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SU9516, a cyclin-dependent kinase 2 inhibitor, promotes accumulation of high molecular weight E2F complexes in human colon carcinoma cells

Bo Yu[a,b], Maureen E. Lane[a,b], Scott Wadler[a,b,*]

[a]*Albert Einstein Comprehensive Cancer Center and the Albert Einstein College of Medicine, Bronx, NY 10463, USA*
[b]*Division of Hematology/Oncology, C606, Weill Medical College of Cornell University, 1300 York Avenue, New York, NY 10021, USA*

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Abstract

The E2F family plays a critical role in the expression of genes required for entry into and progression through S phase. E2F-mediated transcription is repressed by the tumor suppressor retinoblastoma protein (pRb), which results in sequestration of E2F in a multiprotein complex that includes pRb. Derepression of E2F results from a series of complex phosphorylation events mediated by cyclin D/cdk4 and cyclin E/cdk2. We have employed a novel 3-substituted indolinone compound, 3-[1-(3*H*-imidazol-4-yl)-meth-(*Z*)-ylidene]-5-methoxy-1,3-dihydro-indol-2-one (SU9516), which selectively inhibits cdk2 activity (Lane *et al*., Cancer Res 2001;61:6170–7) to investigate these events. Electrophoretic mobility gel shift assays were performed on SU9516-treated and -untreated HT-29, SW480, and RKO human colon cancer cell extracts. Treatment with 5 μM SU9516 prevented dissociation of pRb from E2F1 in all cell lines (HT-29 > RKO > SW480). Treatment effects were time-dependent, demonstrating greater inhibition at 48 hr versus 24 hr in HT-29 cells. Furthermore, E2F species were sequestered in complexes with p107, p130, DP-1, and cyclins A and E. After a 24-hr treatment with 5 μM SU9516, cyclin D1 and cdk2 levels decreased by 10–60%. These findings delineate a previously undescribed mechanism for SU9516-mediated cell growth arrest through down-regulation of cyclin D1, inhibition of cdk2 levels and activity, and pan-sequestration of E2F.

Keywords: Cyclin; Cyclin-dependent kinase; Cyclin-dependent kinase inhibitor; E2F; Retinoblastoma protein

1. Introduction

Progression through the cell cycle is a consequence of a series of phosphorylation events that ensure the accurate synthesis of cellular DNA and the proper distribution of the chromatin to two daughter cells. The commitment to undergo DNA synthesis occurs prior to the G1/S transition [1]. Specific events occurring in this period are therefore of great interest and include sequential phosphorylation events on the retinoblastoma protein by the G1 cyclin-dependent kinases, which result in derepression of the transcription factor E2F (reviewed in [2]). E2F subsequently transactivates genes necessary for the initiation of DNA replication and the maintenance of DNA synthesis (reviewed in [3]).

The E2F transcription factors are heterodimers composed of one protein subunit from the E2F family (E2F1-6) and a second protein from the DP family (DP-1-2). E2F species bind the pocket proteins, pRb, p107, and p130, which repress the transcriptional activity of the E2F family. While all three pocket proteins repress E2F-dependent transcription [4–6], specific pocket proteins bind specific E2F species preferentially. Thus, pRb interacts exclusively with E2F1-3 [7] and has been shown to bind E2F4, p107 binds predominantly to E2F4, and p130 binds both E2F4 and 5 [8–11]. The functional role of E2F6 has yet to be established although it has been reported recently to act as a transcriptional repressor [12].

The E2F and DP proteins cooperate to bind DNA and synergistically transactivate target genes necessary for DNA replication and synthesis. Several lines of evidence support the view that E2F activation is important for the

* Corresponding author. Tel.: +1-212-746-2844; fax: +1-212-746-6645.
E-mail address: scw2004@med.cornell.edu (S. Wadler).

Abbreviations: cdk, cyclin-dependent kinase; DTT, dithiothreitol; EMSA, electrophoretic mobility shift assay; PMSF, phenylmethylsulfonyl fluoride; pRb, retinoblastoma protein; TBS, Tris-buffered saline.

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G1/S transition. Expression levels of E2F transiently increase at the G1/S boundary. Inhibition of E2F activity, either by employing a DP-1 dominant negative mutant [13] or an RNA ligand that inhibits E2F activity [14], prevented entry into S phase. In cycling fibroblasts, E2F3 activity was necessary for progression to S phase [15]. In quiescent cells, overexpression of E2F1-induced chromosomal DNA synthesis [16–19]. Conversely, in cycling cells, a dominant negative E2F was able to prevent S phase entry [20]. Finally, in hematopoietic cells, overexpression of E2F resulted in cytokine-independent proliferation without an increase in cells undergoing apoptosis [21].

Regulation of E2F activity at the G1/S transition is complex. For most of G1, pRb and E2F are tightly associated, and interaction with pRb not only blocks transcriptional activation by E2F, but the complex actively represses transcription of cell cycle genes [22–27]. These functions appear to be separate and independent [26]. Transcriptional repression by pRb/E2F is mediated by pRb-bound components of the hSWI/SNF complex, which is actively involved in chromatin remodeling [28–30], and by histone deacetylases (HDAC), which physically interact with and cooperate with pRb [31–33]. At least two phosphorylation events are necessary for the dissociation of pRb and E2F, releasing E2F to perform its pivotal functions in transactivating the S phase genes [34–38]. In mid G1, cdk4 and 6 interact with the D-type cyclins to form active kinases that phosphorylate pRb. Subsequently, cyclin E forms an active complex with cdk2 to phosphorylate pRb, again in late G1. This disrupts the association with E2F allowing transcription of the S phase genes.

