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A small molecule disruptor of Rb-Raf-1 interaction inhibits cell proliferation, angiogenesis and growth of human tumor xenografts in nude mice

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Abstract

Though it is well established that cyclin-dependent kinases phosphorylate and inactivate Rb, the Raf-1 kinase physically interacts with Rb and initiates the phosphorylation cascade early in the cell cycle. We have identified an orally-active small molecule, RRD-251 (Rb – Raf-1 Disruptor 251), that potently and selectively disrupts the Rb/Raf-1 but not Rb/E2F, Rb/Prohibitin, Rb/Cyclin E and Rb/HDAC binding. The selective inhibition of Rb/Raf-1 binding suppressed the ability of Rb to recruit Raf-1 to proliferative promoters and inhibited E2F1-dependent transcriptional activity. RRD-251 inhibited anchorage-dependent and –independent growth of human cancer cells; and knockdown of Rb with shRNA or forced expression of E2F1 rescued from RRD251-mediated growth arrest. Oral treatment of mice resulted in significant tumor growth suppression only in tumors with functional Rb; and this was accompanied by inhibition of angiogenesis, inhibition of proliferation, decreased phospho-Rb levels, and inhibition of Rb/Raf-1 but not Rb/E2F1 binding *in vivo*. Thus, selective targeting of Rb-Raf-1 interaction appears to be a promising approach for developing novel chemotherapeutic agents.

Introduction

The retinoblastoma tumor suppressor protein, Rb, is a vital regulator of the mammalian cell cycle and its inactivation facilitates S-phase entry (1, 2). Rb is inactivated through multiple waves of phosphorylation during cell cycle progression, mediated by kinases associated with D and E type cyclins in the G1 phase (3, 4). Rb is inactivated in most cancers, either by mutation or deletion of the gene, interaction with viral oncoproteins, or alterations in the levels and activity of upstream regulators of Rb function (5, 6) (7, 8). Rb controls the G1/S boundary by repressing the transcriptional activity of the E2F family of transcription factors, especially E2Fs 1, 2, and 3 (9). Many genes necessary for DNA synthesis and cell cycle progression, such as cyclins A and E, cdc2, thymidylate synthase, DHFR, ORC1 and DNA polymerase α require E2F for their expression (10-13). While cyclins and cdk phosphorylate Rb in mid to late G1 phase releasing transcriptionally active E2F (14-16), Raf-1 kinase binds and phosphorylates Rb early in the G1 phase (17). Disruption of this Rb/Raf-1 interaction by an eight amino acid peptide (corresponding to Raf-1 residues 10-18) prevented Rb phosphorylation even late in the G1 phase, suggesting that the binding of

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Raf-1 is necessary for the eventual inactivation of Rb (18). Further, the level of Rb-Raf-1 interaction was elevated in NSCLC tissue compared to adjacent normal tissue (19), suggesting that this interaction might contribute to oncogenesis. These observations suggested that disruption of the Rb/Raf-1 interaction might have anti-cancer effects and raised the possibility that small molecules that can disrupt the Rb/Raf-1 interaction might be useful as anticancer drugs. Here we report a potent and selective small-molecule disruptor of Rb/Raf-1 interaction that significantly inhibits angiogenesis and tumor growth *in vivo* in an Rb-dependent manner.

Materials and Methods

Cell culture and transfection

The human myelomonocytic leukemia cell line U937 was cultured in RPMI (Mediatech) containing 10% fetal bovine serum (FBS; Mediatech). U2-OS, Saos-2, PANC1, CAPAN2, A375, SK-MEL-5, SK-MEL-28 and MDA-MB-231 cell lines were cultured in Dulbecco modified Eagle Medium (DMEM; Mediatech) containing 10% FBS. A549 cells and A549 shRNA Rb cell lines were maintained in Ham F-12K supplemented with 10% FBS; media for shRNA cells lines contained 0.5µg/ml puromycin. ShRNA cell lines were generated by stably transfecting A549 cells with two different shRNA constructs that specifically target Rb obtained from a shRNAmir library from Open Biosystems. H1650, PC-9, LNCap and Aspc1 cell line were cultured in RPMI (Gibco) containing 10% FBS. Human aortic endothelial cells (HAECs, Clonetics) were cultured in endothelial growth medium, supplemented with 5% FBS. U251MG and U87MG glioma cells were maintained in DMEM supplemented with non-essential amino acids, 50mM β-mercaptoethanol, and 10% FBS. The adenovirus (Ad) constructs Ad-green fluorescent protein (GFP) and Ad-E2F1 were obtained from W.D. Cress. Ad-cyclin D was kindly provided by I. Cozar-Castellano.

