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Huanglian, A Chinese Herbal Extract, Inhibits Cell Growth by Suppressing the Expression of Cyclin B1 and Inhibiting CDC2 Kinase Activity in Human Cancer Cells

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ABSTRACT

Huanglian is an herb that is widely used in China for the treatment of gastroenteritis. We elected to determine whether huanglian could inhibit tumor cell growth by modulating molecular events directly associated with the cell cycle. Huanglian inhibited tumor growth and colony formation of gastric, colon, and breast cancer cell lines in a time- and dose-dependent manner. Cell growth was completely inhibited after 3 days of continuous drug exposure to 10 μg/ml of herb. This degree of growth inhibition was significantly greater than that observed with berberine, the major constituent of the herb. The inhibition of cell growth by huanglian was associated with up to 8-fold suppression of cyclin B1 protein. This resulted in complete inhibition of cdc2 kinase activity and accumulation of cells in G_2. The mRNA expression of cyclin B1 was not changed after huanglian treatment. There was no change in the protein expression of cyclins A or E. Therefore, the effect of huanglian on inhibiting tumor growth seems to be mediated by the selective suppression of cyclin B1, which results in the inhibition of cdc2 kinase activity. Inhibition of cyclin dependent kinase (cdk) activity is emerging as an attractive target for cancer chemotherapy. Huanglian represents a class of agents that can inhibit tumor cell growth by directly suppressing the expression of a cyclin subunit that is critical for cell cycle progression. These results indicate that traditional Chinese herbs may represent a new source of agents designed for selective inhibition of cyclin dependent kinases in cancer therapy.

Herbal medications are currently being promoted for clinical use in cancer therapy. Many of these claims are based on anecdotes in traditional Chinese medicine. Nevertheless, it is conceivable that certain Chinese herbs could have potent anti-cancer properties. Huanglian (*Coptis chinensis*) is an herb that has been widely used in China for several thousand years. It is prepared as an herbal tea from the roots. Huanglian has been used for the treatment of inflammatory conditions accompanied by high fever. This includes pneumonia and infections of the head and face. It is used routinely in China for the treatment of gastroenteritis. Huanglian has been reported to inhibit the growth of *Heliobacter pylori* and the intestinal parasite *Blastocystis hominis* in vitro (Franzblau and Cross, 1986; Yang et al., 1996; Zhang et al., 1997). Extracts of huanglian have been shown to inhibit topoisomerase I activity (Yamashita et al., 1994; Kobayashi et al., 1995). Oral administration of huanglian to laboratory rats inhibits the formation of azoxymethane-induced aberrant crypt foci, a putative preneoplastic lesion for colon cancer (Fukutake et al., 1998).

Huanglian's role as an anticancer agent has not been defined. The demonstration of anticancer effects in vitro and identification of novel targets would provide a rationale for clinical development of this agent as a whole herb in cancer therapy. Our results indicate that huanglian potently inhibits the growth of gastric, breast, and colon cancer cells in vitro in a time- and dose-dependent manner. In addition, this degree of inhibition is associated with suppression of cyclin B1 protein expression and inhibition of cyclin-dependent kinase 1 (cdc2 kinase) activity.

Materials and Methods

Preparation of Huanglian. The raw material used for these studies was originally produced in Sichuan province, People's Republic of China, and was packaged by South Project Ltd. (Hong Kong, China) as *C. chinensis* root. This material was initially a kind gift from Dr. Helen Zhang (NCCA Acupuncturist and Herbalist, New York, NY), and then later purchased from Murray International Trade Co., Inc. (New York, NY). To prepare an herbal extract that approximated the part of huanglian that is consumed as an oral tea, the roots of huanglian were first boiled in distilled water at 100°C for 1 h. Because the insoluble root fibers or "grinds" are not consumed,

ABBREVIATIONS: FBS, fetal bovine serum; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; HPLC, high-performance liquid chromatography; MPM-2, mitotic protein monoclonal-2; CDK, cyclin-dependent kinase; HL, huanglian; cdc2 kinase, cyclin-dependent kinase 1.

we removed them by sterile filtration through 0.45 μm filter paper. To have huanglian suitable for drug studies, the remaining aqueous solution was further concentrated to dryness either by vacuum at room temperature or by boiling until it becomes solid powder. This soluble part was dissolved in distilled water in 1 mg/ml stock solution for experimental use. This stock solution was stable and could be kept at 4°C for 2 months without loss of any effect.

Cell Culture. The human gastric cancer cell line MKN-74 was graciously supplied by Dr. E. Tahara (Hiroshima University, Hiroshima, Japan). The human breast cancer cell lines MCF-7 and MDA-468 and the colon cancer cell line HCT-116 were purchased from American Type Culture Collection (Manassas, VA). MCF-7, MDA-468, and HCT-116 cells were maintained in RPMI medium supplemented with 10% heat-inactivated FBS, penicillin, and streptomycin at 37°C in 5% carbon dioxide. MKN-74 was maintained in Eagle's minimal essential medium supplemented with 10% FBS, glutamine, penicillin, and streptomycin at 37°C in 5% carbon dioxide. All cultures were free of mycoplasma.

