The Cyclin-dependent Kinase Inhibitor CYC202 (R-Roscovitine) Inhibits Retinoblastoma Protein Phosphorylation, Causes Loss of Cyclin D1, and Activates the Mitogen-activated Protein Pathway

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ABSTRACT

Deregulation of the cell cycle commonly occurs during tumorigenesis, resulting in unrestricted cell proliferation and independence from mitogens. Cyclin-dependent kinase inhibitors have the potential to induce cell cycle arrest and apoptosis in cancer cells. CYC202 (R-rroscovitine) is a potent inhibitor of CDK2/cyclin E that is undergoing clinical trials. Drugs selected to act on a particular molecular target may exert additional or alternative effects in intact cells. We therefore studied the molecular pharmacology of CYC202 in human colon cancer cells. Treatment of HT29 and KM12 colon carcinoma cell lines with CYC202 decreased both retinoblastoma protein phosphorylation and total retinoblastoma protein. In addition, an increase in the phosphorylation of extracellular signal-regulated kinases 1/2 was observed. As a result, downstream activation of the mitogen-activated protein kinase pathway occurred, as demonstrated by an increase in ELK-1 phosphorylation and in c-FOS expression. Use of mitogen-activated protein kinase kinase inhibitors showed that the CYC202-induced extracellular signal-regulated kinases 1/2 phosphorylation was mitogen-activated protein kinase kinase 2 dependent but did not contribute to the cell cycle effects of the drug, which included a reduction of cells in G1, inhibition of bromodeoxyuridine incorporation during S-phase, and a moderate increase in G2-M phase. Despite activation of the mitogen-activated protein kinase pathway, cyclin D1 protein levels were decreased by CYC202, an effect that occurred simultaneously with loss of retinoblastoma protein phosphorylation and inhibition of cell cycle progression. The reduced expression of cyclin D1 protein was independent of the p38α MAPK and phosphatidylinositol 3-kinase pathways, which are known regulators of cyclin D1 protein. Interestingly, CYC202 caused a clear reduction in cyclins D1, A, and B1 mRNA, whereas c-FOS mRNA increased by 2-fold. This was accompanied by a loss of RNA polymerase II phosphorylation and total RNA polymerase II protein, suggesting that CYC202 was inhibiting transcription, possibly via inhibition of CDK7 and CDK9 complexes. It can be concluded that although CYC202 can act as a CDK2 inhibitor, it also has the potential to inhibit CDK4 and CDK1 activities in cancer cells through the down-regulation of the corresponding cyclin partners. This provides a possible mechanism by which CYC202 can cause a reduction in retinoblastoma protein phosphorylation at multiple sites and cell cycle arrest in G1, S, and G2-M phases. In addition to providing useful insights into the molecular pharmacology of CYC202 in human cancer cells, the results also suggest potential pharmacodynamic end points for use in clinical trials with the drug.

INTRODUCTION

The cell cycle is governed by the activities of the cyclin-dependent kinases (CDKs) and their regulatory cyclin partners (1–3). Regulation of these complexes occurs by means of the control of cyclin production and destruction, through inhibitory and activating phosphorylation events, and also via the effects of other proteins. In normal cells, initiation of the cell cycle is achieved through stimulation of growth factor receptors, which propel a cell from the quiescent, noncycling state into the active, cycling state. Mitogenic stimulation of GTPase pathways of the RAS and RHO families results in a cascade of serine-threonine kinase activities (4). The mitogen-activated protein kinase (MAPK) pathway has been extensively characterized. Sequential phosphorylation of Raf-1, mitogen-activated protein kinase kinases (MEKs) 1/2, and then extracellular signal-regulated kinases (ERKs) 1/2 follow Ras activation (5, 6). ERK1/2 activation is associated with induction of cyclin D1 transcription, commonly through the activities of FOS and JUN-related proteins (7). Cyclin D1 then complexes with CDK4 and CDK6 to generate active kinase complexes (1) that directly phosphorylate retinoblastoma protein (RB), causing dissociation of histone deacetylase 1 and thereby allowing histone acetylation, which is permissive for transcription (8, 9). Cyclin E is then transcribed, translated, and complexes with CDK2 to further phosphorylate RB with the release of the general transcription factor E2F-1, which then stimulates the transcription of genes involved in DNA replication (8, 10, 11). The cell no longer depends upon mitogens to complete the current cell cycle, and two daughter cells are produced by mitosis.

In cancer, the cell cycle is commonly deregulated, contributing to tumorigenesis. Defects in cell cycle control occur through multiple mechanisms, including constitutive activation of RAS or phosphatidylinositol 3-kinase (PI3) pathways (12–14); amplification or over-expression of D-type cyclins (15); loss, mutation, or methylation of the CDK4/6 inhibitor p16INK4A (16, 17); constitutive activation of CDK4 via mutation (18); viral inactivation of p21WAF1/CIP1 (19, 20), low expression of p27KIP1 (21); and loss or mutation of the tumor suppressor genes RB (20) and/or TP53 (22). All of these oncogenic events confer a greater propensity for progression through the cell cycle (12, 13).

The activity of CDK2/cyclin E is critical for sufficient phosphorylation of RB (8). Hence, inhibition of CDK2 is an attractive therapeutic approach. Small molecule inhibitors of CDK2 should induce cell cycle arrest. In addition, inhibition of CDK2/cyclin A-mediated phosphorylation of E2F-1 by peptides that inhibit the interaction of CDK2/cyclin A might also induce apoptosis in tumor cells but not in normal cells (23, 24). The aminopurine roscovitine is a small molecule agent, developed through a screening program for inhibitors of CDK1/cyclin B that initially yielded olomoucine (25). Structure-activity relationships with synthetic analogues of olomoucine led to the discovery of the more potent roscovitine, which has an IC50 against CDK1/cyclin B1 of 0.7 μM at 15 μM ATP and an average IC50 for growth inhibition against the National Cancer Institute panel of human tumor lines of 16 μM (26, 27). Roscovitine has been shown to cause both G1 and G2-M arrest in the non-small cell lung carcinoma cell line MDR65 and also in the neuroblastoma line CHOP-212, consistent with inhibition of CDK1 and CDK2 (28). The R-isomer of roscovitine (CYC202; Ref. 29) is a more potent and selective inhibitor of CDK2/cyclin E (30), as compared with racemic roscovitine, that is undergoing Phase I and II clinical trials (31).
CYC202 is 7-fold more potent at inhibiting CDK2/cyclin E (IC$_{50}$, 100 nM), nearly 2-fold more potent against CDK2/cyclin A (IC$_{50}$, 540 nM), and 4-fold less potent against CDK1/cyclin B (IC$_{50}$, 12.69 μM). CYC202 also inhibits CDK7/cyclin H with an IC$_{50}$ of 490 nM.

