

9 (*R*)-Roscovitine (CYC202, Seliciclib)

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9.1 INTRODUCTION

Phosphorylation of serine, threonine, and tyrosine residues represents one of the most common post-translational mechanisms used by cells to regulate their enzymatic and structural proteins. Phosphorylation is catalyzed by protein kinases, whereas dephosphorylation is carried out by protein phosphatases. Among the 518 human protein kinases, cyclin-dependent kinases (CDKs) (Malumbres and Barbacid, 2005) have aroused considerable interest because of their essential involvement in cell cycle control (Malumbres and Barbacid, 2001; Meijer, 2003), neuronal cell physiology (Cruz and Tsai, 2004), pain signaling (Pareek et al., 2006), apoptosis (Borgne and Golsteyn, 2003), transcription, and RNA splicing (Garriga and Grana, 2004; Loyer et al., 2005). CDKs are regulated in four different ways: (1) transient association with a regulatory partner (cyclin), (2) various post-translational modifications (phosphorylation, ubiquitin-dependent degradation), (3) transient association with a natural inhibitory protein (CIP1, KIP1/2, or INK4A-D), and (4) intracellular localization. Although the human genome sequencing program has resulted in the detection of about 20 CDKs and 25 cyclins, a more limited number of active CDK/cyclin complexes have been identified (Figure 9.1).

Alterations in the phosphorylation of proteins represent a frequent feature associated with human disease. This is the reason for an exponentially growing investment in the discovery, optimization, and therapeutic evaluation of small molecular weight, pharmacological inhibitors of protein kinases (reviews in Cohen, 2002; Fischer, 2004; Weinmann and Metternich, 2005). It is estimated that 30 to 35% of drug discovery programs in the pharmaceutical industry currently target a protein kinase. Presently, 55 kinase inhibitors are undergoing clinical evaluation against diseases such as cancer, inflammation, diabetes, and neurodegeneration.

Abnormalities in CDK activity and regulation in cancers (Vermeulen et al., 2003), viral infections (Schang, 2004), proliferative renal diseases (Nelson and Shankland, 2005), and neurodegenerative disorders such as Alzheimer's (Tsai et al., 2004), Parkinson's (Smith et al., 2003, 2004), and Nieman-Pick's diseases (Zhang et al., 2004a), ischemia (Wang et al., 2003; Rashidian et al., 2005), or traumatic brain injury (Di Giovanni et al., 2005) have encouraged an intensive search for potent and selective pharmacological inhibitors of these kinases (reviews in Knockaert et al., 2002a; Fischer et al., 2003; Benson et al., 2005; Fischer and Gianella-Borradori, 2005; Shapiro, 2006). Over 100 small-molecular-weight inhibitors of CDKs have been characterized, most of which appear to act by direct competition with ATP for binding to the catalytic site of the kinase. Over 30 of these compounds have been cocrystallized with CDK2 (Noble et al., 2004) or CDK5 (Mapelli et al., 2005), demonstrating their binding in the ATP-binding pocket of CDKs.

The family of 2,6,9-trisubstituted purines encompasses some of the first CDK inhibitors that have been described (review in Haesslein et al., 2002; Meijer and Raymond, 2003). Among these purines, the (*R*)-stereoisomer of roscovitine is one of the most frequently studied and used CDK inhibitors (Figure 9.2). Also referred to as CYC202 or Seliciclib, (*R*)-roscovitine is developed by Cyclacel Pharmaceuticals (<http://www.cyclacel.com>) (Guzi, 2004). It has now reached Phase 2 clinical trials for B-cell malignancies, lung and breast cancer, Phase 1 trials for glomerulonephritis, and Phase 2 trials in IgA nephropathy. The properties and development of roscovitine constitute the object of this review chapter.

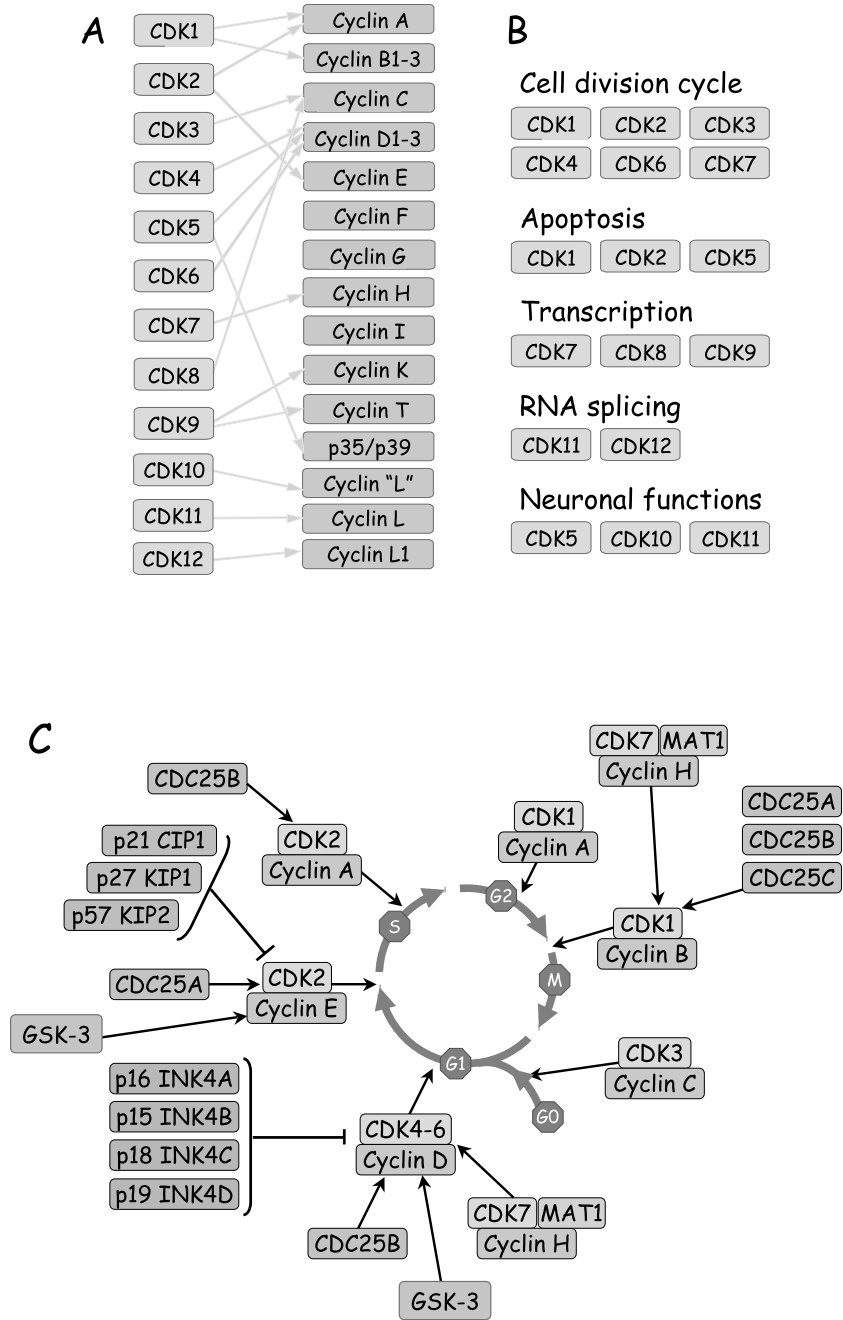


FIGURE 9.1 Cyclin-dependent kinases. CDKs (catalytic subunit) associate with regulatory subunits (cyclins and other) to constitute active protein kinase complexes (A). Different CDKs are involved in various physiological processes (B), including the cell division cycle (C), many regulators of which are altered in human cancer.

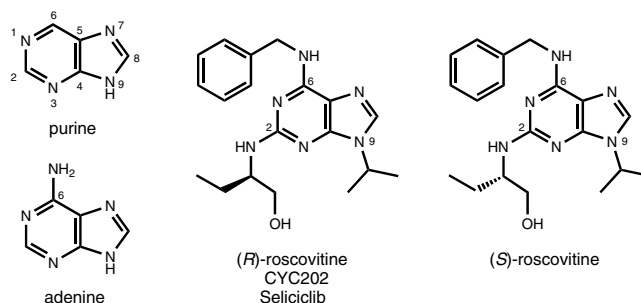


FIGURE 9.2 Structure and atom numbering of the purine ring. Structure of 6-aminopurine (adenine), and the two isomers of 2-(1-ethyl-2-hydroxyethylamino)-6-benzylamino-9-isopropylpurine (roscovitine).

