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CVT-313, a Specific and Potent Inhibitor of CDK2 That Prevents Neointimal Proliferation*

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The activity of cyclin-dependent kinase 2 (CDK2) is essential for progression of cells from G_1 to the S phase of the mammalian cell cycle. CVT-313 is a potent CDK2 inhibitor, which was identified from a purine analog library with an IC_{50} of 0.5 μM *in vitro*. Inhibition was competitive with respect to ATP (K_i = 95 nM), and selective CVT-313 had no effect on other, nonrelated ATP-dependent serine/threonine kinases. When added to CDK1 or CDK4, a 8.5- and 430-fold higher concentration of CVT-313 was required for half-maximal inhibition of the enzyme activity. In cells exposed to CVT-313, hyperphosphorylation of the retinoblastoma gene product was inhibited, and progression through the cell cycle was arrested at the G_1/S boundary. The growth of mouse, rat, and human cells in culture was also inhibited by CVT-313 with the IC_{50} for growth arrest ranging from 1.25 to 20 μM. To evaluate the effects of CVT-313 *in vivo*, we tested this agent in a rat carotid artery model of restenosis. A brief intraluminal exposure of CVT-313 to a denuded rat carotid artery resulted in more than 80% inhibition of neointima formation. These observations suggest that CVT-313 is a promising candidate for evaluation in other disease models related to aberrant cell proliferation.

Cell cycle progression in mammalian cells is regulated by a family of cyclin-dependent protein kinases (CDKs),[1] that include CDK1, CDK2, CDK3, CDK4, and CDK6 (1). CDK2 is a serine/threonine kinase whose activity is essential for the G_1 to S transition during cell division. A number of proteins have been shown to be substrates for CDK2 phosphorylation, and among them are the retinoblastoma gene product (Rb) and other related pocket proteins, members of the E2F transcription factor family, cyclin E, and members of the CDK inhibitory proteins. It is also postulated that CDK2 phosphorylates and regulates proteins involved in DNA replication (2, 3). Two lines of evidence suggest that CDK2 activity is essential for cell proliferation; microinjection of antibodies directed against CDK2 blocks the progression of human diploid fibroblasts into S phase (4, 5), and overexpression of a dominant negative mutant of CDK2 in human osteosarcoma cells has a similar effect (6). The crucial role of CDK2 in controlling cell cycle progression suggests that CDK2 is an attractive target for treatment of aberrant cell proliferation.

Smooth muscle cell proliferation is largely responsible for restenosis following angioplasty (7). A recent study has shown that CDK2 is activated very early after endothelial denudation in the rat carotid artery model of restenosis (8); moreover, antisense oligonucleotides directed against CDK2 were shown to be effective in reducing neointima formation in this model (9, 10). Arguably, the restenosis model can be used as a "proof of principle" for developing CDK2 inhibitors as drug candidates for the treatment of diseases related to aberrant cell proliferation. Olomoucine is a purine analog of ATP and is a specific inhibitor of CDK1 and CDK2 (11). Its potency, however, is relatively low. Using the crystal structure of CDK2 (12) and computer-aided drug design, a combinatorial library strategy (13, 14) generated a large number of purine analogs. While the synthesis and structure activity relations of these compounds will be described elsewhere,[2] the present communication describes the biological effects of CVT-313, a representative compound from this purine-based library.

MATERIALS AND METHODS

Chemicals and Buffers—All chemicals were purchased from Sigma and all tissue culture media and reagents were purchased from Life Technologies, Inc., except where indicated. Baculovirus homogenization buffer was 10 mM HEPES (pH 7.4), 10 mM NaCl, 5 mM EDTA, 1 mM dithiothreitol, 1 mM Pefabloc (Boehringer Mannheim), 1 μg/ml leupeptin, and 5 μg/ml aprotonin. Activation buffer was 10 mM HEPES (pH 7.4), 10 mM $MgCl_2$, and 1 mM ATP. Lysis buffer was 50 mM HEPES (pH 7.4), 250 mM NaCl, 0.1% Triton X-100, 50 mM NaF, 80 mM β-glycerophosphate, 0.1 mM Na_3VO_4, 1 mM Pefabloc, 1 μg/ml leupeptin, 5 μg/ml aprotonin, and 1 mM iodoacetamide.

