HIV Protease Inhibitors Block Akt Signaling and Radiosensitize Tumor Cells Both In vitro and In vivo

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Abstract
In tumor cells with mutations in epidermal growth factor receptor (SQ20B), H-Ras (T24), or K-Ras (MIAPACA2 and A549), the inhibition of Akt phosphorylation increases radiation sensitivity in clonogenic assays, suggesting that Akt is a potential molecular target when combined with therapeutic radiation. Insulin resistance and diabetes are recognized side effects of HIV protease inhibitors (HPIs), suggesting that these agents may inhibit Akt signaling. Because activation of the phosphatidylinositol 3-kinase (PI3K)-Akt signaling pathway is common in human cancers, we hypothesized that HPIs can inhibit Akt activity resulting in increased tumor cell sensitivity to ionizing radiation–induced cell death. First-generation HPIs were subsequently tested and three of the five (amprenavir, nelfinavir, and saquinavir but not ritonavir or indinavir) inhibited Akt phosphorylation at Ser473 at serum concentrations routinely achieved in HIV patients. In both tumor cell colony formation assays and tumor regrowth delay experiments, combinations of drug and radiation exerted synergistic effects compared with either modality alone. In addition, in vivo, doses of amprenavir or nelfinavir comparable with the therapeutic levels achieved in HIV patients were sufficient to down-regulate phosphorylation of Akt in SQ20B and T24 xenografts. Finally, overexpression of active PI3K in cells without activation of Akt resulted in radiation resistance that could be inhibited with HPIs. Because there is abundant safety data on HPIs accumulated in thousands of HIV patients over the last 5 years, these agents are excellent candidates to be tested as radiation sensitizers in clinical trials. (Cancer Res 2005; 65(18): 8256-65)

Introduction
The tendency of tumor cells to gain resistance to multiple forms of therapy concurrently has long been a barrier to effective treatment, including ionizing radiation (IR) that is used to treat ~60% of all cancer patients in the United States (1). This resistance significantly reduces treatment options and makes disease-free recovery difficult (2, 3). Therefore, the ability to discern particular mechanisms governing multimodality tumor cell resistance and subsequently exploit them should result in more effective treatment strategies.

One such factor known to increase cellular resistance to radiation is the presence of overexpressed or activated oncogenes, such as epidermal growth factor receptor (EGFR; refs. 4–6) and Ras (7, 8), or loss of the tumor suppressor gene PTEN (9). One critical observation is that these mutations share a common molecular signaling alteration that ultimately activates the phosphatidylinositol 3-kinase (PI3K)-Akt pathway. In this regard, we and others have shown that blocking PI3K enhances the radiation response in vitro and in vivo. This effect occurs in cells in which this pathway is constitutively activated but does not affect cells quiescent in this pathway (10–12). Down-regulation of Akt using small interfering RNA also radiosensitizes these cells (13). Because this pathway is frequently activated in tumor cells but not in the normal host cells, it is an excellent candidate to target to increase radiation sensitivity. The problem has been to identify inhibitors of this pathway suitable for clinical use. For example, LY294002 and wortmannin, which are widely used to inhibit the PI3K pathway in vitro with significant radiosensitizing effects, however, are poorly tolerated in vivo and of little clinical value (14).

In this study, we explore the possibility that one class of drugs in common use clinically, the HIV protease inhibitors (HPIs), may interfere with PI3K-Akt signaling. These drugs given in combination with reverse transcriptase inhibitors are the mainstays of the current therapeutic regimens for HIV-infected patients. The HPIs are peptidomimetics that inhibit the HIV aspartyl protease, a retroviral enzyme that cleaves the viral gag-pol polyprotein and is necessary for the production of infectious viral particles (15). A prominent side effect of HPI treatment is insulin resistance and diabetes (16, 17). Because Akt, especially the Akt2 isoform (18), plays a key role in the coordinated regulation of growth and metabolism by the insulin/insulin-like growth factor signaling pathway (18, 19), we explored the possibility that HPIs might block the PI3K-Akt signaling axis in tumor cells and hence be used clinically as radiation sensitizers. In fact, Pajonk et al. have shown that one HPI, saquinavir, was a radiation sensitizer in tissue culture (20). Here, we found that three of the five HPIs that we tested were able to inhibit Akt at doses routinely achieved clinically. These compounds also sensitized tumor cells both in vitro and in vivo to radiation. HPIs have been used continuously in patients with well-characterized pharmacokinetics. They are well tolerated; thus, testing these HPIs as radiation sensitizers could easily proceed to clinical trial.

