

## Inhibition of cyclin-dependent kinases by purine analogues

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While testing purines related to the non-specific protein kinase inhibitors *N*<sup>6</sup>-dimethylaminopurine and *N*<sup>6</sup>-( $\Delta^2$ -isopentenyl)adenine as potential inhibitors of the p34<sup>cdc2</sup>/cyclin B kinase, we discovered a compound with high specificity, 2-(2-hydroxyethylamino)-6-benzylamino-9-methylpurine (olomoucine). Kinetic analysis of kinase inhibition reveals that olomoucine behaves as a competitive inhibitor for ATP and as a non-competitive inhibitor for histone H1 (linear inhibition for both substrates). The kinase specificity of this inhibition was investigated for 35 highly purified kinases (including p34<sup>cdc4</sup>/cyclin D1, p40<sup>cdk6</sup>/cyclin D3, cAMP-dependent and cGMP-dependent kinases, eight protein kinase C isoforms, calmodulin-dependent kinase II, myosin light-chain kinase, mitogen-activated S6 kinase, casein kinase 2, double-stranded RNA-activated protein kinase, AMP-stimulated kinase, eight tyrosine kinases). Most kinases are not significantly inhibited. Only the cell-cycle regulating p34<sup>cdc2</sup>/cyclin B, p33<sup>cdk2</sup>/cyclin A and p33<sup>cdk2</sup>/cyclin E kinases, the brain p33<sup>cdk5</sup>/p35 kinase and the ERK1/MAP-kinase (and its starfish homologue p44<sup>mpk</sup>) are substantially inhibited by olomoucine (IC<sub>50</sub> values are 7, 7, 7, 3 and 25  $\mu$ M, respectively). The cdk4/cyclin D1 and cdk6/cyclin D3 kinases are not significantly sensitive to olomoucine (IC<sub>50</sub> values greater than 1 mM and 150  $\mu$ M, respectively). *N*<sup>6</sup>-( $\Delta^2$ -Isopentenyl)adenine is confirmed as a general kinase inhibitor with IC<sub>50</sub> values of 50–100  $\mu$ M for many kinases. The purine specificity of cyclin-dependent kinase inhibition was investigated: among 81 purine derivatives tested, only C2, N6 and N9-substituted purines exert a strong inhibitory effect on the p34<sup>cdc2</sup>/cyclin B kinase. An essentially similar sensitivity to this olomoucine family of compounds was observed for the brain-specific cdk5/p35 kinase. Structure/activity relationship studies allow speculation on the interactions of olomoucine and its analogues with the kinase catalytic subunit. Olomoucine inhibits *in vitro* M-phase-promoting factor activity in metaphase-arrested *Xenopus* egg extracts, inhibits *in vitro* DNA synthesis in *Xenopus* interphase egg extracts and inhibits the licensing factor, an essential replication factor ensuring that DNA is replicated only once in each cell cycle. Olomoucine inhibits the starfish oocyte G2/M transition *in vivo*. Through its unique selectivity olomoucine provides an anti-mitotic reagent that may preferentially inhibit certain steps of the cell cycle.

Phosphorylation of serine, threonine and tyrosine residues by protein kinases represents one of the most common post-translational regulatory modifications of proteins. More than 200 protein kinases have been described, following either purification to homogeneity or molecular cloning [1–4]. As much as 2–3% of eukaryotic genes may encode protein kinases. The importance of protein kinases in physiological processes has stimulated an active search for specific in-

hibitors with potential pharmacological interest [5]. Several classes of compounds have been identified, such as staurosporine, naphthalene sulfonamides (W7, ML-9, SC-9), isoquinoline derivatives (H-7, H-8, KN-62), sphingosine, tyrostatins, and others, but in most cases these inhibitors display broad specificity. Only some pseudosubstrate autoinhibitory peptides show a high degree of specificity.

Cyclin-dependent kinases (cdk) have recently raised considerable interest in view of their essential role in the regulation of the cell division cycle (cdc) [6, 7]. cdk consist of a catalytic subunit, the prototype of which is cdc2, and a regulatory subunit (cyclin). Six human cdk proteins have been described so far [8–10], namely cdk1 (also known as cdc2) and cdk2–6. With the exception of cdk3, for which the regulatory cyclin has not yet been identified [10], all these cdk

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Abbreviations. cdc, cell division cycle; cdk, cyclin-dependent kinase; HMG, high mobility group; MAP kinase, mitogen-activated protein kinase; olomoucine, 2-(2-hydroxyethylamino)-6-benzylamino-9-methylpurine.

proteins are regulated by the transient association with one member of the cyclin family, i.e. cyclin A (*cdc2*, *cdk2*), B1-B3 (*cdc2*), D1-D3 (*cdk2*, *cdk4*, *cdk5*, *cdk6*), E (*cdk2*). Each step of the cell cycle is thought to be regulated by such cdk complexes: G1/S transition (*cdk2/cyclin E*, *cdk3/unknown cyclin*, *cdk4/cyclin D1-D3*, *cdk6/cyclin D3*), S phase (*cdk2/cyclin A*), G2 (*cdc2/cyclin A*), G2/M transition (*cdc2/cyclins B*). Other *cdc2*-related kinases have been sequenced which await identification of their regulatory partners and their cell-cycle regulatory functions [8].

An increasing number of studies are providing data that support the importance of cdk protein deregulation in human tumor development [11]. These data include overexpressions of cyclin D and cyclin E in a large variety of tumors [12–16], overexpression of *cdc2* [17], oncogenicity of the D cyclins [11, 18], oncogenicity of cyclin A in a human hepatic cancer [19], and abnormal temporal expression of cyclins in human tumor cell lines [20]. Very recently, natural cyclin-dependent kinase inhibitors have been directly implicated in the causes of cancer. One transcriptional regulation target of p53 has been identified as *CIP1*, a gene encoding a cdk inhibitor [21]; *INK4*, a *cdk4* inhibitor gene, is deleted at high frequency in human tumors cell lines [22]. These cdk inhibitors have been cloned and sequenced [23] and include p16<sup>INK4</sup> (an inhibitor of *cdk4/cyclin D1-D3*) [24], p21<sup>CIP1</sup> (or SD11, WAF1) (a general inhibitor of cdk proteins) [21, 25–28], and p27<sup>KIP1</sup> (an inhibitor of *cdk2/cyclin E*) [29].

The frequent deregulation of cdk proteins in cancer and the recent discovery of natural inhibitors have stimulated an active search for chemical inhibitors of cdk proteins. Such cdk inhibitors could potentially act by various mechanisms, i.e. by interfering with the binding of substrates (ATP or protein), by affecting the binding of regulatory subunits (cyclins or p9<sup>CKShs</sup>), by interacting with some sites involved in activation (Thr161 in *cdc2*), by interacting with the nuclear/cytoplasmic localization signals or by mimicking the natural inhibitor/ckd interactions. Four types of cdk inhibitors have been described so far. First, using affinity-purified p34<sup>cdc2</sup>/cyclin B kinase as a screening target for new anti-mitotic compounds of potential anti-tumor interest [30], we have identified *N*<sup>6</sup>-(4<sup>2</sup>-isopentenyl)adenine (7) as a kinase inhibitor (underlined numbers refer to compounds in Table 2). This compound is related to the *N*<sup>6</sup> substituted adenine 6-dimethylaminopurine (2), first described as an inhibitor of cell division in embryos [31] and later identified as a p34<sup>cdc2</sup>/cyclin B kinase inhibitor [32, 33]. Despite its lack of specificity, 6-dimethylaminopurine has been largely used in cell cycle studies as a non-specific protein kinase inhibitor. Secondly, the non-specific kinase inhibitor, staurosporine is a powerful p34<sup>cdc2</sup>/cyclin B inhibitor [30, 34]. Thirdly, butyrolactone-I ( $\alpha$ -oxo- $\beta$ -(*p*-hydroxy-*m*-3,3-dimethylallylbenzyl)- $\gamma$ -methoxycarbonyl- $\gamma$ -butyrolactone) has been recently described as a selective inhibitor of *cdc2* and *cdk2* [35, 36]. Finally, the flavone L86-8275 ([-] *cis*-5,7-dihydroxy-2-(2-chlorophenyl)-8[4-(3-hydroxy-1-methyl)-piperidinyl]-4*H*-benzopyran-4-one] has also been described [37]. The first three compounds appear to act as competitive inhibitors for ATP binding.

The effect of purine derivatives on cdk proteins has been investigated in this study. Olomoucine[2-(2-hydroxyethylamino)-6-benzylamino-9-methylpurine] (51), and other C2, N6, and N9-substituted purines, were found to exert strong inhibitory effects on the *cdc2*, *cdk2*, *cdk5* and ERK1 kinases, but not on *cdk4* and *cdk6* (among 35 kinases tested). Analysis of structure/activity relationships for more than 80 purine derivatives allows speculation on the molecular interactions

between these inhibitors and cdk proteins. Olomoucine inhibits both *in vitro* M-phase-promoting factor activity and *in vitro* DNA synthesis in *Xenopus* egg extracts. Olomoucine inhibits the starfish oocyte G2/M transition *in vivo*. By its unique selectivity, olomoucine provides an anti-mitotic reagent which may preferentially inhibit certain steps of the cell cycle.

## MATERIAL AND METHODS

### Chemicals

Olomoucine (51), can be obtained from the ACDC Research Laboratory (Apoptosis/Cell Division Cycle Research Laboratory). Adenine hydrochloride (1), adenosine (29), adenosine-3',5'-cyclic monophosphate (37), 2-aminopurine (72), *N*<sup>6</sup>-benzyl-9-(2-tetrahydropyryl)adenine (28), 6-*n*-butoxypurine (69), caffeine (80), 2,6-diaminopurine (17), *N*<sup>6</sup>,*N*<sup>6</sup>-dimethyladenine (2), guanine (76), guanosine (77), guanosine 5'-monophosphate (78), guanosine 3',5'-cyclic monophosphate (79), *N*<sup>6</sup>-(2-isopentenyl)adenine (7), *N*<sup>6</sup>-(2-isopentenyl)adenosine (30), sodium ortho-vanadate, 1-methyladenine, EGTA, EDTA, Mops, *sn*-glycerol 2-phosphate, dithiothreitol, sodium fluoride, nitrophenyl phosphate, leupeptin, aprotinin, soybean trypsin inhibitor, benzamidine, histone H1 (type III-S), myelin basic protein, casein, polylysine, isopropyl  $\beta$ -D-thiogalactopyranoside, CNBr-activated Sepharose 4B, Luria-Bertani broth base, angiotensin II, and [Val<sup>3</sup>]angiotensin II were obtained from Sigma. 2-Methylamino-9-methyl-6(1*H*)-purinone (59), 6-dimethylamino-9-ethylpurine (24) were obtained from Aldrich. 6-Benzyladenosine 5'-monophosphate (41), 6-benzylamino-7- $\beta$ -D-glucopyranosylpurine (65), 6-benzylamino-9- $\beta$ -D-glucopyranosylpurine (44), 2-benzylthio-6-(2-isopentenyl)adenine (23), 2-chloro-6-benzylamino-9-methylpurine (47), dihydrozeatin (11), dihydrozeatin 9-ribose (32), dihydrozeatin 9-ribose 5'-monophosphate (40), 2-(2-hydroxyethylamino)-6-benzylamino-7-methylpurine (68), 2-(2-hydroxyethylamino)-6-benzylamino-9-methylpurine (51), 2-(2-hydroxyethylamino)-6-isopentenylamino-9-methylpurine (54), 6-isopentenyladenine 9- $\beta$ -D-glucopyranoside (42), 6-(2-isopentenyl)adenosine 5'-monophosphate (38), 2-methylthio-*N*<sup>6</sup>-(2-isopentenyl)adenine (22), 6-(*o*- $\beta$ -D-glucopyranosyl)-zeatin (15), 6-benzylamino-3- $\beta$ -D-glucopyranosylpurine (70), (*trans*)-zeatin (10), zeatin 9-ribose (31), zeatin-7- $\beta$ -D-glucopyranoside (64), zeatin-9- $\beta$ -D-glucopyranoside (43), zeatin-9-ribose 5'-monophosphate (39) were obtained from Apex Organics Ltd. (Honiton, U. K.). Nonidet P-40 was obtained from Fluka. 6-(3-Hydroxybenzylamino)purine (13), *cis*-zeatin (12), 6-(2-hydroxybenzylamino)purine (14), 6-(3-hydroxybenzyl)adenosine (34), 6-(2-hydroxybenzyl)adenosine (35), 6-benzylaminopurine (8), kinetin (9), 6-benzyladenosine (33), kinetin 9-ribose (36), 7-methylpurine (73), and 6-trimethylaminopurine chloride (3) were synthesized as described previously [38–40]. 2-Chloro-6-(5-hydroxypentylamino)-9-methylpurine (49) and 2-chloro-6-(5-hydroxypentylamino)-7-methylpurine (67), were synthesized as described in [41]. 2-Chloro-6-(3-hydroxybenzylamino)-9-methylpurine (48), 2-dimethylaminoethylamino-6-benzylamino-9-methylpurine (60), 2-(2-hydroxyethylamino)-6-benzylamino-9-(2-hydroxyethyl)purine (52), 2-(2-hydroxyethylamino)-6-(3-hydroxybenzyl)amino-9-methylpurine (56), 2-(2-hydroxyethylamino)-6-benzylamino-9-isopropylpurine (53), 2-(2-hydroxyethylamino)-6-(3-hydroxybenzyl)amino-9-isopropylpurine (57), 6-(*o*- $\beta$ -D-glucopyranosyl)-dihydrozeatin (16), 6-(5-hydroxypentylamino)-9-

