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Plant product (R) - Roscovitine valuable inhibitor of CDKs as An anti-cancer agent

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ABSTRACT

Today, cancer is a human tragedy that kills the lives of our beloved people. Cancer in which cell or group of cells display uncontrolled growth, invasion and metastasis. In normal condition, Cell division and death processes are equilibrated to keep tissues at the steady state. For some adult cell renewal is either fast i.e. epithelial, endothelial and certain white blood cells or low i.e. healthy liver cells rarely die and in the adult brain there is a slow loss of cells with little or no replacement. Cell division is a highly organized process since accurate regulation of the cell cycle is essential for normal cell growth and development. CDKs are classic Ser/Thr kinase with molecular weight of 30-40 KDa. Displaying 11 sub domains shared by cell protein kinases. Phosphorylation of serine, Threonine and tyrosine residue represents one of the most common post-translational mechanisms used by cells to regulate their enzymatic and their structural proteins. This review is carried out on natural plant product (R) – Roscovitine which shows inhibitions of CDK activity. It will help to natural product chemist for finding out other sources of (R) - Roscovitine from nature, for chemist to determine various routes of its synthesis its analogues and medicinal chemist/pharmacist to determine its effect towards other CDKs and its pharmacokinetics properties.

Keywords: Plant derived anti-cancer agent, CDKs (cyclin dependent kinase), PDXK (pyridoxal kinase), pRB (retino blastoma protein), and Cell cycle.

INTRODUCTION

Cyclin-dependent kinases (CDKs) are a family of protein kinases first discovered for their role in regulating the cell cycle. They are also involved in regulating transcription, mRNA processing, and the differentiation of nerve cells [1]. They are present in all known eukaryotes, and their regulatory function in the cell cycle has been evolutionarily conserved. In fact, yeast cells can proliferate normally when their CDK gene has been replaced with the homologous human gene [1] [2]. CDKs are relatively small proteins, with molecular weights ranging from 34 to 40 kDa, and contain little more than the kinase domain [1]. By definition, a CDK binds a regulatory protein called a cyclin. Without cyclin, CDK has little kinase activity; only the cyclin-CDK complex is an active kinase. CDKs phosphorylate their substrates on serines and threonines, so they are serine-threonine kinases [1]. The consensus sequence for the phosphorylation site in the amino acid sequence of a CDK substrate is [S/T*]PX[K/R], where S/T* is the phosphorylated serine or threonine, P is proline, X is any amino acid, K is lysine, and R is arginine [1].Cancer is a class of diseases in which a cell, or a group of cells display uncontrolled growth (division beyond the normal limits), invasion (intrusion on and destruction of adjacent tissues), and sometimes metastasis (spread to other locations in the body via lymph or blood). These three malignant properties of cancers differentiate them from benign tumors, which are self-limited, and do not invade or metastasize. Most cancers form a tumor but some, like leukemia, do not. The branch of medicine concerned with the study, diagnosis, treatment, and prevention of

cancer is oncology. Cancer affects people at all ages with the risk for most types increasing with age. It caused about 13% of all human deaths in 2007(7.6 million) [3] [4].

Plant product as the source of anti cancer-agents

Natural products continue to be a major source of pharmaceuticals and for the discovery of new molecular structures [5]. Various plant products like alkaloids, terpenes, sterols, flavanoids, lignans, saponins, Quzssinoids, Ansa Macrolides, Antipyrazoles, Essential oils and Miscelleneous compounds [6].Many of the claims for efficacy in the treatment of cancer, however, should be viewed with some skepticims because cancer, as a specific disease entity, is likely to be poorly defined in terms of folklore and traditional medicines. Natural plant products have been used for the treatment of different diseases for thousands of years. Plants have been used as valuable medicines in India, China, Egypt and Greece from ancient time and effective number of modern drugs has been developed from them. The first written records on the medicinal uses of plants appeared in about 2600 BC from the Sumerians and Akkaidians. The "Ebers Papyrus", the best known Egyptian pharmaceutical record, which documented over 700 drugs, represents the history of Egyptian medicine dated from 1500 BC. The Chinese Materia Medica, Which describes more than 600 medicinal plants, has been well documented with the first record dating from about 1100 BC. Documentation of the Ayurvedic system recorded in Sustra and Charaka Dates from about 10001 BC. The Greek physician described in his work "De material Mediaca" more than 600 medicinal plants. The World Health Organization estimates that approximately 80% of the world's inhibites rely on tradicinal medicinal for their primary health care. Cancer is a human tragedy that strikes and kills the lives of our beloved people in developed and developing countries. It was estimated that there were 10.9 million people's new cases, 6.7 million deaths and 24.6 million persons living with cancer around the world in 2002. Thus, nature origin is defined as natural products, derivatives of natural products or synthetic pharmaceutical based on natural product models [7].

2.1. Extraction protocol of anti-cancer agents from plant specimens

To achieve anticancer agents from plant it must be extracted, concentrated and preserved during storage without being altered through the processes. Then these chemically complex matrix of an extract and available for detection by bioassay [7]. The scheme for production of plant extracts for Biological Active Molecules as shown below figure {1}.

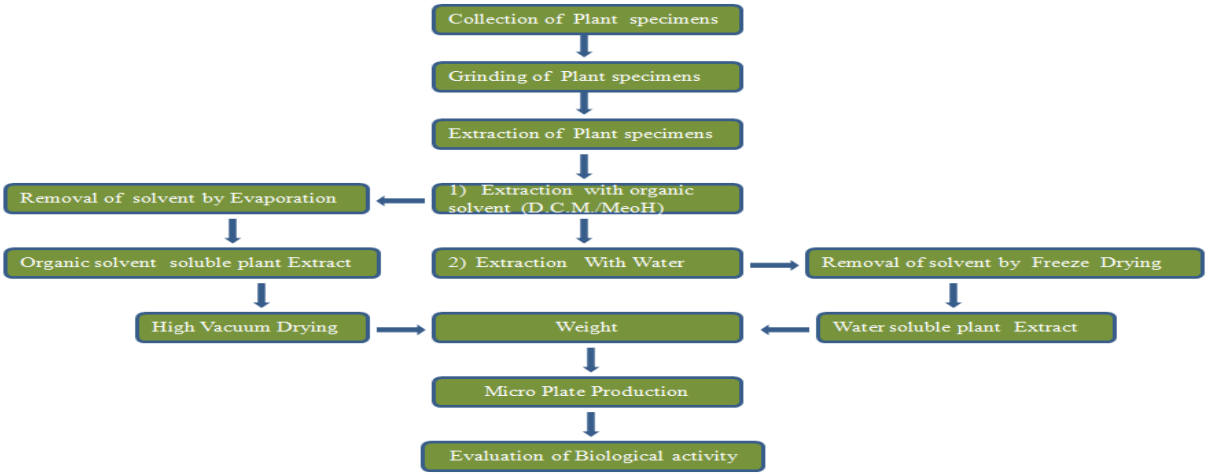


Figure {1}.The scheme for production of plant extracts for Biological Active Molecules.

