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ORIGINAL ARTICLE

Ku Lu · Chuan Shih · Beverly A. Teicher

Expression of pRB, cyclin/cyclin-dependent kinases and E2F1/DP-1 in human tumor lines in cell culture and in xenograft tissues and response to cell cycle agents

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Abstract *Purpose*: Cell cycle regulatory components are interesting targets for cancer therapy. Expression of pRb, cyclin D1, cdk4, cyclin E, cdk2, E2F1 and DP-1 was determined in MCF-7 and MDA-MB-468 breast carcinoma cells, H460 and Calu-6 non-small cell lung carcinoma cells, H82 and SW2 small cell lung carcinoma cells, HCT116 and HT29 colon carcinoma cells and LNCaP and DU-145 prostate carcinoma cells. *Methods*: For Western blotting, the ratio with actin expression was used to normalize the data; all lines were run on the same gels. *Results*: In cell culture, pRb was not detected in MB-468 and H82 was low in SW2 and DU-145 and highest in HCT116; in tumors, pRb was not detected in MB-468, H82, SW2, and DU-145 and was highest in LNCaP and Calu-6. Cyclin D1 was not detected in SW2 cells in culture, was low in MB-468 and H82, and was highest in LNCaP and H460; in tumors, cyclin D1 was low in MB-468, H460, SW2 and DU145, and was highest in LNCaP. In cell culture, cdk4 was lowest in Calu-6, HCT116, HT29 and DU-145 and highest in H82 and SW2; in tumors, cdk4 was low in MCF-7, MB-468, H460, Calu-6 and HCT116 and was very high in the SW2. Expression of cyclin E was very low in MCF-7 and HT29 and high in H460 in culture and was very low in MCF-7, H460, Calu-6, H82, HT29 and DU-145 in tumors and high in HCT116 and LNCaP. In cell culture, E2F1 was lowest in MB-468, Calu-6, HT29 and DU-145 cells and highest in LNCaP cells; in tumors, E2F1 was lowest in MCF-7, MB-468 and Calu-6 and highest in LNCaP. In cell culture, DP-1 was lowest in MB-468, HCT116 and HT29 and highest in SW2. The MCF-7 and MB-468 lines were most resistant to flavopiridol and olmoucine and the H460 and Calu-6 lines were most resistant to genistein. The SW2 tumor was most responsive to flavopiridol and olomoucine. *Conclusions*: There is a high degree of variability in the expression of cell cycle components in human tumor cell lines, resulting in complexity in predicting response to cell cycle directed agents.

Key words Cyclins · Cyclin/cdk · pRB

K. Lu · C. Shih · B. A. Teicher (✉)
Lilly Research Laboratories, Lilly Corporate Center, Indianapolis, IN 46285, USA
e-mail: TEICHER_BEVERLY_A@Lilly.com
Tel.: +1-317-2762739; Fax: +1-317-2776285

Introduction

Cancer is a heterogeneous group of diseases presenting in various forms in various tissues but having in common the characteristic of uncontrolled cell proliferation. Cancer has been recognized as a disease of uncontrolled cell proliferation and the proliferating cell has been the target of cancer chemotherapy since the initial development of that treatment modality. As the basic science of the cell progressed, it was shown that certain anticancer agents were more effective against malignant cells at certain stages of the cell cycle than against cells at other stages of the cell cycle, and attempts were made to develop treatment regimens that took advantage of these observations [59]. Cell replication is now known to be controlled by the transient, sequential, highly-regulated expression of a series of cyclins, which associate with specific cyclin-dependent kinases (cdks) to compose an active enzyme and initiate a cascade of phosphorylations, allowing the cell to progress to the next stage of replication [12, 30]. The Rb protein, pRb, a substrate for the cyclin-cdks is frequently missing or mutated in human tumors [3, 5, 17, 23, 25, 26, 38, 43, 52, 57, 63, 69]. Coordinate with this are the cdk inhibitors (CKIs) that block the actions of specific cyclin-cdk complexes. The cdk inhibitors halt cell cycle progression and cause cells to enter the quiescent G_0 phase. The cdk inhibitors of the INK4 group including p15, p16, p18 and p19 block the cyclin-cdk4 and cyclin-cdk6 complexes and are sometimes mutated or deleted in tumors [30, 37, 55, 66, 70]. However, the most frequent alteration in human malignant disease, thus far recognized, is the overexpression, mutation and/or disregulation of cyclin D [22, 29, 32]. The cyclin D1 gene, *CCND1*, is amplified in

about 20% of breast cancers and the protein, cyclin D1, is overexpressed in about 50% of breast cancers [2, 4, 34, 40, 61]. Overexpression of cyclin D1 has been reported in proliferative breast disease and in ductal carcinoma in situ, indicating that this change is important at the earliest stages of breast oncogenesis [2, 61]. Kamalati et al. (1998) [34] overexpressed cyclin D1 in normal human epithelial cells and found that the transfected cells had reduced growth factor dependency, a shortened cell cycle time, thus providing the cells with a growth advantage. In 123 colorectal carcinoma specimens, those staining strongly for cyclin D1 corresponded to patients with a 5-year survival rate of 53.3%, while those that were negative or staining weakly had 5-year survival rates of 96.2% and 78.8% [41, 49]. Amplification of *CCND1* was found in 25% of dysplastic head-and-neck lesions, 22% of head-and-neck carcinomas and overexpression of cyclin D1 was found in 53% of head-and-neck carcinomas, indicating that in this disease, like breast cancer, alterations in cyclin D1 occur at the very earliest stages of tumorigenesis [39, 51]. In a study of 218 specimens of esophageal squamous cell carcinoma, patients with cyclin D1-positive tumors had significantly worse survival than patients with cyclin D1-negative tumors [53]. In eight human esophageal carcinoma cell lines, seven (87.5%) and six (75%) cell lines had homozygous deletions of the *p16* and *p15* genes [37]. All of the p16-negative cell lines express high levels of cyclin D1 and cdk4. Rustgi et al. [45, 46] developed a transgenic mouse in which the Epstein-Barr virus ED-L2 promoter was linked to human cyclin D1 cDNA. The transgene protein localizes to squamous epithelium in the tongue and esophagus, resulting in a dysplastic phenotype associated with increased cell proliferation and indicating that cyclin D1 overexpression may be a tumor-initiating event. In a series of 84 specimens of soft-tissue sarcomas, there was no amplification of the *CCND1* gene but there was overexpression of cyclin D1 in 29% of cases and the overexpression of cyclin D1 was significantly associated with worse overall survival [36, 70]. Marchetti et al. [42] found that abnormalities of cyclin D1 and/or Rb at the gene and/or expression level were present in more than 90% of of a series of non-small cell lung cancer specimens, indicating that cyclin D1 and/or Rb alterations represent an important step in lung tumorigenesis. In 49 out of 50 pancreatic carcinomas (98%), the Rb/p16 pathway was abrogated exclusively through inactivation of the *p16* gene [55]. Mantle cell lymphoma is defined as a sub-entity of malignant lymphomas characterized by the chromosomal translocation t(11;14)(q13;q32) resulting in overexpression of cyclin D1 and, in addition, about 50% of these tumors have deletion of the *p16* gene [16, 62]. In a series of 17 hepatoblastomas, 76% showed overexpression of cyclin D1 and 88% showed overexpression of cdk4 [35]. There was a correlation between high level cyclin D1 expression and tumor recurrence.