Enzymes—Baculovirus constructs expressing human cyclin AΔ171, CDK2, cyclin H, and CDK7 were a kind gift from David Morgan (University of California, San Francisco). Recombinant proteins were expressed in Sf9 insect cells. Cells were homogenized using a Dounce homogenizer in baculovirus homogenization buffer. CDK2-cyclin A complexes were activated by mixing with a 0.1 volume of CDK7/cyclin H extracts in activation buffer and incubating at 25 °C for 1 h. The CDK2-cyclin A complex was purified as described previously (15). Human cyclin E was amplified from RNA made from MRC-5 cells by reverse transcription-polymerase chain reaction using the following primers: 5′-CGCGGATCCATGAAGGAGGACGGCGGCGC-3′ and 5′-TGCTCTAGATCACGCCATTTCCGGCCCGC-3′. The cDNA was cloned into pFASTBAC HTb (Life Technologies, Inc.), generating a histidine tag at the amino-terminal end of cyclin E. Recombinant cyclin E was expressed in Sf9 cells, mixed with protein extracts of Sf9 cells expressing CDK2, and activated as described above. The CDK2-cyclin E complex was purified by nickel resin chromatography utilizing the histidine tag of cyclin E, as recommended by the vendor (Life Technologies, Inc.). Human CDK4 cDNA was amplified from RNA made from MRC-5 cells by reverse transcription-polymerase chain reaction using the following

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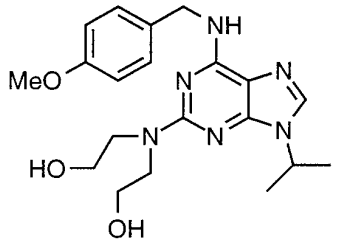
[1] The abbreviations used are: CDK, cyclin-dependent kinase; Rb, retinoblastoma gene product; FACS, fluorescence-activated cell sorter.

[2] S. R. Schow, R. T. Lum, D. Shiffman, R. Mackman, and M. W. Wick, manuscript in preparation.



CVT-313

FIG. 1. **Structure of CVT-313 a CDK2 inhibitor.**

primers: 5′-CGCGGATCCATGGCTACCTCTCGATATGAGCC-3′ and 5′-CCCAAGCTTTCACTCCGGATTACCTTCATCC-3′. The cDNA was cloned into pFASTBAC1 (Life Technologies, Inc.). Human cyclin D1 cDNA (a kind gift from Steven Reed, Scripps Institute) was cloned into a pFASTBAC HTb vector, generating a histidine tag at the amino-terminal end of cyclin D1. CDK4/cyclin D/CDK7/cyclin H were co-expressed in Sf9 cells, and the activated CDK4-cyclin D complex was isolated by nickel resin chromatography. Human CDK1/cyclin B was purchased from New England Biolabs. Rat brain protein kinase C was purchased from Pierce. Bovine protein kinase A was purchased from Boehringer Mannheim. Murine recombinant mitogen-activated protein kinase was purchased from Upstate Biotechnology.

In Vitro Kinase Assays—CDK2/cyclin A, CDK2/cyclin E, and CDK1/cyclin B were incubated with 1 μg of histone H1 (Life Technologies, Inc.) or 1 μg of glutathione *S*-transferase-Rb (Santa Cruz Biotechnology) as indicated, in 10 mM $MgCl_2$ with 50 μM ATP and 0.3 μCi of [γ-^{32}P]ATP (3000Ci/mmol, NEN Life Science Products) in a total volume of 20 μl. Reactions were carried out for 25 min at 30 °C. Reactions were stopped by the addition of 2 μl of 0.5 M EDTA. Samples were blotted onto Whatman P81 phosphocellulose paper and washed three times with 150 mM phosphoric acid. Filters were blotted dry, mixed with scintillation fluid, and quantitated by liquid scintillation spectrometry (Beckman LS 6500). All other *in vitro* kinase assays were performed by the same method, except that the protein kinase C assay contained 0.2 μg of phosphotidyl-L-serine as an activator of proein kinase C. CDK4/cyclin D1 assay used 1 μg of glutathione *S*-transferase-Rb (Santa Cruz Biotechnology) as the substrate in 10 mM Hepes (pH 7.4), 10 mM $MgCl_2$, with 10 μM ATP and 1 μCi of [γ-^{32}P]ATP (3000Ci/mmol). The protein kinase A assay used 50 μM Kemptide (Life Technologies, Inc.) as the substrate in 10 mM $MgCl_2$, 1 μg/μl bovine serum albumin, 12.5 mM Tris (pH 7.5) with 100 μM ATP. The mitogen-activated protein kinase assay contained 10 μg of myelin basic protein (Life Technologies, Inc.) as the substrate in 18.75 mM $MgCl_2$ with 125 μM ATP.

