A Novel cdk2-selective Inhibitor, SU9516, Induces Apoptosis in Colorectal Cancer Cells

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ABSTRACT

Recent studies have indicated that the development of cyclin-dependent kinase (cdk)2 inhibitors that deregulate E2F are a plausible pharmacological strategy for novel antineoplastic agents. We show here that 3-[(3-(4-ylidene)-5-methoxy-1,3-dihydro-indol-2-one (SU9516), a novel 3-substituted indolione compound, binds to and selectively inhibits the activity of cdk2. This inhibition results in a time-dependent decrease (4–64%) in the phosphorylation of the retinoblastoma protein pRb, an increase in caspase-3 activation (5–84%), and alterations in cell cycle resulting in either a G1-G1/s or a G2-M block. We also report here cell line differences in the cdk-dependent phosphorylation of pRb. These findings demonstrate that SU9516 is a selective cdk2 inhibitor and support the theory that compounds that inhibit cdk2 are viable resources in the development of new antineoplastic agents.

INTRODUCTION

Progression through the cell cycle is determined by sequential and specific phosphorylation events by holoenzymes involving cyclins and their catalytic partners, the cdk2.3 Transition through the cell cycle occurs as a result of a well-ordered sequence of events controlled by an orderly cascade of holoenzymes that are specific for each phase of the cell cycle. Dysregulation of this process can result in disordered and uncontrolled cell growth, characteristic of the neoplastic state. One molecular pathway that is often altered in human cancers involves the retinoblastoma tumor suppressor protein, pRb. The phosphorylation of pRb by an active cyclin/cdk complex results in the dissociation of pRb from E2F (4, 5), thus altering the status of E2F-regulated genes from fully repressed to induced (6). The E2F-responsive genes are therefore activated in cancer cells because of the loss of pRb. E2F repressor complexes and the liberation of free E2F.

The expression of E2F-regulated genes, such as the cyclin D family as well as cyclin E, commit the cell to S-phase entry, whereas cyclin A (also an E2F-regulated gene) is important in the progression of cells through the cell cycle (7, 8). Cyclin A/cdk2 complexes, which are largely responsible for S-phase progression, also phosphorylate the E2F family of proteins, causing them to dissociate from their respective DNA binding sites (9, 10). This, in effect, marks the end of the relatively short period of transcription of E2F-regulated genes. The overexpression of E2F-1 results in cells that enter the active growth cycle and progress to S phase (11), thus confirming that a single transcription factor promotes the G1 to S-phase transition and demonstrating the important role of E2F proteins in the cell cycle. Additional experiments show that these E2F overexpressing cells undergo apoptosis after entering S phase (12–14). An increase in cyclin A/cdk2 activity in early S-phase results in the phosphorylation of a number of substrates that inactivate G1 transcription factors (15).

Cyclin E/cdk2 complexes also play an important role in carcinogenesis. Cyclin E amplification has been identified in colon cancer (16) and cdk2 amplification is associated with concurrent cyclin E gene amplification in several tumors (17, 18), including colorectal carcinomas. Furthermore, colorectal tumors have higher levels of cyclin E protein than surrounding normal tissue (19). There is an accumulation of cyclin E as colorectal tumors progress from adenomas to adenocarcinomas (19) suggesting that the overexpression of cyclin E and the subsequent deregulation of normal cell cycle events play an important role in the development and progression of colorectal carcinoma. Moreover, recent evidence (20) suggests that cyclin E/cdk2 may directly regulate E2F activity and may be involved in an autoregulatory mechanism controlling cell cycle-dependent transcription by stimulating the interaction of E2F with the p300/cAMP-response element binding protein family of coactivators.

Such lines of reasoning have led to the investigation and production of novel chemotherapeutic agents that target cyclin/cdk2 activity in an attempt to regulate aberrant cell cycle pathways. Recent studies have identified a short peptide motif that blocks the phosphorylation of substrates by cyclin A/cdk2 or cyclin E/cdk2 and preferentially induces transformed cells to undergo apoptosis when compared with nontransformed cells (21). This study also suggests that even a small decrease in the cyclin A/cdk2-mediated inhibition of E2F will result in apoptosis.

