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# p42/p44 MAPKs are intracellular targets of the CDK inhibitor purvalanol

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**Chemical inhibitors of cyclin-dependent kinases (CDKs) have a great therapeutic potential against various proliferative and neurodegenerative disorders. Intensive screening of a combinatorial chemistry library of 2,6,9-trisubstituted purines has led to the identification of purvalanol, one of the most potent and selective CDK inhibitors to date. In preliminary studies, this compound demonstrates definite anti-mitotic properties, consistent with its nanomolar range efficiency towards purified CDK1 and CDK2. However, the actual intracellular targets of purvalanol remain to be identified, and a method for the determination of its *in vivo* selectivity was developed. In this technique, cell extracts were screened for purvalanol-interacting proteins by affinity chromatography on immobilized inhibitor. In addition to CDK1, p42/p44 MAPK were found to be two major purvalanol-interacting proteins in five different mammalian cell lines (CCL39, PC12, HBL100, MCF-7 and Jurkat cells), suggesting the generality of the purvalanol/p42/p44 MAPK interaction. The Chinese hamster lung fibroblast cell line CCL39 was used as a model to investigate the anti-proliferative properties of purvalanol. The compound inhibited cell growth with a $GI_{50}$ value of 2.5 $\mu$M and induced a G2/M block when added to exponentially growing cells. It did not appear to trigger massive activation of caspase. We next tested whether CDKs and p42/p44 MAPK were actually targeted by the compound *in vivo*. p42/p44 MAPK activity was visualized using an Elk–Gal4 luciferase reporter system and CDK1 activity was detected by the phospho-nucleolin level. When cells were treated with purvalanol, p42/p44 MAPK and CDK1 activities were inhibited in a dose-dependent manner. Furthermore, purvalanol inhibited the nuclear accumulation of p42/p44 MAPK, an event dependent on the catalytic activity of these kinases. We conclude that the anti-proliferative properties of purvalanol are mediated by inhibition of both p42/p44 MAPK and CDKs. These observations highlight the potency of moderate selectivity compounds and encourage the search for new therapeutics which simultaneously target distinct but relevant pathways of cell proliferation.**
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## Introduction

A strategy to inhibit cancer cell proliferation is to interfere with the cell cycle regulators. In this perspective, the family of cyclin-dependent kinases (CDKs), which control cell cycle progression (reviews in Dunphy, 1997; Morgan, 1997; Malumbres *et al*., 2000; Pavletich, 1999), has become an attractive target for the search of small molecular weight inhibitors of the cell cycle. Given the complexity of their regulation, modulation of CDKs activity can be achieved by different approaches: inhibition of the main CDK regulators (cyclins, CDK inhibitors (CKI), or kinases and phosphatases involved in CDK activation) or direct inhibition of CDK catalytic activity. This latter approach is the most extensively studied one and has led to the development of chemical CDK inhibitors with potential pharmacological interest (for review see Fischer and Lane, 2000; Garrett and Fattaey, 1999; Gray *et al*., 1999; Kaubisch and Schwartz, 2000; Meijer, 2000; Meijer and Kim, 1997; Mani *et al*., 2000; Rosania and Chang, 2000; Senderowicz and Sausville, 2000; Sielecki *et al*., 2000; Toogood, 2001; Knockaert *et al*., 2002a).

The search for chemical inhibitors of CDKs has intensified over the past few years, with the development of screening programs using either synthetic compounds or natural product extracts as a source of molecules. So far, numerous specific CDK inhibitors have been identified on the basis of their ability to inhibit CDK1, CDK2 or CDK4: the purines olomoucine (Vesely *et al*., 1994), roscovitine (Meijer *et al*., 1997; de Azevedo *et al*., 1997); purvalanols (Gray *et al*., 1998; Chang *et al*., 1999; Villerbu *et al*., 2002), CVT-313 (Brooks *et al*., 1997), C2-alkylynated purines (Legraverend *et al*., 2000), H717 (Dreyer *et al*., 2001) and NU2058 (Arris *et al*., 2000), piperidine-substituted purines (Shum *et al*., 2001), toyocamycin (Park *et al*., 1996), flavopiridol (Losiewicz *et al*., 1994), indirubins (Hoessel *et al*., 1999; Leclerc *et al*., 2001), paullones (Schultz *et al*., 1999; Zaharevitz *et al*., 1999; Leost *et al*., 2000), $\gamma$-butyrolactone (Kitagawa *et al*., 1993),

hymenialdisine (Meijer *et al*., 2000), indenopyrazoles (Nugiel *et al*., 2001), the pyrimidines NY6027 (Arris *et al*., 2000) and CGP60474 (Zimmermann, 1995), pyridopyrimidines (Barvian *et al*., 2000), the aminopyrimidine PNU 112455A (Clare *et al*., 2001), oxindoles (Kent *et al*., 1999; Davis *et al*., 2001), PD0183812 (Fry *et al*., 2001), cinnamaldehydes (Jeong *et al*., 2000), quinazolines (Shewchuk *et al*., 2000; Sielecki *et al*., 2001), fasclaplysin (Soni *et al*., 2000, 2001), SU9516 (Lane *et al*., 2001) and benzocarbazoles (Carini *et al*., 2001). Despite their chemical diversity, these inhibitors all act by competing with ATP at the ATP-binding site of the kinase. Many have been co-crystallized with CDK2 (for review see Hardcastle *et al*., 2002). These molecules display anti-proliferative properties, arresting the cell cycle in G1 and/or G2/M phases, depending on the cell type. Numerous pre-clinical and clinical studies are currently evaluating their anti-tumor properties (Damiens and Meijer, 2000; Senderowicz and Sausville, 2000).

Efforts to identify chemical CDK inhibitors with pharmacological interest tend to identify molecules with high efficiency and selectivity. Given the large number of ATP-binding proteins present in cells, the *in vivo* selectivity of CDK inhibitors remains an open question. Even though several indirect arguments support the idea that CDK inhibitors act intracellularly by inhibiting CDKs, the real spectrum of intracellular targets of CDK inhibitors remains unknown. We therefore designed an affinity chromatography method to identify the intracellular targets of purvalanol (Knockaert *et al*., 2000), one of the most potent CDK inhibitors to date. The CDK2/purvalanol crystal structure (Gray *et al*., 1998; Chang *et al*., 1999) suggested that a linker could be attached to the carboxylic acid of the 6-anilino substituent of the purine without interfering with the inhibitor/kinase interaction. This side chain was used to couple purvalanol to an agarose matrix. As a negative control, an inactive, N6-methylated, purvalanol was also immobilized on agarose. The immobilized purvalanol beads were used to screen various cell types and tissues for purvalanol-interacting proteins (Rosania *et al*., 1999; Knockaert *et al*., 2000). In most cases, we found that purvalanol interacts with CDKs but also with a few other kinases. In the MCF-7 cell line p42/p44 MAPK were found, along with CDKs, to be the major purvalanol-interacting proteins.