In vitro library screening assays

ELISA 96-well plates (Nunc) were coated with 1µg/ml of GST Raf-1 (1-149aa) overnight at 4°C. Subsequently, the plates were blocked and GST Rb at 20µg/ml was rotated at RT for 30 minutes in the presence or absence of the compounds at 20µM. GST-Rb with or without compounds were then added to the plate and incubated for 90min at 37°C. The amount of Rb bound to Raf-1 was detected by Rb polyclonal antibody (Santa Cruz) 1:1000 incubated for 60 min at 37°C. Donkey-anti-rabbit-IgG-HRP (1:10,000) was added to the plate and incubated at 37°C for 60 minutes. The color was developed with orthophenylenediamine peroxidase substrate tablets (Sigma); the reaction was terminated with 3M H₂SO₄ and absorbance read at 490nm. To determine disruption of Rb to E2F1, Phb, or HDAC1 the above protocol was used with the exception of coating GST Rb on the ELISA plate and adding the drugs in the presence or absence of GST E2F1, Phb, or HDAC1. These interactions were detected with E2F1 monoclonal antibody (Santa Cruz, 1:2000 dilution); Prohibitin monoclonal antibody (NeoMarkers, 1:1000 dilution) and HDAC1 polyclonal antibody (Santa Cruz, 1:1000). For disruption of Mek-Raf-1 binding ELISAs, Raf-1 1µg/ml was coated on the plate and GST-Mek (20µg/ml) was incubated in the presence or absence of compounds for 30 minutes at room temperature. Mek1 polyclonal antibody (Cell Signaling) was used at 1:1000 to detect the binding of Raf-1 to Mek1. The IC₅₀ concentrations for the Rb-Raf-1 inhibitors were determined by plotting with Origin 7.5 software.

Lysate preparation, immunoprecipitation, and Western blotting

Cell lysates were prepared by NP-40 lysis as described earlier (18). Tumor lysates were prepared with T-Per tissue lysis buffer (Pierce) and a Fisher PowerGen 125 dounce homogenizer. Physical interaction between proteins *in vivo* was analyzed by

immunoprecipitation-western blot analyses with 200µg of lysate and 1µg of the indicated antibody (18). Polyclonal E2F1, B-Raf, ASK1, Cyclin D and E were obtained from Santa Cruz Biotechnology. Monoclonal Rb and Raf-1 were obtained from BD Transduction laboratories; and polyclonal antibodies to phospho-Rb (807,811) and Mek1/2 from Cell Signaling.

CAT assays

A549 cells were transfected by calcium phosphate and treated with drug for 24 hours. Assays for chloramphenicol acetyltransferase (CAT) and β -galactosidase were performed using standard protocols (17).

Chromatin Immunoprecipitation (ChIP) assay

A549 cells were serum starved and re-stimulated with serum for 2h or 16h in the presence or absence of RRD 251 at 20µM and ChIP lysates prepared (18). Immunoprecipitations were conducted using antibodies against E2F1, Rb, Raf-1, Brg1, HP1, and HDAC1 and the association with specific promoters detected by PCR. Rabbit anti-mouse secondary antibody was used as the control for all reactions. The sequences of the PCR primers used in the PCRs were as follows: Cdc6 promoter (forward primer), 5'-GGCCTCACAGCGACTCTAAGA-3'; and Cdc6 promoter (reverse primer), 5'-CTCGGACTCACCACAAGC-3'. *TS* promoter (forward primer), and 5'-GAC GGA GGC AGG CCA AGT G-3' *TS* promoter (reverse primer). The *cdc25A* and *c-fos* primers are described in (18).

Real-time PCR

A549 cells were rendered quiescent by serum starvation and subsequently stimulated with serum in the presence or absence of RRD-251. Unstimulated serum starved cells were used as a control. Total RNA was isolated by an RNeasy miniprep kit from QIAGEN following the manufacturer's protocol. One microgram of RNA was DNase treated using RQ1 DNase (Promega), followed by first-strand cDNA synthesis using the iScript cDNA synthesis kit (Bio-Rad). A fraction (1/20) of the final cDNA reaction volume was used in each PCR (20). Primers sequences are as follows: 5'-CTG CCA GCT GTA CCA GAG AT-3' (*TS* forward primer), 5'-ATG TGC ATC TCC CAA AGT GT-3' (*TS* reverse primer), 5'-CCC CAT GAT TGT GTT GGT AT-3' (*Cdc6* forward primer), 5'-TTC AAC AGC TGT GGC TTA CA-3' (*Cdc6* reverse primer), 5'-CTC AAC ACG GGA AAC CTC AC-3' (*18S* forward primer), and 5'-AAA TCG CTC CAC CAA CTA AGA A-3' (*18S* reverse primer). Real-time PCR was performed on an Bio-Rad iCycler.

In vitro kinase assay

The kinase reaction for Raf-1 was carried out with 100ng of Raf-1 (Upstate Signaling), 0.µg of MEK1 (Upstate) as the substrate, 10µM ATP, 10µCi of [γ -³²P] ATP in the kinase assay buffer in the presence or absence of the drugs at 30°C for 30 minutes. 1µM of BAY-43-9006 was used as a control and 20µM RRD-251 was used. Cyclin D and E kinase assays are described in (18).

