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## The HIV Protease Inhibitor Ritonavir Inhibits Lung Cancer Cells, in part, by Inhibition of Survivin

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### Abstract

**Introduction**—Ritonavir is a potential therapeutic agent in lung cancer, but its targets in lung adenocarcinoma are unknown, as are candidate biomarkers for its activity.

**Methods**—RNAi was used to identify genes whose expression affects ritonavir sensitivity. Synergy between ritonavir, gemcitabine and cisplatin was tested by isobologram analysis.

**Results**—Ritonavir inhibits growth of K-ras mutant lung adenocarcinoma lines A549, H522, H23 and K-ras wild type line H838. Ritonavir causes G0/G1 arrest and apoptosis. Associated with G0/G1 arrest, ritonavir down-regulates cyclin dependent kinases, cyclin D<sub>1</sub> and Rb phosphorylation. Associated with induction of apoptosis, ritonavir reduces survivin mRNA and protein levels more than 2-fold. Ritonavir inhibits phosphorylation of c-Src and STAT3, which are important events for survivin gene expression and growth, and induces cleavage of PARP1. While knock down of survivin, c-Src or STAT3 inhibits cell growth, only survivin knock down enhances ritonavir inhibition of growth and survivin over-expression promotes ritonavir resistance. Ritonavir was tested in combination with gemcitabine or cisplatin exhibiting synergistic and additive effects, respectively. The combination of ritonavir/gemcitabine/cisplatin is synergistic in the A549 line and additive in the H522 line, at clinically feasible ritonavir concentrations (<10 μM).

**Conclusions**—Ritonavir is of interest for lung adenocarcinoma therapeutics and survivin is an important target and potential biomarker for its sensitivity. Ritonavir cooperation with gemcitabine/cisplatin might be explained by involvement of PARP1 in repair of cisplatin-mediated DNA damage and survivin in repair of gemcitabine-mediated double stranded DNA breaks (DSB).

### Keywords

Non-small cell lung cancer; ritonavir; survivin; chemotherapy

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## Introduction

A potentially successful approach for cancer drug development consists of re-purposing existing drugs, with known tolerability, toxicity and pharmacology <sup>1</sup>. Ritonavir, originally developed as an HIV drug, exhibits anti-cancer activity against epithelial malignancies including prostate, breast cancer and ovarian cancer <sup>2-4</sup> as well as Kaposi's sarcoma <sup>5</sup> and glioma <sup>6</sup>, but its potential inhibitory activity against lung adenocarcinoma is unknown as are its mechanisms of action. The objective of these studies was to determine whether ritonavir exhibits effects on lung adenocarcinoma growth and survival and, if so, which cancer-associated signaling pathways may be affected in this malignancy. Identification of signaling pathways affected by ritonavir in lung adenocarcinoma could assist the design of clinical trials for recurrent/metastatic disease, where there is an unmet need for new therapeutic options.

One target of particular importance in lung cancer is the anti-apoptotic protein survivin <sup>7</sup>. Survivin regulates multiple cancer-relevant pathways and most notably inhibits apoptosis as an Inhibitor of Apoptosis (IAP) family member, while promoting cell cycle progression through the G1/S and G2/M checkpoints <sup>8-11</sup>. Because of its nodal role in cancer networks, its short half-life <sup>12</sup>, and its exclusive expression in tumor tissues <sup>13, 14</sup>, survivin is a particularly attractive target in cancer therapy <sup>15</sup>. Various approaches to down-regulate survivin expression, including anti-sense oligonucleotides <sup>16</sup>, ribozymes <sup>17</sup>, siRNA <sup>18, 19</sup>, and dominant-negative protein <sup>20</sup>, have been used successfully in pre-clinical setting in NSCLC and other types of cancer. Furthermore a survivin antisense approach, alone or in combination with chemotherapy, is being tested in clinical trials <sup>21, 22</sup>. Survivin is highly expressed in NSCLC <sup>7, 13, 23</sup> and the level of its gene expression may be prognostic of 5 year survival <sup>24</sup>, although correlation between survivin expression and outcomes remains to be determined conclusively <sup>25</sup>. Survivin may also play a role in the development of chemotherapy resistance in NSCLC <sup>26-28</sup> and its down-regulation may sensitize lung cancer to chemotherapy <sup>29</sup>.