The aim of this study was to determine whether p42/p44 MAPK and CDKs are relevant purvalanol targets *in vivo*. We first confirmed with several mammalian cell lines that both p42/p44 MAPK and CDKs actually bind to the purvalanol matrix, suggesting this was not specific to MCF-7 cells. We then used the Chinese hamster lung fibroblast CCL39 cell line to characterize the anti-proliferative properties of purvalanol. The compound inhibits cell proliferation with a $GI_{50}$ of 2.5 $\mu$M and induces a G2/M block when added to exponentially growing cells. In order to demonstrate that p42/p44 MAPK and CDK1 are real *in vivo* targets of purvalanol, we treated CCL39 cells with various purvalanol concentrations and measured its effects on the specific phosphorylation of downstream substrates, an Elk–Gal4 luciferase reporter system for p42/p44 MAPK and phosphonucleolin for CDK1/cyclin B. Purvalanol treatment inhibited both p42/p44 MAPK and CDK1 in a dose-dependent manner. Purvalanol also inhibited the nuclear accumulation of p42/p44 MAPK in response to stimulation of resting cells, a phenomenon shown to be dependent on MAPK catalytic activity. Altogether these data reveal p42/p44 MAPK as an unexpected *in vivo* purvalanol target. The dual contributions of p42/p44 MAPK and CDKs inhibition thus account for the observed anti-proliferative effects of purvalanol.

## Results

A combinatorial chemistry library of 2,6,9-trisubstituted purines has been recently optimized for activity against CDK1/cyclin B (Gray *et al*., 1998; Chang *et al*., 1999). It has yielded the purvalanol inhibitors: purvalanol A (NG-60), purvalanol B (NG-95) and aminopurvalanol (NG-97) (Figure 1). These closely related compounds reversibly inhibit CDKs, by competing with ATP for binding to the catalytic site of the kinase. Addition of a methyl group at the N6 position of these purines, leads to loss of their kinase inhibitory properties as the N6 acts as a donor in an essential hydrogen bond between the inhibitor and the kinase Leu83 residue (review in Gray *et al*., 1999). These CDK-inactive N6-methylated purvalanols (Figure 1) were used as negative controls.

In the present study, the cellular effects of purvalanols were investigated. Cell treatments and kinase assays were performed with aminopurvalanol (NG-97), the most membrane permeable purvalanol, and controls were performed with the corresponding inactive derivative (NG-97M) (Figure 1).

Purification of intracellular proteins interacting with purvalanol was carried out on affinity chromatography resins: a NG-95 matrix derived from purvalanol B (NG-95) and NG-95M matrix derived from methyl-purvalanol B (NG-95M), for negative control.

### *Aminopurvalanol displays anti-proliferative properties and arrests cells in G2/M but does not trigger massive apoptosis*

Exponentially growing Chinese hamster lung fibroblast CCL39 cells were exposed to aminopurvalanol for 48 h and cell proliferation was assayed by cell counting of adherent cells as described in the Materials and methods section (Figure 2a). Proliferation was expressed as the percentage of maximum proliferation 100% corresponding to cell proliferation observed after 48 h without inhibitor. Results clearly show that aminopurvalanol displayed anti-proliferative properties

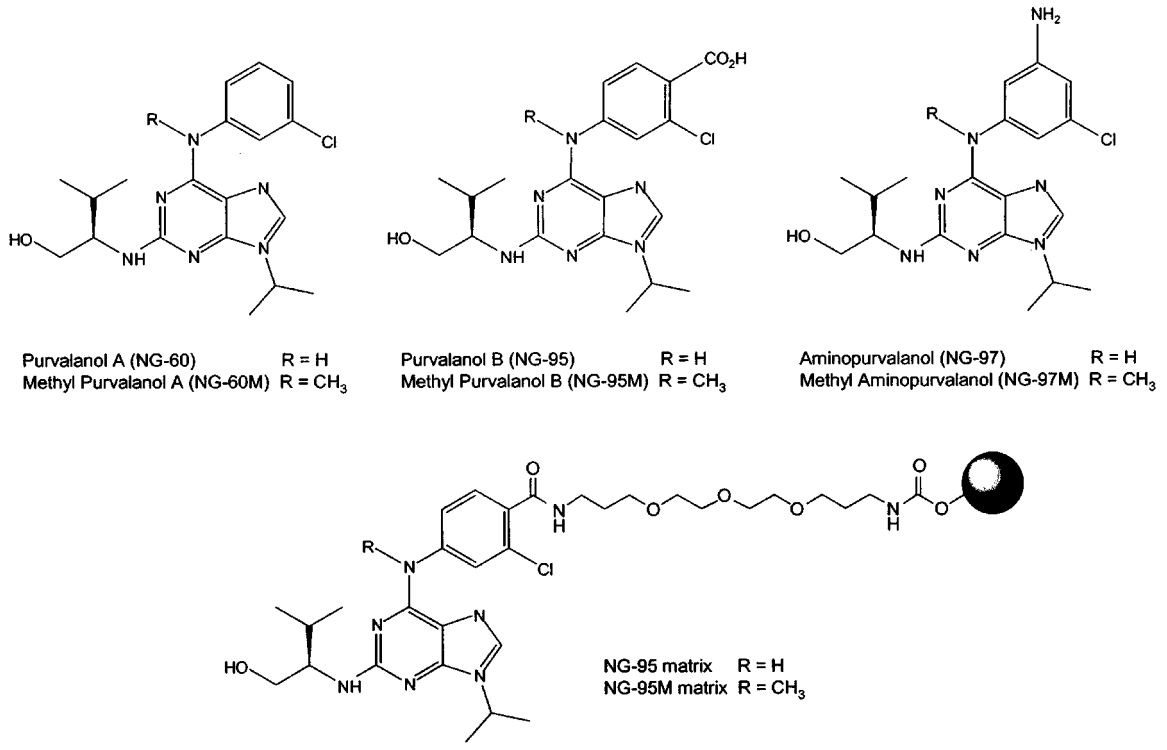


**Figure 1** Structure of purvalanols and immobilized aminopurvalanol. The structures of purvalanol A (NG-60), purvalanol B (NG-95), aminopurvalanol (NG-97), their N6-methylated counterparts (NG-60M, NG-95M, NG-97M) and the immobilized purvalanols (NG-95 and NG-95M) are shown

towards the CCL39 cell line, inhibiting cell proliferation with a $GI_{50}$ of 2.5 $\mu$M.

In parallel, asynchronous cells exposed to aminopurvalanol (Figure 2b, right) or not (Figure 2b, left) were analysed by flow cytometry after PI staining. The 10 $\mu$M aminopurvalanol treatment resulted in an accumulation of cells in G2/M (82.8%) compared to control cells (12.8%). These results are consistent with previous studies reporting the cellular effects of purvalanol on different cell lines (Gray *et al*., 1998; Chang *et al*., 1999). When purvalanol A and aminopurvalanol were tested on the NCI panel of 60 human tumor cell lines, the average $GI_{50}$ values were 2 and 1.8 $\mu$M, respectively (data not shown).

To determine whether aminopurvalanol triggers apoptosis, we investigated the level of caspase activation by monitoring PARP cleavage forms by Western blotting (Figure 2c). An 18 h treatment with 30 and 100 $\mu$M aminopurvalanol did not trigger any substantial poly-(ADP-ribose) polymerase (PARP) cleavage by caspases. In contrast, treatment with the apoptosis-inducing staurosporine resulted in massive PARP cleavage.

### *Aminopurvalanol prevents re-entry into the cell cycle after serum starvation*

We next investigated the cellular effects of aminopurvalanol on growth factor-induced cell cycle entry. Growth-arrested CCL39 cells were induced to re-enter the cell cycle by addition of 10% FCS, in the presence of increasing concentrations of aminopurvalanol. After 18 h, cell cycle re-entry was evaluated by DNA synthesis meausurement (Figure 3a). Aminopurvalanol treatment resulted in a dose-dependent inhibition of cell cycle re-entry ($IC_{50}$: 1.5 $\mu$M). These results were confirmed by the flow cytometry analysis of cell cycle distribution (Figure 3b): while serum-deprived cells exhibited a strong accumulation in G0/G1 (87.7%) (Figure 3b, left), starved cells induced to re-enter cell cycle by addition of 10% FCS (Figure 3b, center) had a cell cycle distribution comparable to exponentially growing cells (Figure 2b, left). In contrast, serum-stimulated cells pretreated with 10 $\mu$M aminopurvalanol (Figure 3b, right), showed a cell cycle profile comparable to serum-deprived cells (90.1% in G0/G1), suggesting that aminopurvalanol maintained a G0/G1 block and prevented cells from re-entering the cell cycle upon FCS addition.