Cell-Growth Inhibition by Huanglian and MTT Assay for Cell Density Measurement. Early log phase cells were trypsinized and regrown in 96-well cell culture plates at the concentration of 5×10^3 cells/ml. Twenty-four hours later, the medium was removed and replaced with fresh medium with or without huanglian (day 0) or berberine (Sigma Chemical, St. Louis, MO). Cell density was measured at day 0 (without huanglian treatment) and on days 1, 2, 3, 4, and 5 by using the MTT (Sigma) assay following the manufacturer's instructions. For these studies, the absorbance of converted dye is measured at a wavelength of 550 nm and the increased absorbance is directly proportional to cell viability. For these studies, all experiments were repeated three or more times.

Colony Formation. Log growth phase cells were trypsinized and plated onto 6-well plates at initial cell concentrations of 1×10^3 cells/ml for both MCF-7 and MDA 468 and 5×10^2 cells/ml for both MKN74 and HCT116 cells. Twenty-four hours later, the medium was removed and fresh mediium was added with or without 1, 10, or 100 μg/ml of huanglian for 24, 48, and 72 h. At end of these treatment intervals, media was discarded and replaced with 4 ml of drug-free media for an additional 2 weeks to allow cells to form colonies. The resulting colonies were stained with 0.01% crystal violet for 30 min and counted. Colony formation was calculated as a percentage of untreated control specimens. Each condition was repeated in at least duplicate.

Reverse HPLC. For these studies, crude huanglian was again solubilized in water, boiled for 1 h, and the insoluble fraction was removed. The aqueous phase was then concentrated to dryness and the huanglian extract was analyzed by reverse HPLC, according to published methods, using an Eclipsed XDB C18 4.6 × 250 mm column with a mobile phase of 25% acetonitrile/25 mM potassium dihydrogen phosphate at a flow of 1 ml/min (Chuang et al., 1996). Arsenic concentrations of the huanglian extract were determined by Huffman Laboratories (Golden, CO), through the use of gaseous hydride atomic absorption.

Western Blot. Treated and nontreated cells were lysed with the lysis buffer at 4°C with 50 mM HEPES-KOH, pH 7.5, 150 mM NaCI, 1 mM EDTA, 2.5 mM EGTA, 1 mM dithiothreitol, 0.1% Tween 20, and 10% glycerol supplemented with the following proteinase inhibitors: 10 mM β-glycerophosphate, 1 mM NaF, 0.1 mM Na vanadate, 0.2 mM phenylmethylsulfonyl fluoride, 10 μg/ml aprotinin, and 10 μg/ml leupeptin (Sigma). Cell lysates (50 μg) were loaded onto 8 or 12% SDS-polyacrylamide gel electrophoresis and transferred to Immobilion-P membranes (Millipore, Bedford, MA). The equal loading of proteins was confirmed by amido black staining (Sigma). The membranes were probed with mouse monoclonal antibodies specific to cyclin B1, cyclin A, and cyclin E (Santa Cruz Biotechnology Inc., Santa Cruz, CA). The primary antibodies were detected with sheep anti-mouse-horseradish peroxide secondary antibody (Amersham Life Sciences, Piscataway, NJ) and subjected to enhanced chemiluminescence reagents (DuPont NEN Life Science Products, Boston, MA). The levels of expression were quantified using a densitometric scanning system. Mouse monoclonal antibody specific to α-tubulin was used as a control antibody to ensure equal protein loading.

Kinase Activity Assay. Kinase assays were performed as described previously (Motwani et al., 1999). In brief, 200 μg of soluble protein was incubated with 1 μg of anti-cyclin B1 (SC-245, Santa Cruz) at 4°C for 2 h. This immunocomplex contains cyclin B1-associated cdc2 kinase. Immune complexes were then precipitated with 40 μl of immobilized rProtein A (RepliGen, Needham, MA) overnight at 4°C, washed three times with lysis buffer, and washed twice with kinase assay buffer. The kinase assay was carried out by combining the washed protein beads with 20 μl of kinase buffer plus 10 μCi of [γ-^{32}P]ATP, 15 μM ATP, and 50 μg/ml Histone H1 (Boehringer Mannheim, Mannheim, Germany). The reaction was allowed to proceed for 20 min at 30°C and was terminated by adding 10 μl of Laemmli sample buffer and boiling for 5 min. Products were resolved by 10% SDS-polyacrylamide gel electrophoresis. The activity levels on autoradiographs were quantified using a densitometric scanning system.

RNA Extraction and Northern Blot Analysis. Total RNA was extracted from MKN-74 cells treated with different schedules and concentrations of huanglian by cesium chloride method as described in Current Protocols in Molecular Biology (Ausubel et al., 1999). Total RNA (20 μg) was then electrophoresed on a 1% agarose-phosphate buffer gel, blotted onto Hybond-N nylon membranes (Amersham), and RNA was crosslinked by UV Stratalinker (Stratagene, LaJolla, CA). The membranes were hybridized with ^{32}P-labeled p21 cDNA probe in Expresshyb hybridization solution (CLONTECH, Palo Alto, CA). The probe was previously labeled by random priming [^{32}P]dCTP incorporation using random-prime labeling kit (Amersham). The probe was purified by passing through Sephadex Quick Spin columns (Boehringer Mannheim). Equal RNA loading was confirmed with β-actin controls.

