

Inhibition of cyclin-dependent kinases by purine analogues Crystal structure of human cdk2 complexed with roscovitine

Walter Filgueira DE AZEVEDO¹, Sophie LECLERC², Laurent MEIJER², Libor HAVLICEK³, Miroslav STRNAD⁴ and Sung-Hou KIM¹

¹ Department of Chemistry and Lawrence Berkeley National Laboratory, University of California, Berkeley CA, USA

² CNRS, Station Biologique, BP 74, Roscoff, France

³ Institute of Toxicology and Forensic Chemistry, Medical Faculty 1, Charles University, Prague, Czech Republic

⁴ Institute of Experimental Botany, Department of Plant Biotechnology, Olomouc, Czech Republic

(Received 6 November 1996) – EJB 96 1646/3

Cyclin-dependent kinases (cdk) control the cell division cycle (cdc). These kinases and their regulators are frequently deregulated in human tumours. A potent inhibitor of cdk2, roscovitine [2-(1-ethyl-2-hydroxyethylamino)-6-benzylamino-9-isopropylpurine], was identified by screening a series of C2,N⁶,N⁹-substituted adenines on purified cdc2/cyclin B. Roscovitine displays high efficiency and high selectivity (Meijer, L., Borgne, A., Mulner, O., Chong, J. P. J., Blow, J. J., Inagaki, N., Inagaki, M., Delcros, J.-G. & Moulinoux, J.-P. (1997) *Eur. J. Biochem.* 243, 527–536). It behaves as a competitive inhibitor for ATP binding to cdc2. We determined the crystal structure of a complex between cdk2 and roscovitine at 0.24-nm (2.4 Å) resolution and refined to an R_{factor} of 0.18. The purine portion of the inhibitor binds to the adenine binding pocket of cdk2. The position of the benzyl ring group of the inhibitor enables the inhibitor to make contacts with the enzyme not observed in the ATP-complex structure. Analysis of the position of this benzyl ring explains the specificity of roscovitine in inhibiting cdk2. The structure also reveals that the (*R*)-stereoisomer of roscovitine is bound to cdk2. The (*R*)-isomer is about twice as potent in inhibiting cdc2/cyclin B than the (*S*)-isomer. Results from structure/activity studies and from analysis of the cdk2/roscovitine complex crystal structure should allow the design of even more potent cdk inhibitors.

Keywords: cell cycle; cyclin-dependent kinase; purine; protein-kinase inhibitor; anti-tumor agent.

Protein kinases are involved in essentially all intracellular regulatory pathways. In view of their essential role in the regulation of the cell division cycle, cyclin-dependent kinases (cdk) have been the object of considerable investigation (reviews in [1–9]). A typical cdk consists of a catalytic subunit [cdk1 (= cdc2)–cdk8] and a regulatory subunit (cyclin A–cyclin H). Cdk proteins are regulated by (a) transcription/translation of their subunits, (b) complex formation, (c) various post-translational modifications (phosphorylation/dephosphorylation), (d) interaction with various protein inhibitors (p16, p21), (e) modifications of their cellular localisation. The crystal structures of cdk2 [10] and of cdk2/cyclin A [11] have been recently determined, allowing a very precise understanding of the mechanisms of enzyme activation and activity.

Numerous examples of cdk deregulation in human primary tumours and in tumour cell lines have been described recently (reviews in [12, 13]). These observations encourage the search for selective chemical inhibitors of cdk proteins and the use of natural inhibitors in potential gene therapy [14, 15]. Prompted by the abundance of cdc2/cyclin B kinase in starfish oocytes, its relatively easy purification by affinity chromatography on p9^{CKS}/p13^{suc1}–Sephacryl and the rapid assay of its activity with histone H1 and [³²P]ATP, a few years ago we set up a simple screening test using purified p34^{cdc2}/cyclin B^{cdc13} as a target [16].

Correspondence to L. Meijer, CNRS, Station Biologique, BP 74, F-29682 Roscoff cedex, France

Fax: +33 2 98 29 23 42.

E-mail: meijer@sb-roscoff.fr

Abbreviations. cdc, cell division cycle; cdk, cyclin-dependent kinase; Me₂SO, dimethylsulfoxide.

The first compound to be identified as a cdc2 inhibitor was 6-dimethylaminopurine (IC₅₀: 120 μM) [17, 18]. This compound was initially synthesized as a puromycin analogue and found to inhibit mitosis of sea urchin embryos without inhibiting protein synthesis [19]. By structural analogy we identified N⁶-(4²-isopentenyl)adenine as a slightly more potent kinase inhibitor (IC₅₀: 55 μM) [16]. Following extensive screening of a series of substituted purines designed for other purposes, olomoucine [2-(2-hydroxyethylamino)-6-benzylamino-9-methylpurine] was identified as a potent cdc2 inhibitor (IC₅₀: 7 μM) [20]. This compound was originally designed as an inhibitor of cytokinin 7-glucosyltransferase from radish cotyledons [21]. The unusual specificity of this compound led us to co-crystallise it with cdk2 [22], and to investigate its cellular effects [23, 24]. Other cdk inhibitors have been described: staurosporine [16, 25], butyrolactone I [26–28], flavopiridol [29–31], suramin [32]. The chemical structures, enzymatic specificities and cellular effects of chemical inhibitors of cdk proteins have been reviewed [33, 34].

The purine olomoucine displays a quite narrow selectivity: among 35 kinases tested, it only inhibits cdc2, cdk2, cdk5 and erk1, to a lesser extent [20]. The position of olomoucine in the ATP binding pocket of cdk2 has been determined by analysis of an olomoucine/cdk2 co-crystal [22]. Interestingly the purine ring of olomoucine and ATP are orientated in a totally different manner. Recently, olomoucine was found to stimulate massive apoptosis in cells which have been arrested in G2 by the use of DNA-damaging agents [35]. These encouraging results led us to investigate other C2,N⁶,N⁹-substituted adenines as potential cdk inhibitors. We here report the identification of a new potent and

selective cdc2 inhibitor, roscovitine [2-(*R*)-(1-ethyl-2-hydroxyethylamino)-6-benzylamino-9-isopropylpurine] (IC_{50} : 0.45 μ M). We also report the analysis of a high-resolution X-ray cdk2/roscovitine complex crystal structure. The enzymatic specificity and cellular effects of roscovitine are described in the following paper [36].