Six distinct classes of small molecules from natural products have been identified as inhibitors of cdks: the purine-based compound olomoucine and analogs, butyrolactone, flavopiridol, staurosporine and UCN-01, suramin and 9-hydroxyellipticine [7, 11, 15, 21, 24, 33, 44, 48]. All of these molecules bind at the ATP-binding site of the enzyme and are competitive with ATP. Olomoucine is an inhibitor of Cdc2, cdk2, cdk5 and MAP kinase in micromolar concentrations and has much weaker effects toward cdk4 and cdk6 [21]. Crystallization of olomoucine with cdk2 showed that its adenine side-chain lay in a completely different orientation from the adenine group of ATP [15]. Olomoucine has been reported to arrest several cell lines in G1 and G2 phases of the cell cycle and to block known cdk-dependent cellular activities.

Flavopiridol, a novel synthetic flavone, potently inhibits several cyclin-dependent kinases including cdk1, cdk2, cdk4 and cdk7 [6, 8, 9, 14, 31, 54, 56, 58]. Exposure to flavopiridol can cause cells to arrest in both the G1 and G2 phases of the cell cycle, at concentrations similar to those required for cell growth inhibition [6, 54]. Flavopiridol inhibits the cdks in a manner competitive with ATP and noncompetitive with the substrate. Flavopiridol also inhibits other protein kinases such as protein kinase C, protein kinase A and epidermal growth factor receptor but at concentrations of 10 μM/l or greater. Flavopiridol is an active antitumor agent in several human tumor xenograft models including Colo-205 colon carcinoma, and DU-145 and LNCaP prostate carcinomas [14, 56]. Flavopiridol has completed phase I clinical trial administered as a 72-h continuous infusion every 2 weeks [58] and phase II trials are planned.

Genistein is a principal isoflavone in soybeans and is a potent inhibitor of the activity of tyrosine kinases such as epidermal growth factor receptors [1, 10, 13, 18, 27, 47]. Genistein specifically inhibits growth of *ras* oncogene-transfected NIH 3T3 cells and diminishes the platelet-derived growth factor-induced c-fos and c-jun expression in CH310T1/2 fibroblasts [47]. Genistein inhibited endothelial cell proliferation and in vitro angiogenesis [18]. Genistein has been found to inhibit cell cycle progression in several human and murine cell lines. In MCF-7 and MDA-MB-231 human breast cancer cells, genistein induced a G2/M arrest that was due in part to inhibition of cdk2 [10].

The current study was undertaken to examine the expression of several cell cycle related proteins in ten human tumor cell lines in culture as compared to expression in the same cell lines grown as xenograft tumors. We also wanted to determine whether response to olomoucine, flavopiridol or genistein in cell culture correlated with expression of the cell cycle proteins and to find out whether response in vivo may be similarly associated.

Materials and methods

Cell lines

The MCF-7 breast adenocarcinoma cell line originated from a pleural effusion of a 69-year-old female patient in 1973; the MDA-

MB-468 breast adenocarcinoma cell line originated from a pleural effusion of a 51-year-old female patient in 1977. The H460 large cell lung carcinoma cell line originated from the pleural fluid of a male patient in 1982 and the Calu-6 anaplastic lung carcinoma cell line originated from a 61-year-old female patient treated with radiation therapy in 1976. The NCI-H82 small cell lung carcinoma cell line originated from a 40-year-old male patient from the pleural fluid in 1978; the SW2 small cell lung carcinoma cell line originated from the pleural fluid in 1979 [19, 20]. The HCT116 colon carcinoma cell line originated from a male patient in 1979; the HT29 colon adenocarcinoma cell line originated from the primary tumor of a 44-year-old female patient in 1964. The LNCaP metastatic prostate carcinoma cell line originated from a needle biopsy of the supraclavicular lymph node of a 50-year-old male patient in 1977; the DU-145 prostate carcinoma cell line originated from a brain lesion of a 69-year-old male patient in 1977. MCF-7, MDA-MB-468, NCI-H460, Calu-6, NCI-H82, HCT116, HT29, LNCaP and DU-145 cells were purchased from ATCC (Rockville, Md., USA).

The MCF-7, MDA-MB-468, NCI-H82, HCT116, HT29, LNCaP and DU-145 cells were grown in RPMI 1640 medium supplemented 10% fetal bovine serum and 1% penicillin-streptomycin (GIBCOBRL, Grand Island, N.Y., USA). The Calu-6 cells were grown in Earle's MEM medium supplemented with 1 × MEM sodium pyruvate, 1 × MEM non-essential amino acids, 10% fetal bovine serum and 1% penicillin-streptomycin (GIBCOBRL). The SW2 cells were grown in RPMI 1640 medium supplemented with 0.2% sodium bicarbonate, 10% fetal bovine serum and 1% penicillin-streptomycin (GIBCOBRL).

Western blot analysis

Adherant cells from near confluent flasks (2 × 75-cm^2 flasks) were suspended by exposure to 0.25% trypsin/1 m*M* EDTA (GIBCOBRL), washed with 0.9% phosphate buffered saline, and collected by centrifugation at 1,000 rpm for 5 min. Freshly collected tumor tissue was snap frozen in liquid nitrogen. The frozen tissue was pulverized in liquid nitrogen using a mortar and pestle, then washed with 0.9% phosphate-buffered saline and the cells collected by centrifugation at 1000 rpm for 5 min. Ice-cold RIPA lysis buffer (0.1 ml/100-mg sample) was added to both the cell and tissue pellets and the samples were shaken at 4 °C for 15 min. The samples were sonicated for 10 s and then separated by centrifugation at 14,000 rpm for 15 min in the cold. The supernatants were collected. The BCA protein assay kit (Pierce, Rockford, Ill., USA) was used to determine protein content. The samples were combined with an equal volume of sample buffer (Tris-glycine SDS; Novex, San Diego, Calif., USA) containing 5% β-mercaptoethanol and then boiled for 3 to 5 min. The protein samples (45 μg) per lane were loaded onto 10% or 12% Tris-glycine denaturing gels (1.5 mm × 15 wells; Pro-Cast SDS-Page; Novex) and electrophoresed. The proteins were transferred from the gel to a PVDF membrane (Novex). The membrane was blocked with 0.9% phosphate buffered saline containing 5% non-fat dry milk and 0.2% Tween-20 overnight at 4 °C. The membrane was washed twice for 10 min each time in washing buffer (0.1% Tween 20, 0.9% phosphate buffered saline) and then incubated at room temperature with the primary antibody of interest in 0.9% phosphate buffered saline containing 5% dry milk for 1 h. After washing three times with washing buffer for 10 min each time, the membrane was incubated at room temperature with secondary antibody of interest in 0.9% phosphate-buffered saline containing 5% dry milk for 1 h. Afterwards the membrane was washed three times with washing buffer for 10 min each time and then exposed to the luminol solution (ECL kit, RPN 2106, Amersham, Arlington Heights, Ill., USA) and the resulting chemiluminescence was detected using Hyperfilm ECL (Amersham).

The primary antibodies used were:

1. Rb monoclonal antibody G3–245 (14001A, Pharmingen, San Diego, Calif.)
2. Cyclin D1(M-20) polyclonal antibody (sc-718, Santa Cruz, Santa Cruz, Calif.)
3. CDK4 polyclonal antibody (06–139, Upstate, Lake Placid, N.Y.)
4. Cyclin E monoclonal antibody HE12, (14591A, Pharmingen)
5. CDK2 polyclonal antibody (06–505, Upstate)
6. E2F1 monoclonal antibody KH95/E2F (14971A, Pharmingen)
7. DP-1 monoclonal antibody TFD10 (66201A, Pharmingen)
8. Actin, Ab-1 Kit monoclonal antibody (CP01, Oncogene Research, Cambridge, Mass., USA).