Cell Culture, Proliferation Assays, and FACS Analysis—All cell lines were purchased from ATCC and grown as recommended except for neonatal rat vascular smooth muscle cells that were a kind gift from Mark Majesky (Baylor College of Medicine). These cells were grown in Dulbecco's modified Eagle's medium containing 5% fetal calf serum. Proliferation assays were carried out using the nonradioactive CellTiter 96 kit (Promega) after 48-h exposure. For FACS analysis of DNA content, cells were trypsinized, fixed in 70% ice-cold ethanol, and treated with 0.1 mg/ml RNase A and 40 μg/ml propidium iodide for 1 h at 37 °C. Samples were sent to Cytometry Associates (San Diego) for analysis.

Western Blot—Proteins were extracted from cells from two 9.6-cm^2 wells using 0.2 ml of lysis buffer. Equal volumes (40 μl) of cleared cell lysate were separated on a 6% polyacrylamide gels, blotted onto nitrocellulose membrane, and probed with Rb antibodies (Santa Cruz Biotechnology). Blots were developed using the BM chemiluminescent system (Boehringer Mannheim).

Rat Restenosis Model—Pairs of male Sprague-Dawley rats (Charles River) aged 2–3 months (400–500 g) were housed under a normal 12-h light/dark cycle in standard plastic "shoe box" cages with standard laboratory chow and water available *ad libitum*. Prior to surgery, the animals were anesthetized with 1.3 ml/kg ketamine/xylazine mixture (58% ketamine hydrochloride, 100 mg/ml; 42% xylazine hydrochloride, 20 mg/ml) injected intraperitoneally. After preparing the ventral cervical area for aseptic surgery, the bifurcation of the left common carotid artery was exposed, and a small vascular clamp was applied to the internal carotid artery. A deflated, saline-filled 2 F Fogarty catheter (Baxter) was inserted via an arteriotomy in the external carotid artery. The catheter was advanced proximally into the common carotid artery up to the aortic arch. To denude and injure the artery, the balloon was

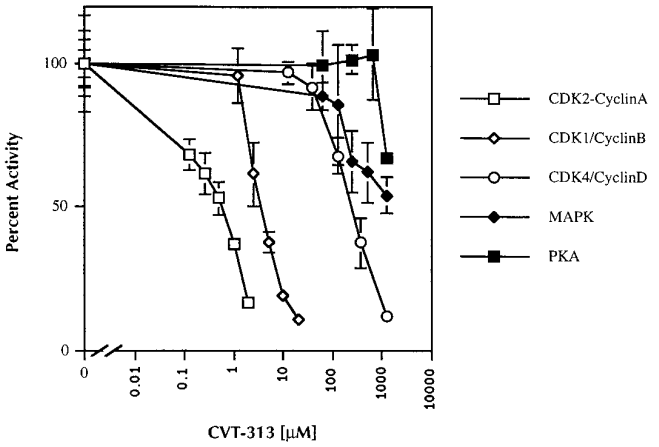


FIG. 2. **CVT-313 selectively inhibits CDK2 activity.** Enzyme activity assays were carried out as described under "Materials and Methods." *Bars* represent the standard error of each data point. The IC_{50} values were interpolated from these plots as follows: CDK2/cyclin A, *open squares*, 0.5 μM; CDK1/cyclin B, *open diamonds*, 4.2 μM; CDK4/cyclin D1, *open circles*, 215 μM; mitogen-activated protein kinase (*MAPK*), *filled diamonds*, >1250 μM; protein kinase A (*PKA*), *filled squares*, >1250 μM.