### In vitro *selectivity of aminopurvalanol*

*In vitro* selectivity studies report that purvalanols display a remarkable efficiency (nanomolar range $IC_{50}$) and selectivity for CDK1, 2 and 5. In the selectivity panel, against which the compounds were screened (about 25 kinases), purvalanols were also reported to inhibit p42/MAPK (ERK2) and p44/MAPK (ERK1), but to a lesser extent (micromolar

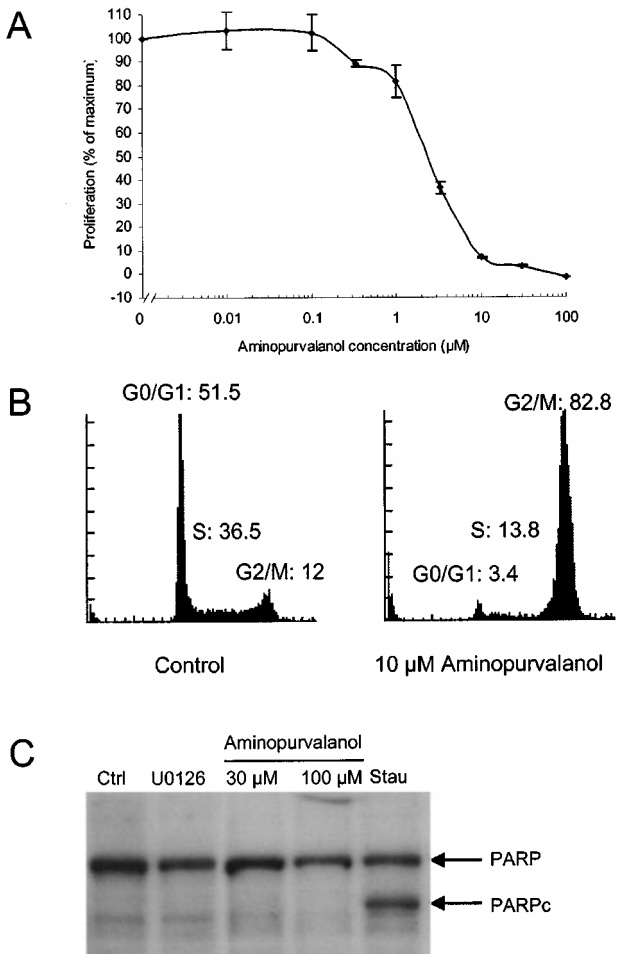


**Figure 2** Anti-proliferative properties of aminopurvalanol in asynchronous cells. (**a**) Exponentially growing CCL39 cells were treated with increasing concentrations of aminopurvalanol for 48 h. Adherent cells were then harvested by trypsinization and counted in a Neubauer cell. Proliferation is expressed as percentage of cell density in control, untreated cells. This graph is the mean ± s.e.m. of one representative experiment carried out in triplicate. (**b**) Aminopurvalanol induces a G2/M block. Asynchronous CCL39 cells were treated with 10 $\mu$M aminopurvalanol for 24 h (right) or left untreated (left). Cells were harvested and the distribution between the cell cycle phases was analysed by flow cytometry. (**c**) Aminopurvalanol, in contrast to staurosporine, does not lead to caspase activation, as monitored by PARP cleavage. Asynchronous CCL39 cells were treated with 30 $\mu$M, 100 $\mu$M aminopurvalanol for 18 h, 1 $\mu$M staurosporine for 12 h or left untreated. Adherent cells and floating cells were harvested by centrifugation and lysed as described in the Materials and methods section. PARP cleavage was analysed by Western blotting following SDS–PAGE

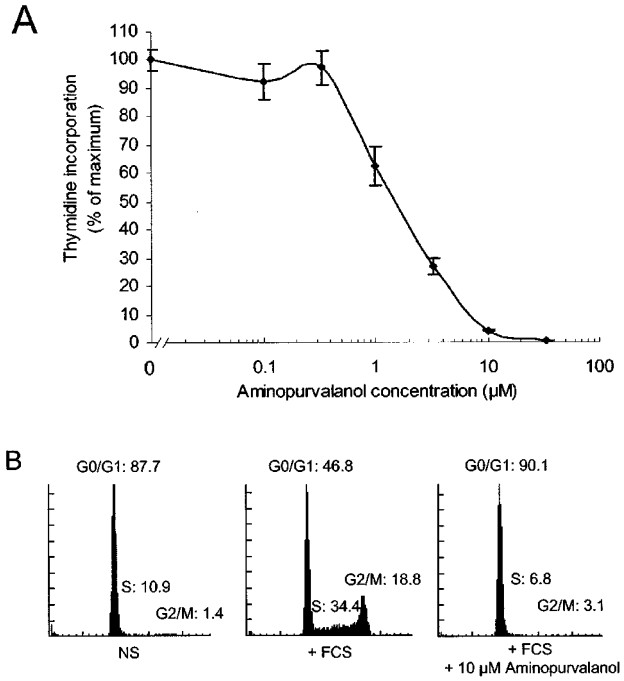


**Figure 3** Anti-proliferative properties of aminopurvalanol in G0/G1 synchronized cells. (**a**) Aminopurvalanol inhibits DNA synthesis. Serum-starved CCL39 were incubated with 0.15 $\mu$Ci $^3$H-thymidine and various concentrations of aminopurvalanol. DNA synthesis was initiated by 10% serum stimulation. After 18 h, cells were harvested and $^3$H-thymidine incorporation was counted. Results are expressed as a percentage of $^3$H-thymidine incorporation in control, untreated cells. This graph is the mean ± s.e.m. of one representative experiment carried out in triplicate. (**b**) Aminopurvalanol induces a G0/G1 block. Serum-starved CCL39 cells were stimulated with 10% FCS (center) and treated with 10 $\mu$M aminopurvalanol (right), or a corresponding volume of DMSO (left). Adherent cells were harvested and the distribution between the cell cycle phases was analysed by flow cytometry

range $IC_{50}$) (Gray *et al*., 1998; Chang *et al*., 1999). However, in these studies, kinase assays were performed using native starfish oocyte CDK1/cyclin B and purified recombinant mammalian p42/MAPK and p44/MAPK produced in bacteria. To allow a better comparison, we purified p42/MAPK, p44/MAPK and CDK1/cyclin B expressed in the fibroblastic cell line CCL39, and determined the inhibitory properties of aminopurvalanol on the purified native kinases. p42/MAPK and p44/MAPK were immunoprecipitated as described under the Materials and methods section and the CDK1/cyclin B complex was purified by affinity chromatography on $p9^{CKShs1}$-sepharose beads (Borgne and Meijer, 1996). Before CDK1/cyclin B purification, cells were synchronized in prophase with the microtubule destabilizing agent nocodazole (Hoebeke *et al*., 1976) in order to increase the level of active CDK1/cyclin B in the cell extract. The results shown in Figure 4 confirmed that aminopurvalanol was more efficient on CDK1/cyclin B ($IC_{50}$: 0.350 $\mu$M) than on p42/MAPK and p44/MAPK ($IC_{50}$: 2.4 $\mu$M and 25 $\mu$M, respectively). The $IC_{50}$ reported here for aminopurvalanol on CDK1/cyclin B (0.350 $\mu$M) differed from the initially reported $IC_{50}$ of aminopurvalanol (0.033 $\mu$M) (Chang *et al*., 1999). This might be explained by the difference in the source of enzyme used in these two experiments: CDK1/cyclin B purified from CCL39 cell line in the present study and from starfish oocytes in the previous study (Chang *et al*., 1999). Alternatively the $p9^{CKShs1}$-sepharose beads may bind inactive, monomeric, unphosphorylated CDKs which are still