Materials and Methods
Cells. SQ20B, T24, MIAPACA2, and A549 cell lines were obtained from American Type Culture Collection (Rockville, MD). The MR4 cells were derived from isogenic rat embryo fibroblasts by transfection with v-myc as described previously (8). Cells were cultured in DMEM (Fisher Scientific, Pittsburgh, PA) supplemented with 10% fetal bovine serum (Atlanta Biologicals, Norcross, GA), penicillin (100 units/mL), and streptomycin (100 mg/mL; Life Technologies, Gaithersburg, MD) at 37°C in humidified 5% CO2/95% air.
The cells were allowed to attach and enter exponential growth and the subsequently dissolved in 100% ethanol.

Indinavir came as solid caplets and were ground into a fine powder and concentrated stock solution for subsequent experiments. Nelfinavir and the viscous liquid inside was dissolved in 100% ethanol to make a and saquinavir came as gelatin capsules. The capsule was punctured and 24 hours after the addition of dexamethasone.

Survival experiments were done and protein samples were harvested Dexamethasone (Sigma, St. Louis, MO) at 1

Vector, a pool of cells was selected with hygromycin for 72 hours. Dexamethasone-inducible plasmid vector. Because this is an episomal vector, a pool of cells was selected with hygromycin for 72 hours. Dexamethasone (Sigma, St. Louis, MO) at 1 μg/mL was added. Radiation survival experiments were done and protein samples were harvested 24 hours after the addition of dexamethasone.

DRUGS. The HPIs were bought for research use from the hospital inpatient pharmacy of the University of Pennsylvania. Ritonavir, amprenavir, and saquinavir came as gelatin capsules. The capsule was punctured and the viscous liquid inside was dissolved in 100% ethanol to make a concentrated stock solution for subsequent experiments. Nelfinavir and indinavir came as solid caplets and were ground into a fine powder and subsequently dissolved in 100% ethanol.

Western blotting. Cells were lysed without trypsinization by rinsing culture dishes once with PBS followed by lysis with reducing Laemmli sample buffer. Samples were boiled, sheared, clarified by centrifugation, and stored at −20°C. Samples containing equal amounts of protein were separated on a 12% SDS-polyacrylamide gel and blotted onto nitrocellulose membranes. Membranes were blocked in PBS containing 0.1% Tween 20 and 5% powdered milk before primary antibody addition. Both polyclonal and monoclonal antibodies detecting total Akt, MAPK, and actin were also used.

Radiation survival determination. Cells in exponential growth phase were counted and plated in 60-mm dishes containing 4 mL medium. The cells were allowed to attach and drugs were added to cultures at least 1 hour before radiation. Cells were irradiated with a Mark I cesium irradiator (J.L. Shepherd, San Fernando, CA) at a dose rate of 1.6 Gy/min. Colonies were stained and counted 10 to 14 days after irradiation. A colony by definition had >50 cells. The surviving fraction was calculated by dividing the number of colonies formed by the number of cells plated times plating efficiency. Each point on the survival curve represents the mean surviving fraction from at least three replicates.

Cell growth curves. Cells (5 × 10^4) were plated in each T25 flask. The cells were allowed to attach and enter exponential growth and the drugs were added. At various times, total cell number was assessed in triplicate.

Tumor generation in nude mice. Pathogen-free female Ncr-nu/nu mice were obtained from Taconic (Germantown, NY) and housed aseptically in the animal facilities of University Laboratory Animal Resources and the Institute for Human Gene Therapy of the University of Pennsylvania. All experiments were carried out in accordance with University Institutional Animal Care and Use Committee guidelines. At 5 to 7 weeks of age, mice were inoculated by s.c. injection into the hind flank with 1 × 10^7 T24 or 1 × 10^6 SQ20B cells resuspended in 100 μL Matrigel (BD Collaborative Research, Franklin Lakes, NJ). SQ20B tumors usually appeared within 1 week and T24 tumors 2 to 3 weeks after injection.

