Synergistic Effects of Acyclic Retinoid and OSI-461 on Growth Inhibition and Gene Expression in Human Hepatoma Cells

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ABSTRACT

Hepatoma is one of the most frequently occurring cancers worldwide. However, effective chemotherapeutic agents for this disease have not been developed. Acyclic retinoid, a novel synthetic retinoid, can reduce the incidence of post-surgical recurrence of hepatoma and improve the survival rate. OSI-461, a potent derivative of exisulind, can increase intracellular levels of cyclic GMP, which leads to activation of protein kinase G and induction of apoptosis in cancer cells. In the present study, we examined the combined effects of acyclic retinoid plus OSI-461 in the HepG2 human hepatoma cell line. We found that the combination of as little as 1.0 μmol/L acyclic retinoid and 0.01 μmol/L OSI-461 exerted synergistic inhibition of the growth of HepG2 cells. Combined treatment with low concentrations of these two agents also acted synergistically to induce apoptosis in HepG2 cells through induction of Bax and Apaf-1, reduction of Bcl-2 and Bcl-x_L, and activation of caspase-3, -8, and -9. OSI-461 enhanced the G_0-G_1 arrest caused by acyclic retinoid, and the combination of these agents caused a synergistic decrease in the levels of expression of cyclin D1 protein and mRNA, inhibited cyclin D1 promoter activity, decreased the level of hyperphosphorylated forms of the Rb protein, induced increased cellular levels of the $p21^{CIP1}$ protein and mRNA, and stimulated $p21^{CIP1}$ promoter activity. Moreover, OSI-461 enhanced the ability of acyclic retinoid to induce increased cellular levels of retinoic acid receptor β and to stimulate retinoic acid response element-chloramphenicol acetyltransferase activity. A hypothetical model involving concerted effects on $p21^{CIP1}$ and retinoic acid receptor β expression is proposed to explain these synergistic effects. Our results suggest that the combination of acyclic retinoid plus OSI-461 might be an effective regimen for the chemoprevention and chemotherapy of human hepatoma and possibly other malignancies.

INTRODUCTION

Hepatoma (hepatocellular carcinoma) is the fifth most frequent malignancy worldwide with an annual incidence of 560,000 cases (1). The development of hepatoma is frequently associated with chronic inflammation of the liver induced by persistent infection with hepatitis B virus or hepatitis C virus. The prognosis for patients with hepatoma is poor because there is no effective treatment of metastatic disease. Furthermore, even in early stage cases where surgical treatment might be expected to be curative, the incidence of recurrence in patients with underlying cirrhosis is ~20 to 25% a year (2, 3). In addition, at present there are no effective chemopreventive agents for hepatoma. Therefore, there is a critical need to develop more effective strategies for the chemoprevention and chemotherapy of hepatoma.

Retinoids, a group of structural and functional analogs of vitamin A, exert fundamental effects on the regulation of epithelial cell growth, differentiation, and development (4). Some retinoids have been shown to have chemopreventive and chemotherapeutic activity for various human cancers (5). Acyclic retinoid, a novel synthetic retinoid, inhibits experimental liver carcinogenesis and induces apoptosis in human hepatoma-derived cells (6–8). A clinical trial showed that the administration of acyclic retinoid reduced the incidence of post-therapeutic recurrence of hepatoma and improved the survival rate of patients (9, 10). These favorable effects were associated with minimal toxicity. We reported recently that in a human hepatoma cell line acyclic retinoid causes an arrest of the cell cycle in G_0-G_1, increased cellular levels of the $p21^{CIP1}$ protein, and decreased levels of the cyclin D1 protein (11). It has been reported that acyclic retinoid acts synergistically with IFN in suppressing the growth and inducing apoptosis in human hepatoma-derived cells (12). Therefore, acyclic retinoid may be a valuable agent in the chemoprevention and chemotherapy of hepatoma, and its efficacy may be enhanced by combination with agents that target other signaling pathway in hepatoma cells.

Retinoids are thought to exert their biological function by regulating gene expression primary through two distinct nuclear receptors, retinoic acid receptors (RARs) and retinoid X receptors (RXRs), both composed of three subtypes (α, β, and γ; ref. 13). RARs modulate the expression of target genes by interacting with the retinoic acid response element (RARE) located in

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the promoter regions of target genes (13). Both RARs and RXRs can play important roles in influencing the growth of various cancers including hepatoma. Thus, RXRα binds to the enhancer element of hepatitis B virus (14). In addition, phosphorylation of RXRα occurs in hepatoma, and this impairs the function of this receptor (15, 16). The *RAR*α gene is located near one of the integration sites of hepatitis B virus, and its expression is induced in hepatoma (17). The *RAR*β gene can also be an integration site of hepatitis B virus (18). The expression of RARβ is markedly decreased in human hepatoma and in human and rat hepatoma-derived cell lines (19, 20). Among the retinoid receptors, RARβ is thought to be one of the most important receptors with respect to regulation of cell growth and apoptosis. Inactivation of the *RAR*β gene abrogates retinoic acid-induced growth arrest (21), and the induction of RARβ expression is associated with strong enhancement of retinoic acid-induced growth inhibition in breast carcinoma and squamous cell carcinoma cell lines (22, 23). Treatment of human hepatoma cells with all-*trans*-retinoic acid causes a marked increase in cellular levels RARβ mRNA but not RARα mRNA (24). Acyclic retinoid also causes an increase in cellular levels of RARβ mRNA, but not RXRα mRNA, and suppresses expression of the *c-myc* oncogene mRNA in PLC/PRF/5 and Huh7 human hepatoma cells (25). We found that acyclic retinoid specifically caused an increase in the levels of expression of the RARβ protein and mRNA in human esophageal squamous cell carcinoma cells (26). These observations suggest that RARβ is an important mediator of the action of acyclic retinoid and related retinoids, and that induction of the expression of RARβ by acyclic retinoid contributes to the inhibition of growth of tumor cells, including hepatoma cells.

OSI-461 is potent derivative of Apotosyn (sulindac sulfone), which inhibits the cyclic GMP-specific phosphodiesterases 2 and 5 (27). This results in persistent increases in cellular levels of cyclic GMP, which leads to activation of protein kinase G and thereby the induction of apoptosis (27). In colon cancer cells, this process is associated with activation of c-Jun NH_2-terminal kinase 1 and reduction of cellular levels of β-catenin and cyclin D1 (27–29). In chronic lymphocytic leukemia cells, OSI-461 causes an arrest of the cell cycle in G_2-M, induces phosphorylation of Bcl-2 protein, and induces apoptosis (30). OSI-461 can also induce G_2-M arrest in colon cancer cells,[4] but the mechanism responsible for this effect is not known. Thus, OSI-461 can exert growth inhibitory effects in a variety of human cancer cells, apparently via diverse mechanisms. However, there are no previous detailed studies on the anticancer effects of OSI-461 or related compounds in hepatoma cells.

The combined use of two or more chemotherapy agents is often advantageous as it may permit lowering of drug dosages and consequently decrease toxicity, reduce the opportunity for the development of drug resistance by cancer cells, and provide the potential for synergistic effects between drugs that act with different mechanisms. There is growing interest in increasing the antitumor activity of retinoids by combination with other agents, including a histone deacetylase inhibitor (31), IFN-β (12), the tumor necrosis factor-related apoptosis-inducing ligand (32), and conventional chemotherapy agents (33). The synergistic effect of the combination of a retinoid with a histone deacetylase inhibitor appears to be because of enhancement of the retinoid receptor signaling pathway (31). However, the molecular mechanisms responsible for the synergistic effects observed with combinations of retinoids with other anticancer agents are not known. The purpose of this study was to examine whether the combination of acyclic retinoid plus OSI-461 exerts synergistic growth inhibitory effects on human hepatoma cells and to examine possible mechanisms for such synergy, focusing on retinoid receptors and cell cycle control molecules.

MATERIALS AND METHODS

Chemicals. Acyclic retinoid (NIK333, Nikken Chemicals Co., Ltd., Tokyo, Japan) was provided by Dr. Hisataka Moriwaki (Gifu University School of Medicine, Gifu, Japan; ref. 6). OSI-461 was supplied by OSI Pharmaceuticals, Inc. (Farmingdale, NY).

Cell Lines and Cell Culture. The HepG2 human hepatoma cell line was obtained from the American Type Culture Collection (Manassas, VA) and was maintained in DMEM supplemented with 10% fetal bovine serum (DF10; Invitrogen, Carlsbad, CA). Cells were cultured in an incubator with humidified air with 5% CO_2 at 37°C.