MPM-2/Propidium Iodide Bivariate Flow Cytometry. MKN-74 cells (1.4×10^6/100 mm dish) were cultured for 48 h and treated with nocodazole (0.1 μg/ml, Sigma) and huanglian (10 μg/ml) as a single agent for 24 h or by treating with nocodazole for 24 h followed by removal of media and addition of either media containing no drug or media containing huanglian for an additional 24 h. The cells were harvested at specific time points by trypsinization and fixed overnight with ice-cold 70% ethanol. After washing with PBS containing 0.05% Tween 20 and 1% FBS, cells were labeled with MPM-2 antibody (final concentration, 6 μg of MPM-2 Ab/ml) (Upstate Biotechnology, Lake Placid, NY) for 1 h at 4°C. Cells were washed twice with PBS and incubated with goat anti-mouse-FITC (Boehringer Mannheim) for 1 h at room temperature in the dark. After washing twice with PBS, cells were resuspended in 5 μg/ml propidium iodide containing 50 μg/ml RNase A. Samples were analyzed on a Becton Dickinson FACScan, and data of 20,000 events for each sample were plotted with CellQuest software (Becton Dickinson, Mountain View, CA). The MPM-2 positive (mitotic cells) show increased green fluorescence, thus shifting above the baseline of the dot plot.

Results

Inhibition of Cell Growth by Huanglian. As shown in Fig. 1, the growth of MKN-74 (Fig. 1A), HCT-116 (Fig. 1B), MCF-7 (Fig. 1C) and MDA468 (Fig. 1D) were inhibited by huanglian after treatment with 10 and 1 μg/ml of huanglian. Huanglian induced a time- and dose-dependent inhibition of cell growth in all four cell lines. By day 3, 10 μg/ml of huanglian induced 100% growth inhibition in each cell line. This effect is independent of the p53 status of the cells, as growth suppression by huanglian was similar for MCF-7, which is the wild-type for p53, and MDA468, which is a mutant of p53. With 10 μg/ml of huanglian, this degree of