Drug treatment of mice. Nelfinavir was formulated as 3-week continuous release pellets containing 12.6 mg drug with a release rate of 0.6 mg/d by Innovative Research of America (Sarasota, FL). Amprenavir was given s.c. by continuous micro-osmotic pump infusion (Alza Corp., Mountain View, CA). Ritonavir, amprenavir, and saquinavir came as gelatin capsules.

Table 1. Surviving fraction after 2 Gy (SF2) in cell lines ± HPIs

<table>
<thead>
<tr>
<th>Cell line</th>
<th>Cancer type</th>
<th>Mutation</th>
<th>Control SF2, n (mean ± SD)</th>
<th>Amprenavir SF2, n (mean ± SD) [p]</th>
<th>Nelfinavir SF2, n (mean ± SD) [p]</th>
</tr>
</thead>
<tbody>
<tr>
<td>T24</td>
<td>Bladder</td>
<td>H-Ras</td>
<td>6 (0.560 ± 0.061)</td>
<td>6 (0.370 ± 0.022) [0.001*]</td>
<td>6 (0.452 ± 0.023) [0.006*]</td>
</tr>
<tr>
<td>SQ20B</td>
<td>Head and neck</td>
<td>EGFR</td>
<td>6 (0.691 ± 0.042)</td>
<td>6 (0.432 ± 0.017) [0.001*]</td>
<td>5 (0.401 ± 0.024) [0.001*]</td>
</tr>
<tr>
<td>MIAPACA2</td>
<td>Pancreatic</td>
<td>K-Ras</td>
<td>4 (0.906 ± 0.086)</td>
<td>5 (0.664 ± 0.051) [0.001*]</td>
<td>5 (0.526 ± 0.040) [0.001*]</td>
</tr>
<tr>
<td>A549</td>
<td>Lung</td>
<td>K-Ras</td>
<td>6 (0.570 ± 0.042)</td>
<td>5 (0.487 ± 0.030) [0.002*]</td>
<td>4 (0.401 ± 0.062) [0.001*]</td>
</tr>
<tr>
<td>REF</td>
<td>Rat fibroblasts</td>
<td>None</td>
<td>5 (0.408 ± 0.040)</td>
<td>4 (0.402 ± 0.136) [0.89*]</td>
<td>5 (0.397 ± 0.054) [0.89*]</td>
</tr>
</tbody>
</table>

*Compared with control SF2 by t test.

Figure 1. Effect of HPIs on phosphorylation of Akt, MAPK, and p70S6K. SQ20B or T24 cells in tissue culture were treated with the indicated HPIs. At the indicated times, cells were harvested and immunoblotted with antibody used to detect the active or phosphorylated forms of Ser 473 Akt, Thr467 Akt, MAPK, and p70S6K. Antibodies detecting total Akt, MAPK, and actin were also used. A, SQ20B cells were treated with 25 μM saquinavir for 5 to 60 minutes. B, SQ20B or T24 cells were treated with 5 μM/L amprenavir for 1 or 3 days. C, SQ20B cells were treated with 10 μM/L amprenavir for 2, 5, or 9 days. D, SQ20B or T24 cells were treated with 5 μM/L nelfinavir for 1 or 3 days.
Palo Alto, CA) of the drug at a dose of 0.8 mg/d. These doses are comparable with that used in HIV patients. Control animals received placebo pellet or pumps with carrier alone.