Cell Proliferation Assays. Cell proliferation was measured by a colony formation assay as described previously (11, 34). Three thousands cells of HepG2 cells were plated into 6 cm-diameter culture dishes. The following day, acyclic retinoid and OSI-461 were added alone or in combination at the indicated concentrations to each plate, and the cells were incubated for an additional 5 days. The colonies were then fixed with 70% EtOH, stained with Giemsa solution, and counted. Results were expressed as % colony formation with 100% representing control cells treated with 0.1% DMSO alone. All assays were done in triplicate, and mean values were plotted. To determine whether the combined effects of acyclic retinoid plus OSI-461 were synergistic, the combination index-isobologram was used in the drug combination assays (35).

Apoptosis Assays. We used DNA fragmentation and caspase activity assays to detect Apoptosis. HepG2 cells were treated with DMSO (Group 1), 5 μmol/L acyclic retinoid alone (Group 2), 0.1 μmol/L OSI-461 alone (Group 3), or the combination of 5 μmol/L acyclic retinoid plus 0.1 μmol/L OSI-461 (Group 4), respectively, for the indicated time (0, 24, 48, and 72 hours), and the cell lysates were used for apoptosis assays. Briefly, the DNA fragmentation assay was done with the Cell Death Detection ELISA^{PLUS} kit (Roche Diagnostics Co., Indianapolis, IN), which detects the presence of histone-associated DNA fragmentation in the cell cytosol, according to the manufacturer's instructions. The levels of DNA fragmentation found in Group 1 at 0 hours was set to 1, and changes were shown as fold activation. Caspase activity assays were done with a fluorometric system as described previously (36). The cell extracts (10 μg of total protein) were incubated with 2 μg of the fluorogenic peptide substrates Ac-DEVD-AFC, Ac-IETD-AFC (PharMingen, San Diego, CA), or Ac-LEHD-AFC (EMD Biosciences Inc., San Diego, CA), the fluorogenic substrates for

[4] Unpublished data.

caspase-3, -8, and -9, respectively, and caspase activities were calculated by release of AFC, which was measured with a Gemini Fluoro/Luminometer (Molecular Devices Co., Sunnyvale, CA). The caspase activities found in Group 1 at each time point were set to 1, and changes were shown as fold activation.

Cell Cycle Assays. HepG2 cells were treated with test drugs for the indicated time (0, 24, 48, and 72 hours). The harvested cells were stained with propidium iodide (Sigma, St. Louis, MO), and the samples were then analyzed for DNA histograms and cell cycle phase distribution by flow cytometer with a FACS Calibur instrument (Becton Dickinson, Franklin Lakes, NJ), and the data were analyzed with the CELL Quest computer program (Becton Dickinson), as described previously (11, 26, 34).

Cyclic GMP Assays. Intracellular levels of cyclic GMP assays were done as described previously (37). HepG2 cells were plated at 1×10^6 cells/10-cm-diameter plates, and on the following day the test drugs were added. After 1 hour of incubation, the cells were lysed, and the cell extracts were assayed for intracellular levels of cyclic GMP with an enzyme-immunoassay system (cGMP EIA system; Amersham Biosciences, Piscataway, NJ) according to the manufacturer's instructions.

Protein Extraction and Western Blot Analysis. HepG2 cells were treated with the four conditions described above for the indicated time (0, 3, 6, 12, 24, and 48 hours). Protein extracts were then prepared as described previously (11, 26). The cell lysates (20 to 80 μg/lane) were separated by SDS-PAGE with 7.5 to 15% polyacrylamide gels. The primary antibodies for cyclin D1, $p16^{INK4A}$, $p21^{CIP1}$, $p27^{KIP1}$, cdk4, cdk6, ppRb, Bax, Bcl-2, Bcl-x_L, β-catenin, RARα, RARβ, RARγ, RXRα, RXRβ, RXRγ, vasodilator-stimulated phosphoprotein (VASP), and actin were obtained as described previously (11, 26, 34, 37). The p53 (C-19) and Apaf-1 (H324) antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Antimouse IgG, antirabbit IgG (Amersham Biosciences), or antigoat IgG (Santa Cruz Biotechnology) antibodies were used as the secondary antibodies. Each membrane was developed with an ECL-enhanced chemiluminescence system (Amersham Biosciences). An antibody to actin served as a loading control. The intensities of blots were quantified with NIH Image software version 1.62. The intensities of blots found at the 0-hour time point in each Group was set to 1, and changes in expression were shown as the fold difference.

RNA Extraction and Semiquantitative Reverse Transcription-PCR Analysis. RNA extraction and semiquantitative reverse transcription-PCR analysis were done as described previously (11, 26). Total RNA was isolated from frozen HepG2 cells with TRIzol reagent as recommended by the manufacturer (Invitrogen). The cDNA was amplified from 1 μg of total RNA with SuperScript one-step reverse transcription-PCR with the platinum Taq system (Invitrogen). PCR was conducted for 25 to 32 cycles in a thermal controller (Programmable Thermal Controller; MJ Research Inc., Watertown, MA). Cyclin D1, $p21^{CIP1}$, RARβ, and actin-specific primer sets and the amplification cycle condition were as described previously (11, 26). Amplified PCR products obtained with actin-specific primers served as internal controls.

Cyclin D1 and $p21^{CIP1}$ Reporter Assays. Reporter assays were done as described previously (38). The cyclin D1 promoter luciferase reporter plasmid-1745CD1LUC was kindly provided by Dr. Richard G. Pestell (Georgetown University, Washington, DC; ref. 39). The $p21^{CIP1}$ promoter luciferase reporter plasmid WWP-Luc was kindly provided by Dr. Kenneth W. Kinzler and Dr. Bert Vogelstein (The Johns Hopkins University Medical Institutions, Baltimore, MD; ref. 40). One microgram of DNA of the cyclin D1 or $p21^{CIP1}$ luciferase reporter plasmids were transfected into HepG2 cells (1×10^5) with Lipofectin reagent (Invitrogen) in opti-MEM I medium (Invitrogen). After 24 hours the medium was changed to DF10 medium containing the specific drugs indicated in the figure legends. The cells were then incubated for indicated times, and luciferase activities were determined with a luciferase assay system (Program Co., Madison, WI). The cells were also co-transfected with a cytomegalovirus-β-galactosidase reporter. Luciferase activities were normalized with respect to the β-galactosidase activities, to correct for differences in transfection efficiency.

RARE Chloramphenicol Acetyltransferase (CAT)-ELISA Assays. The RARE-CAT reporter plasmid was kindly provided by Dr. David A. Talmage (Columbia University, New York, NY; ref. 41). Transient transfection assays with a RARE promoter reporter were done as described above. Two micrograms of a RARE-CAT plasmid was transfected into HepG2. The cells were treated with drugs for 24 hours, and CAT activity was measured with an ELISA kit (CAT ELISA, Roche Diagnostics Co.) according to the manufacturer's instructions, as described previously (26). RARE-CAT activities were normalized with respect to the β-galactosidase activities, as described above.

Statistical Analysis. Statistical analyses of DNA fragmentation assays, caspase activity assays, cyclic GMP assays, cyclin D1 reporter assays, $p21^{CIP1}$ reporter assays, and RARE-CAT assays were analyzed by either Student's or Welch's *t* test. The results were considered statistically significant if *P* was less than 0.05.

RESULTS

Acyclic Retinoid plus OSI-461 Cause Synergistic Inhibition of Growth in HepG2 Cells. We found that acyclic retinoid and OSI-461 inhibited growth of the HepG2 cells with IC_{50} values of about 30.5 and 0.65 μmol/L, respectively, when the cells were grown in DF10 medium (Fig. 1, *A* and *B*). We then examined the effects of combined treatment with a range of concentrations of both agents on the growth of HepG2 cells. We found that the combination of as little as 1.0 μmol/L acyclic retinoid and 0.01 μmol/L OSI-461 (about the IC_{10} value for both compounds) exerted synergistic growth inhibition. Thus, when analyzed by the isobologram method (35), the combination index for 1.0 μmol/L acyclic retinoid plus 0.01 μmol/L OSI-461 was 2+ and for 1.0 μmol/L acyclic retinoid plus 1.0 μmol/L OSI-461 was 4+ (Fig. 1*C*; Table 1). For subsequent mechanistic studies on the combined effects of acyclic retinoid plus OSI-461, we defined the following 4 conditions of treatment: (1) Group 1, control (0.1% DMSO-solvent); (2) Group 2, 5 μmol/L (about IC_{15} value) acyclic retinoid alone; (3) Group 3,