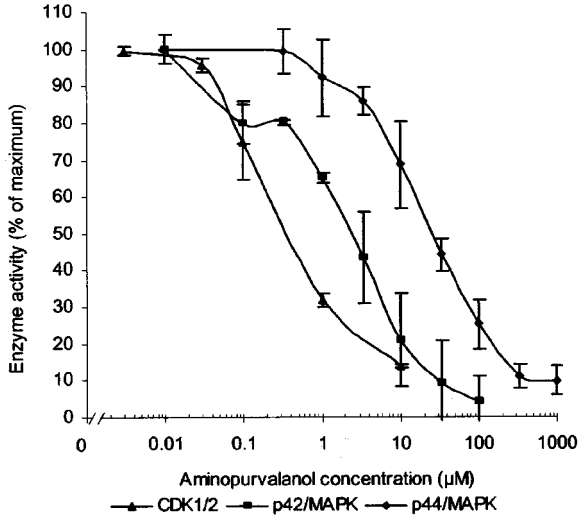


**Figure 4** Inhibitory properties of aminopurvalanol on purified kinases. CCL39 cells were transfected with p44/MAPK-HA or p42/MAPK-VSVG. p42/MAPK and p44/MAPK were immunoprecipitated with HA or VSVG-specific antibodies. Alternatively CCL39 cells were synchronized in G2/M with nocodazole and CDK1/cyclin B was purified with $p9^{CKShs1}$ beads. Assays were performed using either MBP (p42/p44 MAPK) or histone H1 (CDK1/cyclin B) as substrates, in the presence of 15 $\mu$M ATP. Enzymes activities are expressed as a percentage of maximal activity (no inhibitor). Each curve is the mean $\pm$ s.e.m. of one representative experiment carried out in triplicate

able to bind aminopurvalanol, therefore artificially increasing the apparent $IC_{50}$ value.

### *Intracellular targets of purvalanol*

Although *in vitro* studies indicate that purvalanol compounds are selective for CDKs, the actual intracellular targets of these inhibitors remain unverified. To address this issue, we have constructed an affinity matrix in which purvalanol B (NG-95) and the N6-methylated, CDK-inactive derivative (NG-95M) are covalently linked to agarose beads. These resins were used to purify proteins interacting with purvalanol from various extracts of cells and organs (Rosania *et al*., 1999; Knockaert *et al*., 2000). In addition to validating CDKs as intracellular targets of purvalanol, we found a few unexpected protein kinases bound to the matrix. Strikingly, in the human breast cancer epithelial MCF-7 cell line, p42/MAPK and p44/MAPK were, along with CDKs, the two major purvalanol-interacting proteins (Knockaert *et al*., 2000). In order to generalize these observations, extracts from four other cell lines were loaded on the purvalanol matrix (Chinese hamster lung fibroblast CCL39, rat pheochromocytoma PC12, SV40-transformed human breast epithelial HBL-100, and the human haematopoietic Jurkat) as described in the Materials and methods section. Experiments were carried out with nocodazole synchronized cells, in order to enrich the cell extract with Thr14- and Tyr15-dephosphorylated CDK1 as we had previously noticed that these inhibitory phosphorylations prevent CDKs from binding to the purvalanol matrix (Knockaert *et al*., 2000). Crude extracts (100 $\mu$g protein) (Figure 5a) or NG-95 matrix-bound proteins (Figure 5b) were resolved by 10% SDS–PAGE, followed by Western blotting. MAPKs were detected using a polyclonal anti-ERK1 antibody which also cross-reacts with ERK2 (upper panels), and CDKs were visualized with a monoclonal anti-PSTAIRE antibody which recognizes a conserved epitope in CDK1, CDK2 and CDK3 (lower panels). Figure 5a shows that CDKs and both isoforms of MAPKs, p42 and p44, are present in all cell lines tested. Analysis of resin-bound proteins (Figure 5b) revealed that CDKs, but also p42/MAPK and p44/MAPK bound specifically to the NG-95 matrix whereas no signal was detected on the NG-95M-matrix.

Taken together, the *in vitro* selectivity and the results of affinity chromatography experiments suggested that aminopurvalanol might act intracellularly by inhibiting both CDKs and MAPKs. Consequently, we can hypothesize that cellular effects of this compound could be mediated by this dual inhibition.

To challenge this hypothesis, we tested if cell treatment with aminopurvalanol actually resulted in CDK and MAPK inhibition in the cell. Since direct biochemical demonstration of *in vivo* kinase inhibition by the drug presents technical difficulties because the inhibition might be reversible upon cell lysis, we attempted to assay kinase activities in an indirect manner, by evaluating the phosphorylation or activity of their substrates.

### *Aminopurvalanol* in vivo *inhibitory properties*

To evaluate the effects of aminopurvalanol on CDK and MAPK activities, we first investigated the effects of the compound on CDKs and MAPKs protein levels. Exponentially growing CCL39 cells were exposed for 36 h to a range of aminopurvalanol concentrations and proteins were analysed by Western blotting with specific antibodies. CDK1 and cyclin B were purified by affinity chromatography on $p9^{CKShs1}$-sepharose beads (Borgne and Meijer, 1996), and p42/p44 MAPK were purified on purvalanol beads. The level of CDK7 was detected in the crude extract. No major changes in the level of CDK1, CDK7 and p42/p44 MAPK were induced by aminopurvalanol (Figure 6). In contrast, purvalanol exposure resulted in a slight increase in cyclin B level (Figure 6), consistent with the accumulation of cells in G2/M observed at similar concentrations (Figure 2b).

To evaluate CDK1/cyclin B activity *in vivo*, we visualized the level of phosphonucleolin with TG3, an antibody which cross-reacts with nucleolin only when phosphorylated by CDK1/cyclin B (Dranovsky *et al*., 2001). Exponentially growing CCL39 cells were synchronized in metaphase by demecolcine (Sherwood *et al*., 1994; Urbani *et al*., 1995) and treated for 15 min with increasing concentrations of aminopurvalanol. Cells were then lysed and 100 $\mu$g of proteins were resolved by 7.5% SDS–PAGE, followed by immunoblotting with TG3 antibodies (Figure 7). Results clearly

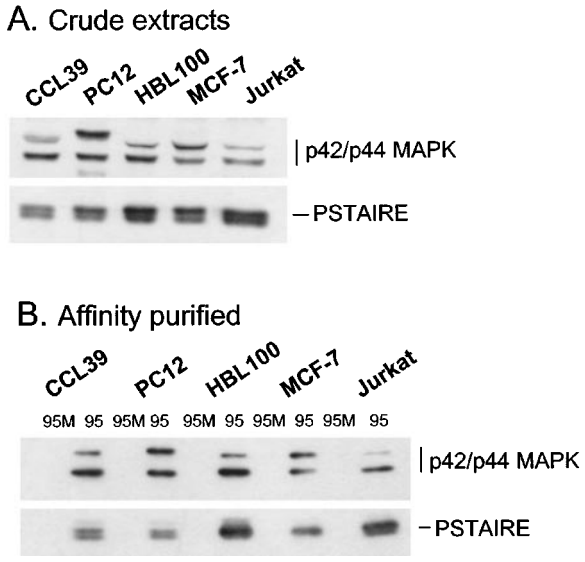


**Figure 5** Purvalanol-binding proteins in cell lines. (**a**) Crude extracts (100 $\mu$g proteins) from different cell lines were resolved by 10% SDS–PAGE followed by Western blotting with anti-ERK1 (upper panel) and anti-PSTAIRE (lower panel) antibodies. (**b**) Two mg of cell line extracts were loaded on control methyl purvalanol (NG-95M) or purvalanol beads (NG-95). Bound proteins were resolved by 10% SDS–PAGE followed by Western blotting with anti p44/MAPK (upper panel) and anti-PSTAIRE (lower panel) antibodies