**Drug measurements in serum.** High-performance liquid chromatography was done using a binary gradient Jasco system. Serum was prepared by adding an equal part of 100% methanol followed by incubation on ice for 1 hour and centrifugation at 13,000 × g for 10 minutes. The supernatant (100 µL) was analyzed using an Alltima C18 column (4.6 × 250 mm, 5 µ) with buffer [0.1 mmol/L ammonium acetate (pH 4.7)] gradient for nelfinavir of 30% to 90% (4 minutes) followed by 90% (8 minutes) and for amprenavir of 30% to 90% (10 minutes) followed by 90% (4 minutes). Nelfinavir or amprenavir content was determined from the area under curve at the drug peak detected at 11 to 12 minutes with E260 for amprenavir and E250 for nelfinavir. Absolute values were calculated from calibration curves.

**Immunohistochemical staining.** Paraffin-embedded tissue sections were stained with antibody for immunohistochemistry to phospho-Ser473 Akt as described by Zhou et al. (22). Slides were graded as 0 to 3+ staining as we have described previously (12).

**In vivo clonogenic assays.** Animals were assigned randomly to treatment groups (control, radiation alone, drug alone, or radiation and drug) when tumors attained a volume of 300 to 400 mm³. Drug treatment was started 3 days (amprenavir) or 5 days (nelfinavir) before radiation. Mice were irradiated with doses of 6 to 8 Gy with a Mark I cesium irradiator under anesthesia. Unirradiated animals were anesthetized and sham irradiated. One hour after irradiation, animals were sacrificed. Tumors were then excised, minced, and dissociated for 30 minutes at 37°C in HBSS containing 166 units/mL collagenase XI, 0.25 mg/mL protease, and 255 units/mL DNase. Cells were recovered after straining through an 80-µm mesh. The cells were pelleted by centrifugation at 500 × g and resuspended in culture medium. Cells were counted by hemocytometer using trypan blue to determine viability, and the counts were verified by Coulter counter analysis. Cells were then plated in 100-mm dishes and cultured for 14 to 21 days.

![Figure 2](https://cancerres.aacrjournals.org)
21 days, after which colonies were stained and counted. Results are expressed as the plating efficiency determined from replicate dishes plated at multiple initial cell densities.

Determinations of tumor regrowth delay. Mice bearing established tumors were randomized and treated with amprenavir/nelfinavir as described above. Irradiation (6 or 8 Gy) of the flank bearing the tumor was done using a 250 kV orthovoltage irradiator (Philips RT 250) at a dose rate of 2.63 Gy/min through a 0.2-mm copper filter. The source-to-tumor target distance was 30 cm with adequate shielding of nontumor sites. Mice were examined twice weekly for evaluation of tumor growth. Tumors were measured with calipers in three mutually perpendicular diameters (a, b, and c) and the volume was calculated as $V = \frac{\pi}{6}a \times b \times c$.

Figure 3. In vivo down-regulation of phosphorylated Ser473 Akt in xenografts with amprenavir or nelfinavir. A, immunohistochemistry (400×) of representative SQ20B tumor from a control mouse (left) and a mouse treated with nelfinavir (right). The tumors were harvested 5 days after being treated with placebo or nelfinavir 0.6 mg/d. B, an immunoblot of the lysate from the tumors shown in A, C, an immunoblot of the lysates from SQ20B tumors treated with amprenavir 0.8 mg/d for 1, 2, 3, or 4 days. The serum concentration of amprenavir corresponding to the treatment is shown below the blot.

The treatment group did not reach a volume of 1,000 mm³ during the observation period. All observations in the radiation plus amprenavir and radiation plus nelfinavir groups were included in the analysis. The increase in time to tumor volume of 1,000 mm³ was analyzed in a similar manner, where a single singularity and/or failure of a Cox regression model to converge. Thus, the interaction term using a one-sided Wald statistic to determine significance between SF²s in Table 1. A regression model was fit to log₁₀-transformed plating efficiency data that included terms to estimate the individual (main) effects of radiation and HPI and the interaction of these two treatments on the mean plating efficiency of tumor cells. Replicate measurements were averaged for each mouse. The linear model took the form:

\[ Y = \beta_0 + \beta_1 (\text{radiation}) + \beta_2 (\text{HPI}) + \beta_3 (\text{radiation} \times \text{HPI}), \]

where \( Y = \log_{10} \text{plating efficiency}, \) radiation, and HPI are indicators for the treatment received (1 = yes, 0 = no) and radiation \( \times \) HPI is an interaction term. The test of synergy between radiation and HPI was conducted on the interaction term using a one-sided Wald statistic to determine whether \( \beta_3 < 0, \) indicating synergy. A negative coefficient for the interaction term indicates a synergistic effect and a more than additive decrease in plating efficiency. \( P = 0.05 \) was considered to be significant.