Ritonavir is known to reduce the levels of survivin in T cell leukemia <sup>30</sup>, but it is unknown whether ritonavir affects the level or function of survivin in lung adenocarcinoma. Because of the critical importance of survivin in NSCLC biology and its outcomes, it was determined whether ritonavir inhibits survivin also in NSCLC, specifically in lung adenocarcinoma cell lines. Survivin transcription is regulated by c-Src and STAT3, both known to be important for NSCLC growth <sup>31-34</sup>. Thus, the effects of ritonavir on these potential targets were also studied. Finally, combinations of ritonavir and chemotherapy that is not expected to interact pharmacokinetically, were tested for efficacy. Specifically, ritonavir was combined with gemcitabine and cisplatin, because both drugs have already been used in combination with ritonavir and other HIV protease inhibitors in case reports and clinical trials <sup>35-37</sup>.

## Materials and Methods

### Antisera

Antibodies raised against human phospho-Rb (#9308), phospho-c-Src (#2101S), STAT3 (clone 124H6; #9139), phospho-STAT3 (clone 3E2; # 9138S), and PARP1 (#9542) were purchased from Cell Signaling Technology (Beverly, MA). Antibodies raised against p53 (clone DO1; #sc-126), CDK2 (#sc-163), CDK4 (#sc-260), CDK6 (#sc-177), cyclin D<sub>1</sub> (sc-718), cyclin A (clone C-19; #sc-596) were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Antibody anti-c-Src (clone GD11; #05-184) was purchased from Millipore (Billerica, MA). Antibodies anti-p27 (#610242), Rb (#554136) and Phospho-Rb were purchased from BD Biosciences (San Jose, CA), antibody anti-survivin (#AF886) from

R&D Systems (Minneapolis, MN), and antibody anti-GAPDH (clone 6C5; 10R-G109A) from Fitzgerald Industries International (Concord, MA).

### Cell lines and drugs

The lung adenocarcinoma lines A549, H522, H23 were obtained from the American Type Culture Collection (ATCC; Manassas, VA). The lung adenocarcinoma line H838 was a kind gift from Dr. Manish Patel (University of Minnesota). Purified ritonavir, gemcitabine and cisplatin were purchased from Sequoia Research Products (Pangbourne, UK).

### siRNA

siRNA duplex SMARTpool upgrades of c-Src (#M-003175-03), STAT3 (#M-003544-02), and survivin (#M-003459-02), and siControl (# D-001210-01) were purchased from Dharmacon Research, Inc (Lafayette, CO). The sequences for siRNA duplexes used in these studies are given in Table S1.

### Western blotting

Cell lysates were prepared with standard methodology. Samples containing the same amount of protein were separated on a 4–20% gradient SDS gel, blotted on a nitrocellulose membrane and probed with specific antibodies. Densitometry analysis of the bands was performed using the software Alpha Imager (Alpha Innotech; San Leandro, CA) and the relative amount of protein was normalized to the corresponding signal for glyceraldehyde 3-phosphate dehydrogenase (GAPDH).

### MTT assay

The cells were cultured in 96-well plates for 48 h in the presence of increasing concentration of ritonavir (ranging from 5 to 60  $\mu$ M) or DMSO. Cell growth was determined by measuring the mitochondrial reduction of the tetrazolium salt, 3, [4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT) (Sigma; St Luis, MO). Each concentration of ritonavir was tested in octuplicate, and each MTT assay was repeated three times.