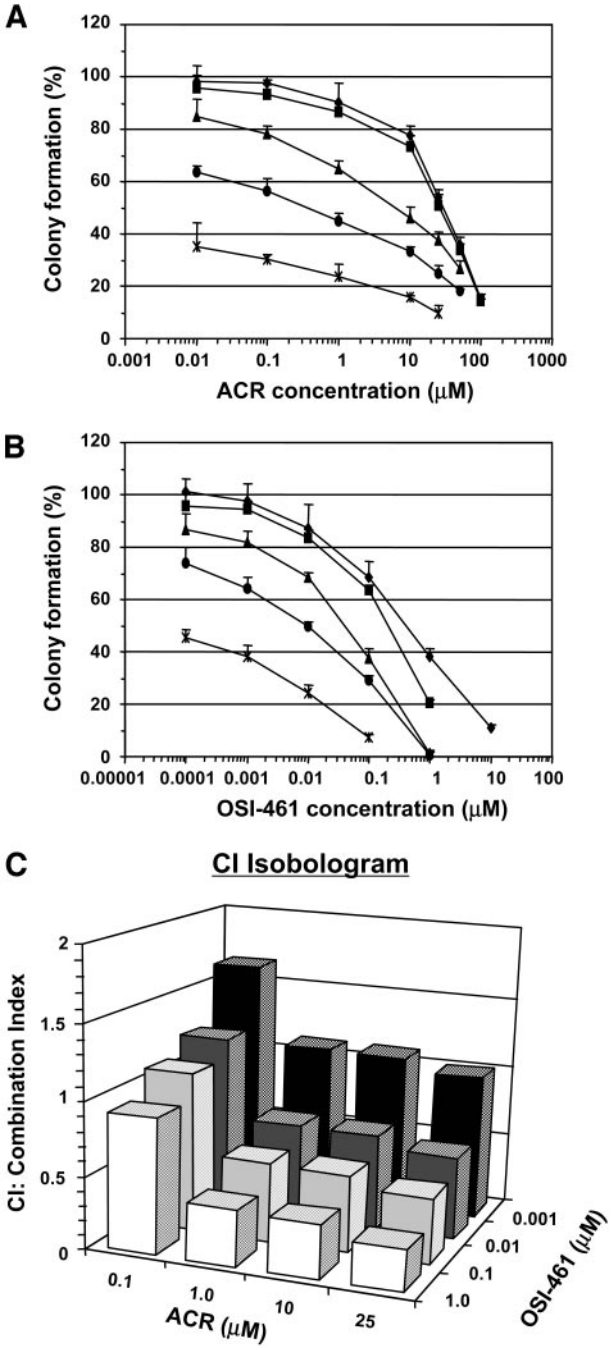

Fig. 1 Inhibition of cell growth by acyclic retinoid alone, OSI-461 alone, and various combinations of these agents in human hepatoma HepG2 cells. *A* and *B*, colony formation assays. HepG2 cells were treated with the indicated concentrations of acyclic retinoid alone, OSI-461 alone, and various combinations of these agents for 5 days, and the numbers of colonies were counted and expressed as percentage of the control value. *C*. The data obtained in *A* and *B* were used to calculate the combination index as described previously (35). Values for triplicate assays; *bars*, ±SD. *A*, ACR alone (♦); ACR + 0.001 μM OSI-461 (■); ACR + 0.01 μM OSI-461 (▲); ACR + 0.1 μM OSI-461 (●); ACR + 1 μM OSI-461 (∗). *B*, OSI-461 alone (♦); OSI-461 + 0.1 μM ACR (■); OSI-461 + 1 μM ACR (▲); OSI-461 + 10 μM ACR (●); OSI-461 + 25 μM ACR (∗). (*ACR*, acyclic retinoid; *CI*, combination index)

0.1 μmol/L (about IC_{32} value) OSI-461 alone; and (4) Group 4, the combination of 5 μmol/L acyclic retinoid plus 0.1 μmol/L OSI-461.

Acyclic Retinoid plus OSI-461 Act Synergistically to Induce Apoptosis in HepG2 Cells. Because both acyclic retinoid (7, 8, 26) and OSI-461 (27, 28) induce apoptosis in cancer cells, we then examined whether the synergistic effect on growth inhibition we observed with combined treatment with these agents (Fig. 1; Table 1) also applied to the induction of apoptosis. To quantify the induction of apoptosis, we measured histone-associated DNA fragmentation with an ELISA system (Fig. 2*A*). We found that the combination of 5 μmol/L acyclic retinoid plus 0.1 μmol/L OSI-461 (Group 4) caused a strong time-dependent induction of apoptosis; the DNA fragmentation ratio increased by 1.6-fold after 24 hours and peaked at 3.8-fold after 48 hours. In contrast, no substantial changes were observed in Groups 2 (5 μmol/L acyclic retinoid alone) or 3 (0.1 μmol/L OSI-461 alone) treated cells during this time course (Fig. 2*A*). We then examined the cellular levels of four apoptosis-related proteins by Western blot analysis (Fig. 2*B*). We found that in Group 4 there was an increase in the levels of expression of the pro-apoptotic proteins Bax and Apaf-1 and a decrease in the levels of the anti-apoptotic proteins Bcl-2 and Bcl-x_L (Fig. 2*B*). In caspase activity assays (Fig. 2*C*), the Group 4 extracts revealed a substantial and time-dependent increase in caspase-9 and -3 activities, which occurred within 24 hours of addition of the two drugs. Caspase-8 activity was also substantially increased after 48 hours (Fig. 2*C*). In contrast, no substantial changes were observed in the levels of apoptosis-related proteins (Fig. 2*B*) or caspase activities (Fig. 2*C*) in the Groups 2 or 3 extracts.

OSI-461 Enhances the G_0-G_1 Arrest Caused by Acyclic Retinoid. Previous studies showed that acyclic retinoid can induce G_0-G_1 cell cycle arrest in HepG2 cells (11) and that OSI-461 can induce G_2-M cell cycle arrest in chronic lymphocytic leukemia cells (30). To determine whether the synergistic effects on growth inhibition (Fig. 1; Table 1) and induction of apoptosis (Fig. 2*A*) caused by combined treatment with these agents were associated with specific changes in cell cycle distribution, cell cycle analysis was done with DNA flow cytometry. The data are summarized in Fig. 2*D*. With the combined treatment (Group 4), the percentage of cells in G_0-G_1 increased by 8.5% after 24 hours and by 17.7% after 48 hours when compared with control untreated cells, and this was associated

Table 1 Combined effects of ACR and OSI-461 on HepG2 cells

OSI-461 concentration (μM)	ACR concentration (μM)			
	0.1 μM	1.0 μM	10 μM	25 μM
0.001 μM	−−	±	±	±
0.01 μM	−	++	++	+++
0.1 μM	±	+++	+++	+++
1.0 μM	±	++++	++++	++++

NOTE: −−, CI > 1.3 antagonism; −, CI 1.1–1.3 moderate antagonism; ±, CI 0.9–1.1 additive effect; +, CI 0.8–0.9 slight synergism; ++, CI 0.6–0.8 moderate synergism; +++, CI 0.4–0.6 synergism; ++++, CI 0.2–0.4 strong synergism.

Abbreviations: ACR, acyclic retinoid; CI, combination index.