Despite demonstrating activity against an isolated molecular target, such as a kinase, determination of the precise cellular mechanism of action of a small molecule inhibitor can be very demanding. Although apparently selective in studies with a range of recombinant or purified proteins, the agent may exert additional or alternative effects in intact cells. One study has shown that theroscovitaine analog purvalanol B may bind to ERK2, in addition to CDK1 and CDK2 (32). We therefore decided to investigate the cellular mechanism of action of CYC202 in human colon cancer cell lines and in particular to examine the comparative effects of the drug on RB phosphorylation and the MAPK pathway.

Our results show that CYC202 not only inhibits RB phosphorylation but also induces activation of the MAPK pathway, leading to positive regulation of downstream transcription factors, ELK-1 and c-FOS. The effects of CYC202 on RB phosphorylation and MAPK pathway activation were separated by concentration dependence and also by differential effects with CYC202 analogs. Perhaps surprisingly, given the activation of the MAPK pathway, CYC202 caused a decrease in the expression of cyclins D1, A, and B1, possibly through the loss of total and phosphorylated RNA polymerase II, which may be an additional mechanism through which CYC202 causes or maintains an arrest in all phases of the cell cycle. The results reported here help to provide a better understanding of the cellular pharmacology of CYC202. In addition, the changes seen provide potential pharmacodynamic markers of the activity of this developmental drug in the ongoing clinical assessment.

**MATERIALS AND METHODS**

**Cell Culture.** HT29, NIH3T3 (American Type Culture Collection, Manassas, VA), and KM12 (National Cancer Institute, Bethesda, MD) cell lines were grown in DMEM (Invitrogen, Paisley, United Kingdom) supplemented with 10% fetal bovine serum (Invitrogen) in an atmosphere of 5% CO$_2$. For drug treatment, cells were counted on a Coulter Z2 (Beckman Coulter, High Wycombe, United Kingdom), and 3 × 10$^6$ cells were seeded into a T175 flask (Corning, Acton, MA) and left to attach for 36 h. Compounds were dissolved in DMSO as a 1000× stock and diluted directly into culture media when required. The total concentration of DMSO in medium did not exceed 0.35%. During treatments, and this had no effect on cell growth. CYC202, SB203580 and LY294002 were purchased from Calbiochem (Nottingham, United Kingdom). PD0184352 was from Upstate Inc. (Buckinghamshire, United Kingdom), the reaction was stopped with 4 mM DTT, 2 mM PMSF, 10 μM sodium orthovanadate, and 1 complete protease inhibitor cocktail tablet (Roche, East Sussex, United Kingdom) per 10 ml of lysis buffer for 30 min on ice. Lysates were centrifuged at approximately 18,000 × g for 10 min at 4°C to remove debris. The supernatant was stored at −80°C before use. The protein concentration of lysates was determined using the BCA protein assay (Pierce, Rockford, IL).

Proteins were separated by SDS-PAGE using Novex precast Tris-glycine gels (Invitrogen, Groningen, The Netherlands) and transferred to Immobilon-P membranes (Millipore, Bedford, MA). Membranes were blocked for 1 h in TBSTM [50 mM Tris (pH 7.5), 150 mM NaCl, 0.1% Tween 20 (Sigma), and 3% milk]. Immunoblotting with primary antibodies diluted in TBSTM was performed at 4°C overnight, followed by a 1-h incubation with horseradish peroxidase-conjugated secondary antibodies at room temperature. Membranes were washed with ECL reagents and exposed to Hyperfilm (Amersham Pharmacia Biotech, Buckinghamshire, United Kingdom). Antibodies used were COOH-terminal control total RB 1:5000, phospho-RB Ser-780 1:5000, phospho-ERK1/2 1:1000, phospho-AKT Ser-473 1:1000, phospho-p38 1:1000, phospho-glycogen synthase kinase (GSK) 3β Ser-9 (Cell Signaling Technologies, Beverly, MA), total RB SC-50 1:2000, total ELK-1 SC-355 1:1000, phospho-ELK-1 SC-8406 1:1000, total p38 SC-7972 1:100, c-FOS SC-8047 1:100 (Santa Cruz Biotechnology, Santa Cruz, CA), total ERK2 1:100000 (kindly provided by Prof. Chris Marshall, Institute of Cancer Research, London, United Kingdom), phospho-RB Ser-608 (34) 1:2000 (Dr. Sibylle Mittnacht, Institute of Cancer Research, London, United Kingdom), phospho-RB Thr-821 1:1000 (Biosource, Nivelles, Belgium), total GSK3β 1:1000, under-phosphorylated RB 1:500 (BD Biosciences, Oxford, United Kingdom), phospho-RB Ser-807/811 1:5000 (Sigma) anti-AKT1/PKBα 1:2000 (Upstate Biotechnology, Lake Placid, NY), cyclin B1 Ab-1 1:200, cyclin D1 Ab-1 1:200, cyclin A Ab-6 1:200, CDK1 Ab-1 1:200, CDK2 Ab-4 1:200, CDK4 1:200 Ab-1 (Neomarkers, Fremont, CA), total RNA polymerase II 1:2000 (Abcam, Cambridge, United Kingdom), goat-anti-rabbit and goat-anti-mouse horseradish peroxidase-conjugated secondary antibodies 1:5000 (Bio-Rad, Hercules, CA), and rabbit anti-sheep horseradish peroxidase-conjugated secondary antibody 1:2000 (Upstate Biotechnology). Western blots are representative of at least two independent experiments. Quantification of Western blots was performed in Image Quant software (Amersham Biosciences, Buckinghamshire, United Kingdom). In the RB studies, phosphorylation at Ser-780 was initially evaluated using the robust antibody for that site. This was followed up using the additional RB antibodies listed above. Observations were initially made after 24 h because preliminary data showed that at least 16 h of exposure were required for an antiproliferative effect and because effects on RB phosphorylation were expected at this time for HT29 and KM12 cells that have cell cycle transit times of 20–22 h. Subsequent experiments were carried out over a time course of 4–48 h. All drug-induced changes were replicated in at least two independent experiments.

