia-Reperfusion Injury (IR)

After a period of ischaemia (lack of oxygen) caused by lack of blood flow, a tissue can be damaged once oxygenation is reinstated. During or following transplant or bypass surgery, stroke or myocardial infaction ischaemia can pose serious problems when inflammation and oxidative stress can occur [257, 258]. Following previously reported anti-inflammatory properties,roscovitine has been tested in a rat model of renal IR and the authors concluded that treatment of the animals with roscovitine before ischaemia resulted in reduced renal damage, as measured by a number of blood chemical markers and histopathological examination [259].

In another study Topaloglu and colleagues [260] investigated the effect of pre-dosing of roscovitine on IR to the right hepatic lobe and noted that roscovitine caused a significant reduction in the number of dead and apoptotic cells in the livers, and a reduction in leukocyte infiltration consistent with protection from injury and inflammation [260].

Summary

As described above roscovitine has been tested pre-clinically in a wide variety of disease models and has shown potential therapeutic benefit in many. Further research is required in orderto ascertain whether the initial signs seen in these model systems are sufficient to warrant the significant financial investment to progress roscovitine or, more likely, a closely related molecule, along the drug development pathway. Initially discovered as a molecule that caused arrest in the cell cycle by blocking CDK2 activity, it is worthy of note how many of the potential therapeutic applications of the compound take advantage of the effects of roscovitine on the transcriptional CDKs.

References:

1. Chiarle R, Pagano M, Inghirami G. The cyclin dependent kinase inhibitor p27 and its prognostic role in breast cancer. *Breast Cancer Res.* 2001; **3**(2): 91–94.
2. Malumbres M, Barbacid M. Cell cycle kinases in cancer. *Curr Opin Genet Dev.* 2007 Feb; **17**(1):60-5.
3. Donnellan R, Chetty R. Cyclin D1 and human neoplasia. *Mol Pathol.* 1998 Feb; **51**(1):1–7.
4. Schafer KA. The cell cycle: A review. *Vet Pathol*. 1998 Nov; **35**(6):461-78.
5. Perez de Castro I, de Carcer G, Malumbres M. A census of mitotic cancer genes: new insights into tumor cell biology and cancer therapy. *Carcinogenesis.* 2007; **28**:899-912.
6. Berthet C, Aleem E, Coppola V, Tessarollo L, Kaldis P. Cdk2 knockout mice are viable. *Curr Biol.* 2003; **13** (20):1775–85.
7. Du J, Widlund HR, Horstmann MA, Ramaswamy S, Ross K, Huber WE, Nishimura EK, Golub TR, Fisher DE. Critical role of CDK2 for melanoma growth linked to its melanocyte-specific transcriptional regulation by MITF. *Cancer Cell* 2004; **6**:565–76.
8. Strock CJ, Park JI, Nakakura EK, Bova GS, Isaacs JT, Ball DW, Nelkin BD. Cyclin-dependent kinase 5 activity controls cell motility and metastatic potential of prostate cancer cells. *Cancer Res* 2006; **66**:7509-7515.
9. Goodyear S, Sharma MC. Roscovitine regulates invasive breast cancer cell (MDA-MB231) proliferation and survival through cell cycle regulatory protein cdk5. *ExpMolPathol*. 2007; **82**:25-32.
10. Cruz JC, Tsai LH. A Jekyll and Hyde kinase: roles for Cdk5 in brain development and disease. *CurrOpinNeurobiol.* 2004; **14**:390-394.
11. Dhavan R, Tsai LH A decade of CDK5. *Nat Rev Mol Cell Biol.* 2001; **2**:749-759.
12. Angelo M, Plattner F, Giese KP. Cyclin-dependent kinase 5 in synaptic plasticity, learning and memory. *J Neurochem.* 2006; **99**:353-370.
13. Bibb JA. Role of Cdk5 in neuronal signaling, plasticity, and drug abuse. *Neurosignals*. 2003; **12**:191-199.
14. Nguyen C, Bibb JA. Cdk5 and the mystery of synaptic vesicle endocytosis. *J Cell Biol*. 2003; **163**:697-699.
15. Tomizawa K, Ohta J, Matsushita M, Moriwaki A, Li ST, Takei K, Matsui H. Cdk5/p35 regulates neurotransmitter release through phosphorylation and downregulation of P/Q-type voltage-dependent calcium channel activity. *J Neurosci.* 2002; **22**:2590-2597.
16. Lee MH, Yang HY. Regulators of G1 cyclin-dependent kinases and cancer.*Cancer and Metastasis Reviews,* 2003; **22**(4): 435-49.

17. Meyerson M, Enders GH, Wu CL, Su LK, Gorka C, Nelson C, Harlow E, Tsai LH. A family of human cdc2-related protein kinases. *EMBO J.* 1992; **11**: 2909-2917.
18. Kwon TK, Buchholz MA,Gabrielson EW,Nordin AA. A novel cytoplasmic substrate for cdk4 and cdk6 in normal and malignant epithelial derived cells.*Oncogene.* 1995; **11**(10):2077-2083.
19. Matsushime H, Ewen ME, Strom DK, Kato JY, Hanks SK, Roussel MF, Sherr CJ. Identification and properties of an atypical catalytic subunit (p34PSK-J3/cdk4) for mammalian D type G1 cyclins.*Cell.* 1992; **71**(2):323-34.
20. Matsushime H,Quelle DE,Shurtleff SA,Shibuya M,Sherr CJ,Kato JY. D-type cyclin-dependent kinase activity in mammalian cells.*Mol Cell Biol.* 1994; **14** (3):2066-76.
21. Meyerson M, Harlow E. Identification of G1 kinase activity for cdk6, a novel cyclin D partner. *Mol Cell Biol.* 1994; **14**:2077-2086.
22. Malumbres M, Sotillo R, Santamaría D, Galán J, Cerezo A, Ortega S, Dubus P, Barbacid M. Mammalian cells cycle without the D-type cyclin-dependent kinases Cdk4 and Cdk6.*Cell.* 2004; **118**:493-504.
23. Takaki T, Fukasawa K, Suzuki-Takahashi I, Semba K, Kitagawa M, Taya Y, Hirai H. Preferences for phosphorylation sites in the retinoblastoma protein of D-type cyclin-dependent kinases, Cdk4 and Cdk6, in vitro.J Biochem.2005; **137**(3):381-6.
24. Zuo L, Weger J, Yang Q, Goldstein AM, Tucker MA, Walker GJ, Hayward N, Dracopoli NC. Germline mutations in the p16INK4a binding domain of CDK4 in familial melanoma.Nat Genet.1996; **12**(1):97-9.
25. Timmermann S,Hinds PW,Münger K.Elevated activity of cyclin-dependent kinase 6 in human squamous cell carcinoma lines.Cell Growth Differ.1997; **8**(4):361-70.
26. Easton J,Wei T,Lahti JM,Kidd VJ. Disruption of the cyclin D/cyclin-dependent kinase/INK4/retinoblastoma protein regulatory pathway in human neuroblastoma.Cancer Res.1998; **58**(12):2624-32.
27. Mahony D,Parry DA,Lees E. Active cdk6 complexes are predominantly nuclear and represent only a minority of the cdk6 in T cells.*Oncogene.* 1998;**16** (5):603-11.
28. Matushansky I,Radparvar F,Skoultchi AI. CDK6 blocks differentiation: coupling cell proliferation to the block to differentiation in leukemic cells.*Oncogene*.2003;**22** (27):4143-9.
29. Ogasawara T, Kawaguchi H, Jinno S, Hoshi K, Itaka K, Takato T, Nakamura K, Okayama H. Bone morphogenetic protein 2-induced osteoblast differentiation requires Smad-mediated down-regulation of Cdk6.Mol Cell Biol.2004; **24**(15):6560-8.