Proliferation assays

Bromodeoxyuridine (BrdU) labeling kits were obtained from Roche Biochemicals. Cells were plated in poly-D-lysine coated chamber slides at a density of 10,000 cells per well and serum starved for 24 hours. Cells were then stimulated with serum in the presence or absence of the indicated drugs for 18h. S-phase cells were visualized by microscopy and quantitated by counting 3 fields of 100 in quadruplicate. For adenovirus experiments, A549 cells were serum starved for 48 hours and subsequently infected with adenovirus in the

presence or absence of RRD-251 for 36 hours. Ad-GFP (6×10^6 pfu/ μ l), Ad-E2F1 (6×10^6 pfu/ μ l), and Ad-Cyclin D (7×10^8 pfu/ μ l) were added at an M.O.I of 150 particles per cell.

Soft Agar colony formation assay

Assays were done in triplicate in 12-well plates (Corning). After allowing the bottom layer of agar (0.6%) to solidify at room temperature, the top layer of agar was (0.3%) was mixed with 5,000 cells per well and the indicated drug and added. Drugs were added twice weekly in complete media to the agar wells. Colonies were quantified by staining with MTT 1mg/ml for 1 hour at 37°C.

Matrigel Assays

Matrigel (Collaborative Biomedical Products) was used to promote the differentiation of HUVECs into capillary tube-like structures (18). A total of 100 μ l of thawed Matrigel was added to 96-well tissue culture plates, followed by incubation at 37°C for 60 minutes to allow polymerization. Subsequently, 1×10^4 HUVECs were seeded on the gels in EGM medium supplemented with 5% FBS in the presence or absence of 20 μ M concentrations of the indicated compounds, followed by incubation for 24 hours at 37°C. Capillary tube formation was assessed using a Leica DMIL phase contrast microscope.

Ex-vivo Rat Aorta Ring Angiogenesis assays

Forty-eight well tissue culture plates were coated with 200 μ l of Matrigel and allowed to polymerize for 1 hour at 37°C. Thoracic aorta was excised from 8-10 week old male Sprague-Dawley Rats (250-300g)(21). After removing fibroadipose tissue, aortas were rinsed several times with EGM-2 (Clonetics), sectioned into 1mm rings and placed on the matrigel-coated wells. The rings were covered with an additional 200 μ l of Matrigel and allowed to polymerize. The rings were cultured in EGM-2 media in the presence or absence of 20 μ M of RRD-251. The media and drug were supplemented twice a week for one week. The Aortic rings were photographed on day 7 using a Leica phase contrast microscope. Quantitation of microvessel growth was done using Image Pro Plus (v.6.0) software and values are reported as microvessel area.

In vivo Matrigel Plug Angiogenesis assays

In vivo matrigel plug assays were carried as previously described (22). Cooled liquid matrigel (300 μ l) was injected subcutaneously into both flanks of nude mice. One group of mice received the vehicle and the second group received RRD-251 50 MPK daily by i.p. injection. At 7 days post matrigel injection, the mice were injected with 100 μ l of 100 MPK FITC-Dextran (Sigma) through the tail vein. 30 minutes later, the mice were euthanized and the matrigel plugs were removed and fixed in buffered formalin. Samples were viewed with a Leica DMI6000 inverted microscope, TCS SP5 confocal scanner, and a 20X/0.7NA Plan Apochromat objective (Leica Microsystems, Germany). An Argon 488 laser line was applied to excite the samples and tunable filters were used to minimize background fluorescence. Image sections at 2.0 μ m were captured with photomultiplier detectors 3D projections were prepared with the LAS AF software version 1.6.0 build 1016 (Leica Microsystems, Germany). Quantification of intensity and angiogenesis was performed using Image Pro Plus 6.2 (Media Cybernetics, Inc., Maryland). Average intensity per pixel is plotted as percent angiogenesis in each image, (n=12). Each image is representative of areas of vessel formation throughout entire matrigel plug. After confocal imaging, samples were paraffin blocked and stained with H&E. H&E images display ¼ of the matrigel plug.

Animal Studies

A549 or H1650 cells were harvested and resuspended in PBS, and implanted subcutaneously (s.c.) into the right and left flanks (10×10^6 cells per flank) of 8-week old female athymic nude mice (Charles River, Wilmington, MA, USA) as described (18, 23). When tumors reached about 100-200mm³, animals were dosed intraperitoneally (i.p.) or orally by gavage with 0.1ml of the drug or the vehicle daily. Tumor volumes were determined by measuring the length (*l*) and the width (*w*) and calculating the volume ($V = lw^2/2$). Statistical significance between control and treated animals were evaluated using Student's *t*-test.