Cell survival experiments

Cells in exponential growth were exposed to various concentrations of flavopiridol (0, 0.1, 0.5, 1 or 2.5 μ*M*), olomoucine (0, 10, 50, 100, 250 or 500 μ*M*) or genistein (0, 10, 50, 100, 250 or 500 μ*M*) for 48 h. After exposure to the agent, the cells were washed with 0.9% phosphate-buffered saline and suspended by exposure to 0.25% trypsin/0.1% EDTA (except H82 and SW2 cells, which were grown in suspension). The cells were plated in duplicate at three or more dilutions for colony formation. After 2 weeks, the colonies were visualized by staining with crystal violet in methanol. Colonies of 50 cells or more were counted. H82 and SW2 cells were treated as single-cell suspensions and growth inhibition was determined using cleavage of the tetrazolium salt, WST-1 staining. The results are expressed as the surviving fraction or growth fraction of treated cells compared with control cultures.

Tumor growth delay experiments

Nude mice, male and female, were purchased from Charles River Laboratories (Wilmington, Mass.) at 5 to 6 weeks of age. When the animals were 7–8 weeks of age they were exposed to 4.5 Gray of total body radiation delivered using a GammaCell 40 irradiator (Nordion, Ottawa, Ontario). Twenty-four hours later, MCF-7, MDA-MB-468, SW2 or NCI-H82 tumor cells (5×10^6) prepared from a brei of several donor tumors were implanted subcutaneously in a 1:1 mixture of RPMI tissue culture media and Matrigel (Collaborative Biomedical Products, Bedford, Mass.) in a hind leg of the animals. Animals bearing the MCF-7 tumor were implanted subcutaneously with 60-day 72-mg estradiol release pellets 2 days prior to tumor cell implantation (Innovative Research of America, Sarasota, FL). MCF-7 tumors grow to 500 mm^3 in 34.7 ± 2.9, MDA-MB-468 tumors grow to 500 mm^3 in 30.3 ± 3.4, SW2 tumors grow to 500 mm^3 in 25.4 ± 4.2 days and NCI-H82 tumors grow to 500 mm^3 in 19.0 ± 0.8 days.

Treatments were initiated on day 7 post tumor cell implantation, when the tumors were approximately 50–100 mm^3 in volume. Animals were treated with flavopiridol (3, 5 or 10 mg/kg) orally by gavage on days 7–11; 14–18; 21–25 or olomoucine (10, 25 or 50 mg/kg) by intravenous injection on days 7–11; 14–18; 21–25 post tumor cell implantation.

The progress of each tumor was measured three times per week until it reached a volume of 2000 mm^3. Tumor growth delay (TGD) was calculated as the time taken by each individual tumor to reach 500 mm^3 compared with the time in the untreated controls. Each treatment group included five animals, and each experiment was repeated twice. TGD times (days) are the means ± SEM for the treatment group compared with those for the control group [64, 65].

Results

Western blot analysis of Rb protein expression in the ten human tumor cell lines revealed a marked heterogeneity in Rb expression relative to the actin content of the cells (Fig. 1). Relatively high Rb levels were found in the MCF-7, Calu-6 and HCT116 cells and non-detectable or very low Rb levels were found in the MB-468, H82 and SW2 cells. There were also marked variations in the

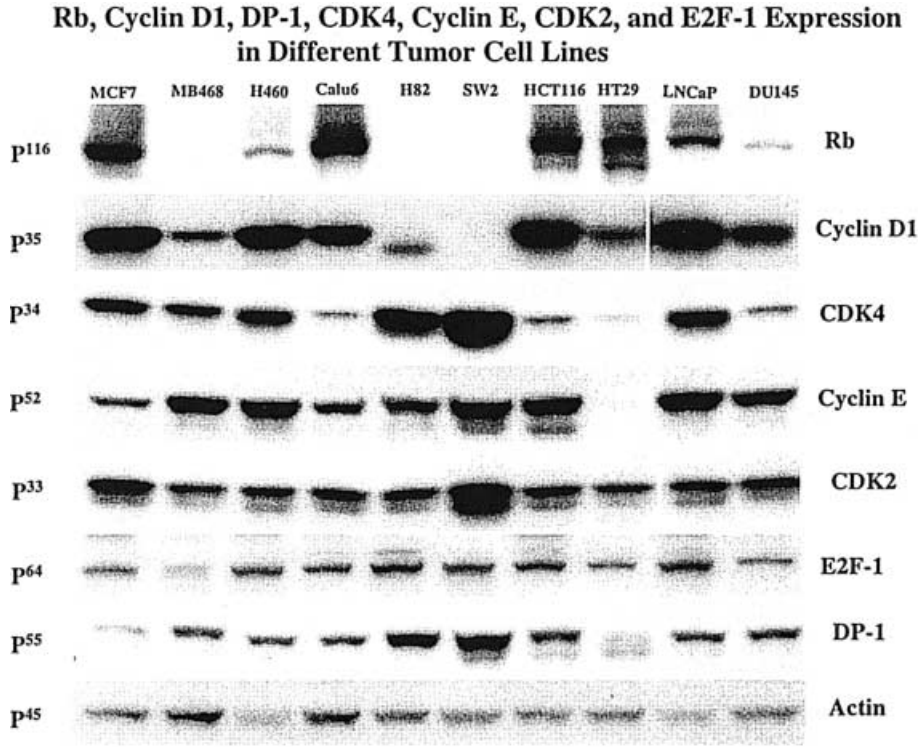


Fig. 1 Western blot for cell cycle components and actin determined from exponentially growing human tumor cell lines in culture. The antibodies used were: Rb, Rb monoclonal G3–245, 14001A, Pharmingen; cyclin D1, cyclin D1(M-20) polyclonal sc-718, Santa Cruz; cdk4, CDK4 polyclonal 06–139, Upstate; cyclin E, cyclin E monoclonal HE12, 14591A, Pharmingen; cdk2, CDK2 polyclonal 06–505, Upstate; E2F-1, E2F1 monoclonal KH95, 14971A, Pharmingen; DP-1, DP-1 monoclonal TFD10, 66201A, Pharmingen and actin, Ab-1 Kit monoclonal CP01, Oncogene Research. Western blotting was carried out using 12% Tris-glycine denaturing Pro-Cast Gels (1.5-mm × 15 wells; NOVEX) except Rb, which used 10% Tris-glycine denaturing Pro-Cast Gels (1.5-mm × 15 wells; NOVEX). Detection was carried out using a luminol-based chemiluminescent reaction for the light detection of horseradish peroxidase labeled antibodies (ECL Western blotting RPN 2106, Amersham)

expression of cyclin D1 in the cells, with highest levels detected in the MCF-7, HCT116 and LNCaP cells and very low or non-detectable levels found in the MB-468, H82 and SW2 cells. The expression of cdk4 also varied markedly in the ten cell lines, with the H82 and SW2 cells exhibiting the highest levels of cdk4 and the Calu-6, HCT116 and DU-145 having the lowest detectable levels of cdk4. The expression of cyclin E was less variable in the cell lines overall. The highest level of cyclin E occurred in the LNCaP cells, while the HT29 cells had no detectable cyclin E. Cdk2 was detectable in all ten cell lines. The SW2 cells had a markedly elevated level of cdk2 relative to actin compared with the other cell lines. Both E2F1 and DP-1 were expressed in all ten cell lines, with much less variability of expression than found with the other cell cycle proteins. The highest expression of E2F1 was found in the H82 cells and the lowest in the MB-468 cells. The highest expression of DP-1 was found in the SW2 cells and the lowest in the MCF-7 cells (Table 1).