3.0. Discovery and synthesis of (R) - Roscovitine

Several plant derived natural products leads to cell cycle modulators i.e. Roscovitine which is derived from olomoucine, originally isolated from the cotylendones of the Radish, Raphanus stavivus L. (Brassieaceae) [8].

3.1 Synthesis of (R) – Roscovitine

(R)-Roscovitine is synthesised by a simple and inexpensive three-step procedure, starting from commercially available 2, 6-dichloropurine (figure 2). The overall yield is 50%. The reactive 6-chloro is first substituted by benzylamine upon heating in butanol. Alkylation with 2-bromo or 2- iodopropane, using K_2CO_3 as a base, is then achieved at 20°C in DMSO. Finally, the less reactive 2-chloro is displaced upon heating with (R)-2-amino-butan-1-ol.This last step is improved when DMSO is used as a solvent. The first two steps of the synthesis can be switched (route B). However, alkylation of 2, 6-dichloropurine leads to the formation of a mixture (82/18) of the 9/7 regioisomers which need to be separated by column chromatography [8].

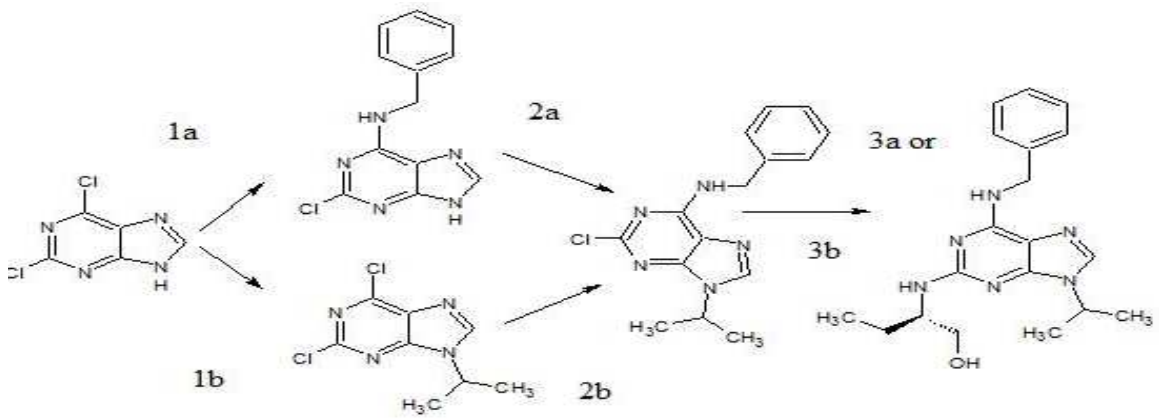


Figure 2. Chemical Synthesis of (R) - Roscovitine.

3.2 Physiochemical Properties of (R) – Roscovitine

The physiochemical properties of (R)-roscovitine are shown in the following table1. [10]

Table 1

Sr. No.	Properties	Functions
1.	Chemical Names	2-(1-ethyl-2-hydroxyethylamino)-6-benzylamino-9 isopropylpurine. 1-butanol, 2-[[-9 (1-methylethyl)-6-[(phenylmethyl)amino]-9H-purin-2yl]amino], (2R)- (R)-2-(6-benzylamino-9-isopropyl-9H-purin-2-ylamino)-butan-1-ol
2.	Other Names	(R) –Roscovitine, CYC202, Seliciclib
3.	State	White Powder
4.	Solubility	Soluble in DMSO (up to 50 mM) and in 50 mM HCL with the pH adjusted to 2.5.
5.	Atomic Composition	$C_{19}H_{26}N_6O$ C= 64.38%; H=7.39%; N=23.71%; O=4.51%
6.	Molecular Weight	354.45
7.	Rotation Values	(R)- Roscovitine: $[\alpha]_{D+}$ 56.3, (S)- Roscovitine: $[\alpha]_D$ -56.3
8.	Melting point	106-108
9.	pKa	4.4
10.	Absorption	λ max: 230nm and 292nm
11.	Chromatographic Analysis	HPLC/UV detection LC-MS/MS
12.	Crystal Structure	Orthorhombic, Space group $P2_12_121$

Table: 2. Selectivity of Roscovitine towards CDKs

Sr. No.	Protein Kinase	IC_{50} (µM)
1.	CDK1/cyclin B	0.65, 2.69,23, 0.45/0.95 (R/S), >80% and 98% at 10µM, 14.1, 1.9,0.67
2.	CDK2/cyclin A	0.7, 0.25, 0.71,1.2/1.8, > 80% inhibition at 10 µM, 2.2, 2.1
3.	CDK2/cyclin E	0.7, 0.95/1.4, 0.10/0.24 (R/S), inhibition at 10 µM,0.13,0.05,0.19
4.	CDK3/cyclin E	1.4/1.5
5.	CDK4/cyclin D1	>100,14.5,75,14.7,14.6,10
6.	CDK5/p25	0.16, >80%inhibiton at10 µM
7.	CDK6/cyclin D1	51
8.	CDK6/cyclin D3	>100, 50
9.	CDK7/cyclin H	0.5-0.6, 0.49, <5, 1.46, 0.51
10.	CDK8/cyclin C	>100, >50
11.	CDK9/cyclin T1	0.6, <5, 0.78

3.3 Selectivity of (R) –Roscovitine towards various Protein Kinases

(R)-Roscovitine has been optimized from the related purine olomoucine using an in vitro CDK1/cyclin B kinase assay [9]. During this initial work, it was realized that (R) - roscovitine displayed rather good selectivity toward CDK1, CDK2, and CDK5 compared to other kinases among a panel of 24 kinases [9]. Since then, the selectivity

has been extensively investigated by various methods first, (R)-roscovitine has been run on other kinase selectivity panels such as Sir Philip Cohen's laboratory kinase selectivity panel (28 kinases) [11]. ProQinase's selectivity panel (85 kinases), Invitrogen's Select Screen TM Kinase Profiling panel (70 kinases), and Cerep's kinase selectivity panel (50 kinases) . A total of 151 protein kinases have been tested for their sensitivity to roscovitine. IC50 values are below 1μM for CDK1, CDK2, CDK5, CDK7, and CDK9 only, whereas CDK4, CDK6, and CDK8 are poorly, if at all, sensitive to roscovitine (Table 2). [10].