**Flow Cytometry.** HT29 cells (3 × 10$^6$) were seeded into a 175-cm$^2$ flask and left overnight to attach to the plastic. Drug treatments were performed as above, and 30 min before harvesting, the cells were pulsed with 10 μM bromodeoxyuridine (BrEdUrd; Sigma). Cells were harvested as above, gently pelleted, resuspended in 1 ml of ice-cold PBS, then fixed by slow addition of 5 ml of ice-cold 70% ethanol while vortexing. For BrEdUrd staining of proliferating cells, cells were resuspended in 2.5 ml of 2 N HCl with 0.2 mg/ml pepsin at room temperature for 20 min to isolate nuclei. Nuclei were then washed twice with PBS before resuspension in 0.5 ml of PBS, 0.5% Tween 20, 1% BSA containing 20 μl of rat anti-BrEdUrd antibody (ImmunoDiagnostics Direct, Cambridge, United Kingdom) for 1 h at room temperature. Nuclei were washed with PBS, before resuspension in 0.5 ml PBS, 0.5% Tween 20, 1% BSA, and 20 μl of goat-anti rat IgG whole molecule FITC conjugate (Sigma) for 30 min at room temperature. Nuclei were further washed with PBS and resuspended in 1 ml of 0.04 mg/ml propidium iodide (Molecular Probes, Cambridge, United Kingdom)/0.25 mg/ml RNase A (Sigma) and incubated at 37°C for 30 min. Samples were analyzed on a Beckman Coulter Elite ESP (Beckman Coulter, High Wycombe, United Kingdom), and bivariate cell cycle
analysis was performed with WinMidi2.8 software (Scripps Research Institute, La Jolla, CA). Gates were set according to Robinson et al. (35). All drug-induced changes were replicated in three independent experiments.

RNase Protection Assay. RNA was prepared from cell pellets using TRIzol (Life Technologies, Inc., Paisley, United Kingdom), washed in 75% ethanol, and dissolved in water before quantification at $A_{260}$. RNase protection assay was carried out using the RiboQuant system (Becton Dickinson, Emmelodegem-Aalst, Belgium) with human multi-probe template sets hCyc-1 and hSTRESS-1. Samples (10 $\mu$g of total RNA) were hybridized and RNase digested as described in the manufacturer’s protocol, followed by analysis on a 6% polyacrylamide gel and quantification on phosphor image screens and Image Quant software.

RESULTS

CYC202 Inhibits RB Phosphorylation. Initially, we investigated the effect of CYC202 on the phosphorylation status of RB protein, as determined by Western blotting, using a phospho-specific antibody to Ser-780 of RB. Treatment of asynchronous HT29 and KM12 human colon cancer cells with CYC202 for 24 h resulted in a concentration-dependent loss of RB phosphorylation. Concentrations of 20 $\mu$M and greater caused a clear reduction in phosphorylation at Ser-780 (Fig. 1, A and B). Densitometric analysis of the Western blots was performed, and phosphorylation at Ser-780 decreased by 81% at 20 $\mu$M, 96% at 50 $\mu$M, and 97% at 100 $\mu$M (Fig. 1 A). A similar response was observed in KM12 cells (Fig. 1 B). In addition to the Ser-780 site, CYC202 reduced phosphorylation at Ser-608 (Fig. 1 C), Ser-807/811 and Thr-821 (Fig. 2, A and B). A general loss of RB phosphorylation was also indicated by a switch from a broad band to a thinner, more rapidly migrating band for total RB in HT29 cells (Fig. 1 A). In addition to loss of phosphorylated RB, a decrease in total RB expression was also noted, especially at 20–100 $\mu$M in HT29 cells (Fig. 1 A).

To further characterize the effect of CYC202 on RB phosphorylation, HT29 cells were treated with 50 $\mu$M CYC202 for 24 h, and lysates were incubated with or without calf intestinal alkaline phosphatase to remove all phosphate residues from RB (Fig. 1 C). CYC202 treatment increased the electrophoretic mobility of RB compared with untreated cells. Importantly, an antibody to nonphosphorylated Ser-608 (36) showed an increase in signal in response to CYC202, of phosphorylated RB, a decrease in total RB expression was also noted, especially at 20–100 $\mu$M in HT29 cells (Fig. 1 A).

Fig. 2. Time course of inhibition of RB phosphorylation and induction of ERK1/2 phosphorylation by CYC202. Asynchronous HT29 (A) and KM12 (B) human colon cancer cells were exposed to 48 and 36 $\mu$M CYC202, respectively (3× the IC$_{50}$) for the indicated times. Cell lysates were prepared, and 25 $\mu$g of protein were resolved by SDS-PAGE and subjected to Western blotting for total RB protein, phosphorylated RB at Ser-780, Ser-607/811, Thr-821, total ERK2, phospho-ERK1/2, and glyceraldehyde-3-phosphate dehydrogenase (GAPDH). Lanes labeled C represent asynchronous control cells harvested at the start of the experiment.

