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Identifying *in vivo* targets of cyclin-dependent kinase inhibitors by affinity chromatography

Marie Knockaert[a], Laurent Meijer[a,b,*]

[a]*Station Biologique de Roscoff, C.N.R.S., BP 74, Cell Cycle Group, Place Georges Teissier, 29682 Roscoff cedex, France*
[b]*Laboratory of Molecular and Cellular Neuroscience, The Rockefeller University, 1230 York Avenue, New York, NY 10021-6399, USA*

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Abstract

Cyclin-dependent kinases (CDKs) regulate the cell division cycle, apoptosis, transcription, differentiation and many functions in the nervous system. The frequent deregulation of CDKs in cancers and in numerous other pathologies justifies the active search for chemical inhibitors capable of reversibly and selectively inhibiting this class of enzymes. Intensive screening of collections of natural and synthetic compounds has led to the identification of several families of ATP competitive CDK inhibitors. As the therapeutic potential of the most promising compounds is currently being evaluated in preclinical and clinical trials, their mechanism of action is still unclear. In particular, the real spectrum of their intracellular targets remains largely unknown. Determination of the selectivity of the compounds and identification of their intracellular targets constitute a prerequisite to understand their cellular effects and to improve their efficiency on a rational basis. The classical method for the determination of a compound's selectivity consists in testing the compound in a panel of purified kinases. However, the selectivity study is then restricted to the panel's enzymes. As a consequence, many, if not most other potential targets are not evaluated. As an alternative way to investigate the range of true targets of CDK inhibitors, we propose an affinity chromatography approach based on immobilized inhibitors. Briefly, the inhibitor is covalently bound to a resin and cellular extracts are batch loaded on this inhibitor matrix. After extensive washing, the bound proteins are resolved by SDS–PAGE and identified by microsequencing. In addition to confirming the interaction of CDK inhibitors with CDKs, this method has led to the identification of additional, sometimes unexpected, targets. We here illustrate the potential of this technique through a few examples.

Keywords: Cyclin-dependent kinases (CDKs); Inhibitors; Selectivity; Affinity chromatography; Purvalanol; Paullone

1. Introduction

Among the estimated 800 human protein kinases, CDKs have been extensively studied because of their essential cellular functions [1–4]. CDKs are low molecular weight serine/threonine kinases (34–40 kDa), and they have the distinctive feature of being inactive by themselves. They constitute the catalytic subunit and they require association with a regulatory subunit, the cyclin, to be active. So far, 10 CDKs and 12 cyclins have been identified in human: the known CDK/cyclin complexes are presented in Fig. 1 (center and left). The CDKs which associate with cyclins F, G, and I have not been identified yet. In addition, there are several ''CDK-related kinases,'' with no identified cyclin partner (Fig. 1, right). They are easily recognized by their sequence homology to *known* CDKs and by the presence of a variation of the conserved ''PSTAIRE'' motif, involved in cyclin binding. Until their associated cyclin is identified, these ''CDK-related kinases'' are named following the sequence of their PSTAIRE motif.

The most extensively studied role for CDKs is cell cycle control. Indeed, the progression through the G1, S, G2, and M phases is regulated by the transient activation of CDK/cyclin complexes. In addition, several other cellular functions of CDKs have been highlighted more recently. They are involved in apoptosis (CDK2, CDK5), in transcription (CDK7, CDK8, CDK9), in neuronal functions (CDK5), and in differentiation (CDK4, CDK5, CDK9).

Deregulations of these CDKs and their activators or inhibitors are frequently observed in cancers, suggesting that CDKs are relevant molecular targets for anticancer

* Corresponding author. Tel.: +33-2-9829-2339; fax: +33-2-9829-2342.
E-mail address: meijer@sb-roscoff.fr (L. Meijer).

Abbreviations: CDKs, cyclin-dependent kinases; GSK-3, glycogen synthase kinase 3; mMDH, mitochondrial malate dehydrogenase.