Only a few kinases are sensitive to roscovitine in the 1 to 40 μM range (CaM Kinase 2, CK1α, CK1 δ, DYRK1A, EPHB2, ERK1, ERK2, FAK, and IRAK4), but most other kinases are insensitive to roscovitine. Based on these data, roscovitine appears to be a reasonably selective kinase inhibitor. However, this panel only reflects 29.2% of the reported 518+ kinases of the human kinome. The second method used to address the selectivity of (R)-roscovitine is based on the identification by mass spectrometry of the roscovitine-binding proteins that can be purified by affinity chromatography on sepharose-immobilized roscovitine from various tissues and cell extracts [12]. This method has been successfully applied to purvalanol and other kinase inhibitors [13, 14]. Roscovitine beads allowed the identification of expected targets such as CDKs, but also various CaM Kinase 2 isoforms, ERK1, ERK2, and CK1α. Surprisingly, pyridoxal kinase (PDXK), the enzyme responsible for the phosphorylation and activation of vitamin B6, a cofactor of many enzymes, was identified as a roscovitine-binding protein in all biological materials tested. This interaction was investigated in detail and further confirmed by the cocrystallization of (R) - roscovitine with sheep brain PDXK [12, 15]. The third method that has been used is a yeast three-hybrid screen [16] based on the reconstitution of an active transcription factor from the close association of a DNA-binding domain (DBD) and the activation domain (AD) of a transcriptional activator (GAL4) expressed separately. The DBD is fused to dihydrofolate reductase (DHFR), and the AD is fused to a library of potential kinase targets. The kinase inhibitor is attached to methotrexate through a polyethylene-glycol linker. The binding of methotrexate to DHFR, on one hand, and the inhibitor to its target, on the other hand, reconstitutes a functional DBD/AD transcription factor, allowing the detection of the inhibitor's targets and their identification [16]. This elegant method was used with purvalanol and roscovitine as proof of principle. The results showed that (R)-roscovitine interacts with its known target CDK2, but also with CK1δ, CK1ε, and the CDK-like kinase PCTK1, and more weakly with CLK1, PAK4, PCTK3, PKWA, and GSK3α [16]. A fourth approach that has been used to investigate the selectivity of (R)-roscovitine is a quantitative competition assay carried out in the absence of ATP or protein substrate in contrast to the classical kinase inhibition assays. It is based on the interaction of a given inhibitor immobilized to biotin with a library of protein kinases expressed as T7 bacteriophage capsid protein fusion proteins [17]. This method has been applied to 20 known, ATP competitive, clinical kinase inhibitors and to 113 kinases. It confirmed the rather good selectivity of (R)-roscovitine, which was found to bind to CDK2, CDK5, PCTK1, CK1γ1, CK1γ2, CK1ε, CLK1, CLK2, CLK4, TTK, and RPS6KA2 (Kinase Domain 1). These and other methods used to identify the targets of inhibitors of CDKs [18] and other kinases [19].

3.4 Biochemical Properties (R)-Roscovitine/Target Co-crystal Structures

Classical enzymology has shown that (R)-roscovitine acts by competing with ATP for binding at the ATP-binding site of CDK1/cyclin B [20]. This binding at the catalytic site was confirmed by direct cocrystallization of (R)-roscovitine with and later with CDK5/p25 (1UNL) and CDK2/cyclin A. A CDK1/roscovitine model has also been described [21]. These crystal structures reveal the interaction between (R) - roscovitine and the amino acids that line up the ATP-binding pocket of the CDK catalytic subunit (Figure {3} A, B and C).

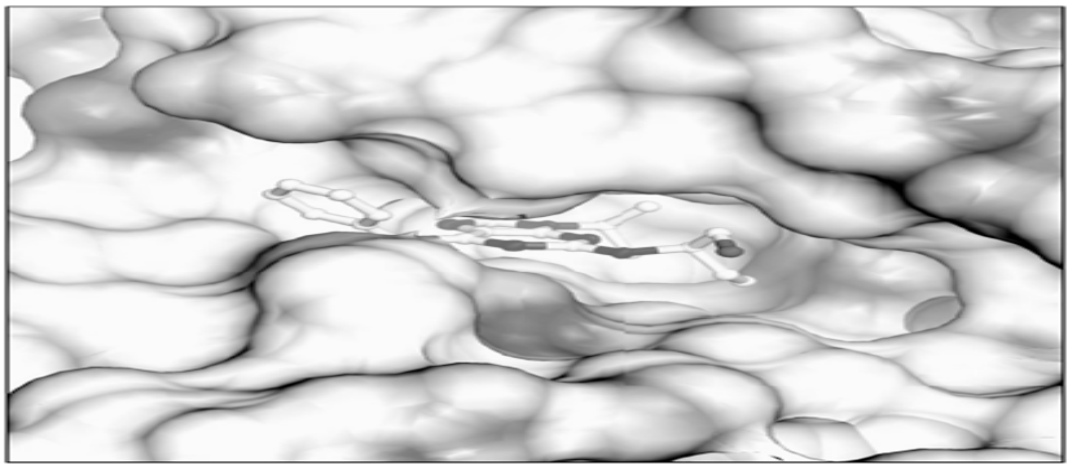
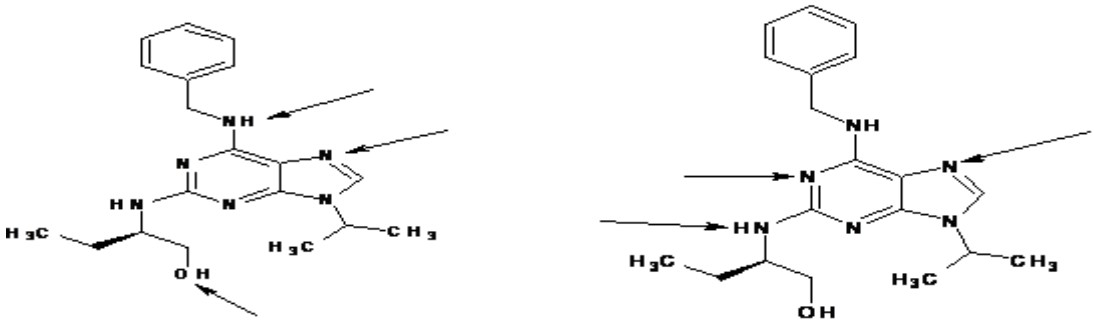


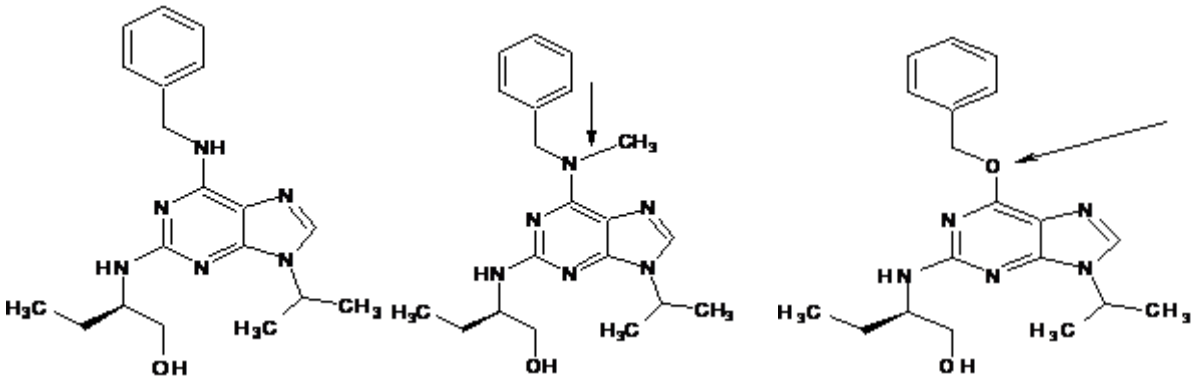
Figure {3} A. Interaction of (R) - roscovitine with tis targets.