Fig. 1. Inhibition of RB phosphorylation and induction of ERK1/2 phosphorylation by CYC202. Asynchronous human colon cancer cells HT29 (A) and KM12 (B) were exposed to increasing concentrations of CYC202 for 24 h. Cell lysates were prepared, and 25 $\mu$g of protein were resolved by SDS-PAGE and subjected to Western blotting for total RB protein, phosphorylated RB at Ser-780, total ERK2, and phospho-ERK1/2. Lanes labeled Control and DMSO represent untreated control cells and vehicle (0.2% DMSO)-treated cells. Densitometric analysis was performed, and the expression of the stated proteins was expressed, relative to controls. C, CYC202 induces a loss of RB phosphorylation and total RB protein. HT29 cells were treated with 50 $\mu$M CYC202 for 24 h, cell lysates were incubated with calf intestinal alkaline phosphatase (CIAP) and then resolved by SDS-PAGE and subject to Western blotting using the stated antibodies. GAPDH, glyceraldehyde-3-phosphate dehydrogenases. D, growth inhibition of HT29 (○) and KM12 (●) colon cancer cells after 96-h exposure to CYC202 as determined by SRB assay. Bars, SD.
demonstrating that loss of total RB protein is not responsible for reduced signal with phospho-specific antibodies. Treatment of cells with calf intestinal alkaline phosphatase increased the mobility of RB for both untreated and CYC202-treated cells, indicating a greater reduction in the phosphorylation of RB than with CYC202 treatment alone. The concentration range over which decreased RB phosphorylation was seen (20–100 μM) was similar to that causing an inhibition of growth in both cancer cell lines, the IC_{50} ± SD for 96 h exposure being 16 (±3) μM in HT29 cells and 17 (±5) μM in KM12 cells (Fig. 1D). These results are consistent with CYC202 acting as a CDK inhibitor, although alternative mechanisms are also possible.

**CYC202 Activates ERK1/2.** To determine the effect of CYC202 on the ERK1/2 MAPK pathway, in relation to the RB changes reported above, cells were exposed to increasing concentrations of the drug, and Western blotting was carried out for total and phosphorylated RB and ERK1/2. Surprisingly, this demonstrated an increase in phosphorylated ERK1/2 in both HT29 and KM12 cells after 24 h of exposure, indicating likely activation of this kinase after drug treatment (Fig. 1, A and B). The increase in ERK1/2 phosphorylation was seen at 50 and 100 μM CYC202. Densitometric analysis (Fig. 1, A and B) showed a 27–36-fold increase in HT29 cells and a 270-fold increase in KM12 cells compared with controls (KM12 cells have very low basal ERK1/2 phosphorylation). Total ERK2 levels were unaffected.

To temporally dissect the events of loss of RB phosphorylation and induction of ERK1/2 phosphorylation by CYC202, HT29 and KM12 cells were exposed to 48 and 36 μM (3× IC_{50} concentrations) CYC202, respectively, for various times (Fig. 2, A and B). The most profound loss of RB phosphorylation at Ser-780 and Ser-807/811 sites occurred from 12 to 24 h after drug exposure. However, loss of RB phosphorylation at Thr-821, the CDK2-specific site, occurred rapidly and peaked at 8–12 h. ERK1/2 phosphorylation remained evident at 24 h. When HT29 cells were treated with 20 μM CYC202, ERK1/2 phosphorylation was still induced at 1–12 h whereas inhibition of RB phosphorylation occurred to a lesser extent. The results suggest that the increase in phosphorylation of ERK1/2 is probably not a direct consequence of inhibition of RB phosphorylation, because ERK1/2 phosphorylation occurs much earlier.

**Induction of ERK1/2 Phosphorylation Is Not Directly Associated with an Inhibition of RB Phosphorylation.** To further investigate whether induction of ERK1/2 phosphorylation might be related to CDK inhibition, HT29 cells were treated with equivalent growth-inhibitory concentrations (3× IC_{50}) of CYC202 and its analogs olomoucine and purvalanol A. Olomoucine is a less potent analog of CYC202 with respect to inhibition of both CDK activity and cell growth (25), whereas purvalanol A is more potent (37). At their respective multiples of the IC_{50} for cell growth, the three tri-substituted aminopurines, CYC202, olomoucine, and purvalanol A, all inhibited RB phosphorylation at 24 h (Fig. 3A). Interestingly, whereas both olomoucine and CYC202 induced ERK1/2 phosphorylation at 24 h, purvalanol A failed to induce ERK1/2 phosphorylation. Total ERK2 protein expression was unchanged (Fig. 3, see also Fig. 1A for CYC202) as assessed by densitometry; olomoucine caused a 24% decrease in ERK2, whereas the other agents did not alter ERK2 (<10% change, data not shown). To confirm that ERK1/2 phosphorylation was independent of inhibition of RB phosphorylation, we investigated the effect of CYC202 in the human osteosarcoma cell line SAOS-2, which lacks RB. After an exposure of 3× IC_{50} for 24 h, a similar increase in ERK1/2 phosphorylation was seen to that in HT29 cells (data not shown). Thus, induction of ERK1/2 phosphorylation by CYC202 is not directly associated with an inhibition of RB phosphorylation.

The above results obtained with the tri-substituted aminopurines are consistent with the view that inhibition of RB phosphorylation is not required or responsible for ERK1/2 activation. The structurally dissimilar CDK inhibitors flavopiridol (38–40) and alsterpaullone (41, 42) were also studied for comparison with CYC202 over a 24-h time course (Fig. 3B). CYC202 gave a similar response to that described earlier in HT29 cells (Figs. 2A and 3B). Flavopiridol caused an inhibition of RB phosphorylation at Thr-821 after 8 h of treatment and very weak inhibition at Ser-780 and Ser-807/811 after 24 h. Alsterpaullone reduced RB phosphorylation at all sites after 8–24 h of treatment, but this was accompanied by a loss of RB protein at 24 h. Flavopiridol and alsterpaullone transiently induced ERK1/2 phosphorylation at 1–4 h after treatment, but this was not sustained compared with CYC202. A similar time course of ERK1/2 activation and inhibition of RB phosphorylation was seen for these compounds in KM12 cells, but with evidence of more extensive inhibition of RB phosphorylation compared with HT29 cells (data not shown).