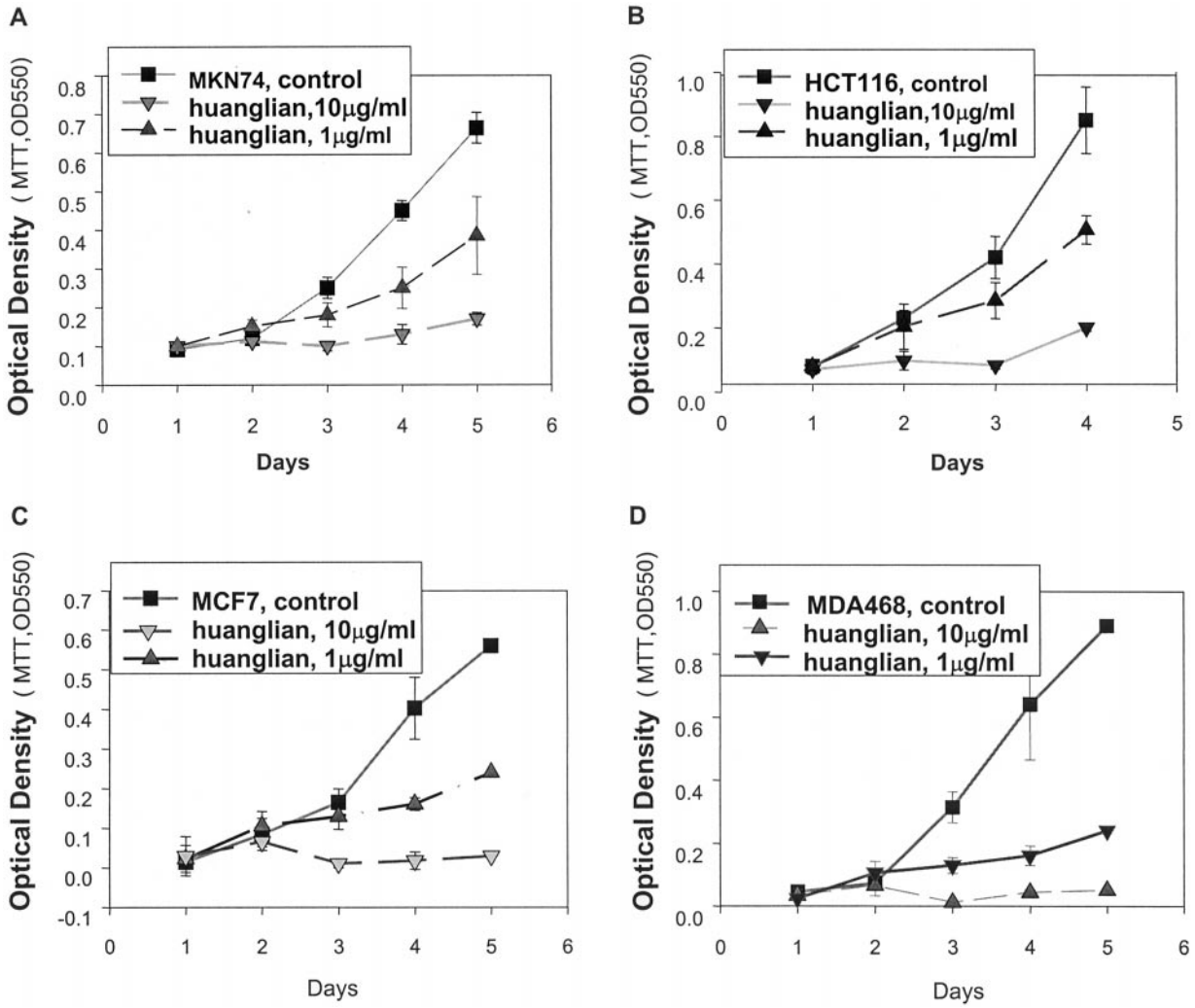

Fig. 1. MKN-74 (A), HCT-116 (B), MCF-7 (C), and MDA468 (D) were treated with drug-free media (control), or media containing 1 or 10 μg/ml of huanglian for 1, 2, 3, 4, and 5 days. Cell growth was as determined by the MTT assay and was directly proportional to the absorbance at a wavelength of 550 nm. Data are representative of at least three independent experiments. Error bars represent mean ± S.D.

growth inhibition persisted until day 5 for the two breast cancer cell lines (MCF-7 and MDA468). In contrast, with the gastric and colon cancer cells lines (MKN-74 and HCT-116), there seemed to be a trend toward increased growth by days 4 to 5. Even though 100% growth inhibition was only observed with a huanglian concentration of 10 μg/ml, similar trends in growth inhibition were still observed with 1 μg/ml of the mixture. In addition, we prepared and tested, in an identical fashion, a series of other Chinese herbal extracts that have been used for medicinal purposes. These included the roots from *Rheum palmatum L.*, the flowers from *Lonicera japonica*, dye from the grass of *Babhicacanthus cusia Bremek*, and the stems of *Arisaima erubescens*. In microgram concentrations, none of these other herbal extracts were shown to be growth inhibitory to these cells (data not shown).

Inhibition of Colony Formation. As shown in Fig. 2, huanglian at concentrations of 1, 10, and 100 μg/ml suppressed colony formation in a dose-dependent manner. At 1 μg/ml, colony formation was inhibited to 10% (MCF-7), 15% (MKN-74), and 25% (MKN-74 and MDA468) of control in the cell lines tested. At a concentration of 10 μg/ml, there was complete growth suppression in all the cell lines except

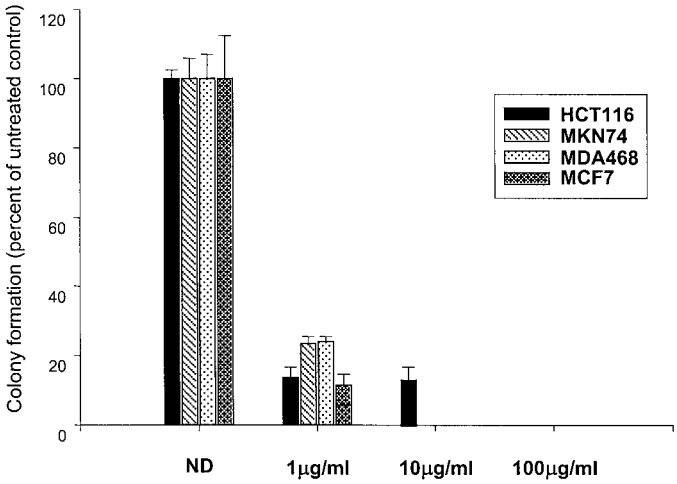

Fig. 2. MKN-74, HCT-116, MCF-7, and MDA468 cells were plated onto 6-well plates and treated with drug free media (control) or media containing 1, 10, or 100 μg/ml of huanglian for 24, 48, or 72 h. The media was then discarded and replaced with drug-free media for an additional 14 days. Colonies were stained with crystal violet and colony formation was calculated as a percentage of untreated control cells. Data are representative of at least three independent experiments. Error bars represent mean ± S.D.

HCT116. The highest concentration of 100 μg/ml proved exceptionally toxic to all the cell lines tested.

Huanglian Assessment by Reverse HPLC. Reverse HPLC was performed on the huanglian extract to determine its constituents. The HPLC profile is shown in Fig. 3. These results indicate that the extract contained seven dominant peaks, constituting 96% of the UV detectable components of the total mixture. The largest peak is berberine (detectable at 15 min). This constitutes 50% of the extract. Five of these other peaks are "berberine-like" in that they yield UV spectra with a λ_{max} of approximately 345 nm, which is characteristic of a chromophore in the berberine family (data not shown). The first peak at 3.2 min has a completely different UV spectra, and identification of this component is in progress.

The Inhibitory Effects of Berberine Depend on the Whole Extract. Huanglian has been reported to contain a wide range of compounds (Chuang et al., 1996). The largest peak of our extract was berberine, which accounted for 50% of huanglian. Therefore, we elected to test the effect of a berberine solution at a concentration that was half that of huanglian on the cell growth of MKN-74 cells. As shown in Fig. 4A, after 3 days of exposure to berberine at 1 μg/ml, tumor cell growth was inhibited by 50%, whereas with huanglian at 2 μg/ml, tumor growth was inhibited by 100% (P = .0013) relative to untreated control cells. A comparable effect was observed when berberine at a concentration of 2.5 μg/ml was compared with huanglian at 5 μg/ml (Fig. 4B). At all time points tested, the inhibitory effect of huanglian was significantly greater than that of berberine ($P < .001$). These studies indicate that the effect of huanglian on inhibiting tumor cell growth can not be explained by the inhibitory effect of berberine alone, but depends on the mixture of the various components present in the huanglian extract.