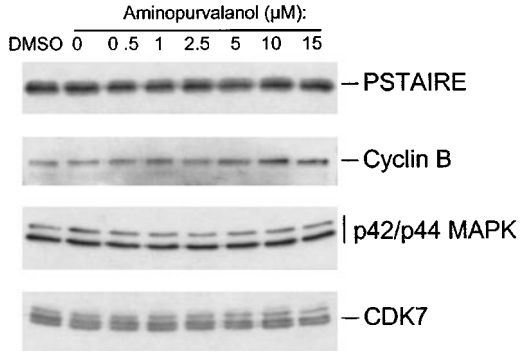


**Figure 6** Aminopurvalanol does not significantly alter the level of CDK1, CDK7, cyclin B or p42/MAPK and p44/MAPK. CCL39 cells were exposed to increasing concentrations of aminopurvalanol for 36 h. Crude extracts (100 $\mu$g proteins) or proteins purified on purvalanol beads (NG-95) or $p9^{CKShs1}$-sepharose (500 $\mu$g initial extract) were resolved by 10% SDS–PAGE followed by Western blotting with anti-PSTAIRE, anti-cyclin $B^{cdc13}$, anti-ERK1, and anti-CDK7 antibodies

showed that aminopurvalanol treatment resulted in a progressive decrease of the phosphonucleolin signal, which became undetectable at 5 $\mu$M ($IC_{50}$: 2.2 $\mu$M). These results indicate a direct inhibition of CDK1/cyclin B by aminopurvalanol at concentrations comparable to those inhibiting proliferation, and support the idea that CDK1 is actually targeted *in vivo* by this compound.

We next evaluated MAPK activity *in vivo* in response to aminopurvalanol treatment. MAPK activity was assessed by two independent methods, a middle term (3 h) and a long term (12 h) response to MAPK activation.

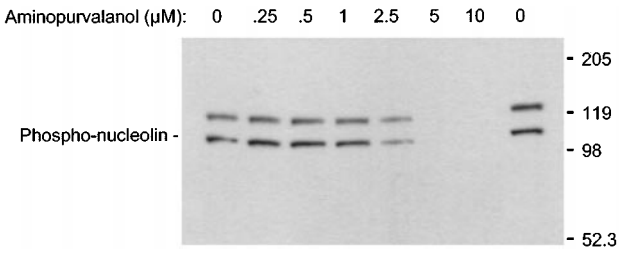


**Figure 7** Inhibition of CDK1-dependent nucleolin phosphorylation in aminopurvalanol-treated CCL39 cells. Demecocline-treated cells were exposed to increasing concentrations of aminopurvalanol for 15 min. Adherent cells were harvested and 100 $\mu$g proteins were resolved by 7.5 SDS–PAGE followed by Western blotting with anti-TG3, an antibody which cross-reacts with nucleolin only when phosphorylated by CDK1/cyclin B

First, we studied the cellular localization of MAPK by immunofluorescence, in response to a 3 h stimulation of resting cells (Figure 8). Indeed, a nuclear accumulation of MAPK following stimulation of resting cells has recently been shown to depend on its catalytic activity (Lenormand *et al*., 1998). To limit the interference a serum stimulation might cause on other pathways and to induce a specific activation of the p42/p44 MAPK signaling module, we used the fibroblastic cell line CCL39–$\Delta$Raf-1:ER stably expressing the chimera $\Delta$Raf-1:ER (Samuels *et al*., 1993). Upon addition of $\beta$-estradiol to these cells, the Raf-1 kinase moiety of the chimera is unmasked and rapidly phosphorylates MEK, which in turn activates p42/p44 MAPK (Lenormand *et al*., 1996). In resting cells p42/p44 MAPK immunostaining is predominantly cytoplasmic (Figure 8a), whereas within 3 h of stimulation by 1 $\mu$M $\beta$-estradiol, it is markedly relocated in the nucleus (Figure 8b). As depicted in Figure 8c,d, the addition of various concentrations of aminopurvalanol (1, 10, 30 $\mu$M) at the time of $\beta$-estradiol addition clearly inhibited p42/p44 MAPK nuclear accumulation in a dose-dependent manner, suggesting a direct effect of aminopurvalanol on the MAPK activity.

We next evaluated MAPK activity after long-term exposure to aminopurvalanol, by measuring indirectly the phosphorylation of the Elk1 transcription factor by MAPK. Indeed Elk1 is a well characterized substrate of MAPK (Gille *et al*., 1992; Janknecht *et al*., 1993; Marais *et al*., 1993) whose transcriptional activity increases when phosphorylated by MAPK. Consequently a plasmid containing Gal4 binding sites upstream of the luciferase reporter gene was co-transfected with a plasmid coding for the gal4-Elk fusion protein. In our cells, we observed that the increase of luciferase activity was dependent on MEK/MAPK activity, since it was inhibited by U0126 treatment (data not shown). Transfected CCL39 cells were deprived of serum for 12 h prior to a 12 h stimulation by 10% FCS. During stimulation, increas-

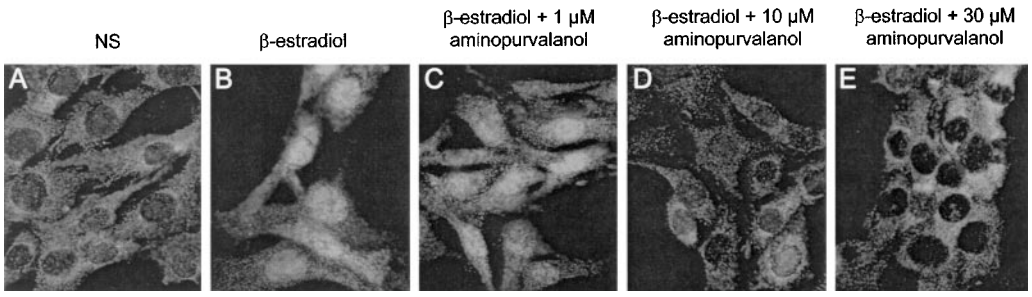


**Figure 8** Inhibition of MAPK-dependent MAPK nuclear accumulation in aminopurvalanol-treated CCL39 cells. CCL39–ΔRaf-1:ER cells were grown for 2 days on glass coverslides and serum deprived for 24 h. Before fixation and p42/p44 MAPK immunolabeling, cells were treated as follows: (**a**) control cells were left unstimulated; (**b**) cells were stimulated with 1 $\mu$M $\beta$-estradiol for 3 h; (**c**) cells were stimulated with 1 $\mu$M $\beta$-estradiol for 3 h in the presence of 1 $\mu$M aminopurvalanol; (**d**) cells were stimulated with 1 $\mu$M $\beta$-estradiol for 3 h in the presence of 10 $\mu$M aminopurvalanol; (**e**) cells were stimulated with 1 $\mu$M $\beta$-estadiol for 3 h in the presence of 30 $\mu$M aminopurvalanol

ing concentrations of aminopurvalanol were added to the culture medium. Transfection efficiency was normalized by co-transfection of a RSV-$\beta$-galactosidase plasmid, in order to eliminate the possible effects of aminopurvalanol on transcription and translation. Results shown in Figure 9 clearly indicated that aminopurvalanol treatment resulted in a dose-dependent inhibition of the p42/p44 MAPK pathway with an $IC_{50}$ of 4 $\mu$M.