### Measurement of clonogenic efficiency

Clonogenic efficiency of lung adenocarcinoma lines was measured by exposing cell monolayers (20% confluent) to varying concentrations of ritonavir (ranging from 10 to 100  $\mu$ M) or DMSO for 24 h. Each ritonavir concentration was tested in triplicate. At the end of the 24 h treatment, cell monolayers were treated with trypsin and re-plated (500 cells per 100 mm plate) in the absence of ritonavir in complete medium (CM) consisting of RPMI 1640 (ATCC; Manassas, VA) supplemented with 10% fetal calf serum (Hyclone/Thermo Fisher Scientific; Logan, UT). The medium was changed every 3 days for 21 days. At the end of the 21-day incubation period, the colonies were fixed, stained with Wright-Giemsa stain (Thermo Fisher Scientific; Waltham, MA) and counted.

### Cell cycle analysis

Lung adenocarcinoma cell lines 30–50% confluent were treated with trypsin, washed and resuspended in CM. Cells were plated at a density of  $5 \times 10^5$  cells/100 mm plate, and grown for 24 and 48 h in CM in the presence of ritonavir ( $IC_{50}$ ) or DMSO. Profiling of propidium iodide (PI) incorporation was performed by FACSscan analysis (Becton Dickinson; San Jose, CA). Adherent and non-adherent cells were included in the profile. Cell cycle distribution was determined using ModFit software (Becton Dickinson; San Jose, CA).

### Measurement of apoptosis

Apoptotic cells were detected using an Annexin-V-FITC/PI apoptosis detection kit (Oncogene; Boston, MA). Cells were plated at a density of  $5 \times 10^5$  cells/100 mm plate, and grown for 48 h in CM in presence of ritonavir ( $IC_{50}$ ) or DMSO. After 48 h, cells were harvested and stained with PI and Annexin-V-FITC. Cells were then acquired by FACScan ( $1 \times 10^4$  cells/assay) and analyzed using CellQuest software (Becton Dickinson; San Jose, CA). Each assay was performed in duplicate.

### Real time PCR

RNA was isolated from A549 and H522 cell lines treated for 48 h with ritonavir (40  $\mu$ M) or DMSO by RNeasy mini kit (QIAGEN; Valencia, CA), following manufacturer's instructions. First strand cDNA was synthesized with high capacity cDNA archive kit (Applied Biosystems; Foster City, CA). The PCR reaction consisted in 40 cycles (2 min at 50°C, 10 min at 95°C, 15 sec at 95°C, 1 min at 60°C).

### siRNA transfection and cell survival assay

Cells were plated in 6 well poly-D-Lysine coated tissue culture plate (Becton Dickinson; Bedford, MA) at  $2.5 \times 10^4$  cells/well, grown for 24 h and then transfected with 50 nmoles/well of siRNA duplexes for c-Src, STAT3, survivin, or control siRNA using Oligofectamine (Invitrogen; Carlsbad, CA) and OPTI-MEM (Invitrogen; Carlsbad, CA). The efficacy of gene silencing was evaluated by examining the protein product with Western blotting 72 h after transfection. Each assay was performed in triplicate. 24 h after the siRNA transfection, the cells were exposed for 48 h to ritonavir (ranging from 0 to 60  $\mu$ M), and cell viability was measured with an MTT assay.

### Generation of survivin expression MIEG3 vectors

Human survivin cDNA was cloned upstream of the internal ribosomal entry site for enhanced green fluorescence protein. The survivin sense orientation expression plasmid (S-IRES-EGFP-MIEG3) was constructed using an *EcoRI* adaptor as described<sup>10</sup>. Empty MIEG3 plasmid was used as control vector. Flow cytometry was used to sort A549 or H522 cells transiently transfected with the S-IRES-EGFP-MIEG3.

### Effects of drug combination on cell growth

Cells were exposed to ritonavir and/or gemcitabine at a fixed constant ratio corresponding to the ratio of the  $IC_{50}$  values for each single drug. The CalcuSyn software (Biosoft; Cambridge, U.K.) – which uses the median-effect analysis method of Chou and Talalay – was used to determine the possible synergic effect of the drugs. According to this method a combination index (CI) can be calculated from dose-response curves obtained using the drugs as single agent or in combination.  $CI < 1.0$  indicates synergy,  $CI = 1.0$  indicates that the drugs act in an additive fashion, and  $CI > 1.0$  indicates antagonism.