Huanglian Suppresses the Expression of Cyclin B1 Protein but not mRNA. The constituents of herbs have been reported to inhibit CDKs and effect the cell cycle (Hoes-

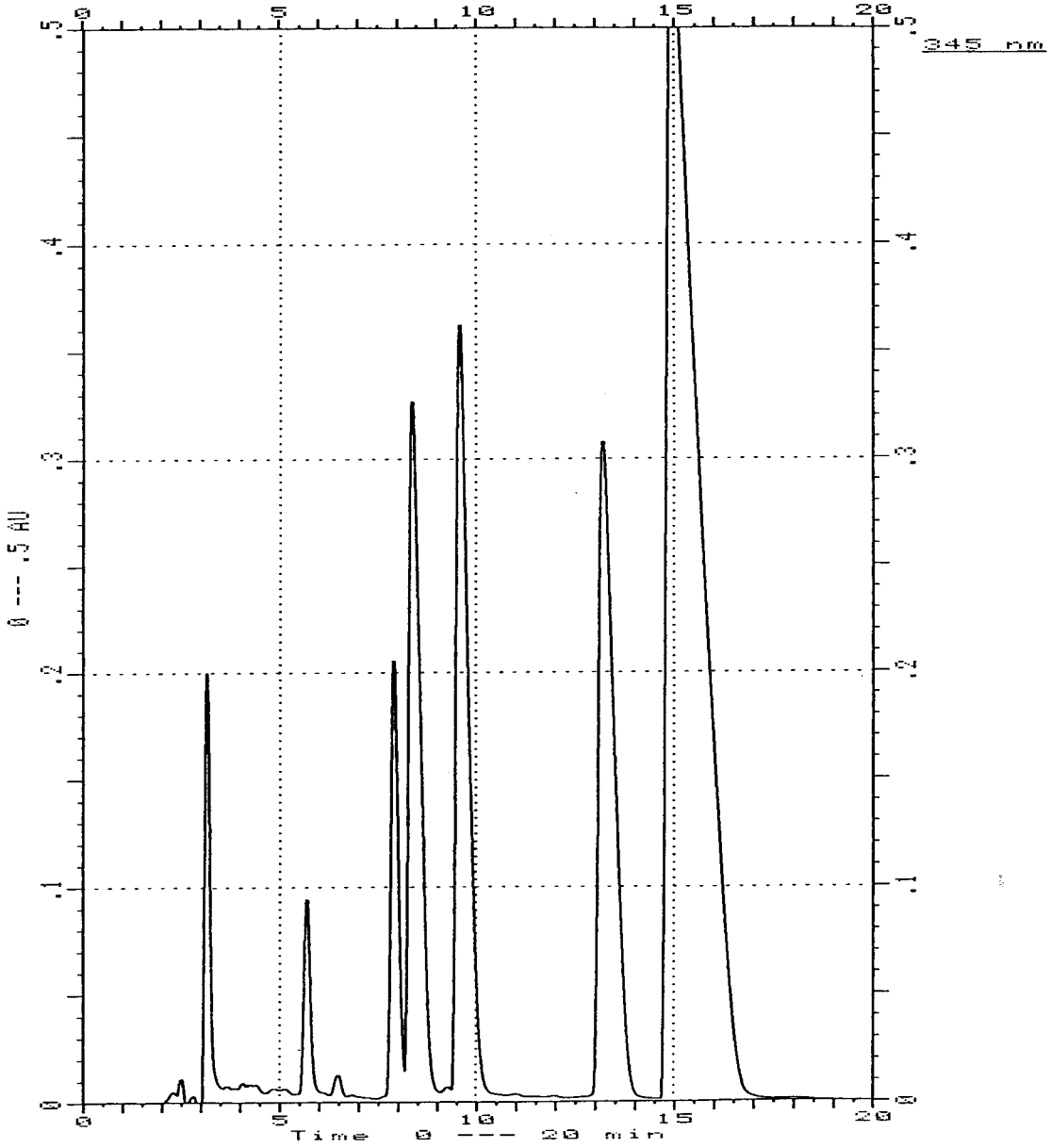

Fig. 3. The huanglian extract was analyzed by reverse HPLC. The resulting chromatograph for the seven dominant peaks is shown.