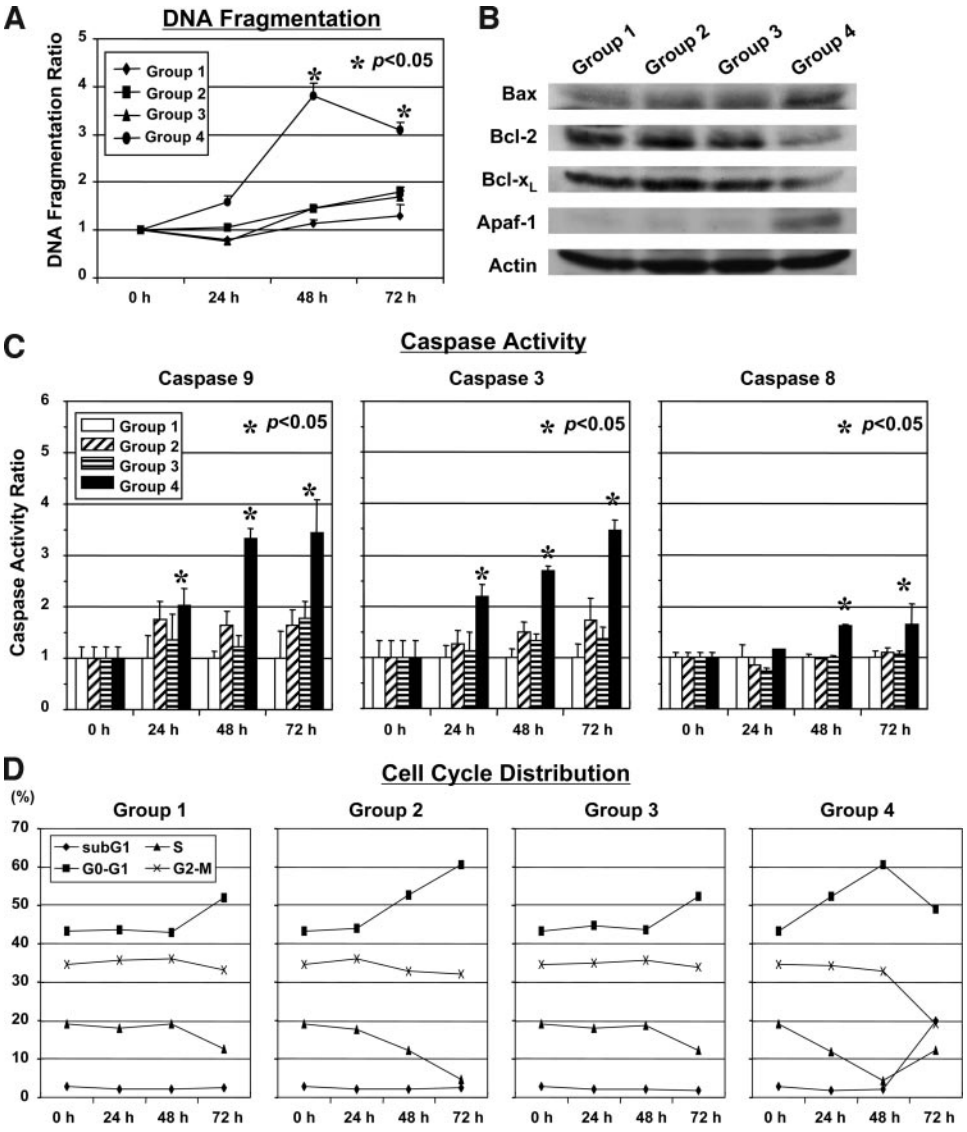

Fig. 2 Effects of the combination of acyclic retinoid plus OSI-461 on apoptosis and cell cycle progression. In this and subsequent figures, Group 1 indicates that cells were treated with DMSO; Group 2, 5 μmol/L acyclic retinoid alone; Group 3, 0.1 μmol/L OSI-461; and Group 4, 5 μmol/L acyclic retinoid plus 0.1 μmol/L OSI-461. *A*, DNA fragmentation assays. HepG2 cells were treated with the indicated drugs as described in Materials and Methods for the indicated times, and DNA fragmentation was determined by an ELISA system. *B*, Western blot analysis for Bax, Bcl-2, Bcl-x_L, and Apaf-1 proteins. HepG2 cells were treated with the indicated drugs for 48 hours, and cell extracts were then examined by Western blot analysis with the respective antibodies. Repeat Western blots gave similar results. *C*, caspase activity assays. HepG2 cells were treated with the indicated drugs for the indicated times. The cells were then lysed, and extracts were assayed for caspase-3, -8, and -9 activities with a fluorometric system, as described in Materials and Methods. *D*, cell cycle assays. The distribution of cells in the sub-G_1, G_0-G_1, S, and G_2-M phases of the cell cycle were calculated and plotted. Each point represents triplicate assays. *Bars*, ±SD.

with a concomitant decrease of cells in the S phase of the cell cycle. Moreover, at 72 hours, there was a marked increase in the sub-G_1 fraction, which is a marker of apoptosis, thus confirming the results on apoptosis obtained in Fig. 2*A*. When compared with the control (Group 1), the Group 2 cells but not the Group 3 cells also displayed an increase in G_0-G_1 cells at 48 hours, but neither group displayed an increase in sub-G_1 cells (Fig. 2*D*).

The Synergistic Combination of Acyclic Retinoid plus OSI-461 Does Not Cause an Increase in Intracellular Levels of Cyclic GMP or the Hyperphosphorylated Form of the VASP Protein. As discussed in the Introduction section, OSI-461 can induce apoptosis in colon cancer cells through a cyclic GMP/protein kinase G pathway (27, 28). To determine whether the induction of apoptosis in HepG2 cells caused by the synergistic combination of acyclic retinoid plus OSI-461 (Fig. 2*A*) was associated with activation of this pathway, we first measured intracellular levels of cyclic GMP (Fig. 3*A*). As a positive control we treated the cells with a relatively high concentration of OSI-461, *i.e.*, 10 μmol/L. As expected from previous studies (37), at 1 hour this substantially increased the intracellular level of cyclic GMP. However, there were no substantial increases in cyclic GMP in Groups 1, 2, 3, or 4 (Fig. 3*A*). We then examined the expression level of the hyperphosphorylated form of the vasodilator-stimulated phosphoprotein protein (VASP-P) because VASP phosphorylation provides a convenient biomarker for the activation of protein kinase G in tumor cells (37). Treatment of HepG2 cells with 10 μmol/L OSI-461 for 1 hour caused a marked increase in VASP-P, but no substantial increase was seen in the extracts from the Groups 1, 2, 3, or 4 cells (Fig. 3*B*). When we examined the time-dependent effect of acyclic retinoid and OSI-461 on cellular levels of VASP-P, across a time period of 0 to 48 hours, there was also no substantial change in Groups 1, 2, 3, or 4 (Fig. 3*C*). Thus, it appears that the growth inhibition (Fig. 1) and apoptosis (Fig.

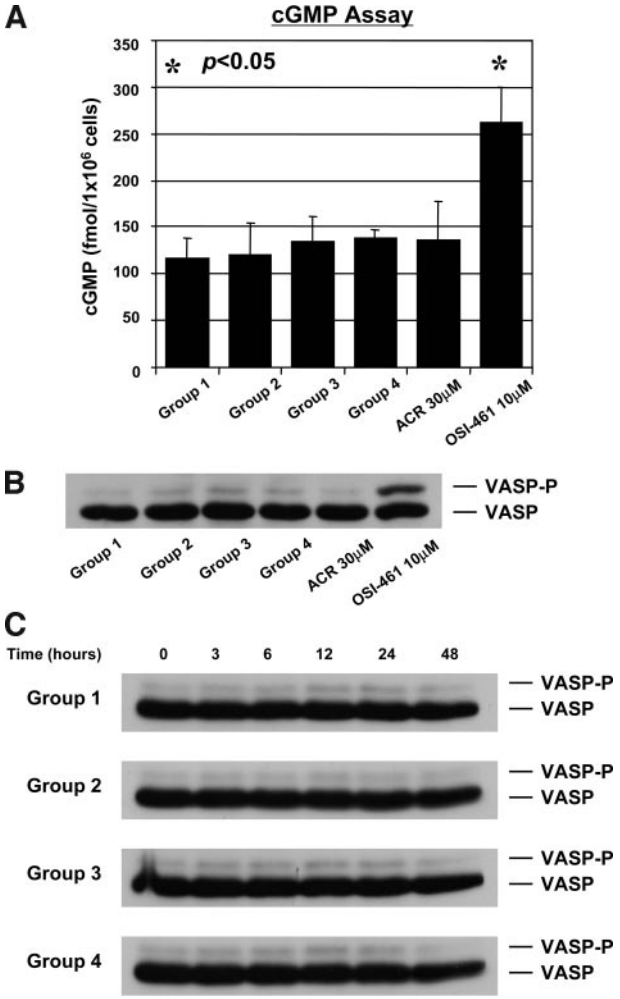

Fig. 3 Effects of the combination of acyclic retinoid plus OSI-461 on intracellular levels of cyclic GMP and the phosphorylated form of the VASP protein (VASP-P). *A*, intracellular levels of cyclic GMP. HepG2 cells were treated with the indicated drugs for 1 hour, and intracellular cyclic GMP levels were then determined by an enzyme-immunoassay system as described in Materials and Methods. Triplicate assays; *bars*, ±SD. *B* and *C*, Western blot analysis for VASP and VASP-P. Cells were treated with the indicated drugs for 1 hour (*B*) or 0 to 48 hours (*C*), and extracts were analyzed by Western blot assays with the respective antibodies. Phosphorylation of the VASP protein causes a shift in the apparent molecular mass of VASP from 46 to 50 kDa (37). Repeat Western blots gave similar results. (*cGMP*, cyclic GMP; *ACR*, acyclic retinoid)

2*A*) induced by the combination of only 0.1 μmol/L OSI-461 and 5 μmol/L acyclic retinoid (Group 4) is not associated with detectable activation of protein kinase G (see Discussion section).