E2F expression is regulated by several autoregulatory feedback loops. E2F1 is a downstream product of *E2F1*, thus inducing its own expression [39]. In addition, *cyclin E* is also an important downstream target for E2F [40]. Up-regulation of *cyclin E* maintains the derepressed form of E2F, which is critical for the G1/S transition.

Cyclin E and E2F interact in a partially redundant, but complicated fashion in late G1. In cells with low expression of E2F, ectopic expression of cyclin E drove quiescent cells into S phase [41], suggesting that cyclin E can replace E2F, at least in part, in the induction of S phase. Likewise, overexpression of E2F has been shown to drive cells into S phase in the presence of a dominant-negative cdk2 or in the absence of cdk2 activity, suggesting that it can, in part, replace the cyclin E/cdk2 function [42]. In cells arrested through expression of a mutant pRb that is constitutively hypophosphorylated, the introduction of cyclin E/cdk2 allowed cells to progress into S phase [41,43]. Further complicating this relationship, events involved in the initiation of DNA replication downstream of E2F induction were shown to be both cdk2-dependent and -independent [44]. Thus, E2F-mediated S phase induction may be comprised of multiple pathways, and may differ among various cell types. Furthermore, cyclin E/cdk2 may have a regulatory role in E2F activity: recently, cyclin E/cdk2 was shown to phosphorylate a site in the activation domain of E2F5 that stimulates binding to the p300/CBP co-activators and augments E2F5-mediated transactivation of *cyclin E* [45].

We have shown previously that treatment of human colon cancer cells *in vitro* with a novel 3-substituted indolinone compound, 3-[1-(3*H*-imidazol-4-yl)-meth-(*Z*)-ylidene]-5-methoxy-1,3-dihydro-indol-2-one (SU9516), that selectively inhibits the kinase activity of cdk2, also decreases the phosphorylation of pRb [46]. Of interest, this compound was also strongly proapoptotic, cytostatic, and antiproliferative. To investigate these downstream effects, we have extended these observations to show that exposure to SU9516 in human colon carcinoma cell lines prevents dissociation of pRb from E2F in a concentration- and time-dependent fashion and, furthermore, results in sequestration of E2F in high molecular weight complexes. This is the first study to show that pharmacologic inhibition of cdk2 can augment sequestration of E2F, thus confirming the activating role of cdk2 for E2F.

2. Materials and methods

2.1. Cell culture

SW480 and HT-29 human colon carcinoma cell lines (ATCC) and RKO cells (a gift of Len Augenlicht) were grown in RPMI-1640 medium supplemented with a 1% penicillin/neomycin/streptomycin (PNS) antibiotic mixture (Gibco BRL) and 10% fetal bovine serum (Gibco BRL). All cells were maintained at 37° in a humidified atmosphere supplemented with 5% CO_2.

2.2. Reagents

SU9516 was supplied by Gerald McMahon, Sugen. NU2058 and NU6027 were provided by R.J. Griffin, University of Newcastle. Flavopiridol was supplied by Edward A. Sausville, National Cancer Institute. Antibodies for Western blot and EMSA, including anti-E2F1(sc-193X), anti-E2F2 (sc-633X), anti-E2F3 (sc-878), anti-E2F4 (sc-866), anti-E2F5 (sc-1063X), anti-E2F1(sc-251), anti-E2F4 (sc-511), anti-Rb (sc-50X), anti-cdk4 (sc-260), and anti-cdk6 (sc-177) rabbit polyclonal antibodies, and anti-cyclin A (sc-239), anti-cyclin B1 (sc-245), anti-cyclin D1 (sc-6281), anti-cyclin E (sc-247), anti-cdc2 (sc-54), and anti-cdk2 (sc-6248) mouse monoclonal antibodies were purchased from Santa Cruz Biotechnology, except as noted. All buffers and salts were obtained from the Sigma Chemical Co., except as noted.

2.3. Total protein extraction and Western blot analysis

This method has been described previously [47]. In brief, following treatment with drugs or buffer, subcon-

fluent cells were washed three times, scraped, and lysed in 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 μg/mL of aprotinin; 10 μg/mL of leupeptin; 1 mM PMSF; 200 μM sodium orthovanadate; 10 mM sodium pyrophosphate; 20 mM NaF) on ice. Lysates were clarified by centrifugation, and protein concentration was determined spectrophotometrically. Lysates were frozen and stored at −80°. For immunoblotting, lysates were loaded at an equal protein concentration, 30–50 μg/lane, and subjected to 10% SDS–PAGE. After electrophoresis, protein was transferred electrophoretically to a nitrocellulose filter at a constant current of 180 A overnight at 4°. Nitrocellulose filters were blocked in 5% nonfat dried milk in TBS-Tween (TBS-T) for 1 hr and then incubated with the anti-target protein, mouse monoclonal antibodies, and anti-actin mouse monoclonal antibody (Sigma) in TBS-T containing 2% nonfat dried milk for 1 hr. After three washes in TBS-T, horseradish peroxidase (HRP)-conjugated anti-mouse secondary antibody was added at a 1:3000 dilution in TBS for 50 min. Detection of signal was performed using the Super Signal Blotting Detection System (Pierce Chemical Co.). The intensities of the autoradiographic bands were quantitated by densitometric scanning. All experiments were repeated at least three times for each cell line, and analyzed as percent of untreated control.