Time to tumor volume of 1,000 mm³ was analyzed in a similar manner, with \( Y \) equal to days to reach a volume of 1,000 mm³ during the observation period. The test of synergy between radiation and HPI was again conducted on the interaction term using a one-sided Wald statistic. For regrowth studies, \( \beta_3 > 0 \) indicates synergy and a more than additive increase in time to tumor volume of 1,000 mm³. In T24 tumors, a single mouse in both radiation plus amprenavir and radiation plus nelfinavir treatment groups did not reach a volume of 1,000 mm³ during the observation period. All observations in the radiation plus amprenavir group and all but one in the radiation plus nelfinavir group exceeded those of the control and single modality groups, which resulted in either singularity and/or failure of a Cox regression model to converge. Thus, each censored was treated as an event on day 35 and linear regression was then employed to test synergy. This approach produces a slightly conservative estimate of treatment effect for the data described above. All analyses were done in SPSS 12.0 (SPSS, Inc., Chicago, IL).

### Results

**Inhibition of Akt signaling by HIV protease inhibitors in tissue culture.** Insulin resistance and diabetes are well-established and recognized side effects of extended HPI use, and it has been suggested that this may be due to inhibition of Akt signaling. Because Akt activity is involved in tumor cell resistance, it seemed logical to determine if HPIs may inhibit Akt. Between 1995 and 1999, five HPIs were approved by the Food and Drug Administration: saquinavir, ritonavir, indinavir, nelfinavir, and amprenavir. We tested these drugs for their effects on Akt phosphorylation. Two cell lines with constitutively active Akt signaling phosphorylation of Akt Ser⁴⁷³ were initially chosen: (a) SQ20B cells with overexpression of activated EGFR and (b) T24 cells that contain an oncogenic mutation in H-Ras and an allelic amplification of that allele. Complete Akt activation requires phosphorylation of Ser⁴⁷³.

SQ20B cells were treated with 25 μmol/L saquinavir from 5 to 60 minutes and Western blot analysis showed a time-dependent decrease in immunoreactive Akt phosphorylation levels with a complete loss of detectable phosphorylation of Akt within 20 minutes (Fig. 1A). There was no change in the total Akt levels using actin as a loading control. At lower concentrations (5-10 μmol/L,

<table>
<thead>
<tr>
<th>Table 2. Test of synergy in SQ20B xenografts for <em>in vivo</em> to <em>in vitro</em> clonogenicity with radiation and amprenavir or radiation and nelfinavir</th>
</tr>
</thead>
<tbody>
<tr>
<td>Log₁₀ plating efficiency mean ± SD (no. measures per mouse)</td>
</tr>
<tr>
<td>Control (<em>n = 4</em>)</td>
</tr>
<tr>
<td>−1.24 ± 0.06 (8-12)</td>
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<tr>
<td>Interaction term / (SE)</td>
</tr>
<tr>
<td>Wald statistic</td>
</tr>
<tr>
<td>Test of synergy (one-sided <em>P</em>)</td>
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</tbody>
</table>

*Mean of the averages for each mouse.

<table>
<thead>
<tr>
<th>Table 3. Test of synergy in T24 xenografts for <em>in vivo</em> to <em>in vitro</em> clonogenicity with radiation and amprenavir or radiation and nelfinavir</th>
</tr>
</thead>
<tbody>
<tr>
<td>Log₁₀ plating efficiency mean ± SD (no. measures per mouse)</td>
</tr>
<tr>
<td>Control (<em>n = 5</em>)</td>
</tr>
<tr>
<td>−0.62 ± 0.03 (12)</td>
</tr>
<tr>
<td>Interaction term / (SE)</td>
</tr>
<tr>
<td>Wald statistic</td>
</tr>
<tr>
<td>Test of synergy (one-sided <em>P</em>)</td>
</tr>
</tbody>
</table>

*Mean of the averages for each mouse.
range), saquinavir took ~24 hours to have the same effect (data not shown). However, saquinavir was highly toxic in tissue culture with cell death within 2 hours of treatment at 25 µmol/L and cell death at 24 to 48 hours for doses between 1 and 10 µmol/L.