### Statistical analysis

Statistical analysis was conducted using two-tailed Student's t test (Excel, Microsoft). A difference was considered significant if  $p < 0.05$ .

## Results

### Ritonavir inhibits growth and colony formation of lung adenocarcinoma lines

The effect of ritonavir on the growth of the A549 and H522 lines was assayed by MTT assay and compared to vehicle (DMSO). Ritonavir inhibits the growth of the lung adenocarcinoma

lines A549 and H522 lines, exhibiting IC<sub>50</sub> values of 35 and 42 μM, respectively (Fig. 1a). Ritonavir also inhibits the growth of the lung adenocarcinoma lines H23 and H838, exhibiting IC<sub>50</sub> values of 44 and 35 μM, respectively, confirming that ritonavir is active across a range of lung adenocarcinoma lines (results for H23 not shown; results for H838 shown in Fig. S1). Ritonavir was also effective at inhibiting adhesion-dependent colony formation of the A549 and H522 lines, exhibiting IC<sub>50</sub> values of 30 and 40 μM, respectively (Fig. 1b).

### Ritonavir induces apoptosis of lung adenocarcinoma lines

To determine whether ritonavir induces apoptosis, the adenocarcinoma lines A549 and H522 were exposed to ritonavir at the IC<sub>50</sub> for 48 h, stained with PI/Annexin-V-FITC and analyzed by flow cytometry. Ritonavir treatment increased the percentage of early apoptotic cells 10-fold from 1.6 to 16%, for the A549 line, and 3-fold, from 3.2 to 10%, for the H522 line (Fig. 1c). The percentage of late apoptotic cells increased 10-fold, from 0.6 to 5.9% for the A549 line, and two-fold from 0.6 to 1.1% for the H522 line.

### Ritonavir induces a G0/G1 cell cycle arrest

To determine whether ritonavir inhibits cell cycle progression of lung adenocarcinoma cells, the A549 and H522 lines were grown in the presence of ritonavir at either its IC<sub>50</sub> (40 μM) or half the IC<sub>50</sub> (20 μM) or in the presence of vehicle (DMSO) for 24 and 48 h. The cycle distribution was determined by flow cytometry. For both lines, incubation with ritonavir for 48 h resulted in a dose-dependent G0/G1 cell cycle block, as demonstrated by increase of the G0/G1 population and by a corresponding reduction of the S and G2/M populations (Fig. 2a, Table S2). Similar results were obtained after 24 h incubation with ritonavir (results not shown).

### Ritonavir down-regulates CDK2, CDK4, CDK6, and cyclin D<sub>1</sub> levels and inhibits pRb, while up-regulating p27<sup>Kip1</sup> and wild type p53

Because ritonavir induces a G0/G1 block in lung adenocarcinoma lines (Fig. 2a), it was investigated whether ritonavir affects the levels of proteins known to regulate the G1/S checkpoint. Both the A549 and H522 lines exhibit reduction of G1/S check point regulators CDK2, CDK4, CDK6, and cyclin D<sub>1</sub>, following 48 h of treatment at the ritonavir IC<sub>50</sub> (Fig. 2b; Table S3). Correlating with reduction of CDKs and cyclin D<sub>1</sub>, phospho-Rb levels were reduced by more than 80% (Fig. 2b; Table S3). Cyclin A, involved in S phase progression, was also decreased by ritonavir (Fig. 2b; Table S3). Furthermore, p27<sup>Kip1</sup>, an important inhibitor of cyclin E/CDK2 complexes was increased in both lines. The A549 line is p53 wild type, while the H522 line is p53 mutant (codon 191) and the effects of ritonavir differed, with p53 increasing in the wild type line and decreasing in the mutant line (Fig. 2b and Table S3).

