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Identification of cytosolic aldehyde dehydrogenase 1 from non-small cell lung carcinomas as a flavopiridol-binding protein

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Abstract The synthetic flavone flavopiridol can be cytostatic or cytotoxic to mammalian cells, depending on the concentration of the drug and the duration of exposure. It has been shown to inhibit the cyclin-dependent kinase (CDK) family of cell cycle regulatory enzymes. However, the existence of additional potential targets for drug action remains a matter of interest to define. To identify cellular targets, flavopiridol was immobilized. CDKs, particularly CDK 4, bound weakly to immobilized flavopiridol when ATP was absent but not in its presence. Two proteins with molecular weights of 40 kDa and 120 kDa had high affinities to the immobilized flavopiridol independent of the presence of ATP. They were present in all cell lines analyzed: cervical (HeLa), prostate and non-small cell lung carcinoma (NSCLC) cell lines. A 60-kDa protein, which was present only in NSCLC cells and bound similarly well to immobilized flavopiridol, was identified as cytosolic aldehyde dehydrogenase class 1 (ALDH-1). The level of this protein correlated with the resistance of NSCLC cell lines to cytotoxicity caused by 500 nM flavopiridol but not higher flavopiridol concentrations. Despite binding to ALDH-1, there was no inhibition of dehydrogenase activity by flavopiridol concentrations as high as 20 μM and flavopiridol was not metabolized by ALDH-1. The results suggest that high cellular levels of ALDH-1 may reduce cytotoxicity of flavopiridol and contribute to relative resistance to the drug. This is the first report that flavopiridol binds to proteins other than CDKs.

Key words: Flavopiridol; CDK; Aldehyde dehydrogenase class 1; NSCLC; Drug resistance

1. Introduction

Recent research in chemotherapeutic development has focused on drugs targeting signal transduction and cell cycle proteins, which are often mutated or deregulated in transformed cells. Cyclin-dependent kinases (CDKs) play a central role in growth regulation as a driving force for cell cycle progression (for reviews see [1–3]). A few drugs have been developed which inhibit CDK activity in vitro. Among those the synthetic flavone flavopiridol is currently in clinical trials as a potential antitumor agent and has already shown therapeutic potential [4,5]. Flavopiridol induces reversible G1 and G2 phase cell cycle arrest independently of the mutational status of the tumor suppressor proteins p53 and pRB. This cytostatic effect was linked to the direct inhibition of various CDKs [6–9]. CAK (cyclin H/CDK 7), that phosphorylates the Thr residue of CDKs resulting in kinase activation [10], is also inhibited by flavopiridol in vitro [11]. This Thr residue is unphosphorylated also in flavopiridol-treated cells, indicating in vivo inhibition of CDK 7 [12]. The binding site for flavopiridol has been mapped to the ATP pocket of the CDKs [13]. However, the action of flavopiridol is more complex than can be explained by CDK inhibition alone. For example, in some cell types flavopiridol readily induces apoptosis [9,14,15], while in others it does not [16]. Also transient CDK activation occurs early after drug treatment correlating likely with loss of the inhibitory Tyr phosphorylation [8]. At the same time, cyclin D1 level decreases [8,12]. These observations indicate that flavopiridol-induced cell cycle arrest may be modulated by targets in addition to CDKs.

Flavopiridol has shown antiproliferative effects in all tumor cell lines analyzed so far [11]. However, the sensitivity of tumor cells to these cytotoxic effects varies. Certain prostate cancer and homeopoetic cells are the most sensitive [9], while some small cell lung cancer cell lines, breast cancer cell lines and bone marrow cells are resistant even up to high levels of flavopiridol [11]. It is unknown why there are these dramatic differences in sensitivity to flavopiridol among cell lines. This variation cannot be simply explained by different CDK activities, since cell death can also be observed in flavopiridol-treated G0/G1 phase endothelial cells, which do not express active CDKs but not in the NSCLC cell line A549 [14]. The involvement of commonly known drug resistance proteins such as the multi-drug resistance P-glycoprotein has also been excluded [11]. Varying growth rates among cell lines may explain some of the differences, although flavopiridol can cause cytotoxic effects on both proliferating and stationary phase cells [16]. In some cell lines, the cytotoxicity has been identified as apoptosis [9,14,15]. However, cell death of the NSCLC cell line A549, for example, shows neither typical apoptotic nor typical necrotic features [16]. It may be possible that there is more than one mechanism of flavopiridol-directed cell death, which may depend on the cell or tumor type.