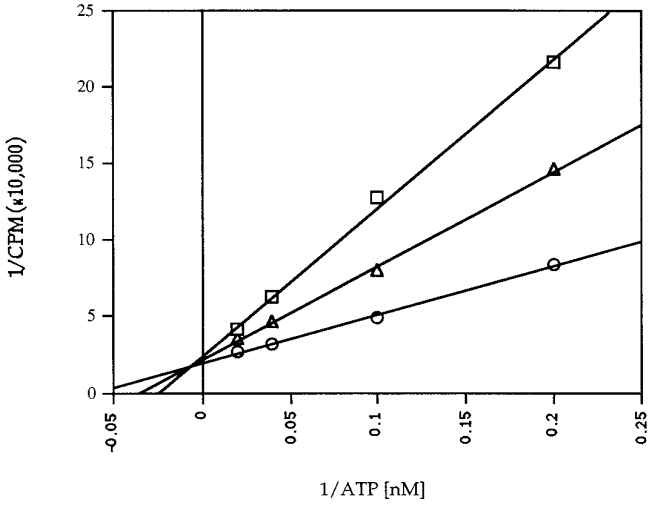


FIG. 3. **Lineweaver-Burke analysis of CVT-313 inhibition.** CDK2/cyclin A activity was assayed using histone HI as substrate. ATP concentrations were 5, 10, 25, and 50 μM. CVT-313 concentrations were 0 (*open circles*), 0.1 (*open triangles*), and 0.2 μM (*open squares*). A double reciprocal plot was extrapolated from the average of triplicate data points.

inflated with 20 μl of saline and then gently pulled back to the bifurcation. The procedure was repeated three times. Immediately after removing the balloon catheter, a delivery cannula (PE10 polyethylene tubing) was inserted, and 65 units of heparin sodium salt (Sigma, H-4898) in 1 ml of saline were injected. Following the heparin injection, a second vascular clamp was used to bisect the common carotid and allow for the infusion of drug or control solutions to one half of the injured artery. A loose silk ligature was used to mark the point where the clamp bisected the artery. This suture mark was later used to determine the treated and untreated sections of the artery when the animal was sacrificed. Before infusing control or drug solutions, the artery was backflushed with 1 ml of sterile saline. 100 μl of control or drug solution were flushed through the artery to wash out the saline and fill the lumen with the solution under study. A 3-0 Prolene ligature was placed around the external carotid to hold the cannula in place and create a closed system. Approximately 1 atm of steady pressure was applied to the delivery syringe over a 15-min incubation period. After the 15-min incubation period, the cannula and vascular clamp bisecting the common carotid artery were removed, to reestablish blood flow and flush residual solution out of the artery, before a 3-0 silk suture was applied to permanently ligate the external carotid artery. Finally, the vascular clamp was removed from the internal carotid artery, the neck

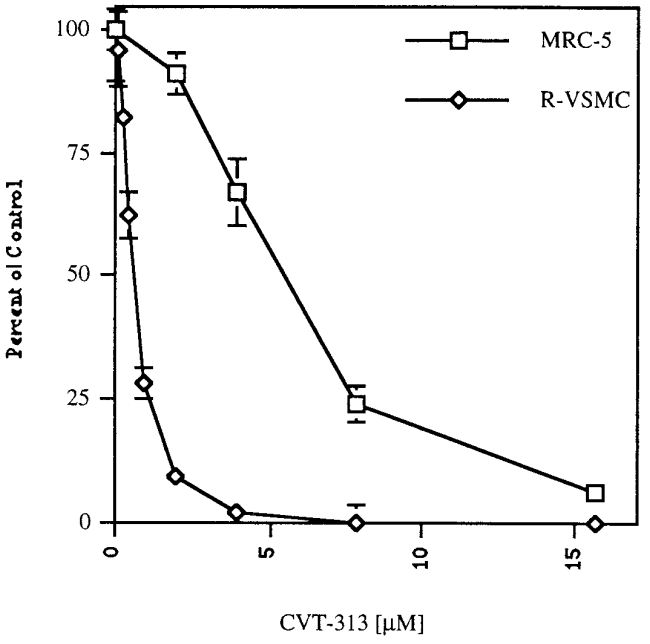


FIG. 4. **CVT-313 inhibits cell proliferation in tissue culture.** CVT313 was added to exponentially growing cells in tissue culture. Cell population was measured using CellTiter 96 system as described under "Materials and Methods." *Bars* represent the standard error of each datum point. IC_{50} were calculated from the linear portion of the inhibition curve, as indicated in Table I.