Immunohistochemistry staining

Tumors were fixed in 10% neutral-buffered formalin before processing into paraffin blocks. 5µm thick paraffin sections were rehydrated to PBS and processed using the following protocols. Sections were rinsed in dH₂O, and then subjected to microwave 'antigen retrieval' in 0.01 M sodium citrate, pH 6.0. Sections were cooled, rinsed 3 times in dH₂O, twice in PBS and incubated in 5% normal goat serum. Sections were incubated in primary antibody in 5% normal goat serum, rinsed 3 times in PBS. For color development, slides were treated with ABC kit from Vector labs rinsed in dH₂O, and developed with DAB. Then, sections were lightly counterstained in hematoxylin, dehydrated, cleared and coverslipped. Tissue sections were stained with hematoxylin and eosin (H&E) using standard histological techniques. Tissue sections were immunostained using Ki-67, CD31 phospho-Rb and CD31 antibodies (BD Biosciences, San Diego, CA, USA) using the avidin-biotin peroxidase complex technique. Mouse monoclonal antibody was used at 1: 50 dilution following microwave antigen retrieval (four cycles of 5 min each on high in 0.1 M citrate buffer). Stained slides were scanned on an Ariol SL-50 Automatic Scanning System and whole tumor sections were quantitated using Image Pro Plus (v.5.1.0) software.

Statistical Analysis

Statistical analysis was performed using one tailed Students *t*-test. Values were considered significant when the *P* value <0.01.

Results

Identification of Rb-Raf-1 disruptor, RRD-251

An ELISA was used to identify compounds that could inhibit the binding of GST-Rb to GST-Raf-1. Screening of the NCI diversity library of 1,981 compounds identified two compounds, NSC-35400 and NSC-35950, which inhibited Rb-Raf-1 interaction 100% and 95% respectively at 20µM concentration. NSC-35400 and NSC-35950 each contained a benzyl-isothioureia derivative and a phenyl-based counter ion (Fig. 1A); to establish whether the benzylisothioureia derivative is the active component, we synthesized RRD-251 (Fig. 1A), which contains chloride as the counter ion. ELISA analysis showed that NSC-35400 disrupted the Rb-Raf-1 interaction with an IC₅₀ of 81 ± 4 nM, NSC-35950 with an IC₅₀ of 283 ± 46 nM and RRD-251 with an IC₅₀ of 77 ± 3.6 nM (Fig. 1B), suggesting that the benzylisothiouronium pharmacophore disrupts the Rb-Raf-1 interaction. ELISAs showed that these disruptors were highly selective for Rb/Raf-1 interaction over Rb/E2F1, Rb/HDAC1, Rb/prohibitin (Fig. 1C) and Raf-1/Mek (Fig. 1D) associations at a concentration of 20µM. Their selectivity in living cells was examined by immunoprecipitation-western blot analysis. A549 cells were serum starved for 48 hours and subsequently serum stimulated for 2 hours in the presence or absence of 20µM of NSC-35400, NSC-35950, and RRD-251; Raf-1 peptide conjugated to penetratin (18) was used as a positive control and a Raf-1 scrambled peptide was used as a negative control. It was found that the compounds inhibited the binding of Raf-1 to Rb (Fig. 2A), while the binding of Rb to E2F1 was not affected. To

further confirm the selectivity of RRD-251, cyclin E was immunoprecipitated from lysates of quiescent cells or those serum stimulated for 8 hours in the presence or absence of RRD-251; western blotting of the immunoprecipitates showed that RRD-251 did not inhibit the binding of Rb to Cyclin E (Fig. 2B). A similar experiment was done on lysates from cells that were serum stimulated for 2 hours; RRD-251 did not inhibit the binding of B-Raf to Rb (Fig. 2C). Similarly, the binding of Raf-1 to Mek1/2 was not affected by RRD-251 (Fig. 2D). Examination of lysates from cells serum stimulated for 2 hours in the presence of RRD-251 showed a reduction in Rb phosphorylation, as seen by western blotting (Fig. 2C, row 5). Interestingly, *in vitro* kinase assays showed that RRD-251 did not affect the kinase activities associated with cyclin D (Fig. 3A, left panel) or cyclin E (Fig. 3A, middle panel) or Raf-1 (Fig. 3A, right panel). These results suggest that the reduction in Rb phosphorylation in cells treated with RRD-251 is due to a disruption of the association of Raf-1 with Rb and that Raf-1 has to physically interact with Rb to inactivate it.