9.2 DISCOVERY OF ROSCOVITINE AND OTHER 2,6,9-TRISUBSTITUTED PURINES

Roscovitine belongs to the family of purines (Figure 9.2), one of the most widely distributed heterocycles in nature, and has the basic ring structure of biologically important molecules such as ATP, cyclic AMP, NAD, FAD, acetyl-coenzyme A, caffeine, and theophylline, to name a few (reviews in Rosemeyer, 2004; Legraverend and Grierson, 2006). Among purines, adenine (6-aminopurine) and guanine constitute two of the four nucleotide building blocks of DNA. The history of the discovery of roscovitine has been reviewed in detail previously (Meijer and Raymond, 2003). Briefly, its development stems from initial studies performed by Lionel Rebhun, who identified 6-dimethylaminopurine (6-DMAP) (Figure 9.3) as an analog of puromycin that was able to prevent cell division of sea urchin embryos, although it had lost puromycin's ability to block protein synthesis (Rebhun et al., 1973). 6-DMAP was later found to inhibit the activity of the so-called "M-phase-specific histone H1 kinase" (Meijer and Pondaven, 1988; Néant and Guerrier, 1988), later to be identified as an equimolar complex between CDK1 (Arion et al., 1988) and cyclin B (Meijer et al., 1989). Following this finding, a small screening assay was established to search for other inhibitors of CDK1/cyclin B (Rialet and Meijer, 1991). Isopentenyladenine (Figure 9.3) was one of the first inhibitors to be identified. However, both 6-DMAP and isopentenyladenine were rather unselective and weakly active. A more extensive screen carried out with Jaroslav Vesely led to the discovery of olomoucine (Figure 9.3), a 2,6,9-trisubstituted purine that displayed promising selectivity toward some of the CDKs, among a panel of over 35 kinases (Vesely et al., 1994). In fact, olomoucine had been initially synthesized by David Letham as an antagonist of plant cytokinin 7-glucosyltransferase. A classical medicinal chemistry and structure–activity relationship study led to the synthesis and extensive characterization of roscovitine (Azevedo et al., 1997; Meijer et al., 1997). Combinatorial chemistry from this lead structure allowed the identification of purvalanols (Gray et al., 1998; Chang et al., 1999). Since then, the family of 2,6,9-trisubstituted purines has been the subject of numerous studies (reviews in Haesslein et al., 2002; Meijer and Raymond, 2003).

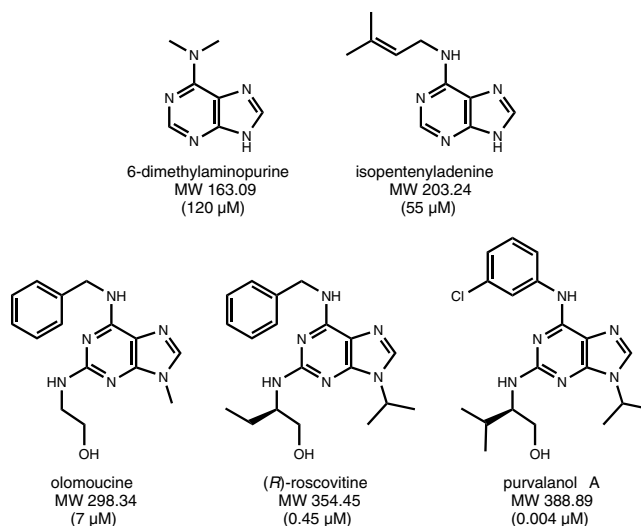


FIGURE 9.3 Structure of 6-dimethylaminopurine, isopentenyladenine, olomoucine, *(R)*-roscovitine, and purvalanol A, with molecular weight and, in parentheses, IC_{50} values for *in vitro* inhibition of CDK1/cyclin B.

9.3 SYNTHESIS OF *(R)*-ROSCOVITINE

(R)-Roscovitine is prepared by a simple and inexpensive three-step procedure, starting from commercially available 2,6-dichloropurine (Figure 9.4) (Havlicek et al., 1997; Wang et al., 2001b). 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

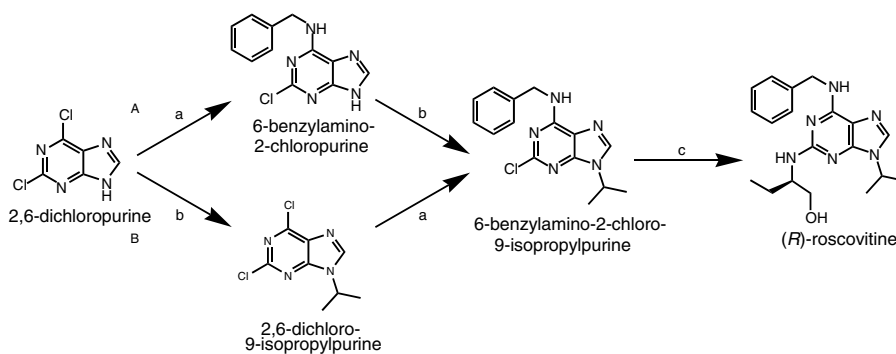


FIGURE 9.4 Chemical synthesis of *(R)*-roscovitine.

TABLE 9.1
Nomenclature and Physicochemical Properties of (*R*)-Roscovitine

Chemical names	2-(1-ethyl-2-hydroxyethylamino)-6-benzylamino-9-isopropylpurine 1-butanol, 2-[[9-(1-methylethyl)-6-[(phenylmethyl)amino]-9 <i>H</i> -purin-2-yl]amino], (2 <i>R</i>)-(2 <i>R</i>)-2-(6-benzylamino-9-isopropyl-9 <i>H</i> -purin-2-ylamino)-butan-1-ol
Other names	(<i>R</i>)-roscovitine, CYC202, Seliciclib
CAS registry number	186692-46-6
NCI number	NSC-701554
Atomic composition	C ₁₉ H ₂₆ N ₆ O C, 64.38%; H, 7.39%; N, 23.71%; O, 4.51%
Molecular weight	354.45
Rotation values	(<i>R</i>)-roscovitine: [α] _D ²⁰ + 56.3 (Wang et al., 2001b) (<i>S</i>)-roscovitine: [α] _D ²⁰ - 56.3
Melting point	106–108°C (Wang et al., 2001b)
pKa	4.4 (Vita et al., 2005a)
Absorption	λ max: 230 nm and 292 nm
Chromatographic analysis	HPLC/UV detection (Vita et al., 2004) LC-MS/MS (Vita et al., 2005c; Raynaud et al., 2005)
Crystal structure	Orthorhombic, space group <i>P</i> 2 ₁ 2 ₁ 2 ₁ (Wang et al., 2001b) Coordinates available at Cambridge Crystallographic Data Centre (deposit@ccdc.cam.ac.uk) CDC 157779, 157780

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.

9.4 CHEMICAL PROPERTIES OF (*R*)-ROSCOVITINE

(*R*)-Roscovitine is a white powder that is soluble in DMSO (up to 50 mM) and in 50 mM HCl with the pH adjusted to 2.5. Its nomenclature and physicochemical properties are summarized in Table 9.1.

9.5 SELECTIVITY AND BIOCHEMICAL PROPERTIES OF (*R*)-ROSCOVITINE

9.5.1 SELECTIVITY

(*R*)-Roscovitine has been optimized from the related purine olomoucine using an *in vitro* CDK1/cyclin B kinase assay (Meijer et al., 1997). 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 (Meijer et al., 1997). 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) (Bain et al., 2003), ProQinase's selectivity panel (85 kinases), Invitrogen's SelectScreen™ Kinase Profiling panel (70 kinases), and Cerep's kinase selectivity panel (50 kinases) (see Bach et al., 2005 (supplementary material) for a compilation of all available data). A total of 151 protein kinases have been tested for their sensitivity to roscovitine. IC₅₀ values are below 1 μM for CDK1, CDK2, CDK5, CDK7, and CDK9 only, whereas CDK4, CDK6, and CDK8 are poorly, if at all, sensitive to roscovitine (Table 9.2). Only a few kinases are sensitive to roscovitine in the 1 to 40 μM range (CaM Kinase 2, CK1α, CK1δ, DYRK1A, EPHB2, ERK1, ERK2, FAK, and IRAK4), but most other kinases are insensitive to roscovitine. Based on these data, roscovitine appears to be

TABLE 9.2
Selectivity of *(R)*-Roscovitine Toward CDKs

Protein Kinase	IC ₅₀ (μM)
CDK1/cyclin B	0.65 ^a , 2.69 ^b , 23 ^c , 0.45/0.95 (<i>R/S</i>) ^d , >80% ^e and 98% ^f inhibition at 10 μM, 14.1 ^g , 1.9 ^h , 0.67 ^m
CDK2/cyclin A	0.7 ^a , 0.25 ^h , 0.71 ^b , 1.2/1.8 ^c , >80% inhibition at 10 μM ^e , 2.2 ^g , 2.1 ^m
CDK2/cyclin E	0.7 ^a , 0.95/1.4 ^c , 0.10/0.24 (<i>R/S</i>) ^b , 98% inhibition at 10 μM ^f , 0.13 ^g , 0.05 ^l , 0.19 ^m
CDK3/cyclin E	1.4/1.5 ^c
CDK4/cyclin D1	>100 ^a , 14.2 ^b , 75 ^c , 14.7 ^g , 14.6 ^l , 10 ^m
CDK5/p25	0.16 ^a , >80% inhibition at 10 μM ^e
CDK6/cyclin D1	51 ^c
CDK6/cyclin D3	>100 ^a , 50 ^g
CDK7/cyclin H	0.5-0.6 ^{ij} , 0.49 ^h , <5 ^k , 0.46 ^g , 0.51 ^m
CDK8/cyclin C	>100 ⁱ , >50 ^k
CDK9/cyclin T1	0.6 ^l , <5 ^k , 0.78 ^g

Note: The purified protein kinases were assayed in the presence of an increasing concentration of *(R)*-roscovitine. IC₅₀ values are presented in μM.

^a From Meijer L. et al. *Eur. J. Biochem.*, 243, 527, 1997.

^b From McClue, S.J. et al. *Int. J. Cancer.*, 102, 463, 2002.

^c Courtesy of ProQinase.

^d From Azevedo, W.F. et al. *Eur. J. Biochem.*, 243, 518, 1997.

^e Courtesy of Invitrogen SelectScreen™ Kinase Profiling Service.

^f Courtesy of Cerep Kinase Selectivity profiling service.

^g From Raynaud, F.I. et al. *Clin. Cancer Res.*, 11, 4875, 2005.

^h From Bain, J. et al. *Biochem. J.*, 371, 199, 2003.

ⁱ From Schang, L.M. et al. *J. Virol.*, 76, 7874, 2002.