EXPERIMENTAL PROCEDURES

Chemicals. The compounds listed in Table 1 were synthesized by one of us (Havlicek et al., unpublished) with the exception of 2-(2-hydroxyethylamino)-6-benzylaminopurine (20) and 2-(2-hydroxyisobutylamino)-6-benzylamino-9-methylpurine (58) which were synthesized by D. S. Letham. Purine analogues were usually dissolved as 10–100 mM stock solutions in dimethylsulfoxide (Me_2SO). Final Me_2SO concentration in the reaction mixture was less than 1% (by vol.). [γ - ^{32}P]ATP (PB 168) was obtained from Amersham.

Buffers. Homogenization buffer: 60 mM glycerol 2-phosphate, 15 mM *p*-nitrophenyl phosphate, 25 mM Mops pH 7.2, 15 mM EGTA, 15 mM $MgCl_2$, 1 mM dithiothreitol, 1 mM sodium vanadate, 1 mM NaF, 1 mM phenyl phosphate, 10 μ g leupeptin/ml, 10 μ g aprotinin/ml, 10 μ g soybean trypsin inhibitor/ml and 100 μ M benzamidine.

Buffer C: homogenization buffer but 5 mM EGTA, no NaF and no protease inhibitors.

Preparation of starfish M phase oocytes extracts. For large-scale oocyte extracts preparations, gonads were removed from ripe *Marthasterias glacialis*, and incubated with 10 μ M 1-methyladenine in Millipore-filtered natural sea water until spawning [37]. By that time all the oocytes had entered the M phase. Oocytes were then removed from the incubation medium by centrifugation, directly frozen in liquid nitrogen and kept at $-80^\circ C$ [20, 38]. M-phase oocytes were homogenized in homogenization buffer at a ratio of 2 g oocytes/ml buffer. After a 45-min centrifugation at 100 000 *g*, the supernatant was recovered and directly used for affinity-chromatography purification of the p34^{cdc2}/cyclin B kinase on p9^{CKShs1}-Sephacryl beads as described [20, 39, 40].

Enzyme preparation and assays. p34^{cdc2}/cyclin B was purified from M-phase starfish (*M. glacialis*) oocytes by affinity chromatography on p9^{CKShs1}-Sephacryl beads, from which it was eluted by free p9^{CKShs1} as described above [38–41]. It was assayed with 1 mg/ml of histone H1 (Sigma, type III-S) in the presence of 15 μ M [γ - ^{32}P]ATP (3000 Ci/mmol; 1 mCi/ml) in a final volume of 30 μ l buffer C [20, 42]. After a 10-min incubation at 30°C, 25- μ l aliquots of supernatant were spotted onto pieces (2.5 \times 3 cm) of Whatman P81 phosphocellulose paper, and, after 20 s, the filters were washed five times (for at least 5 min each time) in a solution of 10 ml phosphoric acid/l water. The wet filters were transferred into 6-ml plastic scintillation vials, 5 ml ACS (Amersham) scintillation fluid was added and the radioactivity measured in a Packard counter. Blank values were subtracted from the data and activities calculated as molar amount of phosphate incorporated/mass histone H1 during a 10-min incubation or as a percentage of maximal activity. Controls were performed with appropriate dilutions of Me_2SO .

To run initial-rate kinetic experiments, the end-point assay system for p34^{cdc2} protein kinase was used as described above except that, on the basis of preliminary trials, appropriate non-saturating substrate concentrations were applied. p34^{cdc2}/cyclin B protein kinase was added such that activity was linear with respect to enzyme concentration and time. In most cases, this required enzyme dilution 3–10-fold into buffer C. Velocity data were expressed in terms of rate of molar amount incorporated

into the substrate/amount of enzyme added. Apparent inhibition constants were determined by graphical analysis.

Human cdk2 was prepared as described by Rosenblatt et al. [43]. Briefly, Sf9 insect cells were infected with baculovirus containing the human cdk2 gene. The supernatant of cell lysate was loaded over a DEAE-Sephacryl column followed by an S-Sepharose column. The flow-through was loaded onto an ATP-affinity column and eluted by a NaCl gradient. The purified protein is fully functional in assays of cdk2 activation *in vitro*, and the resulting complex can be fully activated when incubated with partially purified human cdk-activating kinase.

Inhibitor soaking and co-crystals. We crystallized cdk2 in the conditions described earlier [43]. Briefly, a cdk2 solution was concentrated to 10 mg/ml by dialysis against 20 mM Hepes pH 7.4, 1 mM EDTA. Sitting drops were equilibrated by vapor diffusion at 4°C against reservoirs containing 200 mM Hepes pH 7.4. Diamond and wedge-shaped crystals appeared after 2–4 days. A gradual increase of the Hepes concentration in the reservoirs (up to 800 mM) produced crystals with average dimensions of about 0.2 mm \times 0.3 mm \times 0.3 mm. After cdk2 crystals had formed, small amounts of roscovitine powder were added using a cat's whisker to the crystallisation drops. Most of the crystals cracked after a few days, possibly indicating that binding of the inhibitor to cdk2 induces conformational changes of cdk2. Addition of smaller amounts of inhibitor to the crystallization drops prevented the crystals from cracking. Crystals were soaked for 48 h before data collection. Co-crystallization experiments using the crystallization conditions for cdk2 as well as the sparse matrix method [44] were also undertaken, but so far we have not obtained any X-ray diffracting co-crystals. Preliminary X-ray studies showed that the soaked crystals diffracted to 2.4 Å. The crystals were of the same space group as the apo-enzyme and the cdk2-ATP crystals. The crystallographic parameters are given in Table 1.

Data collection and structure refinement. X-ray diffraction data for the cdk2/roscovitine complex crystal were collected using a Rigaku R-AXIS-II imaging plate area detector (Table 1). Refinement of cdk2/roscovitine complex proceeded with refining the position of the cdk2 apo-enzyme model, without its water molecules, as rigid body against the complex data, using X-PLOR 3.1 [45]. The R_{factor} of the cdk2/roscovitine complex decreased from 0.48 to 0.26 with data from 8.0 Å to 3.0 Å. At this stage $F_o - F_c$ maps were calculated. These maps showed clear density for roscovitine bound to the ATP-binding pocket of cdk2.

The conformation of residues forming the ATP-binding pocket were checked in simulated annealing omit maps before including the inhibitor molecule in the complex structure. Further refinement in X-PLOR continued with simulated annealing using the slow-cooling protocol [46], followed by alternate cycles of positional refinement and manual rebuilding using the program O [47]. Finally, the positions of water molecules were checked and corrected in $F_o - F_c$ omit maps, for which all water molecules were omitted for F_c calculations. Water molecules were modeled into difference densities larger than 3 σ . The final model has a R_{factor} of 0.18 and a R_{free} of 0.27 with good stereochemistry (Table 2).