30. Wallenfang MR,Seydoux G.cdk-7 is required for mRNA transcription and cell cycle progression in Caenorhabditis elegans embryos.ProcNatlAcadSciUSA.2002; **99**(8):5527-32.
31. Larochelle S,Pandur J,Fisher RP,Salz HK,Suter B.Cdk7 is essential for mitosis and for in vivo Cdk-activating kinase activity. *Genes Dev*. 1998; **12**(3):370-81.
32. Wu L, Chen P, Hwang JJ, Barsky LW, Weinberg KI, Jong A, Starnes VA. RNA antisense abrogation of MAT1 induces G1 phase arrest and triggers apoptosis in aortic smooth muscle cells.J Biol Chem.1999; **274**(9):5564-72.
33. Wu L, Chen P, Shum CH, Chen C, Barsky LW, Weinberg KI, Jong A, Triche TJ. MAT1-modulated CAK activity regulates cell cycle G(1) exit. *Mol Cell Biol.* 2001;**21**(1):260-70.
34. Hengartner CJ,Myer VE,Liao SM,Wilson CJ,Koh SS,Young RA. Temporal regulation of RNA polymerase II by Srb10 and Kin28 cyclin-dependent kinases.Mol Cell.1998; **2**(1):43-53.
35. Ramanathan Y, Rajpara SM, Reza SM, Lees E, Shuman S, Mathews MB, Pe'ery T. Three RNA polymerase II carboxyl-terminal domain kinases display distinct substrate preferences.J Biol Chem.2001; **276**(14):10913-20.
36. Rickert P,Corden JL,Lees E. Cyclin C/CDK8 and cyclin H/CDK7/p36 are biochemically distinct CTD kinases.Oncogene.1999; **18**(4):1093-102.
37. Liao SM, Zhang J, Jeffery DA, Koleske AJ, Thompson CM, Chao DM, Viljoen M, van Vuuren HJ, Young RA. A kinase-cyclin pair in the RNA polymerase II holoenzyme.Nature.1995; **374**(6518):193-6.
38. Gold MO,Rice AP.Targeting of CDK8 to a promoter-proximal RNA element demonstrates catalysis-dependent activation of gene expression.Nucleic Acids Res.1998; **26**(16):3784-8.
39. Biddick R,Young ET.Yeast mediator and its role in transcriptional regulation.C R Biol.2005; **328**(9):773-82.
40. Björklund S,Gustafsson CM.The yeast Mediator complex and its regulation.Trends Biochem Sci.2005; **30**(5):240-4.
41. Reeves WM,Hahn S.Activator-independent functions of the yeast mediator sin4 complex in preinitiation complex formation and transcription reinitiation.Mol Cell Biol.2003; **23**(1):349-58.
42. Firestein R, Bass AJ, Kim SY, Dunn IF, Silver SJ, Guney I, Freed E, Ligon AH, Vena N, Ogino S, Chheda MG, Tamayo P, Finn S, Shrestha Y, Boehm JS, Jain S, Bojarski E, Mermel C, Barretina J, Chan JA, Baselga J, Tabernero J, Root DE, Fuchs CS, Loda M, Shivdasani RA, Meyerson M, Hahn WC. CDK8 is a colorectal cancer oncogene that regulates beta-catenin activity.Nature.2008; **455**(7212):547-51.
43. Firestein R,Hahn WC. Revving the Throttle on an oncogene: CDK8 takes the driver seat.Cancer Res.2009; **69**(20):7899-901.

44. Loyer P,Trembley JH,Katona R,Kidd VJ,Lahti JM.Role of CDK/cyclin complexes in transcription and RNA splicing.*Cell Signal*. 2005; **17** (9):1033-51.
45. Yu DS,Cortez D.A role for CDK9-cyclin K in maintaining genome integrity.Cell Cycle.2011; **10**(1):28-32.
46. Malumbres M,Barbacid M.Mammalian cyclin-dependent kinases.Trends Biochem Sci.2005; **30**(11):630-41.
47. Romano G,Giordano A.Role of the cyclin-dependent kinase 9-related pathway in mammalian gene expression and human diseases.Cell Cycle.2008; **7**(23):3664-8.
48. Nechaev S,Adelman K.Pol II waiting in the starting gates: Regulating the transition from transcription initiation into productive elongation.Biochim Biophys Acta.2011; **1809**(1):34-45.
49. Rahl PB, Lin CY, Seila AC, Flynn RA, McCuine S, Burge CB, Sharp PA, Young RA. c-Myc regulates transcriptional pause release.Cell.2010; **141**(3):432-45.
50. Barboric M,Nissen RM,Kanazawa S,Jabrane-Ferrat N,Peterlin BM. NF-kappaB binds P-TEFb to stimulate transcriptional elongation by RNA polymerase II.Mol Cell.2001; **8**(2):327-37.
51. Lee DK,Duan HO,Chang C.Androgen receptor interacts with the positive elongation factor P-TEFb and enhances the efficiency of transcriptional elongation.J Biol Chem.2001; **276**(13):9978-84.
52. Yang Z,Zhu Q,Luo K,Zhou Q.The 7SK small nuclear RNA inhibits the CDK9/cyclin T1 kinase to control transcription.Nature.2001; **414**(6861):317-22.
53. Michels AA, Nguyen VT, Fraldi A, Labas V, Edwards M, Bonnet F, Lania L, Bensaude O. MAQ1 and 7SK RNA interact with CDK9/cyclin T complexes in a transcription-dependent manner.Mol Cell Biol.2003; **23**(14):4859-69.
54. Krueger BJ, Jeronimo C, Roy BB, Bouchard A, Barrandon C, Byers SA, Searcey CE, Cooper JJ, Bensaude O, Cohen EA, Coulombe B, Price DH. LARP7 is a stable component of the 7SK snRNP while P-TEFb, HEXIM1 and hnRNP A1 are reversibly associated.Nucleic Acids Res.2008; **36**(7):2219-29.
55. Chiarle R, Budel LM, Skolnik J, Frizzera G, Chilosi M, Corato A, Pizzolo G, Magidson J, Montagnoli A, Pagano M, Maes B, De Wolf-Peeters C, Inghirami G. Increased proteasome degradation of cyclin-dependent kinase inhibitor p27 is associated with a decreased overall survival in mantle cell lymphoma. *Blood.* 2000 Jan; **95**(2):619-26.
56. Wang W, Wu JF, Zhang ZY, Tong TJ. Characterization of regulatory elements on the promoter region of p16(INK4a) that contribute to overexpression of p16 in senescent fibroblasts. *J Biol Chem.* 2001 Dec; **276**(52):48655-61.

57. Gartel AL, Radhakrishnan SK. Lost in transcription: p21 repression, mechanisms, and consequences. *Cancer Res.*2005; **65**(10):3980-5.
58. Zhelev NZ, Kardalinou E, Hazzalin CA, Cano E, Barratt MJ, Mahadevan LC. Signalling to chromatin and the superinduction of proto-oncogenes. *BiochemSoc Trans.* 1993; **21**:907-11.
59. Merlo A, Herman JG, Mao L, Lee DJ, Gabrielson E, Burger PC, Baylin SB, Sidransky D. 5' CpG island methylation is associated with transcriptional silencing of the tumour suppressor p16/CDKN2/MTS1 in human cancers.*Nat Med*.1995; **1**(7):686-92.
60. Wölfel T, Hauer M, Schneider J, Serrano M, Wölfel C, Klehmann-Hieb E, De Plaen E, Hankeln T, Meyer zum Büschenfelde KH, Beach D. A p16INK4a-insensitive CDK4 mutant targeted by cytolytic T lymphocytes in a human melanoma.Science.1995; **269**(5228):1281-4.
61. Bulavin DV,Tararova ND,Aksenov ND,Pospelov VA,Pospelova TV.Deregulation of p53/p21Cip1/Waf1 pathway contributes to polyploidy and apoptosis of E1A+cHa-ras transformed cells after gamma-irradiation.Oncogene.1999; **18**(41):5611-9.
62. Weinberg RA. The Retinoblastoma protein and cell cycle control. *Cell.* 1995; **81**(3):323-30.
63. Fischer PM, Zhelev NZ, Wang S, Melville JE, Fahraeus R, Lane DP. Structure-activity relationship of truncated and substituted analogues of the intracellular delivery vector Penetratin. *J Pept Res.* 2000; **55**(2):163-172.
64. Fischer PM, Zheleva DI, McInnes C, Gavine A, Zhelev NZ, Lane DP. Peptide inhibitors of cyclin-dependent kinases derived from p21(WAF1): Delineation and structural insight into their interactions with cyclin A. *Clin Cancer Res.* 2001; **7**(11S):3821-3822.
65. Zheleva DI, McInnes C, Gavine AL, Zhelev NZ, Fischer PM, Lane DP. Highly potent p21(WAF1)-derived peptide inhibitors of CDK-mediated pRb phosphorylation: Delineation and structural insight into their interactions with cyclin A. *J Pept Res.* 2002; **60**(5):257-270.
66. Chen YN, Sharma SK, Ramsey TM, Jiang L, Martin MS, Baker K, Adams PD, Bair KW, Kaelin WG Jr. Selective killing of transformed cells by cyclin/cyclin-dependent kinase 2 antagonists. *ProcNatlAcadSciUSA*. 1999; **96**(8): 4325-9.
67. Wada T,Takagi T,Yamaguchi Y,Watanabe D,Handa H. Evidence that P-TEFb alleviates the negative effect of DSIF on RNA polymerase II-dependent transcription in vitro.EMBO J.1998; **17**(24):7395-403.
68. Ljungman M, Paulsen MT. The cyclin-dependent kinase inhibitor roscovitine inhibits RNA synthesis and triggers nuclear accumulation of p53 that is unmodified at Ser15 and Lys382.*MolPharmacol.* 2001; **60**(4): 785-9.
69. Gojo I, Zhang B, Fenton RG. The cyclin-dependent kinase inhibitor flavopiridol induces apoptosis in multiple myeloma cells through

transcriptional repression and down-regulation of Mcl-1.Clin Cancer Res.2002; **8**(11):3527-38.