TABLE I
IC_{50} values for CVT313 added to exponentially growing cells in tissue culture

Cell population was measured using CellTiter 96 system as described under "Materials and Methods." IC_{50} were calculated from the linear portion of the inhibition curve.

Cell line	Description	IC_{50}
		μM
A2058	Human metastatic melanoma	16
Caco-2	Human colon adenocarcinoma	4.5
Capan-1	Human pancreatic adenocarcinoma	10
L1210	Mouse lymphocytic leukemia	6.25
MCF-7	Human breast adenocarcinoma	20
MRC-5	Human normal lung fibroblasts	5
Neonatal RVSMC[a]	Rat neonatal aortic smooth muscle cells	1.25
P-388D1	Mouse lymphoid neoplasm	4
Panc1	Human pancreatic carcinoma	8

[a] Rat vascular smooth muscle cells.

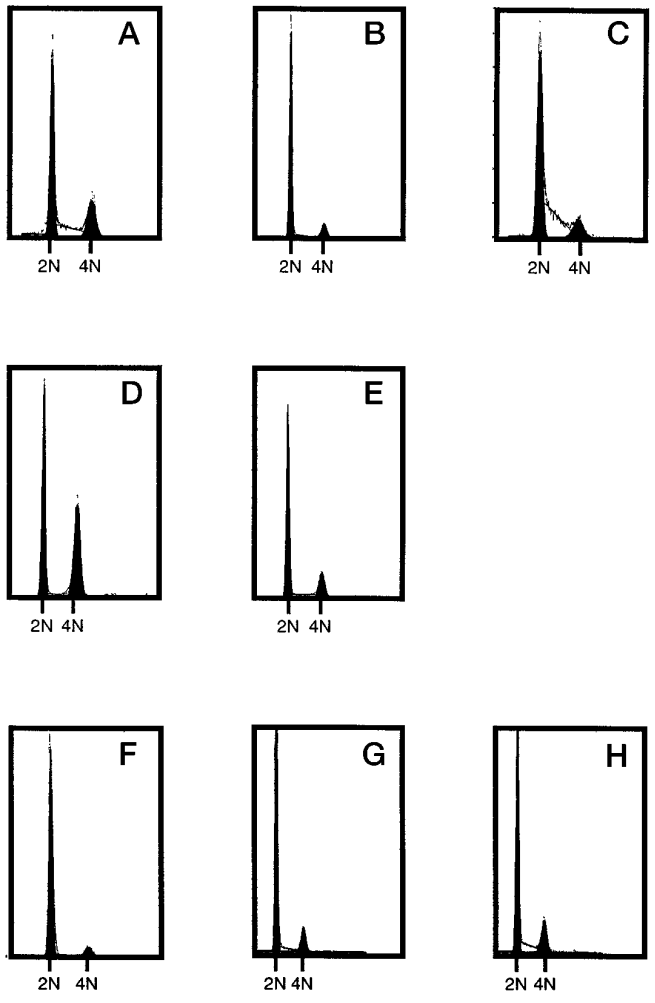


FIG. 5. **DNA content analysis of MRC-5 Cells.** FACS analysis of propidium iodide stained, RNase-treated MRC-5 cells. *A*, nonsynchronized cells. *B*, cells after 72-h serum deprivation. *C*, 72-h serum deprivation followed by 18-h serum stimulation. *D*, 72-h serum deprivation followed by 18-h serum stimulation, at which time CVT-313 at 12.5 μM was added for 24 h. *E*, same as *D* only CVT-313 concentration was 6.25 μM. *F*, unsynchronized cells exposed to CVT-313 at 6.25 μM for 36 h. *G*, same as *F*, only 12 h after drug has been removed. *H*, same as *F*, only 24 h after drug has been removed.