RRD-251 inhibits E2F transcriptional activity

We next reasoned that if the disruption of the Rb/Raf-1 binding has functional consequences on cellular physiology, then RRD-251 should affect the transcriptional activity of E2F1. To examine this, transient transfection experiments were done in control A549 cells as well as A549 cells stably expressing two different shRNA constructs (sh6 and sh8) targeting Rb; these A549 cells had significantly less Rb protein compared to parental A549 cells (Supplementary Fig. 1A). Transfection of E2F1 induced the expression of an E2-CAT reporter; treatment of the transfected cells with RRD-251 repressed E2F1-mediated transcription in a dose dependent manner in wild type A549 cells but not in the A549 cells lacking Rb (Fig. 3B); this suggests that the presence of Rb is necessary for RRD-251 to function. ChIP analysis of the transfected E2 promoter revealed a reduction in the amount of Raf-1 on the promoter with increasing doses of RRD-251 (Supplementary Fig. 1B). We had reported that Raf-1 can be detected on proliferative promoters upon serum stimulation and these results indicate that RRD-251 probably affects E2F-mediated transcription by dissociating Raf-1 from the promoters. The effect of RRD-251 on the expression of two endogenous E2F-regulated proliferative promoters was next examined. A549 cells were serum starved for 72 hours and serum stimulated for 24h in the presence or absence of RRD-251 and the level of thymidylate synthase (*TS*) gene expression was assessed by Real-time PCR. Inhibition of the Rb-Raf-1 interaction led to the silencing of the *TS* promoter (Fig. 3C); similar results were obtained on the *cdc6* gene (Supplementary Fig. 1C). We had reported that the binding of Raf-1 to Rb resulted in the dissociation of the co-repressor Brg-1 from E2F-responsive proliferative promoters (18); ChIP assays were carried out to examine whether RRD-251 affects this process. It was found that the association of Raf-1 to the above promoters upon serum-stimulation was disrupted by pre-treatment of cells with RRD-251 (Fig. 3D). Furthermore, dissociation of the co-repressor Brg-1 from these promoters was also inhibited by RRD-251. This suggests that RRD-251 can modulate the transcriptional regulatory functions of Rb by modulating its phosphorylation status and affecting its interaction with chromatin remodeling proteins like Brg-1. The association of E2F1, HDAC1 and HP1 with these promoters was not affected by RRD-251, as seen by ChIP assays (Fig. 3D).

Inhibition of proliferation by RRD-251 is dependent on Rb status

Given the ability of RRD-251 to inhibit Rb phosphorylation and repress E2F transcriptional activity, it was examined if it could inhibit cell proliferation and whether such an inhibition required a functional Rb gene. 20 μ M RRD-251 was effective at inhibiting serum-induced S-phase entry in parental A549 cells but had no effect on cells stably expressing sh6 and sh8, which lacked Rb (Fig. 4A). We next tested RRD-251 on cancer cell lines that carried natural mutations or deletion of Rb. Proliferation of Saos-2 osteosarcoma cells that have a loss of

Rb (24) was not affected by RRD-251 while the U2-OS osteosarcoma cells carrying wild type Rb were arrested (Fig. 4A). RRD-251 was unable to inhibit proliferation in the Rb mutant DU145 prostate cancer cells, yet could inhibit 60% of S-phase cells in PC3 cells (wt Rb) (Fig. 4A). 20 μ M RRD-251 did not inhibit proliferation of lung cancer cell lines H596 and H2172, both of which harbor mutations in Rb, yet treatment of H1650 and H1299 (wt Rb) with RRD-251 inhibited proliferation by 90% and 70% respectively (Fig. 4A). It was next examined whether RRD-251 could inhibit the proliferation of cells that have mutations in the signaling pathways that regulate Rb function, rather than in the Rb gene itself. RRD-251 could inhibit S-phase entry by 50 – 65% in pancreatic cancer cell lines Aspc1, PANC1, and CAPAN2 that harbor a non-functional *p16INK4a* gene (25) (Fig. 4B). RRD-251 also inhibited S-phase entry of glioblastoma cell lines U87MG and U251MG, both of which are null for *p16* and *PTEN* (26). The metastatic human breast cancer cell line MDA-MB-231 harbors a *K-Ras* mutation and overexpresses EGFR (27); 20 μ M RRD-251 inhibited its proliferation by 56% (Fig. 4B). The A375 melanoma cell line harbors the V600E B-Raf mutation (28) and RRD-251 inhibited S-phase entry by 58%. Prostate cell lines LNCaP and PC3 both contain mutations in *K-Ras* and *PTEN* genes (29), and RRD-251 inhibited proliferation 86% and 35% respectively (Fig. 4B). These results indicate that disruption of Rb-Raf-1 interaction could inhibit the proliferation of cell lines harboring a wide array of mutations in upstream signaling molecules, as long as Rb itself is not altered at the genetic level. Experiments were also carried out to examine the effect of RRD-251 in suppressing the adherence-independent growth of cells in soft agar; it was found that RRD-251 could significantly inhibit the growth of A549, H1650, and SK-MEL-5, SK-MEL-28, and PANC1 cells in soft agar (Fig. 4C).

We next reasoned that if RRD-251 targets selectively the Rb-Raf-1 interaction, the forced expression of a downstream target of Rb such as E2F1, but not of the upstream regulator cyclin D, would rescue the anti-proliferative effects of RRD-251. To this end, A549 cells were serum starved for 48 hours and infected with Ad-E2F1 or Ad-cyclin D, in the presence or absence of 20 μ M of RRD-251 for 36h. Ad-GFP infected cells were used as a control. BrdU incorporation assays showed that ectopic expression of E2F1 efficiently overcame the anti-proliferative activity of RRD-251, whereas over-expression of cyclin D had only a partial effect (Fig. 4D) showing that the growth inhibition by RRD-251 occurs at the level of Rb and its downstream target, E2F1, can rescue the growth suppression.