Several naturally occurring cdk inhibitors that have been identified to date include members of the Cip/Kip (22, 23) and INK4 (23, 24) families. We have previously demonstrated that cyclin E may be a downstream target for cyclin D1 in human colon carcinoma cell lines (25). These data suggest that cdk2 inhibition would be a useful target for the regulation of cancer cell growth. Novel cdk inhibitors are of particular interest in cancer therapy, because many naturally occurring cdk inhibitors are either mutated or deleted in primary tumor cells, abrogating their function as tumor suppressors. Novel, synthetic compounds that can replace the functions of altered tumor suppressor genes are not, surprisingly, prime targets in current cancer research.

SU9516, a novel 3-substituted indolione inhibitor of cdk activity (26) was identified via high-throughput screening with cdk2 and examined to determine its effects on colon cancer cell kinase activity, cell proliferation, cell cycle progression, and apoptosis. We demonstrate here that SU9516 selectively inhibits cdk2 kinase activity, decreases ligand-dependent and -independent cell cycle progression, and increases apoptosis. This class of cdk inhibitors shows promise among other novel chemotherapeutic agents because of its ability to selectively target cdk2 activity.
MATERIALS AND METHODS

Reagents

Reagents were purchased from the following suppliers: HEPES, ATP, MgCl₂, DTT from Sigma Chemical Co., St. Louis, MO; Triton X-100 and glycerol were from Fisher Scientific; purified human recombinant protein kinase Ca, p38, MBP, and histone H1, were from Upstate Biotechnology; GST-pRb was from Santa Cruz Biotechnology (Santa Cruz, CA); TALON Metal Affinity Resin was from Clontech; [γ-33P]ATP was from New England Nuclear; and P30 phosphocellulose filter mat paper was from Wallac. Anti-pRb-Ser-780 (169) antibody for the cyclin D1/cdk4-specific phosphorylated (serine 780) form of the pRb protein and anti-pRb-Thr-356 (172) antibody for the cyclin E/cdk2-specific phosphorylated (threonine 356) form of the pRb protein was kindly supplied by Dr. Richard G. Pestell (Albert Einstein College of Medicine, Bronx, NY) and Dr. Adrian Senderowicz, (NCI, Bethesda, MD) (27).

Synthesis of SU9516

The reaction mixture of 82 mg (0.5 mmol) of 5-methoxyindoxlde, 58 mg (0.6 mmol) of imidazole-4-carboxaldehyde, and 1 drop of piperidine in 2.0 ml of ethanol was heated at 95°C overnight, cooled to room temperature, and concentrated. The residue was purified on silica gel column eluting with ethyl acetate to give 100 mg (83%) of SU9516 as a bright yellow solid: 1H NMR (360 MHz, CDCl₃, 0°C) δ: 13.79 (s, br, 1H, NH-1), 10.77 (s, br, 1H, NH-imidazole), 8.00 (s, br, 1H), 3.45 (s, 3H), 2.67 (2H), and 3.18 (s, 1H), 25.0 (percentage of relative intensity) 242 (M+1)+, 100.

SU9516/cdk2 Docking Model

The SU9516/cdk2 docking model was based on the crystal structure of cdk2 (28) and that of SU3402 in fibroblast growth factor receptor-1 (26). The structures of cdk2 and fibroblast growth factor receptor-1 were superimposed using InsightII (Molecular Simulations, Inc., San Diego, CA.). The indoline portion of SU9516 was then superimposed over that of SU3402. The merged SU9516 and cdk2 complex became the docking model.

Cell Lines

RKO cells (kindly supplied by Dr. Leonard Augenlicht, Bronx, NY) and SW480 cells (ATCC, Rockville, MD), both human colon carcinoma cell lines, were grown in RPMI 1640 supplemented with 1% penicillin/neomycin/streptomycin antibiotic mixture (Life Technologies, Inc., Grand Island, NY) and 10% FBS (Life Technologies, Inc.). A431 (human epidermoid carcinoma), Colo205 (human colorectal adenocarcinoma), and NCI H460 (human large cell lung carcinoma) cells were maintained in complete growth medium under standard conditions in culture media recommended by the ATCC.