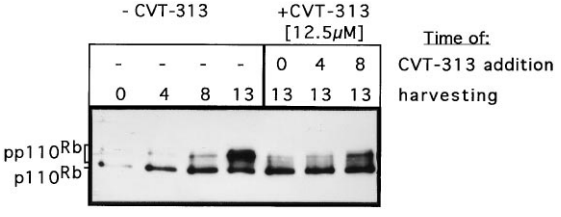


FIG. 6. **Effect of CVT-313 on Rb phosphorylation.** Western blot analysis of MRC-5 protein extracts that were separated on a 7% SDS-polyacrylamide gel using antibodies directed against Rb. Cells were serum deprived for 72 h and then stimulated with serum. CVT-313 at 12.5 μM was added at various times after serum stimulation, and protein was harvested as indicated.

wound was closed, and the animals were allowed to recover. Throughout the 14-day recovery period, a clinically relevant dose of aspirin (162.5 mg/L) was added to the animals' water to prevent thrombosis.

Morphometric Studies—Fourteen days after the balloon catheter injury to the carotid artery, the animals were anesthetized, as described above. Buffered formalin (10%) was perfused at physiological pressure through the aortic arch. The left carotid artery was removed (from the carotid bifurcation to the aortic arch) and bisected at the loose silk ligature that demarked the treated and untreated boundary. The treated and untreated sections were each cut into three equal sections and mounted in cryomolds. The arteries were sectioned in a cryotome into 10-μm slices. Samples were taken randomly at 100-μm intervals and mounted on slides for staining and analysis. Fifteen samples from both treated and untreated segments were used, for a total of 30 samples from each animal. The slides were stained with hematoxylin and eosin and analyzed with the 4× objective of a light microscope (Olympus) and a digitizing tablet (JS-2, Jandel Scientific). Sigmascan software (Jandel Scientific) was used to determine the neointimal area of the digitized image.

RESULTS AND DISCUSSION

CVT-313 Is a Specific and Competitive Inhibitor of CDK2—CVT-313 (2{-bis-(hydroxyethyl)amino}-6-(4-methoxybenzyl-amino)-9-isopropyl-purine, Fig. 1), was prepared by the Medicinal Chemistry department at CV Therapeutics, and details of its synthesis will be described elsewhere.[2] A large library of compounds were synthesized and tested for their ability to inhibit CDK2 activity. CVT-313 was identified as a potent inhibitor of CDK2/cyclin A (Fig. 2, IC_{50} = 0.5 μM). It is equally potent at inhibiting CDK2/cyclin E, using either histone HI or recombinant Rb as a substrate. The phosphorylation of Rb by

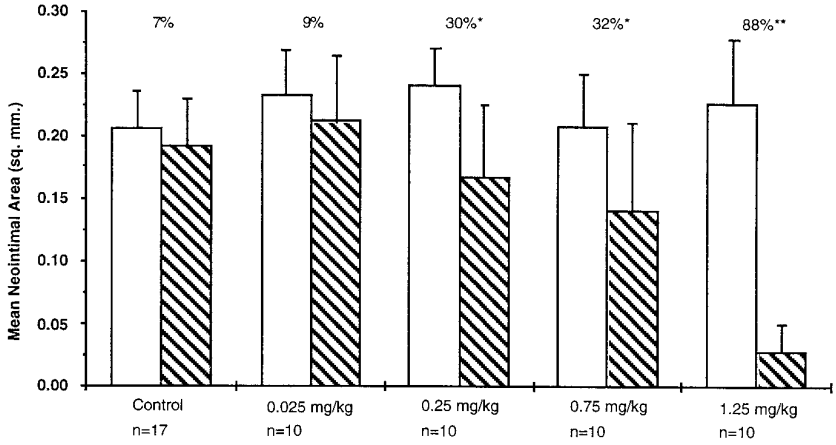


FIG. 7. **Inhibition of neointima formation by CVT 313.** Bar graph represents averages of neointimal areas of treated and untreated segments of rat carotid arteries. *Open bars* represent average neointimal areas of untreated segments. *Hatched bars* represent average neointimal areas of treated segments. Standard deviation of the average is represented by *error bars*. Dosing and group size are indicated *under the bars*. The percentage of reduction in neointimal area between treated and untreated segments are indicated *above the bars*. *Statistically significant with $p < 0.05$ in paired t test. **Statistically significant with $p < 0.001$ in paired t test