Treatment of SQ20B cells with amprenavir (5 µmol/L) resulted in down-regulated phosphorylation of Akt at Ser\(^{473}\) after a 3-day exposure (Fig. 1B) with no effect on total intracellular Akt levels. In addition, treatment of SQ20B cells with amprenavir (10 µmol/L) also resulted in complete dephosphorylation of Akt at Ser\(^{473}\) and this was observed for at least 9 days (Fig. 1C). Exposure to higher doses of amprenavir (20-25 µmol/L) resulted in more rapid down-regulation of Akt at 1 day but not sooner than 16 hours. Finally, at amprenavir concentrations that inhibited Akt phosphorylation (5-10 µmol/L), no effect on cell growth was observed (data not shown). However, at higher doses (40 µmol/L) or longer exposures (>16 hours), significant tumor cell toxicity was observed, suggesting that inhibition of Akt activity has little effect on cell death at these clinically achievable concentrations.

Similar to saquinavir and amprenavir, nelfinavir (5 µmol/L) down-regulated Akt phosphorylation at Ser\(^{473}\) in both SQ20B and T24 cells (Fig. 1D) without change in total intracellular Akt protein levels. Exposure to increasing the concentration of nelfinavir resulted in an earlier onset of this response but resulted in cell toxicity at 20 µmol/L. Finally, similar to the other HPIs tested, no changes in cell growth kinetics or cell death was observed at concentrations that inhibit Akt phosphorylation (data not shown).

**Figure 5.** Regrowth delay of SQ20B xenografts ± nelfinavir after radiation. A, mean tumor volume in control tumors (○), nelfinavir-treated tumors (●), tumors treated with 8 Gy radiation (×), or 8 Gy and nelfinavir-treated tumors (♦). B, data from each individual tumor, which were used to derive the data in A.
through its NH2-terminal region to the p85 subunit (23), which allows the construction of a constitutively active PI3K consisting of the iSH2 region of p85 covalently attached to the NH2 terminus of the p110 subunit (21). Dexamethasone treatment of these transiently transfected cells resulted in the appearance of phosphorylated Akt (Fig. 2B). The presence of phosphorylated Akt as a marker for PI3K activity was specific for cells expressing the activated p110 subunit because neither untransfected cells treated with dexamethasone nor uninduced transfectants showed enhanced Akt phosphorylation.

MR4 cells transfected with active PI3K treated with dexamethasone showed a significant increase in resistance to IR-induced cell death, whereas no change in clonogenic cell survival was seen in control cells. Expression of the vector alone and exposure to dexamethasone did not alter the plating efficiency of the cells. In addition, treatment of transfected cells with amprenavir and nelfinavir in the presence of dexamethasone sensitized them to radiation (Fig. 2C).

HIV protease inhibitors inhibit Akt signaling in vivo. Although in vitro data provide an important preliminary background to advance the potential use of new anticancer agents, these results must be confirmed and validated in an in vivo tumor model system. Before regrowth experiments, we initially determined if nelfinavir and amprenavir can down-regulate Akt in xenografts. As such, nelfinavir was given at 0.6 mg/d via a continuous releasing pellet and it was observed that a minimum of 5 days of exposure was necessary for serum levels of nelfinavir to equilibrate in the 2 to 6 μmol/L range. The tumors were stained with anti-phospho-Ser473 Akt antibody and the phosphorylation level of nelfinavir in the mouse treated with drug was 4.5 μmol/L (Fig. 3B) and Western blotting with anti-phospho-Ser473 Akt confirms the decrease in phospho-Akt seen in tumor slides.