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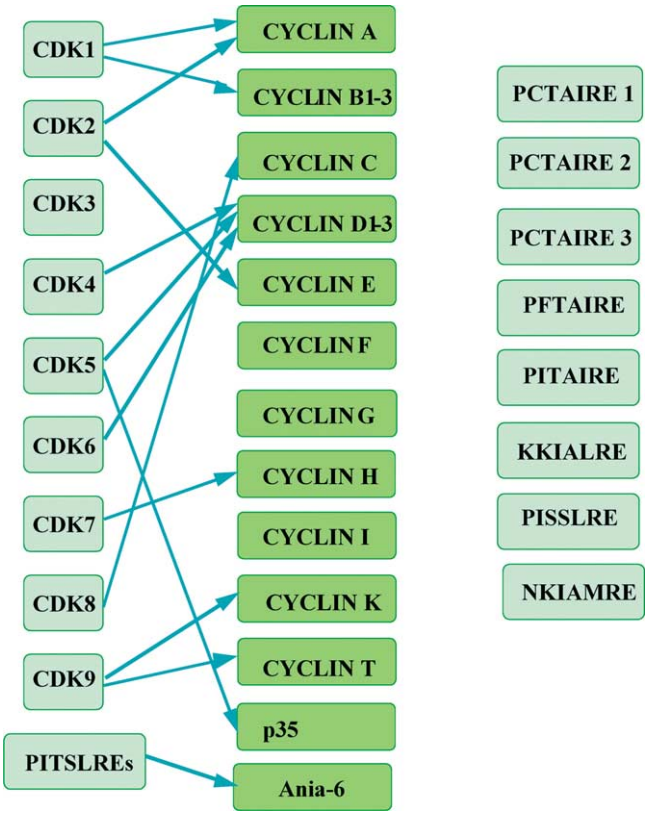

Fig. 1. CDKs, cyclins and CDK-related kinases. Arrows link cyclins to CDKs as found *in vivo*. Left: identified CDKs; centre: identified cyclins; and right: CDK-related kinases (according to sequence homology).

drug design. These observations encouraged the development of screening programmes, aiming at identifying chemical inhibitors of cyclin-dependent kinases, mostly CDK1, CDK2, and CDK4 [5–12]. Intensive screening of collections of natural and synthetic compounds has led to the identification of numerous families of CDK inhibitors. The most studied compounds are presented in Fig. 2. Each compound constitutes a lead structure, further optimized by structure/activity relationship studies, in order to improve its efficiency and selectivity. All chemical CDK inhibitors identified so far share a common mechanism of action: they act by competing with ATP for binding in the ATP-binding pocket of the catalytic subunit. CDK inhibitors display antiproliferative properties, arresting the cell cycle in G1 and G2/M phases, depending on the cell type and on the conditions. With respect to apoptosis, CDK inhibitors appear to have opposite effects depending on whether the cell is cycling or not: in dividing cells, these molecules tend to induce apoptosis, alone or in combination [13,14], whereas in nondividing cells (neurons, cardiomyocytes, thymocytes), CDK inhibitors protect from apoptosis [14–16]. This suggests that CDKs may be involved both in triggering and inhibiting apoptosis. This complex situation is currently under active investigation. The therapeutic potential of the most promising compounds is currently evaluated for cancer treatment [17,18], but the involvement of CDKs in a wide range of cellular functions different from cell cycle control, is likely to extend the CDK inhibitors' field of applications.

1.1. Selectivity of cyclin-dependent kinase inhibitors

Efforts to identify chemical CDK inhibitors with pharmacological interest tend to identify molecules with high efficiency and selectivity, to satisfy the two requirements for the clinical use of a drug, efficiency and harmlessness. Drug candidates are expected to inhibit efficiently the target they have been optimized for (CDKs), but also to be as inefficient as possible on the activity of the other cellular enzymes/proteins, to limit undesired side effects. Determination of the selectivity of the compounds and the identification of their intracellular targets constitute a crucial step in understanding their cellular effects.