^j From Wang, D. et al. *J. Virol.*, 75, 7266, 2001a.

^k From Pinhero, R. et al. *Biol. Procedures Online*, 6, 163, 2004.

^l From Nutley, B.P. et al. *Mol. Cancer Ther.*, 4, 125, 2005.

^m From Byth, K.F. et al. *Mol. Cancer Ther.*, 5, 655, 2006.

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 tissue and cell extracts (Bach et al., 2005). This method has been successfully applied to purvalanol (Knockaert et al., 2000) and other kinase inhibitors (review in Knockaert and Meijer, 2002; Valsasina et al., 2004). Roscovitine beads allowed the identification of expected targets such as CDKs, but also various CaM Kinase 2 isoforms, ERK1, ERK2, and CK1 α . 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 (Bach et al., 2005; Tang et al., 2005).

The third method that has been used is a yeast three-hybrid screen (Becker et al., 2005) 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 (Becker et al., 2004). This elegant method was used with purvalanol and roscovitine as proofs of principle. The results showed that (*R*)-roscovitine interacts with its known target CDK2, but also with CK1 δ , CK1 ϵ , and the CDK-like kinase PCTK1, and more weakly with CLK1, PAK4, PCTK3, PKWA, and GSK3 α (Becker et al., 2004).

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 (Fabian et al., 2005). 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, CK1 γ 1, CK1 γ 2, CK1 ϵ , CLK1, CLK2, CLK4, TTK, and RPS6KA2 (Kinase Domain 1).

These and other methods used to identify the targets of inhibitors of CDKs (Bach et al., 2006) and other kinases (Daub et al., 2004) have been extensively reviewed.

9.5.2 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 (Azevedo et al., 1997). This binding at the catalytic site was confirmed by direct cocrystallization of (*R*)-roscovitine with CDK2 (coordinates available from Dr. S.H. Kim, <shkim@lbl.gov>)

(Azevedo et al., 1997; Dobes et al., 2006; Otyepka et al., 2002), and later with CDK5/p25 (1UNL) (Mapelli et al., 2005) and CDK2/cyclin A (Echallier, Endicott, and Meijer, unpublished). A CDK1/roscovitine model has also been described (Canduri et al., 2004). 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 9.5). Briefly, the interaction involves mostly hydrophobic and van der Waals contacts and two hydrogen bonds (involving N⁷ and N⁶ of the purine) with backbone atoms of Leu83 (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) (Mapelli et al., 2005). The binding mode suggested that N⁶-methyl-*(R)*-roscovitine or O⁶-benzyl-*(R)*-roscovitine (Figure 9.6) 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 N⁶-methyl-*(R)*-roscovitine (Tang et al., 2005).

The cocrystal structures also reveal that the benzyl ring is facing the outside of the ATP-binding pocket. This property has been used to select the place where a linker can be tethered to roscovitine to immobilize it on sepharose beads while still maintaining the potential interaction with its protein kinase targets. A control

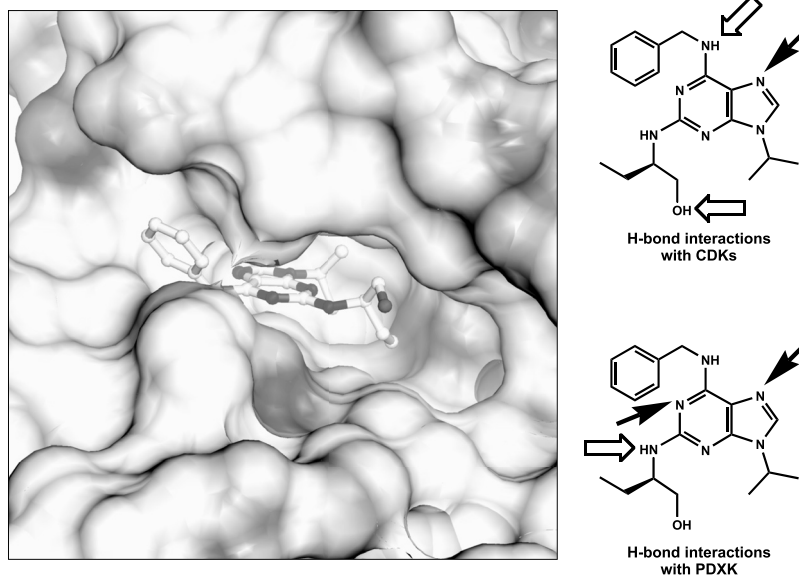


FIGURE 9.5 Interactions of *(R)*-roscovitine with its targets. Left, crystal structure of CDK2 in complex with *(R)*-roscovitine, illustrating the position of *(R)*-roscovitine in the ATP-binding pocket and how its benzyl ring is facing the outside of the kinase. Right, roscovitine and its atoms involved in H-bonds with either CDKs or PDXK (hydrogen acceptors are shown by solid arrows and hydrogen donors by empty arrows).

of roscovitine due to interaction with protein kinases from those due to interaction with PDXX.

These examples illustrate the extraordinary diversity of information that can be drawn from the structure of kinase/inhibitor complexes (Noble et al., 2004).

9.6 CELLULAR EFFECTS OF (*R*)-ROSCOVITINE

(R)-Roscovitine has been evaluated for its effects on a wide variety of cultured cells. Table 9.3 summarizes most of the available data published using mammalian (mostly human) cells. Broadly speaking, two major events have been described: an arrest in cell cycle progression and an induction of cell death.

9.6.1 ANTIMITOTIC EFFECTS

(R)-Roscovitine arrests the cell division cycle in most, if not all, cell lines. Many methods have been used to monitor the effects of the drug on cell proliferation from direct enumeration to FACS analysis, estimation of the number of viable cells, and direct assay of DNA synthesis. The average IC_{50} value for inhibition of proliferation from all available cell line data is about 17 μ M (Table 9.3). This comprises the NCI 60 cell line panel (Meijer et al., 1997) (average IC_{50} = 16 μ M), the McClue et al. (2002) panel (19 cell lines; average IC_{50} = 15.2 μ M), and the Raynaud et al. (2005) panel (24 cell lines; average IC_{50} = 14.6 μ M).

(R)-Roscovitine blocks cell proliferation in G_0 , G_1 , S, G_2 /M, or a combination of these, depending on the cell line studied, the duration of treatment, and the dose of *(R)*-roscovitine. These cell cycle stage arrests can presumably be attributed to a direct inhibition of CDKs (Figure 9.8A): CDK3/cyclin C (inhibition of exit from G_0), CDK2/cyclin E (inhibition of G_1 /S transition), CDK2/cyclin A (inhibition of S phase progression), CDK1/cyclin A (inhibition of G_2 phase), CDK1/cyclin B (inhibition of the prophase-to-metaphase transition in M phase), and, under some conditions, CDK1/cyclin E (inhibition of G_1 /S transition), as recently suggested by Aleem et al. (2005).

In addition there are two indirect mechanisms by which *(R)*-roscovitine can block the cell division cycle (Figure 9.8B). First, by inhibiting the CDK7/cyclin H/MAT1 complex, *(R)*-roscovitine prevents phosphorylation of the T-loop threonine of various CDKs, a step that is essential for activity of the kinases. Consequently, CDK1, CDK2, and CDK4 catalytic activities are expected to decrease. Second, the CDK2/CDK4 natural peptidic inhibitor p27^{KIP1} is phosphorylated by CDK2/cyclin E (Vlach et al., 1997; Bloom and Pagano, 2003). This phosphorylation targets p27^{KIP1} for rapid, ubiquitin-dependent degradation by the proteasome. The presence of *(R)*-roscovitine prevents p27^{KIP1} phosphorylation, leads to its stabilization, accumulation (Zhang et al., 2004b), and enhanced inhibition of its CDK targets and, consequently, to an arrest in G_1 (Figure 9.8B). A p27^{KIP1} luciferase fusion protein has been constructed that provides a convenient and elegant *in vivo* and *in vitro* reporter system for the induction of p27^{KIP1} by CDK inhibitors, including *(R)*-roscovitine (Zhang et al., 2004b).

TABLE 9.3
Cellular Effects of (*R*)-Roscovitine in Mammalian Cells

Cell Line (Cancer Type)	IC ₅₀ (μ M)	Cell Cycle Effect	Cell Proliferation and Death Assay	Reference
501mel (melanoma)	29		15	Du et al., 2004
2008 (ovary) (+ FTI)			9	Edamatsu et al., 2000
A172 (glioma) (+ TRAIL)			3	Kim et al., 2004
A375 (melanoma)	17.5		15	Du et al., 2004
A375 (melanoma)	19.9	θ DNA synthesis		Payton et al., 2006
A431 (epidermoid)	10	θ DNA synthesis	3	Atanasova et al., 2005
A4573 (Ewing's sarcoma)			16	Tirado et al., 2005
A498 (renal)	13.2		3	McClue et al., 2002
A549 (lung)	15.9		3	McClue et al., 2002
A549 (lung)	9.3		2	Raynaud et al., 2005
A2780 (ovarian)	4.9		2	Raynaud et al., 2005
A2780Cis ^R (ovarian)	8.4		2	Raynaud et al., 2005
ACHN (renal)	11.9		3	McClue et al., 2002
AN3CA (endometrial)	14.1		3	McClue et al., 2002
BE (colon)	17.5		2	Raynaud et al., 2005
Bon-1 (carcinoid cell line)	60		3	Goke et al., 2004
CH1 (ovarian)	7.7		2	Raynaud et al., 2005
CH1Cis ^R (ovarian)	9.3		2	Raynaud et al., 2005
CH1Dox ^R (ovarian)	7.4		2	Raynaud et al., 2005
CHAGO-K1 (lung)	30.2		3	McClue et al., 2002
CHP212 (neuroblastoma)			11	van Engeland et al., 1997
Chronic lymphocytic leukemia cells				Hahntow et al., 2004
Chronic lymphocytic leukemia cells				Alvi et al., 2005
COLO-205 (colon)	8.5		2	Raynaud et al., 2005
COLO-205 (colon)	18.6	θ DNA synthesis		Payton et al., 2006
COLO-320 (colon)	15.9	θ DNA synthesis		Payton et al., 2006
Colon 26 (murine colon)	42.5		2	Raynaud et al., 2005
CORL23 (lung)	10.5		2	Raynaud et al., 2005
Daudi (Burkitt's lymphoma)	19.2	θ DNA synthesis		Payton et al., 2006
Dox40 (multiple myeloma)	15		14	Raje et al., 2005
DU145 (prostate)	18.8		3	McClue et al., 2002
Fibroblasts (human fetal lung)		G ₁ , θ S, θ Rb-P		Alessi et al., 1998
GCT27 (testicular)	5.2		2	Raynaud et al., 2005
Granta-519 (mantle cell lymphoma)	25	G ₂ /M	3, 4	Lacrima et al., 2005
H929 (multiple myeloma)	8.84		12	MacCallum et al., 2005
HS27 (foreskin fibroblast)	22.2		2	McClue et al., 2002