The experiments described in Fig. 1 were carried out with proteins collected from near confluent cultures of

Table 1 Relative expression of cell cycle related proteins in human tumor cells/human tumor xenografts and IC_{50}/IC_{99} in culture of human tumor cells exposed to cell cycle related cytotoxic agents. *IC_{99}* 99% inhibitory concentration, *IC_{50}* 50% inhibitory concentration, *ND* not detected

MCF-7	MB-468	H460	Calu-6	H82	SW2	HCT116	HT29	LNCaP	DU145	
8.0/2.1	0/0	1.1/1.0	9.2/2.6	0/0	0.1/0	8/1.6	7/0.8	3.8/1.5	0.5/0	Rb
10.5/12.7	2.7/2.7	9.4/2.7	6.8/5.6	1.9/12.9	0/1.4	10.8/5.7	3.7/19.0	12.4/15.7	6.1/4.1	Cyclin D1
5.3/1.1	3.9/0.6	4.9/0.3	1/0.6	9.8/2.4	15.1/10.9	1.5/0.4	0.3/3.4	6.3/5.1	1.1/1.1	cdk4
0.5/0.6	3.2/2.0	3.4/0.7	0.9/0.8	1.1/0.5	3.1/1.3	3.1/5.7	0/0.2	3.8/2.4	2.2/0.4	Cyclin E
7.7/3.0	4.3/0.6	4.9/0.9	5.6/3.3	5.6/1.8	12.6/9.6	5.9/1.4	4.2/5.4	5.8/5.0	5.8/2.1	cdk2
1.4/1.0	0.7/0.7	1.9/1.3	1.7/0.7	3.6/1.5	2.3/1.5	2.8/1.2	1.2/1.2	3.3/2.3	1.0/0.8	E2F-1
0.7/0.4	1.9/0.4	1.3/0.5	1.4/0.6	3.9/0.5	5.5/1.6	2.7/0.3	0.8/0.4	1.8/0.3	1.9/0.7	DP-1
0.2/1.0	0.3/1.6	0.12/0.65	0.24/0.4	0.3/ND	0.25/ND	0.2/0.04	0.5/2.5	0.16/0.6	0.12/0.5	Flavopiridol
15/ > 120	9.0/48	6.5/33	5.2/19	18/ND	35/ND	7.5/25	10.0/80	2.8/21	8.5/42	Olomoucine
28/148	21/155	16/250	16/85	500/ND	140/ND	30/120	120/420	18/180	25/440	Genistein

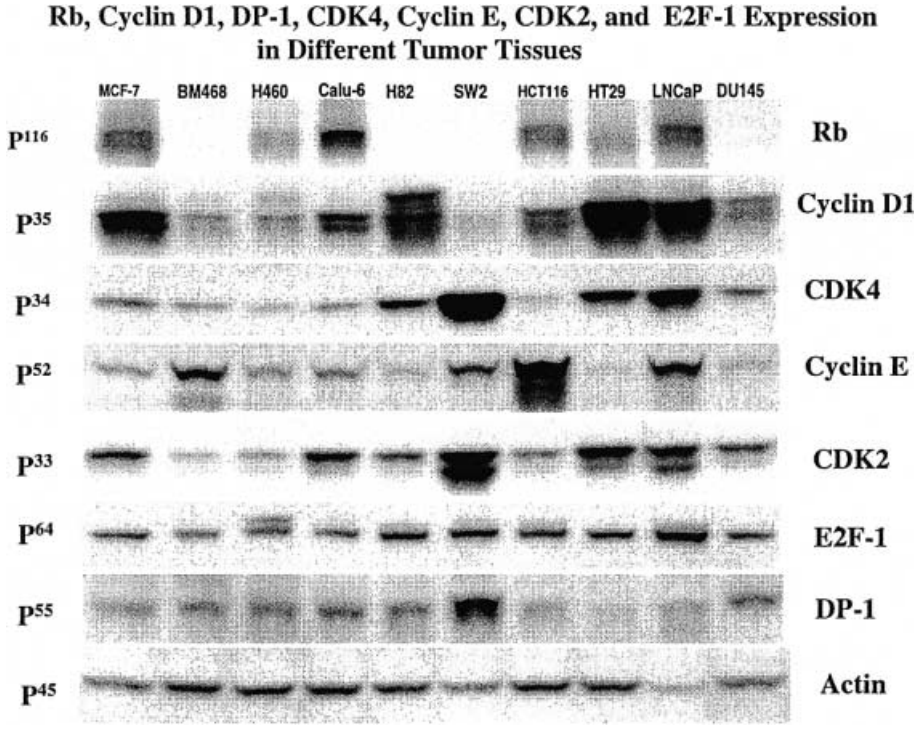


Fig. 2 Western blot for cell cycle components and actin determined from the tumor tissue of human tumor cell lines grown as xenografts in nude mice. The antibodies used were: Rb, Rb monoclonal G3–245, 14001A, Pharmingen; cyclin D1, cyclin D1(M-20) polyclonal sc-718, Santa Cruz; cdk4, CDK4 polyclonal 06–139, Upstate; cyclin E, cyclin E monoclonal HE12, 14591A, Pharmingen; cdk2, CDK2 polyclonal 06–505, Upstate; E2F-1, E2F1 monoclonal KH95, 14971A, Pharmingen; DP-1, DP-1 monoclonal TFD10, 66201A, Pharmingen and actin, Ab-1 Kit monoclonal CP01, Oncogene Research. Western blotting was carried out using 12% Tris-glycine denaturing Pro-Cast Gels (1.5 mm × 15 wells; NOVEX) except Rb, which used 10% Tris-glycine denaturing Pro-Cast Gels (1.5-mm × 15 wells; NOVEX). Detection was carried out using a luminol-based chemiluminescent reaction for the light detection of horseradish peroxidase labeled antibodies (ECL Western blotting RPN 2106, Amersham)

cells. However, in vivo a much lower fraction of the malignant cells will be in cycle and the cells will be in a very different nutritional and molecular environment. Therefore each of the ten human tumor cell lines were grown as subcutaneous xenograft tumors in nude mice, protein was collected from the tumor tissue and the Western blot determination of the cell cycle proteins carried out as above (Fig. 2). As with the cultured cells, there was a marked heterogeneity of expression in Rb protein in the tumor tissues, with an overall diminished relative expression compared with the cell culture expression levels. The highest levels of Rb were found in the Calu-6 tumors and there was no detectable Rb in the MB-468, H82, SW2 or DU-145 tumors. In contrast to Rb, the overall relative expression of cyclin D1 was greater in the tumor tissues than in the cultured cells; although marked heterogeneity of cyclin D1 expression remained, it was detectable in each of the ten tumors. The highest levels of cyclin D1 were found in the MCF-7, Calu-6, HT29 and LNCaP tumors. Cdk4 expression was as variable in the tumor tissues as in the cell cultures and at similar relative levels. Marked overexpression of cdk4 was observed in the SW2 tumors. Overall the expression of cyclin E was decreased in the xenograft tumors relative to the cultured cells and the pattern of expression changed, with the highest levels of cyclin E measured in the HCT116 tumors and low but detectable levels being found in the HT29 tumors. Relative overall expression of cdk2 was also lower in the tumor tissues than in the cell cultures but the marked over-expression of cdk2 in the SW2 tumors was clearly observed. E2F-1 and DP-1 were detectable in each of the ten tumors with highest expression of E2F-1 in the LNCaP tumor and the highest expression of DP-1 in the SW2 tumors (Table 1).

