Selective Cyclin-dependent Kinase 2/Cyclin A Antagonists that Differ from ATP Site Inhibitors Block Tumor Growth

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ABSTRACT

A central function of the tumor suppressor retinoblastoma (Rb) is its ability to repress E2F transcriptional activity. Many cancers harbor inactivated Rb and consequently deregulated E2F. RXL peptides inhibit E2F recruitment and phosphorylation by CDK2/cyclin A. Here we report that RXL peptides selectively kill tumor cells with deregulated Rb/cyclin D pathways. We extend these observations to tumor models and demonstrate inhibition of tumor growth in SV40 large T transformed Balb/c 3T3 grafts and in HER2 transgenic tumors. Moreover, our observations reveal that RXL peptide-treated tumors undergo apoptosis. Our results indicate that RXL motif-based inhibitors will provide selective antiproliferative agents with in vivo efficacy in tumors with deregulated Rb/cyclin D pathways.

INTRODUCTION

Functional inactivation of the tumor suppressor Rb is commonly observed in a variety of cancers (1–4). Mitogen stimulation results in CDK4/cyclin D phosphorylation of Rb and derepression of the transcription factor E2F (5). The transcriptionally active E2F drives gene expression necessary for progression through S phase (5, 6). CDK2/cyclin A phosphorylates the E2F transcription complex and inhibits its DNA-binding activity (7–10), which is mediated by a conserved cyclin A-binding motif (described as Cy or RXL motif) in E2F (10–12). Inactivation of Rb through mutagenesis and deregulation of E2F subverts normal cell cycle progression and engages apoptosis (5, 6, 10, 13–15). Chen et al. (14) have suggested that E2F deregulation and CDK2/cyclin A inactivation are synthetically lethal. Peptides derived from the cyclin A-binding motif were shown to inhibit substrate phosphorylation by CDK2/cyclin A (11, 12, 14, 16) and selectively kill E2F-expressing and -transformed cell lines (14). We tested these peptides in cells with altered cyclin D/Rb pathways. SVT2 cells (17, 18) that harbor functionally inactivated Rb were selectively killed by RXL peptides in comparison with parental BALB/c 3T3. Because cyclin D1 is deregulated by oncogenic activation of Neu/HER2 in mouse mammary tumors (19), we examined the effect of RXL peptides on cell lines derived from these HER2 transgenic tumors and report cell killing. These analyses were further extended to in vivo models, and we report here the RXL peptide-mediated inhibition of tumor growth in SVT2 grafts and HER2 transgenic models. Our in vivo results indicate that RXL peptides target tumor growth driven by deregulation of the Rb/cyclin D pathway with significant ramifications for development of anticancer therapeutics.

MATERIALS AND METHODS

Pep tide Synthesis. Peptides were prepared on a Perceptive Pioneer synthesizer (0.25 mmol, Polyethyleneglycol-polysyrene Rapp resin) using standard Fmoc chemistry protocols (PyBOP/HOBt/DIPEA) and purified via C18 high-performance liquid chromatography with a linear acetonitrile gradient (water/0.1% Trifluoro acetic acid). Products were analyzed by liquid chromatography mass spectrometry (API 150EX; Applied Biosystems, Foster City, CA). The peptides PVKRRFLG, PVKRRRDL, PVKRRRFL (RXL), and ETIQKFLFESS (CONTROL) were synthesized alone or as fusions to the antennapedia sequence PEN (RQIKIWFQNRRMKWKK; Ref. 14).

Cell Culture and Viability Assays. MDA-MB-435, SVT2, BALB/c 3T3, Ratll (American Type Culture Collection, Manassas, VA), and wild-type early passage MEFs were cultured in DMEM containing 10% FCS. U2OS cells were cultured in McCoy’s 5A with 10% fetal bovine serum. The HER2 cells derived from MMTV-HER2 transgenic mouse mammary tumors were cultured on fibronectin-coated plates (F-1141; Sigma, St. Louis, MO) in DMEM/F-12 media containing 2% FCS, insulin, transferrin, heregulin β1 (Genentech, San Francisco, CA), vitamin E acetate (24,817-7; Aldrich, Milwaukee, WI), hydrocortisone (H-0888), progesterone (P-6149), ethanolamine (E-6133), T3 (3,3’,5-Triiodo-l-thyronine sodium salt; T-5516; Sigma), and mouse EGF (35-4001; B.D. Biosciences, Bedford, MA).