Amprenavir was delivered via micro-osmotic pump at 0.8 mg/d. At this dose, the serum concentrations of the drug were in the 1 to 5 μmol/L range, and 2 to 5 μmol/L were sufficient to down-regulate phosphorylation of Akt at Ser473 (Fig. 3C). Thus, both amprenavir and nelfinavir down-regulated phosphorylation of Akt at Ser473 in tumors in vivo at doses comparable with the therapeutic levels achieved in HIV patients.

Radiation sensitization in vivo measured by clonogenic assay. Tumor cell colony formation assays were used to measure radiation response after amprenavir and nelfinavir treatment of SQ20B or T24 tumor-bearing animals. The clonogenicity of tumor cells was compared after isolation from tumors treated with radiation plus amprenavir/nelfinavir, radiation alone, amprenavir/nelfinavir alone, or mock treatment. The radiation dose was 8 Gy for SQ20B tumors and 6 Gy for T24 tumors. The animals were pretreated with nelfinavir pellets for 5 days or with amprenavir pumps for 2 days. Both amprenavir and nelfinavir treatment of...
SQ20B xenografts (Table 3), nelfinavir and radiation exhibited highly statistically significant synergy ($P = 0.003$) and the synergistic effect of amprenavir and radiation nearly reached statistical significance ($P = 0.053$).

**HIV protease inhibitors inhibit xenograft tumor regrowth following irradiation.** Radiation sensitization was also assessed using the tumor regrowth delay assay. Mice bearing SQ20B or T24 tumors were randomly assigned to each treatment arm (radiation plus drug, radiation alone, drug alone, or mock treatment). As with the clonogenic assays, mice were pretreated for 2 days with amprenavir pumps or 5 days with nelfinavir pellets. The radiation dose was 8 Gy for SQ20B tumors and 6 Gy for T24 tumors. The radiation dose was chosen based to yield a growth delay and not a cure to allow better statistical analysis.

SQ20B tumor xenografts treated with nelfinavir and the results for mean tumor volumes (Fig. 5A) and each individual tumors (Fig. 5B) are shown. In the radiation plus nelfinavir group, two slowly growing tumors reached a volume of 1,000 mm$^3$ at 70 and 78 days (data not shown in Fig. 5B). The mean time to tumor volume of 1,000 mm$^3$ was 11 days in the control group and 12 days in the nelfinavir alone group. As expected, mean values increased in both radiation alone (15 days) and radiation and nelfinavir (41 days) groups. As seen in Table 4, a statistically significant synergistic effect between radiation and nelfinavir was detected by linear regression analysis ($P = 0.03$).

In SQ20B xenografts treated with amprenavir, a mean time to tumor volume of 1,000 mm$^3$ was 5.8 days in the control group (Fig. 6A) and 7.1 days in the amprenavir alone group. Mean values increased in both radiation alone (8 days) and radiation and amprenavir (15.8 days) groups. By linear regression analysis, a statistically significant synergistic effect between radiation and amprenavir was detected ($P = 0.0045$; Table 5). Of note is that these tumors were larger at the start of the experiment than the SQ20B tumors with nelfinavir as shown in Fig. 5 (mean starting volume of 300 versus 100 mm$^3$); thus, the time to reach 1,000 mm$^3$ was shorter.

In T24 tumors treated with amprenavir (Fig. 6B), mean time to tumor volume of 1,000 mm$^3$ was 15.8 days in the control group, 13.2 days in the amprenavir alone group, and 14.3 days in the nelfinavir alone group. Mean values increased to 19.3 days with 6 Gy radiation alone and 30.8 days with radiation and amprenavir. In contrast, in T24 tumors treated with nelfinavir, mean value was 28.6 days with radiation, which was statistically identical to that of mice treated with amprenavir. Statistically significant synergy was apparent for both radiation and amprenavir ($P < 0.001$; Table 6) and radiation and nelfinavir ($P = 0.004$; Table 7).

We evaluated six mice for normal tissue toxicity from HPI and radiation: two untreated, two treated with nelfinavir, and two treated with amprenavir. The right leg of each mouse was irradiated with 8 Gy. We assessed the mice weekly for the visual development of skin fibrosis and leg contractures. No difference was seen in unirradiated versus irradiated or drug and irradiated legs. At 60 days, the mice were sacrificed and histologic sections of legs were compared. There was an increase in epidermal thickness with irradiation (mean control, 16 versus 22 $\mu$m/L for 8 Gy). It was, however, impossible to tell which mouse had been given the drug versus control and no additive effect on skin fibrosis was seen with HPI and radiation.