Survival studies were carried out with each of the ten human tumor cell lines exposed to the cell cycle active agents flavopiridol, olomoucine and genistein. The cells were in exponential growth at the time of exposure to each agent and the endpoint of the experiments was colony formation. The human breast carcinoma cells MCF-7 and MB-468 were about equally sensitive to flavopiridol and genistein; however, the MCF-7 cells were relatively highly resistant to olomoucine (Fig. 3). The IC_{99} for MCF-7 cells exposed to flavopiridol for 48 h was 1 μM, for olomoucine it was $\gg$120 μM and for genistein it was 148 μM. The 99% inhibitory concentration (IC_{99}) for MB-468 cells exposed to flavopiridol for 48 h was 1.6 μM, for olomoucine 48 μM, and for genistein 155 μM (Table 1).

The survival of the two human non-small cell lung carcinoma cell lines exposed to flavopiridol, olomoucine and genistein are shown in Fig. 4. While the two cell

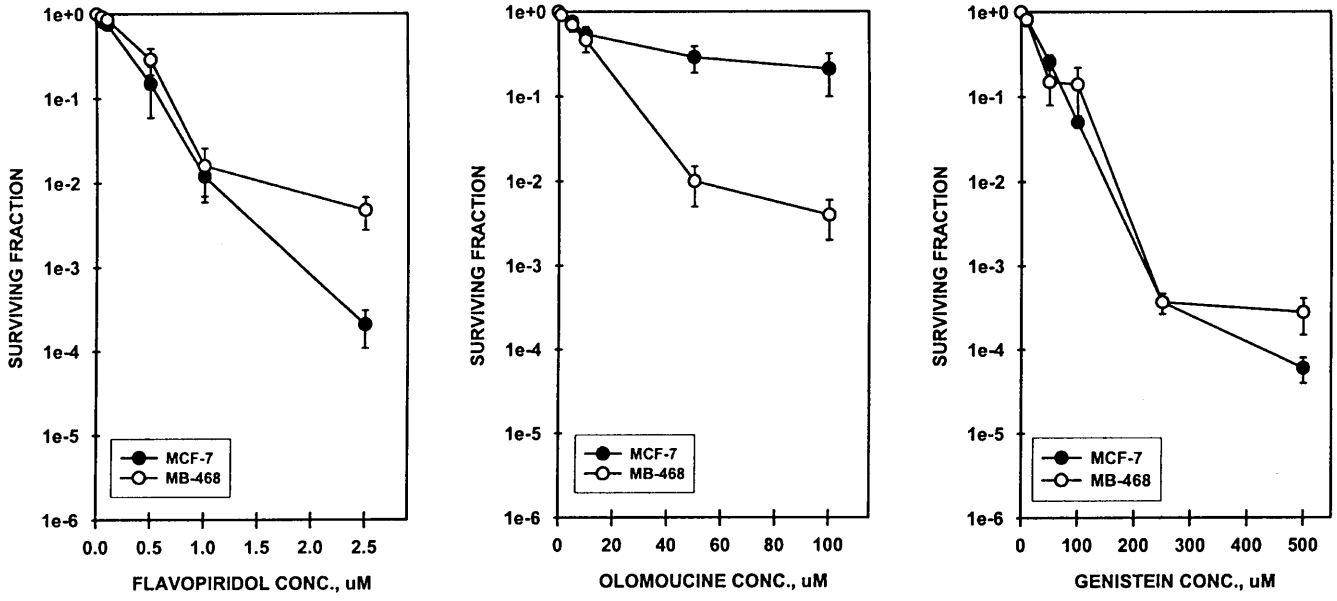
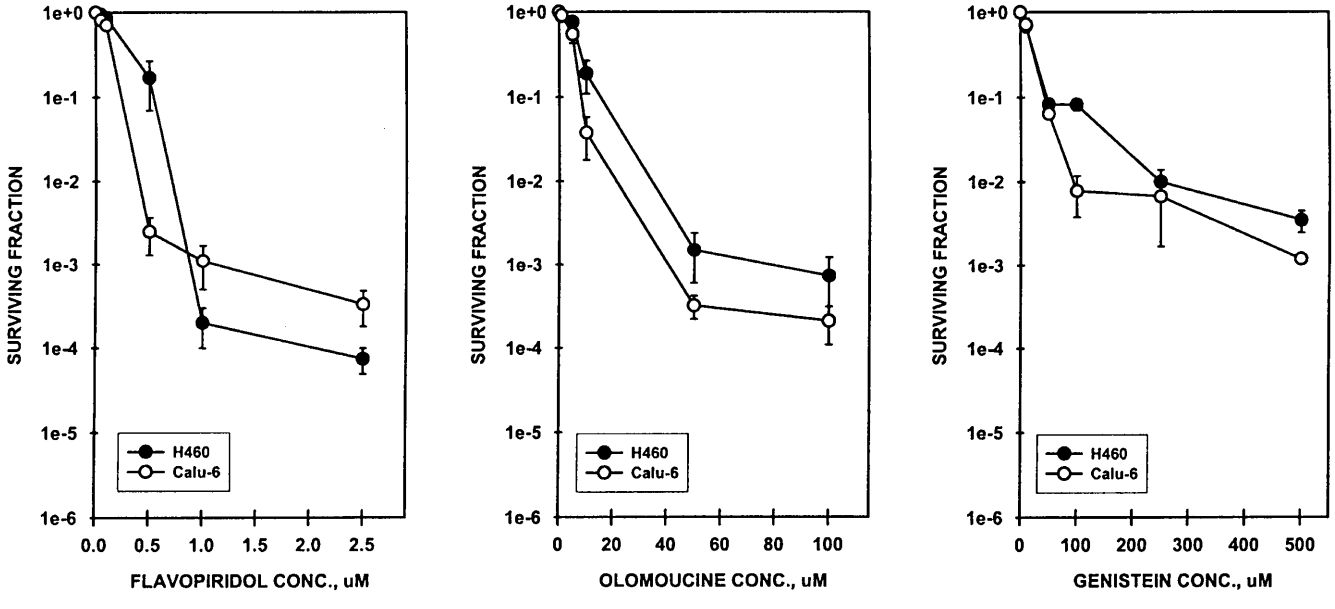


Fig. 3 Survival of exponentially growing human breast carcinoma cells, MCF-7 (●) and MDA-MB-468 (○), exposed for 48 h to various concentrations of flavopiridol, olomoucine or genistein. Survival was determined by colony formation. Points are the means of at least two independent experiments; bars are the standard error of the mean (*SEM*)

lines had a similar response to each of the three cell cycle agents, the Calu-6 cells were more sensitive than were the H460 cells at a level of 2 logs of cell killing to each of the three agents tested. The IC_{99} for H460 cells exposed for 48 h to flavopiridol was 0.65 μ*M*, for olomoucine 33 μ*M* and for genistein 250 μ*M*. The IC_{99} for Calu-6 cells exposed for 48 h to flavopiridol was 0.4 μ*M*, for olomoucine 19 μ*M*, and for genistein 85 μ*M* (Table 1).

Among the ten human tumor cell lines in this study, the SW2 small cell lung carcinoma cells have the most aberrant pattern of cell cycle protein expression. Figure 5 shows the response of the two human small cell lung carcinoma cell lines to exposure to flavopiridol, olomoucine and genistein. While the H82 cells were more sensitive to the higher concentrations of flavopiridol and olomoucine than the SW2 cells, the H82 cells were more resistant to genistein than were the SW2 cells. The 50% inhibitory concentration (IC_{50}) for SW2 cells exposed for 48 h to flavopiridol was 0.25 μ*M*, for olomoucine 35 μ*M* and for genistein 140 μ*M*. The IC_{50} for H82 cells exposed for 48 h to flavopiridol was 0.3 μ*M*, for olomoucine 18 μ*M* and for genistein 500 μ*M* (Table 1).