PKCa Kinase Assay

Purified human recombinant PKCα (25 ng/assay point) was added to a mixture containing lipid activator (0.5 mg/ml phosphatidyleserine and 0.05 mg/ml diglycerides), 20 mM 3-[N-morpholino]propanesulfonic acid buffer (pH 7.2), containing 25 mM NaCl, 0.5 mM EDTA, and 10% glycerol as substrate in a total volume of 25 μl. Reactions were incubated for 20 min at room temperature before stopping with 1% phosphoric acid. Reaction mixture was spotted onto phosphocellulose paper (P81), allowed to dry, and then washed with 1% H₃PO₄ to remove unbound material. The radioactivity in the [ 33 P]stabilized form of 3,3,3-[[3 H], OCH 3 -5] was 10 ng/well, or 1.6 nM. The kinase reaction was initiated by the addition of ATP at a final concentration of 10 μM ATP (twice the experimentally determined Kₚ) and [γ-33P]ATP (1.0 μCi/well) in a 60-μl volume and allowed to proceed at room temperature for 1 h. Reaction was stopped by the addition of 0.1 ml 10% phosphoric acid, and 25 μl of reaction mixture was transferred to P30 phosphocellulose filter mat paper. The filter mat was treated as described for Cdk1/Cdk2 assays.

p38 Kinase Assay

Purified human recombinant p38 was diluted (25 ng/assay point) to a mixture of 20 mM HEPES (pH 7.4) and 20 mM MgCl₂. Kinase reaction was initiated by the addition of 10 μM ATP (final concentration, twice the Kₚ) and [γ-33P]ATP (1.0 μCi/well) in a 60-μl volume and allowed to proceed at room temperature for 1 h. Reaction was stopped by the addition of 1% phosphoric acid. Reaction mixture was spotted onto phosphocellulose paper (P81), washed four times with 1% H₃PO₄, dried, and read as described above.

PDGF Rβ and EGFR Kinase Assays

Solubilized membranes derived from NIH3T3 mouse fibroblasts overexpressing human PDGF-Rβ or EGFR were added to polystyrene 96-well microtiter plates that had been precoated with a monoclonal antibody that recognized either the PDGF-R or EGFR. After a 30-min incubation with lysisate, the plates were washed to remove unbound material, and serial dilutions of chemical inhibitors were added to the immunolocalized receptor. The kinase reaction was started by the addition of ATP to the wells at a final concentration of 20 μM for PDGF-R and 3 μM for EGFR, twice the experimentally determined Kₚ values for ATP. The kinase reaction was allowed to proceed for 30 min for PDGF-Rβ and for 5 min for EGFR at room temperature and then stopped by the addition of EDTA. The amount of phosphorylated kinase present on the receptors in the individual wells was determined by incubating the immuno-localized receptor with a biotinylated monoclonal antibody directed against phosphorylated tyrosine. After removal of the unbound anti-phosphotyrosine antibody, avidin-conjugated horseradish peroxidase was added to the wells. A stabilized form of 3,3',5',5'-tetramethyl benzidine dihydrochloride and H₂O₂ was added to the wells. The color readout of the assay was allowed to develop for ~30 min and the reaction was stopped with H₂SO₄.
Cell Proliferation/Toxicity Assays

**SRB Assay for Cell Proliferation.** RKO cells and SW480 cells were seeded in triplicates \((n = 6)\) in 96-well plates (Becton Dickinson, Lincoln Park, NJ) at \(1 \times 10^{4}\) cells/well and allowed to attach overnight. SU9516 (Sugen, San Francisco, CA) was added in concentrations from 0.05 \(\mu M\) to 50.00 \(\mu M\) for 24 h, the cells were then washed twice with PBS, and cells were replenished with complete media. The cells were fixed on 0, 4, and 7 days post-drug removal and assayed for protein levels using a modified SRB cytotoxicity assay (31). The cells were fixed in 10% trichloroacetic acid for 1 h, washed in distilled H\(_2\)O, and stained in 0.4% SRB/acidic acid for 30 min. The cells were then washed in 0.1% acetic acid, solubilized in 10 \(\mu M\) Tris (pH 9), and analyzed on a Bio-Rad 360 microplate reader (Bio-Rad) at 595 nm. All experiments were repeated at least three times.