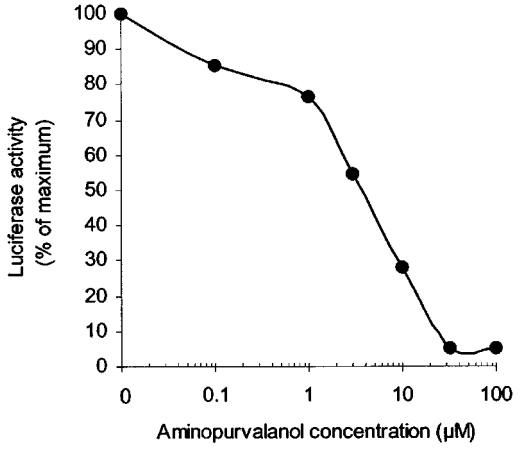


**Figure 9** Inhibition of MAPK-dependent Elk-1 phosphorylation in aminopurvalanol-treated CCL39 cells. CCL39 cells were transiently co-transfected with the vectors encoding $\beta$-galactosidase, Gal4-Elk1 fusion protein, and Gal4-luciferase reporter constructs by the calcium phosphate technique. Cells were then treated with increasing concentrations of aminopurvalanol and luciferase and $\beta$-galactosidase activities were assayed. For each concentration, values measured for luciferase activities were normalized with the values obtained for $\beta$-galactosidase activities. This graph is a representative of three experiments carried out in triplicate

## Discussion

### *Purine as inhibitors of CDKs*

Our first efforts to identify CDK inhibitors date back to 1988, when 6-dimethylaminopurine was discovered as an inhibitor of the M phase specific histone H1 kinase (later known as CDK1/cyclin B) ($IC_{50}$: 120 $\mu$M) (Meijer and Pondaven, 1988; Neant and Guerrier, 1988). Screening through a few related purines, we found that isopentenyladenine was slightly more active ($IC_{50}$: 55 $\mu$M) (Rialet and Meijer, 1991). Unfortunately both compounds were of limited interest due to their lack of specificity. Isopentenyladenine is a widely studied plant hormone (cytokinin). In a search for antagonists of this hormone, David Letham had identified 2-(2-hydroxyethylamino)-6-benzylamino-9-methylpurine as an inhibitor of cytokinin 7-glucosyltransferase from radish cotyledons (Parker *et al*., 1986). This compound was renamed olomoucine when we identified it as an efficient CDK1/cyclin B inhibitor among a series of 81 variously substituted purines ($IC_{50}$: 7 $\mu$M) (Vesely *et al*., 1994). Interestingly, olomoucine seemed to be rather selective for CDKs with some inhibitory activity on MAPK at higher concentrations (Vesely *et al*., 1994). Semi-random synthesis of olomoucine analogues by classical medicinal chemistry lead to the identification of inhibitory C2,N6,N9-trisubstituted purines, from which we selected 2-(R)-(1-ethyl-2-hydroxyethylamino)-6-benzylamino-9-isopropylpurine (named roscovitine for convenience) for further investigation (de Azevedo *et al*., 1997; Meijer *et al*., 1997). Roscovitine was also rather potent at inhibiting CDKs ($IC_{50}$: 0.450 $\mu$M with CDK1/cyclin B) and still quite selective. This compound generated a wide interest and was the start point of a combinatorial chemistry effort to generate even more efficient purine analogues. These efforts were also guided by the co-crystal structures of CDK2 with olomoucine (Schultze-Gahmen *et al*., 1995) and roscovitine (de Azevedo *et al*., 1997). They ultimately led to the identification of purvalanols which are both very potent *in vitro* ($IC_{50}$ in the 0.004–0.04 $\mu$M range) and selective (Gray *et al*., 1998; Chang *et al*., 1999; Rosania *et al*., 1999).

### *Purine inhibitors of CDKs, the selectivity problem*

The selectivity of these purines has been based on *in vitro* assays on a panel of 30–35 purified, often recombinant, kinases, which obviously represent only a very minor fraction of the reported 800+ kinases

present in the human genome. In addition, there are a number of other ATP-binding proteins, or even nucleotide-binding proteins, which might bind the purine CDK inhibitors. Therefore selectivity data should be interpreted with caution. In an effort to investigate the range of purvalanol's targets we recently designed an affinity chromatography technique to purify intracellular proteins interacting with the inhibitor (Rosania *et al*., 1999; Knockaert *et al*., 2000). In this method, purvalanol was covalently linked to an agarose matrix and extracts from various cell types and tissues were screened for purvalanol-binding proteins.

Using the same affinity chromatography approach, several unexpected targets of CDK inhibitors have been identified: glycogen phosphorylase for flavopiridol (Schnier *et al*., 1999; Oikonomakos *et al*., 2000; Kaiser *et al*., 2001), mitochondrial malate dehydrogenase and glycogen synthase kinase-3 for paullones (Knockaert *et al*., 2002b), casein kinase 1 in *Plasmodium falciparum* for purvalanol (Knockaert *et al*., 2000). In all cases, the unexpected target enzymes were inhibited by the free compound. This argument validates the affinity chromatography technique to identify the real targets of a biologically active compound whose mechanism of action is (partially) unknown.

### *Purvalanol inhibits CDKs* in vivo

We found that CDKs interacted with purvalanol, in most models tested. CDK1/2 was recovered from the matrix in starfish oocytes, sea urchin eggs, *Xenopus* oocytes (Knockaert *et al*., 2000). CDK1, 2 and 7 were found to bind purvalanol beads in seven different mammalian cell lines (Figure 5b; Knockaert *et al*., 2000; Schang *et al*., 2001). CDK5 was recovered on the purvalanol matrix from porcine and human brain extracts (Knockaert *et al*., 2000; data not shown) and sea urchin eggs (Knockaert *et al*., 2002b). This is not surprising, as purvalanols are potent CDK inhibitors that are highly selective for CDK1, 2, 5 and 7 over other members of the CDK family (Gray *et al*., 1998; Chang *et al*., 1999; Rosania *et al*., 1999). We next tested whether purvalanol was able to inhibit CDKs *in vivo*, and here we present clear evidence that this is the case. CCL39 cells were treated with the inhibitor and the phosphorylation of nucleolin was analysed with TG3, an antibody that cross-reacts with nucleolin only when phosphorylated by CDK1/cyclin B (Figure 6). Purvalanol treatment resulted in a dose-dependent inhibition of nucleolin phosphorylation ($IC_{50}$: 2.2 $\mu$M). The difference between this $IC_{50}$ and $IC_{50}$ found on purified CDK1/cyclin B (0.350 $\mu$M, Figure 4), can be explained by the fact that purvalanol efficiency on CDK1/cyclin B is measured in a cellular context in one case, and on the purified kinase, in the other case. Indeed, several factors are likely to interfere with the cellular effects of a given compound: cell permeability, intracellular metabolism of the compound, competition with high intracellular concentration of ATP, and interaction with other targets. Nevertheless, these results are in agreement with arguments supporting the idea that CDK inhibitors act intracellularly by inhibiting CDKs: firstly, the reported cell cycle effects are compatible with CDK inhibition (G1 arrest: inhibition of CDK2; G2/M arrest: inhibition of CDK1) (review in Meijer and Kim, 1997). Secondly, similar effects are obtained with structurally different inhibitors. Thirdly, a generally good correlation is observed between the *in vitro* efficiency of inhibitors on CDKs and their *in vivo* efficacy on cells.