TABLE 9.3 (CONTINUED)
Cellular Effects of (R)-Roscovitine in Mammalian Cells

Cell Line (Cancer Type)	IC ₅₀ (μ M)	Cell Cycle Effect	Cell Proliferation and Death Assay	Reference
HCT15 (colon)	18.3	NCC	3, 4	McClue et al., 2002
HCT116 (colon)	10.7		3	McClue et al., 2002
HCT116 (colon)		θ Rb S608-P		Barrie et al., 2003
HCT116 (colon)	6.9	G ₂ /M	2	Raynaud et al., 2005
HCT116 p53+ (colon)	13.7	θ DNA synthesis		Payton et al., 2006
HCT116 p53- (colon)	23.7	θ DNA synthesis		Payton et al., 2006
HEK293 (embryonic kidney)	48.9		13	Bettayeb and Meijer, unpubl.
HepG2 (hepatic)	11.3		3	McClue et al., 2002
HL-60 (leukemia)	20.5	θ DNA synthesis		Payton et al., 2006
HL-60 (leukemia) (+ FTI)			9, 10	Edamatsu et al., 2000
HNSCC cell lines	9.8 – 25.0	G ₂ /M	6	Mihara et al., 2002
HS294 (melanoma)	16.6	θ DNA synthesis		Payton et al., 2006
HT29 (colon)	14.6		3	McClue et al., 2002
HT29 (colon)		θ Rb S608-P		Barrie et al., 2003
HT29 (colon)	20.3		2	Raynaud et al., 2005
HT29 (colon)	16		2	Whittaker et al., 2004
HT29 (colon) (+ FTI)			9	Edamatsu et al., 2000
HT1376 (bladder)	18.4		3	McClue et al., 2002
HX147 (lung)	19		2	Raynaud et al., 2005
IMR-90 (fetal lung fibroblast)	>100		2	McClue et al., 2002
INR1-G9 (glucagonoma)	80		3	Goke et al., 2004
INS-1 (insulinoma)	90		3	Goke et al., 2004
JeKo-1 (mantle cell lymphoma)	25	G ₂ /M	3, 4	Lacrima et al., 2005
Jurkat (acute T-cell leukemia)	27.8	θ DNA synthesis		Payton et al., 2006
K562 (chronic myelogenous leukemia)		G ₂ /M, θ S		Penuelas et al., 1998
K562 (chronic myelogenous leukemia)	35	θ DNA synthesis		Payton et al., 2006
K562 (leukemia) (+ FTI)			9	Edamatsu et al., 2000
KM12 (colon)	17		2	Whittaker et al., 2004
KM12 (colon)	15		2	Raynaud et al., 2005
Keratinocytes (confluent)	35		3	Atanasova et al., 2005
Keratinocytes (subconfluent)	17		3	Atanasova et al., 2005
L1210 (mouse leukemia)	40	G ₂ /M	1	Meijer et al., 1997
L1210 (mouse leukemia)	47	G ₂ /M		Somerville and Cory, 2000

(continued)

TABLE 9.3 (CONTINUED)
Cellular Effects of (*R*)-Roscovitine in Mammalian Cells

Cell Line (Cancer Type)	IC ₅₀ (μ M)	Cell Cycle Effect	Cell Proliferation and Death Assay	Reference
L1210 (Y8) (mouse leukemia)	52	G ₂ /M	8	Somerville and Cory, 2000
LNcaP (pancreas)			17	Mohapatra et al., 2005
LNcaP (pancreas) (+ FTI)			9	Edamatsu et al., 2000
LP-1 (multiple myeloma)	12.68		12	McCallum et al., 2005
LoVo (colon)	9.3		3	McClue et al., 2002
LoVo (colon)	20		2	Raynaud et al., 2005
LR5 (multiple myeloma)	25		14	Raje et al., 2005
M14 (melanoma)	17		15	Du et al., 2004
MALME-3M (melanoma)	30		15	Du et al., 2004
Mawi (colon)	18		2	Raynaud et al., 2005
MCF-7 (breast)	10.9		3	McClue et al., 2002
MCF-7 (breast)			5	Mgbonyebi et al., 1998
MCF-7 (breast)	14.7	G ₂ /M, θ DNA synthesis	7	Wojciechowski et al., 2003
MCF-7 (breast)	14	G ₂ /M	7	Wesierska-Gadek et al., 2003
MCF-7 (breast)	7.8		2	Raynaud et al., 2005
MCF-10F (breast)			5	Mgbonyebi et al., 1998
MDA-MB-231 (breast)		θ DNA synthesis	5	Mgbonyebi et al., 1998, 1999
MDA-MB-231 (breast)		G ₂ /M (+ irradiation)		Maggiorella et al., 2003
MDA-MB-231 (breast)	20.8	θ DNA synthesis		Payton et al., 2006
MDA-MB-231 (breast)	15		2	Raynaud et al., 2005
MDA-MB-435S (breast)	17.5		3	McClue et al., 2002
MES-SA (uterine)	11.9		3	McClue et al., 2002
MES-SA/Dx5 (MDR+) (uterine)	7.9		3	McClue et al., 2002
MES-SA (uterine)	25	θ DNA synthesis		Payton et al., 2006
MES-SA/Dx5 (MDR+) (uterine)	12.5	θ DNA synthesis		Payton et al., 2006
MiaPaCa2 (pancreatic)	13.9		3	McClue et al., 2002
MiaPaCa2 (pancreatic)	12.6	θ DNA synthesis		Payton et al., 2006
MKN45 (stomach) (+ FTI)			9	Edamatsu et al., 2000
MM.1r (multiple myeloma)	25		14	Raje et al., 2005
MM.1s (multiple myeloma)	23		14	Raje et al., 2005
MOR (lung)	12.5		2	Raynaud et al., 2005
MR65 (NSC-lung)			11	van Engeland et al., 1997

TABLE 9.3 (CONTINUED)
Cellular Effects of *(R)*-Roscovitine in Mammalian Cells

Cell Line (Cancer Type)	IC ₅₀ (μ M)	Cell Cycle Effect	Cell Proliferation and Death Assay	Reference
MT-2 (leukemia)			8	Mohapatra et al., 2003
NCI 60 cell line panel	Mean: 16		2	Meijer et al., 1997
NCI-H69 (lung)	26.0		3	McClue et al., 2002
NCI-H460 (lung)	13.1		3	McClue et al., 2002
NCEB-1 (mantle cell lymphoma)	50	G ₂ /M	3, 4	Lacrima et al., 2005
OPM2 (multiple myeloma)	18.46		12	MacCallum et al., 2005
OPM2 (multiple myeloma)	15		14	Raje et al., 2005
PC-3 (prostate) (+ FTI)			9	Edamatsu et al., 2000
PC-3 (prostate)	12	θ DNA synthesis		Payton et al., 2006
REC (mantle cell lymphoma)	25	G ₂ /M	3, 4	Lacrima et al., 2005
RPMI (multiple myeloma)	23		14	Raje et al., 2005
RPMI 8226 (multiple myeloma)	19.5		12	MacCallum et al., 2005
Sa-SO2 (osteosarcoma)	16.5		2	Raynaud et al., 2005
Sa-SO2 (osteosarcoma)	17.1	θ DNA synthesis		Payton et al., 2006
SH-SY5Y (neuroblastoma)	25		13	Ribas and Boix, 2004
SH-SY5Y (neuroblastoma)	16.1		13	Bettayeb and Meijer, unpubl.
SKMEL2 (melanoma)	21		15	Du et al., 2004
SKMEL5 (melanoma)	23		15	Du et al., 2004
SKMEL28 (melanoma)	25		15	Du et al., 2004
SKOV-3 (ovarian)	31		2	Raynaud et al., 2005
SW480 (colon carcinoma)	30		15	Du et al., 2004
SW620 (colon)	23		2	Raynaud et al., 2005
TC-71 (Ewing's sarcoma)			16	Tirado et al., 2005
T98 (glioma) (+ TRAIL)			3	Kim et al., 2004
U2-OS (osteosarcoma)	15		2	Raynaud et al., 2005
U2-OS (osteosarcoma)		G ₂ /M	15	Maude and Enders, 2005
U-2 OS (osteosarcoma)	28.3	θ DNA synthesis		Payton et al., 2006
WI38 (fetal lung)	24.0		2	McClue et al., 2002
U87MG (glioma) (+ TRAIL)			3	Kim et al., 2004
U266 (multiple myeloma)	17.93		12	MacCallum et al., 2005
U266 (multiple myeloma)	25		14	Raje et al., 2005
U251 (glioma) (+ TRAIL)			3	Kim et al., 2004

(continued)

TABLE 9.3 (CONTINUED)
Cellular Effects of (*R*)-Roscovitine in Mammalian Cells

Cell Line (Cancer Type)	IC ₅₀ (μ M)	Cell Cycle Effect	Cell Proliferation and Death Assay	Reference
U937 (leukemia)			11	Rosato et al., 2006
UACC62 (melanoma)	12		15	Du et al., 2004
UACC257 (melanoma)	25		15	Du et al., 2004

Note: The IC₅₀ values for inhibition of proliferation are provided in μ M. Cell cycle effects are monitored by FACS analysis, [³H]-thymidine uptake, or BrdU incorporation. Cell proliferation and cell death are monitored by various methods.