**Ligand-induced BrdUrd Incorporation Assay.** NIH3T3 mouse fibroblasts overexpressing the EGF, PDGFR, or IGF-1 receptors were engineered using retroviral vectors. Cell lines were grown in DMEM with 10% calf serum and 2 mM glutamine. Confluent cells in 96-well plates were made quiescent by serum-deprivation for 24 h and then stimulated with EGF (4 nm), PDGF (3.8 nm), or IGF-1 (1.5 nm), in the presence of a serial dilution of SU9516 for 20 h. BrdUrd was added for a 2-h labeling period, and the cells were fixed. The amount of BrdUrd incorporation was determined with peroxidase-conjugated anti-BrdUrd antibody using an ELISA kit (Roche Molecular, Indianapolis, IN). IC\(_{50}\) values were calculated as the concentration of compound that inhibited the growth of cells by 50% as compared with control cells grown in the absence of an inhibitor.

**BrdUrd Incorporation into Synchronously Growing Cells.** SW480 and RKO colon cancer cell lines were seeded in six-well plates and serum starved in RPMI (0.1% FBS) for 24 h and then induced with full medium (RPMI with 10% FBS) and treated with 5 \(\mu M\) SU9516 for 20 h. Cells were pulsed with BrdUrd for 2 h and then harvested and analyzed via flow cytometry using a FITC-conjugated antibody set (PharMingen, San Diego, CA).

**BrdUrd Incorporation into Asynchronously Growing Cells.** Asynchronously growing A431, Colo205, or NCI H460 cells in a 96-well plate in complete growth medium under standard conditions in culture media recommended by the ATCC were exposed to serial dilutions of SU9516 for 24 h. BrdUrd was then added for a 2-h labeling period, and the cells were fixed. The amount of BrdUrd incorporation was determined with peroxidase-conjugated anti-BrdUrd antibody using an ELISA kit (Roche Molecular). IC\(_{50}\) values were calculated as the concentration of compound that inhibited the growth of cells by 50% as compared with control cells grown in the absence of inhibitor.

**Apoptosis/Cell Cycle.** Exponentially growing cells treated with 5 \(\mu M\) SU9516 (SUGEN) for 0, 24, 48, and 72 h were harvested from six-well plates with trypsin/EDTA (0.05% trypsin, 0.53 mM EDTA-4Na; Life Technologies, Inc.) and collected via centrifugation. Samples were washed with PBS, fixed in ice-cold 70% ethanol, and stored at -80\(^\circ\)C until analysis. Samples were analyzed for total sub-G\(_0\) population by first decanting the ethanol and resuspending the samples in 1.0 ml of HBSS. Next, 0.5 ml of phosphate-citric acid buffer (0.192 \(m\) Na\(_2\)HPO\(_4\), 0.004 \(m\) citric acid) was added to ensure that apoptotic cells were separated from G\(_1\) cells. Cells were centrifuged, the supernatant removed, and resuspended in 20 \(\mu g\)/ml of PI with 0.25 mg/ml RNase in HBSS. Samples were then analyzed for sub-G\(_0\) population and cell cycle via flow cytometry using a laser excitation of 488 nm and measuring DNA-linked red fluorescence (PI) through a 600-nm wavelength filter as described previously (32). Cell cycle data and apoptotic population were determined using ModFit LT for windows (Verity Software House, Inc.)

**Active Caspase-3 Staining.** Cells treated as above were collected after trypsinization and washed with PBS. Pellets were resuspended in 200 \(\mu l\) Cytofix/CytoPerm solution (PharMingen) and incubated on ice for 30 min. Cells were washed in 1.0 ml of Perm/Wash buffer (PharMingen) and then resuspended in 100 \(\mu l\) of the same buffer. Samples were incubated with antiactive caspase-3 (PharMingen 67354X) for 30 min at 4\(^\circ\)C in the dark. Next, the samples were washed in Perm/Wash buffer and then resuspended in 0.5 ml of Perm/Wash buffer. Active caspase-3 levels were determined using a phycocyanin-linked anti-caspase-3 antibody following the manufacturers instructions for flow cytometry (PharMingen) and analyzed using WinList software (Verity Software House, Inc.).