70. MacCallum DE, Melville J, Frame S, Watt K, Anderson S, Gianella-Borradori A, Lane DP, Green SR. Seliciclib (CYC202, R-Roscovitine) induces cell death in multiple myeloma cells by inhibition of RNA polymerase II-dependent transcription and down-regulation of Mcl-1.Cancer Res.2005; **65**(12):5399-407.
71. Raje N, Kumar S, Hideshima T, Roccaro A, Ishitsuka K, Yasui H, Shiraishi N, Chauhan D, Munshi NC, Green SR, Anderson KC. Seliciclib (CYC202 or R-roscovitine), a small-molecule cyclin-dependent kinase inhibitor, mediates activity via down-regulation of Mcl-1 in multiple myeloma.Blood.2005; **106**(3):1042-7.
72. Santo L, Vallet S, Hideshima T, Cirstea D, Ikeda H, Pozzi S, Patel K, Okawa Y, Gorgun G, Perrone G, Calabrese E, Yule M, Squires M, Ladetto M, Boccadoro M, Richardson PG, Munshi NC, Anderson KC, Raje N. AT7519, A novel small molecule multi-cyclin-dependent kinase inhibitor, induces apoptosis in multiple myeloma via GSK-3beta activation and RNA polymerase II inhibition.Oncogene.2010; **29**(16):2325-36.
73. Lam LT, Pickeral OK, Peng AC, Rosenwald A, Hurt EM, Giltnane JM, Averett LM, Zhao H, Davis RE, Sathyamoorthy M, Wahl LM, Harris ED, Mikovits JA, Monks AP, Hollingshead MG, Sausville EA, Staudt LM. Genomic-scale measurement of mRNA turnover and the mechanisms of action of the anti-cancer drug flavopiridol.Genome Biol.2001; **2**(10):RESEARCH0041.
74. Wang S, Fischer PM. Cyclin-dependent kinase 9: a key transcriptional regulator and potential drug target in oncology, virology and cardiology. *Trends PharmacolSci.* 2008; **29**(6):302-13.
75. Sano M, Schneider MD.Cyclin-Dependent Kinase-9: An RNAPII Kinase at theNexus of Cardiac Growth and Death Cascades. *Circulation Research.* 2004; **95**:867-876.
76. Meijer L, Guerrier P.maturation and fertilization in starfish oocytes.Int Rev Cytol.1984; **86**:129-96.
77. Meijer L, Pondaven P, Guerrier P, Moreau M. A starfish oocyte users guide. *Cah Biol.Mar.* 1984; **25**:457-80.
78. Meijer L, Pondaven P, Tung HY, Cohen P, Wallace RW.Protein phosphorylation and oocyte maturation. II. Inhibition of starfish oocyte maturation by intracellular microinjection of protein phosphatases 1 and 2A and alkaline phosphatase.Exp Cell Res.1986; **163**(2):489-99.
79. Meijer L, Mordret G. Starfish oocyte maturation: From prophase to metaphase. *Semin Cell Dev Biol.* 1994; **5**(3):165-71.
80. Kanatani H, Shirai H, Nakanish K, Kurokawa T. Isolation and identification of meiosis inducing substance in starfish asterias amurensis. *Nature* 1969; **221**(5177): 273-8.

81. Okumura E, Fukuhara T, Yoshida H, Hanada Si S, Kozutsumi R, Mori M, Tachibana K, Kishimoto T. Akt inhibits Myt1 in the signalling pathway that leads to meiotic G2/M-phase transition. *Nat Cell Biol.* 2002 Feb; **4**(2):111-6.
82. Pondaven P, Meijer L.Protein phosphorylation and oocyte maturation. I. Induction of starfish oocyte maturation by intracellular microinjection of a phosphatase inhibitor, alpha-naphthylphosphate.Exp Cell Res.1986; **163**(2):477-88.
83. Pelech SL, Meijer L, Krebs EG. Characterization of maturation-activated histone H1 and ribosomal S6 kinases in sea star oocytes.Biochemistry.1987; **26**(24):7960-8.
84. Meijer L, Pelech SL, Krebs EG. Differential regulation of histone H1 and ribosomal S6 kinases during sea star oocyte maturation.Biochemistry.1987; **26**(24):7968-74.
85. Arion D, Meijer L, Brizuela L, Beach D.cdc2 is a component of the M phase-specific histone H1 kinase: evidence for identity with MPF.Cell.1988; **55**(2):371-8.
86. Meijer L, Arion D, Golsteyn R, Pines J, Brizuela L, Hunt T, Beach D. Cyclin is a component of the sea urchin egg M-phase specific histone H1 kinase.EMBO J.1989; **8**(8):2275-82.
87. Pondaven P, Meijer L, Beach D.Activation of M-phase-specific histone H1 kinase by modification of the phosphorylation of its p34cdc2 and cyclin components. *Genes Dev.* 1990; **4**(1):9-17.
88. Masui Y.A quest for cytoplasmic factors that control the cell cycle.Prog Cell Cycle Res.1996; **2**:1-13.
89. Kishimoto T.Activation of MPF at meiosis reinitiation in starfish oocytes.Dev Biol.1999; **214**(1):1-8.
90. Labbé JC, Capony JP, Caput D, Cavadore JC, Derancourt J, Kaghad M, Lelias JM, Picard A, Dorée M. MPF from starfish oocytes at first meiotic metaphase is a heterodimer containing one molecule of cdc2 and one molecule of cyclin B.EMBO J.1989; **8**(10):3053-8.
91. Rialet V, Meijer L.A new screening test for antimitotic compounds using the universal M phase-specific protein kinase, p34cdc2/cyclin Bcdc13, affinity-immobilized on p13suc1-coated microtitration plates.Anticancer Res.1991; **11**(4):1581-90.
92. Rebhun LI, White D, Sander G, Ivy N.Cleavage inhibition in marine eggs by puromycin and 6-dimethylaminopurine.Exp Cell Res.1973; **77**(1):312-8.
93. Meijer L, Pondaven P, Guerrier P, Moreau M. A starfish oocyte users guide. *Cahiers de biologie marine.* 1984; 25(4) 457.
94. Neant I, Guerrier P.6-Dimethylaminopurine blocks starfish oocyte maturation by inhibiting a relevant protein kinase activity.Exp Cell Res.1988; **176**(1):68-79.

95. Veselý J, Havlicek L, Strnad M, Blow JJ, Donella-Deana A, Pinna L, Letham DS, Kato J, Detivaud L, Leclerc S, *et al.* Inhibition of cyclin-dependent kinases by purine analogues.Eur J Biochem.1994; **224**(2):771-86.
96. Havlícek L, Hanus J, Veselý J, Leclerc S, Meijer L, Shaw G, Strnad M. Cytokinin-derived cyclin-dependent kinase inhibitors: synthesis and cdc2 inhibitory activity of olomoucine and related compounds.J Med Chem.1997; **40**(4):408-12.
97. De Azevedo WF, Leclerc S, Meijer L, Havlicek L, Strnad M, Kim SH. Inhibition of cyclin-dependent kinases by purine analogues: crystal structure of human cdk2 complexed with roscovitine.Eur J Biochem.1997; **243**(1-2):518-26.
98. Wang SD, McClue SJ, Ferguson JR, Hull JD, Stokes S, Parsons S, Westwood R, Fischer PM. Synthesis and configuration of the cyclin-dependent kinase inhibitor roscovitine and its enantiomer. *Tetrahedron: Asymmetry*. 2001; **12**:2891-4.
99. Meijer L, Borgne A, Mulner O, Chong JPJ, Blow JJ, Inagaki N, Inagaki M, Delcros JG, Moulinoux JP. Biochemical and cellular effects of roscovitine, a potent and selective inhibitor of the cyclin-dependent kinases cdc2, cdk2 and cdk5. *Eur J Biochem.* 1997 Jan; **243**(1-2):527-36.
100. McClue SJ, BlakeD, Clarke R, Cowan A, Cummings L, Fischer PM, MacKenzie M, Melville J, Stewart K, Wang S, Zhelev N, Zheleva D, Lane DP. In vitro and in vivo antitumor properties of the cyclin dependent kinase inhibitor CYC202 (R-roscovitine). *Int J Cancer*. 2002 Dec; **102**(5):463–8
101. Biglione S, Byers SA, Price JP, Nguyen VT, Bensaude O, Price DH, Maury W. Inhibition of HIV-1 replication by P-TEFb inhibitors DRB, seliciclib and flavopiridol correlates with release of free P-TEFb from the large, inactive form of the complex.*Retrovirology* 2007; **4**:47.
102. Bain J, McLauchlan H, Elliott M, Cohen P. The selectivity of protein kinase inhibitors: a further update. *Biochem J*. 2003; **371**(1):199-204.
103. Bain J, Plater L, Elliott M, Shpiro N, Hastie CJ, McLauchlan H. The selectivity of protein kinase inhibitors: a further update. *Biochem J*. 2007; **408**(3):297-315.
104. Raynaud FI, Fischer PM, Nutley BP, Goddard PM, Lane DP, Workman P. Cassette dosing pharmacokinetics of a library of 2,6,9-trisubstituted purine cyclin-dependent kinase 2 inhibitors prepared by parallel synthesis. *Mol Cancer Ther*. 2004; **3**:353-362.
105. Schutte B, Nieland L, van Engeland M, Henfling ME, Meijer L, Ramaekers FC. The effect of the cyclin-dependent kinase inhibitor olomoucine on cell cycle kinetics. *Exp Cell Res.* 1997 Oct 10; **236**(1):4-15.
106. Alessi F, Quarta S, Savio M, Riva F, Rossi L, Stivala LA, Scovassi AI, Meijer L, Prosperi E. The cyclin-dependent kinase inhibitors olomoucine