{B} Left **{C} Right**

Figure 3. {B} Left, crystal structure of CDK2 in complex with (R)-roscovitine, illustrating the position of (R)-roscovitine in the ATP-binding pocket and how its benzyl ring is facing the outside of the kinase. {C} Right, roscovitine and its atoms involved in H-bonds with either CDKs or PDXK.

Briefly, the interaction involves mostly hydrophobic and van der Waals contacts and two hydrogen bonds (involving N^7 and N^6 of the purine) with backbone atoms of Leu 83 (CDK2). In addition, a weak hydrogen bond is formed between O1 and a water molecule. A similar binding mode is observed with CDK5 (involving Cys83) [21]. The binding mode suggested that N6-methyl-(R)-roscovitine or O^6-benzyl-(R)-roscovitine (Figure 4) would be unable to interact at the ATP site, and would therefore constitute useful kinase-inactive control. This was confirmed experimentally by kinase assays and also by affinity chromatography on immobilized N^6-methyl-(R)-roscovitine [15].



(R)- roscovitine (CDK1: 0.45 µM) (CDK2: 0.40 µM) **N6-Methyl-(R)- roscovitine (CDK1: 45 µM) (CDK2: 250 µM)** **O6-Methyl-(R)roscovitine (CDK1: 100 µM) (CDK2: 250 µM)**

Figure 4. (R)-roscovitine and its control, protein kinase inactive analogs, (N6-methyl-(R)-roscovitine), and (O6- benzyl-(R)-roscovitine). Arrows point to the chemical changes introduced in the roscovitine analogs. The IC_{50} values for in vitro inhibition of CDK1/cyclin B and CDK2/cyclin E are shown in parentheses.

The binding mode suggested that N^6-methyl-(R)-roscovitine or O^6-benzyl-(R)-roscovitine (Figure 5) would be unable to interact at the ATP site, and would therefore constitute useful kinase-inactive controls. This was confirmed experimentally by kinase assays and also by affinity chromatography on immobilized N^6-methyl-(R)-roscovitine [15]. The cocrystal structures also reveal that the benzyl ring is facing the outside of the ATP-binding pocket. This property selects the place where a linker can be together to roscovitine to immobilize it on sepharose beads while still maintaining the potential interaction with its protein kinase targets. A control matrix is obtained when N^6-methyl-(R)-roscovitine is immobilized to sepharose beads (Figure 4). Affinity chromatography with sepharose-immobilized roscovitine revealed that roscovitine interacts with PDXK from all species and tissues that have been tested [12]. (R)-Roscovitine has been cocrystallized with sheep brain PDXK and the interaction investigated in detail. Surprisingly, (R)-roscovitine is located in the pyridoxal-binding site, rather than at the ATP-binding site. Furthermore, the atoms of roscovitine involved in the binding to PDXK are not the same as those implicated in the binding to CDKs. This has allowed us to synthesize two compounds (Figure 4B & 4C) that interact with PDXK — they were actually cocrystallized with PDXK [15] but that do not bind to CDKs. We are currently synthesizing roscovitine analogs that should bind CDKs without interacting with PDXK. The two sets of molecules should allow us to distinguish the cellular effects of roscovitine due to interaction with protein kinases from those due to

interaction with PDXK. These examples illustrate the extraordinary diversity of information that can be drawn from the structure of kinase/inhibitor complexes [22].

3.5 Functions of (R)-Roscovitine

(R)-Roscovitine is the natural Cdk inhibitors which play an important role in the regulation of the cell cycle and involved in signaling pathways required for several aspects of cell division and proliferation E.g. transcription, apoptosis and neural development. Mal functions of these enzymes have been associated to several diseases [23, 24].

(**1) Cdks and cell cycle:** Several Cdks control the major steps of the cell cycle phase by proliferation of distinct cell proteins such as transcription factors, histones, cytosketal proteins, tumor suppressor genes [25]. They associate with cyclin-subunits, whose concentrations soscillate along the cell cycle, causing the stage specific timing of the Cdks [26], as described in (figure 5).

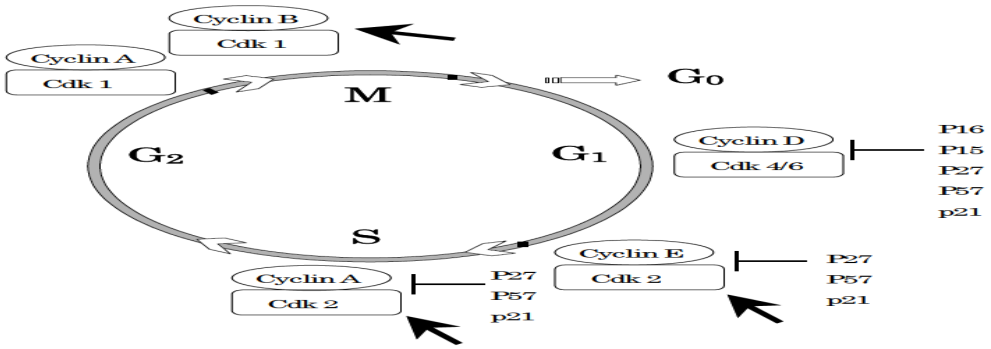


Figure 5. Schematic representation of the cell cycle and its regulatory components. Black arrows indicate the possible action for roscovitne.

In higher eukaryotic organisms, cell cycle control involves a complex combination of Cdks and cyclins. Cdk2-cyclin E complex acts at the beginning of S phase and induces initiation of DNA synthesis, phospholates the retinoblastoma protein (pRB) causing the release of transcription factors and regulates centrosome replication. Cdk-cyclin B complex participates in mitosis initiation, phosphorylates different substrate, e.g. nuclear lamins and anaphase promoting factor and Cdk1 binds to cyclin A and contributes to preparation for mitosis [23]. During G1 Phase, Cdk4 and Cdk6 complexes can phosphorylated and inhibit pRB resulting in release of transcription factors [27]. These complexes can phosphorylate and inhibit pRB resulting in release of transcription factors.