**Discussion**

The PI3K-Akt pathway is frequently constitutively activated in human tumor cells versus normal cells, suggesting that factors in this cascade are potential molecular targets (reviewed in ref. 24). We and others have shown that activation of this pathway results in...
Table 5. Test of synergy for regrowth delay in time to reach tumor volume of 1,000 mm³ in SQ20B xenografts ± amprenavir after radiation

<table>
<thead>
<tr>
<th>Days to tumor volume of 1,000 mm³ mean ± SD (range)</th>
<th>Control (n = 5)</th>
<th>Amprenavir (n = 7)</th>
<th>8 Gy (n = 5)</th>
<th>8 Gy + Amprenavir (n = 5)</th>
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<tbody>
<tr>
<td></td>
<td>5.8 ± 1.6 (4-7)</td>
<td>7.1 ± 2.0 (4-11)</td>
<td>8.0 ± 3.0 (4-11)</td>
<td>15.8 ± 3.4 (11-20)</td>
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<td>Interaction term / (SE)</td>
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<tr>
<td>Wald statistic</td>
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<td>6.457 (2.211)</td>
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<td>Test of synergy (one-sided P)</td>
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<td></td>
<td>2.920</td>
<td>0.0045</td>
</tr>
</tbody>
</table>

NOTE: All animals reached tumor volume of 1,000 mm³ before sacrifice.

Table 6. Test of synergy for regrowth delay in time to reach tumor volume of 1,000 mm³ in T24 xenografts ± amprenavir after radiation

<table>
<thead>
<tr>
<th>Days to tumor volume of 1,000 mm³ mean ± SD (range)</th>
<th>Control (n = 5)</th>
<th>Amprenavir (n = 6)</th>
<th>6 Gy (n = 6)</th>
<th>6 Gy + Amprenavir (n = 5)</th>
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</thead>
<tbody>
<tr>
<td></td>
<td>15.8 ± 3.2 (11-18)</td>
<td>13.2 ± 4.4 (7-18)</td>
<td>19.3 ± 2.1 (18-22)</td>
<td>30.8 ± 4.0 (26-35*)</td>
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<td>Interaction term / (SE)</td>
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<td>Wald statistic</td>
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<td>14.100 (3.006)</td>
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</tr>
<tr>
<td>Test of synergy (one-sided P)</td>
<td></td>
<td></td>
<td>4.691</td>
<td>&lt;0.001</td>
</tr>
</tbody>
</table>

*One animal did not reach a tumor volume of 1,000 mm³ during observation period.
It nevertheless remains that three of the five HPIs tested resulted in down-regulation of Akt phosphorylation at Ser473 at clinically achievable concentrations. We tested two of the HPIs (amprenavir and nelfinavir) as adjuvant antitumor agents in vitro and saw synergistic sensitization with HPI and radiation. Because there is safety data on both amprenavir and nelfinavir for the last 5 years, they should be tested as radiation sensitizers in clinical trial.

### Table 7. Test of synergy for regrowth delay in time to reach tumor volume of 1,000 mm³ in T24 xenografts + nelfinavir after radiation

<table>
<thead>
<tr>
<th>Days to tumor volume of 1,000 mm³ mean ± SD (range)</th>
<th>Nelfinavir (n = 6)</th>
<th>6 Gy (n = 6)</th>
<th>6 Gy + Nelfinavir (n = 5)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control (n = 5)</td>
<td>15.8 ± 3.2 (11-18)</td>
<td>14.3 ± 3.1 (11-18)</td>
<td>19.3 ± 2.1 (18-22)</td>
</tr>
<tr>
<td>Interaction term β (SE)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Wald statistic</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Test of synergy (one-sided P)</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

*One animal did not reach a tumor volume of 1,000 mm³ during observation period.

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### References

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