**CYC202-induced ERK1/2 Activation Results in ELK-1 Phosphorylation and c-FOS Expression.** To determine the functional significance of ERK1/2 activation in response to CYC202, HT29 cells were treated with drug, and the phosphorylation of ELK-1 and expression of c-FOS were assessed by Western blotting. Phosphorylation of ELK-1 and increased expression of c-FOS are associated with functional activation of ERK1/2 (7). As described earlier, CYC202 again caused a loss of RB phosphorylation at 24 h, and an induction of ERK1/2 phosphorylation was seen at 4–24 h (Fig. 4A). A strong induction of ELK-1 phosphorylation was observed after 4 h of CYC202 treatment, as shown by a mobility shift in total ELK-1 and confirmed by use of a phospho-specific antibody to Ser-383 on ELK-1 (Fig. 4A). Induction of c-FOS protein, which is transcriptionally regulated by ELK-1 (43–45), was also seen at 24 h in HT29 colon cancer cells treated with CYC202 (Fig. 4A). A time-course experiment showed that although ERK1/2 phosphorylation was induced at 1 h and beyond, a 4-h exposure was required to induce ELK phos-
were prepared, and 50 μM CYC202, 10 μM U0126, or a combination of both agents for the times indicated. Cell lysates were prepared, and 50 μg of protein were resolved by SDS-PAGE and subjected to Western blotting for the stated proteins. Lanes labeled C and D represent untreated control cells and vehicle (0.2% DMSO)-treated cells. C, cell cycle distribution of HT29 cells exposed to DMSO, 50 μM CYC202, 10 μM U0126, and a combination of both CYC202 and U0126 for 24 h, as assessed by propidium iodide staining and analysis by flow cytometry. The number of cells in each phase of the cell cycle is expressed as a percentage of gated events.

Cell cycle effects, 50 μM CYC202 caused a reduction in G1 phase in HT29 cells and also a modest increase in the G2-M phase at 24 h (Fig. 4B), as did flavopiridol and alsterpaullone (data not shown). U0126 at 10 μM caused a tight G1 arrest at 24 h, consistent with inhibition of the ERK1/2 MAPK pathway (4, 50). After combined exposure to U0126 and CYC202 for 24 h, the cell cycle profile was much more similar to that for CYC202 alone than with U0126 alone. The same result was confirmed with PD0184352 (data not shown). Therefore, activation of the ERK1/2 MAPK pathway does not appear to be essential for the cell cycle changes induced by CYC202. In addition, no change in CYC202-induced cell death was observed, as demonstrated by assessment of the sub-G1 population on flow cytometry or by measurement of total cell counts (data not shown).

CYC202 Induces a Loss of Cyclin D1. Because activation of the ERK1/2 MAPK pathway is known to stimulate production of cyclin D1 and hence promote cell cycle progression (4, 50), HT29 colon cancer cells were treated with CYC202 to determine the effect upon cyclin D1 protein levels. Contrary to expectations, based on the effects reported herein on ERK1/2, ELK-1, and c-FOS, treatment with CYC202 did not increase cyclin D1 but rather resulted in a marked reduction (Fig. 5A). Densitometric analysis showed a decrease of 13% at 4 h, 85% at 8 h, 84% at 12 h, and 74% at 24 h (Fig. 5B). Fig. 5A shows that this occurred in the same time frame as the loss of RB phosphorylation, which decreased by 40% at 8 h, 58% at 12 h, and 74% at 24 h (Fig. 5B). Importantly, U0126 also prevented the induction of ERK1/2 phosphorylation in response to CYC202 and in addition blocked the associated CYC202-induced phosphorylation of ELK-1 and the increase in c-FOS (Fig. 4A). Similar results were obtained with the structurally dissimilar MEK1/2 inhibitor PD0184352 (Refs. 48, 49; data not shown). Hence, it can be concluded that both ERK1/2 phosphorylation and activation of the down-stream ERK1/2 pathway in response to CYC202 are MEK1/2 dependent in HT29 colon cancer cells.

In terms of cell cycle effects, 50 μM CYC202 caused a reduction in G1 phase in HT29 cells and also a modest increase in the G2-M phase at 24 h (Fig. 4B), as did flavopiridol and alsterpaullone (data not shown). U0126 at 10 μM caused a tight G1 arrest at 24 h, consistent with inhibition of the ERK1/2 MAPK pathway (4, 50). After combined exposure to U0126 and CYC202 for 24 h, the cell cycle profile was much more similar to that for CYC202 alone than with U0126 alone. The same result was confirmed with PD0184352 (data not shown). Therefore, activation of the ERK1/2 MAPK pathway does not appear to be essential for the cell cycle changes induced by CYC202. In addition, no change in CYC202-induced cell death was observed, as demonstrated by assessment of the sub-G1 population on flow cytometry or by measurement of total cell counts (data not shown).

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70% at 24 h. Total RB was unchanged at 4 h, decreased by 16% at 8 h, 31% at 12 h, and 37% at 24 h. To investigate how these signaling events relate to the induction of cell cycle arrest by CYC202, HT29 cells were treated with 50 μM CYC202 and harvested at various times for analysis of the ability of cells to incorporate BrdUrd by flow cytometry (Fig. 5C). Approximately 56% of vehicle-treated cells incorporated BrdUrd into their DNA, demonstrating active progression through S-phase. After 8 h of treatment with CYC202, 0% of cells were BrdUrd positive, and at 24 h only 4% of cells were labeled. Thus, the cells were losing the ability to progress through S-phase at the same time that RB phosphorylation was inhibited, and levels of cyclin D1 protein were decreased. CYC202 (3 × IC50 for 24 h) also decreased cyclin D1 levels in SAOS-2 cells (data not shown). Thus, the loss of cyclin D1 was not a consequence of the inhibition of RB phosphorylation.

**CYC202 Induces Phosphorylation of p38SAPK**. Phosphorylation of cyclin D1 at Thr-286 by p38SAPK has been suggested as a mechanism by which cyclin D1 is targeted for degradation by the proteasome to mediate a cell cycle arrest after osmotic stress (51). Therefore, the effect of CYC202 on the activation of p38SAPK by phosphorylation was assessed in HT29 cells using Western blotting. Fifty μM CYC202 caused an increase in p38SAPK phosphorylation at 1 h. This increased up to 8 h, falling slightly at 12 and 24 h but still remaining above basal phosphorylation levels (Fig. 5A).

**Induction of p38SAPK Phosphorylation and Cyclin D1 Depletion Are Independent of Inhibition of RB Phosphorylation.** To determine whether p38SAPK activation and loss of cyclin D1 protein were related to CDK inhibition, HT29 cells were exposed to 3 × IC50 concentrations of CYC202, olomoucine, purvalanol A, flavopiridol, and alsterpaullone for 24 h. Only olomoucine and CYC202 caused induction of p38SAPK phosphorylation and marked loss of cyclin D1 at 24 h (Fig. 6A). As before, flavopiridol and alsterpaullone were also studied for comparison with CYC202 over a 24-h time course (Fig. 6B). CYC202 induced p38SAPK phosphorylation from 4 to 24 h and again caused the consistent decrease in cyclin D1 protein from 4 h and greater after treatment. In HT29 cells, flavopiridol had no effect on p38SAPK phosphorylation but reduced cyclin D1 protein, particularly at 8 h. Alsterpaullone induced p38SAPK phosphorylation from 1 to 8 h, but this decayed by 24 h. No loss of cyclin D1 was observed in response to alsterpaullone.