All cell lines were seeded in 96-well dishes at 1 × 10^5–1.5 × 10^5 cells/well. After an overnight incubation cells were rinsed with PBS and over laid with 100 μl of Opti-MEM (31985–062; Invitrogen, Carlsbad, CA). Peptides diluted in Opti-MEM were added in duplicate to cells for a final concentration of 2.5–80 μM and incubated for 18 h (48 h for the HER2 transgenic tumor cells) at 37°C/5% CO2. Cell viability was detected using the MTT assay (1465007; Roche Molecular Biochemicals, Indianapolis, IN), according to the manufacturer’s protocol. MTT assays were carried out in duplicate in at least three independent experiments. Cell viability was calculated as the percentage of treated to untreated controls.

CDK2/Cyclin A in Vitro Kinase Assays. U2OS lystate immunoprecipitated with anti-cyclin A (SC-239; Santa Cruz Biotechnology, Santa Cruz, CA) or 25 ng of recombinant CDK2/cyclin A complex were incubated for 20 min at 30°C in a kinase reaction as described previously (14). RXL or CONTROL peptides ranging from 0.1 to 33 or 33 μM Purvalanol A (540500; Calbiochem, San Diego, CA) were included in the reactions. MBP-Rb (701–928) fusion protein at 500 ng (6022S; Cell Signaling Technology, Beverly, MA) and 1 μg of Histone H1 (223549; Roche Molecular Biochemicals) were added as substrate. Kinase reactions were resolved by SDS-PAGE and transferred to nitrocellulose membranes. 32P uptake by substrate was detected by exposure to BioMax-MR film (895-2855; Kodak, Rochester, NY). Western blots for cyclin A (SC-751; Santa Cruz Biotechnology) were carried out as before (14).

Mouse Tumor Studies. For tumor studies, 0.5 million SVT2 cells were injected into 8-week-old female BALB/c nude mice (Charles River Labs, Wilmington, DE). Once tumors reached ~100 mm3, mice were randomly assigned to groups (n = 5) and injected intra and peritumorally with 0.4, 0.1, and 0.04 mg of PEN-PVKRRLDL or 0.4 mg of PEN-CONTROL peptide in a 100-μl volume of DMSO/PBS daily for 5 days. In the second study, mammary tumors derived from HER2 transgenic mice were transplanted into 8-week-old syngeneic FVB mice. When tumors were ~100 mm3, mice were randomly grouped (n = 5) and injected with 1 and 0.4 mg of PEN-PVKRRFLG or 1 mg of PEN-CONTROL peptide, as before. Tumor volume was measured every 2 days and calculated using the equation π/6 × length × breadth2. Two mice in the 1-mg PEN-PVKRLFLG dose group did not appear healthy after the first dose and were removed from the study. All tumors were harvested and fixed for histopathology evaluation after the final dose. In a separate experiment, SVT2 and HER2 transgenic tumor-bearing mice (n = 5) were injected intra tumoral with 1 mg of PEN-PVKRRFLG or PEN-CONTROL peptide. Tumors were harvested 6 h postinjection and fixed for histopathology evaluation.

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2 The abbreviations used are: Rb, retinoblastoma; CDK, cyclin-dependent kinase; MTT, 3-(4,5-dimethylthiazol-2-y)-2,5-diphenyltetrazolium bromide; MEF, mouse embryonic fibroblast; SVT2, SV40 large T transformed Balb/c 3T3; MBP, maltose-binding protein; TUNEL, terminal deoxynucleotidyl transferase-mediated nick end labeling; PEN, penetratin; MMTV, mouse mammary tumor virus.

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Animal protocols were approved by the Genentech Institutional Animal Care and Use Committee.

Histology and Assessment of Apoptotic Activity in Peptide-treated Tumors. Tumors were harvested 5 days or 6 h after peptide treatment and fixed in neutral-buffered formalin overnight. Sections were stained with H&E for histology evaluation. Apoptotic activity in the tumor sections was determined using TUNEL Assay (Oncor, Gaithersburg, MD) or an immunohistochemical stain for cleaved caspase-3 (Cell Signaling Technology), according to the manufacturer’s protocol, with simultaneous methylene green staining to visualize tumor cells.