2.4. Nuclear protein extraction and EMSA

Cells were treated either with media or one of various kinase inhibitors for 24–48 hr. Nuclear extracts from cells were prepared as described previously with the following modifications [48]. Subconfluent cells on 10 cm^2 dishes were washed twice in phosphate-buffered saline, scraped into 1.5 mL of phosphate-buffered saline, microcentrifuged for 15 s at 4°, then resuspended in 400 μL of lysis buffer (10 mM HEPES, pH 7.9; 0.1 mM EGTA; 0.1 mM EDTA; 10 mM KCl; 0.5 mM PMSF; 1 mM DTT) on ice for 15 min. Twenty-five microliters of 10% Nonidet P-40 was added, and the mixture was vortexed vigorously for 10 s, followed by microcentrifugation for 30 s. The pellets were resuspended in 50–75 μL of ice-cold buffer (20 mM HEPES, pH 7.9; 1 mM EGTA; 1 mM EDTA; 400 mM NaCl; 1 mM PMSF; 1 mM DTT) on ice for 15 min, followed by microcentrifugation for 5 min. The nuclear protein concentration was determined spectrophotometrically from the supernatants, and the supernatants were frozen in aliquots at −80° for EMSA. The reaction mixture for the E2F binding assay contained 6–10 μg of nuclear extract in 20 μL of probe mix (20 mM HEPES, pH 7.9; 0.2 mM EDTA; 20% glycerol; 20 mM KCl; 0.5 mM DTT; 0.5 ng of ^{32}P-labeled oligonucleotide probe; 0.8–2 μg polydeoxyguanylic-deoxycytidylic acid (poly(dG-dC)·poly(dG-dC) double strand); and 1.2–2 μg polydeoxyinosinic-deoxycytidylic acid (poly(dI-dC)·poly(dI-dC) double strand)). Reaction mixtures were incubated at room temperature for 20 min and separated on a 5% polyacylamide (37.5:1 ratio of acrylamide to bis-acrylamide) gel in 0.25× TBE (22.5 mM Tris-borate, 0.5 mM EDTA) for 80 min at 180 V. For antibody perturbation experiments, 2.5 μg of antibody was added 50 min before the addition of the oligonucleotide probe and incubated on ice. Antibodies to irrelevant proteins, including Sp1, RXR, and c-*myc*, were employed to ensure the specificity of the method, and resulted in no band shifts. The double-stranded DNA oligonucleotide for E2F was synthesized by the oligonucleotide facility at the Albert Einstein College of Medicine, and contains the consensus binding site for E2F1 (5′-ATTTAAGTTTCGCGCCCTTTCTCAA-3′/3′-T). Internal controls included an unlabelled competitor oligonucleotide probe and a mutant competitor oligonucleotide probe to ensure the specificity of the results. All experiments were performed at least three times for each cell line.

3. Results

3.1. Prevention of pRb/E2F dissociation by SU9516 treatment

We have shown previously that SU9516 inhibits cdk2-specific phosphorylation of pRB [46]. To assess the effect of decreased pRb phosphorylation, we investigated whether treatment with SU9516 inhibits dissociation of the E2F complex. As shown in Fig. 1 (arrow), treatment of HT-29 cells with 5 μM SU9516 (lane 6) resulted in enhanced pRb/E2F complex formation as compared with untreated HT-29 cells (lane 5).

Furthermore, treatment with SU9516 promoted formation of other high molecular weight E2F complexes (Fig. 1, arrow). Supershifted bands were observed with antibodies to p130 (lane 10), p107 (lane 8), cyclin A (lane 12), cyclin E (lane 14), and DP-1 (lane 16), but not cyclin D1 or cyclin B (not shown). The heterogeneity of the bands suggests that either the constituents of these multiprotein complexes are different, or it may reflect different binding specificities of the antibodies employed. We expanded this observation to include two other human colon cancer cell lines, RKO and SW480 cells. These cell lines were tested following treatment with 5 μM SU9516 for 24 hr. Accumulation of pRb/E2F complex was observed, but was cell line specific as it was lower in both RKO and SW480 than in the HT-29 cell nuclear extracts (data not shown).

We next sought to determine the predominant E2F species sequestered in these complexes. Treatment with SU9516 resulted in sequestration of all five E2F species (Fig. 2, lanes 6, 8, 10, 12, and 14, top arrow), although there was obvious disparity between the complexes formed, which again may reflect heterogeneity among these complexes or different binding specificities of the antibodies. Of interest, the absence of two specific supershifted bands