**CYC202 Mediated Loss of Cyclin D1 Is p38SAPK Independent.** To determine whether activated p38SAPK was phosphorylating cyclin D1 at Thr-286 and targeting it for proteolytic degradation in response to CYC202, HT29 cells were treated with 50 μM CYC202, 20 μM SB203580 (an inhibitor of p38SAPK activity; Ref. 52), or both compounds simultaneously for a 24-h period (Fig. 6C). As reported earlier in this paper, CYC202 again caused an increase in p38SAPK phosphorylation and a loss of cyclin D1 protein. However, SB203580 had no effect on CYC202-induced phosphorylation of p38SAPK or on CYC202-induced decrease in cyclin D1 protein. Therefore, loss of cyclin D1 in response to CYC202 is not mediated through p38SAPK activity.

**CYC202 Does Not Inhibit PI3 Kinase.** Cyclin D1 levels can also be regulated by the PI3 kinase pathway (53). To determine whether CYC202 reduced cyclin D1 levels through modulation of this pathway, the widely used NIH3T3 cell line model was used initially because this gives a clear readout of the PI3 kinase pathway after mitogenic stimulation (54). Cells were serum starved and then treated with either vehicle, 50 μM CYC202, 10 μM U0126, or 20 μM of the PI3 kinase inhibitor LY294002 (55) for 30 min. Cells were then harvested immediately or stimulated with 100 ng/ml of IGF-I for 15 or 30 min, followed by lysis and analysis by Western blotting (Fig. 7). Starved NIH3T3 cells exhibited no detectable phosphorylated AKT at Ser-473; however, IGF-I treatment resulted in phosphorylation of AKT. Neither CYC202 nor the MEK1/2 inhibitor U0126 had affected AKT phosphorylation at 15 or 30 min after stimulation, confirming that they do not inhibit the PI3 kinase pathway in these cells. As expected, the PI3 kinase inhibitor LY294002 blocked phosphorylation of AKT. Also as predicted, the MEK1/2 inhibitor U0126 abolished basal ERK1/2 phosphorylation before and after IGF-I stimulation. Consistent with inhibition of PI3 kinase and subsequent loss of AKT phosphorylation, GSK3β phosphorylation at Ser-9 was inhibited in response to LY294002. This site on GSK3β is phosphorylated by AKT and is an inactivating event (56). In agreement with its lack of effect on AKT phosphorylation, CYC202 did not alter GSK3β phosphorylation. In addition, cyclin D1 was unaffected after this short exposure. However, 50 μM CYC202 caused a loss of cyclin D1 protein after 8 h of treatment, demonstrating that inhibition of cyclin D1 was occurring in NIH3T3 cells and that this cell line is a valid model for determining the effects of CYC202 upon cyclin D1 protein expression (data not shown).

To relate these findings to HT29 colon cancer cells, the effect of CYC202 on constitutive AKT phosphorylation was also determined in these cells. Treatment with 50 μM CYC202 for up to 24 h did not inhibit AKT phosphorylation at Ser-473; in fact, at some time points an increase was seen (Fig. 7B).

**CYC202 Causes Down-Regulation of Cyclins D1, A, and B1 and Up-Regulation of c-FOS at the mRNA Level.** Having excluded both p38SAPK and the PI3 kinase pathway as mediators of the loss of
cyclin D1 protein in response to CYC202, expression of cyclin D1 mRNA in response to 50 μM CYC202 was determined by RNase protection assay. HT29 cells were treated with CYC202 for 24 h, and mRNA expression was determined (Fig. 8A). Interestingly, cyclin D1 mRNA expression decreased by 70% after drug treatment. Both cyclin A and cyclin B1 mRNA levels were also markedly decreased, with almost no detectable mRNA remaining at 24 h (Fig. 8A). In contrast, c-FOS mRNA expression was found to increase in response to CYC202 by up to 2-fold as compared with controls (Fig. 8A). Expression of p21WAF1/CIP1 mRNA was slightly decreased. The decrease in cyclin D1 mRNA and increase in c-FOS mRNA are consistent with the CYC202-induced changes in cyclin D1 and c-FOS at the protein level, reported earlier herein. Fig. 8D also shows that decreases in cyclin A and B1 mRNA are observed at the protein level. In contrast, no changes in CDK1, CDK2, or CDK4 expression were seen (Fig. 8A).

Roscovitine was reported to inhibit mRNA synthesis by up to 65%, possibly through inhibition of CDK7/cyclin H or even CDK9/cyclin T1 (57, 58). To determine whether inhibition of these CDKs could mediate a loss of cyclin mRNAs, expression and phosphorylation of the COOH-terminal domain of RNA polymerase II were assessed. Fig. 8C shows that 48 μM CYC202 caused a time-dependent loss of both total and phosphorylated RNA polymerase II from 4 h onwards and that this occurred within the same time frame as loss of cyclin D1 protein.

**DISCUSSION**

We have demonstrated that CYC202 reproducibly inhibits RB phosphorylation in a concentration- and time-dependent manner in human colon cancer cell lines in cell culture. These effects were seen at pharmacologically relevant exposures (3× IC50). The most profound loss of RB phosphorylation occurred at 12–24 h. Interestingly, multiple phosphorylation sites on RB were inhibited, Ser-780 and Ser-807/811 have been reported to be phosphorylated solely by CDK2 complexed with either cyclin A or E (59). Loss of Thr-821 phosphorylation at 4–8 h after treatment preceded inhibition of phosphorylation observed at Ser-780 and Ser-807/811, which occurred after 12–24 h. Therefore, selective inhibition of the putative CDK2-exclusive phosphorylation site was observed initially, followed by inhibition of other phosphorylation sites after a more prolonged exposure to CYC202.