### *Purvalanol inhibits MAPKs* in vivo

In addition to CDKs, p42/p44 MAPKs were found to interact with the purvalanol matrix, in most models: lugworm oocytes, *Xenopus* oocytes, porcine and human brains (Knockaert *et al*., 2000; data not shown), rat tissues (Knockaert *et al*., 2000; data not shown), rat hepatocyte primary cultures (data not shown) and in seven different mammalian cell lines (Figure 5b; Knockaert *et al*., 2000; Schang *et al*., 2002). These observations are quite striking, given the large difference between the $IC_{50}$ values of purvalanol for CDKs and MAPKs *in vitro* (Figure 4). We believe that these results may in part reflect the relative abundance of the two groups of kinases. In other words, purvalanol interacts, in the cell, with targets for which it has a strong affinity but which are not very abundant (CDKs). It also interacts with targets for which it has a moderate affinity but which are more abundant (MAPKs). To test whether MAPKs are really targeted *in vivo* by purvalanol, we measured the phosphorylation of one of its downstream substrate, the transcription factor Elk1. Aminopurvalanol treatment resulted in a dose-dependent inhibition of Elk1 phosphorylation (Figure 9) ($IC_{50}$: 4 $\mu$M), suggesting that the MAPK pathway is actually targeted *in vivo* by the inhibitor. This hypothesis was further confirmed by the observation that comparable concentrations of aminopurvalanol block MAPK-dependent nuclear accumulation of p42/p44 MAPK (Figure 8).

### *Anti-mitotic properties of purvalanol, a dual mechanism of action*

The major conclusion that can be drawn from this study is that aminopurvalanol acts intracellularly by inhibiting both CDKs and MAPKs. The remarkable selectivity of purvalanol for CDKs, initially reported following *in vitro* studies, is thus challenged. Their cellular effects, initially attributed to an inhibition of CDKs, are probably accounted for by mechanisms more complex than anticipated. This raises a number of questions. How does MAPK inhibition contribute to the anti-mitotic and anti-tumor properties of purvalanol? We can predict that inhibition of this new target does not impair the anti-proliferative properties of purvalanol, and may even facilitate them. Indeed, several arguments support the idea that p42/p44 MAPK are relevant targets for anti-cancer chemother-

apy. Firstly, MAPKs are directly necessary for cell cycle progression (Pages *et al*., 1993; Edelmann *et al*., 1996; Shapiro *et al*., 1998; Wright *et al*., 1999). Furthermore the nucleocytoplasmic translocation of CDK2 is regulated by MAPK (Keenan *et al*., 2001), and therefore inhibition of MAPK inhibits a functional property of CDK2. Secondly, constitutive activation of MAPKs is observed in a large number of tumor cell lines as well as in primary human tumors (Hoshino *et al*., 1999). Thirdly, numerous anti-cancer therapies target the MAPK pathway (reviews in Sebolt-Leopold, 2000; Weinstein-Oppenheimer *et al*., 2000; Herrera and Sebolt-Leopold, 2002). Fourthy, inhibition of the MAPK pathway seems to potentiate apoptosis induced by paclitaxel (Yu *et al*., 2001). Thus purvalanol might be used to enhance the therapeutic effects of taxanes. However, to our knowledge, no direct inhibitor of MAPK has been reported. The clinical use of MAPK pathway inhibitors for anti-cancer chemotherapy will require a thorough evaluation, as MAPKs are implicated in transducing a wide range of signals, like differentiation or apoptosis.

### *Highly selective versus 'dirty' compounds*

It is clear that the more we investigate a compound's selectivity, the less it appears selective! It is also clear that some families of compounds (purvalanols, paullones) are more selective than others (flabopiridol, staurosporine). As research tools, protein kinase inhibitors are bringing a lot, as long as one remains aware of the lack of absolute selectivity and as we interpret the data on the basis of an extensive knowledge on the range of their targets. Does absolute selectivity exist? Does it constitute the good answer to cure hyperproliferative disorders, in which multiple pathways are deregulated? Or, at the contrary, is a 'dirty' compound best? It is worth noting that many clinically active compounds are not very selective. However we believe that knowing the real targets of an active compound will help to improve its clinical efficiency: (1) by providing additional screening parameters to improve further analogues; (2) by providing information on the range of targets which need to be hit to obtain the desired anti-tumor effect. Searching for a combination of enzymatic effects rather than a single partial effect is likely to yield more efficient compounds; (3) by providing information on the enzymes which should not be targeted, it also should allow the anticipation and possible circumvention of potential undesired toxic side effects.

In conclusion, we feel that the affinity chromatography approach illustrated here represents a straightforward and simple method to identify the (sometimes unexpected!) targets of a given family of compounds with definitive pharmacological interest. Here we show that purvalanol, initially identified as a CDK inhibitor, also acts as a MAPK inhibitor *in vivo*. This dual effect is likely to underlie the potent anti-mitotic effects and the promising anti-tumor properties of the C2, N6, N9-trisubstituted purines.

## Materials and methods

### *Cell culture and treatments*

The Chinese hamster lung fibroblast cell line CCL39, the corresponding p44/MAPK-HA and p42/MAPK-VSVG transfected cells, the human breast cancer epithelial cell line MCF-7 and the SV40-transformed human breast epithelial cell line HBL-100 were cultured in Dulbecco's modified Eagle medium (DMEM) (Eurobio) supplemented with 2 mM L-glutamine (Eurobio), 7.5% fetal calf serum (FCS), and gentamycin (Gibco BRL) at 37°C in an atmosphere of 95% air, 5% $CO_2$. The CCL39–ΔRaf-1:ER clonal cell line (Samuels *et al*., 1993) was maintained in DMEM without phenol red and supplemented with 2 mM L-glutamine, 7.5% FCS, gentamycin and glucose to reach the concentrations of normal DMEM, at 37°C in an atmosphere of 95% air, 5% $CO_2$. The rat pheochromocytoma cell line PC12 was maintained in DMEM supplemented with 10% FCS, 5% horse serum, 4 mM L-glutamine and gentamycin at 37°C in an atmosphere of 95% air, 5% $CO_2$. The human haematopoietic Jurkat cell line was grown in RPMI 1640 medium (Gibco BRL) supplemented with 10% FCS, 2 mM L-glutamine and gentamycin in an atmosphere of 95% air, 5% $CO_2$. Growth arrested cells were obtained by total serum deprivation of confluent cultures for 24 h. Nocodazole (200 ng/ml for 18 h) and demecolcine (400 ng/ml for 6 h) treatments were performed on 70–80% confluent cultures. Aminopurvalanol (NG-97) treatment was performed on 50–60% confluent cultures (time and concentrations as indicated). Control experiments were carried out with appropriate dilutions of DMSO.