RRD-251 inhibits angiogenesis in vitro and in vivo

Raf-1 kinase plays a role in facilitating angiogenesis (30, 31) and it has been suggested that Raf-1-mediated inactivation of Rb is involved in the process (18). To examine whether angiogenic tubule formation could be inhibited by RRD-251, Human umbilical vein endothelial cells (HUVECs) were grown in matrigel in the presence or absence of 20 μ M RRD-251; RRD-251 significantly inhibited the angiogenic tubule formation (Fig. 5A). These results were confirmed in an *ex-vivo* experiment using rat aortic rings. As shown in Figure 5B, 20 μ M RRD-251 was able to inhibit angiogenic sprouting from rat aortic rings grown in matrigel for 7 days. Quantitation of vessel area showed a significant reduction in angiogenesis ($p=0.000007$, Supplementary Fig. 2A). We next examined whether RRD-251 could inhibit angiogenesis in matrigel plugs *in vivo* (22). Aythmic nude mice were injected with cold matrigel in both flanks. Mice were administered either vehicle or RRD-251 50 mg/kg body weight by intraperitoneal injection daily for one week. Mice were injected with 100 MPK FITC-Dextran via the tail vein prior to euthanasia and matrigel plugs were fixed in formalin. Angiogenesis in the entire plugs were assessed by confocal imaging. FITC images showed growth of angiogenic tubules in plugs from mice that received vehicle; in contrast, there was a remarkable inhibition of angiogenic vessel formation in the matrigel plugs from mice treated with RRD-251 (Fig. 5C). Quantitation of vessel intensity plotted as relative

angiogenesis per image shows significant inhibition, $p=0.0004$ ($n=12$, Supplementary Fig. 2B). Further examination of the matrigel plugs by H&E staining showed a complete inhibition of cells migrating into the matrigel for vessel formation (Fig. 5D). It was also examined if RRD-251 could inhibit VEGF levels in cell culture. A549 cells treated with RRD-251 for 24 hours displayed decreased VEGF levels compared to control (Supplementary Fig. 2C). Further, treatment with RRD-251 in the presence of VEGF for 12 hours could prevent Rb phosphorylation in HAEC cells (Supplementary Fig. 2D).

Antitumor activity of RRD-251

Given the ability of RRD-251 to inhibit cell proliferation, adherence-independent growth and angiogenesis, we examined whether RRD-251 could inhibit tumor growth *in vivo* in nude mouse xenograft models. Athymic nude mice were implanted s.c. with 1×10^7 A549 cells bilaterally and the tumors were allowed to reach 200 mm^3 in size before oral or i.p. administration of RRD-251 or vehicle (18) (23). Tumors from vehicle treated mice grew to an average size of $1040 \pm 128 \text{ mm}^3$; in contrast, tumors in mice treated with RRD-251 did not grow and even regressed slightly (50 MPK-i.p.: $145 \pm 20 \text{ mm}^3$; 150 MPK-oral $148 \pm 32 \text{ mm}^3$) (Fig. 6A, left). Oral dose response experiments were carried out on A549 xenografts, which resulted in RRD-251 100 MPK and 150 MPK completely inhibiting tumor growth (Figure 6A, middle). Tumors from vehicle treated mice reached an average size of $996 \pm 180 \text{ mm}^3$; contrast, tumors in mice treated with RRD-251 (oral) responded in a dose dependent manner. Complete inhibition was seen in 100 MPK-oral: $293 \pm 44 \text{ mm}^3$ and 150 MPK-oral: $237 \pm 67 \text{ mm}^3$ (Fig. 6A, middle). Similar results were observed with H1650 xenograft tumors; RRD-251 inhibited tumor growth significantly ($2185 \pm 326 \text{ mm}^3$ in vehicle treated animals compared to $557 \pm 76 \text{ mm}^3$ in RRD-251 (50 MPK-i.p.) treated animals) (Fig. 6A, right).

Tumors were harvested at the end of the treatment and analyzed by immunohistochemical staining with hematoxylin and eosin, and antibodies to Ki-67, phospho-Rb (807,811), and CD-31. A significant inhibition of proliferation was observed in tumors from RRD-251 treated animals, as seen by Ki-67 staining (Fig. 6B); phosphorylation of Rb was also reduced as seen by staining with an antibody to phospho-Rb (Fig. 6B). Tumors also showed a reduction in microvasculature, as seen by CD31 staining (Fig. 6B). Quantitation of Ki-67 staining, phospho-Rb staining and CD31 staining is shown in supplementary Fig. 3A-C. To assess whether RRD-251 affected its target, tumors lysates were prepared from vehicle treated and RRD-251 treated mice and Rb-Raf-1 interaction assessed by IP-western blots. RRD-251 treatment had led to a reduction in Rb-Raf-1, but not Rb/E2F1 interaction in tumor xenografts (Fig. 6C).