methylpurine (25), 6-benzylamino-9-(3-hydroxypropyl)purine (27), 2-amino-6-benzylaminopurine (18), 2-(2-hydroxyethylamino)-6-aminopurine (19), 2-dimethylaminoethylamino-6-benzylaminopurine (21), 6-benzylamino-9-methylpurine (26), 2-amino-6-benzylamino-9-methylpurine (45), 2-chloro-6-amino-9-methylpurine (46), 2-(2-hydroxyethylamino)-6-amino-9-methylpurine (50), 2-(2-hydroxyethylamino)6-isopentenylamino-9-isopropylpurine (55), 6-benzylamino-7-methylpurine (66) and 9-methylpurine (74) were synthesized in our laboratory (Havlicek, L., unpublished results). 6-Allylaminopurine (4), 6-(*N*-allylmethylamino)purine (5), 6-diallylaminopurine (6), 2-(2-hydroxyethylamino)-6-benzylaminopurine (20), 2-(2-hydroxyisobutylamino)-6-benzylamino-9-methylpurine (58) were also synthesized (Letham, D. S., unpublished results). 1-Methyl-6-amino-8-bromopurine (75) was synthesized as described earlier [42]. Purine analogues were usually dissolved as 100 mM stock solutions in dimethylsulfoxide. The final dimethylsulfoxide concentration in the reaction mixture was less than 1% (by vol.). Protein-A-Sepharose and [ $\gamma$ - $^{32}$ P]ATP were obtained from Amersham.

### Buffers

The following buffers were used: 60 mM *sn*-glycerol 2-phosphate, 15 mM *p*-nitrophenyl phosphate, 25 mM Mops, pH 7.2, 15 mM EGTA, 15 mM MgCl<sub>2</sub>, 1 mM dithiothreitol, 1 mM sodium vanadate, 1 mM NaF, 1 mM phenylphosphate, 10  $\mu$ g/ml leupeptin, 10  $\mu$ g/ml aprotinin, 10  $\mu$ g/ml soybean trypsin inhibitor and 100  $\mu$ M benzamidin (buffer A); 50 mM Tris, pH 7.4, 5 mM NaF, 250 mM NaCl, 5 mM EDTA, 5 mM EGTA, 0.1% Nonidet P-40, 10  $\mu$ g/ml leupeptin, 10  $\mu$ g/ml aprotinin, 10  $\mu$ g/ml soybean trypsin inhibitor and 100  $\mu$ M benzamidin (buffer B); buffer A with 5 mM EGTA, no NaF and no protease inhibitors (buffer C); 200 mM NaHCO<sub>3</sub>, pH 8.2, 200 mM NaCl (buffer D).

### Preparation of starfish M-phase oocytes

For large scale oocyte extract preparations, gonads were removed from ripe *Marthasterias glacialis*, and were incubated with 10  $\mu$ M 1-methyladenine in Millipore-filtered natural sea water for 30 min. By this time all the oocytes had entered the M phase. The empty gonads were removed, oocytes were centrifuged at 5000 g for 2 min and directly frozen in liquid nitrogen and kept at  $-80^{\circ}\text{C}$  [43]. M-phase oocytes were homogenized in buffer A at a ratio of 1 g/2 ml gonads. After a 45-min centrifugation at 100000 g, the supernatant was recovered and directly used for affinity-chromatography of the p34<sup>cdc2</sup>/cyclin B kinase.

### Preparation and use of p9<sup>CKShs1</sup>-Sepharose beads

p9<sup>CKShs1</sup> was purified from an overproducing strain of *Escherichia coli* [44, 45]. The bacterial extract was diluted to 1 mg/ml protein with distilled water prior to mixing for 30 min at 4°C with a slurry of S Sepharose beads; these beads were packed into a column and washed with 20 mM sodium bicarbonate; p9<sup>CKShs1</sup> was eluted with buffer D and further purified on a 100 cm  $\times$  2.6 cm Sephacryl S-200 column. P9<sup>CKShs1</sup> was conjugated to CNBr-activated Sepharose 4B according to the manufacturer's instructions. Unreacted groups on the resin were quenched with 1 M ethanolamine (pH 8.0). The concentration of coupled proteins/ml gel was 3.9 mg p9<sup>CKShs1</sup>, i.e. 0.5  $\mu$ mol/ml gel. p9<sup>CKShs1</sup>-Sepharose

beads were kept at 4°C as a 20% (by vol.) suspension in buffer B. The oocytes extract supernatant was added to the beads (400  $\mu$ l/10  $\mu$ l p9<sup>CKShs1</sup>-Sepharose beads) and the tube was kept under constant rotation at 4°C for 30 min. After a centrifugation at 10000 g for 10-s and removal of the supernatant, the beads were washed three times with buffer B and active p34<sup>cdc2</sup>/cyclin B was eluted with free p9<sup>CKShs1</sup>.

### Enzymes

Kinases activities were assayed at 30°C in triplicate, in buffer C (unless otherwise specified). Control values were subtracted from the data and activities calculated as pmol phosphate incorporated into protein acceptor for a 10-min incubation. Controls were performed with appropriate dilutions of dimethylsulfoxide. In some cases phosphorylation of the substrate was assessed by autoradiography after SDS/PAGE.

p34<sup>cdc2</sup>/cyclin B was purified from M-phase starfish (*M. glacialis*) oocytes by affinity chromatography on p9<sup>CKShs1</sup>-Sepharose beads, from which it was eluted by free p9<sup>CKShs1</sup> [43-46]. The enzyme was assayed as described [44] with 1 mg/ml histone H1 (Sigma type III-S), in the presence of 15  $\mu$ M [ $\gamma$ - $^{32}$ P]ATP (3000 Ci/mmol; 1 Ci/l) in a final volume of 30  $\mu$ l. After a 10-min incubation at 30°C, 25- $\mu$ l aliquots of supernatant were spotted onto 2.5 cm  $\times$  3 cm pieces of Whatman P81 phosphocellulose paper, and, after 20 s, the filters were washed five times (for at least 5 min each time) in 10 ml phosphoric acid/1 water. The wet filters were transferred into 6 ml plastic scintillation vials, 5 ml ACS (Amersham) scintillation fluid was added prior to measuring the radioactivity in a Packard counter. The kinase activity was expressed in pmol phosphate incorporated into histone H1/10-min incubation or as a fraction of the maximal activity (%).

To run initial-rate kinetic experiments, the end-point assay system for p34<sup>cdc2</sup> protein kinase was used as described above except that appropriate non-saturating substrate concentrations were applied on the basis of preliminary trials. p34<sup>cdc2</sup>/cyclin B protein kinase was added such that activity was linear with respect to enzyme concentration and time. In most cases, this required 3-10-fold dilution of enzyme with buffer C. Velocity data were expressed in pmol incorporated into the substrate /s for the amount of enzyme added. Apparent inhibition constants were determined by graphical analysis.

p33<sup>cdk2</sup>/cyclin A, p33<sup>cdk2</sup>/cyclin E and p33<sup>cdc2</sup>/cyclin A were reconstituted from extracts of Sf9 insect cells infected with various baculoviruses (provided by D. Morgan) [47]. A C-terminal epitope label from hemagglutinin was present on the human cdk2 and cdc2. Human cyclins A and E were not labeled. Cdk proteins-containing lysates (5 mg/ml protein) were mixed with equal amounts of cyclin A/E lysates and incubated for 45 min at 20°C. After activation, the complexes were immunoprecipitated with 12CA5 antibody (1  $\mu$ g/assay) and protein-A-Sepharose and assayed for histone H1 kinase activity with 50  $\mu$ g histone H1, in the presence of 15  $\mu$ M [ $\gamma$ - $^{32}$ P]ATP, for 5 min (p33<sup>cdc2</sup>/cyclin A) or 20 min (p33<sup>cdk2</sup>/cyclin A and p33<sup>cdk2</sup>/cyclin E) at 37°C. The phosphorylated histone H1 was resolved by 12.5% polyacrylamide SDS/PAGE and analysed by autoradiography.

p33<sup>cdk5</sup>/p35 (provided by J. Lew and J. H. Wang) was purified from bovine brain [48], excluding the Mono S chromatographic step. The active fractions from the Superose 12 column were pooled and concentrated to a final concentra-

tion of approximately 25 µg enzyme/ml. The kinase was assayed with 1 mg/ml histone H1 (Sigma, type III-S), in the presence of 15 µM [ $\gamma$ - $^{32}$ P]ATP, in a final volume of 30 µl, as described for the p34<sup>cdc2</sup>/cyclin B kinase.

p33<sup>cdk4</sup>/cyclin D1, obtained from insect cell lysates [49], was assayed with purified glutathione-S-transferase-retinoblastoma protein in the presence of 15 µM [ $\gamma$ - $^{32}$ P]ATP, in a final volume of 30 µl. After 10-min incubation at 30°C, 30 µl Laemmli sample buffer were added. The phosphorylated substrate was resolved by 10% polyacrylamide SDS/PAGE and analysed by autoradiography with Hyperfilm  $\beta$ Max and densitometry. p34<sup>cdk4</sup>/cyclin D was also purified from human Hep3B cells by affinity chromatography on p15<sup>cdk-BP</sup>-Sephrose beads [50]. Both cyclins D1 and D2 were detected in this preparation (data not shown). Kinase activity was assayed with the high mobility proteins HMG-I and P1 (33 µg/ml) in the presence of 15 µM [ $\gamma$ - $^{32}$ P]ATP. Phosphorylated substrates were resolved by 12.5% polyacrylamide SDS/PAGE and analysed by autoradiography and densitometry.

p33<sup>cdk6</sup>/cyclin D (provided by M. Meyerson) was obtained from insect cell lysates [9]. It was assayed with purified glutathione-S-transferase-retinoblastoma protein in the presence of 15 µM [ $\gamma$ - $^{32}$ P]ATP, in a final volume of 30 µl. After 15 min incubation at 30°C, 30 µl sample buffer were added. The phosphorylated substrate was resolved by 10% polyacrylamide SDS/PAGE and analysed by autoradiography and densitometry.

The mitogen-activated protein kinases (MAP kinases) starfish p44<sup>mapk</sup> [51] and GST-erk1 [52] (cloned from a human HepG2 library, provided by D. Charest and S. L. Pelech) were assayed with 1 mg/ml myelin basic protein in the presence of 15 µM [ $\gamma$ - $^{32}$ P]ATP as described above for the p34<sup>cdc2</sup>/cyclin B kinase.

Protein kinase C isoforms [53] were purified from baculovirus-infected Sf9 insect cells and were kindly assayed by T. Meyer and J. Loretan with protamine sulfate in the presence of 10 µM [ $\gamma$ - $^{32}$ P]ATP.

The catalytic subunit of cAMP-dependent protein kinase [54] (provided by S. Lohmann), purified from bovine heart, was assayed with 1 mg/ml histone H1, in the presence of 15 µM [ $\gamma$ - $^{32}$ P]ATP as described above for the p34<sup>cdc2</sup>/cyclin B kinase.

cGMP-dependent protein kinase [55] (provided by F. Hofmann), purified to homogeneity from bovine tracheal smooth muscle, was assayed with 1 mg/ml histone H1, in the presence of 15 µM [ $\gamma$ - $^{32}$ P]ATP as described above for the p34<sup>cdc2</sup>/cyclin B kinase.