Combined Treatment with Acyclic Retinoid plus OSI-461 Causes a Synergistic Decrease in Cyclin D1 Protein and mRNA, a Decreases in ppRb, and Inhibition of Cyclin D1 Promoter Activity, without Decreasing Cellular Levels of β-Catenin. Because treatment of HepG2 cells with acyclic retinoid plus OSI-461 caused an increase of cells in the G_0-G_1 phase of the cell cycle (Fig. 2*D*), we examined by Western blot analysis the effects of this combination on cellular levels of the G_1 cell cycle control proteins cyclin D1, cdk4, cdk6, and the cyclin-dependent kinase inhibitors $p16^{INK4a}$ and $p27^{KIP1}$ (Fig. 4, *A* and *D*). We also examined cellular levels of the β-catenin protein because its accumulation transactivates Lef/Tcf transcription, thus enhancing the expression of cyclin D1 (42), and OSI-461 can cause a decrease in cellular levels of both the cyclin D1 and β-catenin proteins in SW480 human colon cancer cells (29). We found that the level of cyclin D1 protein displayed a moderate decrease at 24 hours, and a marked decrease at 48 hours in the combined treatment Group 4 cells. There was also a moderate decrease in level of the cyclin D1 protein at 48 hours in Group 2, but no substantial change in cells treated with only 0.1 μmol/L OSI-461 (Group 3). No appreciable changes in the cellular levels of β-catenin protein were found in Groups 1, 2, 3, or 4 at these time points (Fig. 4*A*). The decreases in cyclin D1 protein were paralleled by similar decreases in its mRNA (Fig. 4*B*). In addition, in transient transfection reporter assays (Fig. 4*C*) there were substantial decreases in cyclin D1 promoter activity in Group 4, especially, in assays done for 48 hours. Cells treated with 5 μmol/L acyclic retinoid alone (Group 2) also displayed a substantial decrease in cyclin D1 promoter at 48 hours, but this effect was less than that seen with the combination of drugs. No substantial inhibition was seen in cells treated with only 0.1 μmol/L OSI-461 (Fig. 4*C*). Taken together, with the above studies on mRNA levels (Fig. 4*B*) these results provide evidence that the combined inhibitory effects of acyclic retinoid plus OSI-461 on expression of cyclin D1 are exerted at the level of transcription of the *cyclin D1* gene.

The tumor suppressor protein retinoblastoma protein (pRb) inhibits the G_1 to S transition, but when it becomes hyperphosphorylated (designated ppRb) it no longer exerts this inhibitory effect (43, 44). Therefore, we also examined whether treatment with these agents alters the cellular level of ppRb (Fig. 4*D*). We found that the level of ppRb displayed a marked decrease in Group 2 cells and was virtually undetectable in Group 4 cells at 48 hours after addition of the drugs. However, no change was observed in the Group 3 cells. No detectable change was seen in cellular levels of the cdk4, cdk6, $p16^{INK4a}$, or $p27^{KIP1}$ proteins in Groups 1, 2, 3, or 4 (Fig. 4*D*).

Combined Treatment with Acyclic Retinoid and OSI-461 Causes a Synergistic Increase in $p21^{CIP1}$ and p53. In view of our finding that the combination of acyclic retinoid plus OSI-461 caused a decrease in the expression of cyclin D1 and ppRb (Fig. 4) and an increase of cells in the G_0-G_1 phase of the cell cycle (Fig. 2*D*), we then examined cellular levels of $p21^{CIP1}$ because it binds to cyclin D1-cdk4/6 complexes, thus inhibiting phosphorylation of pRb and delaying transition from the G_1 phase to the S phase of the cell cycle (Fig. 5, *A–C*; refs. 45, 46). We also examined cellular levels of p53 because activation of p53 induces the expression of $p21^{CIP1}$ (Fig. 5*D*; ref. 40). When we examined the effects of acyclic retinoid and OSI-461 on cellular levels of $p21^{CIP1}$ during a 48 hours treatment period, there was a marked increase in the $p21^{CIP1}$ protein at 6 to 12 hours in both Groups 2 and 3 cells. This effect was even stronger in the Group 4 cells, and the increase appeared within 3 hours and persisted for at least 48 hours (Fig. 5*A*, *top* and *bottom* panels). Somewhat similar effects were seen with respect to levels of $p21^{CIP1}$ mRNA, when determined by reverse transcrip-

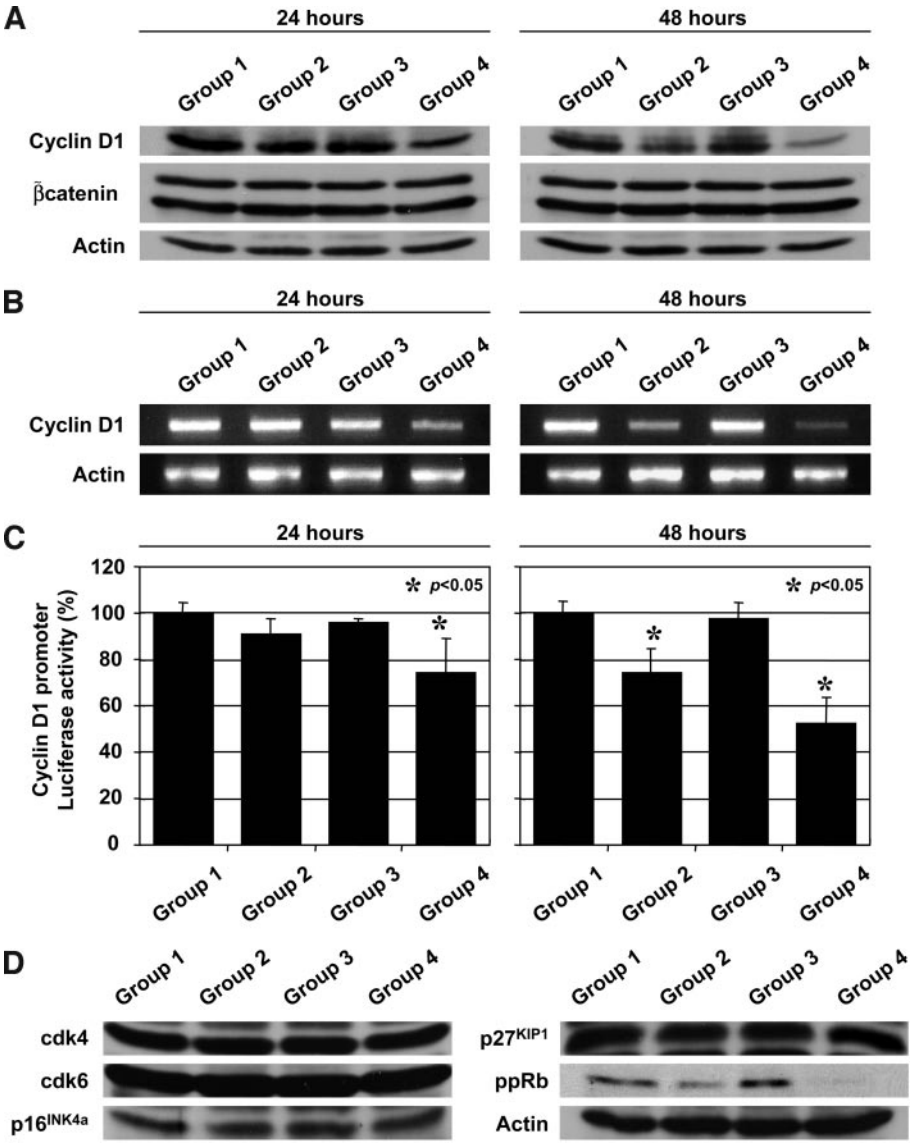

Fig. 4 Effects of the combination of acyclic retinoid plus OSI-461 on expression of cell cycle control proteins and β-catenin. *A* and *B.* HepG2 cells were treated with the indicated drugs for 24 or 48 hours. *A.* The extracted proteins were examined by Western blot analysis with anti-cyclin D1 or anti-β-catenin-specific antibodies. *B.* The extracted mRNAs were examined by reverse transcription-PCR analysis with cyclin D1-specific primers, and actin primers were used as a control. PCR was conducted for 30 cycles. *C,* transient transfection reporter assays for cyclin D1 promoter activity. The cyclin D1 promoter luciferase reporter plasmid −1745CD1LUC was transfected into HepG2 cells, and the transfected cells were treated with the indicated drugs for 24 or 48 hours. The extracts were then examined for luciferase activity. Luciferase activity obtained with cells treated with 0.1% DMSO was defined as 100% at each time point. Triplicate assays; *bars,* ±SD. *D,* Western blot analysis for cdk4, cdk6, $p16^{INK4a}$, $p27^{KIP1}$, and ppRb. HepG2 cells were treated with the indicated drugs for 48 hours, and cell extracts were then examined by Western blot analysis with the respective antibodies. Repeat Western blots and reverse transcription-PCR assays gave similar results.

tion-PCR analysis (Fig. 5*B*). In transient transfection reporter assays (Fig. 5*C*), we also found that 5 μmol/L acyclic retinoid alone (Group 2) and 0.1 μmol/L OSI-461 alone (Group 3) caused an increase in transcriptional activity of $p21^{CIP1}$ promoter when assayed at 6 hours. Moreover, the combination of these agents (Group 4) caused a synergistic increase; the relative luciferase activity increased by 3.9-fold compared with the Group 1 control cells (Fig. 5*C*). Taken together, with the above studies on mRNA levels (Fig. 5*B*) these results provide direct evidence that the *$p21^{CIP1}$* gene was regulated transcriptionally by the combined treatment of acyclic retinoid plus OSI-461. When we examined time-dependent effects of these agents on cellular levels of the p53 protein, we found that there was an increase of this protein at 6 to 24 hours in Groups 3 and 4 cells. However, no substantial changes were observed in cells treated with 5 μmol/L acyclic retinoid alone (Group 2; Fig. 5*D*, *left* and *right panels*).