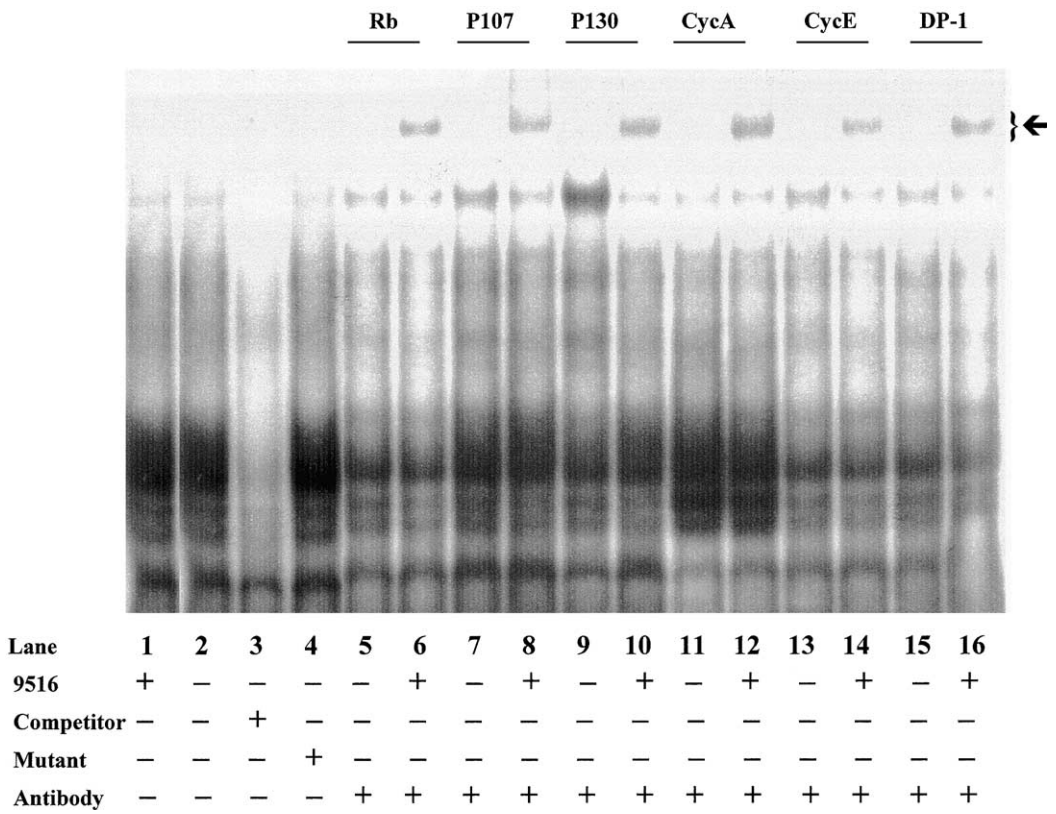


Fig. 1. Effects of SU9516 treatment on the binding of E2F to Rb and other components of the multiprotein complex. HT-29 cells were incubated with 5 μM SU9516 or buffer for 24 hr. Cell extracts were analyzed by EMSA. Treatment of cell extracts is indicated in the figure. Lane 1, SU9516-treated cell extracts; lane 2, untreated cell extracts; lane 3, competitor, unlabelled wild-type oligonucleotide; lane 4, mutant, mutated competitor oligonucleotide; lanes 5–16, specific antibodies for gel shift experiments, indicated above the lanes. Arrow: supershifted E2F complexes are present in SU9516-treated cell extracts. Enhancement of bands for p130 and cyclin A were reproducible across all experiments for HT-29 cells, and may represent stabilization of complexes by these antibodies without a shift. The blot shown is representative of three experiments.

was observed with antibodies to E2F1 (A) and E2F4 (B) (Fig. 2, bottom arrow and insets).

To test the time–response effects of SU9516, cells were treated with 5 μM SU9516 for either 0, 24, or 48 hr. Treatment of HT-29 cells for 48 hr resulted in greater accumulation of pRb/E2F complex than did treatment for 24 hr (Fig. 3), while in RKO and SW480 cells, there was no difference observed between samples treated for 24 or 48 hr (data not shown).

3.2. Effects of other protein kinase inhibitors on pRb/E2F

To determine whether treatment with other inhibitors of cdk2 would result in comparable effects on the formation of pRb/E2F containing complexes, gel shift assays were performed on nuclear extracts from cells treated with other compounds. A novel purine, O^6-cyclohexylmethylguanine (NU2058), and a novel pyrimidine, 2,6-diamino-O^4-cyclohexylmethyloxy-5-nitrosopyrimidine (NU6027), are inhibitors of cdk1 and cdk2 (K_i for cdk2, 12 ± 3 and 1.3 ± 0.2 μM, respectively) [49]. Flavopiridol (L86-8275), an *N*-methylpiperidinyl, chlorophenyl flavone, has demonstrated potent and specific *in vitro* inhibition of cdks 1, 2, 4, and 7, with a preferential specificity for cdk4 [50,51]. Incubation of HT-29 cells with 10 μM NU2058, 1 μM NU6027, or 10 μM flavopiridol for 24 hr resulted in enhanced formation of pRb/E2F containing complexes (Fig. 4, arrow), observed consistently in three separate assays. Treatment of cells with *N*-[4-(trifluoromethyl)-phenyl] 5-methylisoxazole-4-carboxamide, an inhibitor of platelet-derived growth factor receptor signaling with no identifiable effect on cdk2/pRb/E2F pathways, resulted in no supershifts (not shown).

3.3. Effects of SU9516 treatment on levels of cyclins

To determine whether the augmented binding of pRb to E2F observed in the EMSA studies above resulted from repression of cyclin E, western analysis of extracts from cells treated with 5 μM SU9516 for 24 hr was performed. As shown in Fig. 5, the levels of cyclin E increased by 40–75% at 24 hr in all three cell lines, and then returned to baseline by 72 hr. Since increased cyclin E activity has been associated with an increase in phosphorylation of pRb and derepression of E2F, it is unlikely that the increased levels of cyclin E accounted for enhanced binding of pRb and E2F. In contrast, the levels of cyclin D1, A, and B1