(2) Cdks and cancer: Normal Cdks activities are important for the ordered execution of the processes that govern cell growth, such as accurate and complete DNA replication, RNA transcription and mitotic transfer of the genome to new daughter cells [28, 29]. Abnormal expression and activity of Cdks, their regulators and substrates have been associated with cancer development and progression [30, 24]. Some of these aberrations comprise over expression of cyclin D and CDk 4 encoding genes, mutations in INK4a gene (Cdk inhibitor) and inactivation of pRB, which produce alterations in the RB pathway [31]. Amplification of MDM2, mutations on members of Cio/Kip family of Cdk inhibitors, and mutations or deletions in P53 gene pathway. Since several cancerous cells have corrupted control of the cell cycle and various Cdks are activated in many tumors compared to normal cells, targeting Cdks would be an intelligent strategy to block and/or interfere with tumor cell proliferation as an alternative to classical cytotoxic drugs.

(3) Effects on transcription: Roscovitine was reported to inhibit RNA synthesis in human neonatal fibroblast and in human colon carcinoma cell line by partially inhibiting the Phosphorylation of the C-terminal domain of RNA polymerases II [32]. The transcription of human immunodeficiency virus type 1 (HIV-1) and other viruses were inhibited after exposure to roscovitine. HIV-1 production in virally integrated lymphocytic and monocytic cell lines and activated peripheral blood mononuclear cells infected with different HIV-1 strains were inhibited by exposure to roscovitine [9].

(4) Effects on apoptosis: Roscovitine seems to have contradictory effects towards apoptosis depending on the cycling status of the cell. In highly dividing cells, roscovitine alone or in combination with other therapies stimulate apoptosis [33, 34, 35, 36, 37]. In contrast, in non-dividing or differentiating cells such as neurons and thymocytes, roscovitine exerted a protective effect. Active cytoplasmic Cdk2 has been associated with in apoptosis in several studies [38, 39]. A decrease in Cdk2 activity by roscovitine was related to reduction in apoptosis mesangial cells

exposed to stress conditions such as serum deprivation or UV exposure [40]. Roscovitine was also shown to block thermocyte negative selection through CDK2 inhibition.

(5) Effects on brain tissue: Cdk5 is mostly expressed in brain. Binding of Cdk5 with p35 and p39 has been reported to play an important role in brain development [41]. Roscovitine was found to potent CDK5 inhibitor [42, 7]. Roscovitine was also shown to activate specifically P/Q calcium channels in central neurons and to modify transmitter release [43].

(6) Effects on viral replication: Cdks are involved in the replication of many clinically important viruses including papilloma, HIV-1, human cytomegalovirus (HCMV) and herps simplex virus (HSV) type1 and type2. These make Cdk inhibitors a promo sing alternative for the treatment of viral infection. In this context, roscovitine has been shown to inhibit the replication of HSV via inhibition of cellular Cdks and the replication of HCMV through inhibition of cellular Cdk2 activity [44].

(7) Effects on tumor cells: Roscovitine was shown to inhibit the proliferation of several tumor cell lines e.g. head and neck, squamous-cell carcinoma [36] uterine sarcoma, lung and colorectal carcinoma [45]. Additionally, roscovitine was reported to inhibit DNA synthesis in human gliomas [46] and human cervical tumors [47].

(8) Effects in combination therapy: The synergistic and sensitizing effect of roscovitine in combination with chemotherapy and radiotherapy was demonstrated in several studies. The selective sensitization of pRB-deficient tumor cell lines to exposure of doxorubicin together with radiotherapy was demonstrated in several studies. The selective sensitization of of pRB –deficient tumor cell lines to exposure of doxorubicin together with roscovitine and flavopiridol has been demonstrated [48]. This effect is relevant considering the abnormal activity of pRB observed in human tumors. Roscovitine in combination with campothecin, a topoisomerase I inhibitor, showed in tumor cell lines synergestic activation of p53, nuclear accumulation of wild-type p53 and apoptosis [49]. The synergestic effect of farnesyl-transferaser inhibitors in combination with roscovitine have been reported to cause apoptosis in human cancer cell lines [50]. The radio-sensitization effect of roscovitine was described in in vitro in human breast cancer cell-lines (MDA-MB 231), which lacks functional p53 protein and in vivo in mice xenografted with MDA-MB 231 [51].

(9) Effects in vitro reproduction: Several studies have described the effects of roscovitine on mammalian oocytes maturating, because of its ability to inhibit Cdks [52, 53] reported an enhancement in cloned calf and fetus survival after embryo transfer of roscovitine- treated adult somatic cells. Roscovitine synchronized donor cell cycle and increased the nuclear programming capacity of the cells [54]. Many Cdk inhibitors are currently being evaluated for the in vivo synchronization of oocyte cell cycle, achievement of fertilization and development of the embryos and improvement of survival rate of cloned domestic animals.

3.6 Mechanisms of action of (R) - Roscovitine towards cell cycle and cell death.

(R)-roscovitine acts in different ways, all of which converge towards cell cycle arrest and cell death, thereby providing the observed anti-tumor effects. Induction of cell cycle arrest originates from both a direct inhibition of cell cycle- regulating CDKs (Figure 6A).

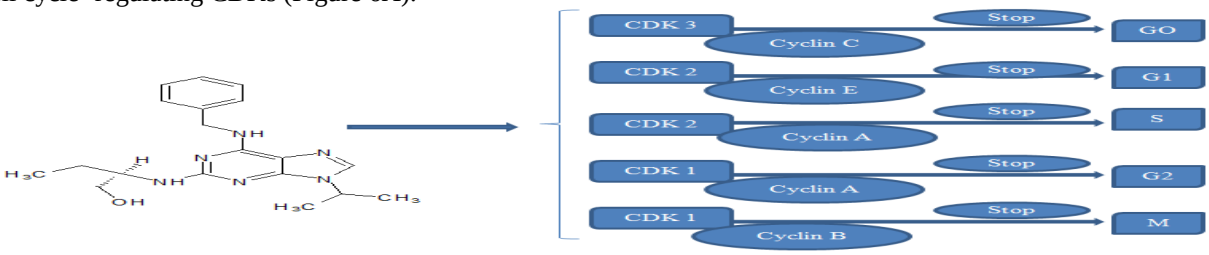


Figure {6}A. Direct stochiometric interaction with CDKs leads to inhibition of the catalytic activity of various CDK/cyclin complexes with a direct effect on various cell cycle phases.

An indirect effect by inhibition of the upstream CDK-activating CDK7 and an increased level of the CDK inhibitory p27KIP1 (Figure 6B).