### Effects of ritonavir on survivin, c-Src, STAT3, and cleaved PARP levels in lung adenocarcinoma lines

The potential roles of survivin, its up-stream regulatory proteins c-Src and STAT3 and their phosphorylated forms, and its down-stream signaling molecule PARP were studied in the A549 and H522 lines. Ritonavir treatment significantly reduced survivin expression in both cell lines (Fig. 3a; Table S4a). In both lines, ritonavir reduced fractional phosphorylation of c-Src by more than half (Fig. 3a; Table S4a). Total c-Src was also reduced in the H522 line, but not A549 (Fig. 3a; Table S4a). Ritonavir treatment reduced the fractional phosphorylation of STAT3 by 40% in both cell lines, but did not affect STAT3 levels (Fig. 3a; Table S4a). In both lines, ritonavir significantly increased the levels of cleaved PARP (Fig. 3a; Table S4a).

### Effects of ritonavir on survivin mRNA levels in lung adenocarcinoma lines

To determine whether reduction in survivin protein levels by ritonavir corresponds to a down-regulation of its message, survivin mRNA levels were measured by real-time PCR. Survivin mRNA was reduced by 70 to 80% in ritonavir-treated lung adenocarcinoma lines (Fig. 3b). These results indicate that ritonavir-mediated reduction of survivin is mediated largely through reduction of survivin mRNA.

### RNAi profiling of survivin or its regulators c-Src and STAT3 reveals that survivin is the most important ritonavir target

Recently, RNAi silencing has been used to identify genes whose expression affects drug responses in cancer<sup>38</sup>. In the present study, the regulatory pathway for survivin expression, which is mediated by c-Src and STAT3, was profiled by RNAi with the purpose of testing the relative importance of the members of this pathway as ritonavir targets. To verify the effectiveness of the RNAi approaches, the protein levels of survivin, c-Src and STAT3 were determined. Knock down of c-Src, STAT3 and survivin reduced their corresponding proteins by 50, 70 and 50%, respectively (Fig. 4a, Table S4b). Knock down of c-Src significantly reduced survivin and STAT3 levels in both lines, indicating that there is a direct relationship between c-Src levels and its downstream signaling proteins (Fig. 4a, Table S4b). Knock down of STAT3 reduced survivin in the H522 line only (Fig. 4a, Table S4b). These results indicate that survivin levels are determined, in part, by c-Src and STAT3 levels, confirming a model of a signaling hierarchy between these regulatory proteins in NSCLC. Inhibitory effects of survivin, c-Src and STAT3 knock down on the ritonavir-sensitive lung adenocarcinoma lines were verified by MTT assay. siRNA targeting c-Src, STAT3, or survivin significantly inhibits the growth of both the A549 and H522 lines at 48 h (Fig. 4b). After establishing the effectiveness of the RNAi silencing of c-Src, STAT3 and survivin, this approach was used to determine the relative importance of the survivin pathway members as ritonavir targets. Only survivin knock down shifted the MTT dose response curve significantly to the left for the A549 and H522 lines, indicating the importance of survivin levels for ritonavir sensitivity (Fig. 4c). In contrast, despite the hierarchy placing c-Src and STAT3 up-stream of survivin, c-Src, STAT3 and non-target RNAi failed to affect the ritonavir IC<sub>50</sub> for either line. After survivin RNAi treatment, the ritonavir IC<sub>50</sub> decreased to 25  $\mu$ M for both lines (Fig. 4c). Testing ritonavir at its IC<sub>50</sub> in combination with survivin siRNA confirmed that reduction of survivin sensitizes lung cancer lines to ritonavir (Fig. 4d).

### Over-expression of survivin induces resistance to ritonavir in lung adenocarcinoma lines

Because survivin knock down sensitizes lung adenocarcinoma lines to ritonavir, it was determined whether survivin over-expression leads to ritonavir resistance. Survivin expression was increased three-fold in both lines by the S-IRES-EGFP-MIEG3 transfection (Fig. 5a). Transfection with the survivin expression plasmid resulted in significant increases of the ritonavir IC<sub>50</sub> for both lines (10 to 20  $\mu$ M) (Fig. 5b). Therefore survivin over-expression causes resistance to ritonavir while survivin knock down causes sensitivity, identifying survivin as a critical target for this drug.