Casein kinase 2 was isolated from rat liver cytosol [56] and assayed with 1 mg/ml casein and 15 µM [ $\gamma$ - $^{32}$ P]ATP. The substrate was spotted on Whatmann 3MM filters and washed with 10% (mass/vol.) trichloroacetic acid.

Calmodulin-dependent protein kinase II (rat brain  $\alpha$  subunit; provided by K. P. Lu and A. Means) was expressed in *E. coli*, purified as described [57], and assayed, in the presence of calcium and bovine brain calmodulin (provided by R. Wallace), with 1 mg/ml casein and 15 µM [ $\gamma$ - $^{32}$ P]ATP. The substrate was spotted on Whatmann 3MM filters and washed with 10% (mass/vol.) trichloroacetic acid.

Myosin light-chain kinase (chicken gizzard) [58] (provided by T. J. Lukas and M. Watterson) was assayed in the presence of 100 nM calmodulin, 100 µM CaCl<sub>2</sub>, 50 mM Hepes, 5 mM MgCl<sub>2</sub>, 1 mM dithiothreitol and 0.1 mg/ml BSA at pH 7.5 using a synthetic peptide based on the smooth muscle myosin light-chain phosphorylation site (KKRPQR-ATSNVFAM, 50 µM) and in the presence of 15 µM [ $\gamma$ -

$^{32}$ P]ATP, in a final volume of 50 µl. Incorporation of radioactive phosphate was monitored on phosphocellulose filters as described above.

Rat mitogen-activated S6 kinase (p70<sup>s6k</sup>) (provided by M. Siegmund and G. Thomas) was purified from baculovirus-infected Sf9 insect cells and assayed for autophosphorylation and phosphorylation towards rat liver 40S ribosomal subunits [59], in the presence of 15 µM [ $\gamma$ - $^{32}$ P]ATP and protein kinase inhibitor. S6 phosphorylation was analysed by autoradiography after 12.5% polyacrylamide SDS/PAGE.

ASK- $\gamma$  (Arabidopsis shaggy-related protein kinase) was expressed as a glutathione S-transferase fusion protein in *E. coli* [60] (provided by M. W. Bianchi and M. Kreis), purified on glutathione-Sephrose and assayed, for 10 min at 30°C, with 5 µg myelin basic protein, in the presence of 15 µM [ $\gamma$ - $^{32}$ P]ATP, in a final volume of 30 µl. The phosphorylated myelin basic protein was recovered on Whatman P81 phosphocellulose paper as described for the p34<sup>cdc2</sup>/cyclin B kinase.

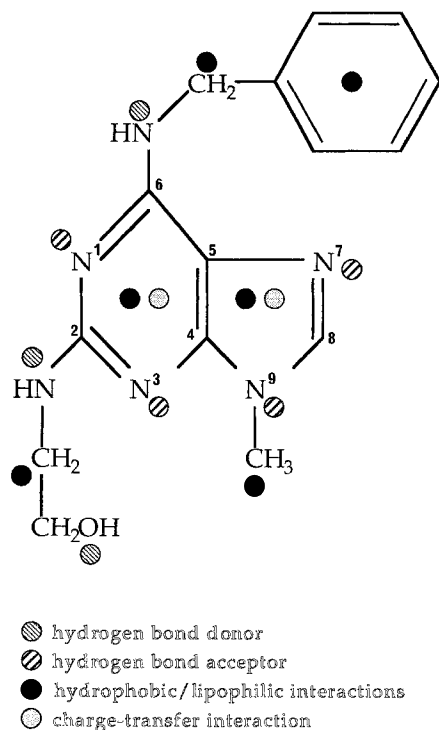
p56<sup>lck</sup>, the lymphocyte cytokine-activated tyrosine kinase (provided by J. Watts and R. Aebersold), was purified from baculovirus-infected Sf9 insect cells [61] and assayed with 1 mg/ml myelin basic protein in the presence of 15 µM [ $\gamma$ - $^{32}$ P]ATP. Incorporation of radioactive phosphate was monitored on phosphocellulose filters as described above.

The src-related tyrosine kinases proto-oncogenes *lyn* (TPK-IIA) [62] *fgr* (TPK-III/Q1) [63] and TPK-IIB [64] were purified to homogeneity from the particulate fraction of rat spleen; the c-src kinase [64], expressed in *E. coli* was a generous gift of Dr S. Fischer and Dr R. Benarous. Enzymes (10 U) were assayed at 30°C, in 50 µl 50 mM Tris/HCl, pH 7.5, 5 mM MnCl<sub>2</sub> (MgCl<sub>2</sub> in the case of *fgr*-TPK), 10 µM sodium vanadate with 2 mM angiotensin II (DRVYIHPF) in the presence of 15 µM [ $\gamma$ - $^{32}$ P]ATP. 1 µM polylysine was present in the *lyn*-TPK-IIA assay. 1 U enzyme is capable of transferring 1 pmol phosphate/min to 2 mM angiotensin II. Reactions were stopped after 10 min by addition of 30% acetic acid (final concentration);  $^{32}$ P incorporation was evaluated by ion-exchange-chromatography and isobutanol/benzene extraction [65].

Insulin receptor tyrosine kinase domain (CIRK-41) [66] was overexpressed in a baculovirus system and purified to homogeneity (provided by H. Y. L. Tung). The kinase activity of this protein was assayed, for 10 min at 30°C, with 5 µg Raytide (Oncogene Sciences), in the presence of 15 µM [ $\gamma$ - $^{32}$ P]ATP, in a final volume of 30 µl. The phosphorylated Raytide was recovered on Whatman P81 phosphocellulose paper as described for the p34<sup>cdc2</sup>/cyclin B kinase.

The intracellular domain of the epidermal growth factor receptor (EGF-R ICD) [67], expressed in Sf9 cells, was purified and assayed in 20 mM Tris/HCl, pH 7.6, 10 mM MgCl<sub>2</sub>, 1 mM dithiothreitol, 10 µM Na<sub>3</sub>VO<sub>4</sub>, 1.5 µM [ $\gamma$ - $^{32}$ P]ATP, (1.3 Ci/mmol), 50–200 ng enzyme protein (activated by preincubation for 10 min at 20°C in the presence of 50 µM ATP before the assay) and 1 mM angiotensin II in a final volume of 50 µl. Reactions were carried out at 20°C, for 10–15 min and were terminated by addition of 25 µl 25% trichloroacetic acid and 25 µl 0.2% bovine serum albumin. Aliquots of the supernatant after centrifugation were spotted onto P81 phosphocellulose papers and processed as described previously [67] (assays were kindly performed by Dr Helmut Mett).

AMP-stimulated kinase was purified from rat liver and assayed as described [68] with the synthetic SAMS-containing peptide with 15 µM ATP and in the presence of 15 µM



**Fig. 1. Olomoucine and its potential interactions with a binding site.**

AMP (assays were kindly performed by Dr S. Dale and Dr D. G. Hardie).

v-abl kinase was expressed in *E. coli* and affinity purified on IgG-Affigel 10 [69]. The activity was assayed using 1 mM [Val<sup>5</sup>]angiotensin II and 10 μM [ $\gamma$ -<sup>32</sup>P]ATP as substrates [69] (assays were kindly performed by Dr Helmut Mett).

Double-stranded RNA-activated protein kinase [70] was purified from interferon-treated human Daudi cells, by affinity chromatography on immobilized monoclonal antibodies directed against the p68 kinase. The protein was assayed with 25 μg calf thymus histone in the presence of 0.2 μM [ $\gamma$ -<sup>32</sup>P]ATP [71] (assays were kindly performed by Dr J. Galabru).

Nucleoside diphosphate kinase from *Dictyostelium discoideum* was expressed in bacteria, purified and assayed as described [72] (assays were kindly performed by Dr A. A. Bominaar and M. Véron).

The glutathione-S-transferase-pyp3 tyrosine phosphatase [73] (provided by J. B. A. Millar and P. Russell) and the glutathione-S-transferase-cdc25A threonine/tyrosine phosphatase (provided by K. Galaktionov and D. Beach) were prepared and assayed as previously described [43].

DNA topoisomerase I (calf thymus) and DNA topoisomerase II (*Saccharomyces cerevisiae*) activities were measured by relaxation of supercoiled plasmid pBR322 DNA. 5 μM suramin was used as a positive control to inhibit relaxation completely [74] (assays were kindly performed by Dr A. K. Larsen).

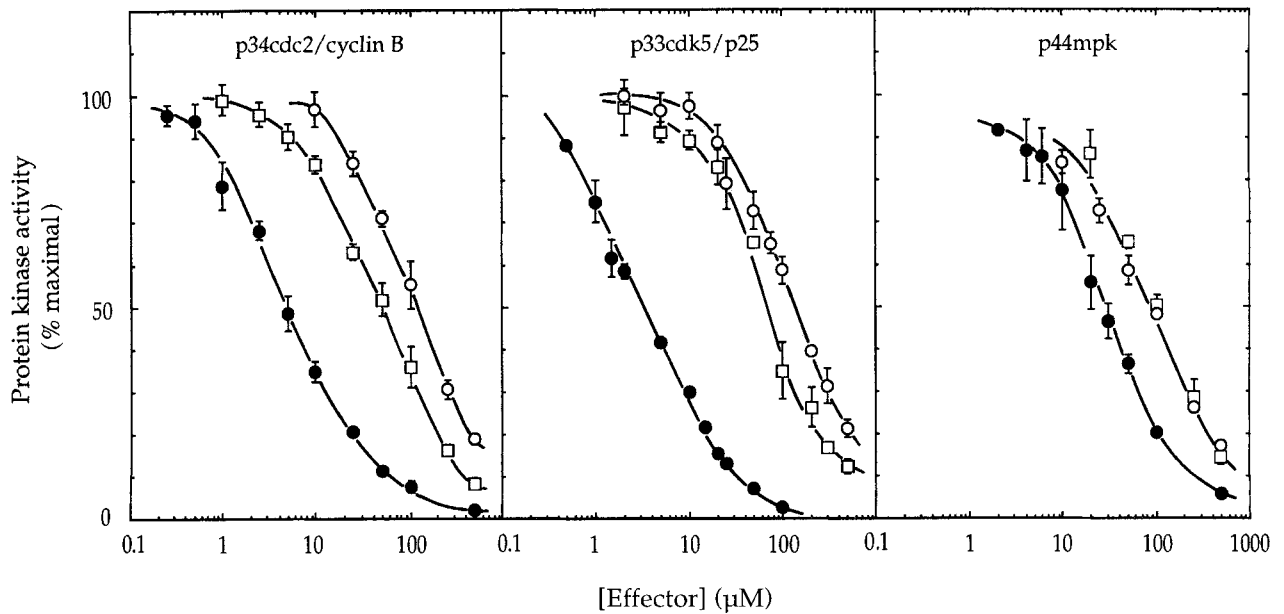
DNA polymerases  $\alpha$  and  $\delta$  (in the presence of proliferating cell nuclear antigen) were purified from calf thymus and assayed by incorporation of [<sup>3</sup>H]dTTP into (dT)<sub>12-18</sub> primer on a poly(dA) matrix as described [75] (assays were kindly performed by Dr G. Baldacci).

**Table 1. IC<sub>50</sub> values for olomoucine and N<sup>6</sup>-( $\Delta^2$ -isopentenyl)adenine added to various purified enzymes.** Enzyme activities were assayed as described in the Material and Methods section, in the presence of increasing concentrations of olomoucine (51) and N<sup>6</sup>-( $\Delta^2$ -isopentenyl)adenine (7). IC<sub>50</sub> values were calculated from the dose/response curves. When no inhibitory effect was observed, the highest concentration tested is given in parentheses; —, not tested.