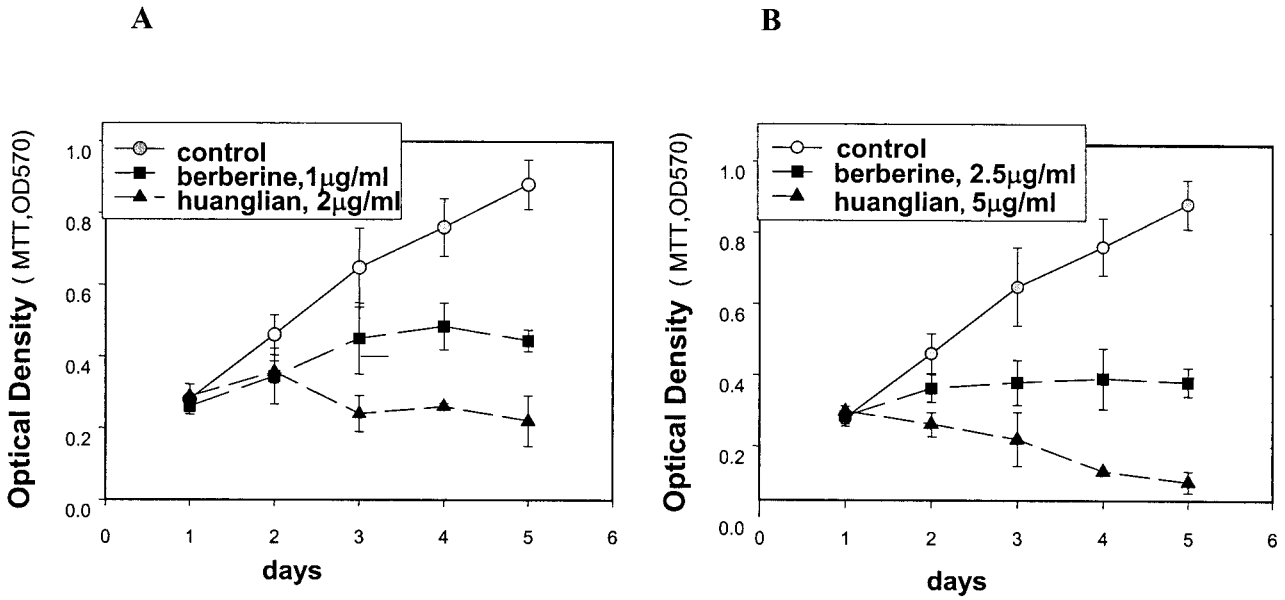

Fig. 4. MKN-74 cells were treated with drug free media (control), or media containing 2 μg/ml of huanglian or 1 μg/ml of berberine (A) and 5 μg/ml of huanglian or 2.5 μg/ml of berberine (B) for 1, 2, 3, 4, and 5 days. Cell growth was as determined by the MTT assay and was directly proportional to the absorbance at a wavelength of 570 nm. Data are representative of at least three independent experiments. Error bars represent mean ± S.D.

sel et al., 1999). Therefore, we elected to test the effect of huanglian on the expression of cell cycle-specific cyclins in MKN-74 cells. Protein lysates were prepared after 24-, 48-, and 72-h exposure to 10 μg/ml of huanglian and Western blots for cyclin B1 and other related proteins were performed. As shown in Fig. 5A, 10 μg/ml of huanglian (HL) suppressed cyclin B1 protein expression 5- to 8- fold after 48 and 72 h, respectively, of drug exposure, compared with untreated control cells (−). This correlates to the drug concentration (10 μg/ml) and the duration of drug exposure (2 to 3 days) at which there was 100% growth inhibition.

To determine whether the loss of cyclin B1 protein was caused by a decrease in mRNA expression at the transcriptional level, we evaluated the MKN-74 cells for the expression of cyclin B1 mRNA under identical treatment conditions. These results are shown in the Northern blot (Fig. 5B). Even though the overall mRNA content of the cells seemed to decrease over time, there was no difference in cyclin B1 mRNA with 10 μg/ml of huanglian at 24, 48, or 72 h, compared with the untreated control cells at similar time points. These results would indicate that huanglian decreases the expression of cyclin B1 at either a translational or post-translational level.

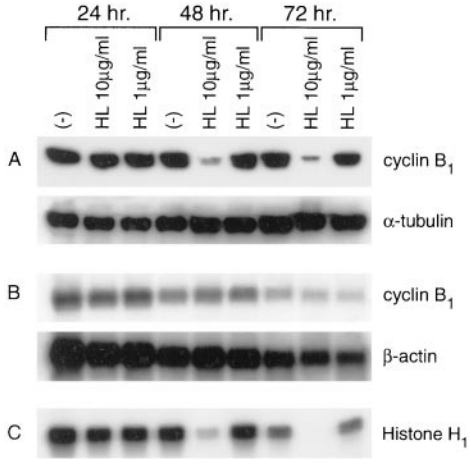

Fig. 5. MKN-74 cells were treated drug free media (−) or media containing huanglian (HL) at concentrations of 10 μg/ml for 24, 48, or 72 h. Cell lysates were then prepared for Western blot analysis of cyclin B1 with α–tubulin controls (A), Northern blot analysis for cyclin B1 with β–actin controls (B), and cyclin B1-associated cdc2 kinase activity (C). Anti-cyclin B1 antibody was used to immunoprecipitate kinase complexes, and histone H1 was used as a substrate. Data are representative of three independent experiments.

Huanglian Inhibits cdc2 Kinase Activity. Cyclin B1 is the cyclin that binds to and activates cyclin dependent kinase 1 (cdc2 kinase) (Nurse, 1990). Therefore, we hypothesized that the loss of cyclin B1 protein should result in a decrease in the enzymatic activity of cdc2 kinase. The results are shown in Fig. 5C. As predicted, the suppression of cyclin B1 protein resulted in a decrease in cdc2 kinase activity (reflected as a decrease in H1 phosphorylation) after exposure to 10 μg/ml of huanglian for 48 and 72 h. In fact, by 72 h, cdc2 kinase activity was completely inhibited compared with the untreated control cells.