Fig. 4 Survival of exponentially growing human non-small cell lung carcinoma cells, H460 (●) and Calu-6 (○), exposed for 48 h to various concentrations of flavopiridol, olomoucine or genistein. Survival was determined by colony formation. Points are the means of at least two independent experiments; bars are the standard error of the mean (*SEM*)



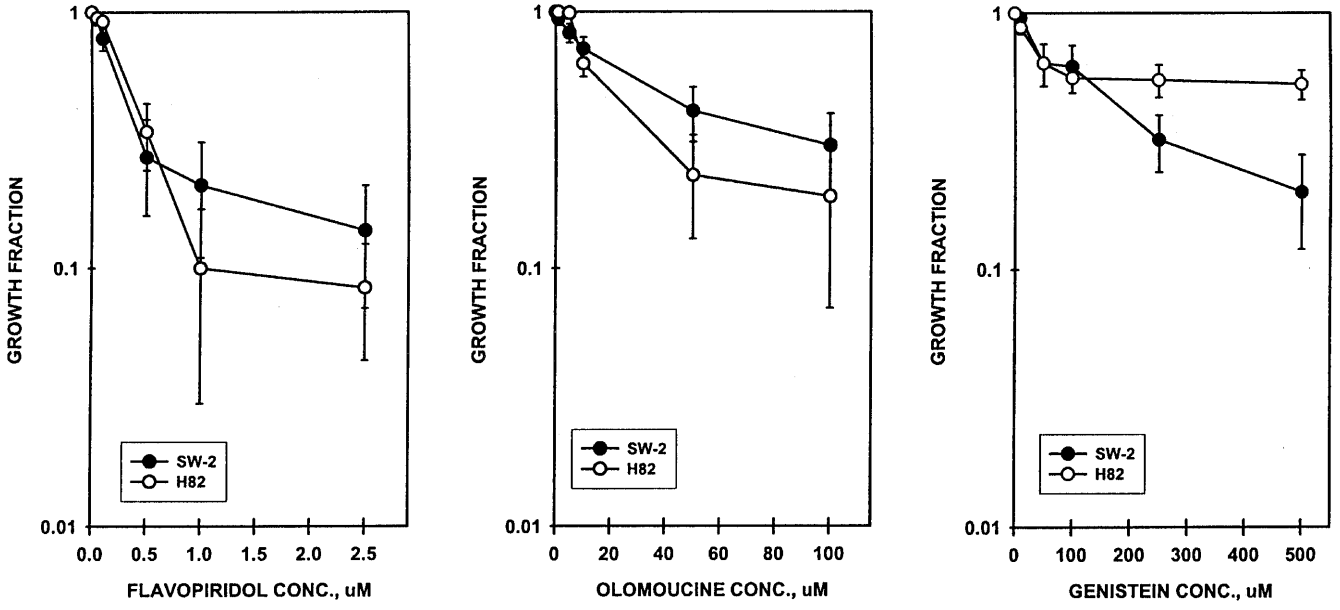
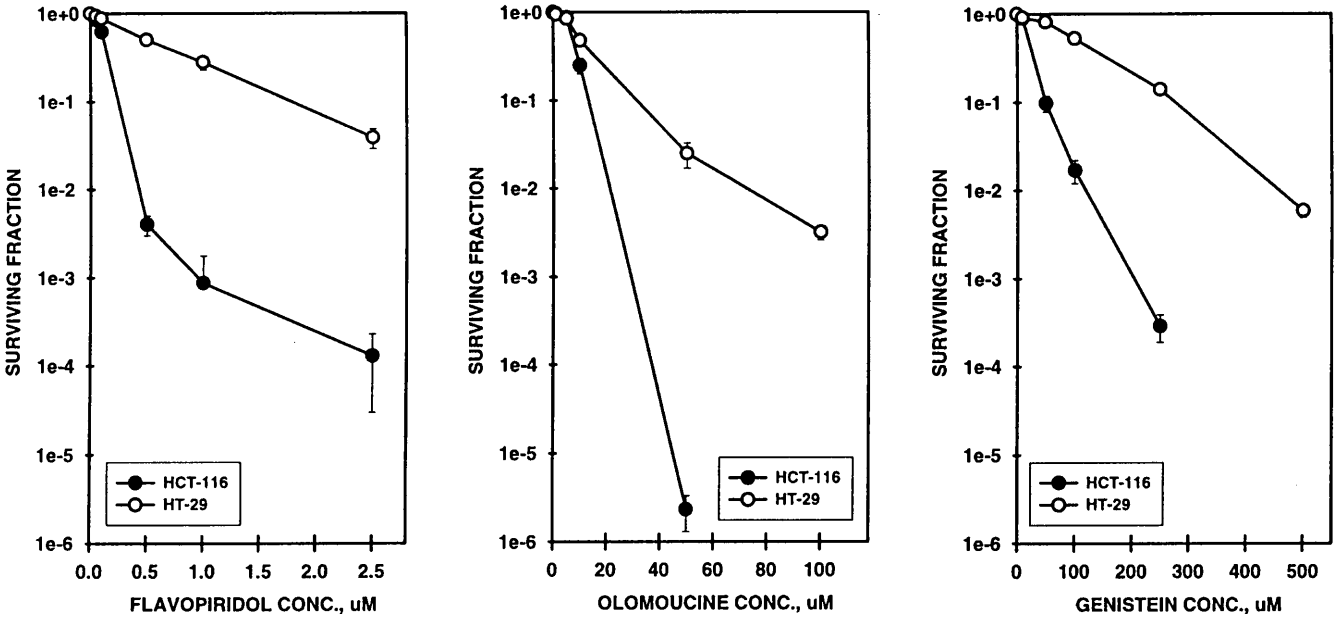


Fig. 5 Growth of exponentially growing human small cell lung carcinoma cells, SW2 (●) and H82 (○), exposed for 48 h to various concentrations of flavopiridol, olomoucine or genistein. Growth was determined by staining with WST. Points are the means of at least two independent experiments; bars are the standard error of the mean (*SEM*)

The survival of human colon carcinoma cells, HCT116 and HT29, exposed to flavopiridol, olomoucine or genistein are shown in Fig. 6. The HCT116 cells were more sensitive to each of the three-cell cycle agents than were the HT29 cells. Amongst the ten cell lines, the HT29 cells were most resistant to flavopiridol with an IC_{50} of 0.5 μM and an IC_{99} much greater than 2.5 μM, while the HCT116 cells were among the most sensitive with an IC_{50} of 0.2 μM and an IC_{99} of 0.4 μM. Although the IC_{50}s for the HCT116 and HT29 cells exposed to olomoucine were not very different, (7.5 μM and 10 μM, respectively), the survival curve for the HCT116 cells was steeper than the survival curve for the HT29 cells, so that the IC_{99}s for the cells were 25 μM for the HCT116 cells and 80 μM for the HT29 cells. The HCT116 cells also more sensitive to genistein than were the HT29 cells. The IC_{99} for the HCT116 cells exposed for 48 h to genistein was 120 μM and the IC_{99} for HT29 cells exposed for 48 h to genistein was 420 μM (Table 1).