**Protein Extraction and Western Blot Analysis.** Subconfluent cells were washed three times in ice-cold PBS, scraped, and lysed in lysis buffer [50 mM HEPES (pH 7.5), 150 mM NaCl, 10% (v/v) glycerol, 1% Triton X-100, 1.5 mM MgCl\(_2\), 1 mM EGTA, 10 \(\mu g/ml\) aprotinin, 10 \(\mu g/ml\) leupeptin, 1 mM phenylmethylsulfonyl fluoride, 200 \(\mu M\) Na orthovanadate, 10 mM Na PP\(_3\), and 20 mM NaF] on ice (33). Lysates were clarified by centrifugation, and protein concentration was determined spectrophotometrically. The lysates were immediately frozen and stored at -80\(^\circ\)C, until further analysis. For immunoblotting, lysates were loaded at an equal protein concentration, 30–50 \(\mu g\)/lane, and subjected to 10% SDS-PAGE. After electrophoresis, proteins were electrophoretically transferred to a nitrocellulose filter at a constant current of 180 mA overnight at 4\(^\circ\)C. Nitrocellulose filters were blocked in 5% nonfat dried milk in TBS-Tween buffer for 1 h and then incubated with the anti-target

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\text{Fig. 1. a. Structure of SU9516. SU9516 is a 3-substituted indolinone compound. Fig. 1b, model of SU9516 docked in the catalytic domain of cdk2. The receptor is rendered by secondary structure with }\alpha\text{-helices shown in red cylinders and } \beta\text{-sheets shown in blue-green plate. Atoms of SU9516 are shown in spheres, and its carbon atoms are yellow. 1c, interactions with cdk2. }\sim\text{, potential hydrogen bonds.}
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Table 1  Inhibition of cdk activity by SU9516

<table>
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RESULTS

SU9516 Is a Selective cdk2 Inhibitor. As shown in Fig. 1a, SU9516, is a 3-substituted indolinone compound with a planar structure. Potential hydrogen bonds are depicted in Fig. 1c. These interactions can explain the potent inhibition of cdk2 activity by SU9516. Table 1 shows that SU9516 is a potent inhibitor for cdk2 and that it is 9-fold and 1.8-fold more selective for cdk2 than cdk4 and cdk1, respectively.

SU9516 Decreases cdk2-specific Phosphorylation of pRB. Additional evidence for the selective nature of SU9516 was determined by studying the effects on pRB phosphorylation. Continuous exposure to SU9516 (5 μM) for 24, 48, or 72 h resulted in a 4–52% decrease (P ≤ 0.05) in cdk2-specific phosphorylation of pRB but not cdk4-specific phosphorylation in RKO cells (Fig. 2, a–d). In contrast, treatment with SU9516 (5 μM) inhibited (P ≤ 0.05) both cdk2-specific (27–64%) and cdk4-specific (26–49%) phosphorylation of pRB in SW480 cells at all (24, 48, and 72 h) time points (Fig. 2, e–h).

Cytokine Effects of SU9516. The effects of SU9516 on cytokinetics were investigated in asynchronously growing SW480 and RKO cells. As expected, inhibition of cell cycle progression with 5 μM SU9516 occurred in RKO cells (Fig. 3, a–b) and in SW480 cells (Fig. 3, c–d). This is consistent with the effects observed on cell proliferation. Surprisingly, cell cycle arrest occurred in G2-M rather than in G0/G1, with a >3-fold increase in cells in G2-M (P ≤ 0.0001) at 48 h in RKO cells and a >2.5-fold increase in G2-M in SW480 cells.

SU9516 Induces Apoptosis. As shown in Fig. 1a, SU9516-induced apoptosis was observed in SW480 cells within 24 h (Fig. 3c). The percentage of apoptotic cells increased with time and reached a maximum at 72 h (Fig. 3c).

protein, murine monoclonal antibody and anti-actin murine monoclonal antibody (Sigma Chemical Co.) in 2% nonfat-TBS-Tween 20 buffer for 1 h. After three washes in TBS-Tween 20 buffer, horseradish peroxidase-conjugated antimurine secondary antibody (Santa Cruz Biotechnology) was added at 1:3000 dilution in TBS-buffer for 50 min. Detection of the immune signal was performed using the Super Signal Western Blotting Detection System (Pierce Chemical Co., Rockford, IL). The intensities of the autoradiographic bands were quantitated by densitometric scanning.
The effects of cdk2-mediated inhibition on cell cycle progression was characterized further in EGF-induced 3T3 cells and is summarized in Table 2. SU9516 produced a dose-dependent G1 accumulation in EGF-stimulated cells. As shown in Table 3, comparable concentrations of SU9516 were required to inhibit the proliferation of growth-factor stimulated cells (PDGF, IGF-1, and EGF) as compared with asynchronously growing cells.