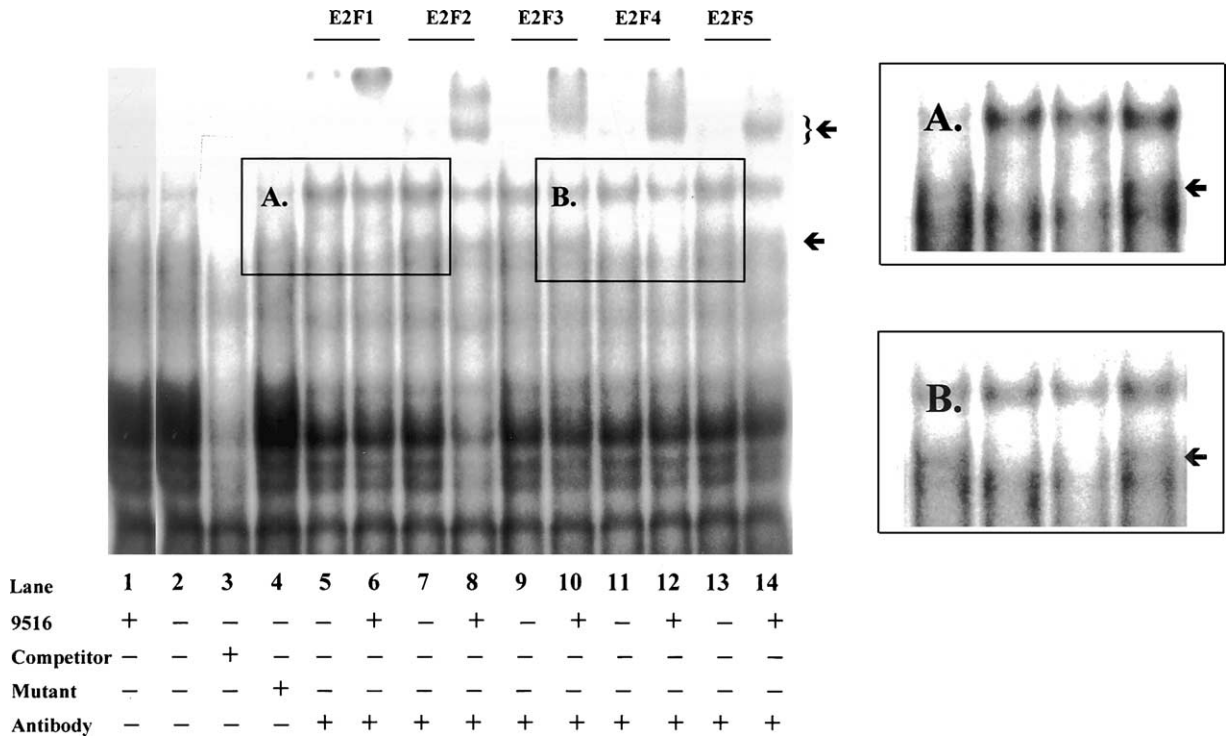


Fig. 2. Presence of E2F species in multiprotein complexes enhanced by SU9516 treatment. HT-29 cells were incubated with 5 μM SU9516 or buffer for 24 hr. Cell extracts were analyzed by EMSA. Treatment of cell extracts is indicated in the figure. Lane 1, SU9516-treated cell extracts; lane 2, untreated cell extracts; lane 3, competitor, unlabelled wild-type oligonucleotide; lane 4, mutant, mutated competitor oligonucleotide; lanes 5–14, specific antibodies for gel shift experiments, indicated above the lanes. Top arrow: supershifted E2F complexes are present in SU9516-treated cell extracts. Bottom arrow and insets: supershifted bands for E2F1 (A), and E2F4 (B) are absent. This figure is representative of three blots.

decreased by 10–30% at 24 hr, and then continued to decrease by 4-fold at 72 hr (Fig. 5).

3.4. Effects of SU9516 treatment on levels of cdks

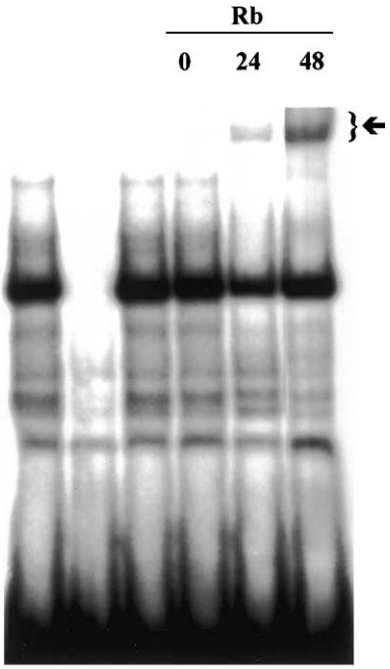
Expression of cdk2 was also studied to determine whether changes in cdk2 contributed to the decreased phosphorylation of pRb and enhanced formation of the pRb/E2F complex. The cdk2 levels decreased by about 20–50% in all three cell lines at 24 hr (Fig. 6), with a 4-fold change by 72 hr. Levels of cdk1 decreased in a similar fashion, which was of interest considering that SU9516 inhibits cdk1 activity, although with less specificity than cdk2. In contrast, as shown in Fig. 6, the levels of cdk4 and cdk6 were unchanged.

3.5. Effects of SU9516 treatment on levels of E2F1-5

We have shown previously that treatment with SU9516 resulted in decreased cdk2-specific phosphorylation of pRb, without changes in protein levels of pRb [46]. Therefore, the increase in pRb/E2F containing complexes observed following SU9516 treatment did not result from an increase in protein levels of pRb. To determine whether the increase in pRb/E2F complex resulted from increased levels of E2F1-5 protein, western analysis was performed on extracts from cells treated for 24 hr with 5 μM SU9516. There was a transient 45% increase in E2F1 observed in HT-29 cells, a transient 75% increase in E2F3 observed in RKO cells, and a transient 50% increase in E2F5 levels observed in SW480 cells at 24 hr, all of which returned to baseline at 48 hr ($N \geq 3$ for all; data not shown). E2F2 and 4 changed by <20% at 24–48 hr as did E2F1, 3 and 5 in the other cell lines. Overall, the small magnitude, the lack of consistency, and the transiency of these changes make them unlikely to fully account for the enhanced pRb/E2F binding observed on EMSA.