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Abbreviations: BME, β-mercaptoethanol; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis; NSCLC, non-small cell lung carcinoma

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In order to identify proteins, which could potentially modulate the actions of flavopiridol, we immobilized flavopiridol and used this reagent as an affinity matrix to isolate proteins from different cell lines. One protein was identified as cytosolic aldehyde dehydrogenase class 1 from the NSCLC cell line A549. Despite its affinity to this enzyme, flavopiridol does not inhibit the enzymatic activity nor is flavopiridol a substrate for ALDH-1.

2. Materials and methods

2.1. Cell culture

HeLa cells were grown in RPMI 1640 medium supplemented with 10% fetal bovine serum (FBS) and antibiotics (Gibco). Human non-small cell carcinoma cell lines (NSCLC) H23, HOP62, H460 and A549 were obtained from NCI Repository, Frederick, MD, USA. Cells were maintained in RPMI 1640 medium supplemented with 5% FBS, 2 mM glutamine, 100 units/ml penicillin and 100 μg/ml streptomycin. All cultures were maintained at 37°C in a 5% CO_2 incubator. For affinity chromatography experiments cells were harvested with Trypsin centrifuged and washed twice with phosphate buffered saline (PBS) and stored in a freezer at −80°C until use.

2.2. Colony-forming growth assay

Exponentially growing cells (5×10^5) were treated with graded concentrations of flavopiridol for 4 h and 24 h. Following drug treatment, cells were washed with serum free RPMI 1640 and trypsinized. Aliquots containing 250 (H460, A549) or 500 (HOP62, H23) cells were plated in triplicates in 6-well plates containing 4 ml medium. Plates were incubated for additional 7 days (H460, A549) or 10 days (HOP62, H23) in a CO_2 incubator. The resulting colonies were then washed with PBS, fixed with methanol, stained with trypan blue (0.04%), and were counted manually. Vehicle-treated control plates typically contained 150–200 colonies (HOP62, H23) or 250–400 colonies (H460 and A549). Cloning efficiency of vehicle-treated control cells were 42% for H23, 33% for HOP62, 50% for H460 and 74% for A549.

2.3. Affinity chromatography

The flavopiridol affinity resin was synthesized as follows: 0.33 g epoxy-activated Sepharose 6B (Pharmacia, equivalent to about 1 ml swollen resin) was washed according to the manufacturer's protocol. The resin was added to 5 ml 0.1 M $NaHCO_3$ (pH 10.5) containing 6 μMol flavopiridol and incubated at 37°C for 24 h. The soluble concentration of flavopiridol was determined at 260 nm in a photometer before and after the coupling procedure. 87% flavopiridol was coupled corresponding to 5.25 μMol per ml resin. To block unreacted groups, the resin was incubated for 4 h with 1 M ethanolamine. We expect that flavopiridol is linked to the resin via the hydroxyl groups.