RESULTS

The RXL peptides tested were fused to an antennapedia sequence, PEN, which facilitates entry of peptides into cells. To confirm previous observations (14) and analyze our synthesized peptides, PEN-PVKRRLDL was tested in an in vitro kinase assay. Cyclin A immunoprecipitates of U2OS cell lysate were subjected to a kinase reaction with 0.1, 1, and 10 μM PEN-PVKRRLDL peptide in the presence of γ-32P-ATP. The reactions were analyzed by detecting phosphorylation of an RXL motif containing substrate (Rb-MBP fusion protein). PEN-PVKRRLDL peptide inhibition of cyclin A-associated kinase activity was observed in a dose-dependent manner, whereas PEN-CONTROL or the absence of peptide did not affect kinase activity (Fig. 1a).

In addition, PEN-PVKRRLDL and PEN-PVKRRLFG peptides were tested for the ability to block phosphorylation of substrate that does not contain an RXL motif. As anticipated, RXL peptides did not block Histone H1 phosphorylation, although Rb phosphorylation was inhibited. In contrast, Purvalanol A (20), a CDK2 selective ATP analogue, inhibited phosphorylation of Histone H1 and Rb (Fig. 1b). We tested the PEN-PVKRRLDL and PEN-PVKRRLFG peptides in a standard cell viability assay, U2OS and MDA-MB-435 cells, which are wild type and mutant for p53, respectively, were killed by PEN-RXL peptides in a concentration-dependent manner (Fig. 2, a and b). In contrast, Purvalanol A did not kill cells as effectively as the RXL peptides, although a dose dependence was observed (Fig. 2a). This may be attributable to less efficient uptake of Purvalanol A, compared with PEN-RXL peptide by U2OS cells. The uptake of peptide was confirmed by exposure of cells to fluorescein-labeled PEN-PVKRRLDL (data not shown).

Because cyclin D1 regulates mammary tumor development in HER2/neu transgenic mice (19), we tested cell lines obtained from mammary tumors of MMTV-HER2 transgenic mice, which are wild type for p53. Four cell lines were derived from discrete MMTV-HER2 mammary tumors.3 Cells were treated with RXL peptides, and cell killing was assayed by MTT analysis. All HER2 transgenic lines tested were killed by the PEN-PVKRRLDL and PEN-PVKRRLFG peptides in a dose-dependent manner (Fig. 2, c–f).

Nontransformed MEFs and Rat1a cells were also exposed to RXL peptides. Early passage MEFs and Rat1a cells treated with increasing doses of PEN-PVKRRLDL and PEN-PVKRRLFG did not show cell killing, compared with the transformed cells U2OS and MDA-MB-435 (Fig. 2, a, b, g, and h). We also examined the effect of the RXL peptides on SVT2 cells with inactivated Rb and p53 function (17, 18), in comparison with nontransformed parental BALB/c 3T3 cells. We observed cell killing in the transformed SVT2 cell line in a dose-dependent manner, whereas parental BALB/c 3T3 cells did not show a similar profile (Fig. 2, c and d). These in vitro data demonstrate that transformed cells with deregulated cyclin D or Rb are killed by exposure to RXL peptides. We extended these analyses in vivo, and PEN-RXL peptides were tested in a SVT2 mouse graft model and HER2 transgenic tumor model.

To examine a tumor model with nonfunctional Rb, we inoculated 0.5 × 106 SVT2 cells s.c. into nude mice. Once tumors were established (∼100 mm3), mice were treated with PEN-PVKRRLDL peptide. Intra and peritumoral injections of 0.4, 0.1, and 0.04 mg of PEN-PVKRRLDL peptide or 0.4 mg of PEN-CONTROL peptide were administered daily for 5 days. Tumor volume measurements indicate a dose-dependent inhibition of tumor growth, compared with the control group (Fig. 3a). The most striking observation by histological analysis of the tumors was that PEN-PVKRRLDL-treated tumors had large areas of apoptosis and necrosis, particularly at the tumor periphery (Fig. 4b). In contrast, PEN-CONTROL-treated tumors showed limited apoptosis, and the tumor periphery remained intact (Fig. 4a). TUNEL staining of tumors examined 6 h postinjection revealed staining of apoptotic cells at the tumor periphery in PEN-PVKRRLDL-treated tumors (Fig. 4d) but not in the PEN-CONTROL-treated group (Fig. 4c). These data extend the previous observations of apoptosis in tumor cell lines treated with RXL peptide (14). Collectively, these data indicate that RXL peptides inhibit tumor growth most likely by an apoptotic mechanism.