and roscovitine arrest human fibroblasts in G1 phase by specific inhibition of CDK2 kinase activity. *Exp Cell Res.* 1998 Nov 25; **245**(1):8-18.

107. Yakisich JS, Boethius J, Lindblom IO, Wallstedt L, Vargas VI, Sidén A, Cruz MH. Inhibition of DNA synthesis in human gliomas by roscovitine. *Neuroreport.* 1999 Aug 20; **10**(12):2563-7.
108. Iseki H, Ko TC, Xue XY, Seapan A, Hellmich MR, Townsend CM Jr. Cyclin-dependent kinase inhibitors block proliferation of human gastric cancer cells. *Surgery*. 1997 Aug; **122**(2):187-94; discussion 194-5.
109. Iseki H, Ko TC, Xue XY, Seapan A, Townsend CM Jr. A novel strategy for inhibiting growth of human pancreatic cancer cells by blocking cyclin-dependent kinase activity. *J Gastrointest Surg.* 1998 Jan-Feb; **2**(1):36-43.
110. Mgbonyebi OP, Russo J, Russo IH. Roscovitine inhibits the proliferative activity of immortal and neoplastic human breast epithelial cells. *Anticancer Res.* 1998 Mar-Apr; **18**(2A):751-5.
111. Mgbonyebi OP, Russo J, Russo IH. Roscovitine induces cell death and morphological changes indicative of apoptosis in MDA-MB-231 breast cancer cells. *Cancer Res.* 1999 Apr 15; **59**(8):1903-10.
112. Lee HR, Chang TH, Tebalt MJ 3rd, Senderowicz AM, Szabo E. Induction of differentiation accompanies inhibition of Cdk2 in a non-small cell lung cancer cell line. *Int J Oncol*. 1999 Jul; **15**(1):161-6.
113. Wesierska-Gadek J, Gueorguieva M, Horky M. Roscovitine-induced up-regulation of p53AIP1 protein precedes the onset of apoptosis in human MCF-7 breast cancer cells. Mol Cancer Ther 2005; **4**:113-124.
114. Wesierska-Gadek J, Wandl S, Kramer MP, Pickem C, Krystof V, Hajek SB. Roscovitine up-regulates p53 protein and induces apoptosis in human HeLaS(3) cervix carcinoma cells. *J Cell Biochem*. 2008; **105**:1161-1171.
115. Lacrima K, Valentini A, Lambertini C, Taborelli M, Rinaldi A, Zucca E, Catapano C, Cavalli F, Gianella-Borradori A, Maccallum DE, Bertoni F. In vitro activity of cyclin-dependent kinase inhibitor CYC202 (Seliciclib, R-roscovitine) in mantle cell lymphomas. *Ann Oncol*. 2005; **16**:1169-1176.
116. Mohapatra S, Chu B, Zhao X, Pledger WJ. Accumulation of p53 and reductions in XIAP abundance promote the apoptosis of prostate cancer cells. *Cancer Res*. 2005; **65**:7717-7723.
117. Kim EH, Kim SU, Shin DY, Choi KS. Roscovitine sensitizes glioma cells to TRAIL-mediated apoptosis by downregulation of survivin and XIAP. *Oncogene*. 2004; **23**:446-456.
118. Alvi AJ, Austen B, Weston VJ, Fegan C, MacCallum D, Gianella-Borradori A, Lane DP, Hubank M, Powell JE, Wei W, Taylor AM, Moss PA, Stankovic T. A novel CDK inhibitor, CYC202 (R-roscovitine), overcomes the defect in p53-dependent apoptosis in B-CLL by down-regulation of genes involved in transcription regulation and survival. *Blood*. 2005; **105**:4484-4491.

119. Wesierska-Gadek J, Gueorguieva M, Horky M. Dual action of cyclin-dependent kinase inhibitors: induction of cell cycle arrest and apoptosis. A comparison of the effects exerted by roscovitine and cisplatin. *Pol J Pharmacol*. 2003; **55**:895-902.
120. Meijer L BK, Galons H. (2006) Roscovitine (CYC202, Seliciclib). In: Smith PJ and Yue E (eds). Monographs on Enzyme Inhibitors. CDK Inhibitors and their Potential as Anti-Tumor Agents.187-226 p.
121. Lu W, Chen L, Peng Y, Chen J (2001) Activation of p53 by roscovitine-mediated suppression of MDM2 expression. *Oncogene*. 2001; **20**:3206-3216.
122. Lambert LA, Qiao N, Hunt KK, Lambert DH, Mills GB, Meijer L, Keyomarsi K. Autophagy: a novel mechanism of synergistic cytotoxicity between doxorubicin and roscovitine in a sarcoma model. Cancer Res 2008; **68**:7966-7974.
123. Abal M, Bras-Goncalves R, Judde JG, Fsihi H, De Cremoux P, Louvard D, Magdelenat H, Robine S, Poupon MF. Enhanced sensitivity to irinotecan by Cdk1 inhibition in the p53-deficient HT29 human colon cancer cell line. *Oncogene*. 2004; **23**:1737-1744.
124. Raynaud FI, Whittaker SR, Fischer PM, McClue S, Walton MI, Barrie SE, Garrett MD, Rogers P, Clarke SJ, Kelland LR, Valenti M, Brunton L, Eccles S, Lane DP, Workman P.In vitro and in vivo pharmacokinetic-pharmacodynamic relationships for the trisubstituted aminopurine cyclin-dependent kinase inhibitors olomoucine, bohemine and CYC202. *Clin Cancer Res*. 2005; **11**(13):4875-87.
125. Fleming IN, Hogben M, Frame S, McClue SJ, Green SR. Synergistic inhibition of ErbB signaling by combined treatment with seliciclib and ErbB-targeting agents. *Clin Cancer Res*. 2008; **14**(13):4326-35
126. Hui AB, Yue S, Shi W, Alajez NM, Ito E, Green SR, Frame S, O'Sullivan B, Liu FF. Therapeutic efficacy of seliciclib in combination with ionizing radiation for human nasopharyngeal carcinoma. *Clin Cancer Res*. 2009; **15**(11):3716-24
127. Nair BC,Vallabhaneni S,Tekmal RR,Vadlamudi RK. Roscovitine confers tumor suppressive effect on therapy-resistant breast tumor cells. *Breast Cancer Res.* 2011; **13**(3):R80
128. Cheng CK, Gustafson WC, Charron E, Houseman BT, Zunder E, Goga A, Gray NS, Pollok B, Oakes SA, James CD, Shokat KM, Weiss WA, Fan QW. Dual blockade of lipid and cyclin-dependent kinases induces synthetic lethality in malignant glioma. *Proc Natl Acad Sci USA*. 2012; **109**(31):12722-7.
129. Tirado OM, Mateo-Lozano S, Notario V. Roscovitine Is an Effective Inducer of Apoptosis of Ewing's Sarcoma Family Tumor Cells In vitro and In vivo. *Cancer Res.* 2005; **65**(20):9320-7