Root-mean-square (rms) differences from ideal geometries for bond lengths and angles were calculated using X-PLOR 3.1 [45]. Protein superpositions based on $C\alpha$ atoms were obtained using the program LSQKAB from CCP4 [48]. Hydrogen bonds and van der Waal's contacts were assigned with the program CONTACTSYM and HBPLUS [49, 50]. The cutoff for hydrogen bonds and salt-bridges was 3.4 Å and up to 4.11 Å for van der Waal's contacts, depending on the atom type and using standard van der Waal's radii. Comparisons of the common contacts among the different ligands and cdk2 were made by a program

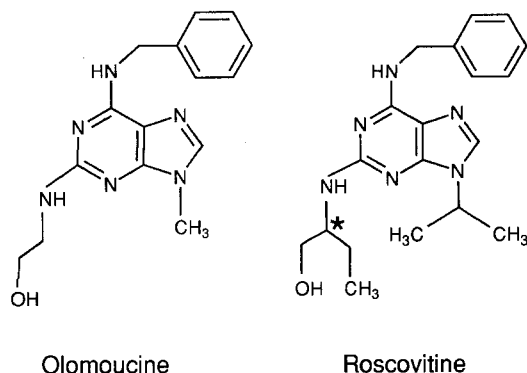


Fig. 1. Chemical structure of roscovitine [2-(*R*)-(1-ethyl-2-hydroxyethylamino)-6-benzylamino-9-isopropylpurine] (**102**) and olomoucine [2-(2-hydroxyethylamino)-6-benzylamino-9-methylpurine] (**51**). An asterisk on roscovitine marks the asymmetric carbon.

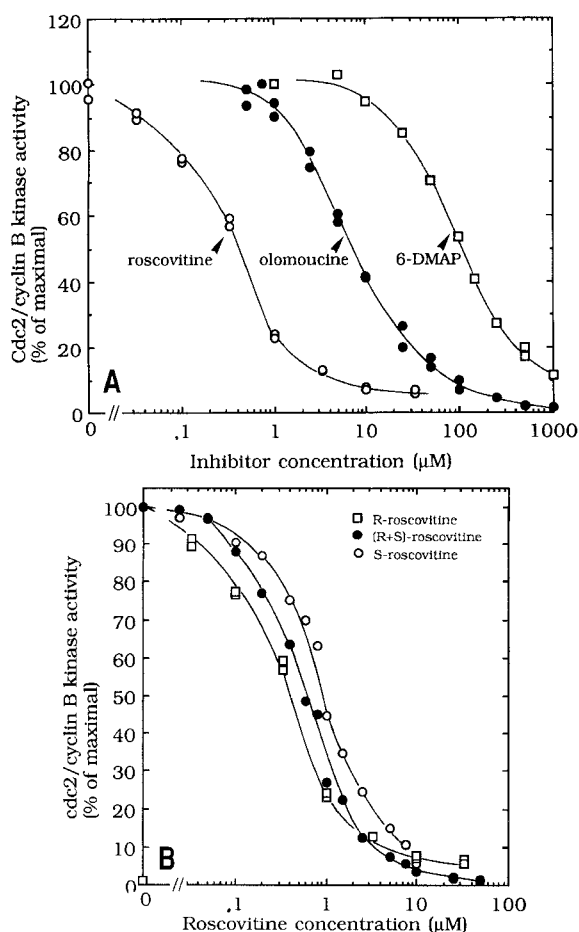


Fig. 2. Inhibition of p34^{cdc2}/cyclin B by roscovitine. (A) Roscovitine, olomoucine and 6-dimethylaminopurine (6-DMAP) dose/response curves for p34^{cdc2}/cyclin B. Enzyme activity was assayed as described in Experimental Procedures in the presence of increasing concentrations of inhibitors. Activity is presented as a percentage of maximal activity, i.e. measured in the absence of inhibitors. (B) (*R*)-Roscovitine is a better inhibitor of p34^{cdc2}/cyclin B than (*S*)-roscovitine. The racemic mixture displays intermediate inhibitory potency. Enzyme activities were assayed as described in Experimental Procedures in the presence of increasing concentrations of inhibitors.

developed in our laboratory, LIGSTAT. This simple program uses the outputs of the program CONTACTSYM and summarises the common hydrogen bonds and van der Waals contacts among ligands and enzyme, in two different files. The solvent

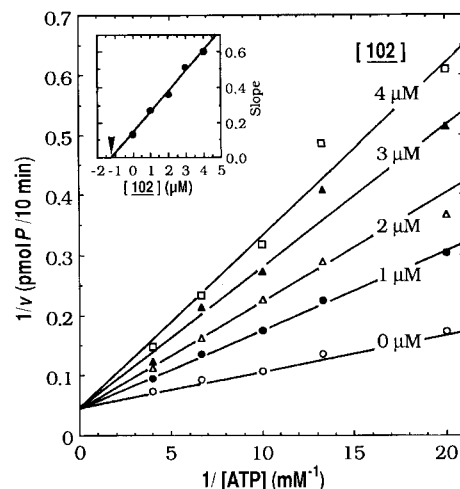


Fig. 3. Double-reciprocal plots of kinetic data from assays of p34^{cdc2}/cyclin B protein kinase activity at different concentrations of roscovitine (**102**). Enzyme activities were assayed as described under Experimental Procedures. (A) 1/*v* versus 1/[ATP] primary plot. ATP concentrations in the reaction mixture varied over 0.05–0.25 mM; concentration of histone H1 was kept constant at 0.7 mg/ml. Inset displays secondary replots of slopes versus concentration from primary plots. Apparent inhibition constant (*K_i*) is indicated by an arrow.

accessibility of individual residues was assessed using the program MS [51] with a 1.7-Å radius for the solvent probe.

RESULTS

Roscovitine, an olomoucine-related purine, inhibits cdc2/cyclin B by competition for ATP binding. 6-Dimethylaminopurine was the first-described cdc2 inhibitor of the purine family (Fig. 2 A) [17, 18]. We recently described the inhibitory properties of C2,*N*⁶,*N*⁹-substituted adenines towards cyclin-dependent kinases and mitogen-activated protein (MAP) kinases [20]. Among the compounds tested, olomoucine [2-(2-hydroxyethylamino)-6-benzylamino-9-methylpurine] (**51**) (Fig. 1) was the most active inhibitor (IC₅₀: 7 μM and 30 μM, for cdc2/cyclin B and erk-1, respectively) (Fig. 2A). A series of related compounds were synthesized and tested as potential inhibitors of the cdc2/cyclin B kinase (Table 1). The chemistry of these purines will be described elsewhere (Havlicek et al., unpublished).