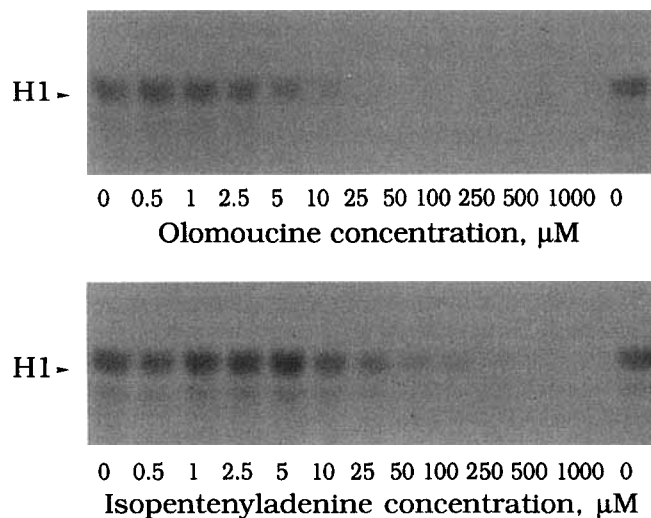
Enzyme	IC <sub>50</sub> for	
	olomoucine	N <sup>6</sup> -( $\Delta^2$ -isopentenyl)adenine
	μM	
p34 <sup>cdc2</sup> /cyclin A	~ 50	—
p34 <sup>cdc2</sup> /cyclin B	7	45
p34 <sup>cdc2</sup> /cyclin E	~ 10	—
p33 <sup>cdk2</sup> /cyclin A	7	50
p33 <sup>cdk2</sup> /cyclin E	7	—
p34 <sup>cdk4</sup> /cyclin D	>1000	200
p33 <sup>cdk5</sup> /35	3	80
p40 <sup>cdk6</sup> /cyclin D3	> 250	> 100
p44 <sup>mpk</sup>	25	95
GST-erk-1	30	90
c-protein kinase C $\alpha$	>1000	43
c-protein kinase C $\beta$ 1	>1000	96
c-protein kinase C $\beta$ 2	>1000	100
c-protein kinase C $\gamma$	800	43
n-protein kinase C $\delta$	>1000	> 100
n-protein kinase C $\epsilon$	>1000	> 100
n-protein kinase C $\eta$	930	78
a-protein kinase C $\zeta$	>1000	> 100
Cyclic AMP-dependent kinase	>2000	50
Cyclic GMP-dependent kinase	>2000	50
Calmodulin-dependent kinase II	(1000)	—
Myosin light-chain kinase	>1000	>1000
Mitogen-activated S6 kinase (p70 <sup>sk</sup> )	(1000)	—
Casein kinase 2	>2000	600
AMP-activated protein kinase (–AMP)	230	18
AMP-activated protein kinase (+AMP)	220	11
ASK- $\gamma$ (plant GSK-3)	130	95
dsRNA-activated protein kinase (PKR)	> 500	>1000
Insulin-receptor tyrosine kinase	400	140
Epidermal-growth-factor-receptor tyrosine kinase	440	—
p56 <sup>lck</sup> (lymphocyte-specific tyrosine kinase)	>2000	90
p56 <sup>lyn</sup> (TPK-IIA)	>1000	>2000
TPKIIB	>1000	>2000
p55 <sup>src</sup> (TPK-III/Q1)	>1000	78
c-src	(1000)	1000
v-abl	( 100)	—
Nucleoside-diphosphate kinase	(1000)	(1000)
Phosphatase GST-cdc25A	(1000)	—
Phosphatase GST-pyp3	(1000)	—
DNA topoisomerase I	( 250)	—
DNA topoisomerase II	( 250)	—
DNA polymerase $\alpha$	( 500)	—
DNA polymerase $\delta$	( 500)	—

### M-phase promoting-factor activity, DNA synthesis and replication licensing factor activity in *Xenopus* egg extracts

Metaphase-arrested *Xenopus* egg extracts were prepared as described previously [76] and stored in liquid nitrogen. Demembrated *Xenopus* sperm nuclei were prepared as de-



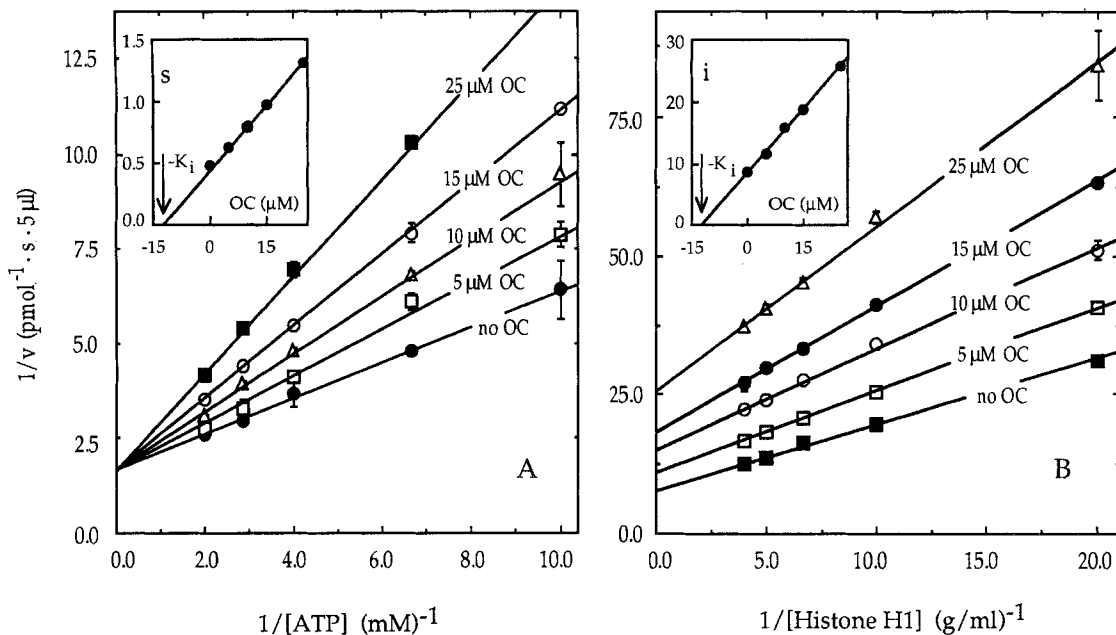
### p33cdk2/cyclin A



**Fig. 2.** 6-Dimethylaminopurine,  $N^6$ -( $\Delta^2$ -isopentenyl)adenine and olomoucine dose response curves for p34<sup>cdc2</sup>/cyclin B, p33<sup>cdk5</sup>/p35, p44<sup>mpk</sup> and p33<sup>cdk2</sup>/cyclin A protein kinases. Enzyme activities were assayed in triplicate as described in the Materials and Methods section in the presence of increasing concentrations of olomoucine (●) (51),  $N^6$ -( $\Delta^2$ -isopentenyl)adenine (□) (7) and 6-dimethylaminopurine (○) (2). Maximal (100%) activities were 19.5, 44.1 and 5.8 pmol/10 min for 5  $\mu$ l enzyme, respectively. In the case of p33<sup>cdk2</sup>/cyclin A kinase, the phosphorylated substrate, histone H1, was resolved by 12.5% polyacrylamide SDS/PAGE and autoradiography.

scribed [77]. After thawing, extracts were supplemented with 25 mM phosphocreatine, 5  $\mu$ g/ml creatine phosphokinase, 250  $\mu$ g/ml cycloheximide, [ $\alpha$ -<sup>32</sup>P]dATP (for DNA synthesis assays). Demembrated sperm nuclei were added to a final sperm concentration of 3 ng/ $\mu$ l DNA extract and olomoucine was then added at different concentrations. M-phase promoting factor inhibition by olomoucine was monitored 1.5 h after addition by assessing the amount of sperm nuclei that had been assembled into interphase nuclei, possessing a complete phase-dense nuclear envelope. DNA synthesis was assessed by releasing extract into interphase by the addition of

0.3 mM CaCl<sub>2</sub> and measuring the total amount of [ $\alpha$ -<sup>32</sup>P]dATP incorporation after 3 h by trichloroacetic acid coprecipitation [77]. Licensing factor activity was assessed by releasing extract into interphase by the addition of 0.3 mM CaCl<sub>2</sub> and isolating chromatin after 15 min as described [76]. The extent of licensing was determined by the ability of chromatin that had been previously incubated with olomoucine to replicate in extracts lacking licensing factor. The extract lacking licensing factor was prepared by a treatment with 3 mM 6-dimethylaminopurine [76]. The extent of DNA synthesis in 6-dimethylaminopurine extracts was



**Fig. 3. Double reciprocal plots of kinetic data from assays of p34<sup>cdc2</sup>/cyclin B protein kinase activity at different concentrations of olomoucine.** Enzyme activities were assayed in triplicates as described in the Materials and Methods section. (A)  $1/v$  versus  $1/ATP$  primary plot. ATP concentrations in the reaction mixture varied from 0.1–0.5 mM; the concentration of histone H1 was kept constant at 0.7 mg/ml. (B)  $1/v$  versus  $1/histone\ H1$  primary plot. Histone H1 concentrations in the reaction mixture varied from 0.05–0.25 mg/ml; the ATP concentration was kept constant at 0.07 mM. The insets show secondary replots of slopes (s) and intercepts (i) from primary plots. Apparent inhibition constants ( $K_i$ ) are indicated (↓). OC (olomoucine).

expressed as a fraction of the amount of DNA synthesis occurring in control extracts with the same chromatin templates.

### Starfish oocyte maturation

Starfish oocytes were prepared, maturation was induced and p34<sup>cdc2</sup> was analysed as described previously [46]. The level of tyrosine phosphorylation of p34<sup>cdc2</sup> was analysed by Western blotting with anti-phosphotyrosine IgG (provided by J. Wang) after SDS/PAGE of the p9<sup>CKShs1</sup>-affinity bound proteins, as described [50, 78].

## RESULTS

### Olomoucine, a purine derivative, inhibits the p34<sup>cdc2</sup>/cyclin B kinase

In an effort to detect potential inhibitors of affinity-purified p34<sup>cdc2</sup>/cyclin B kinase we have tested compounds structurally related to the non-specific kinase inhibitors *N*<sup>6</sup>-( $\Delta^2$ -isopentenyl)adenine (1) and 6-dimethylaminopurine (2). Most compounds had little inhibitory activity ( $IC_{50}$  values greater than 500 μM).