This effect of huanglian on inhibition of cdc2 kinase activity is considered indirect because it is mediated by suppression of the cdc2 activator, cyclin B1. We elected to determine whether huanglian could directly inhibit cdc2 kinase. For these studies, we immunoprecipitated cyclin B1-associated cdc2 kinase from untreated cell lysates and then added 10 μg/ml huanglian directly to the cdc2 kinase assay. Under these conditions, we could detect no inhibition of histone H1 phosphorylation (data not shown), supporting the observation that the inhibition of cdc2 kinase by huanglian must be mediated by suppression of cyclin B1.

Effect of Huanglian on G_2/M Transition. The transition from the G_2 to the M phase of the cell cycle requires the activation of cyclin B1 associated cdc2 kinase (Nurse, 1990). The decreased kinase activity of this complex after huanglian treatment should result in a decreased number of cells entering the M phase. We elected to test this in the MKN-74

TABLE 1

Flow cytometric analysis of MKN-74 cells synchronized with nocodazole (0.1 μg/ml) for 12 h (Noc_{12}), or nocodazole for 12 h followed by either drug-free media for 24 h ($Noc_{12}\rightarrow(-)_{24}$) or nocodazole for 12 h followed by huanglian for 24 h ($Noc_{12}\rightarrow HL_{24}$). The cells were harvested at indicated time points and 20,000 gated events were analyzed for DNA content of each sample. Fixed cells were labeled with primary MPM-2 monoclonal antibody followed by secondary goat anti-mouse-fluorescein isothiocyanate antibody to determine the M phase fraction. Data are representative of three independent experiments.

Treatment	G_1 (%)	S (%)	G_2 (%)	M (%)
(−)	34	47	17	2
NOC_{12}	5	30	24	41
$NOC_{12}\rightarrow(-)_{24}$	20	34	30	16
$NOC_{12}\rightarrow HL_{24}$	6	37	54	3

cells by flow cytometry and labeling of cells with the MPM-2 antibody, which identifies cells in mitosis. In the asynchronous population the number of cells in M phase are very low. Therefore, to better document accumulation of cell in G_2, we first elected to synchronize the cell with nocodazole in the M phase (e.g., mitotic block). As shown in Table 1, treatment with nocodazole for 12 h (NOC_{12}) resulted in 41% of the cells remaining in M phase. When cells were first treated with nocodazole for 12 h and then treated with drug-free medium for an additional 24 h ($NOC_{12}\rightarrow(-)_{24}$), the cells were released from mitosis, although 16% of the cells still remained in M phase. In contrast, when the nocodazole therapy was followed by 24 h of 10 μg/ml of huanglian ($NOC_{12}\rightarrow HL_{24}$), the cells exited out of M phase (now only 3%) and 54% of the cells accumulated in the G_2 phase of the cycle. Under these conditions, huanglian suppressed cyclin B1 protein expression by 70% and inhibited cdc2 kinase activity by 90% (data not shown). These results indicate that huanglian induced a cell cycle block at G_2 by suppressing cdc2 kinase activity in association with loss of cyclin B1 protein.

Huanglian Does Not Suppress the Protein Expression of Cyclins A or E. To determine the specificity of huanglian for cyclin B1, we also examined the effect of huanglian on protein expression of other cyclins. As shown in Fig. 6, huanglian at 10 μg/ml huanglian did not suppress the protein expression of cyclin E after 24, 48, and 72 h of drug exposure. Cyclin A protein expression was decreased by 1.5-fold with 10 μg/ml of huanglian relative to the untreated control cells, but only after 72 h of continuous exposure.

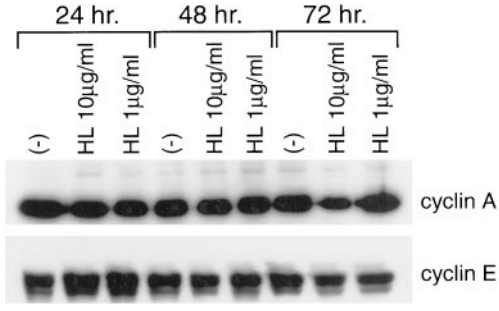

Fig. 6. MKN-74 cells were treated with drug free media (−) or media containing huanglian (HL) at a concentrations of 10 μg/ml for 24, 48, or 72 h. Cell lysates were then prepared for Western blot analysis of cyclins A and E1.

Discussion

Chinese herbs have been used for the treatment of human diseases for thousands of years. However, over the past several years, there has been considerable interest in these agents in the treatment of human cancers. Despite the reported benefits of these herbs in individual patients, it still remains unclear whether these agents have anticancer properties and whether there is a molecular basis to their inhibitory effects on cell growth. Recently, several agents that inhibit the CDKs have been identified from plants and marine invertebrates. For example, flavopiridol is a CDK inhibitor (Kaur et al., 1992; Losiewicz et al., 1994; Carlson et al., 1996) isolated from *Dysoxylum binectariferum*, a plant indigenous to India. Flavopiridol directly inhibits CDK1, -2, -4, and -6 by binding to the ATP-binding domain of the enzyme. Flavopiridol has been shown to inhibit tumor cell growth and, in fact, has entered clinical trials with promising preliminary results (Senderowicz et al., 1998). Therefore, botanicals represent a potential source for drugs that inhibit the CDKs and have anticancer properties.