For chromatography experiments, cells were lysed in 3 ml of lysis buffer (50 mM Tris/HCl (pH 7.5), 250 mM NaCl, 0.1% NP40, 5 mM EDTA, 50 mM NaF) per 1 ml of packed cells (about 1×10^8 cells). Protease inhibitors were added in concentration of 1 mM for phenylmethyl-sulfonyl-fluoride, 25 μg/ml for aprotinin and 25 μg/ml for leupeptin. The cells were lysed in a homogenizer or by pipetting through a 26-gauge size needle. The suspension was centrifuged for 10 min at 15 000 rpm in a SS34 rotor in a Sorvall centrifuge. The protein amount of the supernatant was determined with a Bradford protein determination assay (Biorad). The cell extract was diluted with equal volume of binding buffer (20 mM Tris/HCl (pH 8.0), 250 mM NaCl, 5 mM $MgCl_2$). 1.5 ml of this cell extract was passed over a 1 ml flavopiridol affinity resin. The resin was washed with 16 column volumes binding buffer. Bound proteins were eluted with either a saturated solution of flavopiridol in binding buffer (Note: flavopiridol has low solubility in H_2O) or with dimethyl formamide (DMF) concentrations up to 20% in binding buffer. Proteins were concentrated by precipitation in 10% trichloro acetic acid and centrifugation. The protein pellets were washed with acetone. Proteins were analyzed by SDS-PAGE [17].

2.4. Preparation of proteins for mass spectral analysis

Approximately 20 pmoles of the affinity-purified p60 protein was precipitated with 5% TCA and washed twice with acetone (−20°C). The protein was dissolved in 20 ml 10 mM Tris/HCl (pH 8.2) with 1 mM BME. After 1 h, iodoacetamide was added at a final concentration of 3 mM. After 30 min at 30°C, BME was added to 6 mM. The pH was raised to pH 8.6 with Tris (30 mM), 100 ng Trypsin (modified sequencing grade, Boehringer Mannheim) added, and digestion carried out at 37°C for 12 h. Peptides were separated on a Michron HPLC with a microbore reverse phase column and analyzed by MS/MS on a Finnigan MAT LCQ. The MS/MS data was analyzed by 'Sequest' software based on a FASTA Swiss Protein database and individual tryptic peptides matches with predicted fragmentation patterns of y (C-terminal) and b (N-terminal) ions.

2.5. ALDH-1 enzyme assays

For ALDH-1 enzyme assays either purified or partially purified ALDH-1 was used. For ALDH-1 purification, 2 ml of packed A549 cells were lysed as described and cell extract was passed over flavopiridol affinity columns. ALDH-1 was eluted with 20% DMF in binding buffer and BME was added to a final concentration of 10 mM. The effluents were concentrated by centrifugation in concentrators (FILTRON) with 30 kDa molecular weight cut off. The final volume was 0.2 ml. The proteins were further separated by FPLC using a Superose 12 column. Partially purified ALDH-1 was prepared as described using $100\,000\times g$ supernatant [18]. The enzyme assays were carried out as described [18]. Flavopiridol, dissolved in DMF, was added to test inhibition of ALDH-1. The highest DMF concentration in the assay was 2%, which did not have any effect on the enzyme activity.

2.6. Determination of flavopiridol concentration following incubation with ALDH-1

To test the modification of flavopiridol by ALDH-1, flavopiridol was incubated with ALDH-1 as described above and the flavopiridol concentration was determined as described [19]. Briefly, flavopiridol was isolated from the samples, following alkalinization, by extraction with *t*-butylmethyl ether. The extract was evaporated; the residue was reconstituted in mobile phase and analyzed by reversed phase HPLC using electrochemical detection. All samples were analyzed in duplicate. Samples, whose duplicate determinations did not agree within 10%, were reanalyzed until acceptable reproducibility was obtained.

2.7. Immunoblot analysis

Anti-CDK 1 (CDC 2) antibodies have been described [20]. Anti-CDK 2, 4 and 7 antibodies were purchased from Santa Cruz Biotechnology. Polyclonal anti-ALDH-1 antibodies were prepared by 'Genemed' against the unique peptide (C)SSSGTPDLPVLLTDL-COOH. The immunoblot analysis was carried out as described [21].