Given the large number of ATP-binding proteins present in cells, the *in vivo* selectivity of these ATP competitive CDK inhibitors remains an open question. The most currently used method to assess the selectivity of a compound consists in testing this compound against a panel of purified, often recombinant kinases as illustrated in [19–21]. However, this approach appears to be rather unsatisfying for several reasons. First, it requires the expression, purification and assay of each individual kinase, a very tedious process. Second, the number of enzymes we can test only represents a minor fraction of the total number of kinases in the cell. Third, potential nonkinase targets are not evaluated. To overcome these drawbacks and to address the selectivity question in a wider manner, we recently developed an alternative method for the investigation of intracellular targets of CDK inhibitors. This technique is based on purification by affinity chromatography on immobilized inhibitors. It is summarized in Fig. 3. Briefly, a linker (polyethylene glycol) is attached to an appropriate place on the inhibitor (the crystal structure data of the inhibitor with CDK2 provides helpful information on the most suitable positions) and this linker is then covalently bound to an agarose matrix. Extracts from various cell types and tissues are batch loaded on the inhibitor matrix. After extensive washing of the resin, bound proteins are then eluted, separated on polyacrylamide gel electrophoresis, and identified by microsequencing. Using this technique, we investigated the intracellular targets of two CDK inhibitors, purvalanol and paullones.

Purvalanol was identified as a CDK inhibitor in 1998, after intensive screening of a combinatorial chemistry library of 2,6,9-trisubstituted purines [10,22,23]. This compound demonstrates a remarkable inhibitory efficiency towards CDK1, CDK2, and CDK5 (nanomolar IC_{50} values) and its selectivity is outstanding (Fig. 4, left). In the kinase panel against which it was screened (24 enzymes), besides CDKs, purvalanol only inhibits a few other kinases (including p44/MAPK (Erk1) and p42/MAPK (Erk2)), but to a lesser extent (micromolar range IC_{50}). For the