Tumor Growth Inhibition by RRD-251 is Rb-dependent

Since RRD-251 did not inhibit the proliferation of A549 cells lacking Rb *in vitro*, experiments were done to assess whether tumors generated from these cells can respond to RRD-251 *in vivo*. An experiment as in Figure 5A was carried out on nude mice carrying tumors from A549 cells stably expressing shRNAs for Rb (sh6 and sh8). Interestingly, these tumors did not respond to RRD-251 and continued to grow at the rate of the vehicle treated tumors (Fig. 6D left and right panels). To examine whether the sh6 and sh8 tumors maintained downregulation of Rb, lysates were made from the sh6 and sh8 tumors at the end of the experiment and a western blot was done for Rb. It was found that these tumors lacked Rb, further confirming that RRD-251 specifically targets the Rb-Raf-1 protein interaction to inhibit cell proliferation and tumor growth (Supplementary Fig. 3D).

Discussion

The Ras/Raf/Mek/MAPK cascade is a proliferative pathway induced by a wide array of growth factors and is activated in many human tumors (32-34) and is an attractive target for the development of anti-cancer drugs (35, 36) (30, 31). Raf-1 kinase itself has been targeted for cancer therapy and two clinical attempts have been made to inhibit Raf-1 activity in patients (37) (38, 39). It has been shown that signaling events involving the MAP kinase cascade do not proceed in a linear fashion and instead they have substrates outside the cascade (17, 40, 41). In this context, the Rb protein appears to be a cellular target of the Raf-1 kinase outside the MAP kinase cascade. In addition, Rb-Raf-1 binding was elevated in tumor compared to adjacent normal controls (19), suggesting that Raf-1 mediated inactivation of Rb might contribute to oncogenesis. While it is established that Rb gene itself is mutated in cancers like retinoblastoma, osteosarcoma and small-cell lung carcinoma, majority of tumors harbor mutations in the upstream regulators of Rb function (7, 8). These include genes like Ras, PTEN, p16INK4 as well as receptor tyrosine kinases (42-44). Our results show that the disruption of the Rb-Raf-1 interaction can be fruitfully utilized to inhibit the proliferation of cells harboring such mutations in the Rb regulatory pathway. Thus we believe that these molecules have the potential to target a wide variety of human cancers.

While inhibitors of cell proliferation, DNA damaging agents as well as microtubule disruptors have widely been used as anticancer agents, developments in the past decade have demonstrated that targeting angiogenesis is also an effective way of combating tumor growth (45). In this context, our results show that RRD-251 can not only inhibit cell proliferation, but also inhibit neoangiogenesis *in vitro* and *in vivo*. Given the published reports that Raf-1 kinase contributes to angiogenesis and that VEGF can induce Rb phosphorylation, it is likely that RRD-251 is inhibiting angiogenesis by affecting these molecules (31, 46). The ability of RRD-251 to inhibit both cell proliferation as well as angiogenesis might be acting in a two-pronged manner to inhibit the growth of tumors *in vivo*; as can be imagined, these are desirable features in anti-cancer drugs.

While it has been difficult to generate small molecule inhibitors of protein-protein interactions that are clinically active (39), recent success in disrupting the hdm2-p53 (47) interaction shows that this is a viable strategy to develop novel anti-cancer drugs. Identification of RRD-251 as a cell-permeable, orally available, and highly selective inhibitor of the Rb-Raf-1 interaction is an example of the practicality of targeting protein-protein interaction for cancer therapy. Although we find that RRD-251 inhibits Rb-Raf-1 *in vitro* at nM concentrations in an *in vitro* ELISA assay, higher concentrations are needed to inhibit cell proliferation as well as growth of cells in soft agar; this finding is similar to what has been observed with other anti-cancer drugs such as BAY-43-9006, R547, and Iressa (48-50). At the same time, our *in vivo* studies show that concentrations can be achieved *in vivo* where RRD-251 has a significant therapeutic benefit.

The finding that RRD-251 is effective in inhibiting the proliferation of cells harboring a wide variety of mutations in signaling cascades that inactivate Rb, but does not affect cells carrying mutated Rb or no Rb, shows the specificity of this agent. Rb protein has been reported to interact with about one hundred proteins in the cell; it can be imagined that small molecules that can maintain the tumor suppressor functions of Rb by disrupting its physical interaction with other proteins would be a fruitful avenue to develop novel anti-cancer drugs.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1.