3. Results

One possible approach to identify in vivo drug targets is to isolate proteins with the highest affinity to these drugs using affinity chromatography. Flavopiridol has two hydroxyl groups, which are chemically reactive. In a typical experiment, about 5 μMols flavopiridol were coupled to 1 ml of Sepharose 6B. For chromatography experiments, HeLa cell extract was prepared in lysis buffer and diluted with binding buffer containing 0.5 M NaCl, to eliminate unspecific salt-sensitive binding of proteins to the affinity resin. Elution was done either with a saturated flavopiridol solution in buffer or with increasing amount of organic solvent such as dimethyl formamide (DMF) or dimethyl sulfoxide (DMSO). The eluted proteins were concentrated and then separated by SDS-PAGE. In HeLa cells the most prominent band after staining the gel with silver nitrate corresponded to a 120-kDa protein followed by a 40-kDa and a 28-kDa protein (Fig. 1). While all proteins were in the same fractions when eluted with flavopiridol (Fig. 1A), they were eluted stepwise with increasing amounts of DMF (Fig. 1B). This is strong evidence for specific binding of these proteins to the resin-bound flavopiridol. There were some background bands, but these could be degradation products of the 120-kDa protein.

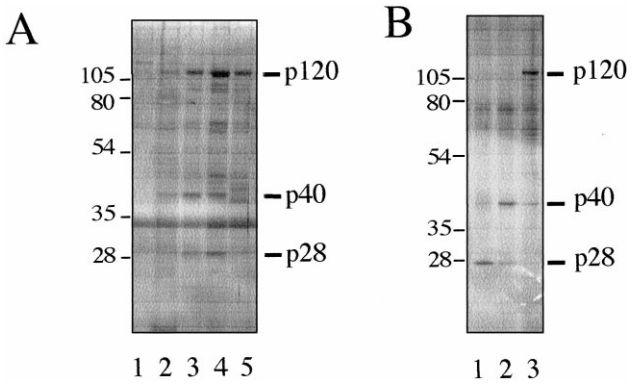

Fig. 1. Affinity chromatography with flavopiridol as ligand. A: Elution with flavopiridol. Lane 1, last buffer wash; lanes 2–5, 1 ml saturated flavopiridol each. B: Elution with DMF; lane 1, 10% DMF; lanes 2–3, 20% DMF. The proteins were separated by SDS-PAGE and stained with silver nitrate.

Flavopiridol inhibits CDKs' activity in vitro and the binding site has been determined to be the ATP pocket of these kinases [11,13]. We tested the presence of CDK 1, 2, 4 and 7 in wash and effluent fractions by immunoblot with the corresponding antibodies after separation of proteins by SDS-PAGE (Fig. 2, upper panel). CDK 1 was detected only in the flow through and the first wash fraction. CDK 2, 4 and 7 were also detected in further wash fractions. Unlike the 40- and 120-kDa proteins, the CDKs were not significantly retarded by the column. This result indicated that the CDKs