130. Maggiorella L, Deutsch E, Frascogna V, Chavaudra N, Jeanson L, Milliat F, Eschwege F, Bourhis J. Enhancement of radiation response by roscovitine in human breast carcinoma in vitro and in vivo.*Cancer Res.* 2003; **63**:2513-2517.
131. Vita M, Abdel-Rehim M, Olofsson S, Hassan Z, Meurling L, Sidén A, Sidén M, Pettersson T, Hassan M. Tissue distribution, pharmacokinetics and identification of roscovitine metabolites in rat. *Eur J Pharm Sci*. 2005; **25**:91-103.
132. Vita M, Meurling L, Pettersson T, Cruz-Sidén M, Sidén A, Hassan M. Analysis of roscovitine using novel high performance liquid chromatography and UV-detection method: pharmacokinetics of roscovitine in rat. *J Pharm Biomed Anal*. 2004; **34**:425-431.
133. Vita M, Abdel-Rehim M, Nilsson C, Hassan Z, Skansen P, Wan H, Meurling L, Hassan M. Stability, pKa and plasma protein binding of roscovitine. *J Chromatogr B Analyt Technol Biomed Life Sci*. 2005; **821**:75-80.
134. Gherardi D, D'Agati V, Chu TH, Barnett A, Gianella-Borradori A, Gelman IH, Nelson PJ. Reversal of collapsing glomerulopathy in mice with the cyclin-dependent kinase inhibitor CYC202. *J Am Soc Nephrol.* 2004; **15**:1212-1222.
135. Nutley BP, Raynaud FI, Wilson SC, Fischer PM, Hayes A, Goddard PM, McClue SJ, Jarman M, Lane DP, Workman P. Metabolism and pharmacokinetics of the cyclin-dependent kinase inhibitor R-roscovitine in the mouse. *Mol Cancer Ther*. 2005; **4**:125-139.
136. de la Motte S, Gianella-Borradori A. Pharmacokinetic model of R-roscovitine and its metabolite in healthy male subjects. *Int J Clin Pharmacol Ther*. 2004; **42**:232-239.
137. Benson C, White J, De Bono J, O'Donnell A, Raynaud F, Cruickshank C, McGrath H, Walton M, Workman P, Kaye S, Cassidy J, Gianella-Borradori A, Judson I, Twelves C. A phase I trial of the selective oral cyclin-dependent kinase inhibitor seliciclib (CYC202; R-Roscovitine), administered twice daily for 7 days every 21 days. *Br J Cancer*. 2007; **96**:29-37.
138. Cervenková K, Belejová M, Chmela Z, Rypka M, Riegrová D, Michnová K, Michalíková K, Súrová I, Brejcha A, Hanus J, Cerný B, Fuksová K, Havlícek L, Veselý J. In vitro glycosidation potential towards olomoucine-type cyclin-dependent kinase inhibitors in rodent and primate microsomes. *Physiol Res.* 2003; **52**:467-474.
139. McClue SJ, Stuart I. Metabolism of the Trisubstituted Purine Cyclin-Dependent Kinase Inhibitor Seliciclib (R-Roscovitine) in Vitro and in Vivo. *Drug Metab Dispos.* 2008 Mar;36(3):561-70. Epub 2007 Nov 29.
140. Iurisci I, Filipski E, Reinhardt J, Bach S, Gianella-Borradori A, Iacobelli S, Meijer L, Lévi F. Improved tumor control through circadian clock induction by Seliciclib, a cyclin-dependent kinase inhibitor. *Cancer Res*. 2006; **66**:10720-10728.

141. Rossi AG, Sawatzky DA, Walker A, Ward C, Sheldrake TA, Riley NA, Caldicott A, Martinez-Losa M, Walker TR, Duffin R, Gray M, Crescenzi E, Martin MC, Brady HJ, Savill JS, Dransfield I, Haslett C. Cyclin-dependent kinase inhibitors enhance the resolution of inflammation by promoting inflammatory cell apoptosis. *Nat Med*. 2006; **12**:1056-1064.
142. Duffin R, Leitch AE, Sheldrake TA, Hallett JM, Meyer C, Fox S, Alessandri AL, Martin MC, Brady HJ, Teixeira MM, Dransfield I, Haslett C, Rossi AG. The CDK inhibitor, R-roscovitine, promotes eosinophil apoptosis by down-regulation of Mcl-1. *FEBS Lett*. 2009; **583**:2540-2546.
143. Li L, Wang H, Kim J, Pihan G, Boussiotis V. The cyclin dependent kinase inhibitor (R)-roscovitine prevents alloreactive T cell clonal expansion and protects against acute GvHD. *Cell Cycle*. 2009; **8**:1794 - 1802.
144. Song H, Vita M, Sallam H, Tehranchi R, Nilsson C, Sidén A, Hassan Z. Effect of the Cdk-inhibitor roscovitine on mouse hematopoietic progenitors in vivo and in vitro. *Cancer Chemother Pharmacol*. 2007; **60**:841-849.
145. Björkman S. Prediction of cytochrome p450-mediated hepatic drug clearance in neonates, infants and children : how accurate are available scaling methods? *Clin Pharmacokinet*. 2006; **45**(1):1-11.
146. Yu C, Rahmani M, Dai Y, Conrad D, Krystal G, Dent P, Grant S. The lethal effects of pharmacological cyclin-dependent kinase inhibitors in human leukemia cells proceed through a phosphatidylinositol 3-kinase/Akt-dependent process. *Cancer Res*. 2003; **63**:1822-1833.
147. Wellwood J, Taylor K. Central nervous system prophylaxis in haematological malignancies. *Intern Med*. 2002; **J32**:252-258.
148. Sallam H, Jimenez P, Song H, Vita M, Cedazo-Minguez A, Hassan M. Age-dependent pharmacokinetics and effect of roscovitine on Cdk5 and Erk1/2 in the rat brain. *Pharmacol Res*. 2008; **58**:32-37.
149. Nouws JF. Pharmacokinetics in immature animals: a review. *J Anim Sci*. 1992; **70**:3627-3634.
150. Rich KJ, Boobis AR. Expression and inducibility of P450 enzymes during liver ontogeny. *Microsc Res Tech*. 1997; **39**:424-435.
151. Alcorn J, McNamara PJ. Pharmacokinetics in the newborn. *Adv Drug Deliv Rev*. 2003; **55**:667-686.
152. Kearns GL, Abdel-Rahman SM, Alander SW, Blowey DL, Leeder JS, Kauffman RE. Developmental pharmacology--drug disposition, action, and therapy in infants and children. *N Engl J Med*. 2003; **349**:1157-1167.
153. Butt AM, Jones HC, Abbott NJ. Electrical resistance across the blood-brain barrier in anaesthetized rats: a developmental study. *J Physiol*. 1990; **429**:47-62.
154. Zheng YL, Li BS, Kanungo J, Kesavapany S, Amin N, Grant P, Pant HC. Cdk5 Modulation of mitogen-activated protein kinase signaling regulates neuronal survival. *Mol Biol Cell*. 2007; **18**:404-413.

155. Nikolic M, Tsai LH. Activity and regulation of p35/Cdk5 kinase complex. *Methods Enzymol*. 2000; **325**:200-213.
156. Patrick GN, Zhou P, Kwon YT, Howley PM, Tsai LH. p35, the neuronal-specific activator of cyclin-dependent kinase 5 (Cdk5) is degraded by the ubiquitin-proteasome pathway. *J Biol Chem.* 1998; **273**:24057-24064.
157. Kesavapany S, Amin N, Zheng YL, Nijhara R, Jaffe H, Sihag R, Gutkind JS, Takahashi S, Kulkarni A, Grant P, Pant HC. p35/cyclin-dependent kinase 5 phosphorylation of ras guanine nucleotide releasing factor 2 (RasGRF2) mediates Rac-dependent Extracellular Signal-regulated kinase 1/2 activity, altering RasGRF2 and microtubule-associated protein 1b distribution in neurons. *J Neurosci*. 2004; **24**:4421-4431.
158. Meijer L, Raymond E. Roscovitine and other purines as kinase inhibitors. From starfish oocytes to clinical trials. *Acc Chem Res.* 2003; **36**:417-425.
159. Phelps MA, Lin TS, Johnson AJ, Hurh E, Rozewski DM, Farley KL, Wu D, Blum KA, Fischer B, Mitchell SM, Moran ME, Brooker-McEldowney M, Heerema NA, Jarjoura D, Schaaf LJ, Byrd JC, Grever MR, Dalton JT. Clinical response and pharmacokinetics from a phase 1 study of an active dosing schedule of flavopiridol in relapsed chronic lymphocytic leukemia. *Blood*. 2009; **113**:2637-2645.
160. Daldrup-Link HE, Link TM, Rummeny EJ, August C, Könemann S, Jürgens H, Heindel W. Assessing permeability alterations of the blood-bone marrow barrier due to total body irradiation: in vivo quantification with contrast enhanced magnetic resonance imaging. *Bone Marrow Transplant.* 2000; **25**:71-78.
161. Bhatia M, Militano O, Jin Z, Figurski M, Shaw L, Moore V, Morris E, Tallamy B, van deVen C, Ayello J, Baxter-Lowe L, Satwani P, George D, Bradley MB, Garvin J, Cairo MS. An age-dependent pharmacokinetic study of intravenous and oral mycophenolate mofetil in combination with tacrolimus for GVHD prophylaxis in pediatric allogeneic stem cell transplantation recipients. *Biol Blood Marrow Transplant.* 2010; **16**:333-343.
162. Burger DM, Verweel G, Rakhmanina N, Verwey-Van Wissen CP, La Porte CJ, Bergshoeff AS, Lyall H, Hartwig NG, Green H, Soldin S, Gibb DM, de Groot R. Age-dependent pharmacokinetics of lamivudine in HIV-infected children. *Clin Pharmacol Ther*. 2007; **81**:517-520.
163. Hassan M, Ljungman P, Bolme P, Ringdén O, Syrůcková Z, Békàssy A, Starý J, Wallin I, Kållberg N. Busulfan bioavailability. *Blood*. 1994; **84**:2144-2150.
164. Hassan M, Oberg G, Bekassy AN, Aschan J, Ehrsson H, Ljungman P, Lönnerholm G, Smedmyr B, Taube A, Wallin I, et al. Pharmacokinetics of high-dose busulphan in relation to age and chronopharmacology. *Cancer Chemother Pharmacol*. 1991; **28**:130-134.
165. Palle J, Frost BM, Gustafsson G, Hellebostad M, Kanerva J, Liliemark E, Schmiegelow K, Lönnerholm G; Nordic Society of Paediatric Haematology