Identification of highly selective Rb-Raf-1 inhibitors. (A) Compounds identified in the NCI diversity set that showed the highest inhibition of Rb-Raf-1 by ELISA. Highest scoring compounds NSC-35400 and NSC-35950 are both benzyl isothiourea derivatives. RRD-251 was synthesized to determine activity based on isothiourea structure. (B) NSC35400, NSC35950 and RRD-251 disrupt the Rb-Raf-1 interaction with high potency. IC₅₀ values (81nM, 283nM and 77nM, respectively) were determined using ELISA. (C) Rb-Raf-1 inhibitors at 20μM concentration do not inhibit other binding partners to Rb (E2F1, prohibitin and HDAC1) and to Raf-1 (Mek).

Figure 2.

RRD-251 is selective for Rb-Raf-1 interaction *in vivo*. (A) Serum-stimulated binding of Raf-1 to Rb is inhibited by Rb-Raf-1 disruptors as well as a Raf-1 peptide conjugated to penetratin, the drugs do not inhibit the binding of E2F1 to Rb. Specificity of the disruption was assessed by IP-western blots (B) RRD-251 does not inhibit Rb-Cyclin E interaction in cell serum-stimulated for 8 hours. (C) RRD-251 does not disrupt the Rb-B-Raf binding in IP-Western Blots. (D) Treatment of cells with RRD-251 for 5 minutes in the presence of serum does not affect the binding of MEK1/2 to Raf-1.

Figure 3.

RRD-251 does not inhibit kinase activity (A, left) RRD-251 does not inhibit cyclin D kinase activity in *in vitro* kinase assays. (A, middle) RRD-251 does not inhibit cyclin E kinase activity in *in vitro* kinase assays. (A, right) RRD-251 treatment does not inhibit Raf-1 kinase activity in *in vitro* kinase assays; BAY-43-9006 was used as a control. (B) RRD-251 inhibits E2F1 mediated E2CAT transcription in CAT reporter assays. (C) RRD-251 inhibits *TS* gene expression in real time PCR experiments. (D) ChIP assays show that Brg1, not Raf-1 is present on quiescent A549 *cdc6*, *cdc25A* and *TS* promoters. Upon serum stimulation, Brg1 is dissociated from both promoters, correlating with Raf-1 binding. Serum stimulation in the presence of RRD-251 causes the dissociation of Raf-1 and retention of Brg1 on E2F1 responsive promoters. Serum stimulation for 16 hours causes dissociation of Rb, Raf-1, HP1, Brg1, and HDAC1 from the promoters. An irrelevant antibody was used as a control for immunoprecipitations; *c-fos* promoter was used as a negative control.

Figure 4.

RRD-251 inhibits S-phase entry dependent on Rb status. (A) BrdU incorporation assay showing that 20μM of RRD-251 does not inhibit the proliferation of A549 cells over-expressing shRNA constructs to Rb, but arrests wild-type A549 cells and a non-homologous control shRNA. RRD-251 also does not inhibit S-phase entry in cancer cell lines that contain mutant Rb. (B) BrdU incorporation assays showing the growth arrest mediated by RRD-251 in a variety of tumor cell lines harboring various mutations. RRD-251 could effectively arrest cells with mutations in EGFR, p16, PTEN, K-Ras, and p53 but not Rb. (C) RRD-251 inhibits adherence-independent growth of several cell lines in soft agar. (D) Over expression of E2F1 was sufficient to overcome cell cycle arrest mediated by RRD-251, while cyclin D over expression had only a partial effect.

Figure 5. RRD-251 inhibits angiogenesis *in vitro* and *in vivo*. (A) RRD-251 inhibits Human Umbilical Vein Endothelial cell angiogenic tubule formation in matrigel. (B) RRD-251 inhibits angiogenesis in a rat aorta matrigel model. (C) Confocal FITC images of matrigel plugs from nude mice treated with Vehicle or RRD-251 50 MPK daily for one week. (D) H&E staining of matrigel plugs from nude mice treated with Vehicle or RRD-251 50 MPK. H&E images display 1/4 of matrigel plug.

Figure 6.

Intraperitoneal (i.p.) and oral administration of RRD-251 inhibits human tumor growth in nude mice. (A, left) A549 cells xenotransplanted bilaterally into the flanks of athymic nude mice were allowed to grow for 14 days until tumor volume reached 200mm³; daily administration of RRD-251 at 50 MPK-i.p. and 150 MPK-oral completely inhibited tumor growth. (A, middle) Dose response of RRD-251 administered by oral gavage, 100mpk and 150 MPK could completely inhibit tumor growth. (A, right) RRD-251 inhibited H1650 xenograft tumor growth in nude mice. (B) Immunohistochemical staining of tumors from mice treated with RRD-251. Tumors were stained with Ki-67 for proliferation, pRb for cell cycle, and CD31 for angiogenesis. (C) Both doses of RRD-251 inhibit the Rb-Raf-1 interaction in tumor lysates without inhibiting Rb-E2F1 interaction, as seen by IP-Western blots. (D, left and right) Inhibition of tumor growth is dependent on a functional Rb protein. A549-sh6 and A549-sh8 cells were implanted into the flanks of nude mice. RRD-251 was unable to inhibit tumor growth in tumors lacking Rb protein.