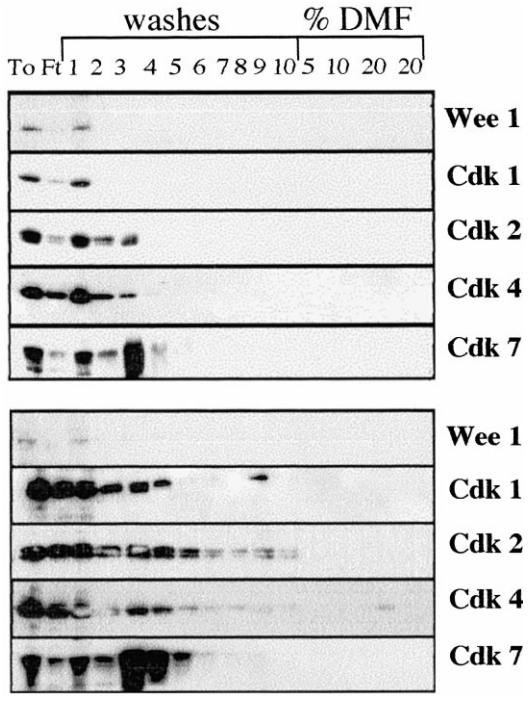

Fig. 2. Immunoblots of proteins from flavopiridol affinity chromatography with anti-CDK antibodies. Upper panel: The chromatography was performed as described in Fig. 1B except that the washes were collected in ten 1 ml wash fractions. The flow through, the first and second washes were not concentrated. All other fractions were concentrated by TCA precipitation. Lower panel: The chromatography was performed as in the upper panel, except that the cell extract was incubated with 5 μg/ml apyrase for 1 h. To, total cell extract; FT, flow through; lanes 1–10, washes.

do not bind tightly to the immobilized flavopiridol under these conditions. It is likely for steric reasons that the coupling of flavopiridol to Sepharose 6B blocks or partially blocks the binding site for CDKs, since the crystal structure of flavopiridol bound to CDK 2 suggests contact of one of the -OH residues with the enzyme [13]. Alternatively it is also possible that high levels of ATP in the cell extract compete with flavopiridol for binding to CDKs. To test this, we added apyrase to the HeLa cell extract to degrade ATP. When the binding of CDKs to the column was analyzed, we found that CDK 1, 2 and 4 were much more retarded by the flavopiridol resin than in the presence of ATP (Fig. 2, lower panel). A small fraction of CDK 4 was found in the 20% DMF effluent. This result confirms previous findings that flavopiridol binds to the ATP pocket of CDKs [13]. However, ATP easily abrogates this binding. The binding of the 28-, 40- and 120-kDa proteins was not affected whether ATP was present or not.

NSCLC cell lines, in particular the A549 cell line, are among those which are rather resistant to the cytotoxic effect of flavopiridol [11,14]. Affinity chromatography with cell extract from the A549 cell line using the flavopiridol affinity column showed that in addition to the 40- and 120-kDa proteins there was a protein of about 60 kDa present (Fig. 3). Because of its abundance and possible involvement in flavopiridol resistance, p60 was further purified by HPLC. The mass of a total of 25 peptides was determined after Trypsin digestion of the protein by ion trap mass spectroscopy analysis. The masses and spectra of all peptides showed a match with a single protein, which was identified as cytosolic aldehyde dehydrogenase class 1 (ALDH-1). The ALDH family of proteins functions in metabolism of aldehydes to protect cells from their toxicity [22–24]. Multiple ALDH isoforms have been characterized and have different substrate specificity. They have been classified according to their amino acid sequence homology as ALDH-1 (cytosolic), ALDH-2 (mitochondrial) and ALDH-3 (cytosolic and mitochondrial).

Among four NSCLC cell lines, A549 was found to be more resistant to cell death by flavopiridol compared to three other cell lines despite the fact that cell death in NSCLC cell lines is independent from the expression of bcl-2 [25]. This is also

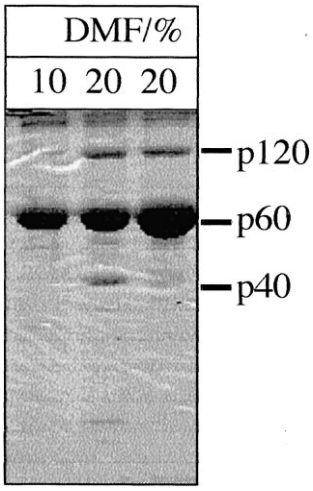

Fig. 3. Proteins bound to immobilized flavopiridol from A549. The chromatography conditions are as in Fig. 1. Elution was done with 10 and 20% DMF. The concentrated proteins were separated by SDS-PAGE and stained with Coomassie blue.