the CDK2-cyclin A complex was about 3-fold less sensitive to inhibition by CVT-313 (IC_{50} = 1.5 μM; data not shown). It is unclear whether this is because Rb is a natural substrate of CDK2/cyclin E but not of CDK2/cyclin A, or because we used a deletion derivative of cyclin A in our assay system. CVT-313 was tested for its effect on two related cyclin-dependent kinases. For inhibition of CDK1 activity, a 8.5-fold higher concentration was required (IC_{50} = 4.2 μM) and for inhibition of CDK4 activity, a 430-fold higher concentration was required (IC_{50} = 215 μM). To ascertain the specificity of inhibition of CVT-313, three other serine/threonine ATP dependent kinases were also tested. The concentration of CVT-313 required for half maximal inhibition of mitogen-activated protein kinase and protein kinase A was at least a 2500-fold higher than that required to inhibit CDK2, whereas protein kinase C was not inhibited at all at 1250 μM of CVT313 (data not shown). Thus, the inhibition of CDK2 activity by CVT-313 appears to be highly specific. The kinetics of inhibition of CDK2 by CVT-313 were examined. Results shown in Fig. 3 indicate that the inhibition was competitive with respect to ATP and the K_i was 95 nM.

CVT-313 Reversibly Inhibits Cell Proliferation at the G_1/S or G_2/M Boundary—Using normal and tumor human/murine cell lines, the effects of CVT-313 on cell proliferation was measured (Fig. 4 and Table I). The IC_{50} for growth inhibition ranged from 1.25 to 20 μM. To examine whether the growth inhibition by CVT-313 was cell cycle-specific, MRC-5 cells (human diploid lung fibroblasts) were exposed to CVT-313. Unlike nonsynchronized MRC-5 cells, a large number of MRC-5 cells that had been synchronized by serum deprivation for 72 h contained 2 N DNA (Fig. 5, *A versus B*). After 18 h of serum stimulation, a relatively large proportion of the cells progressed into S phase, with their DNA content intermediate between 2 and 4 N. (Fig. 5*C*). If CVT-313 (12.5 μM) was added to cells 18 h after serum stimulation, the DNA content of most of the cells was either 2 or 4 N with very few cells (less than 10%) entering S phase (Fig. 5, *D versus C*). If under similar culture conditions the concentration of CVT-313 was decreased to 6.25 μM, FACS analysis showed most cells with 2 N DNA, fewer cells with 4 N DNA, and very few cells in S phase. These data suggest that cells arrest at the G_1/S and G_2/M boundary at a higher concentration of CVT-313, but at the lower concentration of CVT-313, most of the cells are arrested at the G_1/S boundary. These observations are consistent with the established role of CDK1 and CDK2 in controlling G_2/M and G_1/S transition, respectively, and with our findings that, at lower concentrations, CVT-313 inhibits only CDK2 activity, but a higher concentration of CVT-313 is needed to inhibit CDK1 activity. We were also interested in ascertaining whether CVT-313 could be used to synchronize cells at the G_1/S boundary. Nonsynchronized MRC-5 cells were