and Oncology. Etoposide pharmacokinetics in children treated for acute myeloid leukemia. *Anticancer Drugs.* 2006; **17**:1087-1094.

166. Palle J, Frost BM, Petersson C, Hasle H, Hellebostad M, Kanerva J, Schmiegelow K, Lönnerholm G; Nordic Society for Paediatric Haematology and Oncology. Thioguanine pharmacokinetics in induction therapy of children with acute myeloid leukemia. *Anticancer Drugs.* 2009; **20**:7-14.
167. Le Tourneau C, Faivre S, Laurence V, Delbaldo C, Vera K, Girre V, Chiao J, Armour S, Frame S, Green SR, Gianella-Borradori A, Diéras V, Raymond E. Phase I evaluation of seliciclib (R-roscovitine), a novel oral cyclin-dependent kinase inhibitor, in patients with advanced malignancies. *Eur J Cancer*. 2010 Dec; **46**(18):3243-50.
168. Siegel-Lakhai WS, Rodenstein DO, Beijnen JH, Gianella-Borradori A, Schellens JHM, Talbot DC. Phase I study of seliciclib (CYC202 or R-roscovitine) in combination with gemcitabine (gem)/cisplatin (cis) in patients with advanced non-small cell lung cancer (NSCLC). *J Clin Oncol.* 2005 Jun; **23**(16S):150
169. Yeo W, Goh B, Le Tourneau C, Green SR, Chiao JH, Siu LL. A phase II randomized study of oral seliciclib in patients with previously treated nasopharyngeal carcinoma. *J Clin Oncol.* 2009 May; **27**(15S):6026.
170. Hsieh WS, Soo R, Peh BK, Loh T, Dong D, Soh D, Wong LS, Green S, Chiao J, Cui CY, Lai YF, Lee SC, Mow B, Soong R, Salto-Tellez M, Goh BC. Pharmacodynamic effects of seliciclib, an orally administered cell cycle modulator, in undifferentiated nasopharyngeal cancer. *Clin Cancer Res.* 2009 Feb 15; **15**(4):1435-42.
171. Kim E, Arnould T, Sellin LK, Benzing T, Fan MJ, Grüning W, Sokol SY, Drummond I, Walz G. The polycystic kidney disease 1 gene product modulates Wnt signaling. J Biol Chem. 1999 Feb 19;274(8):4947-53.
172. Babich V, Zeng WZ, Yeh BI, Ibraghimov-Beskrovnaya O, Cai Y, Somlo S, Huang CL. The N-terminal extracellular domain is required for polycystin-1-dependent channel activity. J Biol Chem. 2004 Jun 11;279(24):25582-9.
173. Ikeda M, Guggino WB. Do polycystins function as cation channels? Curr Opin Nephrol Hypertens. 2002 Sep;11(5):539-45.
174. Parnell SC, Magenheimer BS, Maser RL, Rankin CA, Smine A, Okamoto T, Calvet JP. The polycystic kidney disease-1 protein, polycystin-1, binds and activates heterotrimeric G-proteins in vitro. Biochem Biophys Res Commun. 1998 Oct 20;251(2):625-31.
175. Russo RJ, Husson H, Joly D, Bukanov NO, Patey N, Knebelmann B, Ibraghimov-Beskrovnaya O. Impaired formation of desmosomal junctions in ADPKD epithelia. Histochem Cell Biol. 2005 Dec;124(6):487-97.
176. Wilson PD, Sherwood AC, Palla K, Du J, Watson R, Norman JT. Reversed polarity of Na(+) -K(+) -ATPase: mislocation to apical plasma membranes in polycystic kidney disease epithelia. Am J Physiol. 1991 Mar;260(3 Pt 2):F420-30.

177. Wilson PD. Apico-basal polarity in polycystic kidney disease epithelia. Biochim Biophys Acta. 2011 Oct;1812(10):1239-48.
178. Bukanov NO, Smith LA, Klinger KW, Ledbetter SR, Ibraghimov-Beskrovnaya O. Long-lasting arrest of murine polycystic kidney disease with CDK inhibitor roscovitine. Nature. 2006 Dec 14;444(7121):949-52.
179. Pippin JW, Qu Q, Meijer L, Shankland SJ. Direct in vivo inhibition of the nuclear cell cycle cascade in experimental mesangial proliferative glomerulonephritis with Roscovitine, a novel cyclin-dependent kinase antagonist. J Clin Invest. 1997 Nov 15;100(10):2512-20.
180. Milovanceva-Popovska M, Kunter U, Ostendorf T, Petermann A, Rong S, Eitner F, Kerjaschki D, Barnett A, Floege J. R-roscovitine (CYC202) alleviates renal cell proliferation in nephritis without aggravating podocyte injury. Kidney Int. 2005 Apr;67(4):1362-70.
181. Sheryanna AM, Smith J, Bhangal G, Barnett A, McClue S, Tam FW, Cook T, Pusey CD. Treatment with a cyclin-dependent kinase inhibitor, seliciclib, is effective in reducing glomerular macrophage numbers and the severity of established experimental glomerulonephritis. Nephrology (Carlton). 2011 May;16(4):410-6.
182. Berger SP, Roos A, Daha MR. Complement and the kidney: what the nephrologist needs to know in 2006? Nephrol Dial Transplant. 2005 Dec;20(12):2613-9.
183. Kewalramani R, Singh AK.Immunopathogenesis of lupus and lupus nephritis: recent insights. Curr Opin Nephrol Hypertens. 2002 May;11(3):273-7.
184. Berden JH, Licht R, van Bruggen MC, Tax WJ. Role of nucleosomes for induction and glomerular binding of autoantibodies in lupus nephritis. Curr Opin Nephrol Hypertens. 1999 May;8(3):299-306.
185. Howie JB, Helyer BJ. The immunology and pathology of NZB mice. Adv Immunol. 1968;9:215-66.
186. Theofilopoulos AN, Dixon FJ. Murine models of systemic lupus erythematosus. Adv Immunol. 1985;37:269-390.
187. Zoja C, Casiraghi F, Conti S, Corna D, Rottoli D, Cavinato RA, Remuzzi G, Benigni A. Cyclin-dependent kinase inhibition limits glomerulonephritis and extends lifespan of mice with systemic lupus. Arthritis Rheum. 2007 May;56(5):1629-37.
188. Griffiths PD. CMV as a cofactor enhancing progression of AIDS. J Clin Virol. 2006 Apr;35(4):489-92.
189. Adler SP, Nigro G, Pereira L. Recent advances in the prevention and treatment of congenital cytomegalovirus infections. Semin Perinatol. 2007 Feb;31(1):10-8.
190. Kapasi AJ, Spector DH. Inhibition of the cyclin-dependent kinases at the beginning of human cytomegalovirus infection specifically alters the levels and localization of the RNA polymerase II carboxyl-terminal domain