### *Cell proliferation, FACS analysis, DNA synthesis measurement*

Exponentially growing CCL39 cells were seeded at a density of $7.5 \times 10^4$/well into 24-well plates (Falcon). For cell proliferation assays, they were treated for 48 h with complete medium containing either DMSO alone or 0–100 $\mu$M aminopurvalanol (NG-97) dissolved in DMSO. Cell proliferation was then estimated by direct counting of adherent cells with a Neubauer cell as described: the increase in cell number between $t=0$ and $t=48$ h is calculated for each concentration and results are expressed as percentage of maximum proliferation, 100% proliferation corresponding to cell proliferation observed after 48 h without inhibitor. $GI_{50}$ (the dose inducing 50% growth inhibition) was determined from the dose-response curve. In parallel, cells were fixed in cold 70% ethanol for 6 h, washed with PBS, incubated with 5 $\mu$g RNAse A $ml^{-1}$ and stained with 25 $\mu$g $ml^{-1}$ propidium iodide (PI) for 30 min at 37°C. The stained cells were analysed on a Becton Dickinson FACScan cytofluorimeter using the cell FIT Software program (Becton Dickinson immunocytometry system). For DNA synthesis assays, CCL39 cells were seeded as described for proliferation and when they reached confluency, they were starved for 24 h in a medium without FCS. Cells were then pre-incubated with 0.15 $\mu$Ci [$^3$H]-thymidine and treated or not with various concentrations of aminopurvalanol, prior to 10% FCS stimulation. After 18 h, cells were washed with PBS, fixed with 10% TCA, washed twice with TCA and acid-precipitable material was solubilized in 0.1 M NaOH. Radioactivity was then measured in liquid scintillation counter.

### *Western blot analysis*

For affinity chromatography experiments, cells were washed twice with cold phosphate buffer saline (PBS) and lysed in

homogenization buffer containing 60 mM $\beta$-glycerophosphate, 15 mM p-nitrophenyl phosphate, 25 mM MOPS (pH 7.2), 15 mM EGTA, 15 mM $MgCl_2$, 1 mM DTT, 1 mM sodium orthovanadate, 1 mM sodium fluoride, 1 mM phenyl phosphate, 10 $\mu$g leupeptin/ml, 10 $\mu$g aprotinin/ml, 10 $\mu$g soybean trypsin inhibitor/ml and 100 $\mu$M benzamidine. After sonication, insoluble material was removed by centrifugation at 12 000 *g* for 15 min at 4°C. Protein concentration was determined by the Bradford assay (Biorad). For crude extract analysis, proteins were heat denatured in Laemmli sample buffer and resolved by 7.5 or 10% SDS–PAGE (0.75 mm thick gels) followed by transfer on 0.1 $\mu$M nitrocellulose filters (Schleicher and Schuell). These were blocked with 5% low fat milk in Tris-Buffered Saline-Tween-20 for 1 h at room temperature, then incubated with anti-ERK1 (1 : 4000, 1 h) (Sigma #M7927), anti-PSTAIRE (1 : 3000, 1 h) (Sigma #P7962), anti-PARP (1 : 2000, 1 h) (BIOMOL #SA-250), anti-cyclin $B^{cdc13}$ (1 : 1000, 1 h) (kind gift from Dr M Yamashita), anti-CDK7 (1 : 500, 1 h) (Santa Cruz #sc-529) or TG3 antibodies (1 : 750, overnight) (kind gift from P Davies, New York, USA), and analysed by Enhanced Chemiluminescence (ECL, Amersham). When necessary, the signals on Western blots were quantified using NIH Image.

### *Compounds and affinity matrices*

Aminopurvalanol (NG-97), N6-methylated derivative (NG-97M) and corresponding affinity matrices were synthesized as described (Chang *et al*., 1999; Rosania *et al*., 1999; Knockaert *et al*., 2000). Just before use, 10 $\mu$l of settled beads were washed in 1 ml of Bead Buffer (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, 100 $\mu$M benzamidine) and resuspended in 400 $\mu$l of this buffer. Cell extracts prepared as described above were then added (2 mg total protein) and the tubes were rotated at 4°C for 30 min. After a brief spin, the supernatant was removed and the beads were washed four times with 1 ml Bead Buffer before addition of 50 $\mu$l of Laemmli sample buffer. Following heat denaturation for 3 min, the bound proteins were resolved by SDS–PAGE and analysed by Western blotting as described above.

### *Protein kinase assays*

*MAP Kinase assay* Two CCL39 clones stably transfected with p44/MAPK-HA (Meloche *et al*., 1992) and p42/MAPK-VSVG (Lenormand *et al*., 1993) were serum starved for 24 h, and stimulated with 10% FCS for 5 min. Cell lysates for the two clones were incubated 1 h at 4°C with 10 $\mu$l of the appropriate antibody, anti-HA (Babco #16B12) or anti-VSVG (Roche #1667351) followed by a 1 h incubation with 10 $\mu$l of protein A-Sepharose. Immune complexes were washed four times with homogenization buffer and once with kinase buffer (homogenization buffer with 5 mM EGTA, no NaF and no protease inhibitors). Kinase activity was assayed by resuspending the final pellet in 100 $\mu$l of kinase buffer containing the substrate myelin basic protein (MBP (0.1 mg $ml^{-1}$), ATP (15 $\mu$M) and [$\gamma$-$^{32}$P]ATP (0.15 $\mu$l) and increasing concentrations of aminopurvalanol or DMSO alone. After incubation at 30°C for 20 min, reactions were stopped by addition of 30 $\mu$l of 4× Laemmli sample buffer. The samples were boiled for 5 min and separated by 10% SDS–PAGE. The gels were then stained with Coomassie blue, dried and subjected to autoradiography. Radioactivity was quantified on a Fuji Phosphorimager.

*CDK1/cyclin B kinase assay* Nocodazole-treated CCL39 cells were lysed in homogenization buffer and CDK1/cyclin B was purified by affinity chromatography on $p9^{CKShs}$-sepharose beads. Its activity was assayed with histone H1 (Sigma, type III-S) as a substrate, as previously described (Borgne and Meijer, 1996).

Activities are expressed as the percentage of maximum activity (i.e. in the absence of inhibitor). $IC_{50}$ are determined from the dose-response curves.

### *Transfection and luciferase assays*

The expression vectors encoding the Gal4-Elk1 fusion protein and the Gal4-luciferase reporter gene have been described previously (Brunet *et al*., 1999) and were kindly provided by Dr Brunet. The control vector was the RSV-$\beta$-galactosidase plasmid.

Exponentially growing CCL39 cells were seeded in 24-well plates at a density of $10^5$ cells per well and co-transfected with 0.5 $\mu$g 5× Gal4, 1 $\mu$g of the Gal4–Elk1 fusion construct and 0.1 $\mu$g $\beta$-galactosidase plasmid using the calcium phosphate technique. After a 6 h incubation with the calcium–phosphate–DNA precipitate, cells were allowed to recover for 12 h in fresh medium containing 7.5% FCS. Cells were then deprived of serum for 12 h prior to a subsequent 12 h long stimulation by 10% FCS in the presence of aminopurvalanol or DMSO. Cells were lysed in 200 $\mu$l lysis buffer and luciferase and $\beta$-galactosidase activities were assayed as described (Milanini *et al*., 1998).

### *Indirect immunofluorescence*

Indirect immunofluorescence analysis was performed as previously described (Lenormand *et al*., 1998). CCL39–ΔRaf-1 : ER cells were fixed at −20°C for 10 min with methanol/acetone (70 : 30, vol/vol) without previous PBS wash. After a 10 min rehydration at 25°C in PBS containing 10% FCS (PBS : FCS), fixed cells were then incubated with anti-ERK antibody from Upstate Biotechnologies, Inc., (#06-182) for 60 min at 25°C in PBS/FCS (dilution 1 : 2000). Cells were then washed five times with PBS and incubated in PBS/FCS for 60 min at 25°C with biotin coupled anti-rabbit from Amersham (1 : 500) and with FITC coupled streptavidin from Amersham (1 : 500). Finally, cells were washed five times with PBS, mounted under glass coverslides with CITIFLUOR media, and examined under epifluorescent illumination.

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