**DISCUSSION**

The loss of cdk regulatory function and cell cycle checkpoint function has been directly linked to the molecular pathology of cancer (34). Several investigators are currently evaluating the role of cdk inhibition as a plausible strategy for therapeutic intervention in cancer therapy (35–38). Flavopiridol is a cdk inhibitor that has completed Phase 1 clinical trials (39), but its specificity for cdk inhibition is limited in enzyme inhibition studies because it inhibits cdks 1, 2, and 4 with equal sensitivity (36, 40). Roscovitine, olomucine and other 6-aminopurine derivatives have been shown to bind competitively with respect to ATP (41–43), but these compounds do not produce the hydrogen-bonding characteristics of the ATP molecule (41–43). Presently, there is a need for more selective cdk inhibitors to evaluate the effectiveness of these compounds in aberrant cell cycle regulation. SU9516, a 3-substituted indolione, was identified from high throughput screening with cdk2. SU9516 was found to be a potent and selective inhibitor of the catalytic activity of cdk2. In the model depicted in Fig. 1c, the oxindole group of SU9516 can form hydrogen bonds with the peptide backbone of cdk2. As would be expected, inhibition of cdk2 activity was associated with inhibition of cdk2-inhibited proliferation of both RKO and SW480 cells, and induced with full media (10% serum) at the time of drug treatment. RKO cells remained blocked in G2-M at 20 h post-serum induction and addition of SU9516 (Fig. 4a). SW480 cells, however, were now blocked in G1-G2 under the same conditions (Fig. 4b).

SU9516 Induces Apoptosis in RKO and SW480 Cells. An explanation for decreased proliferation in asynchronously grown cells was investigated by examining the effects of SU9516 on apoptosis. After continuous exposure to 5 μM SU9516 for 24, 48, and 72 h, both RKO (10–15%) and SW480 (4–22%) cells demonstrated an increase (P ≤ 0.01) in apoptosis as detected by PI cell staining (Fig. 5, a and b). Using an anti-caspase 3 antibody, similar results were seen after 48 h (P ≤ 0.05) and 72 h (P ≤ 0.05) exposure to SU9516 (5 μM) in both RKO (15–50% at 5 μM) and SW480 (5–23% at 5 μM) cells (Fig. 6, a and b). However, the increase in apoptosis seen after 24-h drug treatment using the PI stain (Fig. 5, a and b) was not demonstrated in either cell line using the anti-caspase 3 antibody.

SU9516 Inhibits Proliferation in Exponentially Growing and Ligand-induced Tumor Cells. As expected, with inhibition of pRb phosphorylation, proliferation in both RKO and SW480 cells decreased in a dose-dependent manner at 20 h after treatment with SU9516 (Fig. 7a and Table 1). Furthermore, antiproliferative effects in both RKO cells and SW480 cells were still evident 4 days post-drug removal (Fig. 7b). However, at 7 days post-drug removal, RKO cells continued to demonstrate the antiproliferative effects of SU9516, whereas the SW480 cells recovered to near control levels (Fig. 7c).

Fig. 4. Cell cycle analysis in serum-starved cells after induction with complete media and SU9516 treatment for 20 h. 4a, the addition of SU9516 (0.0–50 μM) produced a 1.3–17-fold decrease in RKO cell S-phase populations compared with untreated cells in concentrations ranging from 1.5 μM to 50 μM, respectively. A G2-M block was observed in RKO cells peaking at 12.5 μM SU9516 and resulting in a 3.8-fold increase in G2-M populations when compared with untreated cells. 4b, the addition of SU9516 (3.0–50 μM) produced a 1.3–1.8-fold increase, respectively in SW480 G0-G1 cell populations compared with untreated cells. The addition of 6.0–50.0 μM resulted in depletion of the SW480 S-phase population ranging from 1.1- to 55-fold decreases, respectively. A G2-M block was observed in SW480 cells peaking at 12.5 μM SU9516 and resulting in a 2.4-fold increase in G2-M populations when compared with untreated cells.