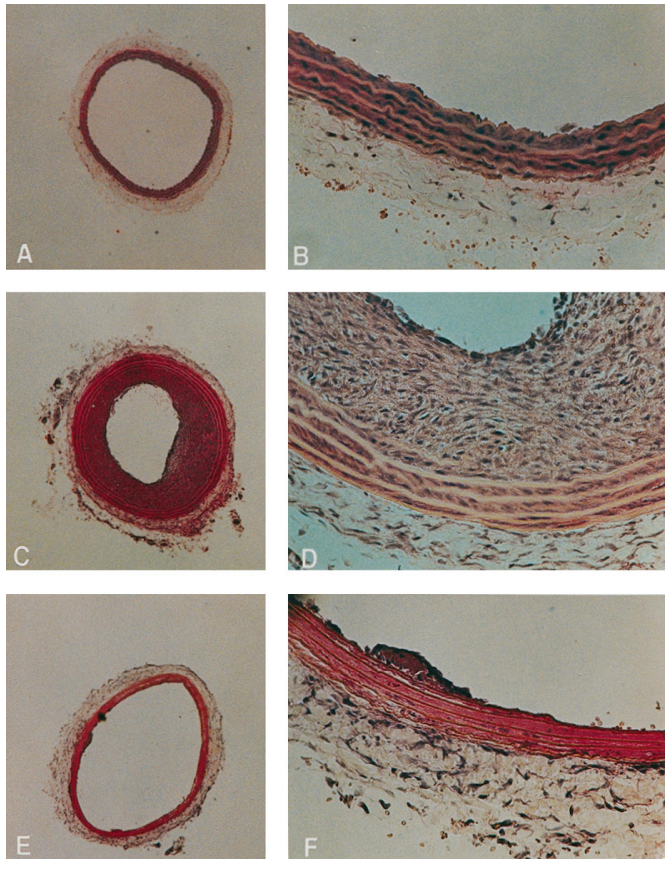


FIG. 8. **Representative slices of treated, untreated, and normal rat carotid.** *A*, normal rat carotid, × 40. *B*, same as *A*, × 200. *C*, rat carotid 14 days after endothelial denudation, × 40. *D*, same as *C*, × 200. *E*, same as *C* and locally treated with 2.5 mg/kg CVT-313 as described in the text, × 40. *F*, same as *E*, × 200.

treated with CVT-313 (6.25 μM) for 36 h and then analyzed by FACS. Fig. 5*F* shows that most of these cells had a 2 N DNA content, consistent with a G_1/S arrest. After removal of CVT-313 from the growth medium (Fig. 5, *G* and *H*), cells reentered the cell cycle, suggesting that the inhibition of cell proliferation was reversible.

CVT-313 Inhibits Rb Hyperphosphorylation—One of the *in vivo* targets of CDK2 is Rb. We wanted to ascertain whether CVT-313 inhibited Rb hyperphosphorylation in MRC-5 cells that had been synchronized by serum starvation. Western blot analysis was used to determine the phosphorylation status of Rb after serum stimulation (Fig. 6). At 4, 8, and 13 h after stimulation with serum, a time-dependent hyperphosphorylation of Rb could be demonstrated. FACS analysis of cells that

were processed in parallel indicated that these cells almost exclusively contained 2 N DNA (data not shown) which, consistent with published data, indicated Rb hyperphosphorylation prior to entry into S phase (16). If CVT-313 (6.25 μM) was added at 0, 4, or 8 h after serum stimulation, Rb hyperphosphorylation was inhibited. The effect on Rb hyperphosphorylation was less striking when CVT-313 was added 8 h after stimulation, presumably because some phosphorylation of Rb by CDK2 had already occurred. We have also detected some Rb hyperphosphorylation even when CVT-313 was added at the time of serum stimulation. These phosphorylation events could be the result of CDK4 activity, consistent with our finding that CVT-313 does not significantly inhibit CDK4 activity *in vitro*.

CVT-313 Inhibits Restenosis in Rats—To test the *in vivo* efficacy of CVT-313, we chose the injured rat carotid artery model of restenosis. In this model, luminal narrowing is due to neointima formation by smooth muscle cell migration and proliferation (7). Analysis of drug efficacy in this animal model has two potential problems. First, since the response to endothelial denudation varies from animal to animal, control groups could fortuitously show a higher proliferative response compared with treated animals. We addressed this problem by treating only one half of the injured carotid, and used the other half as an internal untreated control. Another potential problem relates to the nonuniformity of the stenotic lesion along the carotid, that can generate erroneous data when only a few sections from each carotid are analyzed. We therefore, routinely analyzed 15 randomly selected tissue slices throughout each segment of the carotid.

Using this methodology, we demonstrated that exposure of the denuded carotid artery to the hydrochloride salt of CVT-313 in saline solution (1.25 mg/kg) for 15 min under pressure, reduced neointima formation by 80% (Fig. 7). Moreover, in each individual animal treated with CVT-313 there is at least 70% inhibition of the neointimal area, demonstrating efficacy in every treated animal. Two lower doses of CVT-313 (0.75 and 0.25 mg/kg) were less efficacious, reducing mean neointimal area by about 30%, whereas the lowest dose tested (0.025 mg/kg) did not achieve any significant reduction in neointimal area. Representative sections from experimental rat carotid arteries 14 days after endothelial denudation (Fig. 8) demonstrate the efficacy of CVT-313 in blocking restenosis in the rat carotid model. These "proof of principle" studies validate the use of CDK2 as an antiproliferative target and indicate that CVT-313 is an ideal candidate worthy of evaluation in other animal models of proliferative diseases.

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