Abbreviations: LDH = lactate dehydrogenase; MTS = 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxy methoxyphenyl)-2-(4-sulfophenyl)-2*H*-tetrazolium salt; MTT = 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide; NCC = no apparent increase in a specific cell cycle phase; θ Rb S608-P = inhibition of retinoblastoma protein phosphorylation on Serine 608; θ DNA synthesis = inhibition of BrdUrd or [³H]-thymidine incorporation; θ S = reduction in S phase cells.

Cell proliferation and cell death assays: 1 = microculture tetrazolium assay; 2 = sulforhodamine B assay; 3 = MTT assay; 4 = sub-G₁ + TUNEL assay; 5 = Cell proliferation reagent WST-1 (Boehringer); 6 = DNA fragmentation, PARP cleavage; 7 = CellTiter-Glo Luminescent Viability assay (Promega); Trypan blue dye exclusion; TUNEL assay; PARP cleavage; 8 = annexin V, caspase-3, PARP cleavage; 9 = caspase-3 activation; 10 = sub-G₁, cytochrome C release; 11 = annexin-V/PI; 12 = Alamar blue, TUNEL, PARP cleavage; 13 = MTS assay; DNA fragmentation, caspase activation, TUNEL assay, LDH release; 14 = MTT assay, [³H]-thymidine incorporation, sub-G₁, caspase-3, PARP cleavage; 15 = WST-1 cell proliferation assay (Roche); 16 = Trypan blue dye exclusion, TUNEL, caspase-3 activation; 17 = DNA fragmentation, PARP and keratin 18 cleavage.

A decrease in the phosphorylation of substrates at sites that are specifically phosphorylated by CDKs is a clear demonstration of the direct or indirect inhibition of the CDK catalytic activity by (*R*)-roscovitine (Table 9.4). Furthermore, these molecular events constitute tools that can be used as surrogate markers to monitor the efficacy of (*R*)-roscovitine in animal models and during human clinical trials.

Besides these direct targets, there are many biochemical and morphological events that rely on CDK activity, and are, therefore, sensitive to (*R*)-roscovitine. Among those, DNA replication initiation depends on CDK2. *In vitro* initiation of DNA replication is inhibited by (*R*)-roscovitine (Krude, 2000) as monitored in cell cultures by inhibition of [³H] thymidine uptake and incorporation in DNA. Nucleolus formation and functions rely on active CDKs and are inhibited by (*R*)-roscovitine (Sirri et al., 2002). Centrosome duplication depends on CDK2 and is also prevented by (*R*)-roscovitine (Matsumoto et al., 1999). Golgi fragmentation is inhibited by (*R*)-roscovitine through dephosphorylation of GM130 on Ser25 (Lowe et al., 1998). Nuclear envelope breakdown at the end of prophase is a consequence of lamins phosphorylation by CDK1/cyclin B, and is consequently inhibited by (*R*)-roscovitine treatment. (*R*)-Roscovitine treatment induces Erk1/2 activation in HT29 and KM12 colon carcinoma cell lines (Whittaker et al., 2004).

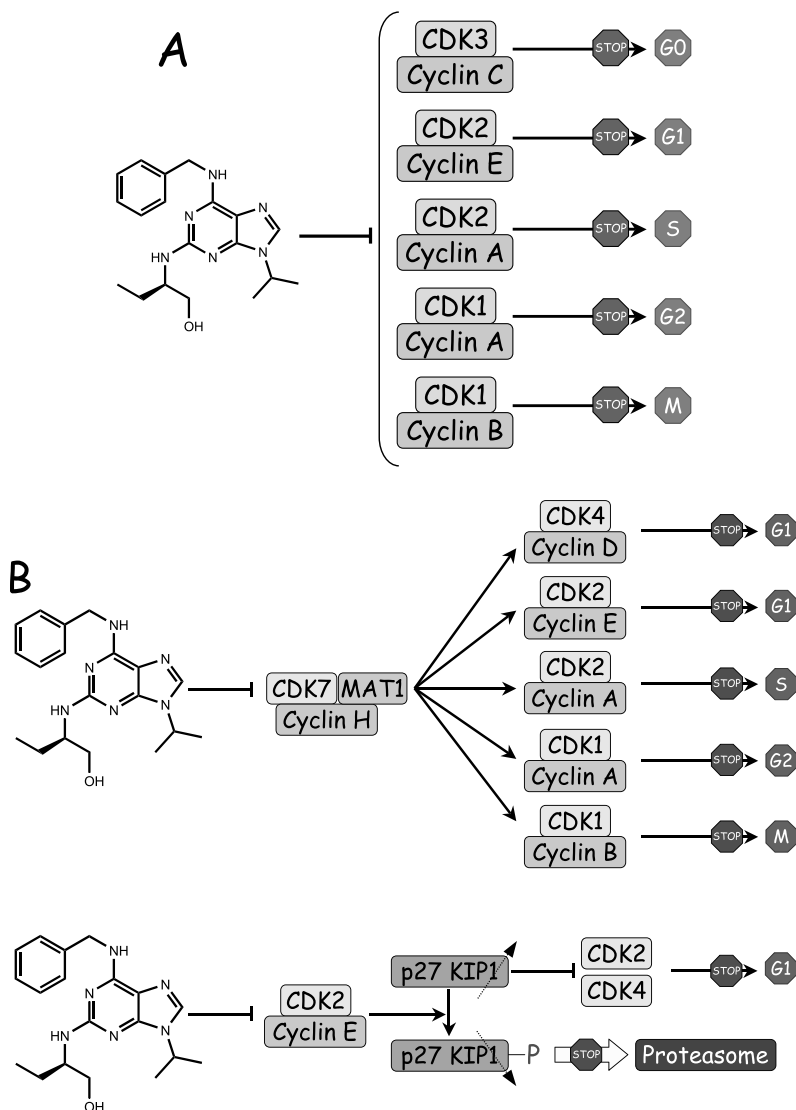


FIGURE 9.8 Multiple mechanisms of action of *(R)*-roscovitine and their cellular consequences. (A) Direct stoichiometric interaction with CDKs leads to inhibition of the catalytic activity of various CDK/cyclin complexes with a direct effect on various cell cycle phases (indicated by a “STOP” sign). (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. Consequently, the activity of various CDK/cyclin complexes are reduced; (ii) inhibition of CDK2/cyclin E prevents phosphorylation and subsequent proteolytic degradation of p27^{KIP1}, a natural CDK2/CDK4 inhibitor. p27^{KIP1} Accumulation thus contributes to an arrest in G₁.