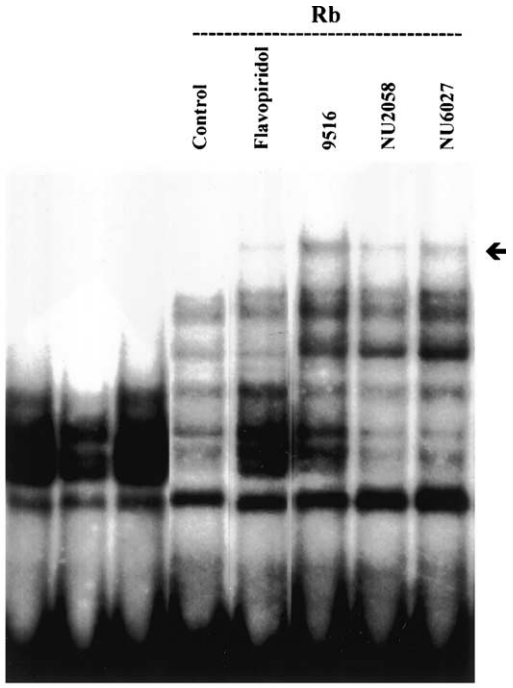
4. Discussion

SU9516 was derived from the synthetic optimization of a lead compound identified by high throughput screening with cdk2 [52]. We have demonstrated recently that SU9516 is 2-fold more selective for cdk2 than for cdk1 and 9-fold more selective for cdk2 than for cdk4 [46]. SU9516 was a potent inhibitor of cdk2-specific pRb phosphorylation in SW480 and RKO human colon carcinoma cells, suggesting a potentially specific role for this agent as a modulator of cell growth. Furthermore, in our tumor model system, SU9516-induced deregulation of the pRb/E2F pathway resulted in antiproliferative, cytostatic, and proapoptotic effects, similar to those achieved with peptide antagonists to cdk2, which were designed to block the docking of cyclins E and A to cdk2 [53].



Lane	1	2	3	4	5	6
Competitor	–	+	–	–	–	–
Mutant	–	–	+	–	–	–
Antibody	–	–	–	+	+	+

Fig. 3. Enhanced accumulation of E2F complexes with extended exposure to SU9516. HT-29 cells were treated with 5 μM SU9516 for 0, 24, or 48 hr. Lane 1, unlabelled wild-type oligonucleotide; lane 2, mutated competitor oligonucleotide; lane 3, no antibody. Lanes 4–6: extracts incubated with antibody to pRb. There was greater accumulation of high molecular weight E2F/pRb complexes with longer exposure to drug. This figure is representative of three blots.



Lane	1	2	3	4	5	6	7	8
Competitor	–	+	–	–	–	–	–	–
Mutant	–	–	+	–	–	–	–	–
Antibody	–	–	–	+	+	+	+	+

Fig. 4. Effects of treatment with SU9516, purine or pyrimidine kinase inhibitors, or flavopiridol on formation of high molecular weight E2F complexes. HT-29 cells in exponential growth were treated with kinase inhibitors for 24 hr. Extracts from cells treated with the indicated kinase inhibitors were analyzed by EMSA. Lane 1, unlabelled wild-type oligonucleotide; lane 2, mutated competitor oligonucleotide; lane 3, no antibody. Lanes 4–8, extracts incubated with antibody to pRb. Arrow: high molecular weight E2F complex. The figure is overexposed to better show supershifted bands, specifically in the flavopiridol-treated cells, and is representative of three blots.

The current investigations were intended to extend these observations to effects on E2F/pRb binding. Since phosphorylation of pRb results in derepression of E2F [4,34,54], our hypothesis was that SU9516-induced inhibition of cdk2-specific phosphorylation of pRb would enhance binding and inactivation of E2F by pRb and result in accumulation of pRb/E2F complex. This was observed in three human colon cancer cell lines. Treatment of cells with NU2058 and NU6027, purine and pyrimidine cdk2 inhibitors, also produced this effect, as did flavopiridol, a pan-cdk inhibitor, reproducing the effects observed with SU9516. This study clearly demonstrates that pharmacologic intervention targeting cdk2 inhibition can prevent dissociation of pRb/E2F containing complexes.

What was somewhat surprising was that treatment with SU9516 resulted in accumulation of E2F in high molecular weight multiprotein complexes with the other pocket proteins, p107 and p130, as well as with DP-1, cdk2, and cyclins A and E. It is known that both cyclin E/cdk2 and cyclin A/cdk2 kinases associate with E2F and with p107 in a temporally distinct fashion [55,56]. Furthermore, p130 also binds and modifies E2F activity in a manner different from p107 [57]. In addition, a dominant negative cdk2 prevented the dissociation of p130/E2F4 complexes [58]. E2F is known to exist as part of a multiprotein complex. However, we have now expanded this observation to demonstrate pan-sequestration of E2F in such complexes following pharmacologic inhibition of cdk2. Furthermore, this supports the broad range of antigrowth effects observed in our previous studies, which included apoptosis, cytostasis, and inhibition of proliferation.