kinases cdk9 and cdk7 at the viral transcriptosome. J Virol. 2008 Jan;82(1):394-407.
191. Sanchez V, Spector DH. Cyclin-dependent kinase activity is required for efficient expression and posttranslational modification of human cytomegalovirus proteins and for production of extracellular particles. J Virol. 2006 Jun;80(12):5886-96.
192. Tamrakar S, Kapasi AJ, Spector DH. Human cytomegalovirus infection induces specific hyperphosphorylation of the carboxyl-terminal domain of the large subunit of RNA polymerase II that is associated with changes in the abundance, activity, and localization of cdk9 and cdk7. J Virol. 2005 Dec;79(24):15477-93.
193. Sanchez V, McElroy AK, Yen J, Tamrakar S, Clark CL, Schwartz RA, Spector DH. Cyclin-dependent kinase activity is required at early times for accurate processing and accumulation of the human cytomegalovirus UL122-123 and UL37 immediate-early transcripts and at later times for virus production. J Virol. 2004 Oct;78(20):11219-32.
194. Bain M, Sinclair J. The S phase of the cell cycle and its perturbation by human cytomegalovirus. Rev Med Virol. 2007 Nov-Dec;17(6):423-34.
195. Sanchez V, McElroy AK, Spector DH. Mechanisms governing maintenance of Cdk1/cyclin B1 kinase activity in cells infected with human cytomegalovirus. J Virol. 2003 Dec;77(24):13214-24.
196. Schang LM, St Vincent MR, Lacasse JJ. Five years of progress on cyclin-dependent kinases and other cellular proteins as potential targets for antiviral drugs. Antivir Chem Chemother. 2006;17(6):293-320.
197. Rechter S, Scott GM, Eickhoff J, Zielke K, Auerochs S, Müller R, Stamminger T, Rawlinson WD, Marschall M. Cyclin-dependent Kinases Phosphorylate the Cytomegalovirus RNA Export Protein pUL69 and Modulate Its Nuclear Localization and Activity. J Biol Chem. 2009 Mar 27;284(13):8605-13.
198. Greene WC, Peterlin BM. Charting HIV's remarkable voyage through the cell: Basic science as a passport to future therapy. Nat Med. 2002 Jul;8(7):673-80.
199. Karn J. Tackling Tat. J Mol Biol. 1999 Oct 22;293(2):235-54.
200. Price DH. P-TEFb, a cyclin-dependent kinase controlling elongation by RNA polymerase II. Mol Cell Biol. 2000 Apr;20(8):2629-34.
201. Adams M, Sharmeen L, Kimpton J, Romeo JM, Garcia JV, Peterlin BM, Groudine M, Emerman M. Cellular latency in human immunodeficiency virus-infected individuals with high CD4 levels can be detected by the presence of promoter-proximal transcripts. Proc Natl Acad Sci U S A. 1994 Apr 26;91(9):3862-6.
202. Antoni BA, Stein SB, Rabson AB. Regulation of human immunodeficiency virus infection: implications for pathogenesis. Adv Virus Res. 1994;43:53-145.

203. Dingwall C, Ernberg I, Gait MJ, Green SM, Heaphy S, Karn J, Lowe AD, Singh M, Skinner MA, Valerio R. Human immunodeficiency virus 1 tat protein binds trans-activation-responsive region (TAR) RNA in vitro. Proc Natl Acad Sci U S A. 1989 Sep;86(18):6925-9.
204. Feng S, Holland EC. HIV-1 tat trans-activation requires the loop sequence within tar. Nature. 1988 Jul 14;334(6178):165-7.
205. Weeks KM, Crothers DM. RNA recognition by Tat-derived peptides: interaction in the major groove? Cell. 1991 Aug 9;66(3):577-88.
206. Weeks KM, Crothers DM. RNA binding assays for Tat-derived peptides: implications for specificity. Biochemistry. 1992 Oct 27;31(42):10281-7.
207. Herrmann CH, Rice AP. Lentivirus Tat proteins specifically associate with a cellular protein kinase, TAK, that hyperphosphorylates the carboxyl-terminal domain of the large subunit of RNA polymerase II: candidate for a Tat cofactor. J Virol. 1995 Mar;69(3):1612-20.
208. Baskaran R, Dahmus ME, Wang JY. Tyrosine phosphorylation of mammalian RNA polymerase II carboxyl-terminal domain. Proc Natl Acad Sci U S A. 1993 Dec 1;90(23):11167-71.
209. Zhang J, Corden JL. Phosphorylation causes a conformational change in the carboxyl-terminal domain of the mouse RNA polymerase II largest subunit. J Biol Chem. 1991 Feb 5;266(4):2297-302.
210. Boeing S, Rigault C, Heidemann M, Eick D, Meisterernst M. RNA polymerase II C-terminal heptarepeat domain Ser-7 phosphorylation is established in a mediator-dependent fashion. J Biol Chem. 2010 Jan 1;285(1):188-96.
211. Meinhart A, Cramer P. Recognition of RNA polymerase II carboxy-terminal domain by 3'-RNA-processing factors. Nature. 2004 Jul 8;430(6996):223-6.
212. Wei P, Garber ME, Fang SM, Fischer WH, Jones KA. A novel CDK9-associated C-type cyclin interacts directly with HIV-1 Tat and mediates its high-affinity, loop-specific binding to TAR RNA. Cell. 1998 Feb 20;92(4):451-62.
213. Yang X, Gold MO, Tang DN, Lewis DE, Aguilar-Cordova E, Rice AP, Herrmann CH. TAK, an HIV Tat-associated kinase, is a member of the cyclin-dependent family of protein kinases and is induced by activation of peripheral blood lymphocytes and differentiation of promonocytic cell lines. Proc Natl Acad Sci U S A. 1997 Nov 11;94(23):12331-6.
214. Zhu Y, Pe'ery T, Peng J, Ramanathan Y, Marshall N, Marshall T, Amendt B, Mathews MB, Price DH. Transcription elongation factor P-TEFb is required for HIV-1 tat transactivation in vitro. Genes Dev. 1997 Oct 15;11(20):2622-32.
215. Garber ME, Mayall TP, Suess EM, Meisenhelder J, Thompson NE, Jones KA. CDK9 autophosphorylation regulates high-affinity binding of the

human immunodeficiency virus type 1 tat-P-TEFb complex to TAR RNA. Mol Cell Biol. 2000 Sep;20(18):6958-69.

216. Akoulitchev S, Mäkelä TP, Weinberg RA, Reinberg D. Requirement for TFIIH kinase activity in transcription by RNA polymerase II. Nature. 1995 Oct 12;377(6549):557-60.
217. Lu H, Zawel L, Fisher L, Egly JM, Reinberg D. Human general transcription factor IIH phosphorylates the C-terminal domain of RNA polymerase II. Nature. 1992 Aug 20;358(6388):641-5.
218. Zhou M, Halanski MA, Radonovich MF, Kashanchi F, Peng J, Price DH, Brady JN. Tat modifies the activity of CDK9 to phosphorylate serine 5 of the RNA polymerase II carboxyl-terminal domain during human immunodeficiency virus type 1 transcription. Mol Cell Biol. 2000 Jul;20(14):5077-86.
219. Clark E, Santiago F, Deng L, Chong S, de La Fuente C, Wang L, Fu P, Stein D, Denny T, Lanka V, Mozafari F, Okamoto T, Kashanchi F. Loss of G(1)/S checkpoint in human immunodeficiency virus type 1-infected cells is associated with a lack of cyclin-dependent kinase inhibitor p21/Waf1. J Virol. 2000 Jun;74(11):5040-52.
220. Agbottah E, de La Fuente C, Nekhai S, Barnett A, Gianella-Borradori A, Pumfery A, Kashanchi F. Antiviral activity of CYC202 in HIV-1-infected cells. J Biol Chem. 2005 Jan 28;280(4):3029-42.
221. Schang LM. Effects of pharmacological cyclin-dependent kinase inhibitors on viral transcription and replication. Biochim Biophys Acta. 2004 Mar 11;1697(1-2):197-209.
222. Schang LM, Phillips J, Schaffer PA. Requirement for cellular cyclin-dependent kinases in herpes simplex virus replication and transcription. J Virol. 1998 Jul;72(7):5626-37.
223. Schang LM, Rosenberg A, Schaffer PA. Transcription of herpes simplex virus immediate-early and early genes is inhibited by roscovitine, an inhibitor specific for cellular cyclin-dependent kinases. J Virol. 1999 Mar;73(3):2161-72.
224. Hirst M, Kobor MS, Kuriakose N, Greenblatt J, Sadowski I. GAL4 is regulated by the RNA polymerase II holoenzyme-associated cyclin-dependent protein kinase SRB10/CDK8. Mol Cell. 1999 May;3(5):673-8.
225. Sun X, Zhang Y, Cho H, Rickert P, Lees E, Lane W, Reinberg D. NAT, a human complex containing Srb polypeptides that functions as a negative regulator of activated transcription. Mol Cell. 1998 Aug;2(2):213-22.
226. Cismowski MJ, Laff GM, Solomon MJ, Reed SI. KIN28 encodes a C-terminal domain kinase that controls mRNA transcription in Saccharomyces cerevisiae but lacks cyclin-dependent kinase-activating kinase (CAK) activity. Mol Cell Biol. 1995 Jun;15(6):2983-92.