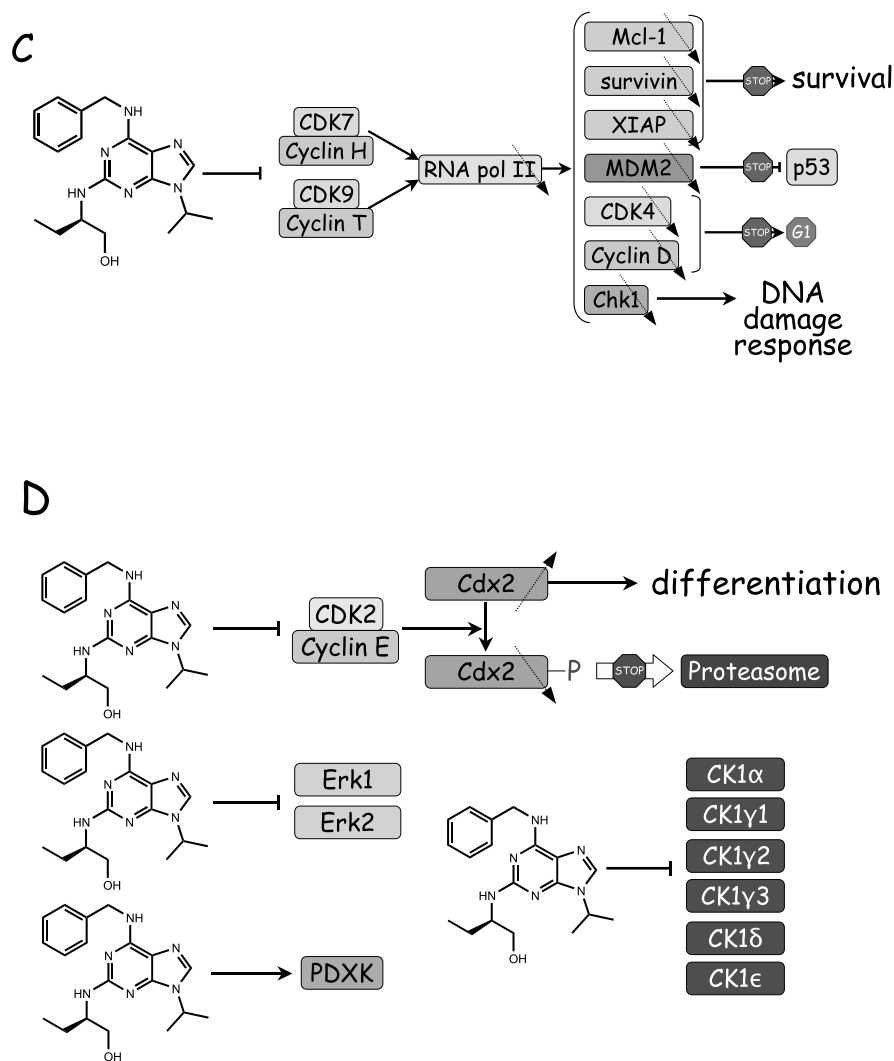


FIGURE 9.8 (CONTINUED) (C) Direct interaction with CDK7/cyclin H and CDK9/cyclin T leads to inhibition of RNA polymerase II (by lack of serine 2 and 5 phosphorylation). Consequently, transcription is reduced and short-lived proteins are rapidly downregulated. In particular, the reduction of survival factors such as Mcl-1, survivin, and XIAP contributes to cell death, the reduction of MDM2 level upregulates p53 level, reduced CDK4 and cyclin D contribute to a G_1 arrest, and downregulation of Chk1 leads to activation of a DNA damage response. Furthermore, CDK7/CDK9 inhibition by (*R*)-roscovitine leads to a downregulation of RNA polymerase II expression. (D) Other possible mechanisms of action. Inhibition of CDK2/cyclin E prevents phosphorylation and subsequent proteolytic degradation of Cdx2, a transcription factor involved in intestinal cell differentiation. (*R*)-Roscovitine also inhibits the MAP-kinases Erk1 and Erk2, and members of the casein kinase 1 (CK1) family, contributing to its antiproliferative effects. Finally, (*R*)-roscovitine binds to pyridoxal kinase (PDXK).

TABLE 9.4
Phosphorylation Sites That Are Sensitive to (*R*)-Roscovitine in Mammalian Cells

Phosphorylated Substrate	CDK-Specific Site	P-Specific Antibody	Reference
Vimentin	Ser55 (CDK1)	4A4 antibody	Meijer et al., 1997
Nucleolin	—	TG3 antibody	Knockaert et al., 2002b
GM130 Golgi protein	Ser25 (CDK1)	—	Lowe et al., 1998
Survivin	Thr34 (CDK1)	—	O'Connor et al., 2000, 2002; Wall et al., 2003
Retinoblastoma protein	Ser608	51B7 antibody	Barrie et al., 2003 Raynaud et al., 2005
Retinoblastoma protein	Ser249/Thr252	Antibody 44-584 (Biosource)	MacCallum et al., 2005
p27 ^{KIP1}	Thr187 (CDK2)	—	Vlach et al., 1997 Bloom and Pagano, 2003 Gherardi et al., 2004 Zhang et al., 2004b
PP-1 phosphatase	Thr320 (CDK1)	P-Thr320 antibody (Cell Signaling Technology)	Kwon et al., 1997 Payton et al., 2006
Inhibitor-2 (at centrosomes)	Thr72 (CDK1)	P-Thr72 antibody	Leach et al., 2000
Peroxiredoxin I	Thr90 (CDK1)	P-Thr90 PrxI antibody	Chang et al., 2002

9.6.2 CELL DEATH EFFECTS

(R)-Roscovitine induces cell death in many cell lines (Table 9.3). Roscovitine-induced cell death can occur at all phases of the cell cycle (McClue et al., 2002). It displays all the characteristics of apoptosis: chromatin condensation, nuclear DNA fragmentation, accumulation in the sub-G₁ compartment as detected by FACS analysis, DNA laddering, release of cytochrome C, activation of caspases, PARP cleavage, positive TUNEL staining, and LDH release (see, for example, Ribas and Boix, 2004).

Functional p53 does not appear to be necessary for cell death induction, but roscovitine has a slightly greater potency in p53 wild-type cells compared to cells bearing mutant p53 (Meijer et al., 1997; McClue et al., 2002; Raynaud et al., 2005). Little (Payton et al., 2006) or no (Raynaud et al., 2005) difference in sensitivity to roscovitine was observed between HCT-116 (wild-type p53) and an HCT-116 isogenic p53^{-/-} variant. Nevertheless, roscovitine induces an increased expression (David-Pfeuty, 1999; Blaydes et al., 2000; Kotala et al., 2001; Lu et al., 2001; Wojciechowski et al., 2003; Wesierska-Gadek et al., 2003; Mohapatra et al., 2005), Ser46 phosphorylation (in MCF-7 cells) (Wesierska-Gadek et al., 2005), and nuclear accumulation (Ljungman and Paulsen, 2001; David-Pfeuty, 1999; David-Pfeuty et al.,

2001) of wild-type p53. An increase of p53-dependent transcription is therefore observed, leading, for example, to the accumulation of p21^{CIP1} (MCF-7 cells) (Kotala et al., 2001; Lu et al., 2001) or p53AIP1 (Wesierska-Gadek et al., 2005). The effect may be due to downregulation of MDM2, which would contribute to stabilize p53 and induce p53-dependent transcription as monitored in a p53-responsive BP100-luciferase reporter (Lu et al., 2001). This effect is not observed with expression of p16, p21, p27, p57, or dominant negative mutants of CDK1, CDK2, CDK3, CDK4, and CDK6, suggesting that another target of roscovitine is involved. Interestingly, the removal of roscovitine results in superactivation of p53 (Lu et al., 2001).

Although roscovitine-induced cell death was initially thought to result directly from cell cycle arrest, it is increasingly considered to be the consequence of an inhibition of CDK7/CDK9-dependent transcription (Figure 9.8C). Indeed, transcription largely rests on the activity of RNA polymerase II, the activity of which relies on phosphorylation of its C-terminal domain by CDK7/cyclin H, CDK8/cyclin C, and CDK9/cyclin T. Both CDK7 and CDK9 are potently inhibited by roscovitine (Table 9.2). (*R*)-Roscovitine inhibits the phosphorylation of the C-terminal domain of RNA polymerase II (by CDK7), reducing mRNA synthesis in both human fibroblasts and HCT-116 colon cancer cells (Ljungman and Paulsen, 2001). In multiple myeloma cells, (*R*)-roscovitine induces the dephosphorylation of RNA polymerase II (Ser2, Ser5) and consequently decreases transcription (MacCallum et al., 2005). In contrast to flavopiridol, which inhibits gene expression globally, the effects of (*R*)-roscovitine on transcription are rather limited (Lam et al., 2001). The expression of only a small number of proteins is found to be severely reduced. One would predict the greatest effect to be observed on gene products with mRNA and protein short half-lives. This would result in a rapid decline in the level of these proteins. This appears to be true for important survival factors such as Mcl-1, a member of the antiapoptotic Bcl-2 family, XIAP, and survivin. Induction of cell death by roscovitine thus seems to correlate rather well with inhibition of transcription of essential cell survival factors (Figure 9.8C). Furthermore, among the genes that are downregulated by treatment of cells with (*R*)-Roscovitine is RNA polymerase II, as well as cyclin D1 and CDK4 in HCT-116 (Raynaud et al., 2005), and cyclin D1 in some mantle cell lymphoma cell lines (Lacrima et al., 2005). Treatment with (*R*)-roscovitine also leads to the downregulation of Chk1 and activation of a DNA damage response marked by an activation of ATM and Chk2 (Maude and Enders, 2005).

Mcl-1 expression is strongly reduced in mantle cell lymphoma cell lines exposed to (*R*)-roscovitine (Lacrima et al., 2005). Mcl-1 downregulation is sufficient by itself to induce apoptosis in multiple myeloma cells, as demonstrated by the use of siRNA (McCallum et al., 2005). Mcl-1 downregulation is also observed in multiple myeloma cells (Raje et al., 2005) and in U937 leukemia cells (Rosato et al., 2005).

(*R*)-Roscovitine reduces the level of the antiapoptotic protein XIAP by downregulating XIAP mRNA expression (Mohapatra et al., 2003, 2005). It also decreases the tyrosine phosphorylation and consequent activation of STAT5a, an upstream regulator of XIAP. (*R*)-Roscovitine downregulates survivin and XIAP, which contributes to the activation of caspase cascades, overcoming glioma cell resistance to TRAIL-mediated apoptosis (Kim et al., 2004). Combined treatment of glioma cells

with TRAIL and *(R)*-roscovitine leads to a decrease in expression of XIAP and survivin, and activation of caspases and cell apoptosis (Kim et al., 2004).

Chronic lymphocytic leukemia (CLL) B-lymphocytes are noncycling, G₀-arrested cells in which CDK2 is expressed but inactive. Nevertheless, *(R)*-roscovitine induces caspase-dependent cell death in these CLL cells to a significantly much higher level than in peripheral blood mononuclear cells, purified normal B-lymphocytes (Hahntow et al., 2004; Alvi et al., 2005), or normal T-lymphocytes (Alvi et al., 2005). Again, this effect is associated with downregulation of Mcl-1 and XIAP proteins (Hahntow et al., 2004; Alvi et al., 2005) and other proteins such as RNA polymerase II (Alvi et al., 2005). In CLL cells, roscovitine does not trigger an increase in p53 or its nuclear translocation, and roscovitine-induced cell death is independent of the p53 and ATM status (Alvi et al., 2005).