One of the compounds, 2-hydroxymethylpropylamino-6-benzylamino-9-isopropylpurine or 2-(1-ethyl-2-hydroxyethylamino)-6-benzylamino-9-isopropylpurine (**102**) (Fig. 1), renamed roscovitine for convenience, displayed a 10-fold increase in efficacy (IC₅₀: 0.65 μM) when compared to olomoucine (Fig. 2A). As roscovitine contains an asymmetric carbon (Fig. 1), the two optical isomers were separately synthesized and tested for their kinase inhibitory activity (Fig. 2 B). The (*R*) isomer displays a slightly higher (IC₅₀: 0.45 μM) activity than the (*S*) isomer (IC₅₀: 0.95 μM). The racemic (*R*+*S*) mixture shows intermediate activity (Fig. 2B). This isomeric difference was barely detectable with cdk2/cyclin E (data not shown).

To investigate the mechanism of roscovitine action, kinetic experiments were performed in the presence of increasing roscovitine concentrations, with varying ATP levels (Fig. 3). Double-reciprocal plotting of the data demonstrates that roscovitine acts as a competitive inhibitor for ATP. The linearity of the slope versus roscovitine concentration replots qualifies roscovitine as a linear inhibitor (Fig. 3, inset). The apparent inhibition constant (*K_i*) was 1.2 μM. Olomoucine was recently co-crystallized with cdk2 [22]. Resolution of the crystal structure showed

Table 1. IC₅₀ values for various purines added to purified p34^{cdc2}/cyclin B kinase. Enzyme activities were assayed as described in Experimental Procedures in the presence of increasing concentrations of purines. IC₅₀ values were calculated from the dose/response curves. The numbering of purines from Table 2 of Vesely et al. [20] is continued in the present table. Numbers on the left, new compounds; numbers on the right, a few reference purines originally presented by Vesely et al. [20].

Compound		Structure	IC ₅₀
Type	new ref.		
			μM
C2 and N ⁶ substituted purines	<u>82</u>	2-chloro-6-aminopurine	>1000
	<u>83</u>	2-methyl-6-aminopurine	320
		<u>18</u> 2-amino-6-benzylaminopurine	90
		<u>19</u> 2-(2-hydroxyethylamino)-6-aminopurine	200
		<u>20</u> 2-(2-hydroxyethylamino)-6-benzylaminopurine	25
N ⁶ and N9 substituted purines	<u>84</u>	6-chloro-9-tetrahydropyranilpurine	>1000
	<u>85</u>	6-amino-9-methylpurine	330
		<u>26</u> 6-benzylamino-9-methylpurine	40
C2,N ⁶ and N9 substituted purines		<u>46</u> 2-chloro-6-amino-9-methylpurine	70
	<u>86</u>	2-chloro-6-isopentenylamino-9-methylpurine	40
	<u>87</u>	2-chloro-6-isopentenylamino-9-isopropylpurine	3.3
		<u>49</u> 2-chloro-6-(5-hydroxypentylamino)-9-methylpurine	20
		<u>88</u> 2-chloro-6-cyclohexylmethylamino-9-methylpurine	130
		<u>47</u> 2-chloro-6-benzylamino-9-methylpurine	12
		<u>89</u> 2-chloro-6-benzylamino-9-isopropylpurine	17
		<u>48</u> 2-chloro-6-(3-hydroxybenzylamino)-9-methylpurine	5.2
		<u>90</u> 2-chloro-6-(2-hydroxyethyl)benzylamino-9-isopropylpurine	40
		<u>45</u> 2-amino-6-benzylamino-9-methylpurine	40
		<u>91</u> 2-ethylamino-6-(4-hydroxy-3-methyl-trans-2butenylamino)-9-methylpurine	48
		<u>92</u> 2-(2-aminoethylamino)-6-benzylamino-9-isopropylpurine	1
		<u>60</u> 2-(2-diethylaminoethylamino)-6-benzylamino-9-methylpurine	> 100
		<u>93</u> 2-(3-aminopropylamino)-6-benzylamino-9-isopropylpurine	7.5
		<u>94</u> 2-(<i>n</i> -heptylamino)-6-benzylamino-9-isopropylpurine	>1000
		<u>95</u> 2-dimethylamino-6-benzylamino-9-isopropylpurine	30
		<u>50</u> 2-(2-hydroxyethylamino)-6-amino-9-methylpurine	50
		<u>54</u> 2-(2-hydroxyethylamino)-6-isopentenylamino-9-methylpurine	65
		<u>55</u> 2-(2-hydroxyethylamino)-6-isopentenylamino-9-isopropylpurine	8.5
		<u>96</u> 2-(<i>RS</i>)-2-hydroxypropylamino)-6-isopentenylamino-9-isopropylpurine	1.2
		<u>97</u> 2-(2-hydroxyethylamino)-6-cyclohexylmethylamino-9-methylpurine	6
		<u>98</u> 2-(<i>RS</i>)-(2-hydroxypropylamino)-6-cyclohexylmethylamino-9-methylpurine	4
		<u>51</u> 2-(2-hydroxyethylamino)-6-benzylamino-9-methylpurine	7
		<u>52</u> 2-(2-hydroxyethylamino)-6-benzylamino-9-(2-hydroxyethyl)purine	8
		<u>53</u> 2-(2-hydroxyethylamino)-6-benzylamino-9-isopropylpurine	2
		<u>56</u> 2-(2-hydroxyethylamino)-6-(3-hydroxybenzylamino)-9-methylpurine	5
		<u>57</u> 2-(2-hydroxyethylamino)-6-(3-hydroxybenzylamino)-9-isopropylpurine	6
	<u>99</u> 2-[bis(2-hydroxyethyl)amino]-6-benzylamino-9-isopropylpurine	1.2	
	<u>100</u> 2-(<i>RS</i>)-(2-hydroxypropylamino)-6-benzylamino-9-isopropylpurine	0.9	
	<u>101</u> 2-(3-hydroxypropylamino)-6-benzylamino-9-isopropylpurine	1	
	<u>102</u> 2-(<i>RS</i>)-(1-ethyl-2-hydroxyethylamino)-6-benzylamino-9-isopropylpurine	0.65	
	<u>103</u> 2-(4-hydroxy-3-methylbutylamino)-6-benzylamino-9-isopropylpurine	4.5	
	<u>58</u> 2-(2-hydroxyisobutylamino)-6-benzylamino-9-methylpurine	6	
	<u>104</u> 2-(5-hydroxypentylamino)-6-benzylamino-9-isopropylpurine	7.5	
C2, N ⁶ and N7 substituted purines	<u>105</u>	2-chloro-6-benzylamino-7-methylpurine	100
	<u>106</u>	2-(2-hydroxypropylamino)-6-benzylamino-7-methylpurine	1000
		<u>68</u> 2-(2-hydroxyethylamino)-6-benzylamino-7-methylpurine	1000
Other substituted purines	<u>107</u>	1-methyl-6-aminopurine	>1000
	<u>108</u>	2-methyl-6-aminopurine	320
	<u>109</u>	3-methyl-6-aminopurine	400
	<u>110</u>	1-methyl-6-benzylaminopurine	1000
	<u>111</u>	6-benzylamino-7-methylpurine	>1000