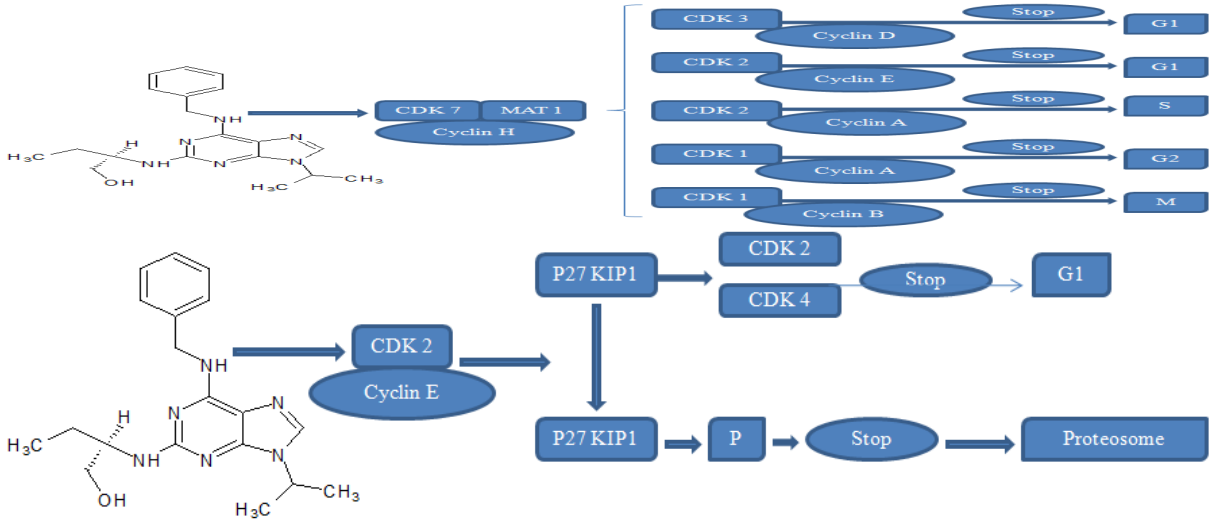


Figure {6}B. Indirect inhibition of cell cycle progression

(i) Interaction with CDK7/Cyclin H MAT1 prevents the Phosphorylation of a key activating threonine residue located on the T-loop of the substrate CDKs.
(ii) Inhibition of CDK2/Cyclin E prevents Phosphorylation and subsequent proteolytic degradation of p27 KIP1, a natural CDK2/CDK4 inhibitor.

Induction of cell death originates from a transient reduction in transcription due to direct inhibition of CDK7 and CDK9, leading to the down regulation of essential, short-lived survival factors that are typically expressed in cancer cells such as Mcl-1, XIAP, surviving (Figure 6C).

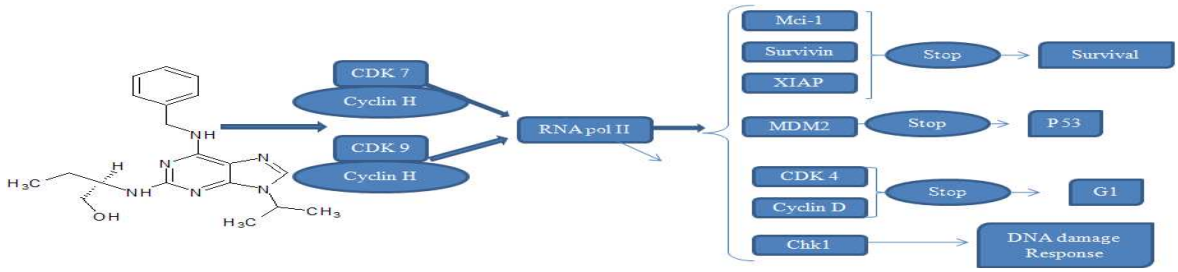


Figure {6}C. Indirect interaction with CDK7/cyclin H and CDK9/cyclin T leads to inhibition of RNA polymerase

Furthermore, we believe that the short half-life of (R)-roscovitine and the lack of activity of its metabolites together prevent a long-term and massive inhibition of transcription, which is likely to be deleterious to normal cells. We hypothesize that a brief inhibition of transcription selectively affects tumor cells that are highly dependent on short-lived survival factors. The transient down regulation of these survival factors then triggers an irreversible activation of apoptosis that can proceed even after roscovitine has been metabolized away. In contrast, normal cells, which do not rely on these survival factors, are only transiently and reversibly arrested in their cell cycle progression. In addition to these direct anti-tumor effects, roscovitine appears to display synergistic properties with a number of anti-tumor treatments. These effects are highly dependent on the sequence of drug treatment. These additive or synergistic effects have strong implications for the use of (R)-roscovitine in chemotherapy. In contrast with the cell-death-inducing properties described earlier, (R)-roscovitine, similar to other CDK inhibitors, has well-established antiapoptotic properties, mostly but not exclusively [55], in non dividing cells such as neural cells [56]. These properties are being extensively investigated for their applications in the neurodegeneration diseases field [28]. It is still largely unknown how CDK inhibitors are able to protect cells from apoptosis induced by various factors. Depending on the model, CDK1 [55, 57, 58], CDK2 [59, 60] or CDK5 [61] is involved. The contribution of CDKs varies according to cell type, conditions, nature and concentration of the apoptosis-inducing drug. The antiapoptotic properties of CDK inhibitors may reduce the use of these compounds as anti-tumor drugs. Nevertheless, CDK inhibitors might diminish their own side effects and even be used to counteract damaging effects of other anti-tumor drugs. For example, roscovitine could be used as therapy for cisplatin induced nephrotoxicity [62]. In addition, these

antiapoptotic properties of roscovitine might provide some protection to normal cells. Understanding the paradoxical apoptosis-inducing and apoptosis-preventing properties of roscovitine and other CDK inhibitors is a major challenge for current research. CDK2 has been closely linked with melanoma growth [63]. The CDK2 gene overlaps with the melanocyte-specific gene SILVER/PMEL17, which encodes an antigen commonly used for melanoma diagnosis and immune therapy. Both genes are regulated by the melanocyte lineage transcription factor MITF. CDK2 appears to be an essential target gene for MITF, which is important for survival of melanocytes and melanoma. Mutations in MITF lead to melanocyte defects. Expression of MITF and CDK2 are tightly correlated in human melanoma samples and melanoma cell lines, and their levels predict sensitivity to (R)-roscovitine [63]. Finally, interesting studies have shown that cyclin E is expressed as low molecular weight (LMW) forms in breast and melanoma cancers [64, 65]. Expression of LMW cyclin E strongly correlates with poor prognosis. CDK2 associated with these LMW forms is quite active and resistant to inhibition by protein inhibitors such as p27KIP1 and, therefore, constitutes an attractive target in these clinical settings.