The survival of human prostate carcinoma cells, LNCaP and DU-145, exposed to flavopiridol, olomou-

Fig. 6 Survival of exponentially growing human colon carcinoma cells, HCT116 (●) and HT29 (○), exposed for 48 h to various concentrations of flavopiridol, olomoucine or genistein. Survival was determined by colony formation. Points are the means of at least two independent experiments; bars are the standard error of the mean (*SEM*)



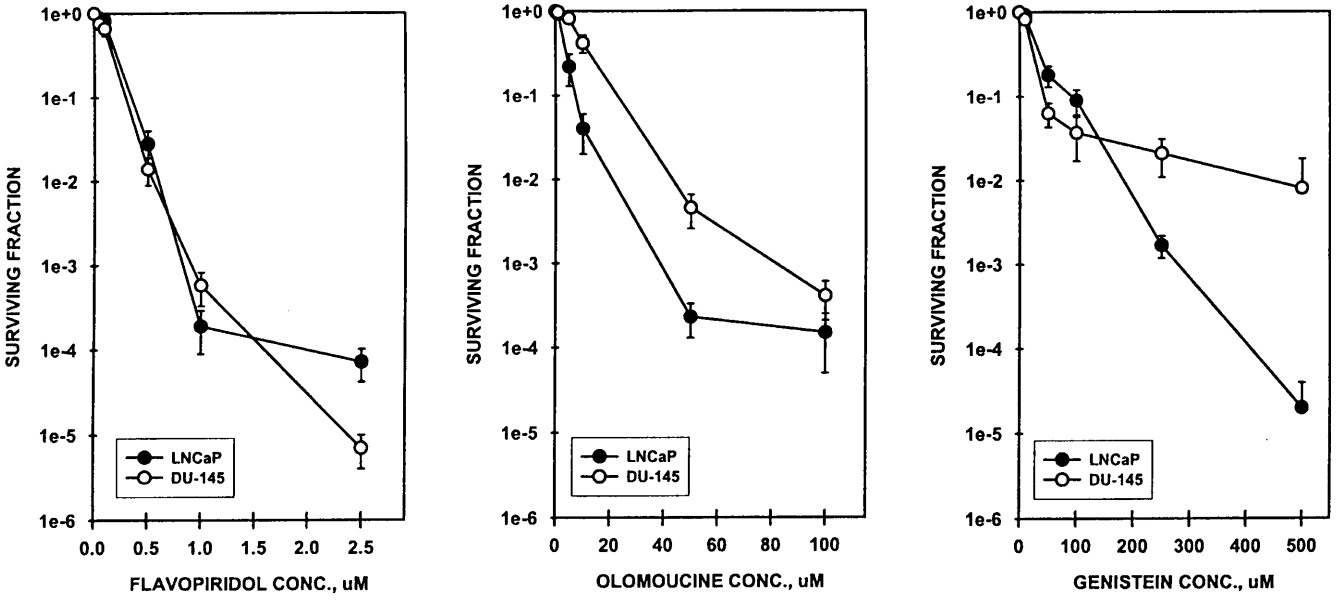


Fig. 7 Survival of exponentially growing human prostate carcinoma cells, LNCaP (●) and DU-145 (○), exposed for 48 h to various concentrations of flavopiridol, olomoucine or genistein. Survival was determined by colony formation. Points are the means of at least two independent experiments; bars are the standard error of the mean (*SEM*)

cine or genistein are shown in Fig. 7. The LNCaP and DU-145 cells were equally sensitive to flavopiridol, but the LNCaP cells were more sensitive to olomoucine and genistein than were the DU-145 cells. The IC_{99} for LNCaP cells exposed to flavopiridol for 48 h was 0.6 μM, for olomoucine 21 μM and for genistein 180 μM. The IC_{99} for DU-145 cells exposed to flavopiridol for 48 h was 0.5 μM, for olomoucine 42 μM and for genistein 440 μM (Table 1).

Tumor growth delay was used as the in vivo assay to determine the sensitivity of human tumor xenografts to the cell cycle agents, flavopiridol and olomoucine. The human breast carcinoma cell lines, MCF-7 and MB-468 were grown as xenograft tumors in female nude mice (Fig. 8). Flavopiridol was administered orally to the mice over a dosage range once daily for 5 days per week for 3 weeks beginning on day 7 post tumor cell implantation. The MB-468 tumor was more responsive to treatment with flavopiridol than was the MCF-7 tumor. The tumor growth delay at the dose of 10 mg/kg of flavopiridol in the MCF-7 tumor was 6.2 days, while the tumor growth delay for the same treatment in the MB-468 tumor was 10.9 days. Olomoucine was administered to animals bearing the MCF-7 tumor or the MB-468 tumor by intravenous injection over a dosage range once daily for 5 days for 3 weeks beginning on day 7 post tumor cell implantation. The MB-468 tumor was also more responsive to olomoucine than was the MCF-7

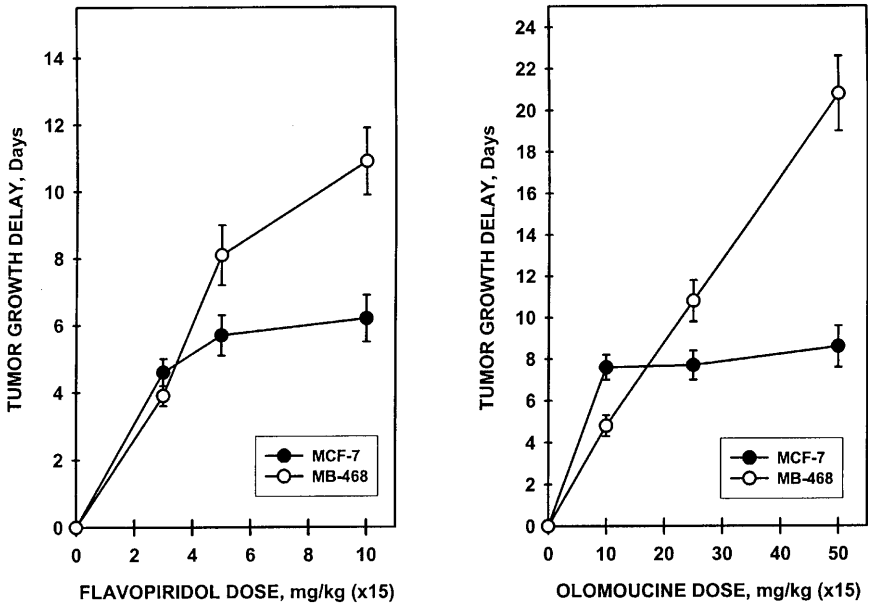


Fig. 8 Growth delay of the human MCF-7 breast carcinoma (●) and the MDA-MB-468 breast carcinoma (○) grown as xenografts in female nude mice after treatment with flavopiridol (3, 5 or 10 mg/kg) orally, days 7–11, 14–18, 21–25 or olomoucine (10, 25 or 50 mg/kg) intravenously, days 7–11, 14–18, 21–25. Points are the means of at least two independent experiments; bars are the standard error of the mean (*SEM*)