that olomoucine binds in the ATP-binding pocket. However, the purine rings of olomoucine and ATP are orientated in a totally different manner [22]. Roscovitine and cdk2 were next co-crystallized. Analysis of the cdk2/roscovitine complex crystal structure confirms that roscovitine binds in the ATP-binding pocket.

Cdk2/roscovitine complex crystal structure: overall protein conformation binding. The cdk2/roscovitine complex structure is almost identical to the cdk2 structure found in cdk2 apoenzyme [10], ATP complex [10], olomoucine complex [22] and isopentenyl adenine complex [22], and very similar to that in cdk2-des-chloro-flavopiridol complex [52]. As observed in sev-

Table 2. Diffraction data collection statistics of cdk2/roscovitine complex. $R_{\text{sym}} = \sum |I(h) - \langle I(h) \rangle| / \sum I(h)$, with $I(h)$, observed intensity and $\langle I(h) \rangle$, mean intensity of reflection h over all measurement of $I(h)$.

Parameter	Value
Space group	P2 ₁ 2 ₁ 2 ₁
Cell dimensions (Å)	$a = 72.31$ $b = 73.07$ $c = 54.28$
Number of measurements ($I/s(I) > 1.0$)	33 067
Unique reflections	11 134
Completeness of data to 2.4 Å (%)	88.5
R_{sym} (%)	0.069

Table 3. Refinement statistics for cdk2 complex with roscovitine. $R_{\text{factor}} = \sum |F_o - F_c| / \sum F_o$, the sums being taken over all reflections with $F/s(F) > 2$ cutoff. $R_{\text{free}} = R_{\text{factor}}$ for 10% of the data, which were not included during crystallographic refinement. B values are average B values for all non-hydrogen atoms.

Parameter	Value
Resolution (Å)	8.00–2.4
R_{factor} (%)	0.18
R_{free} (%)	0.27
B values (Å ²)	
main chain	20.6
side chains	24.3
inhibitor	37.1
waters	31.2
Deviations observed	
rms, bond lengths (Å)	0.011
rms, bond angles (°)	1.5
Number of water molecules	82

eral of the cdk2 structures, electron density is weak in one region in the enzyme, spanning residues 36–47 and 148–159.

The enzyme is folded into the typical bilobal structure, with the smaller N-terminal domain consisting predominantly of β -sheet structure and the larger C-terminal domain consisting primarily of α -helices (Fig. 4). There are no significant differences in the domain orientations between the inhibitor-enzyme complex and the ATP-enzyme complex. The inhibitor binds, as seen for ATP and the purine derivative inhibitors, in the deep cleft between the two domains (Fig. 4).

Conformation of bound roscovitine and ligand binding pocket. The electron density for all atoms of the inhibitor roscovitine is clear and strong (Fig. 5A). Roscovitine binds in the ATP-binding pocket, with the purine ring of roscovitine occupying approximately the same region as the purine ring of ATP (Fig. 5B). The two ring systems overlap roughly in the same plane. However the purine ring in the roscovitine complex has a different orientation than ATP with respect to the protein (Fig. 5 B). In this orientation, the N7 of roscovitine is close to the position of the N1 in purine of ATP. In the roscovitine molecule the benzyl ring points towards the outside of the ATP-binding pocket and occupies a region not occupied by any parts of the ATP in the ATP complex. All atomic interactions between roscovitine and cdk2 are schematically shown in Fig. 6B.

There is one chiral center in roscovitine (Fig. 1). The electron density indicates that the bound inhibitor is the (*R*)-stereoisomer of roscovitine, which is more potent than the (*S*)-isomer (Fig. 2B).

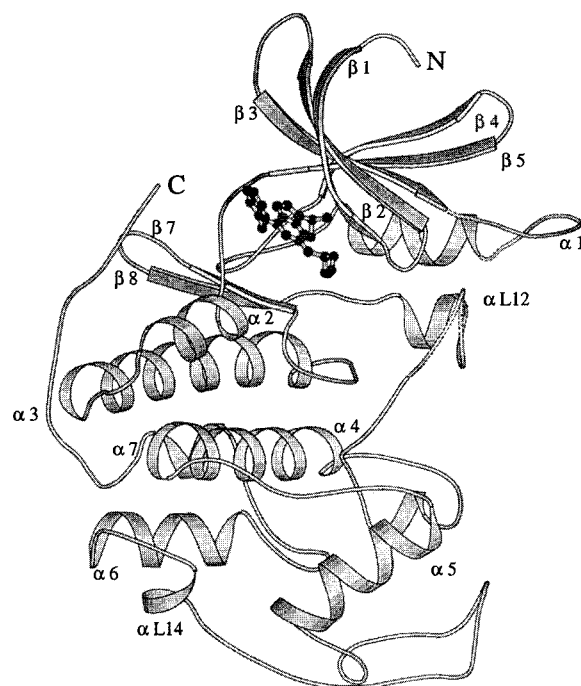


Fig. 4. Backbone drawing of cdk2 with the inhibitor roscovitine in the ATP-binding pocket between the smaller N-terminal domain and the larger C-terminal domain. Secondary structural elements are indicated by arrows for β -strands and coils for α -helices, and labelled as in the apoenzyme [10]. Residues 36–47 and 148–159, which have weak electron densities, are drawn with dotted lines.

Table 4. Buried areas of cdk2 and ligands. Complementary = (buried area in ligand/buried area in cdk2) \times 100.