Cyclin-dependent kinase 2 has multiple roles in the cell growth regulatory process in addition to phosphorylation of pRb. In association with cyclin E, cdk2 regulates $p220^{NPAT}$-mediated histone transcription [59], down-regulates p27 [60], blocks DNA replication by preventing MCM proteins from associating with chromatin [61], and is essential for centrosome duplication [62] and for nucleosome assembly [63]. In association with cyclin A, cdk2 inhibits origin replication complex (ORC) binding [61], regulates cyclin B activity, and phosphorylates E2F1, which in turn eliminates

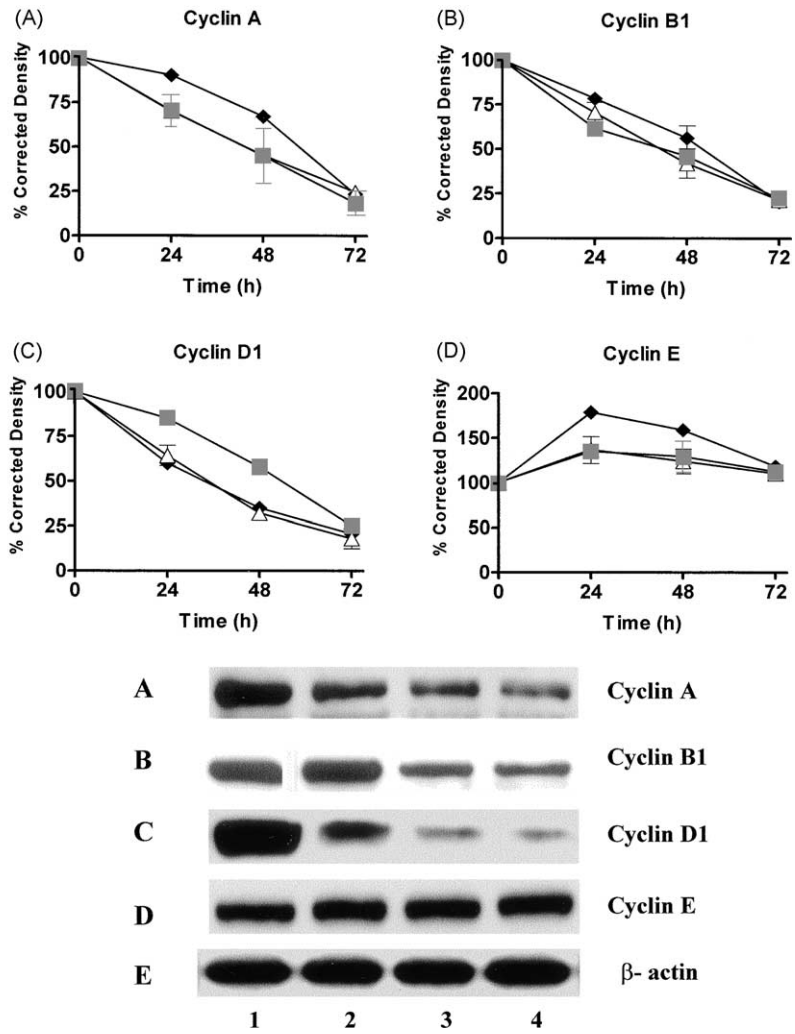


Fig. 5. Effects of treatment with 5 μM SU9516 on protein levels of specific cyclins. HT-29, SW480, or RKO cells in exponential growth were treated with SU9516 for 24 hr. Extracts were analyzed for protein levels by Western blot. Top panel: levels of cyclins A, B1, D1, and E at 0, 24, 48, and 72 hr. Key: (◆) SW480 cells; (■) RKO cells; and (△) HT-29 cells. Each point is the mean of three experiments. Error bars represent the SEM. Bottom panel: representative blots of HT-29 cell extracts for panels A–D. Lane 1, no treatment; lanes 2–4, SU9516 treatment for 24, 48, and 72 hr, respectively. β-Actin, internal control. This figure is representative of at least three blots.

E2F1 DNA binding function [64,65]. Suppression of E2F1 DNA-binding activity by cyclin A/cdk2 is linked to orderly S phase progression. Conversely, disruption of this linkage, for example, by inhibition of cdk2, could result in S phase delay or arrest followed by regrowth or, alternatively, apoptosis, depending upon whether the DNA-bound E2F1 could transactivate [66]. Therefore, the proapoptotic [46] and antiproliferative effects of SU9516 observed may be attributable to effects downstream of the inhibition of cyclinE/cdk2-specific phosphorylation of pRb.

We also sought to determine whether the accumulation of the pRb/E2F complex could result from increased total expression of either pRb or E2F, without an actual change in the percentage of protein found in the complex. We had shown previously that levels of pRb are not increased following treatment with SU9516 [46]. In the current studies, we examined levels of various E2F species following drug treatment. While there was a transient increase in E2F3 in RKO cells at 24 hr following drug treatment, this was relatively small in comparison with the markedly enhanced binding observed on EMSA. Furthermore, EMSA analysis demonstrated that E2F1 was the predominant complexed E2F species in the human colon carcinoma cell lines studied, and levels of total E2F1 did not increase, suggesting that the increased level of E2F/pRb complex observed was not due to increased E2F expression levels.