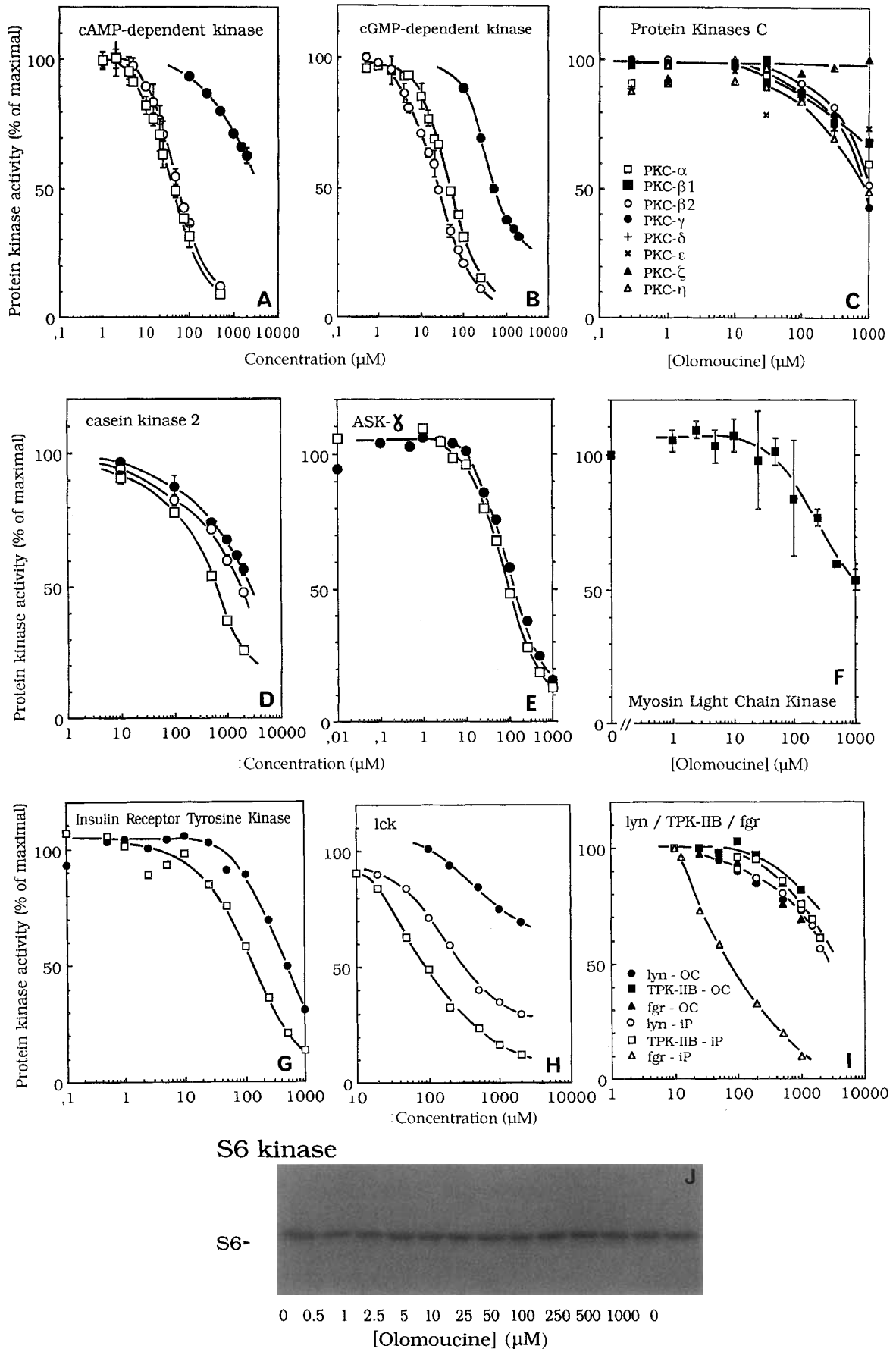
One of the compounds, 2-(2-hydroxyethylamino)-6-benzylamino-9-methylpurine (51) (Fig. 1), renamed olomoucine for convenience, displayed significant inhibitory activity towards the cdc2 kinase ( $IC_{50}$  7 μM; Fig. 2, Table 1). Olomoucine also inhibited autophosphorylation on the cyclin B subunit with a similar  $IC_{50}$  value (data not shown). The  $IC_{50}$  values of *N*<sup>6</sup>-( $\Delta^2$ -isopentenyl)adenine (1) and 6-dimethylaminopurine (2) on cdc2 kinase were, respectively, 55 μM and 120 μM (Fig. 2, Table 1). Olomoucine had first been described as an inhibitor of cytokinin 7-glucosyltransferase from radish cotyledons [41].

### Olomoucine acts as a competitive inhibitor for ATP

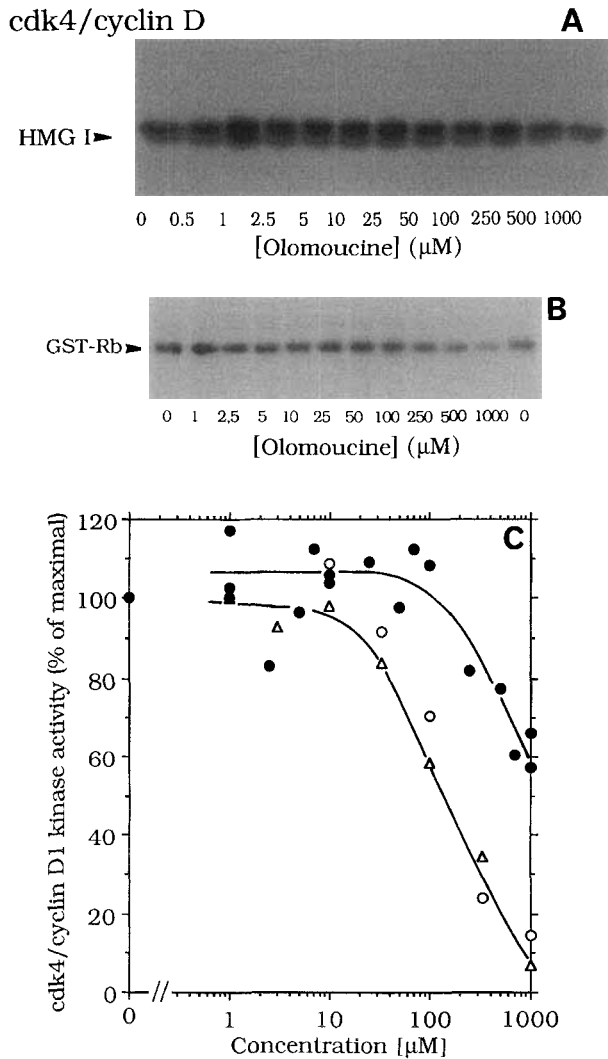
To investigate the mechanism of olomoucine action in detail, kinetic experiments were performed in the presence of increasing olomoucine concentrations, with varying ATP or histone H1 levels (Fig. 3). Appropriate double-reciprocal plotting of the data demonstrates that olomoucine acts as a competitive inhibitor for ATP and as a non-competitive inhibitor for histone H1 (Fig. 3). The linearity of the intercepts or slopes versus olomoucine concentration replots (Fig. 3) classifies olomoucine as a linear inhibitor for both substrates. Analogous results were obtained with *N*<sup>6</sup>-( $\Delta^2$ -isopentenyl)adenine and 6-dimethylaminopurine (data not shown). Apparent inhibition constants ( $K_i$ ), determined graphically, for olomoucine, *N*<sup>6</sup>-( $\Delta^2$ -isopentenyl)adenine and 6-dimethylaminopurine were 14, 78 and 184 μM, respectively.

### Kinase specificity of the olomoucine inhibitory effect

We next investigated the effects of olomoucine and *N*<sup>6</sup>-( $\Delta^2$ -isopentenyl)adenine on a variety of highly purified kinases (Table 1, Fig. 2, Figs 4–6). Kinase activities were assayed with appropriate substrates (histone H1, myelin basic protein, casein, S6 subunit and others), at 10–15 μM ATP and in the presence of increasing concentrations of inhibitors.  $IC_{50}$  values calculated from the dose/response curves are presented in Table 1. As already suggested, *N*<sup>6</sup>-( $\Delta^2$ -isopentenyl)adenine turned out to be a general kinase inhibitor with  $IC_{50}$  values of 50–100 μM for many kinases. In contrast, most kinases which are not cyclin-dependent were poorly sensitive to olomoucine, if at all (Fig. 4, Table 1); however ASK- $\gamma$ , the plant of homologue of glycogen-synthase kinase (GSK-3) [59] was inhibited to some extent by olomoucine ( $IC_{50}$  130 μM; Fig. 4). Olomoucine did not affect the enzymic activity of several cell cycle important enzymes such as nucleo-



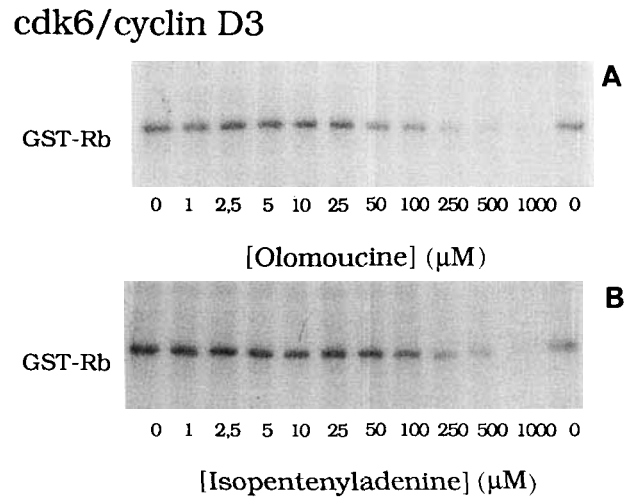
**Fig. 4. Effects of olomoucine on several kinases.** Enzyme activities were assayed as described in the Materials and Methods section in the presence of increasing concentrations of olomoucine (●) (51),  $N^6$ -( $A^2$ -isopentenyl)adenine (□) or 6-dimethylaminopurine (○) (2). (A) cAMP-dependent kinase; (B) cGMP-dependent kinase; (C) protein kinase C isoforms; (D) casein kinase 2; (E) ASK- $\gamma$ , the plant homologue of GSK-3; (F) myosin light-chain kinase; (G) insulin receptor tyrosine kinase domain; (H) p56<sup>lck</sup>; (I) tyrosine kinases lyn, fgr and TPK-IIB (closed symbols, olomoucine; open symbols,  $N^6$ -( $A^2$ -isopentenyl)adenine); (J) S6 kinase.



**Fig. 5. Effects of olomoucine and its analogues on the cdk4/cyclin D kinase.** Enzyme activities were assayed as described in the Materials and Methods section in the presence of increasing concentrations of olomoucine and analogues. (A) p15<sup>cdk-BP</sup>-bound cdk4/cyclin D from Hep3B cells, assayed with high-mobility group protein I (HMG I). The phosphorylated HMG I was resolved by 12.5% polyacrylamide SDS/PAGE and analysed by autoradiography. (B) Recombinant cdk4/cyclin D1 assayed with glutathione-*S*-transferase–retinoblastoma protein (GST-Rb). The phosphorylated GST-Rb was resolved by 10% polyacrylamide SDS/PAGE and analysed by autoradiography. (C) Recombinant cdk4/cyclin D1 assayed with GST-Rb in the presence of increasing concentrations of olomoucine (●) (51), *N*<sup>6</sup>-( $\Delta^2$ -isopentenyl)adenine (○) (7) or 2-(2-hydroxyethylamino)-6-(3-hydroxybenzyl)amino-9-methylpurine (△) (56). The phosphorylated GST-Rb was resolved by 10% polyacrylamide SDS/PAGE, analysed by autoradiography and phosphorylation was quantified by densitometry.

side diphosphate kinase, DNA topoisomerase I and II, DNA polymerases  $\alpha$  and  $\delta$ , tyrosine phosphatases cdc25A and pyp3 (Table 1).

Only five out of 35 protein kinases tested were significantly inhibited by olomoucine, namely p33<sup>cdk2</sup>/cyclin A, p33<sup>cdk2</sup>/cyclin E, p34<sup>cdc2</sup>/cyclin B, p33<sup>cdk5</sup>/p35 and erk1 (and its starfish homologue, p44<sup>mpk</sup>; Fig. 2, Table 1). IC<sub>50</sub> values are respectively 7, 7, 3 and 25  $\mu$ M for these kinases. Interestingly, the cdk4/cyclin D kinase is not sensitive to olomoucine (IC<sub>50</sub> greater than 1000  $\mu$ M; Fig. 5). This was observed



**Fig. 6. Effects of olomoucine and *N*<sup>6</sup>-( $\Delta^2$ -isopentenyl)adenine on the cdk6/cyclin D3 kinase.** Recombinant cdk6/cyclin D3 was assayed with glutathione-*S*-transferase–retinoblastoma protein (GST-Rb) as described in the Materials and Methods section in the presence of increasing concentrations of *N*<sup>6</sup>-( $\Delta^2$ -isopentenyl)adenine (7) (A) and olomoucine (51) (B). The phosphorylated GST-Rb was resolved by 10% polyacrylamide SDS/PAGE and analysed by autoradiography.

with recombinant mammalian cdk4/cyclin D1 (Fig. 5B and C), with p15<sup>cdk-BP</sup>-purified cdk4/cyclin D from Hep3B cells (Fig. 5A) and with the kinase from starfish oocytes which binds to p15<sup>cdk-BP</sup>–Sepharose [50] (data not shown). The recently described p40<sup>cdk6</sup>/cyclin D3 [9] is also not very sensitive to olomoucine and somewhat more sensitive to *N*<sup>6</sup>-( $\Delta^2$ -isopentenyl)adenine (IC<sub>50</sub> greater than 150  $\mu$ M and 100  $\mu$ M, respectively) (Fig. 6).

### Purine specificity of cdk inhibition

To elucidate the structural features required for N6-substituted purines to exert inhibitory effects on different cdk proteins, we next tested more than 80 purine derivatives on p34<sup>cdc2</sup>/cyclin B, p33<sup>cdk5</sup>/p35 and p34<sup>cdk4</sup>/cyclin D1 (Table 2, Figs 5–8).

### Inhibition of p34<sup>cdc2</sup>/cyclin B by purines

Some of the tested purines displayed significant inhibitory activity towards the kinase (Table 2; Fig. 7). The active compounds can essentially be classified in three categories.