Ligand	Buried areas in		Comple- mentarity	IC_{50} μM
	cdk2	ligand		
	Å		%	
ATP	435	352	81	—
Roscovitine	419	326	78	0.4
L868276	399	301	75	1.7
Olomoucine	360	261	73	7.0
Isopentenyladenine	290	203	70	50

In the roscovitine complex structure, binding to cdk2 is characterized by predominantly hydrophobic and van der Waal's interactions with the same hydrophobic enzyme residues that form the pocket for the adenine base in the ATP complex structure. There are 12 van der Waal's contacts with the purine ring, and 20 with the benzyl ring. The total contacts between roscovitine and cdk2 are 53. Many of the contacts between roscovitine and cdk2 are made by only three residues (Ile10, Leu83 and Leu134) which form a total of 20 contacts, corresponding to 38% of the observed contacts.

Geometric complementarity and buried surfaces areas. The specificity and affinity of a protein and its cognate ligand depend on directional hydrogen bonds, ionic interactions, as well as on shape complementarity of the contact surfaces of both partners [53, 54]. The shape complementarity is best described as solvent-accessible surfaces that become buried upon ligand binding. If the complementarity is good, the size of the buried

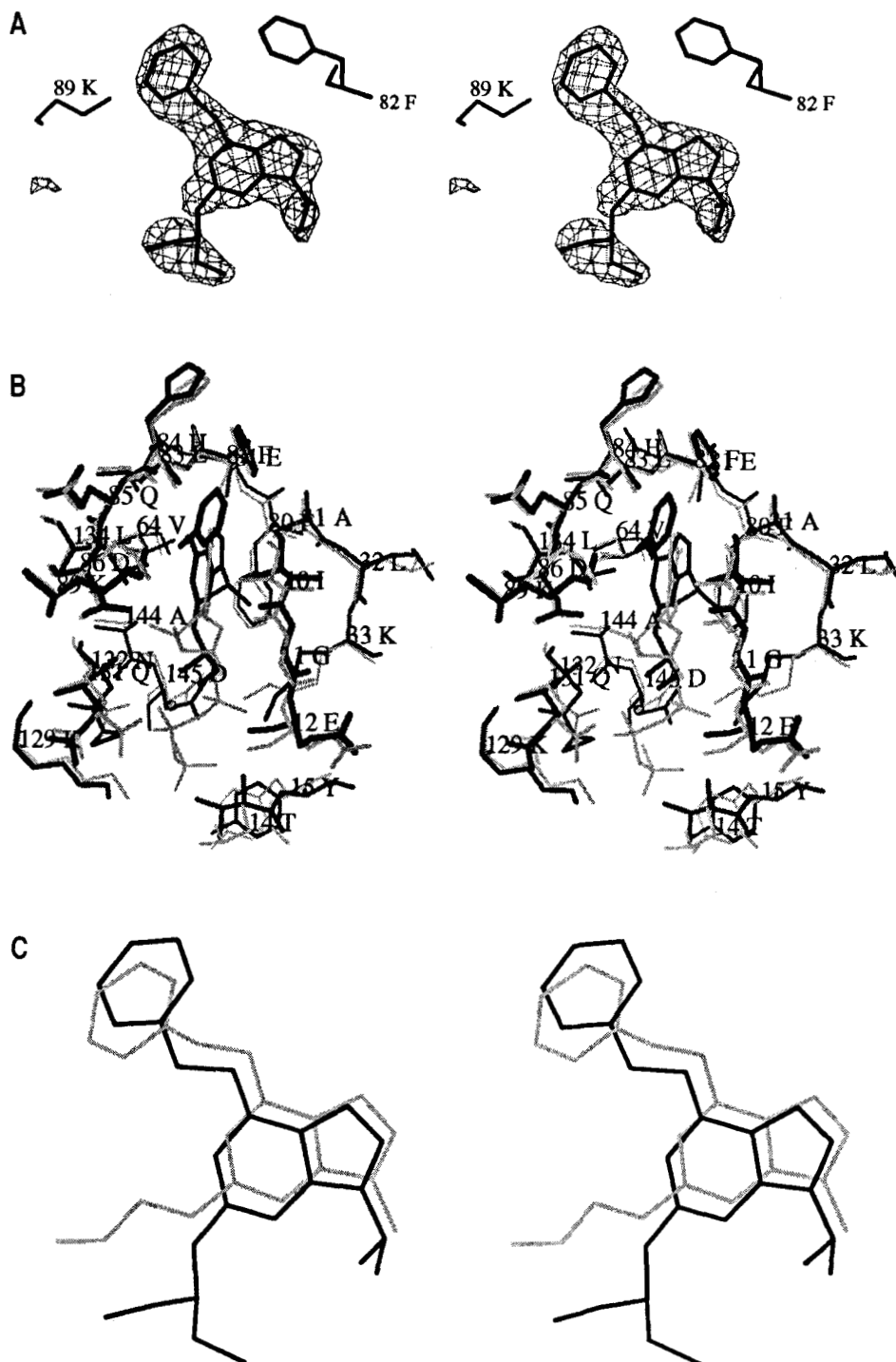


Fig. 5. Stereo view of (A) the electron density for roscovitine, (B) the superposed binding pockets of cdk2-ATP and cdk2/roscovitine complexes and (C) superposition of roscovitine and olomoucine as they are bound to cdk2. The electron density of roscovitine in difference electron density maps was calculated after simulated annealing refinement. The maps are contoured at 2.5σ and displayed with the computer program O [47]. In B, the binding pocket of cdk2-ATP complex is shown in grey; the binding pocket of cdk2/roscovitine complex is drawn in black. In C, roscovitine is in black and olomoucine in grey.

areas in a protein and its cognate ligand should be similar. In the cdk2-ATP complex the buried areas of ATP (352 \AA^2) and cdk2 (435 \AA^2) show a close fit. ATP is almost completely inaccessible to solvent and its buried surface amounts to 80% of the buried surface in cdk2. Corresponding values are 326 \AA^2 and 419 \AA^2 for roscovitine and cdk2, respectively, and the buried surface for roscovitine amounts to 78% of the buried surface in cdk2. The cdk2/roscovitine complex shows good complementarity in the area of the purine and benzyl rings.

DISCUSSION

In this paper we describe some new purine inhibitors of cdc2/cyclin B and especially roscovitine, a novel highly efficient C2,N⁶,N9-substituted adenine which interacts with the ATP-binding site of cdk. The enzymatic specificity and cellular effects of roscovitine are presented in the following paper [36].

By classical enzymological analysis (Fig. 3) we have shown that roscovitine acts as a competitive inhibitor for ATP binding.

interaction. They provide the structural basis for understanding specificity and potency of roscovitine compared to other inhibitors as well as the protein's authentic ligand ATP.