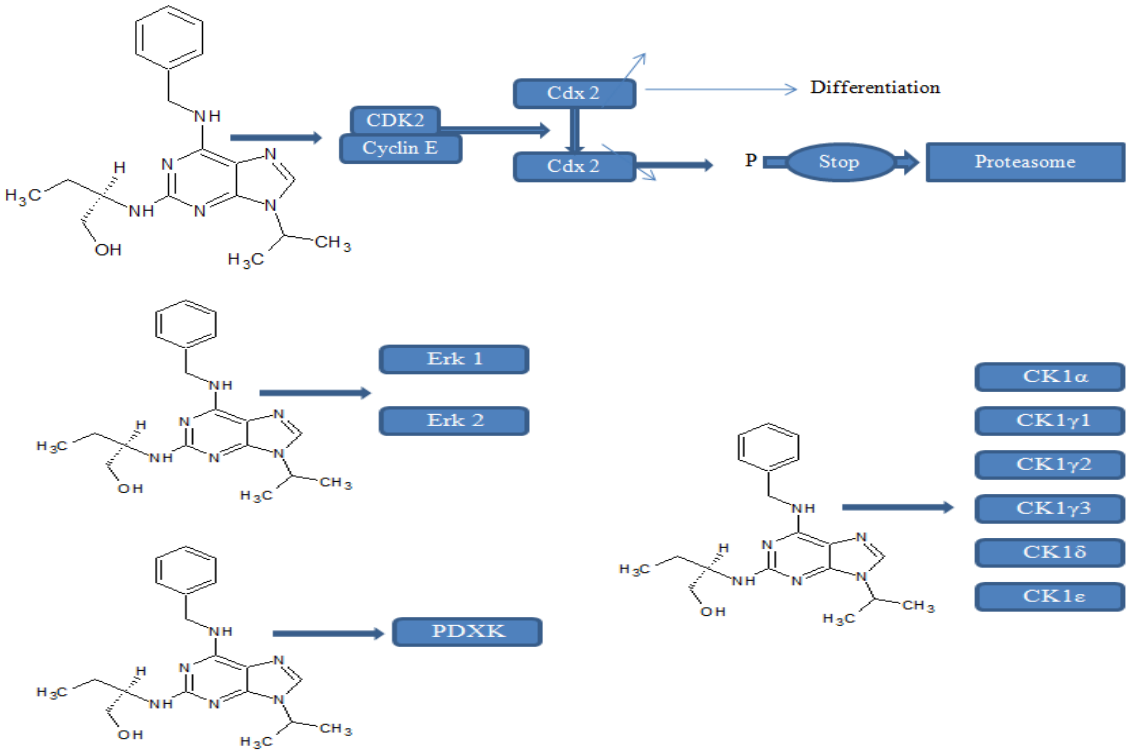


Figure {6}D. Other possible mechanism of action. Inhibition of CDK2/cyclin E prevents phophorylation and subsequent proteolytic degradation of cdx2, a transcription factor involved in intentional cell differentiation.

3.7 Pharmacology of (R)-Roscovitine

A correlation has been established between some chemical parameters of a molecule and its absorption or permeation properties [66]. (R)-roscovitine appears to meet all required parameters for favorable absorption.

(1) Quantification of (R)-Roscovitine: Two methods have been developed to quantify (R)-roscovitine. The first is high performance liquid chromatography, associated with detection at 292 nm, and provides a working linear range of detection between 100 mg/ml to 5000 mg/ml (i.e., 0.28 to 14 µM) [67]. The second is liquid chromatography coupled to tandem mass spectrometry (LC-MS/MS) and allows the detection and quantification of (R)-roscovitine over a range of 0.5 to 2000 mg/ml (i.e., 0.0014 to 5.6 µM) [67, 68, 69].

(2) Pharmacokinetics: Pharmacokinetic studies of (R)-roscovitine injected in rat (25 mg/kg body weight) show a rapid, biphasic elimination of the drug with a 5 min and a <30 min half-life, in accordance with a two-compartment open model [67, 70]. In mouse, the plasma level of roscovitine also rapidly drops within 30 min to <1% of the I.V. injected dose (40 mmol/g, i.e., about 15 mg/kg) [68, 69, 72]. Detailed pharmacokinetic studies performed in BALB/c mice [50] and Tg26 mice. They show rapid, bi exponential clearance of (R)-roscovitine from plasma following I.V., I.P., or oral administration [68, 72, 73]. (R)-Roscovitine uptake into the general circulation was fast and its plasma half-life was 1.19 h. Plasma concentrations could be maintained above 15 µM (the average IC_{50}

values obtained with various tumor cell lines) for 4, 12, and 24 h following oral administration at 50, 500, and 2000 mg/kg, respectively [69]. The pharmacokinetics of (R)-roscovitine was recently studied in humans [75]. Following oral administration of a single dose (50, 100, 200, 400, and 800 mg) in healthy men, (R)-roscovitine and its carboxylated metabolite were measured in plasma and urine. (R)-Roscovitine undergoes rapid passage into the blood, distribution in tissues, and metabolism.

(3) Metabolism: When (R)-roscovitine was injected I.V. at 100 mg/kg in mouse [72] or at 25 mg/kg in rat [70] several metabolites were identified in the plasma (Figure 7). (R)-Roscovitine undergoes a rapid loss of the isopropyl group (M1), several oxidations (M2–M7), or conjugation of a glucose residue (M8). M3 is the most abundantly produced metabolite, which is then excreted in urine [70, 71, 72]. When (R)-roscovitine was incubated in microsomal preparations, M1 to M6 metabolites were generated, the COOH-(R)-roscovitine M3 being the most abundant. Sensitivity to the absence of NADPH and to SKF-525A demonstrates that this main metabolite is produced through an NADPH- and cytochrome-P450-dependent process. Glycosidation is also a major pathway observed in rodent and primate microsomes [75]. Both M3 and M6 were synthesized and found to be much less potent than the parent (R)-roscovitine at inhibiting CDK2 [72]. In humans, the carboxylated derivative was also the main metabolite formed following oral administration of (R)-roscovitine as shown in figure {7} [74].