OSI-461 Enhances the Induction of RARβ Protein and mRNA Produced by Acyclic Retinoid Alone. Because retinoids are thought to exert most of their effects on regulating gene expression by binding to specific retinoid nuclear receptors (13), we then examined effects of the combination of acyclic retinoid plus OSI-461 on cellular levels of specific retinoid receptors (Fig. 6, *A–C*). We focused on RARβ because of our previous evidence, which suggests that acyclic retinoid acts mainly through this receptor (26). Fig. 6*A* indicates that when we examined the time-dependent effect of 5 μmol/L acyclic retinoid (Group 2), we found a small increase in the levels of the RARβ protein after 6 hours, but no substantial effect of 0.1 μmol/L OSI-461 (Group 3). The combination of these two drugs (Group 4) produced a stronger and more persistent induction of RARβ than that seen with acyclic retinoid alone (Fig. 6*A*, *top* and *bottom panels*). Qualitatively similar results were seen with respect to levels of RARβ mRNA in Groups 2 and 4. For

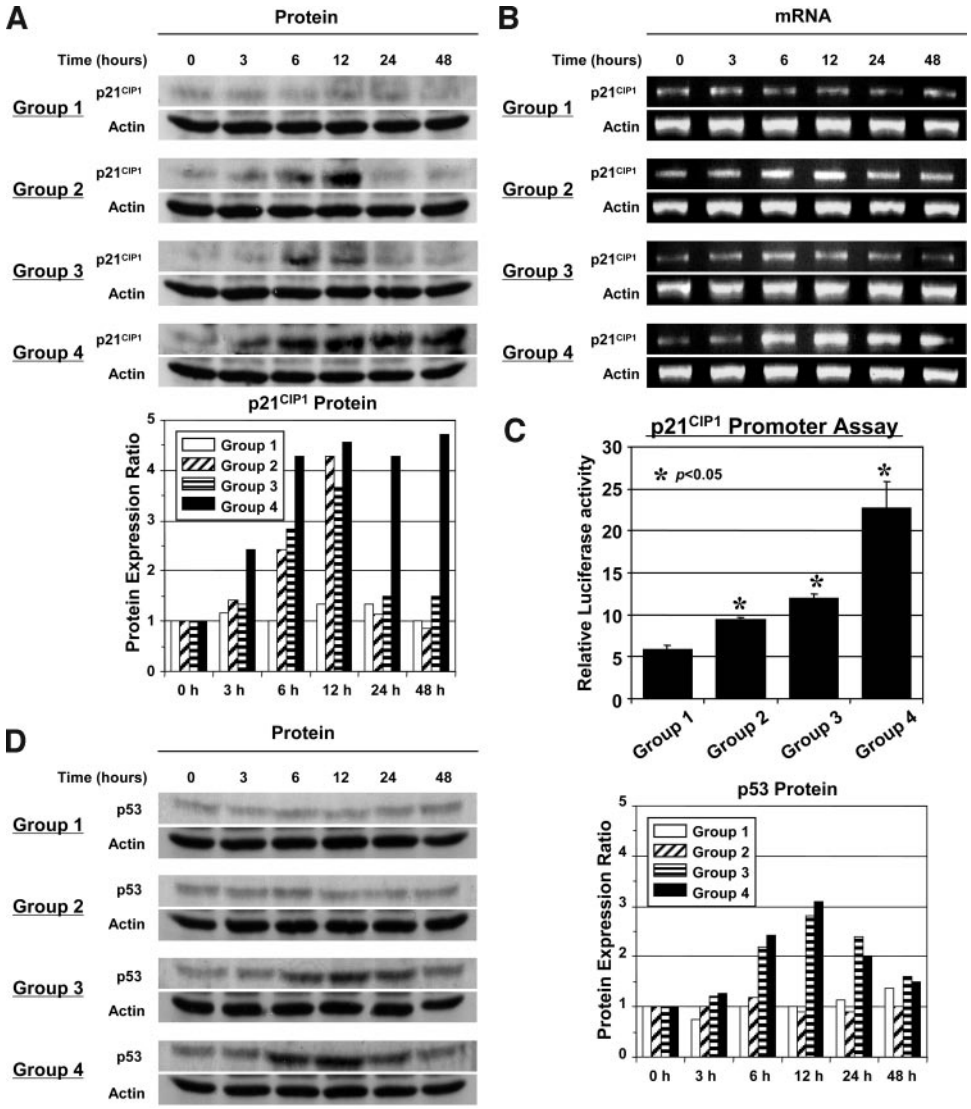

Fig. 5 Effects of the combination of acyclic retinoid plus OSI-461 on expression of $p21^{CIP1}$ and p53. *A*, *B*, and *D*, HepG2 cells were treated with the indicated drugs for the indicated times. The extracted proteins were examined by Western blot analysis with anti-$p21^{CIP1}$–specific (*A*) or anti-p53–specific (*D*) antibodies. The extracted mRNAs were examined by reverse transcription-PCR analysis with the $p21^{CIP1}$-specific primers, and actin primers were used as a control (*B*). PCR was conducted for 25 cycles. The results obtained from Western blot analysis of the $p21^{CIP1}$ and p53 proteins were quantitated by densitometry and are displayed in the (*bottom panel*) of *A* and the (*right panel*) of *B*, respectively. Repeat Western blots and reverse transcription-PCR assays gave similar results. *C*, transient transfection reporter assays for $p21^{CIP1}$ promoter activity. The $p21^{CIP1}$ promoter luciferase reporter plasmid WWP-LUC was transfected into HepG2 cells, and the transfected cells were treated with the indicated drugs for 6 hours. The extracts were then examined for luciferase activity. Triplicate assays; *bars*, ±SD.

reasons that are not apparent, the levels of RARβ appeared to decline in the Group 1 samples at 24 and 48 hours (Fig. 6*B*). No appreciable changes were found in the levels of the other retinoid receptor proteins RARα, RARγ, RXRα, RXRβ, and RXRγ when assayed at 12 hours in the Groups 1 to 4 cells (Fig. 6*C*).

OSI-461 Enhances the Stimulation of RARE-CAT Reporter Activity Produced by Acyclic Retinoid Alone. RARs modulate the expression of target genes by interacting with RARE elements located in the promoter regions of target genes (13). In view of our finding that the combination of acyclic retinoid plus OSI-461 caused a rapid and marked increase in the levels of expression of both RARβ protein and mRNA (Fig. 6, *A* and *B*), we then examined whether the combination of these agents stimulates the transcriptional activity of a RARE-CAT reporter in transient transfection reporter assays (Fig. 6*D*). We found that 30 μmol/L acyclic retinoid caused a marked (2.4-fold) increase of RARE reporter activity, whereas a high concentration of OSI-461 (10 μmol/L) had no effect. When HepG2 cells were treated with 5 μmol/L acyclic retinoid (Group 2) or 0.1 μmol/L OSI-461 (Group 3), there was no substantial stimulation. However, when these two treatments were combined (Group 4), there was a substantial 1.7-fold stimulation of receptor activity (Fig. 6*D*).