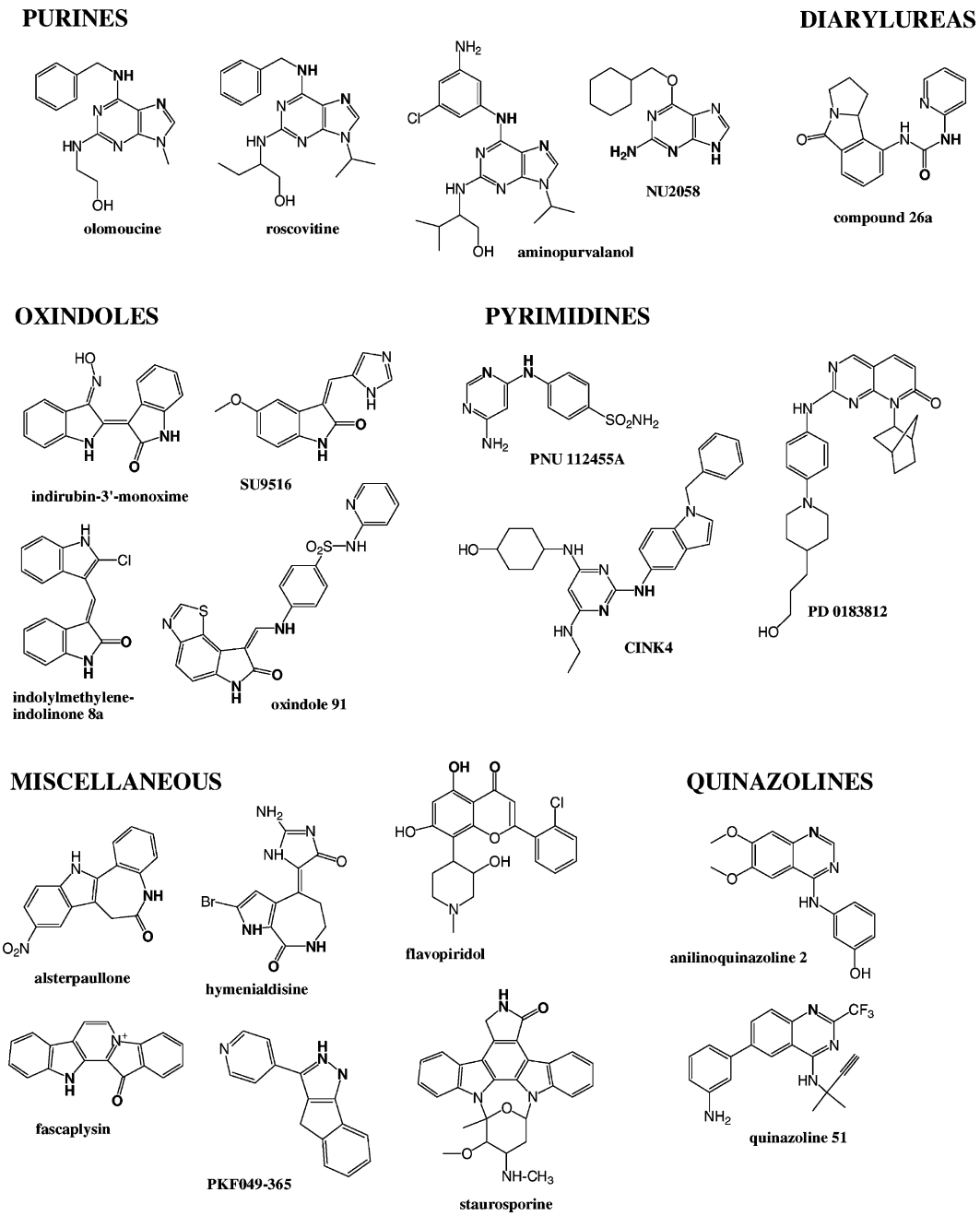

Fig. 2. Structures of some of the most studied pharmacological inhibitors of CDKs. CDK inhibitors share several common properties: a molecular weight below 600, they are flat, hydrophobic heterocycles, and they act by competing with ATP.

reasons mentioned earlier, this selectivity study was completed by an affinity chromatography approach, to investigate the real intracellular targets of purvalanol. The CDK2/purvalanol crystal structure [20,22], 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 (Fig. 3). This side chain was used to couple purvalanol to an agarose matrix. As a negative control, an inactive, N^6-methylated, purvalanol was also immobilized on agarose. The immobilized purvalanol beads were used to screen various cell types and tissues for purvalanol-interacting proteins [23,24]. An example of the porcine brain proteins recovered from the purvalanol matrix is shown in Fig. 4, right.

As expected, CDKs were found to interact with purvalanol matrix, in most models tested: starfish oocytes, sea urchin eggs, *Xenopus* oocytes, porcine brain, and mammalian cell lines. Nevertheless, in some cases, a few other kinases were also recovered from the purvalanol matrix, in addition to CDKs. In particular, p42/p44 MAPKs were identified as major purvalanol-interacting proteins in a large number of models: lugworm oocytes, *Xenopus* oocytes, porcine brain (Fig. 4, right), and mammalian cell lines. In the MCF-7 cell line, p42/MAPK was