Fig. 5. Apoptosis assay (sub-G0-G1 population) after continuous exposure to SU9516. 5a, RKO colon cancer cells treated with 5 μM SU9516 exhibited a significant increase in the level of apoptotic cells when compared with untreated cells as determined by using PI-staining and flow cytometry to analyze the sub-G0 population. Apoptosis in RKO cells increased significantly over time when compared with untreated cells and exhibited a 20.7-fold increase over controls at 24 h posttreatment as well as a 15.1- and a 14.6-fold increase over controls at 48 h and 72 h, respectively. 5b, SW480 cells exhibited similar increases in apoptosis, with sub-G0 cell levels ranging from a 2.9-fold increase over controls at 24 h to a 72.6-fold increase at 72 h post-drug addition when compared with untreated cells (** P ≤ 0.01).
not cdk4-specific phosphorylation of pRb in RKO cells. In SW480 cells, inhibition of cdk2 by SU9516 was also associated with inhibition of cdk4-specific phosphorylation of pRb. Although SU9516 is a selective cdk2 inhibitor, it also exhibits weak activity toward cdk4, and this may reflect the decrease in cdk4 activity in SW480 cells. Expression levels of cdk4 and cyclin D1 between RKO cells and SW480 cells are comparable (data not shown), so the difference in cdk4 inhibition between the two cell lines suggests an alternate mechanism for cdk4 activation.

At 4 days post-drug removal, proliferation was inhibited in both cell lines. This is consistent with both the inhibitory effects on cdk2 and the downstream inhibition of cdk2-specific phosphorylation of pRb. Although SU9516 is a selective cdk2 inhibitor, it also exhibits weak activity toward cdk4, and this may reflect the decrease in cdk4 activity in SW480 cells. Expression levels of cdk4 and cyclin D1 between RKO cells and SW480 cells are comparable (data not shown), so the difference in cdk4 inhibition between the two cell lines suggests an alternate mechanism for cdk4 activation.

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The observed antiproliferative effects could be attributable to either the generalized slowing of cell growth or, alternatively, an increase in cell death. In fact, both effects were seen. Apoptosis increased in

The cyclin E/cdk2 complex plays a collaborative role in induction of S phase, functioning downstream of E2F; this role is also likely altered by the inhibition of cdk2. Interestingly, antiproliferative effects were observed in both ligand-stimulated cells and unstimulated, asynchronously growing cells. Because PDGF, EGFR, and IGF may work through induction of cyclin D1 (32, 50, 51), the antiproliferative effects of SU9516 are consistent with an effect downstream of cyclin D1, which would be predicted from data supporting sequential phosphorylation of pRb by cyclin D1/cdk4 and then cyclin E/cdk2.

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Fig. 6. Apoptosis assay measuring active caspase-3. RKO (a) and SW480 (b) colon cancer cells treated with 5 and 10 μM SU9516 exhibited a significant dose-dependent increase in the level of active caspase-3 mediated apoptosis compared with untreated cells at the same time point. RKO cell levels of active caspase-3, determined by flow cytometry, increased over time from 26% (24 h) to 52% (48 h). SW480 cells exhibited increased levels of active caspase-3 as well, ranging from 9% (24 h) to 26% (72 h), (+, P ≤ 0.05; ***, P ≤ 0.001).