DISCUSSION

In the present study, we found that the combination of low concentrations of acyclic retinoid and OSI-461 caused synergistic inhibition of growth of human hepatoma HepG2 cells and that this was associated with arrest of the cell cycle in G_0-G_1 and induction of apoptosis (Figs. 1 and 2; Group 4). In previous studies, we found that when acyclic retinoid was tested alone at its IC_{40} concentration (20 μmol/L) it markedly inhibited growth of HepG2 cells and that this inhibition was associated with arrest of the cell cycle in G_0-G_1, increased cellular levels of the

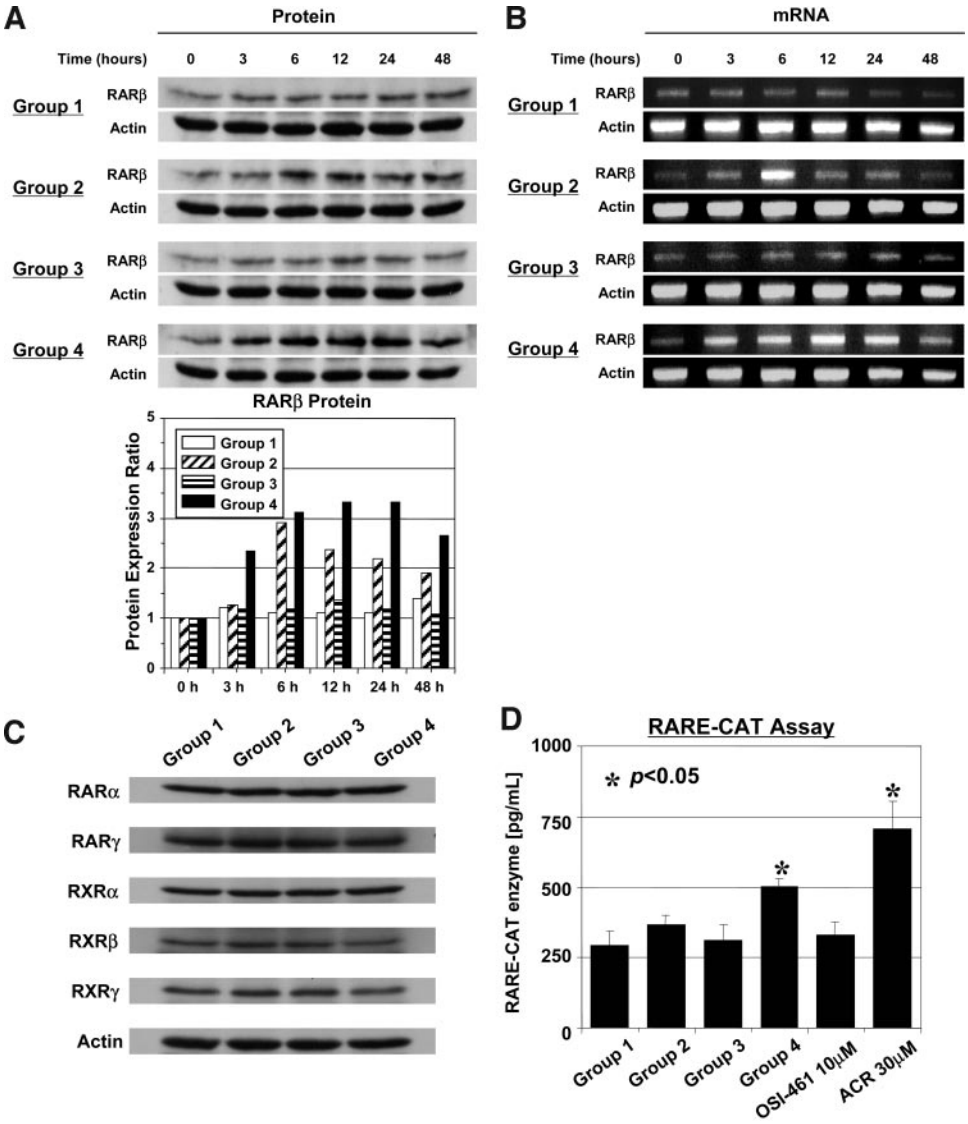

Fig. 6 Effects of the combination of acyclic retinoid plus OSI-461 on expression of nuclear retinoid receptors and on RARE-CAT activity. *A* and *B*, expression levels of RARβ. HepG2 cells were treated with the indicated drugs for the indicated times. The extracted proteins (*A*) or mRNAs (*B*) were examined by Western blot analysis (*A*) or by reverse transcription-PCR analysis (*B*). PCR was conducted for 32 cycles. The results obtained from Western blot analysis of the RARβ protein were quantitated by densitometry and are displayed in the (*bottom panel*) of *A*. *C*, Western blot analysis for other retinoid receptors. HepG2 cells were treated with the indicated drugs for 12 hours, and cell extracts were then examined by Western blot analysis with the respective antibodies. *D*, RARE-CAT activity. The RARE-CAT reporter plasmid was transfected into HepG2 cells, and the cells were then treated with the indicated drugs for 24 hours. RARE-CAT activities were measured with an ELISA system as described in Materials and Methods. Triplicate assays; *bars*, ±SD. Repeat Western blots and reverse transcription-PCR assays gave similar results. (*ACR*, acyclic retinoid)

$p21^{CIP1}$ protein, decreased levels of cyclin D1, and inhibition of the transcriptional activity of the cyclin D1 promoter (11). We also found that acyclic retinoid inhibits the growth of esophageal squamous cell carcinoma cells and that this inhibition is associated with an increase in cellular levels of RARβ (26). Presumably, the latter effect enhances the expression of retinoid responsive genes and inhibition of AP-1 activity, thus contributing to the growth inhibitory effects of acyclic retinoid (26). In the present study, the combination of low concentrations of acyclic retinoid and OSI-461 had a synergistic effect in increasing cellular levels of RARβ and $p21^{CIP1}$, decreasing the expression of cyclin D1, and decreasing the level of ppRb (Figs. 4–6). This drug combination also enhanced induction of the proapoptotic proteins Bax and Apaf-1, reduced the levels of the antiapoptotic proteins Bcl-2 and Bcl-x_L, and caused activation of caspases-3, -8, and -9 (Fig. 2, *B* and *C*). Presumably, the latter effects play a role in the induction of apoptosis (Fig. 2*A*).

A major question posed by the above findings is the molecular mechanisms by which the combination of low concentrations of acyclic retinoid and OSI-461 induce these diverse cellular and biochemical effects. A hypothetical scheme that addresses this question is shown in Fig. 7. This scheme emphasizes positive feedback interactions between the expression of $p21^{CIP1}$ and RARβ, but it is likely that other mechanisms also play a role. We will first focus on the promoter region of the $p21^{CIP1}$ gene. Nuclear retinoid receptors are ligand-dependent transcription factors that bind to RARE elements in the promoter regions of retinoid responsive target genes (13). The promoter region of the $p21^{CIP1}$ gene contains a variety of positive *cis*-acting elements, including both a RARE and a p53-responsive element (47). Indeed, previous studies indicate that all-*trans*-retinoic acid induces transcription of the $p21^{CIP1}$ gene by enhancing binding of the retinoid receptor complex to this RARE in the $p21^{CIP1}$ gene (48). Presumably, our finding that 5 μmol/L acyclic retinoid alone induces expression of $p21^{CIP1}$ at the level of transcription in HepG2 cells is explained

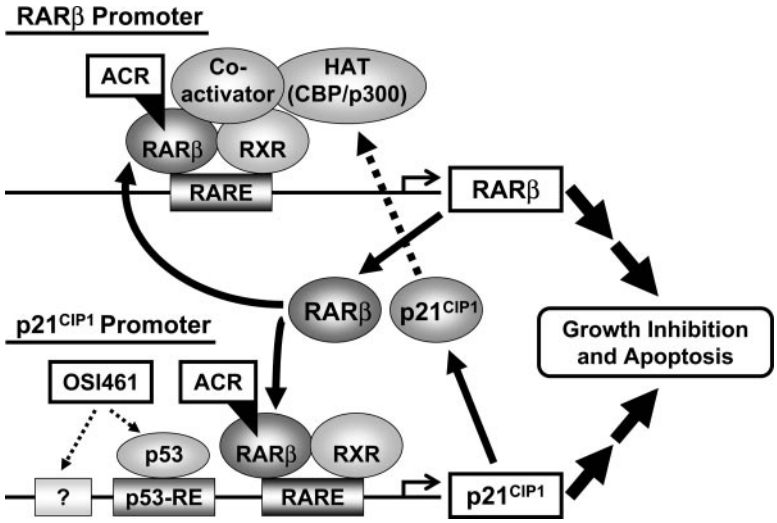

Fig. 7 A hypothetical scheme to explain how reciprocal interactions between RARβ and $p21^{CIP1}$ might contribute to the synergistic effects of acyclic retinoid plus OSI-461 on growth inhibition and apoptosis. This figure indicates that acyclic retinoid binds directly to and activates RARβ, but the mechanism by which OSI-461 increases the expression of p53 is not known. OSI-461 might increase the expression of $p21^{CIP1}$ by an unknown p53-independent pathway. Also shown is the ability of $p21^{CIP1}$ to stimulate histone acetyltransferase activity. For additional details see the Discussion section. (*HAT*, histone acetyltransferase; *ACR*, acyclic retinoid; *CBP*, CAMP responsive element-binding protein; *p53*-RE, p53-responsive element.)