Huanglian is a botanical that is used today in traditional Chinese medicine for febrile illnesses associated with gastroenteritis and pneumonia. Our results indicate that huanglian inhibits the growth of gastric, breast, and colon cancer cell lines, apparently through inhibition of cdc2 kinase activity by suppression of cyclin B1 protein expression. There was no effect on direct enzyme inhibition. We also observed no effects on the expression of other cyclins, including cyclins A and E. This is in contrast to flavopiridol, which directly inhibits the CDK enzymatic activity. It has also has been shown to suppress the activity of CDK4 by inhibiting the transcription of cyclin D (Carlson et al., 1999).

Our results indicate that the suppression of cyclin B1 by huanglian occurs at a translational level, because there was no suppression of cyclin B1 mRNA. Ubiquitin-mediated proteolysis underlies the regulation of the cell cycle and is particularly important in progression through the cell cycle (King et al., 1996). A multisubunit ubiquitin ligase called the 20S anaphase-promoting complex or cyclosome controls the destruction of B-type cyclins (Glotzer et al., 1991). Once "marked" with a polyubiquitin chain, cyclin B is then degraded by a large multimeric protease called the proteasome (Hershko and Ciechanover, 1998; Tanaka, 1998). Preliminary data from our laboratory indicates that the treatment of MKN-74 cells with huanglian in the presence of the proteasome inhibitor PS-341 prevents the loss of cyclin B1 protein (data not shown). This would indicate that huanglian activates the ubiquitin proteasome cascade and promotes the post-translation degradation of the protein.

Huanglian has been shown to be safe for human consumption. Administration of oral doses ranging from 3 to 10 g/day has been associated with no side effects in adults (Quin et al., 1994). Although the maximum tolerated dose in humans is not known, huanglian has been administered to mice at a dose of 27 g/kg/day with no toxicity (Ozaki et al., 1993). In newborn infants, huanglian has been shown to increase unconjugated bilirubin in newborns presumably by displacing bilirubin from serum binding proteins. The authors advised against the use of this herb in neonates in Southern China,

where neonatal hyperbilirubinemia is prevalent (Yeung et al., 1990).

In the development of huanglian for cancer therapy, there remains a concern that the inhibitory effects are attributable to a single component rather than the whole herb. Huanglian is a complex mixture and the active components of this herb are unknown. The major component of huanglian is berberine, an alkaloid of the protoberberine family, but other constituents, including coptisine, palmatine, jatrorrhizine, bauerenol, and epiberberine have also been identified (Fang et al., 1989). It has been reported that continuous exposure of HepG2 hepatoma cells to berberine (1 to 50 uM) inhibits tumor cell growth in a dose-dependent manner (Chi et al., 1994). This was associated with a decrease in both the S-phase fraction of the cells and in the secretion of α-fetoprotein.

In the Chinese literature, berberine was reported to constitute only 2 to 3% of huanglian. However, our herbal extract of huanglian was determined to contain 50% berberine. Aqueous extracts of huanglian, when administered orally to laboratory rats, result in levels of berberine that are measurable by HPLC (Ozaki et al., 1993). In patients with severe underlying cardiac disease, rapid infusion of intravenous berberine has been reported to cause cardiac conduction abnormalities, including QTc prolongation (Marin-Neto et al., 1988; Zeng and Zeng, 1999). Therefore, we believed it was important to determine the degree to which berberine, at half the concentration of huanglian, would inhibit tumor cell growth in vitro. The results indicate that 100% growth inhibition can only be achieved with the whole herbal extract. This indicates that there are constituents in the herb other than berberine that are critical for its growth inhibitory effects. In view of the safety of the herb, these results support the concept of developing the whole herbal extract, rather than its dominant peaks, for cancer therapy.

Many traditional Chinese herbs are contaminated with arsenic (Chan, 1994). For example the major constituent of "yiu-chen" is arsenic oxide. Arsenic is now being used to treat acute promyelocytic leukemia (Soignet et al., 1998). Because arsenic could not be detected by our UV monitor, the huanglian extract was tested for arsenic by atomic absorption. This method revealed only 0.1 to 0.2 μg of arsenic in each gram of solid extract (data not shown). This amount of arsenic is considerably below levels that would be considered to have any biologic effect on these cells.

Huanglian, therefore, joins a class of novel agents that inhibit tumor growth by targeting the inhibition of CDKs. Indirubin, the active constituent of a Chinese antileukemic drug, has been reported to inhibit CDK2 activity by directly interacting with its ATP-binding site (Hoessel et al., 1999). Huanglian seems to be distinct from these other agents in that it inhibits cdc2 kinase at the G_2/M transition; this is achieved indirectly by suppressing cyclin B1 protein expression. This results in loss of cell viability and a decrease in clonogenicity. Because the whole herb seems to be important for the maximal effect of this agent in vitro, we plan to investigate the potential of huanglian as an oral anticancer drug. A phase I clinical trial is planned to define maximum tolerated dose of the herb and to determine whether this agent will down-regulate the expression of cyclin B1 in tumor biopsies obtained from patients on the study. Thus, it is possible to develop a clinical strategy, using a laboratory-based approach to identify botanicals in traditional Chinese herbal medicines, that inhibit tumor cell growth by inhibiting the activity of cell cycle-specific kinases.

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