Other mechanisms may be involved in the effects of *(R)*-roscovitine on cell proliferation and cell death (Figure 9.8D). Inhibition of the MAP-kinases Erk1 and Erk2 (Meijer et al., 1997), and of several members of the casein kinase 1 (CK1) family (Ferandin et al., unpublished), certainly contributes to the antiproliferative effects of *(R)*-roscovitine. As discussed previously, *(R)*-roscovitine binds to pyridoxal kinase (PDXK) (Bach et al., 2005; Tang et al., 2005) with undetermined effects on the cell cycle. Finally, inhibition of CDK2/cyclin E prevents Ser281 phosphorylation and subsequent proteolytic degradation of Cdx2, a transcription factor involved in the balance between proliferation and differentiation of intestinal cells (Boulanger et al., 2005; Gross et al., 2005; Gespach, 2005).

9.6.3 ANTI-TUMOR EFFECTS: CONVERGENCE OF DIFFERENT MECHANISMS OF ACTION

We have seen how *(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 9.8A) and an indirect effect by inhibition of the upstream CDK-activating CDK7 and an increased level of the CDK inhibitory p27^{KIP1} (Figure 9.8B). Induction of cell death originates from a transient reduction in transcription due to direct inhibition of CDK7 and CDK9, leading to the downregulation of essential, short-lived survival factors that are typically expressed in cancer cells (such as Mcl-1, XIAP, survivin) (Figure 9.8C). 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 downregulation 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 (Table 9.5). More examples are available in the patent literature and should be published soon. These effects are

TABLE 9.5
Additive and Synergistic Effects of Various Treatments with (*R*)-Roscovitine
in Mammalian Cells

Treatment	Target	Cells	Reference
Irradiation	DNA	Breast carcinoma	Maggiorella et al., 2003
SCH56582	Farnesyltransferase	Leukemic and prostate cancer	Edamatsu et al., 2000
Camptothecin	Topoisomerase I	MCF-7 breast tumor	Lu et al., 2001
Irinotecan	Topoisomerase I	p53-mutated colon cancer	Abal et al., 2004
Doxorubicin	Topoisomerase II	A549 and HEC1B adenocarcinoma HCT116 and H1299	Crescenzi et al., 2005
Etoposide	Topoisomerase II	U2-OS osteosarcoma	Maude and Enders, 2005
LY294002	Phosphatidylinositol 3-kinase	U937 monocytic leukemia	Yu et al., 2003
TRAIL ^a		U87MG, T98, A172, U251 glioma cells	Kim et al., 2004
LAQ824	Histone deacetylase	U937, Jurkat and HL-60 leukemia	Rosato et al., 2005
nutlin-3	MDM2/p53 binding	SH-SY5Y neuroblastoma	Ribas et al., 2006

^a Tumor-necrosis-factor-related apoptosis-inducing ligand.

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 (Borgne et al., 2006), in nondividing cells such as neural cells (review in Borgne and Golsteyn, 2003). These properties are being extensively investigated for their applications in the neurodegeneration diseases field (Knockaert et al., 2002a). It is still largely unknown how CDK inhibitors are able to protect cells from apoptosis induced by various factors. Depending on the model, CDK1 (Konishi et al., 2002; Park et al., 2005; Borgne et al., 2006), CDK2 (Gil-Gomez et al., 1998; Hakem et al., 1999), or CDK5 (Sandal et al., 2002) is involved. The contribution of CDKs varies according to cell type, conditions, and the 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 (Price et al., 2004). 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 (Du et al., 2004). 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 (Du et al., 2004).

Finally, interesting studies have shown that cyclin E is expressed as low molecular weight (LMW) forms in breast and melanoma cancers (review in Akli and Keyomarsi, 2004; Hunt and Keyomarsi, 2005). 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 p27^{KIP1} and, therefore, constitutes an attractive target in these clinical settings.

9.7 PHARMACOLOGY OF *(R)*-ROSCOVITINE

A correlation has been established between some chemical parameters of a molecule and its absorption or permeation properties (Lipinski et al., 1997). *(R)*-roscovitine appears to meet all required parameters for favorable absorption (Table 9.6).

9.7.1 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 ng/ml to 5000 ng/ml (i.e., 0.28 to 14 μ M) (Vita et al., 2004). 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 ng/ml (i.e., 0.0014 to 5.6 μ M) (Vita et al., 2005c; Raynaud et al., 2004, 2005).

9.7.2 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 (Vita et al., 2004, 2005b).

TABLE 9.6
***(R)*-Roscovitine and the Lipinski Rule of 5**

Favorable Parameters According to the Lipinski Rule of 5	<i>(R)</i> -Roscovitine
Molecular mass: ≤ 500	354.45
Log P ^a : < 5	3.244
Number of H-bond donors (OH and NH): ≤ 5	3
Number of H-bond acceptors (O and N): ≤ 10	4

^a P is the octanol-water partition coefficient.

In mouse, the plasma level of roscovitine also rapidly drops within 30 min to <1% of the I.V. injected dose (40 nmol/g, i.e., about 15 mg/kg) (Chmela et al., 2003). Detailed pharmacokinetic studies performed in BALB/c mice (Nutley et al., 2005; Raynaud et al., 2004, 2005) and Tg26 mice (Gherardi et al., 2004) have been recently published. They show rapid, biexponential clearance of (*R*)-roscovitine from plasma following I.V., I.P., or oral administration (Nutley et al., 2005; Raynaud et al., 2004, 2005; Gherardi et al., 2004). (*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 μ M (the average IC₅₀ values obtained with various tumor cell lines) for 4, 12, and 24 h following oral administration at 50, 500, and 2000 mg/kg, respectively (Raynaud et al., 2005).

The pharmacokinetics of (*R*)-roscovitine were recently studied in humans (de la Motte and Gianella-Borradori, 2004). 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.

9.7.3 METABOLITES

When (*R*)-roscovitine was injected I.V. at 100 mg/kg in mouse (Nutley et al., 2005) or at 25 mg/kg in rat (Vita et al., 2005b), several metabolites were identified in the plasma (Figure 9.9). (*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 (Chmela et al., 2003; Nutley et al., 2005; Vita et al., 2005b).

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 (Cervenkova et al., 2003).

Both M3 and M6 were synthesized and found to be much less potent than the parent (*R*)-roscovitine at inhibiting CDK2 (Nutley et al., 2005).

In humans, the carboxylated derivative was also the main metabolite formed following oral administration of (*R*)-roscovitine (de la Motte and Gianella-Borradori, 2004).

9.8 ANTI-TUMOR EFFECTS OF (*R*)-ROSCOVITINE

9.8.1 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) (McClue et al., 2002). Nor could MTD be reached when (*R*)-roscovitine was given orally (maximal achievable dose was 2000 mg/kg) (McClue et al., 2002). When administered intraperitoneally, three doses of 100 mg/kg were well tolerated (McClue et al., 2002). In BALB/c mice,

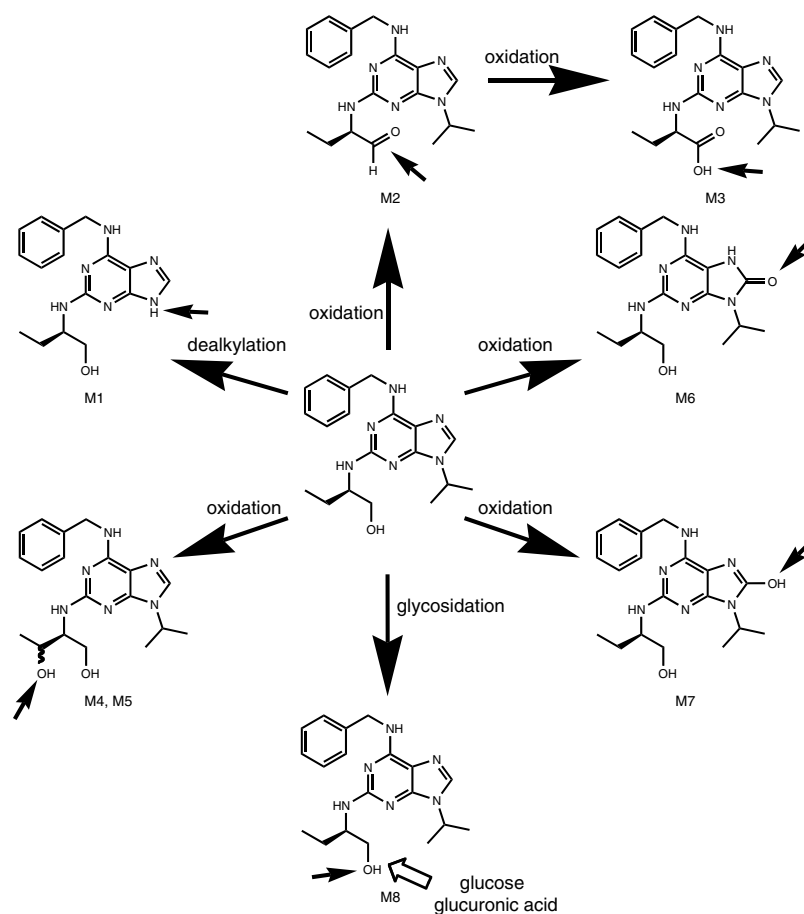


FIGURE 9.9 *(R)*-Roscovitine and its metabolites. Small arrows point to the chemical changes introduced in the initial roscovitine structure.

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 (Raynaud et al., 2005).