by this mechanism (Fig. 5, *A–C*). We found that 0.1 μmol/L OSI-461 alone caused an increase in cellular levels of both $p21^{CIP1}$ and p53 (Fig. 5). It is known that p53 itself is a potent transcriptional activator of $p21^{CIP1}$ expression (40). This combined effect may explain the rapid, marked, and sustained increase in the levels of expression of both $p21^{CIP1}$ protein and mRNA at the level of transcription that we observed after treating HepG2 cells with acyclic retinoid plus OSI-461 (Fig. 5, *A–C*). OSI-461 can also induce $p21^{CIP1}$ expression in other cell types (30), but the mechanism is not known. This aspect is additionally discussed below. We found a marked decrease in cellular levels of the hyperphosphorylated form of the pRb protein in the combined treatment Group 4 cells (Fig. 4*D*). The pRb protein plays a key role as a negative regulator of the G_1-S transition of the cell cycle by binding to and inhibiting the transcription factor E2F (43, 44). Activation of cyclin D-cdk4 or -cdk6 complexes results in hyperphosphorylation of pRb, thus relieving this inhibitory activity (43, 44). The $p21^{CIP1}$ protein can bind to these cyclin-cdk complexes and inhibit their kinase activity (43, 44). Thus, the marked and sustained induction of $p21^{CIP1}$ by the combination of acyclic retinoid plus OSI-461 may contribute to the arrest in G_0-G_1 that we observed in the Group 4 treated cells (Fig. 2*D*). The other factor that could contribute to this effect is the marked decrease in cyclin D1 expression in the Group 4 treated cells (Fig. 4, *A–C*). Previous studies indicate that when tested alone at higher concentrations both acyclic retinoid and OSI-461 can inhibit the expression of cyclin D1 (11, 26, 29), and in the present study a low concentration of acyclic retinoid caused some inhibition of the expression of cyclin D1 (Fig. 4, *A–C*). The precise mechanism by which these two drugs inhibit cyclin D1 expression is not known.

We will next consider the *RAR*β gene. The promoter region of this gene contains a RARE (24, 49), which explains why 5 μmol/L acyclic retinoid alone can cause a transient increase in cellular levels of both the RARβ protein and mRNA (Fig. 6, *A* and *B*). The transcription of retinoid responsive target genes is also controlled by co-activators (5). When a ligand binds to RAR-RXR heterodimers, co-activators recruit the histone acetyltransferases, cAMP-responsive element-binding protein (CBP) and/or p300 (5). The acetylation of histone H4 by histone acetyltransferases results in nucleosomal repulsion, chromatin decondensation, and enhanced transcription (5). The transcriptional activation by both cAMP-responsive element binding protein (CBP) and p300 can be stimulated by co-expression of $p21^{CIP1}$ (50). Furthermore, introduction of the $p21^{CIP1}$ gene into cells transcriptionally activates the upstream promoter region of the *RAR*β gene (51). Therefore, our finding that the combination of acyclic retinoid plus OSI-461 increased cellular levels of RARβ and RARE-CAT activity (Fig. 6, *A, B,* and *D*) may be due, at least in part, to stimulation of the activities of CPB and/or p300 by the increased cellular levels of $p21^{CIP1}$. This sustained induction of RARβ and its activation by the ligand acyclic retinoid could produce a positive feedback effect on the promoter region of both the *RAR*β and $p21^{CIP1}$ genes, thus enhancing growth inhibition in HepG2 cells. Our findings that the $p21^{CIP1}$ promoter activity was synergistically stimulated by the combined treatment of acyclic retinoid plus OSI-461 is consistent with our hypothesis of a positive feedback effect between the *RAR*β and $p21^{CIP1}$ genes. Overexpression of RARβ in squamous cell carcinoma cells can inhibit cell proliferation in a ligand independent manner, and this inhibition is associated with induction of p300 and CBP (23). These effects as well as other putative growth inhibitory effects of RARβ (21, 22) could additionally contribute to the growth inhibition observed in the present study.

HepG2 cells contain a wild-type *p53* gene (52). The p53 protein plays a critical role in induction of apoptosis in tumor cells by inducing the expression of proteins involved in the mitochondrial death receptor (DR) independent pathway and also in the DR-dependent pathway (53, 54). The induction of apoptosis by acyclic retinoid plus OSI-461 was associated with increased levels of the pro-apoptotic proteins Bax and Apaf-1 and decreased levels of the anti-apoptotic proteins Bcl-2 and Bcl-x_L (Fig. 2, *A* and *B*). RARβ appears to be the most important retinoid receptor with respect to induction of apoptosis

(21–23). Transfection of the *RAR*β gene into RARβ-negative breast cancer cells in the presence of retinoic acid induces apoptosis (22), and transfection of RARβ into human squamous cell carcinoma cells in the presence of 9-*cis*-retinoic acid induces apoptosis, and this is associated with increased cellular levels of Bax and Apaf-1 (23). Therefore, it seems likely that the marked and sustained induction of RARβ in HepG2 cells treated with acyclic retinoid plus OSI-461 (Fig. 6, *A* and *B*) plays an important role in inducing apoptosis in these cells, and that this effect is mediated, at least in part, through the mitochondrial DR-independent pathway. Because we found an increase in caspase-8 activity in HepG2 cells treated with acyclic retinoid plus OSI-461 (Fig. 2*C*), these agents may also activate the DR-dependent pathway of apoptosis (55). OSI-461 could exert this effect through the induction of p53 (Fig. 5*B*). Acyclic retinoid also increases caspase-8 activity in human hepatoma JHH-7 cells (8). Furthermore, Sun *et al.* (32, 56) found that another synthetic retinoid 6-[3-(1-adamantyl)-4-hydroxyphenyl]-2-naphthalene carboxylic acid (CD437) can increase the expression of Fas, DR4, and DR5 in human lung cancer cells. Therefore, in future studies it will be of interest to additionally examine the effects of acyclic retinoid plus OSI-461 on activating the DR-dependent pathway.

As mentioned in the Introduction section, there is evidence that OSI-461-induced growth inhibition and apoptosis involve activation of the cyclic GMP-dependent enzyme protein kinase G (27, 28). Our laboratory found that in colon cancer cells phosphorylation of the cellular protein VASP by protein kinase G provides a convenient endogenous marker of the activation of this pathway (37). Treatment of chronic lymphocytic leukemia (30) and colon cancer cells[5] with OSI-461 can cause G_2-M arrest of the cell cycle, but the mechanism responsible for this effect is not known. In the present study we found that treatment of HepG2 cells with 10 μmol/L OSI-461 (about the IC_{90} concentration) induced an increase in the cellular level of cyclic GMP (Fig. 3*A*), an increase in VASP-P (Fig. 3*B*), and G_2-M arrest of the cell cycle (data not shown). However, the low dose of OSI-461 (0.1 μmol/L), which acted synergistically with acyclic retinoid to induce growth inhibition and apoptosis (Figs. 1 and 2*A*), when used in combination with 5 μmol/L acyclic retinoid in HepG2 cells did not cause a detectable increase in the cellular level of cyclic GMP (Fig. 3*A*), an increase in VASP phosphorylation (Fig. 3, *B* and *C*), or G_2-M arrest (Fig. 2*D*). As mentioned above, this treatment did, however, cause an increase in cellular levels of both $p21^{CIP1}$ and p53 (Fig. 5). Because previous studies indicate that OSI-461 also induces $p21^{CIP1}$ expression in p53-mutated chronic lymphocytic leukemia (30) and SW480 colon cancer cells[6], the induction of $p21^{CIP1}$ by OSI-461 might occur via the p53-independent pathway. However, the precise mechanism by which a low concentration of OSI-461 induces the expression of $p21^{CIP1}$ and p53 remains to be determined. Because it apparently occurs via a cyclic GMP/protein kinase G independent pathway, it may involve a yet to be discovered relatively high affinity target for this drug.

We should emphasize that the hypothetical scheme shown in Fig. 7 and the above discussion on possible molecular mechanisms do not exclude other possible explanations for our findings on the synergistic effects produced by acyclic retinoid plus OSI-461 on growth inhibition and apoptosis in HepG2 cells. Thus, RXRs may also play roles in regulating the promoter activity of the $p21^{CIP1}$ gene because transcriptional activation of this gene is preferentially stimulated by RAR-RXR heterodimers compared with RAR-RAR homodimers (48). Nevertheless, our results suggest that this combination of drugs, or combinations of similar drugs, may be useful in the chemoprevention and/or therapy of hepatoma and possibly other types of cancer. Indeed, in recent unpublished studies we found that the combination of all-*trans*-retinoic acid plus OSI-461 exerts synergistic inhibition of the growth of head and neck squamous cell carcinoma cells. These findings suggest the combination of specific retinoids with OSI-461 might be an effective regimen for the chemoprevention and/or chemotherapy of various types of human malignancies.

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