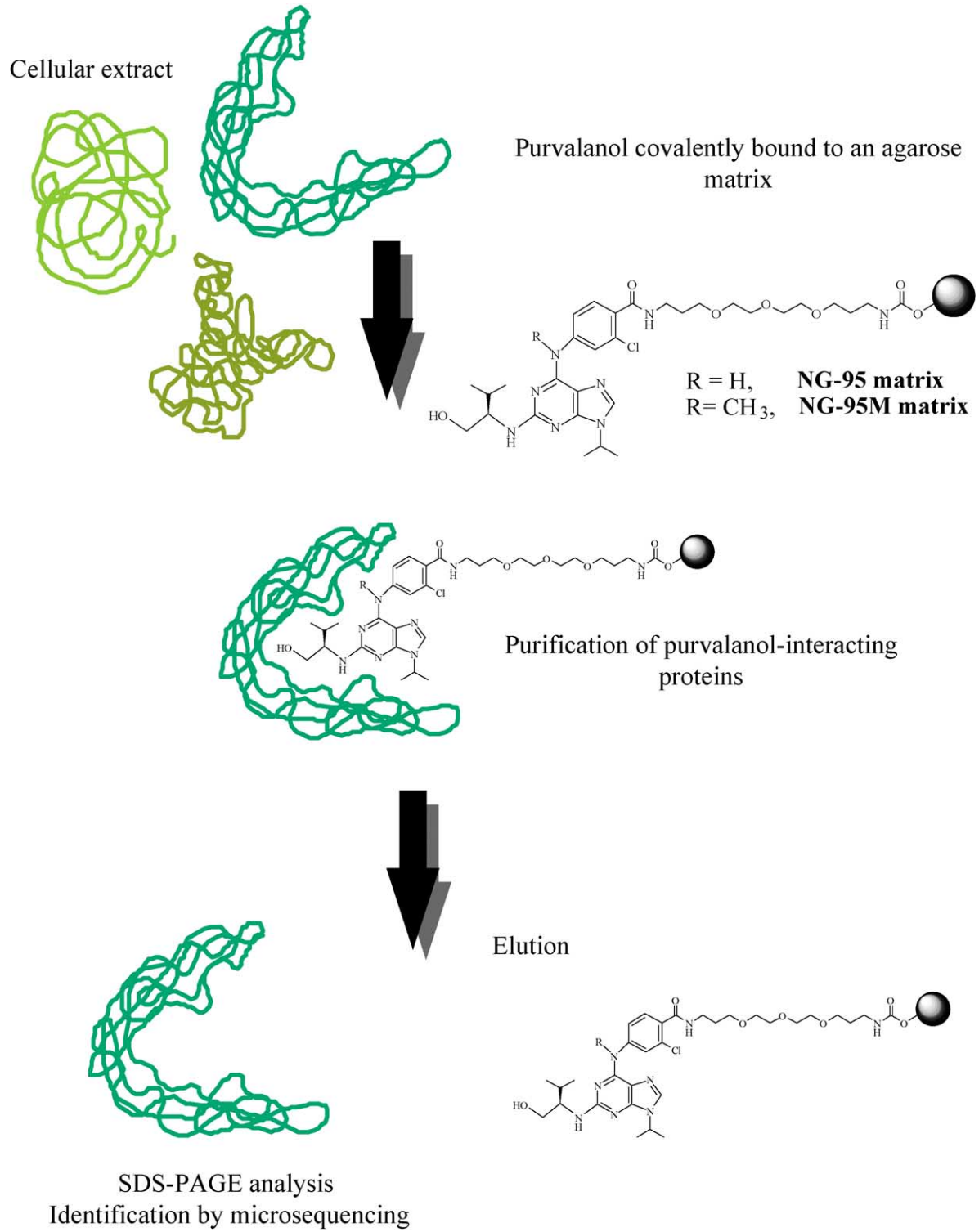

Fig. 3. Affinity chromatography purification of the targets of CDK inhibitors. The inhibitors, purvalanol (NG-95) in this example, is immobilized to agarose beads through a polyethylene glycol linker. A control resin is also prepared using N^6-methyl-purvalanol (95 M), a kinase-inactive compound. Cell/tissue extracts are then run on the matrices. After extensive washing, the affinity matrix-bound proteins are resolved by SDS–PAGE. The proteins are then excised from the gel and identified by microsequencing.

even found to be the major purvalanol-interacting protein [24].