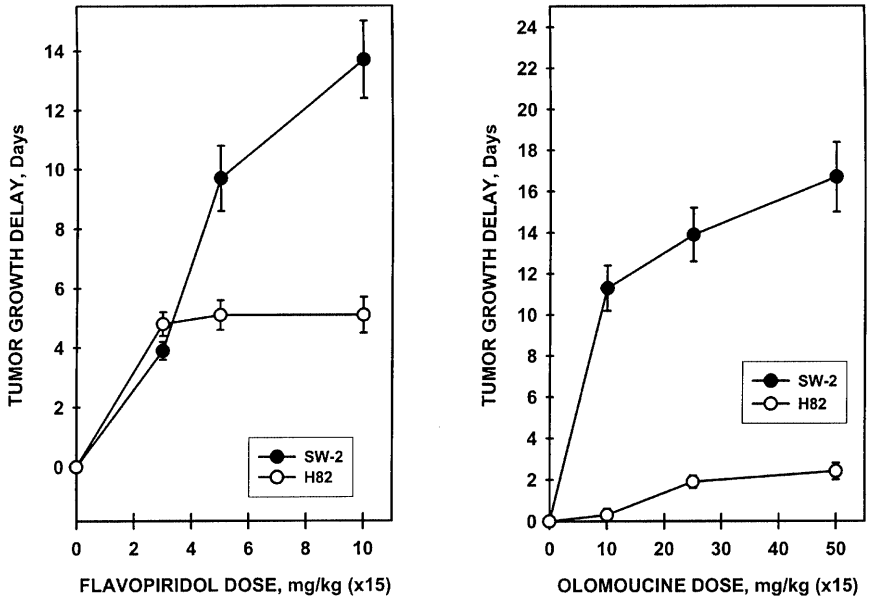


Fig. 9 Growth delay of the human SW2 small cell lung carcinoma (●) and the human H82 small cell lung carcinoma (○) grown as xenografts in female nude mice after treatment with flavopiridol (3, 5 or 10 mg/kg) orally, days 7–11, 14–18, 21–25 or olomoucine (10, 25 or 50 mg/kg) intravenously, days 7–11, 14–18, 21–25. Points are the means of at least two independent experiments; bars are the standard error of the mean (*SEM*)

tumor. The MCF-7 tumor showed no dose response to olomoucine, with a tumor growth delay of 7.6–8.6 days over the dosage range of 10–50 mg/kg of the agent. The MB-468 tumor, on the other hand, showed a marked dose response to olomoucine, such that the tumor growth delay was 4.8 days at a dose of 10 mg/kg olomoucine and 20.8 days at a dose of 50 mg/kg olomoucine.

The human small cell lung carcinoma cell lines, SW2 and H82, grow very well as xenograft tumors in male nude mice. When animals bearing the SW2 tumor or the H82 tumor were treated with flavopiridol over a dosage range orally once daily for 5 days per week for 3 weeks, the SW2 tumor was markedly more responsive to treatment with flavopiridol than was the H82 tumor (Fig. 9). The tumor growth delay with flavopiridol over the dosage range tested in the H82 tumor was 4.8–5.1 days. The tumor growth delay of the SW2 tumor over the same dosage range of flavopiridol was 3.9–13.7 days. A similar pattern pertained when animals bearing the SW2 tumor or the H82 tumor were treated with intravenously administered olomoucine over a dosage range once daily for 5 days per week for 3 weeks. The SW2 tumor was very responsive to olomoucine, with tumor growth delays ranging from 11.3 days to 16.7 days over the dosage range of olomoucine assayed, while the H82 tumor was very marginally responsive to olomoucine, with tumor growth delays ranging from 0.3 days to 2.4 days over the dosage range assayed.

Discussion

Understanding the driving force of proliferation in malignant cells is a major goal of cancer research, thus oncogenes, tumor suppressor genes and regulatory components of the cell cycle are of prime interest. The cyclins and cyclin-dependent kinases, which are the regulatory switches of the cell cycle, are of special interest in cancer since it appears that the expression of these proteins is often disregulated in malignant disease [28, 60, 67]. Specifically, during the G1 phase of the cell cycle, cells commit to divide, to become quiescent or to differentiate. It is at this molecular 'restriction point' in cell cycle control that malignant cells pass through, resulting in neoplastic growth [50]. The D-type cyclins acting in association with cdk4 and/or cdk6 to phosphorylate pRb have been identified as the molecular switches of the G1 phase restriction point [68].

In the current study, ten human tumor cell lines derived from solid tumors were examined for expression of seven G1 phase related proteins. There was marked variability in the expression of the cyclins and cdks and of their substrate protein pRb; however, there was much less variability in the expression of the transcription factor partner proteins E2F1 and DP-1. Despite the variability in the cyclins, cdks and pRb, all of these cell lines grow very well in cell culture and all are tumorigenic in animals. The human SW2 small cell lung carcinoma was very abnormal, being negative for the expression of pRb and cyclin D1 and highly overexpressing cdk4. The SW2 cell line grows well in culture and animals. Therefore it is likely that other cyclins can compensate for the lack of cyclin D1 or that the cell line has an altered cyclin D1 that was not detectable with the primary antibody used.

The two most sensitive cell lines in cell culture were the Calu-6 non-small cell lung carcinoma and the HCT116 colon carcinoma. Both of these cell lines expressed high levels of Rb protein, high levels of cyclin D1 and moderate to low levels of cdk4. However, no consistent pattern of protein expression emerged that correlated with response of the cells to the cell cycle agents. The overall most resistant cell line was the HT29

colon carcinoma, which expressed high levels of Rb protein, moderate levels of cyclin D1 and low levels of cdk4. Since the cells cycle associated cytotoxic agents used in this study lack selectivity for a particular molecular target, the response of cells to these cytotoxic molecules may reflect a complexity targets in the cell cycle regulatory mechanisms as well as, potentially, kinases in addition to cell cycle regulators.

For four of the cell lines, a comparison can be made of response of the cells in culture to flavopiridol and olomoucine and response of the same cells grown as xenograft tumors in nude mice. In cell culture at higher concentrations, the MCF-7 breast carcinoma cells were more sensitive to flavopiridol than were the MB-468 breast carcinoma cells. When grown as tumors, the MCF-7 tumors were less responsive to flavopiridol than were the MDA-MB-468 tumors. In cell culture, MCF-7 cells were quite resistant to the cytotoxic actions of olomoucine, while the MB-468 cells were moderately responsive to olomoucine. The human tumor xenografts of the two cell lines reflected a similar pattern of response, in that the MCF-7 tumors were much less responsive to olomoucine treatment than were the MB-468 tumors. There was no significant difference in the sensitivity of the SW2 and H82 small cell lung carcinoma cell lines to flavopiridol in cell culture, with a trend toward the H82 cells being more sensitive to flavopiridol, when grown as xenograft tumors. However, the SW2 tumors were significantly more responsive to treatment of the tumor-bearing animals with flavopiridol than were the H82 tumors. Similarly, there was no significant difference in the response of the SW2 cell and the H82 cells to olomoucine in cell culture. In vivo, however, the H82 human tumor xenografts were highly resistant to olomoucine when the tumor-bearing animals were treated with the compound, while the SW2 tumors were very responsive to olomoucine.

While each of the three compounds examined herein have been described as cell cycle anticancer agents, none of them is selective for a particular target within cell cycle regulation. Flavopiridol appears to inhibit many cdks with equal potency. Olomoucine has been shown to inhibit cdk2 but has also been shown to block both the G1 and G2/M phases of the cell cycle, indicating inhibitory activity in addition to cdk2. Genistein has been shown to inhibit cdk2 but also to block cells in the G2/M phase of the cell cycle [1, 6, 7, 8, 9, 10, 11, 13, 14, 15, 18, 21, 24, 27, 31, 33, 44, 47, 48, 54, 56, 58]. It may be possible that agents designed to be highly selective for a specific cell cycle target will allow some correlation between expression of the target molecule(s) and response of tumor cells in culture and tumors in vivo to those agents. Further, it is likely that when agents are acting selectively on the activity of the cyclin D/cdk4(6), thus preventing the passage of cell through the G1 phase restriction point (a cell cycle switch shown to be abnormal in the majority of human tumors), chemotherapeutic agents with increased selectivity for malignant cells will result.

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