Fig. 7. Cell recovery after 24 h exposure to 5 μM SU9516. Data represented is from cell proliferation assays, with each point representing the mean (n = 6) percentage of control relative to untreated cells. a. RKO cells (●) and SW480 (▲) cells seeded at the same density were treated with SU9516 for 24 h (day 0 post-drug removal), washed with PBS, and assayed for proliferation. The effects of SU9516 elicited an antiproliferative effect in both cell lines at this time point. b. RKO cells (●) and SW480 cells (▲), seeded at the same density, were treated with SU9516 for 24 h, washed with PBS, and allowed to recover in complete medium for 4d post drug removal. The effects of SU9516 still elicited an antiproliferative effect in both cell lines at this time point. c. RKO cells (●), and SW480 cells (▲), seeded at the same density, were treated with SU9516 for 24 h, washed with PBS, and allowed to recover in complete medium for 7 days post-drug removal. A persistent antiproliferative effect was only evident in RKO cells at this time point.
both cell lines after SU9516 treatment. In addition, there was a slowing of cell cycle progression in both cell lines. The absence of p53 checkpoint function in SW480 cells may render them independent of pRB-mediated pathways for cell cycle regulation, and at least partially independent of cyclinE/cdk2 inhibitory effects. One interesting observation was that asynchronous RKO and SW480 cells accumulated in G2-M, rather than G1-G2, as would be expected from inhibition of cyclin E/cdk2 phosphorylation of pRb. Inasmuch as earlier studies have shown that senescent cells accumulate inactive cyclin E-cdk2 complexes (52), it was a surprise that RKO cells retained the G2-M block even when the cells were first serum deprived and then treated with SU9616 when induced to reenter the cycle. SW480 cells that were induced to reenter the cell cycle and treated with SU9516 exhibited the expected G2/G1 block when treated under the same serum-deprived cell culture conditions. We expected both cell lines to arrest in G1, because studies with p16-derived peptides demonstrated that the effect of the peptide was limited to a small window after serum induction (53). SU9516 then, even at relatively high concentrations, does not cause RKO cells to accumulate in G2. These cells are able to bypass the G1 restriction point and proceed to accumulate in G2. Because SU9516 has a selective advantage of only 1.8 for cdk2 in relation to cdk1, the lack of a G2-G1 arrest in RKO cells may reflect the inhibition of cdk1. Cdk2-dependent kinase 2 function has been associated with several growth regulatory mechanisms in addition to the phosphorylation of pRb. These include the regulation of histone-modified transcription (54), disruption of DNA replication by virus infection via prevention of MCM proteins associating with chromatin (55), sequestration of E2F (56), and centrosome duplication (56). Differences in cell cycle response to SU9516 are, therefore, likely to be attributed to cell line-specific differences that trigger alternate cdk2 related molecular pathways in growth regulation.

Our results are consistent with the effects observed with oligopeptides designed to block the phosphorylation site of cyclinA/cdk2 and cyclin E/cdk2 complexes, which would be expected to have similar effects to those of SU9516, which inhibits the enzymatic activity of cdk2. As with treatment with SU9516, treatment with E2F-derived oligopeptides (PVKRRRLDL) resulted in antiproliferative and apoptotic effects in multiple cell lines. In addition, as with SU9516 treatment, slowing of the cell cycle was observed, but with an accumulation of cells in S phase (21). The apoptotic effects observed with SU9516 may relate to effects on the cyclin A/cdk2 complex rather than on cyclin E/cdk2. Because cyclin Ac/cdk2 can negatively regulate E2F (9, 10, 57, 58), the inhibition of cdk2 allows an increase in free E2F, which can induce both cell proliferation and apoptosis (11–14). It is interesting that subthal level inhibition of cdk2 by SU9516 triggers both growth arrest and apoptosis in a cell line-specific manner and that sustained (>24 h) exposure is not required for the inhibition of proliferation in RKO cells. Of additional interest, and consistent with our findings in both RKO (p53 function) and SW480 (p53 mutant) cells, E2F induces apoptosis in either a p53-dependent or–independent fashion (59–61).

Our data support the targeting of cdk2 as a viable pharmacological strategy for the development of antineoplastic pharmacophores (21). Cdk2 plays a central role in the regulation of pRb and E2F (49). Additional studies using recently described purine and pyrimidine cdk inhibitors (62) are currently underway in our laboratory. These compounds form triplet hydrogen bonds with the cdk2-ATP binding site. Furthermore, we have recently demonstrated a tight interactive relationship between cyclin D1/cdk4 and cyclin E/cdk2 (25). The substrates for the cyclin E/cdk2 complexes associated with S-phase induction have yet to be determined, but the top candidates include pRb (63, 64), Cdc45 and its associated proteins (65), and the nuclear protein mapped to the ATM locus (66). We are currently expanding our studies of the combined inhibition of the cyclin D1/cdk4 and cyclin E/cdk2 pathways and investigating the downstream effects of such novel therapeutic strategies on S-phase induction.

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A Novel cdk2-selective Inhibitor, SU9516, Induces Apoptosis in Colon Carcinoma Cells

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