These observations are quite striking, given the large difference between the IC_{50} values of purvalanol for CDKs and MAPKs *in vitro*. 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) but also with targets for which it has a moderate affinity, but which are more abundant

In vitro selectivity (panel of purified kinases)

Kinases	IC_{50} (nM)
CDK1/Cyclin B	6
CDK2/Cyclin A	6
CDK2/Cyclin E	9
CDK5/p35	6
p44mapk/ Erk1	3 333
p42mapk/ Erk2	1 000
Casein kinase 1	3 333
Insulin-receptor tyrosine kinase 2	2 200
cAMP-dependent protein kinase	3 800
CDK4/Cyclin D1	>10 000
c-jun NH2-terminal kinase	>10 000
Protein kinase C α	>100 000
Protein kinase C β1	>100 000
Protein kinase C β2	>100 000
Protein kinase C γ	>100 000
Protein kinase C δ	>100 000
Protein kinase C ε	>100 000
Protein kinase C η	>100 000
Protein kinase C ζ	>100 000
cGMP-dependent protein kinase	>100 000
GSK-3 β	>10 000
Casein kinase 2	>10 000
V-abl	>100 000
Raf kinase	>10 000

In vivo selectivity (affinity chromatography on immobilised inhibitor)

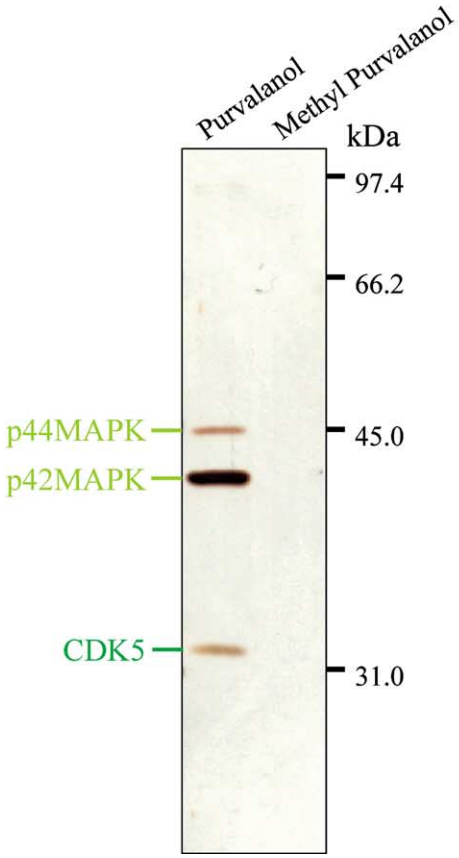

Fig. 4. Methods for testing the selectivity of CDK inhibitors: the example of purvalanol. Left: the classical method consists in testing the compound against a panel of purified kinases. The selectivity data of purvalanol is represented with a color gradient: dark green, nanomolar IC_{50}; light green, micromolar IC_{50}; black, insensitive kinases. Right: affinity chromatography on immobilized inhibitors. In this example, using a porcine brain extract, the targets of purvalanol were identified as p44MAPK/Erk1, p42MAPK/Erk2, and CDK5. They were absent from control beads (methyl-purvalanol).

(MAPKs). As a consequence, the cellular effects of purvalanol are probably accounted for mechanisms more complex than we initially anticipated and in particular, inhibition of p42/p44/MAPKs may significantly contribute to its observed cellular effects. To challenge this hypothesis, we analyzed if purvalanol actually induced an inhibition of the MAPK pathway in the cell. We found that a purvalanol treatment resulted in dose-dependent inhibition of the p42/p44/MAPK pathway, thus validating MAPKs as relevant purvalanol targets [25].