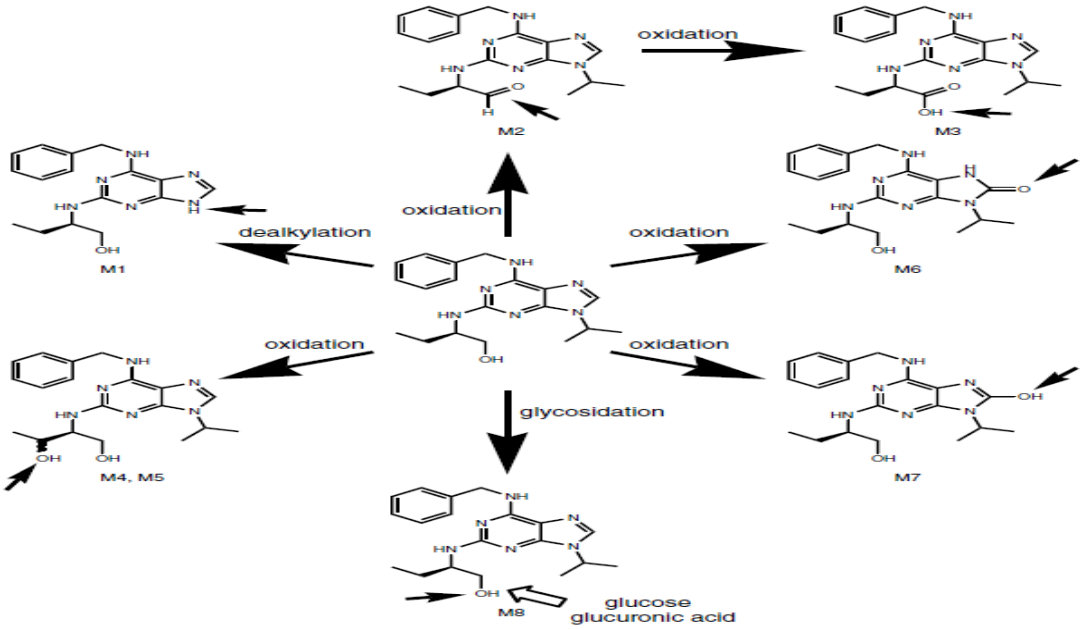


Figure {7}. (R)-Roscovitine and its metabolites. Small arrows point to the chemical changes introduced in the initial roscovitine structure.

(4) Toxicity: (R)-Roscovitine appears to be well tolerated. The maximum tolerated dose (MTD) in mice could not be reached when (R)-roscovitine was delivered intravenously; owing to poor solubility (maximal achievable dose was 20 mg/kg) [45]. Nor could MTD be reached when (R)-roscovitine was given orally (maximal achievable dose was 2000 mg/kg) [45]. When administered intraperitoneally, three doses of 100 mg/kg were well tolerated [45]. In BALB/c mice, the MTD was 100 mg/kg for intravenous injection. For intraperitoneal injections, 150 mg/kg was well tolerated. Finally, (R)-roscovitine was well tolerated up to 2000 mg/kg when administered as a single oral dose [69].

(5) Storage: Roscovitine is a rather stable compound as raw material or in nonaqueous solutions. For long-term storage, we recommend storage as a dry powder at −20°C [10].

(6) Dilution: Roscovitine is usually dissolved in dimethylsulfoxide (DMSO) as a stock solution of 10 to 50 mM. It can be aliquoted and stored at −20°C. However, some precipitation is sometimes observed, probably because of the absorption of water by DMSO.

(7) Concentrations: When tested in in vitro enzymatic assays, we suggest the following range of roscovitine concentrations for initial testing: 0–0.01–0.025–0.05–0.1–0.25–0.5– 1–2s.5–5–10–25–50–100 µM (a final ATP

concentration of 15 μM ATP has been routinely used in our kinase assays). In initial cellular tests, we recommend the following range of concentration: 0–0.1–0.25–0.5–1–2.5–5–10–25–50–100 μM. For in vivo testing, several routes have been used to deliver roscovitine. Experimental data show that roscovitine displays high bioavailability when delivered intraperitoneally or orally [10].

(8) Affinity towards chromatography: For affinity chromatography on immobilized roscovitine [12], extracts are first prepared in a homogenization buffer and centrifuged for 10 min at 14,000 g at 4°C. The supernatant is assayed for protein content and immediately loaded batch wise on the affinity matrix. Just before use, packed roscovitine beads are washed with bead buffer and resuspended in this buffer. The cell or tissue extract supernatant (up to 3 mg total protein) or purified protein is then added. The tubes are rotated at 4°C for 30 min. After a brief spin and removal of the supernatant, the beads are washed four times with bead buffer before addition of 2X Laemmli sample buffer. Following heat denaturation, the bound proteins are analyzed by SDS-PAGE and Western blotting or silver staining. Protein bands can then be excised and digested in gel with trypsin. The resulting peptides are purified and concentrated prior to mass spectrometry analysis.

CONCLUSION

Considerable review carried out from the early discovery of roscovitine starting from the isopentenyladenine and olomoucine structures and using native CDK1/cyclin B kinase purified from the highly synchronous starfish M phase oocytes. (R)-Roscovitine appears to be a rather selective inhibitor of a few CDKs, but it interacts with a few other protein kinases and pyridoxal kinase. The antimitotic and proapoptotic properties of (R)-roscovitine are to be accounted for by a favorable combination of effects converging both to multiple cell cycle arrest points and to induction of cell death by several parallel mechanisms. This multitarget effect of (R)-roscovitine constitutes a weakness when the drug is used as a pharmacological tool in cell biology studies, and results should be interpreted with care. In contrast, this diversity of molecular actions becomes an advantage when (R)-roscovitine is investigated as a potential drug for the treatment of cancer. Moreover, with such complexity of cellular targets, we do not expect rapid resistance to develop following (R)-roscovitine treatment. This is supported by the fact that, despite several serious efforts, no (R)-roscovitine-resistant cell lines have been reported. Also, despite the genetic demonstration that CDK2 is dispensable for mitotic division [76], the sensitivity of CDK2−/− cells to (R)-roscovitine is only slightly lower than that of CDK2+/+ wild-type cells [12]. The artificial absence of CDK2 appears to be compensated by CDK1 [77], another target of (R)-roscovitine. The contribution of the interaction between (R)-roscovitine and PDXK to its anti-tumor effects remains an open question. To address this issue, we are currently designing roscovitine derivatives deprived of interaction with PDXK but still inhibiting CDKs. Protein kinase-inactive but PDXK binding (R)-roscovitine derivatives are not completely devoid of antiproliferative effects, but these effects may be unspecific as they require very high doses. The pharmacological parameters of (R)-roscovitine certainly could be improved by slight modifications of the parent structure which becomes a challenge for chemists to synthesize its analogues for anti-cancer (inhibitors of cyclin dependent kinases) screening for future years. Whether the relatively short half-life of (R)-roscovitine constributes an advantage or a disadvantage is still to be determined. One way to address this issue would be to generate more metabolically stable (R) - roscovitine derivatives with identical biochemical properties. The oral bioavailability of (R)-roscovitine certainly constitutes a great advantage. Dosing frequency of administration and circadian optimization of drug delivery need to be investigated further to obtain the most suitable drug exposure. Furthermore, the combination of (R)-roscovitine with currently used cancer treatments represents a promising field of investigation. Finally, the identification of the cancer subtypes that are most sensitive to (R)-roscovitine remains an open field. Currently, B-cell malignancies, lung cancer, breast cancer, and melanoma seem to be promising clinical targets.

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