The transient rise in E2F3 is of interest in the context of the proapoptotic effects of SU9516. E2F3$^{-/-}$ murine embryonic fibroblasts expressed lower levels of cyclins E and A, Cdc2, b-Myb, Cdc6, RRM2 and PCNA, supporting a critical role for E2F3 in S phase entry and progression [67]. Excess unbound E2F3 has been associated with proapoptotic effects. Therefore, SU9516-mediated apoptosis may be related to sequestration of E2F1 and transiently enhanced

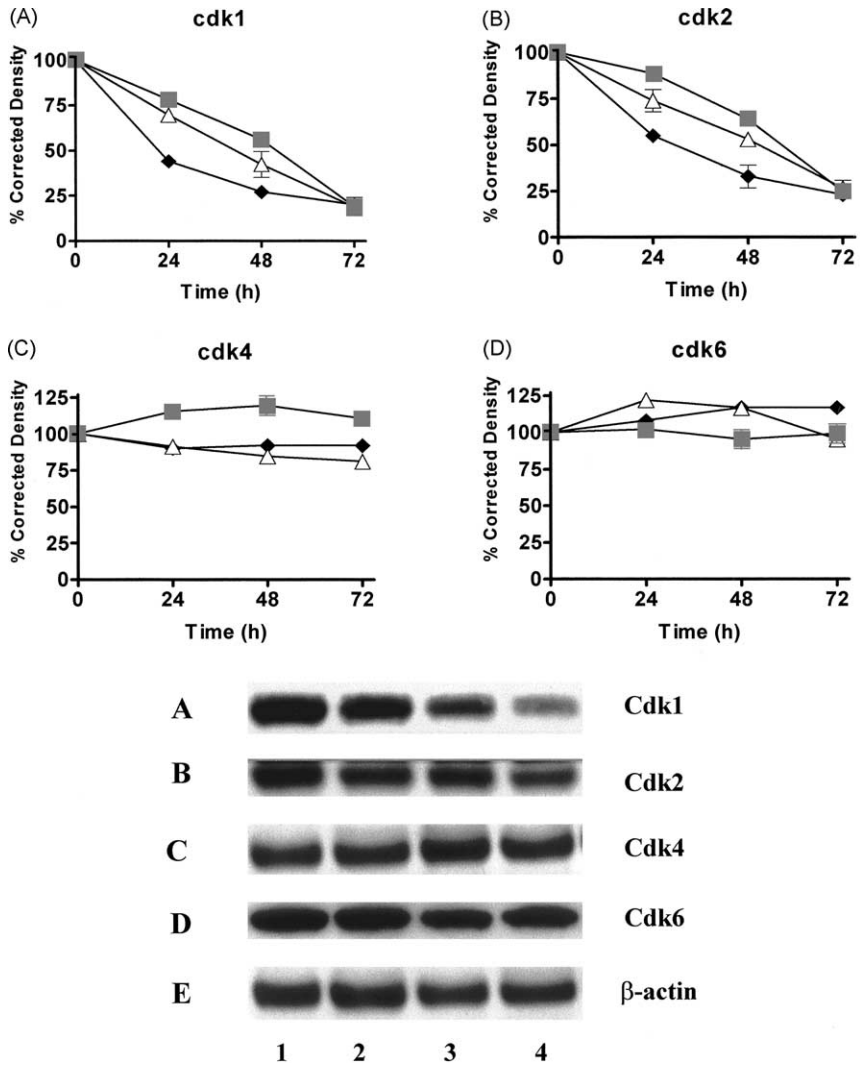


Fig. 6. Effects of SU9516 treatment on protein levels of specific cdks. HT-29, SW480, or RKO cells in exponential growth were treated with 5 μM SU9516 for 24 hr. Extracts were analyzed for protein levels by Western blot. Top panel: levels of cdks at 0, 24, 48, and 72 hr. Key: (◆) SW480 cells; (■) RKO cells; and (△) HT-29 cells. Each point is the mean of three experiments. Error bars represent the SEM. Bottom panel: representative blots of HT-29 cell extracts for panels A–D. Lane 1, no treatment; lanes 2–4, SU9516 treatment for 24, 48, and 72 hr. β-Actin, internal control. This figure is representative of at least three blots.

levels of unbound E2F3. E2F5 levels were also transiently increased in SW480 cells. However, E2F5 binds p130 predominantly, and therefore is unlikely to have accounted for the increased levels of E2F/pRb binding.

As pRb binding to E2F decreases with increased levels of cyclin E/cdk2, we also asked whether perturbations in protein levels could have accounted for the increase in levels of the pRb/E2F complex, irrespective of effects on cdk2 kinase activity. Small changes in cyclin E levels were observed, but are unlikely to fully account for a marked increase in levels of the pRb/E2F complex, particularly since the changes occurred in the wrong direction, that is, would be more likely to cause derepression of E2F1. The decrease in cyclin D1 levels at 24 hr by >50% and the continued decrease at 48 hr could be associated with decreased phosphorylation of pRb and an increase in the accumulation of the pRb/E2F complex, even in the absence of observed changes in the levels of cdk4 and 6. The decreased levels of cdk2 protein appear to be a more significant factor, and may represent a downstream effect of cdk2 inhibition by SU9516. This is in contrast to the effects of treatment with the pan-cdk inhibitor flavopiridol, which results in decreased cdk2 activity, but no change in protein levels [68]. Nevertheless, the relative contributions of SU9516-mediated cyclin D1 down-regulation and cdk2 inhibition cannot be determined from the current studies.

In addition to SU9516, several other pharmacologic inhibitors of cdk2 are in development [69] (for review, see [70]). Our study demonstrates that strategies aimed at inhibiting cdk2 activity are feasible anti-tumor strategies. Specifically, in our system, inhibition of cdk2 kinase activity resulted in sequestration of E2F, which has numerous effects on tumor cell cycle regulation, including antiproliferative and proapoptotic effects.

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