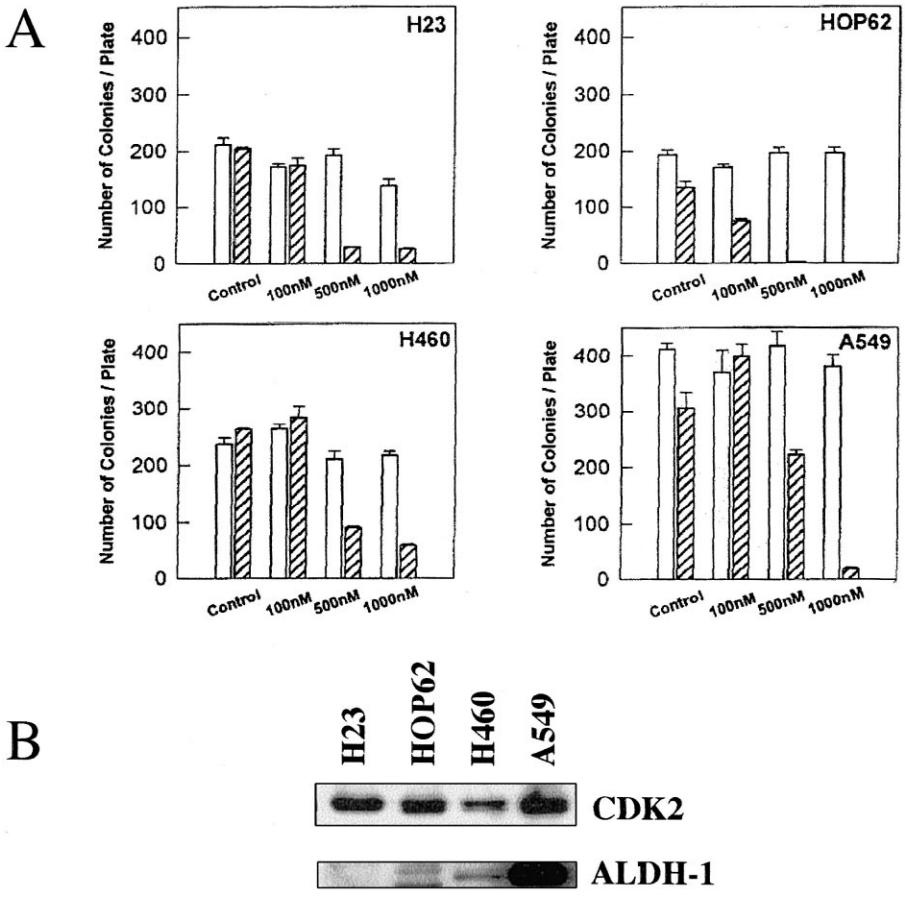

Fig. 4. Correlation of cytotoxicity of flavopiridol on NSCLC cells with ALDH-1 level. A: Colony-forming assay: cells were exposed to flavopiridol (100, 500, or 1000 nM) or vehicle for 4 h (open bar) or 24 h (hatched bar). Colonies formed after drug removal were enumerated as described in Section 2. Values are means ± S.E.M. of three experiments. B: Immunoblot with anti-ALDH-1 antibodies and as control with anti-CDK 2 antibodies.

shown by a colony-forming assay (Fig. 4). Flavopiridol diminished the colony-forming ability of the lung cancer cell lines after 24 h but not 4 h of drug exposure (Fig. 4A). H23 and HOP62 were most sensitive, with colony counts reduced to 13 and 0.9% of control; H460 was less sensitive and A549 most resistant, with 34% and 72% of control colony number after exposure to 500 nM flavopiridol for 24 h. We determined the ALDH-1 levels in the four cell lines by immunoblots with anti-ALDH-1 antibodies and as control with anti-CDK 2 antibodies (Fig. 4B). A549 cells had the highest concentration of ALDH-1 followed by H460. H23 and HOP62 had undetectable levels of ALDH-1 compared to these two cell lines. The ALDH activities including ALDH-1 had been determined in the 60 cell lines from the NCI drug screen and the relative ALDH-1 activities of the four NSCLC cell lines are: H23 and HOP62 < H460 < A549, which correlates with the colony formation assay and the immunoblot data [26]. We analyzed if the ALDH-1 level was affected in A549 cells upon flavopiridol treatment, but the level was not changed (data not shown). Although we find correlation of ALDH-1 content (level and activity) with the colony formation assay, the results indicate that there must be additional mechanisms, which interfere with flavopiridol resistance at higher flavopiridol concentrations than 500 nM.