227. Serizawa H, Mäkelä TP, Conaway JW, Conaway RC, Weinberg RA, Young RA. Association of Cdk-activating kinase subunits with transcription factor TFIIH. Nature. 1995 Mar 16;374(6519):280-2.
228. Shiekhattar R, Mermelstein F, Fisher RP, Drapkin R, Dynlacht B, Wessling HC, Morgan DO, Reinberg D. Cdk-activating kinase complex is a component of human transcription factor TFIIH. Nature. 1995 Mar 16;374(6519):283-7.
229. Marshall NF, Price DH. Purification of P-TEFb, a transcription factor required for the transition into productive elongation. J Biol Chem. 1995 May 26;270(21):12335-8.
230. Marshall NF, Peng J, Xie Z, Price DH. Control of RNA polymerase II elongation potential by a novel carboxyl-terminal domain kinase. J Biol Chem. 1996 Oct 25;271(43):27176-83.
231. Peng J, Marshall NF, Price DH. Identification of a cyclin subunit required for the function of Drosophila P-TEFb. J Biol Chem. 1998 May 29;273(22):13855-60.
232. Gebara MM, Sayre MH, Corden JL. Phosphorylation of the carboxy-terminal repeat domain in RNA polymerase II by cyclin-dependent kinases is sufficient to inhibit transcription. J Cell Biochem. 1997 Mar 1;64(3):390-402.
233. Leresche A, Wolf VJ, Gottesfeld JM. Repression of RNA polymerase II and III transcription during M phase of the cell cycle. Exp Cell Res. 1996 Dec 15;229(2):282-8.
234. Schang LM, Hwang GJ, Dynlacht BD, Speicher DW, Bantly A, Schaffer PA, Shilatifard A, Ge H, Shiekhattar R. Human PC4 is a substrate-specific inhibitor of RNA polymerase II phosphorylation. J Biol Chem. 2000 Mar 3;275(9):6071-4.
235. Wang L, Deng L, Wu K, de la Fuente C, Wang D, Kehn K, Maddukuri A, Baylor S, Santiago F, Agbottah E, Trigon S, Morange M, Mahieux R, Kashanchi F. Inhibition of HTLV-1 transcription by cyclin dependent kinase inhibitors. Mol Cell Biochem. 2002 Aug;237(1-2):137-53.
236. Bhattacharjee RN, Banks GC, Trotter KW, Lee HL, Archer TK. Histone H1 phosphorylation by Cdk2 selectively modulates mouse mammary tumor virus transcription through chromatin remodeling. Mol Cell Biol. 2001 Aug;21(16):5417-25.
237. Leitch AE, Lucas CD, Marwick JA, Duffin R, Haslett C, Rossi AG. Cyclin-dependent kinases 7 and 9 specifically regulate neutrophil transcription and their inhibition drives apoptosis to promote resolution of inflammation. Cell Death Differ. 2012 Dec;19(12):1950-61.
238. McLuckie, A. (editor), Respiratory disease and its management., New York, Springer, p. 51, ISBN 978-1-84882-094-4, 2009
239. Leach, R.E., Acute and Critical Care Medicine at a Glance (2nd ed.), Wiley-Blackwell, ISBN 1-4051-6139-6, 2009

240. Pommerville, J.C., Alcamo's Fundamentals of Microbiology (9th ed.), Sudbury MA, Jones & Bartlett., p. 323, ISBN 0-7637-6258-X, 2010
241. Hoogendijk AJ, Roelofs JJ, Duitman J, van Lieshout MH, Blok DC, van der Poll T, Wieland CW. R-roscovitine reduces lung inflammation induced by lipoteichoic acid and Streptococcus pneumoniae. Mol Med. 2012 Sep 25;18:1086-95.
242. Steinman RA, Robinson AR, Feghali-Bostwick CA. Antifibrotic effects of roscovitine in normal and scleroderma fibroblasts. PLoS One. 2012;7(11):e48560.
243. Chavez JC, Hurko O, Barone FC, Feuerstein GZ. Pharmacologic interventions for stroke: looking beyond the thrombolysis time window into the penumbra with biomarkers, not a stopwatch. Stroke. 2009 Oct;40(10):e558-63.
244. Knockaert M, Greengard P, Meijer L. Pharmacological inhibitors of cyclin-dependent kinases. Trends Pharmacol Sci. 2002 Sep;23(9):417-25.
245. Rashidian J, Iyirhiaro GO, Park DS. Cell cycle machinery and stroke. Biochim Biophys Acta. 2007 Apr;1772(4):484-93.
246. Timsit S, Menn B. Cerebral ischemia, cell cycle elements and Cdk5. Biotechnol J. 2007 Aug;2(8):958-66.
247. Cheung ZH, Fu AK, Ip NY. Synaptic roles of Cdk5: implications in higher cognitive functions and neurodegenerative diseases. Neuron. 2006 Apr 6;50(1):13-8.
248. Byrnes KR, Faden AI. Role of cell cycle proteins in CNS injury. Neurochem Res. 2007 Oct;32(10):1799-807.
249. Katchanov J, Harms C, Gertz K, Hauck L, Waeber C, Hirt L, Priller J, von Harsdorf R, Bruck W, Hortnagl H, Dirnagl U, Bhide PG, Endres M. Mild cerebral ischemia induces loss of cyclin-dependent kinase inhibitors and activation of cell cycle machinery before delayed neuronal cell death. J Neurosci. 2001 Jul 15;21(14):5045-53.
250. Kato H, Takahashi A, Itoyama Y. Cell cycle protein expression in proliferating microglia and astrocytes following transient global cerebral ischemia in the rat. Brain Res Bull. 2003 May 15;60(3):215-21.
251. Timsit S, Rivera S, Ouaghi P, Guischard F, Tremblay E, Ben-Ari Y, Khrestchatisky M. Increased cyclin D1 in vulnerable neurons in the hippocampus after ischaemia and epilepsy: a modulator of in vivo programmed cell death? Eur J Neurosci. 1999 Jan;11(1):263-78.
252. Menn B, Bach S, Blevins TL, Campbell M, Meijer L, Timsit S. Delayed treatment with systemic (S)-roscovitine provides neuroprotection and inhibits in vivo CDK5 activity increase in animal stroke models. PLoS One. 2010 Aug 12;5(8):e12117.
253. Kasai H, Imamura T, Tsuruma K, Takahashi Y, Kurasawa T, Hirata H, Shimazawa M, Hara H. Effects of roscovitine, a cell cyclin [correction of

cycling]-dependent kinase inhibitor, on intraocular pressure of rabbit and retinal ganglion cell damage. Neurosci Lett. 2013 Feb 22;535:95-9.

254. Saunders, Dorland's Illustrated Medical Dictionary 31edn., Elsevier, Philadelphia, p1405, ISBN 978-1-4160-2364-7, 2007
255. Ricker, J.H. (ed), *Differential Diagnosis in Adult Neuropsychological Assessment*., Springer Publishing Company., p. 109, ISBN0-8261-1665-5, 2003
256. Ivanov A, Tyzio R, Zilberter Y, Ben-Ari Y. (R)-roscovitine, a cyclin-dependent kinase inhibitor, enhances tonic GABA inhibition in rat hippocampus. Neuroscience. 2008 Oct 2;156(2):277-88.
257. Clavien PA, Harvey PR, Strasberg SM. Preservation and reperfusion injuries in liver allografts. An overview and synthesis of current studies. Transplantation. 1992 May;53(5):957-78.
258. Langdale LA, Flaherty LC, Liggitt HD, Harlan JM, Rice CL, Winn RK. Neutrophils contribute to hepatic ischemia-reperfusion injury by a CD18-independent mechanism. J Leukoc Biol. 1993 May;53(5):511-7.
259. Aydemir A, Abbasoglu O, Topaloglu S, Ertoy D, Ayhan A, Kilinç K, Karabulut E, Sayek I. Protective effect of roscovitine on renal ischemia-reperfusion injury. Transplant Proc. 2002 Sep;34(6):2027-8.
260. Topaloglu S, Abbasoglu O, Ayhan A, Sokmensuer C, Kilinc K. Antiapoptotic and protective effects of roscovitine on ischemia-reperfusion injury of the rat liver. Liver Int. 2003 Aug;23(4):300-7.