#### *N*6-substituted adenines

These compounds include the previously described non-specific kinases inhibitors 6-dimethylaminopurine (2) and *N*<sup>6</sup>-( $\Delta^2$ -isopentenyl)adenine (7). The most active compounds were 6-(*N*-allylmethylamino)purine (5) and 6-diallylamino-purine (6) (IC<sub>50</sub> values of 12  $\mu$ M and 22  $\mu$ M, respectively). The specificities of these compounds towards other kinases were not investigated.

#### *N*6-substituted and *N*9-substituted adenines

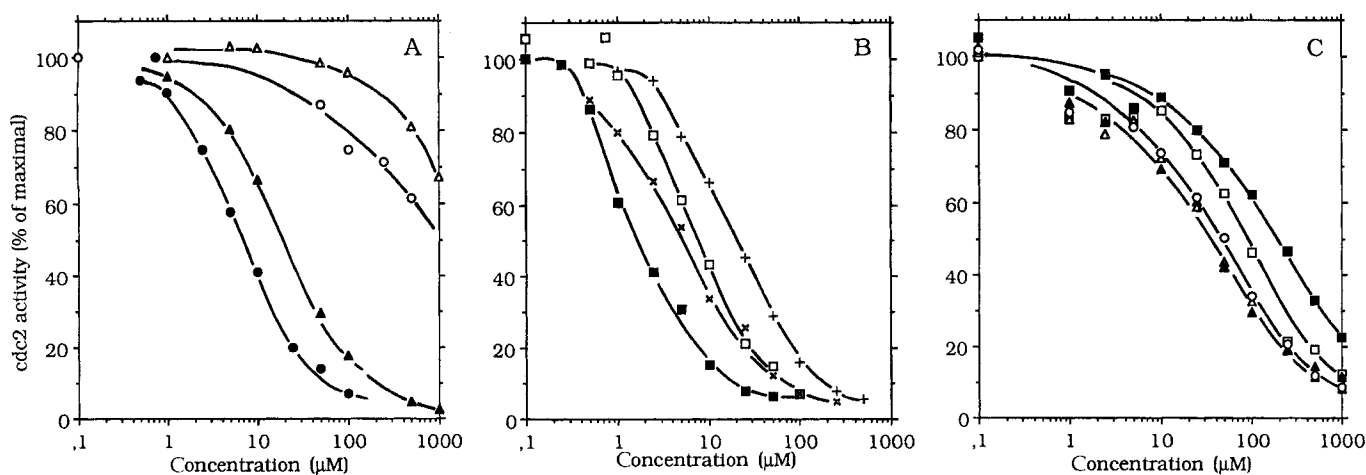
The most active compounds were adenosine (29), 6-(5-hydroxypentylamino)-9-methylpurine (25), 6-benzylamino-9-methylpurine (26) and 6-benzylamino-9-(3-hydroxypro-

**Table 2. IC<sub>50</sub> values for various purines added to purified cdc2, cdk5 and cdk4 kinases.** Enzyme activities were assayed as described in the Material and Methods section, in the presence of increasing concentrations of purines. IC<sub>50</sub> values were calculated from the dose/response curves. —, not tested. \*, precipitation at the highest concentration tested.

No.	Compounds	IC <sub>50</sub> for			
		cdc2	cdk5	cdk4	
		μM			
1	N6 substituted purines	6-aminopurine (adenine)	200	—	—
2		6-dimethylaminopurine ( <i>N</i> <sup>6</sup> , <i>N</i> <sup>6</sup> -dimethyladenine)	120	120	—
3		6-trimethylaminopurine	> 500	—	750
4		6-allylaminopurine	50	100	500
5		6-( <i>N</i> -allylmethylamino)purine	12	42	200
6		6-diallylaminopurine	22	15	200
7		6-( $\gamma,\gamma$ -dimethylallylamino)purine [ <i>N</i> <sup>6</sup> -(2-isopentenyl)adenine]	55	70	200
8		6-benzylaminopurine ( <i>N</i> <sup>6</sup> -benzyladenine)	200	80	—
9		6-furfurylaminopurine (kinetin)	180	—	—
10		6-(4-hydroxy-3-methyl- <i>trans</i> -but-2-enylamino)purine ( <i>trans</i> -zeatin)	70	150	>1000
11		6-(4-hydroxy-3-methyl-butylamino)purine (dihydrozeatin)	80	—	—
12		6-(4-hydroxy-3-methyl- <i>cis</i> -but-2-enylamino)purine ( <i>cis</i> -zeatin)	150	110	—
13		6-(3-hydroxybenzylamino)purine ( <i>m</i> -topolin)	70	—	—
14		6-(2-hydroxybenzylamino)purine ( <i>o</i> -topolin)	200*	—	—
15		6-( <i>o</i> - $\beta$ -D-glucopyranosyl)-zeatin	850	700	—
16		6-( <i>o</i> - $\beta$ -D-glucopyranosyl)-dihydrozeatin	500	600	—
17	C2 and N6 substituted purines	2,6-diaminopurine	> 100*	—	—
18		2-amino-6-benzylaminopurine	90	—	—
19		2-(2-hydroxyethylamino)-6-aminopurine	200	—	—
20		2-(2-hydroxyethylamino)-6-benzylaminopurine	25	50	200
21		2-dimethylaminoethylamino-6-benzylaminopurine	500	—	—
22		2-methylthio-6-( $\gamma,\gamma$ -dimethylallylamino)purine	> 100*	> 100*	—
23	2-benzylthio-6-( $\gamma,\gamma$ -dimethylallylamino)purine	> 500*	> 500*	—	
24	N6, N9 substituted purines	6-dimethylamino-9-ethylpurine	300	>1000	>1000
25		6-(5-hydroxypentylamino)-9-methylpurine	50	—	—
27		6-benzylamino-9-(3-hydroxypropyl)purine	45	—	—
28		6-benzylamino-9-(2-tetrahydropyranyl)purine	200	160	—
29		9- $\beta$ -D-ribofuranosyladenine (adenosine)	55	—	—
30		6-(2-isopentenyl)adenosine	> 500	—	—
31		zeatin-9- $\beta$ -D-ribofuranoside	> 500	>1000	—
32		dihydrozeatin-9- $\beta$ -D-ribofuranoside	> 500	> 500	—
33		6-benzyladenosine	> 500	> 500	—
34		6-(2-hydroxybenzyl)adenosine	> 500	—	—
35		6-(3-hydroxybenzyl)adenosine	> 500	—	—
36		6-furfuryladenosine (kinetin-9- $\beta$ -D-ribofuranoside)	> 500	—	—
37		adenosine 3',5'-cyclic monophosphate (cAMP)	260	—	—
38		6-(2-isopentenyl)adenosine 5'-monophosphate	> 500	—	—
39		zeatin-9- $\beta$ -D-ribofuranoside 5'-monophosphate	> 500	—	—
40		dihydrozeatin-9- $\beta$ -D-ribofuranoside 5'-monophosphate	> 500	—	—
41	6-benzyladenosine 5'-monophosphate	> 500	> 500	—	
42	6-isopentenyladenine-9- $\beta$ -D-glucopyranoside	> 500	—	—	
43	zeatin-9- $\beta$ -D-glucopyranoside	> 500	> 500	—	
44	6-benzylamino-9- $\beta$ -D-glucopyranosylpurine	> 500	>1000	—	
45	C2, N6 and N9 substituted purines	2-amino-6-benzylamino-9-methylpurine	40	—	—
46		2-chloro-6-amino-9-methylpurine	70	—	—
47		2-chloro-6-benzylamino-9-methylpurine	12	30	—
48		2-chloro-6-(3-hydroxybenzylamino)-9-methylpurine	5.2	9.5	—
49		2-chloro-6-(5-hydroxypentylamino)-9-methylpurine	20	22	>1000
50		2-(2-hydroxyethylamino)-6-amino-9-methylpurine	50	—	—
51		2-(2-hydroxyethylamino)-6-benzylamino-9-methylpurine (olomoucine)	7	3	>1000
52		2-(2-hydroxyethylamino)-6-benzylamino-9-(2-hydroxyethyl)purine	8	30	—
53		2-(2-hydroxyethylamino)-6-benzylamino-9-isopropylpurine	2	3	—
54		2-(2-hydroxyethylamino)-6-isopentenylamino-9-methylpurine	65	13	>1000
55		2-(2-hydroxyethylamino)-6-isopentenylamino-9-isopropylpurine	8.5	—	—
56		2-(2-hydroxyethylamino)-6-(3-hydroxybenzyl)amino-9-methylpurine	5	2.6	150
57		2-(2-hydroxyethylamino)-6-(3-hydroxybenzyl)amino-9-isopropylpurine	6	10	150
58		2-(2-hydroxyisobutylamino)-6-benzylamino-9-methylpurine	6	9	700
59		2-methylamino-9-methyl-6(1 <i>H</i> )-purinone	>1000	—	—
60		2-dimethylaminoethylamino-6-benzylamino-9-methylpurine	> 100	140	—

Table 2. (Continued).

No.	Compounds	IC <sub>50</sub> for		
		cdc2	cdk5	cdk4
		μM		
64	N6 and N7 substituted purines	zeatin-7-β-D-glucopyranoside	> 500	—
65		6-benzylamino-7-β-D-glucopyranosylpurine	> 500	> 500
66		6-benzylamino-7-methylpurine	>1000	—
67	C2, N6 and N7 substituted purines	2-chloro-6-(5-hydroxypentylamino)-7-methylpurine	>1000	>1000
68		2-(2-hydroxyethylamino)-6-benzylamino-7-methylpurine	> 500	>1000
69	Other purine derivatives	6- <i>n</i> -butoxypurine	100	60
70		6-benzylamino-3-β-D-glucopyranosylpurine	>1000	>1000
71		6-mercaptopurine	850	—
72		2-aminopurine	> 500	>1000
73		7-methylpurine	> 500	—
74		9-methylpurine	350*	—
75		1-methyl-6-amino-8-bromopurine	> 500	—
76		guanine, 2-amino-6-hydroxypurine	> 100*	—
77		guanosine, 9-β-D-ribofuranosylguanine	200	—
78		GMP, guanosine-5'-monophosphate	> 500	—
79		cGMP, guanosine-3',5' cyclic monophosphate	> 500	—
80		caffeine, 1,3,7-trimethyl-2,6-dihydroxypurine	> 500	>1000
81		pyridopurine	>1000	700



**Fig. 7. Effects of olomoucine and other purines on the cdc2/cyclin B kinase.** Enzyme activities were assayed as described in the Materials and Methods section in the presence of increasing concentrations of olomoucine (51) and analogues. (A) Effect of N7 substitution [2-(2-hydroxyethylamino)-6-benzylamino-9-methylpurine (●) (51); 2-(2-hydroxyethylamino)-6-benzylamino-7-methylpurine (○) (68); 2-chloro-6-(5-hydroxypentylamino)-9-methylpurine (▲) (49); 2-chloro-6-(5-hydroxypentylamino)-7-methylpurine (Δ) (67)]. (B) Effect of some of the most active purines [2-(2-hydroxyethylamino)-6-benzylaminopurine (+) (20); 2-(2-hydroxyisobutylamino)-6-benzylamino-9-methylpurine (×) (58); 2-(2-hydroxyethylamino)-6-(3-hydroxybenzyl)amino-9-methylpurine (□) (56), 2-(2-hydroxyethylamino)-6-benzylamino-9-isopropylpurine (■) (53)]. (C) effect of N6 and C2 substitutions [2-(2-hydroxyethylamino)-6-aminopurine (■) (19); 2-amino-6-benzylaminopurine (□) (18); 6-benzylamino-9-methylpurine (Δ) (26); 2-amino-6-benzylamino-9-methylpurine (▲) (45); 2-(2-hydroxyethylamino)-6-amino-9-methylpurine (○) (50)].