More recently, we immobilized another class of very potent CDK inhibitors, the paullones [26]. Paullones were first identified as CDK1, CDK2, and CDK5 inhibitors in 1999, using the COMPARE analysis of a database of compounds tested in the NCI *in vitro* cancer cell line panel [27,28]. During a classical selectivity study performed on 25 purified kinases, we found that paullones not only inhibited GSK-3 [29] but also were even more potent on GSK-3 than on CDKs. Nevertheless, the true selectivity of paullones needed to be defined and we undertook to investigate their intracellular targets by affinity chromatography. Paullones have not been co-crystallised with CDK2. However, they have been docked into the ATP-binding pocket of a homology model of CDK1/cyclin B developed from the crystal structure of CDK2/cyclin A [30]. This has allowed us to select the most suitable position on the inhibitor, where the attachment of the linker would not hamper the kinase/inhibitor interaction. The linker was then covalently bound to an agarose matrix and the resin was processed as previously described for purvalanol beads. Ethanolamine beads (with no inhibitor) were used as a negative control. As expected, GSK-3 was recovered from the paullone matrix, in most models tested, thus confirming the strong interaction between paullones and this family of enzymes. Surprisingly, CDKs were not detected on paullone beads, loaded with extracts rich in CDK1 (starfish oocytes) and CDK5 (porcine brain), or even with purified CDKs. The fact that CDKs are less abundant than GSK-3 and about 10 times less sensitive to paullones is unlikely to explain their lack of binding to immobilized paullones. We believe that CDKs are unable to bind to paullone matrix because of some steric hindrance due to the proximity of the beads which may be too close from the ligand. Extension of the linker may therefore be a

requirement to allow CDK binding. Quite unexpectedly, in a wide range of tissues and cells, including mammalian tissues and cell lines, sea urchin eggs, *Xenopus* oocytes, and a parasitic protozoan *Leishmania mexicana*, mMDH was found to interact with the paullone matrix. These results prompted us to investigate the molecular basis for the interaction between paullones and mMDH; we thus tested the effects of various paullones on the activity of purified porcine heart mMDH obtained from a commercial source. Paullones inhibit purified mMDH in a dose-dependent manner (micromolar IC_{50} in the values) and kinetic studies revealed that this inhibition is due to competition with NAD/NADH binding. Whether paullones are able to inhibit mMDH in a cellular context and the consequences of mMDH inhibition need to be determined, in the perspective of the therapeutic use of paullones.

Using an affinity chromatography approach similar to ours, glycogen phosphorylase was recently identified as a major flavopiridol-binding protein [31,32]. This CDK inhibitor indeed inhibits glycogen phosphorylase by binding to the purine-inhibitory site of the enzyme [32,33]. This unexpected result suggests that the antiproliferative (review [34]) and antitumoral properties (reviews: [18,35]) of flavopiridol may be due to inhibition of intracellular mechanisms other than CDK activity. These include inhibition of glycogen phosphorylase [32,33], GSK-3α/β [36], and cytosolic aldehyde dehydrogenase [31] as well as interaction with multidrug resistance protein 1 [37,38].

2. Conclusions

Efforts to identify CDK inhibitors first start with *in vitro* studies, using purified, often recombinant enzymes. Following this *in vitro* study, the properties of the most promising compounds are then evaluated in a cellular context. In the perspective of the therapeutic use of CDK inhibitors, performing this thorough cellular evaluation is crucial, as 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, intracellular localization of targets and/or compounds, and interaction with other targets. Affinity chromatography studies, illustrated here with purvalanol, paullones and flavopiridol have confirmed, in most cases, the interaction between the anticipated target(s) (CDKs and/or GSK-3) and the inhibitor. This validation step is crucial, as their optimization as potent *in vitro* CDK inhibitors does not imply they are potent *in vivo* CDK inhibitors. This approach has also revealed unexpected targets of CDK inhibitors, and these new targets were tested for their sensitivity to the original compound. Whether their inhibition participates to the pharmacological properties of the compounds needs to be investigated, in the perspective of their clinical use.

We feel that the development of this affinity chromatography approach to any other molecules under therapeutic development may facilitate a better understanding of their cellular effects. On one hand, it confirms that the enzyme the compound is optimized against, is indeed targeted, and on the other hand, it allows the identification of possibly important additional targets. Inhibition of these unexpected targets may either participate to the desired pharmacological effect or, in contrast, contribute to undesired side effects. In any case identification of these additional targets will help in improving the therapeutic properties of the initial kinase inhibitors.

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