Next we investigated whether flavopiridol inhibits ALDH-1 enzyme activity. We used either partially purified ALDH-1 ($100\,000 \times g$ fraction) [18], or affinity-purified ALDH-1, which was purified further by Superose 12 column chromatography. ALDH-1 activity (enzyme kinetics) was tested alone or in the presence of flavopiridol; however, there was no inhibition of the ALDH-1 activity. The enzyme activity was determined as 1.53×10^{-4} Mol NAD^+ reduced/min/mg protein in the absence or presence of 20 μM flavopiridol in enzyme assays using partially purified ALDH-1. When purified ALDH-1 was used, the activity was determined as 5.4×10^{-3} Mol NAD^+ reduced/min/mg protein in the absence or presence of 10 μM flavopiridol. Due to solubility problems we did not use higher flavopiridol concentrations. Similarly, reducing the substrate (propionaldehyde) concentration 3–10-fold or cofactor (NAD^+) concentration up to 15-fold without changing the drug concentrations did not result in increased inhibition of ALDH-1 by flavopiridol either (data not shown). Thus, flavopiridol binds to ALDH-1 without affecting its activity. We also tested if flavopiridol is a substrate for ALDH-1. Flavopiridol was incubated for up to 1 h with purified ALDH-1 in the absence and presence of NAD^+. However, no change in the concentration of flavopiridol was observed. Thus flavopiridol is not a substrate for ALDH-1.

4. Discussion

We have isolated several flavopiridol interacting proteins.

We expected to find CDKs among the binding proteins, because their enzymatic activities were reported to be inhibited by flavopiridol in vitro with IC_{50} values of 200–400 nM and flavopiridol was shown to bind to the ATP pocket of CDK 2 [13]. CDKs showed indeed a certain affinity for the immobilized flavopiridol but only when the ATP in the cell extract was degraded confirming that ATP competes with flavopiridol for binding to CDKs. The question arises whether these binding conditions also reflect the in vivo binding of flavopiridol to CDKs, since the intracellular ATP concentration is around 5 mM. Our results suggest that flavopiridol has higher affinity to the 28-, 40- and 120-kDa proteins and to ALDH-1, which are retarded on the column throughout all the wash steps independent of ATP, than to CDKs.

One of the proteins we identified was ALDH-1, which was present only in NSCLC cell lines but not in the other two cell lines tested. In enzyme assays we found that ALDH-1 is not inhibited by flavopiridol concentrations up to 20 μM. This would indicate that flavopiridol binds to a unique site on ALDH-1, which is not important for its enzymatic activity. We could further exclude the possibility that ALDH-1 modifies flavopiridol. There are differences in the cytotoxicity of flavopiridol among NSCLC cell lines, which contain ALDH-1, and our results suggest that ALDH-1 may contribute to this. High cellular ALDH-1 levels could bind a considerable amount of flavopiridol in the cytosol and change the sensitivity level of cells to flavopiridol. Since flavopiridol has cytostatic and cytotoxic effects on all cell lines independent of the cell type, binding of flavopiridol to ALDH-1 is, however, unlikely to be the only cause for these effects. A screen of the 60 cell lines used by the NCI in its Developmental Therapeutics Program to test new drugs for the presence of ALDH-1 has shown that NSCLC cell lines have the highest levels of this enzyme [26]. It would be interesting and important to test CDK inhibitors unable to bind to ALDH-1, which might be more useful anticancer agents, owing to their not binding to other cellular components.

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