A second tumor model was studied using HERCEPTIN-resistant mammary tumors from HER2 transgenic mice.4 Tumor implants were grown in syngeneic FVB mice, and when tumors were established, mice were injected with 1 and 0.4 mg of PEN-PVKRRLFG peptide as before. Tumor measurements indicate an inhibition of tumor growth in the PEN-PVKRRLFG-treated group, compared with that of PEN-CONTROL (Fig. 3b). Cleaved caspase-3 staining, another marker of apoptosis, reveals that HER2 transgenic tumors treated with PEN-PVKRRLF peptide undergo extensive apoptosis, compared with the control group (Fig. 4, e and f). Our tumor model data extend the in vitro observations that both PEN-PVKRRLFG and PEN-PVKRRLDL peptides are capable of inhibiting tumor growth by apoptosis. Inflam-
mation and edema were observed at the sites of injection and peritumorally in control and PEN-RXL-treated groups in both tumor studies and are attributed to mechanical disruption by daily injections and the vehicle used for peptide suspension. In both tumor models, proliferating cells in hair follicles and skin were monitored for apoptosis on administration of peptides. Extensive apoptosis was not observed in these proliferating tissues (data not shown), compared with adjacent tumor cells. Although unlikely, it is formally possible that the peptides did not reach these tissues.

**DISCUSSION**

The utility of chemotherapeutics that targets the cell cycle is limited by toxicity to normal cells. Although selective modulators that exploit the deregulated cell cycle machinery of tumors have shown promising results in vitro (21), few candidate therapeutics have shown extended in vivo efficacy. We demonstrate that RXL peptides that inhibit CDK2/cyclin A phosphorylation not only kill transformed cells but also mediate apoptosis in two independent in vivo models of tumorigenesis. This is the first such report of in vivo efficacy demonstrated by RXL peptides. Previous studies have examined RXL peptide-mediated inhibition of CDK2/cyclin A phosphorylation of substrates in vitro and in cells (14, 22). However, the outcome of this inhibition on tumor growth was not examined. Often, inhibitors tested in cells with known mechanisms of action do not demonstrate extended efficacy in tumor studies. The observed apoptosis in RXL peptide-treated tumors, compared with control tumors, strongly suggests that
the mechanism of cell death is consistent both in vivo and in vitro and is essential to the validation of new therapeutics based on synthetic lethality.

The crystal structure of p27 KIP1 and RXL peptide bound to CDK2/cyclin A complex indicates that the peptide binds at a hydrophobic site on the surface of cyclin A (23, 24). The RXL peptides inhibit phosphorylation of cyclin A-binding proteins while maintaining the ability to phosphorylate Histone H1, a substrate that does not
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contain an RXL motif (Fig. 1b; Ref. 24). The structure-based prediction of inhibiting CDK2/cyclin A phosphorylation of substrates, such as E2F, suggests that cell killing in vitro and inhibition of tumor growth in vivo could be driven by E2F-mediated apoptosis. Previous studies demonstrate that E2F-driven apoptosis occurs in cells independent of p53 status (25, 26). Our observations of RXL peptide-mediated cell killing in vitro and in vivo occur in both p53 wild-type and mutant backgrounds, suggesting that apoptosis occurs through an E2F-mediated mechanism. Because p53 is mutated in approximately half of all human cancers, this mechanism of targeting tumors has an obvious therapeutic advantage. It is interesting to note that perturbations of the different components of the Rb/CDK4/cyclin D pathway, which impact E2F deregulation, are consistently affected by RXL peptides both in cells and the tumor models examined. Furthermore, these results suggest that design-based peptidomimetics derived from RXL peptides could act as potentially selective antineoplastic agents in vivo and provide a strong rationale for intervention at this point in the cell cycle, in contrast to ATP site inhibitors of CDK that are not tumor selective.

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