Because RB is a direct substrate of the CDKs, the decrease in RB phosphorylation is consistent with CYC202 inhibition of CDK activity in cells, especially at early time points. On the other hand, multiple mechanisms are potentially capable of reducing RB phosphorylation, including inhibition of upstream RAS or PI3 kinase pathways. Roscovitine and its R-enantiomer CYC202 are well characterized with respect to their action as ATP-competitive inhibitors of purified or recombinant CDKs (26, 30, 60). However, as with all developmental and even established anticancer drugs, there is potential for additional or alternative modes of action in cells. The observation that a close analog of CYC202, purvalanol B, can bind to ERK2 (32) prompted us...
to investigate the effects of CYC202 on the ERK1/2 pathway (Fig. 2, A and B). Surprisingly, at concentrations that inhibit RB phosphorylation and cell proliferation in HT29 and KM12 colon cancer cells, CYC202 caused rapid induction of ERK1/2 phosphorylation. This effect peaked at 8–12 h, although it remained elevated compared to controls after 24 h of treatment. It was possible that this unusually sustained activation of the ERK1/2 pathway could be significant in the induction of cell cycle arrest. Several studies have demonstrated that although transient ERK1/2 phosphorylation precedes G1-S entry, sustained ERK1/2 phosphorylation can induce cell cycle arrest and possibly also differentiation (45, 61–63). ERK1/2 activation was additionally seen with iso-effective concentrations of the less potent CYC202 analog olomoucine. However, induction of ERK1/2 phosphorylation was not a general response to CDK inhibition and reduced RB phosphorylation, because the more potent analogue purvalanol A inhibited RB phosphorylation but failed to induce ERK1/2 phosphorylation at equivalent growth-inhibitory concentrations. This result suggests that substitution at position 6 of the benzylamino group of the tri-substituted aminopurines may be involved in ERK1/2 induction, because introduction of chlorine at this position, as in purvalanol A, caused loss of this property while increasing inhibitory potency on CDK2. Both flavopiridol and alsterpaullone inhibited RB phosphorylation in a time- and cell line-dependent manner.

It is clear from our results that induction of sustained ERK1/2 phosphorylation can be dissociated temporally, as well as in terms of structure-activity relationships, from CDK inhibition and loss of RB phosphorylation. Further evidence dissociating induction of ERK1/2 phosphorylation from inhibition of RB phosphorylation came from the demonstration of induction of ERK1/2 phosphorylation in RB-deficient SAOS-2 cells.

To determine the functional significance of ERK1/2 phosphorylation in response to CYC202, the status of proteins regulated downstream of the ERK1/2 pathway was assessed. At concentrations of CYC202 that induced phosphorylation of ERK1/2, an increase in the phosphorylation of ELK-1, together with an elevation in c-FOS protein, occurred from 4 to 24 h. Hence, ERK1/2 was indeed functionally activated after CYC202 treatment with the predicted downstream consequences. The functional activation of the ERK1/2 MAPK pathway may be surprising, given that CYC202 inhibited recombinant ERK2 in cell-free assays, albeit at an IC50 value (1.17 μM) that is much higher than for CDK2/cyclin E. The lack of ERK1/2 inhibition may relate in part to likely lower inhibitory activity in the presence of high intracellular ATP. However, the precise reasons remain unclear.

It should be highlighted that the activation of the ERK1/2 pathway by CYC202 is more sustained compared with mitogenic stimulation (64). Although induction of ERK1/2 phosphorylation is relatively quick, the increase in ELK phosphorylation and c-FOS is comparatively slow. Recent data suggest that c-FOS acts as a sensor of ERK1/2 signal duration (65). Sustained ERK1/2 and p90RSK activity cause c-FOS stabilization by direct phosphorylation, extending the half-life of c-FOS protein. Such effects may contribute to the delayed increase in c-FOS protein expression in response to CYC202. The FOS family of proteins is involved in the induction of cell proliferation (43, 44); therefore, it could be speculated that activation of the MAPK pathway might represent an attempt by the cell to overcome the growth-inhibitory effects of CYC202. Alternatively, this may be part of a MAPK pathway-induced stress response, leading to cell cycle arrest.

It is known that the CYC202 analog purvalanol A can bind to and inhibit ERK1/2 (26); hence, it is possible that such binding may induce a conformational change that promotes its phosphorylation by MEK1/2. The potent and selective MEK1/2 inhibitor U0126 prevented CYC202-induced phosphorylation of ERK1/2 in human colon cancer cells. Similar results were obtained with the chemically dissimilar and even more selective MEK1/2 inhibitor PD0184352. Thus, it can be concluded that MEK1/2 activity is required for CYC202 to induce ERK1/2 phosphorylation. Furthermore, we showed clearly that the MEK1/2 to ERK1/2 pathway is also responsible for the drug-induced activation of the transcription factors ELK-1 and c-FOS. Interestingly, U0126 did not substantially alter the CYC202-induced changes in the cell cycle distribution, which involved a reduction in G1 and a modest increase in G2-M phase. U0126 did not potentiate CYC202-induced cell death, as measured by the sub-G1 peak on flow cytometry and cell counts, in contrast to reports for combinations of UCN-01 and MEK1/2 inhibitors (66), nor was any protection observed. This suggests that CYC202-induced ERK1/2 phosphorylation does not modulate the cell cycle or apoptotic effects of the drug. Interestingly, activation of ERK1/2 by cisplatin and phenethyl isothiocyanate was critical for the induction of apoptosis, and inhibitors of MEK1/2 have blocked apoptosis induced by these agents (67, 68).

That CYC202-induced cell death is not modulated by MEK1/2 inhibitors demonstrates the complex relationship between ERK1/2 phosphorylation induced by various agents and cellular outcome.

Activation of the MAPK pathway is known to induce the synthesis of cyclin D1 (7). Therefore, we measured expression of cyclin D1 in HT29 colon cancer cells in response to 50 μM CYC202. Surprisingly, at the same time as the induction of ERK1/2 phosphorylation, cyclin D1 protein expression was almost completely lost. This was unexpected given the activation of the ERK1/2 pathway by CYC202. On the other hand, loss of cyclin D1 could potentially contribute to the loss of CDK activity toward RB, particularly through inhibition of CDK4 activity. Furthermore, this effect could contribute to the cell cycle effects of CYC202, especially the G1 arrest (69), and also the loss of phosphorylation at sites on RB believed to be targets of CDK4 (such as Ser-780). Therefore, although capable of acting as a selective CDK2 inhibitor, CYC202 may also reduce the activity of CDK4/ cyclin D1 complexes through loss of the cyclin partner.