General features. The buried surface areas of cdk2 and the inhibitors show higher complementarity for the highly specific cdk2 inhibitors (olomoucine, L868276 and roscovitine), and a lower complementarity for the less-specific cdk2 inhibitor (isopentenyladenine) which agrees with the observed IC_{50} . The number of van der Waal's contacts between cdk2 and inhibitors also agrees with the observed IC_{50} values; the high-specificity inhibitors (low IC_{50}) show approximately the same number of van der Waals contacts with cdk2 while isopentenyladenine (high IC_{50}) shows far fewer contacts with cdk2.

Purine ring. The purine ring of roscovitine is located in the cdk2 structure in a similar orientation as the purine ring of olomoucine (Fig. 5C) [22]. This orientation is completely different from the orientation of the purine ring of ATP (Fig. 5B). A very interesting common feature stands out in the analysis of the common contacts between cdk2 and different ligands: one hydrogen bond, involving the N of Leu83, is present in all five ligands. This structural feature should be considered when designing new cdk2 inhibitors. Leu83 interacts with roscovitine through two hydrogen bonds, one with N7 of the purine, the other with N⁶, as was also observed with olomoucine [22]. Structure/activity studies confirm that N7 of the purine ring must remain free of substitution (compare 51 with 68, 100 with 106 and 47 with 105).

N⁶-Benzyl substituent. Especially interesting is the region of cdk2 occupied by the benzyl ring of the roscovitine molecule that is pointing away from the binding pocket (Fig. 5B). This region is not occupied by any parts of the ATP in the ATP complex but contributes with 20 van der Waal's contacts to the benzyl ring of the inhibitor in the cdk2/roscovitine complex. The main contact residues are Ile10, Phe82 and His84, as observed with olomoucine. The position of the benzyl ring of roscovitine is also responsible for the different position of the side-chain of His84 (Fig. 5B) that is moved away from the binding pocket in the roscovitine complex. The buried surface of cdk2 and the inhibitor shows a very close fit on the area around the benzyl ring suggesting that the benzyl ring is a moiety which increases the specificity for cdk2. Other hydrophobic substituents can replace the benzyl ring (isopentenyl (compare 53 and 55), cyclohexylmethyl (compare 97 and 51), 3-hydroxybenzyl (compare 56 and 51)), with essentially conserved inhibitory efficiencies.

C2 side chain. A side chain at C2 appears to be essential: hydroxylated ethylamino and propylamino substitutions provide the most active compounds (96, 99, 100, 101, 102). Isobutyl (58), pentyl (104) and heptyl (94) amino substitutions lead to less active compounds. The C2 substituent binds to an area of the ATP-binding pocket occupied by the ribose in the cdk2/ATP complex [22]. In addition to the hydroxyl group providing a hydrogen bond, a hydrophobic environment in the C2 substituent appears to be important (Fig. 6B).

N9 side chain. N9 substitution by a hydrophobic residue is important (compare 82 with 46, 20 with 51, 19 with 50, 86 with 87, 54 with 55 and 51 with 53): isopropyl appears to be the most active substituent.

In summary, the comparison of the three-dimensional structures of the cdk2/roscovitine complex with the cdk2-ATP complex shows that the hydrophobic purine-binding pocket has a surprising ability to accommodate new molecular structures that

are completely different from purine derivatives. Furthermore, specificity of the inhibitor for the cell-cycle kinases over non-cell-cycle kinases can be obtained by a moiety such as a benzyl group interacting on a protein surface of the ligand-binding pocket common among cell-cycle kinases. This discovery opens the possibility of testing new inhibitor families, in addition to new substituents for the already known lead structures such as flavone and purine derivatives.

We are grateful to D. S. Letham for providing compounds 20 and 58. We thank the fishermen of the *Station Biologique de Roscoff* for collecting the starfish. This research was supported by a fellowship from the *Conselho Nacional de Pesquisas* (Brazil) (to W. F. A. Jr) and by grants from the Office of Health and Environmental Research, US Department of Energy (DE-AC03-76SF00098; to S. H. K.), National Institutes of Health (to S. H. K.), the *Association pour la Recherche sur le Cancer* (ARC 6268; to L. M.), the *Groupement des Entreprises Françaises dans la Lutte contre le Cancer* (GEFLUC; to L. M.), the *Conseil Régional de Bretagne* (to L. M.) and the Czech Grant Agency (20619410641; to M. S.). We also gratefully acknowledge the gift of funds from Asahi Chemical Industry Co. Ltd (Japan), for the support of the research described here. This work on roscovitine is dedicated to the memory of Alain Maron.

REFERENCES

- Murray, A. & Hunt, T. (1993) in *The cell cycle – an introduction*, W. H. Freeman and Company, New York.
- Fantes, P. & Brooks, R. (eds) (1994) in *The cell cycle – a practical approach*, IRL Press, Oxford.
- Glover, D., Hall, A. & Hastie, N. (eds) (1994) *J. Cell Sci. Suppl.* 18.
- Grana, X. & Reddy, E. P. (1995) *Oncogene* 11, 211–219.
- Gould, K. L. (1995) in *Protein kinases* (Woodget, J. R., ed.) ch. 5, pp. 149–176, IRL Press, Oxford.
- Meijer, L., Guidet, S. & Tung, H. Y. L. (eds) (1995) in *Progress in cell cycle research*, vol. 1, Plenum Press, New York.
- Morgan, D. O. (1995) *Nature* 374, 131–134.
- Pines, J. (1995) *Biochem. J.* 308, 697–711.
- De Bondt, H. L., Rosenblatt, J., Jancarik, J., Jones, H. D., Morgan, D. O. & Kim, S. H. (1993) *Nature* 363, 595–602.
- Jeffrey, P. D., Russo, A. A., Polyak, K., Gibbs, E., Hurwitz, J., Masagué, J. & Pavletich, N. P. (1995) *Nature* 376, 313–320.
- Cordon-Cardo, C. (1995) *Am. J. Pathol.* 147, 545–560.
- Karp, J. E. & Broder, S. (1995) *Nat. Med.* 1, 309–320.
- Harper, W. (1996) *Trends Cell Biol.* 6, 388–392.
- Yang, Z. Y., Perkins, N. D., Ohno, T., Nabel, E. G. & Nabel, G. J. (1995) *Nat. Med.* 1, 1052–1056.
- Rialet, V. & Meijer, L. (1991) *Anticancer Res.* 11, 1581–1590.
- Meijer, L. & Pondaven P. (1988) *Exp. Cell Res.* 174, 116–129.
- Néant, I. & Guerrier, P. (1988) *Exp. Cell Res.* 176, 68–79.
- Rebhun, L. I., White, D., Sander, G. & Ivy, N. (1973) *Exp. Cell Res.* 77, 312–318.
- Vesely, J., Havlicek, L., Strnad, M., Blow, J. J., Donella-Deana, A., Pinna, L., Letham, D. S., Kato, J. Y., Détivaud, L., Leclerc, S. & Meijer, L. (1994) *Eur. J. Biochem.* 224, 771–786.
- Parker, C. W., Entsch, B. & Letham, D. S. (1986) *Phytochemistry* 25, 303–310.
- Schulze-Gahmen, U., Brandsen, J., Jones, H. D., Morgan, D. O., Meijer, L., Vesely, J. & Kim, S. H. (1995) *Proteins: Struct. Funct. Genet.* 22, 378–391.
- Abraham, R. T., Acquarone, M., Andersen, A., Asensi, A., Bellé, R., Berger, F., Bergounioux, C., Brunn, G., Buquet-Fagot, C., Fagot, D., Glab, N., Goudeau, H., Goudeau, M., Guerrier, P., Houghton, P. J., Hendriks, H., Kloareg, B., Lippai, M., Marie, D., Maro, B., Meijer, L., Mester, J., Mulner-Lorillon, O., Poulet, S. A., Schierenberg, E., Schutte, B., Vaulot, D. & Verlhac, M. H. (1995) *Biol. Cell* 83, 105–120.
- Glab, N., Labidi, H., Qin, L.-X., Trehin, C., Bergounioux, C. & Meijer, L. (1994) *FEBS Lett.* 353, 207–214.
- Gadbois, D. M., Hamaguchi, J. R., Swank, R. A. & Bradbury, E. M. (1992) *Biochem. Biophys. Res. Commun.* 184, 80–85.