9.8.2 ANIMAL MODELS

The anti-tumor activity of *(R)*-roscovitine has been investigated in various human tumor xenografts (summarized in Table 9.7) (review in Meijer and Raymond, 2003; Guzi, 2004). An interesting additivity of irradiation and *(R)*-roscovitine treatments of breast carcinoma *in vitro* and *in vivo* was reported (Maggiorella et al., 2003). Irinotecan and *(R)*-roscovitine treatments also showed some additivity in a colon cancer xenograft model (Abal et al., 2004). Other results were reported in meeting abstracts and have not been published in detail at the time of writing this chapter (Guzi, 2004; Fischer and Gianella-Borradori, 2005).

TABLE 9.7
***In Vivo* Anti-Tumor Activity of (*R*)-Roscovitine in Nude Mice Models**

Tumor Cells	Delivery Mode	Concentration	Frequency	Results	Reference
LoVo (colon carcinoma)	Intraperitoneal	100 mg/kg	3×/d/5 d	44.8% reduction in tumor size T/C: 58.3%	McClue et al., 2002
MES-SA/Dx5 (uterine carcinoma)	Oral	200 mg/kg	3×/d/10 d	62% reduction in tumor size T/C: 47.3%	McClue et al., 2002
		500 mg/kg	3×/d/4 d	35% reduction in tumor size T/C: 85.7%	
MDA-MB 231 (breast cancer)	Intraperitoneal	100 mg/kg (± 7.5 Gy irradiation)	Single dose	54% (irradiation) or 73% (irradiation + roscovitine) reduction in tumor size	Maggiorella et al., 2003
HT29 (colon cancer)	Oral	200 mg/kg (± irinotecan)	2×/d/3–4 d	Additivity	Abal et al., 2004
HCT116 (colon cancer)	Oral	500 mg/kg	2×/d/5 d	T/C: 65%	Raynaud et al., 2005
PC-3	Subcutaneous infusion with osmotic minipump	10 mg/ml	2 mg/week for 3 weeks	35% tumor growth inhibition	Payton et al., 2006
A4573 (Ewing's sarcoma)	Intraperitoneal	50 mg/kg	5 d or two 5-d series with a 2-d break	11.5-fold difference in tumor volume of treated vs. control animals 1 d after 5-d treatment Overall 5-fold reduction in tumor growth	Tirado et al., 2005

9.8.3 HUMAN CANCERS

(*R*)-Roscovitine has been evaluated in several Phase I clinical trials. A total of 70 patients have been evaluated for anti-tumor activity. One partial response (hepatocellular carcinoma) and 10 stable diseases lasting for more than 4 months were reported. Two of these patients had non-small-cell lung cancers that remained stable for more than a year.

(R)-Roscovitine is currently under investigation in several Phase 2 clinical trials in four different clinical situations as a monotherapy against B-cell malignancies (chronic lymphocytic leukemia, mantle cell lymphoma, and multiple myeloma) and in combination trials against non-small-cell lung cancer (+ gemcitabine/cisplatin or docetaxel) and metastatic breast cancer (+ capecitabine).

Unfortunately, the results of the Phase 1 and 2 clinical trials of *(R)*-roscovitine in human patients are only available as meeting abstracts and have not been published in detail at the time of writing this chapter. Overviews of the clinical data are available in recent reviews (Guzi, 2004; Fischer and Gianella-Borradori, 2005).

9.9 USING ROSCOVITINE

9.9.1 STORAGE, DILUTION, CONCENTRATIONS, AND AFFINITY CHROMATOGRAPHY

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 . 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.

When tested in *in vitro* enzymatic assays, we suggest the following range of roscovitine concentrations for initial testing: 0–0.01–0.025–0.05–0.1–0.25–0.5–1–2.5–5–10–25–50–100 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. These methods are summarized in Table 9.8. Experimental data show that roscovitine displays high bioavailability when delivered intraperitoneally or orally.

For affinity chromatography on immobilized roscovitine (Bach et al., 2005), 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 batchwise 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.

9.9.2 USING ROSCOVITINE: A “ROSCOVITINE USER’S KIT”

With an assumed selectivity for CDKs, *(R)*-roscovitine is frequently used to evaluate the contribution of CDKs to cellular processes. Although this assumption of selectivity is partially confirmed, the use of *(R)*-roscovitine and the interpretation of its

TABLE 9.8
Methods Used to Deliver Roscovitine to Animals and Humans

Animal	Delivery Mode	Concentration	Frequency	Vehicle	Reference
Mouse	Intravenous	20 mg/kg		PEG400:10% Kollidon 17PF (7:13)	McClue et al., 2002
Mouse	Oral	500 mg/kg	3x/d/4 d	50 mM HCl	McClue et al., 2002
		200 mg/kg	3x/d/10 d		
Mouse	Intraperitoneal	100 mg/kg	3x/d/5 d	50 mM HCl pH 2.5	McClue et al., 2002
Mouse	Intraperitoneal	100 mg/kg		0.25% Tween 20 in 0.9% NaCl	Maggiorella et al., 2003
Mouse	Intraperitoneal	20–75 mg/kg	2x/d/20 d	DMSO:PBS (4:6)	Gherardi et al., 2004
Mouse	Oral	500 mg/kg	2x/d/5 d	DMSO: Tween 20:50 mM HCl/saline (10:5:85)	Raynaud et al., 2005
Mouse	Intravenous	100 mg/kg	—	—	Nutley et al., 2005
Mouse	Intraperitoneal	2.8 mg/kg	1x/d/5 or 14 d	DMSO:PBS (2:8)	Griffin et al., 2005
Mouse	Intraperitoneal	50 mg/kg	1x/d/5 d, 2-d interval, then 1x/d/5 d	Dissolve in ethanol or DMSO. Dilute in Tween 80, <i>N,N</i> - dimethylacetamide, polyethylene glycol 400 (Fisher Scientific) (1:2:7)	Tirado et al., 2005
Rat	Intraperitoneal	2.8 mg/kg	1x/d/3–10 d	DMSO	Pippin et al., 1997
Rat	Oral	100 mg/kg	1x/d/10 d then daily I.P.	30 mM HCl, then DMSO	Milovanceva- Popovska et al., 2002
Human	Oral	50–800 mg	1 dose	50 mg capsules	de la Motte and Gianella- Borradori, 2004

cellular effects has limitations. First, (*R*)-roscovitine inhibits CDK1, CDK2, CDK5, CDK7, and CDK9 almost equally. Second, the effects on Erk1/2, CK1, and CaM kinase 2 cannot be neglected as they may significantly contribute to the cellular response to (*R*)-roscovitine. Third, interference with PDXK might, under some circumstances, lead to a significant reduction in intracellular vitamin B₆ level, and this is expected to alter a large number of metabolic enzymes, with numerous cellular

consequences. In addition, high levels of PDXK in some tissues may reduce the availability of CDKs to roscovitine.

Nevertheless, *(R)*-roscovitine can provide valuable and convincing information if used in conjunction with a “kit” of other molecules:

1. *(S)*-Roscovitine, apparently much less active on PDXK than *(R)*-roscovitine, in most models (except primates); in addition, *(S)*-roscovitine is slightly less efficient on CDKs than *(R)*-roscovitine, and this difference should be observed in CDK-dependent cellular events (Bach et al., 2005).
2. *O*⁶-Benzyl-*(R)*-roscovitine or *N*⁶-methyl-*(R)*-roscovitine, two compounds closely related to *(R)*-roscovitine, which are essentially inactive on CDKs but still interact with PDXK (Tang et al., 2005).
3. *O*lomoucine (Vesely et al., 1994) and *aminopurvalanol* (Chang et al., 1999), which are less and more active on CDKs, respectively.
4. *U0126*, *PD184352*, or *PD98059*, three MEK1 inhibitors that prevent the activation of Erk1/2 and thus mimic the Erk1/2 inhibiting properties of *(R)*-roscovitine, but do not inhibit CDKs.
5. *IC261*, *D4476*, and *SB203580*, three CK1 inhibitors that mimic the CK1-inhibiting properties of *(R)*-roscovitine, but do not inhibit CDKs.
6. At least another structurally unrelated CDK inhibitor. For example, the combined use of both kenpaullone and roscovitine has been recommended to help identify substrates and physiological roles of CDKs, whereas the combined use of kenpaullone and lithium could be useful for identifying substrates and physiological roles of glycogen synthase kinase-3 (GSK-3) (Bain et al., 2003).

In addition, it is highly recommended to complement the use of *(R)*-roscovitine with molecular biology methods that prevent or reduce the expression and activity of specific CDKs or cyclins such as siRNAs, antisense oligonucleotides, peptidic nucleic acids, expression of dominant negative mutants, and CDK or cyclin knockouts.

Finally, the use of specific antibodies that cross-react with sites that are specifically phosphorylated by CDKs (Table 9.4) provides further support for specific inhibition of CDKs.

9.10 FUTURE PROSPECTS

Considerable work has been carried out since 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. But how can roscovitine be improved?

(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 (see Subsection 9.9.2). In contrast, this diversity of molecular actions becomes an advantage when (*R*)-roscovitine is investigated as a potential drug for the treatment of cancer. Indeed, 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 (Ortega et al., 2003), the sensitivity of CDK2^{-/-} cells to (*R*)-roscovitine is only slightly lower than that of CDK2^{+/+} wild-type cells (Bach et al., 2005). The artificial absence of CDK2 appears to be compensated by CDK1 (Aleem et al., 2005), 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 (Figure 9.6) are not completely devoid of anti-proliferative effects, but these effects may be unspecific as they require very high doses (Meijer et al., unpublished).

The pharmacological parameters of (*R*)-roscovitine certainly could be improved by slight modifications of the parent structure. Whether the relatively short half-life of (*R*)-roscovitine constitutes 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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