It was possible that the CYC202-induced decrease in cyclin D1 levels could occur through proteasomal degradation or via inhibition of cyclin D1 mRNA or protein synthesis. Two signaling pathways are implicated in targeting cyclin D1 for destruction by the ubiquitin-dependent proteasome machinery, i.e., involving PI3 kinase and p38SAPK (5, 73). Although p38SAPK phosphorylation was increased by CYC202 with a concomitant decrease in cyclin D1 protein expression, the p38SAPK inhibitor SB203580 (52) failed to block the CYC202-induced loss of cyclin D1 (47). Thus, the possibility that p38SAPK was responsible for cyclin D1 depletion can be excluded.

In addition to p38SAPK, Thr-286 of cyclin D1 can also be phosphorylated by GSK3β, which is in turn regulated by AKT in the PI3 kinase pathway (53, 70). Our results showed that CYC202 failed to inhibit PI3 kinase or AKT kinase activation after acute stimulation of NIH3T3 cells by IGF-I. In addition, CYC202 had no effect on constitutive AKT phosphorylation at Ser-473 in HT29 colon cancer cells, confirming that the decrease in cyclin D1 levels was not attributable to PI3 kinase inhibition.

To determine whether the loss of cyclin D1 induced by CYC202 might be occurring as a result of decreased mRNA levels, HT29 colon cancer cells were treated with CYC202, and cyclin D1 mRNA was quantified by an RNase protection assay. We demonstrated that CYC202 caused a down-regulation of cyclin D1 mRNA after 24 h of treatment. In addition, we found that mRNA levels for both cyclins A and B1 were also reduced. Interestingly, p21WAF1/CIP1 mRNA was unaltered, in contrast to the reported effect for roscovitine (71). However, c-FOS mRNA was increased by CYC202. This suggests that the accumulation of c-FOS protein may at least in part be
mediated at the mRNA level. The above trends in altered mRNA expression were confirmed at the protein level by Western blotting. A clear loss of cyclin D1, A, and B1 proteins was observed alongside the increase in c-FOS. We also showed that CYC202 inhibits expression of both total RNA polymerase II and the COOH-terminal phosphorylated form of RNA polymerase II in HT29 cells. It is likely that this decrease mediates a repression of transcription that may contribute to or cause the loss of the cyclins. However, there is evidence that, unlike flavopiridol, roscovitine does not bring about a global loss of mRNA transcription (72). In support, roscovitine has been shown to reduce mRNA synthesis by only 65%, with the remaining population of mRNAs being resistant to inhibition (57). Furthermore, the fact that p21WAF1/CIP1 mRNA expression was not reduced and that c-FOS mRNA expression was increased by CYC202 demonstrated that reduced gene expression was not universal. Additional microarray studies with CYC202 are under way in our laboratory that may shed light on the mechanisms underlying the drug-induced depletion of particular mRNAs and proteins. A decrease in cyclin D1 expression by CYC202 was seen in the RB-deficient SAOS-2 cells. This demonstrates that the mechanism of action of CYC202 is not RB dependent; however, it does not exclude CDKs from being targets of CYC202, because CDK2, for example, has multiple substrates (73–75).

The observation of reduced levels of cyclins D1, A, and B1 provides further insights into the potential mechanism of the non-phase-specific cell cycle arrest by CYC202. Thus, direct inhibition of CDK2/cyclin E kinase activity coupled with reduced activities of other CDK/cyclin complexes by depletion of the cyclin partners could provide combinatorial blockade of multiple CDKs. This hypothesis is consistent with our observation that although initially selective for the Thr-821 CDK2 phosphorylation site, multiple sites on RB were subsequently inhibited by CYC202. However, it should be noted that the direct CDK target of CYC202, CDK2, is in fact involved in all phases of the cell cycle, in addition to its established functions in G1 and S. A role for CDK2 in the G2-M phase has been described recently (76). In addition, CYC202 has inhibitory activity against CDK1/cyclin B1 complexes, which could also contribute to inhibition of RB phosphorylation and accumulation in G2-M (3). Recently, the suitability of CDK2 as a target for therapeutic intervention has been questioned because colon cancer cell lines can proliferate in the absence of CDK2.

In summary, we have reported a number of new findings on the cellular effects of the developmental anticancer agent CYC202. Our results should help further understand the potentially complex cellular mechanism of action of CYC202, which is now undergoing clinical trials (31). The observed dose- and time-dependent inhibition of RB phosphorylation is consistent with inhibition of CDKs. Although early inhibition of the Thr-821 CDK2 phosphorylation site was clearly seen, the decrease in levels of cyclins D1, A, and B1 could explain the subsequently reduced phosphorylation of RB at multiple sites, as well as the effects of the drug at various phases of the cell cycle. Although the effects were seen at 3× IC50 (50 μM) concentrations of CYC202 and therefore may contribute to the pharmacological effects at this dose, direct inhibition of CDK2 was also seen at around the IC50 (20 μM). Thus, the latter effect may predominate at lower exposures. Concentrations of up to 50 μM have been achieved at therapeutic doses in mice (78, 79). An interesting induction of the ERK1/2 pathway is seen with CYC202, but the results suggest that this is probably not involved in the major downstream cellular effects of the drug. In addition to contributing to the understanding of the molecular pharmacology of CYC202, several of the observed cellular changes induced by CYC202, including phosphorylation of ERK1/2 and ELK-1, induction of c-FOS, and the reduced expression of cyclins D1, A, and B1, should now be evaluated as potential pharmacodynamic markers to measure the achievement of pharmacologically active concentrations in clinical trials, alongside decreased phosphorylation of RB.

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The Cyclin-dependent Kinase Inhibitor CYC202 (R-Roscovitine) Inhibits Retinoblastoma Protein Phosphorylation, Causes Loss of Cyclin D1, and Activates the Mitogen-activated Protein Kinase Pathway

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