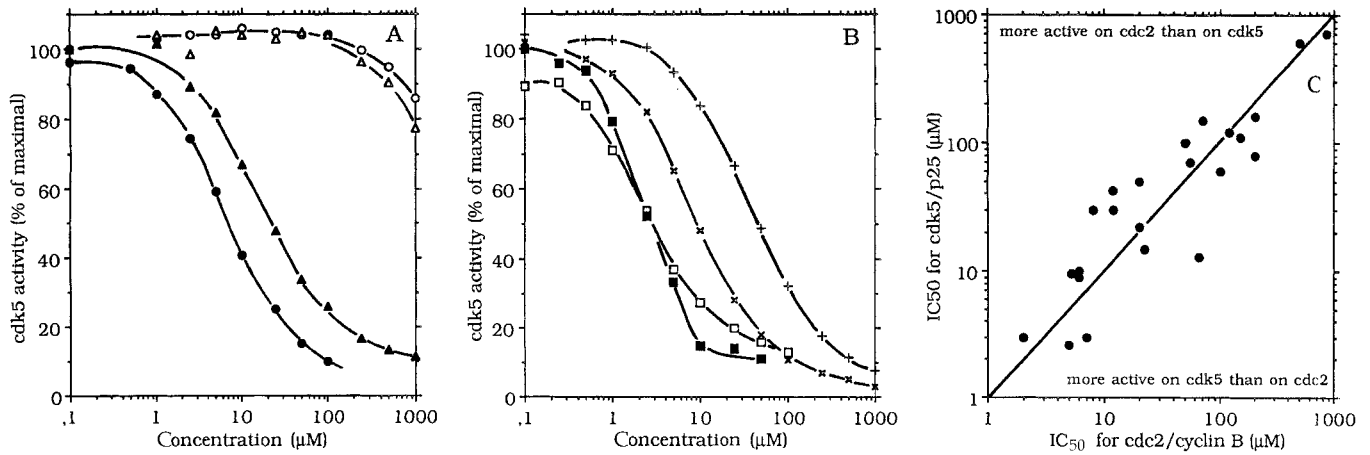
pyl)purine (27) (IC<sub>50</sub> values of 55, 50, 40 and 45 μM, respectively).

#### C2, N6, and N9-substituted adenines

Olomoucine (51), is the prototype of this family. Among these compounds, 2-chloro-6-(3-hydroxybenzylamino)-9-methylpurine (48), 2-(2-hydroxyethylamino)-6-benzylamino-9-isopropylpurine (53), 2-(2-hydroxyethylamino)-6-(3-hydroxybenzylamino)-9-methylpurine (56) and 2-(2-hydroxyethylamino)-6-(3-hydroxybenzylamino)-9-isopropylpurine

(57) were the most effective inhibitors (IC<sub>50</sub> values of 5.2, 2, 5 and 6 μM, respectively; Fig. 7).

Removal of the 2-hydroxyethylamino substitution at C2 reduced the inhibitory effect (Table 2, compare 26 and 51, 8 and 20). C2 substitutions by a 2-hydroxyethylamino group did not decrease the efficiency of inhibition. Replacement of this C2 substituent by a chloro or an amino residue reduced the inhibitory activity (compare 51, 47, 45), while a dimethylaminoethylamino moiety caused a very marked reduction of inhibition (Table 2, compare 20 and 21, 51 and 60). However, replacement of the hydroxyethylamino group of olo-



**Fig. 8. Effects of olomoucine and other purines on the cdk5/p35 kinase.** Enzyme activities were assayed as described in the Materials and Methods section in the presence of increasing concentrations of olomoucine (51) and analogues. (A) effect of N7 substitution [2-(2-hydroxyethylamino)-6-benzylamino-9-methylpurine (●) (51); 2-(2-hydroxyethylamino)-6-benzylamino-7-methylpurine (○) (68); 2-chloro-6-(5-hydroxypentylamino)-9-methylpurine (▲) (49); 2-chloro-6-(5-hydroxypentylamino)-7-methylpurine (△) (67)]. (B) Effect of some of the most active purines [2-(2-hydroxyethylamino)-6-benzylaminopurine (+) (20); 2-(2-hydroxyisobutylamino)-6-benzylamino-9-methylpurine (×) (58); 2-(2-hydroxyethylamino)-6-(3-hydroxybenzyl)amino-9-methylpurine (□) (56), 2-(2-hydroxyethylamino)-6-benzylamino-9-isopropylpurine (53)]. (C) Plot of the purines IC<sub>50</sub> values for cdk5/p35 versus the purines IC<sub>50</sub> values for cdc2/cyclin B.

olomoucine with the related substituent 2-hydroxyisobutylamino, did not alter activity appreciably (compare 51 and 58). Removal of the N6 benzyl substitution reduced the inhibitory activity (Table 2, compare 19 and 20, 46 and 47, 50 and 51). Though N6 substitutions by 3-hydroxybenzyl and benzyl residues provided approximately equally inhibitory compounds (Table 2, compare 51 and 56, 47 and 48, 57 and 53), substitution by an isopentenyl residue reduced the activity (Table 2, compare 54 and 51, 55 and 53). Substitution at the N7 position dramatically reduced the inhibitory activity (Table 2, Fig. 7A; compare 49 and 67, 51 and 68, 20 and 68, 26 and 66, 8 and 66). Removal of the methyl group at the N9 position reduced the inhibitory potency (Table 2, compare 19 and 50, 20 and 51, 18 and 45). Replacement of the N9 methyl group by an isopropyl group increased the inhibitory activity (Table 2, compare 53 and 51, 55 and 54). Hydroxyalkyl and alkyl residues at the N9 position provided equivalent activities (Table 2, compare 26 and 27, 51 and 52). N9 substitutions by sugars abolished the inhibitory activity.

### Inhibition of p33<sup>cdk5</sup>/p35 by purines

A selection of purines was next tested on the brain p33<sup>cdk5</sup>/p35 kinase (Table 2, Fig. 8). Essentially similar sensitivities to purines were observed with the cdk5 and cdc2 kinases. Again, an exceptional sensitivity to C2, N6, and N9-substituted adenines was observed. Among these compounds, olomoucine (51), 2-(2-hydroxyethylamino)-6-benzylamino-9-isopropylpurine (53) and 2-(2-hydroxyethylamino)-6-(3-hydroxybenzylamino)-9-methylpurine (56) were the most powerful inhibitors (IC<sub>50</sub> values of 3, 3 and 2.6 μM, respectively; Table 2, Fig. 7B). Purines IC<sub>50</sub> values for the cdc2 and cdk5 kinases were directly compared (Fig. 8C). A high correlation ( $R$  0.974) was observed, suggesting strong structural analogies within the purine binding domains for both kinases.

C2 substitution by a chloro-residue resulted in a substantial reduction of cdk5 inhibition (Table 2, compare 51 and 47). The same was found for the C2 substitution by a dimeth-

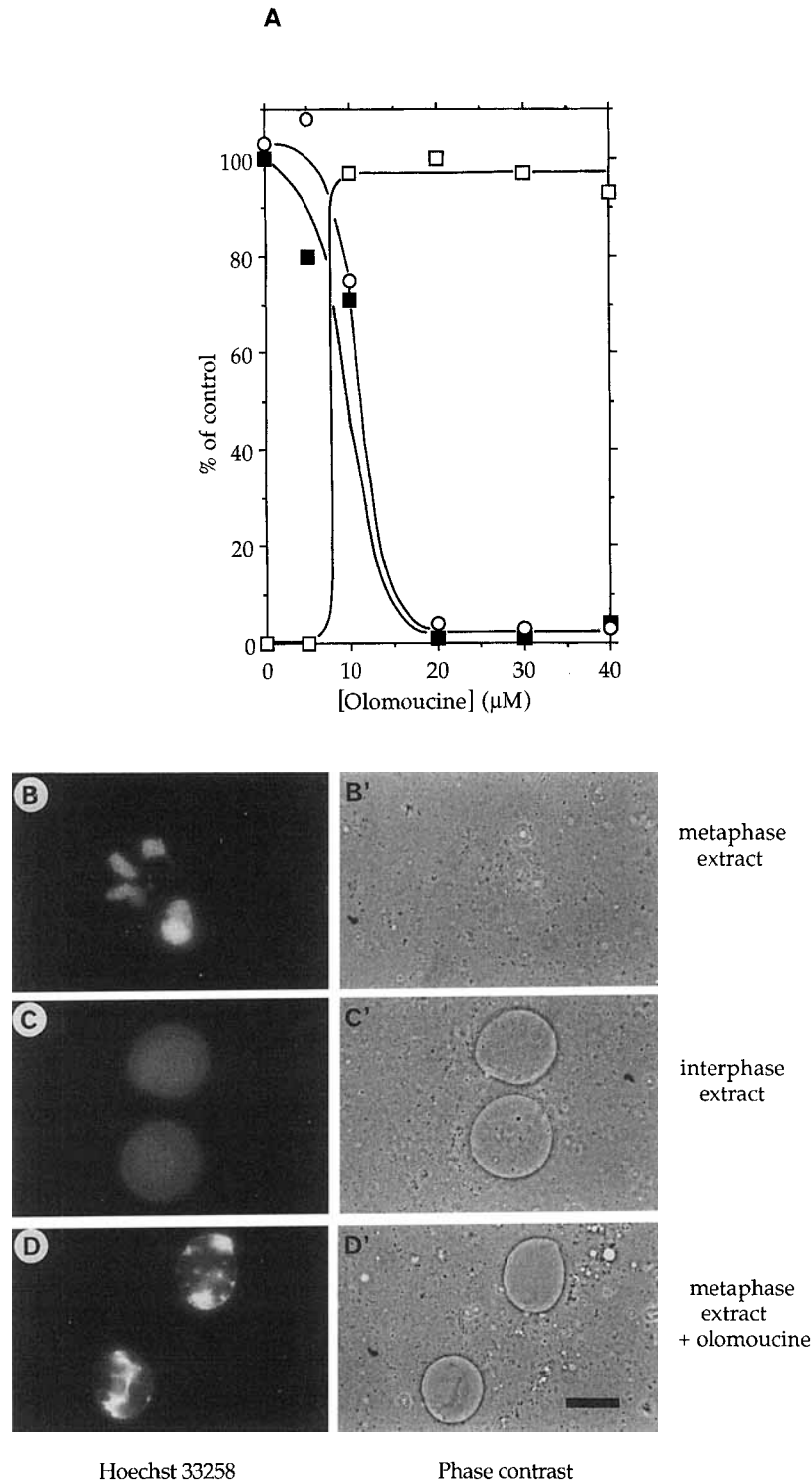
ylaminoethylamino residue (Table 2, compare 51 and 60). N6 substitution by a 3-hydroxybenzyl instead of a benzyl residue provided a somewhat better inhibitor (Table 2, compare 47 and 48, 56 and 51). Substitution at the N7 position significantly reduced the inhibitory activity by a large extent (Table 2, Fig. 8A; compare 49 and 67, 51 and 68, 26 and 66). Removal of the methyl group at the N9 position reduced the inhibitory activity (Table 2, compare 20 and 51; Fig. 8B). N9 substitutions by sugars abolished the inhibitory activity.

### Inhibition of p34<sup>cdk4</sup>/cyclin D1 by purines

The very low sensitivity of the cdk4 kinase towards olomoucine was consistently observed (Fig. 5). Therefore a selected series of purines was tested to assess the generality of this observation. Clearly, we were unable to find any significant inhibitor of cdk4 among N6 substituted adenines (Table 2, Fig. 5).