26. Kitagawa, M., Higashi, H., Suzuki-Takahashi, I., Okabe, T., Ogino, H., Taya, Y., Nishimura, S. & Okuyama, A. (1994) *Oncogene* 9, 2549–2557.
27. Kitagawa, M., Okabe, T., Ogino, H., Matsumoto, H., Suzuki-Takahashi, I., Kokubo, T., Higashi, H., Saitoh, S., Taya, Y., Yasuda, H., Ohba, Y., Nishimura, S., Tanaka, N. & Okuyama, A. (1993) *Oncogene* 8, 2425–2432.
28. Someya, A., Tanaka, N. & Okuyama, A. (1994) *Biochem. Biophys. Res. Commun.* 198, 536–545.
29. Kaur, G., Stetler-Stevenson, M., Sebers, S., Worland, P., Sedlacek, H., Myers, C., Czech, J., Naik, R. & Sausville, E. (1992) *J. Natl. Cancer Inst.* 84, 1736–1740.
30. Worland, P. J., Kaur, G., Stetler-Stevenson, M., Sebers, S., Sartor, O. & Sausville, E. A. (1993) *Biochem. Pharmacol.* 46, 1831–1840.
31. Losiewicz, M. D., Carlson, B. A., Kaur, G., Sausville, E. A. & Worland, P. J. (1994) *Biochem. Biophys. Res. Commun.* 201, 589–595.
32. Bojanowski, K., Nishio, K., Fukuda, M., Larsen, A. K. & Saijo, N. (1994) *Biochem. Biophys. Res. Commun.* 203, 1574–1580.
33. Meijer, L. (1995) in *Progress in cell cycle research* (Meijer, L., Guidet, S. & Tung, H. Y. L., eds) vol. 1, pp. 351–363, Plenum Press, New York.
34. Meijer, L. (1996) *Trends Cell Biol.* 6, 393–397.
35. Ongkeko, W., Ferguson, D. J. P., Harris, A. L. & Norbury, C. (1995) *J. Cell Sci.* 108, 2897–2904.
36. Meijer, L., Borgne, A., Mulner, O., Chong, J. P. J., Blow, J. J., Delcros, J. G. & Moulinoux, J.-P. (1997) *Eur. J. Biochem.* 243, 527–536.
37. Meijer, L., Pondaven, P., Guerrier, P. & Moreau, M. (1986) *Cah. Biol. Mar.* 25, 457–480.
38. Baratte, B., Meijer, L., Galaktionov, K. & Beach, D. (1992) *Anticancer Res.* 12, 873–880.
39. Azzi, L., Meijer, L., Reed, S. I., Pidikiti, R. & Tung, H. Y. L. (1992) *Eur. J. Biochem.* 203, 353–360.
40. Azzi, L., Meijer, L., Ostvold, A. C., Lew, J. & Wang, J. H. (1994) *J. Biol. Chem.* 269, 13279–13288.
41. Richardson, H. E., Stueland, C. S., Thomas, J. & Reed, S. I. (1990) *Genes & Dev.* 4, 1332–1344.
42. Meijer, L., Azzi, L. & Wang, J. Y. J. (1991) *EMBO J.* 10, 1545–1554.
43. Rosenblatt, J., De Bondt, H., Jancarick, J., Morgan, D. O. & Kim, S.-H. (1993) *J. Mol. Biol.* 230, 1317–1319.
44. Jancarick, J. & Kim, S.-H. (1991) *J. Appl. Crystallogr.* 24, 409–411.
45. Brünger, A. T. (1991) *X-PLOR, a system for crystallography and NMR, version 3.1*, Yale University Press, New Haven CT.
46. Brünger, A. T. & Krukowski, A. (1990) *Acta Crystallogr. A* 46, 585–593.
47. Jones, T. A., Zou, J. Y., Cowan, S. W. & Kjeldgaard, M. (1991) *Acta Crystallogr. A* 47, 110–119.
48. Collaborative Computational Project, no. 4 (1994) *Acta Crystallogr. D* 50, 760–763.
49. Sheriff, S., Hendrickson, W. A. & Smith, J. L. (1987) *J. Mol. Biol.* 197, 273–296.
50. McDonald, I. K. & Thornton, J. M. (1994) *J. Mol. Biol.* 238, 777–793.
51. Connolly, M. L. (1983) *J. Appl. Crystallogr.* 16, 548–558.
52. Azevedo, W. F. Jr, Mueller-Dieckmann, H. J., Schulze-Gahmen, U., Worland, P. J., Sausville, E. & Kim, S.-H. (1996) *Proc. Natl. Acad. Sci. USA* 93, 2735–2740.
53. Janin, J. & Chothia, C. (1990) *J. Biol. Chem.* 265, 16027–16030.
54. Wilson, I. A. & Stanfield, R. L. (1993) *Curr. Opin. Struct. Biol.* 3, 113–118.