### Olomoucine inhibits both M-phase promoting factor activity and DNA synthesis in *Xenopus* egg extracts

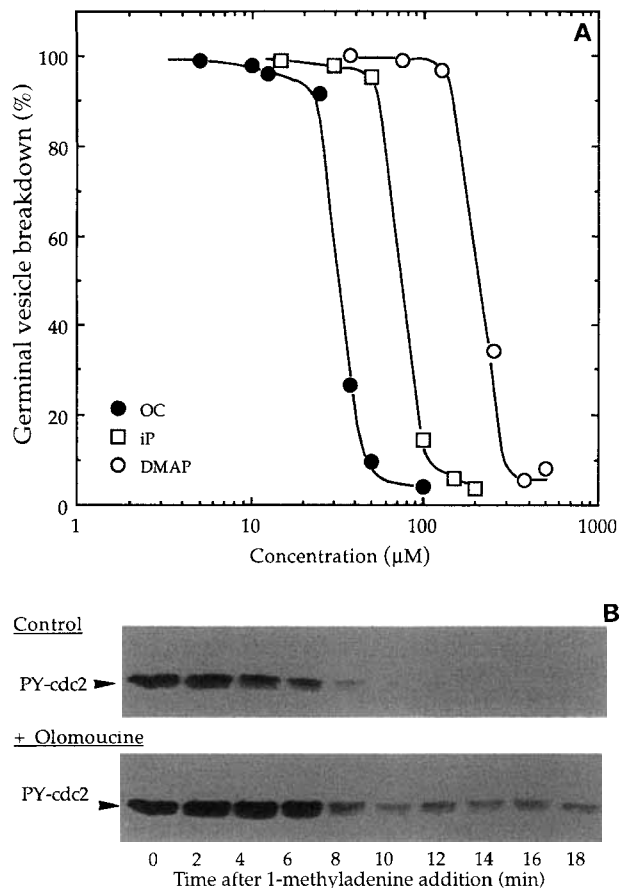
The cellular effects of olomoucine were next assessed using *Xenopus* egg extracts [76]. Metaphase-arrested *Xenopus* egg extracts were incubated with olomoucine and sperm chromatin. At olomoucine concentrations ranging from 0–5 μM, chromosomes remained highly condensed and no nuclear envelope was visible (Fig. 9A, Fig. 9B and B'). At 10 μM olomoucine and higher concentrations, interphase nuclei appeared (Fig. 9A, Fig. 9D and D') with partially decondensed chromatin and an intact nuclear envelope, showing that M-phase promoting factor activity is inhibited. Inhibition of DNA synthesis was assessed as follows: olomoucine and sperm chromatin were added to a metaphase-arrested *Xenopus* egg extract. The extract was released into interphase (Fig. 9A, Fig. 9C and C') by addition of CaCl<sub>2</sub> [76] and, 3 h later, total DNA synthesis was measured by [ $\alpha$ -<sup>32</sup>P]dATP incorporation in trichloroacetic-acid-precipitable material. Replication was significantly inhibited at 10–15 μM olo-



**Fig. 9. Effects of olomoucine on *in vitro* DNA synthesis and M-phase promoting factor activity.** (A) Three different titrations were performed on *Xenopus* extracts (see text), namely inhibition of M-phase promoting factor activity (□, inhibition of amount of assembled nuclei), inhibition of DNA synthesis (■) and inhibition of DNA licensing (○). Sperm nuclei were incubated in metaphase extract (B, B'), in interphase extract (i.e. metaphase extract plus 0.3 mM CaCl<sub>2</sub>; C, C') or in metaphase extract containing 20 μM olomoucine (51) (D, D'). Nuclei were stained with the DNA stain Hoechst 33258 and observed either under ultraviolet fluorescence (B–D) or in phase contrast (B'–D'). The scale bar is 20 μm.

moucine (Fig. 9A). For the inhibition effect to become detectable, the first 15-min incubation of the interphase extract is probably sufficient. Within that interval, DNA replication is thought to require a phosphorylation-dependent factor, the

so-called licensing factor, that seems to support only a single initiation event [76]. The effect of olomoucine on this factor was measured as follows: olomoucine and sperm chromatin were added to metaphase-arrested *Xenopus* egg extract. The



**Fig. 10. Effects of olomoucine on starfish oocyte maturation and on *in vivo* tyrosine dephosphorylation of p34<sup>cdc2</sup>.** G2-arrested oocytes were treated for 15 min with increasing concentrations of olomoucine (●) (51), N<sup>6</sup>-(Δ<sup>2</sup>-isopentenyl)adenine (□) (7) and 6-dimethylaminopurine (○) (2) prior to addition of 1 μM 1-methyladenine. After 30 min, germinal vesicle breakdown was scored (A). B, starfish oocytes were treated or not treated with 100 μM olomoucine for 15 min prior to 1 μM 1-methyladenine addition at zero time. Extracts prepared at various times were loaded onto p9<sup>CKShs1</sup>-Seph-rose beads. The bound proteins were resolved by SDS/PAGE prior to immunoblotting with antibodies directed against phosphotyrosine (PY). The immunoblots at the level of p34<sup>cdc2</sup> are presented.

extract was then released into interphase by CaCl<sub>2</sub> and incubation was carried on for 15 min (by which time the licensing reaction is normally complete) [76]. Chromatin was isolated and assayed for its ability to replicate in a licensing-factor-deficient medium (see Material and Methods section). Licensing is significantly inhibited at 10–15 μM olomoucine (Fig. 9A).

#### Olomoucine inhibits the G2/M transition *in vivo*

Olomoucine was next tested *in vivo*. G2-arrested starfish oocytes were treated with various concentrations of olomoucine prior to exposure to the hormone 1-methyladenine. Olomoucine inhibited oocyte maturation with an IC<sub>50</sub> value of 30 μM (Fig. 10A). Although olomoucine did not inhibit the cdc2-activating phosphatase cdc25 (Table 1, data not shown), it reduced the *in vivo* tyrosine dephosphorylation of p34<sup>cdc2</sup> (Fig. 10B). This dephosphorylation is exerted by cdc25 and normally precedes activation of cdc2 kinase at the G2/M transition [46]. The positive feedback loop by which the cdc2 kinase phosphorylates and hyperactivates the cdc25 phosphatase

may thus have been interrupted by olomoucine at the cdc2 kinase level.

## DISCUSSION

In this study we describe the inhibitory effects of purine derivatives on cyclin-dependent kinases. We report the discovery of olomoucine, a new kinase inhibitor with unusual specificity.

#### Interactions between olomoucine and cyclin-dependent kinases

By classical enzymic analysis, we show that olomoucine acts as a competitive inhibitor for ATP binding. The linear character of this inhibition argues against multiple binding sites. The atomic structure of cdk2 has been recently described [79]. The adenine base of ATP is positioned in a hydrophobic pocket between the β sheet of the small lobe and the L7 loop between β5 and α2. Preliminary analysis of cdk2/olomoucine and cdk2/N<sup>6</sup>-(Δ<sup>2</sup>-isopentenyl)adenine crystals shows that, indeed, the purine group of olomoucine is located in the pocket where ATP binds in cdk2 (Schulze-Gahmen, U., Jones, H. D., Meijer, L., Vesely, J., Morgan, D. O. and Kim, S. H., unpublished results).

Structure/action relationship studies (Table 1, Figs 7 and 8) allow some speculation on the nature of the interactions of C2, N6, and N9-substituted adenines with cdk proteins. Fig. 1 summarizes the potential interactions of olomoucine with a receptor structure. We have no data regarding the involvement of N1 and N3 in binding. N7 must remain free (see Results section) probably for a direct interaction, where it behaves as a hydrogen bond acceptor. N9 substitution by a hydrophobic residue (methyl, hydroxyethyl, isopropyl), but not by a sugar, is important. This N9 substitution may provide a direct hydrophobic interaction with the cdk. N6 substitution by a hydrophobic residue (benzyl, hydroxybenzyl, isopentenyl) is also important. The hydrophobic interaction could occur not only with the cdk but also within olomoucine, i.e. with its purine ring. Finally, a side chain at C2 appears to be essential. There are possibilities of hydrogen bonds and hydrophobic interactions at this site.

Olomoucine can be considered as a lead compound for the design of new cdk inhibitors. Analysis of cdk2/olomoucine and cdk2/N<sup>6</sup>-(Δ<sup>2</sup>-isopentenyl)adenine crystals will allow a full understanding of the interactions but may also provide some hints for the design of more efficient inhibitors. Based on our present knowledge of olomoucine/cdc2 interactions, we are presently designing suicide-type inhibitors which establish a covalent bond with their cdk targets and irreversibly inhibit the kinases. We expect these second-generation inhibitors to be required at concentrations close to the cellular concentration of the kinase.

#### Kinase specificity of inhibition by olomoucine

Among the 35 kinases tested, only the proline-directed kinases of the cyclin-dependent kinase family, and, to a minor extent, the erk1-MAP kinase, are inhibited by micromolar concentrations of olomoucine (Table 1). This specificity once more demonstrates that a high specificity can be reached even with a competitive inhibitor for ATP. The crystal structures of the protein and inhibitor complexes reveal more information on the molecular basis of this exceptional kinase specificity of olomoucine. The only other enzyme exhibiting some sensitivity to olomoucine, the plant homologue

of GSK-3, is also phylogenetically the closest to the cdk family. We are presently testing the effects of olomoucine on mammalian GSK-3.

Olomoucine sensitivity separates the cdk proteins into two subfamilies, *cdc2/cdk2/cdk5* (sensitive to olomoucine) and *cdk4/cdk6* (low sensitivity to olomoucine). The lack of effect of olomoucine on *cdk4/cyclin D1*, observed for three different types of preparations, is particularly evident. This insensitivity of *cdk4* and poor sensitivity of *cdk6* may be related to the relative evolutionary distance of *cdk4* and *cdk6* from *cdc2* [8]; the *cdc2* sequence displays 65, 57, 44 and 47% identity with *cdk2*, *cdk5*, *cdk4* and *cdk6*, respectively. *cdk4* and *cdk6* share 71% identity. Molecular models of *cdk4* and *cdk6*, based on the crystallographic coordinates of *cdk2*, may help to understand why olomoucine has such a low affinity for *cdk4* and *cdk6*. This point is of interest when designing *cdk4/cdk6*-specific inhibitors.

### Cellular effects of olomoucine

The unusual specificity of olomoucine towards cell cycle controlling enzymes suggests that olomoucine could be used to inhibit specifically certain steps of the cell cycle. Indeed, in this study we show that both M-phase promoting factor and DNA synthesis are inhibited *in vitro* (Fig. 9). Furthermore, olomoucine inhibits 1-methyladenine-induced G2/M transition in starfish oocyte *in vivo* at a step distal to the dephosphorylation of *p34<sup>cdc2</sup>* (Fig. 10). In collaboration with a number of colleagues we have tested the cellular effects of olomoucine (unpublished results). Olomoucine inhibits *Fucus* zygote development ( $IC_{50}$  32  $\mu$ M); *Arabidopsis thaliana* cell suspensions are arrested in late G1 and G2 by olomoucine; stimulated *Petunia hybrida* mesophyll protoplasts are arrested in G1 by olomoucine ( $IC_{50}$  25  $\mu$ M); olomoucine slows down the prophase/metaphase transition in cleaving sea urchin embryos, but does not affect the duration of the metaphase/anaphase and anaphase/telophase transitions; *Xenopus laevis* oocyte maturation is also inhibited by olomoucine ( $IC_{50}$  100  $\mu$ M); growth of the rhabdomyosarcoma cell lines Rh1, Rh18, Rh28 and Rh30 is inhibited by olomoucine ( $IC_{50}$  values 47, 86, 40, 18  $\mu$ M, respectively); DNA synthesis in interleukin-2-stimulated T lymphocytes (CTLL-2 cells) is inhibited by olomoucine ( $IC_{50}$  50  $\mu$ M). Furthermore, the *cdc2* kinase (immunoprecipitated from nocodazole-treated CTLL-2 cells) and the *cdk2* kinase (immunoprecipitated from hydroxyurea-treated CTLL-2 cells) are inhibited by olomoucine. Finally, the National Cancer Institute has tested olomoucine against 60 human tumor cell lines comprising nine tumor types. The average olomoucine concentration that causes 50% growth inhibition is 60.3  $\mu$ M. These data show that olomoucine arrests cells both at the G1/S and the G2/M boundaries, consistent with the hypothesis of a prevalent effect on *cdk2* and *cdc2*, respectively.

In numerous human tumors, cyclins and cyclin-dependent kinases are abnormally regulated. The recent discovery of p21 [21, 25–28], a natural universal inhibitor of cyclin-dependent kinases, as a component of cyclin-dependent kinases complexes in normal, but not in transformed, mammalian cells, and the fact that p21 overexpression inhibits proliferation strongly support the search for specific cdk inhibitors. It is possible that, through its specificity, olomoucine may lead to a compound which will preferentially inhibit the proliferation of certain tumor cells. The high frequency deletion of a natural inhibitor of *cdk4* in human tumor cell lines [22] also strongly supports the screening for *cdk4*-specific inhibi-

tors. Understanding why olomoucine does not interact with *cdk4* may be of importance in this respect.

The discovery of olomoucine may also have some implications in the field of Alzheimer's disease research. The brains of patients afflicted by this disease contains intracellular deposits of abnormally phosphorylated  $\tau$  protein [80]. Many of the phosphorylation sites and the corresponding kinases have been identified. Among these sites are those that are phosphorylated by *cdk5/p35* [81–83], *erk1* [80] and GSK-3, the three best targets of olomoucine. We are now investigating whether olomoucine can contribute to reduce the abnormal hyperphosphorylation of the  $\tau$  protein observed in Alzheimer's disease.

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