### Ritonavir exhibits enhanced anti-cancer activity when combined with gemcitabine and or cisplatin

After identifying ritonavir inhibitory activity in lung adenocarcinoma lines and survivin as its major target, it was important to learn effective ways of combining ritonavir with other chemotherapy agents. This goal can be accomplished by identifying chemotherapy drugs that do not interact pharmacokinetically with ritonavir, thereby avoiding drug interactions that could cause unpredictable toxicity. Ritonavir is known to interact with drugs

metabolized by CYP3A4, by either decreasing or increasing their metabolism<sup>39</sup>. For instance, as a CYP3A4 inhibitor, ritonavir is known to boost the levels of chemotherapy drugs, such as taxanes, vinca alkaloids, and etoposide, making toxicity difficult to predict<sup>40, 41</sup>. In contrast, the widely used lung cancer drug gemcitabine is not known to interact with ritonavir and as such it represents a promising candidate for combined therapy. When gemcitabine was combined with ritonavir at the respective IC<sub>50</sub> for each drug, reduction of survivin was still observed, indicating that gemcitabine does not interfere with ritonavir-mediated reduction of survivin (Fig. 6a, Table S5). Furthermore, in the A549 gemcitabine alone significantly reduced survivin levels and such reduction was enhanced by the combination ritonavir/gemcitabine. When tested for synergy by Chou Talalay isobologram analysis<sup>42</sup>, the combination ritonavir/gemcitabine exhibited strong synergy (Fig. 6b) with combinatorial index (CI) values for the A549 and H522 lines of  $0.57 \pm 0.33$  and  $0.16 \pm 0.09$ , respectively. Ritonavir exhibits an IC<sub>50</sub> of 15–20  $\mu\text{M}$  in combination with gemcitabine concentrations of 15 to 60 nM, which are well below C<sub>max</sub> concentrations attained clinically with gemcitabine monotherapy<sup>43, 44</sup>. Also cisplatin, which is commonly used in lung cancer chemotherapy, is not known to interact with ritonavir. The ritonavir/cisplatin combination was less active in inhibiting the growth of lung adenocarcinoma lines than the ritonavir/gemcitabine combination, exhibiting CI values of  $1 \pm 0.55$  and  $1.1 \pm 1.16$  for the A549 and H522 lines, respectively (Fig. 6b). These CI values indicate that cisplatin and ritonavir are additive rather than synergistic for the A549 and H522 lines<sup>42</sup>. Similarly to what we observed for the combination ritonavir/gemcitabine, ritonavir IC<sub>50</sub> was reduced to ~20  $\mu\text{M}$  in combination with cisplatin at 4.5  $\mu\text{M}$ .

Since the combination gemcitabine/cisplatin is a standard regimen for the treatment of lung adenocarcinoma<sup>45</sup>, we also tested the effect of ritonavir in combination with gemcitabine/cisplatin in the A549 and H522 lines. The ritonavir/gemcitabine/cisplatin combination acted synergistically in the A549 line (CI=0.6  $\pm$  0.2) and was slightly superior to the combination gemcitabine/cisplatin (Fig. 6c). On the other hand, in the H522 line, the combination ritonavir/gemcitabine/cisplatin acted additively (CI=0.9  $\pm$  0.2) but it was inferior to the gemcitabine/cisplatin combination (Fig. 6c). Importantly, in both cell lines, ritonavir IC<sub>50</sub> was < 10  $\mu\text{M}$  in combination with concentrations of gemcitabine and cisplatin well below their IC<sub>50</sub>.

## Discussion

Survival of recurrent/metastatic NSCLC with palliative chemotherapy fails to exceed 1 y and there is an unmet need for new drugs and drug combinations that work through novel mechanisms<sup>46</sup>. In the present study, we propose ritonavir as a candidate drug for metastatic lung adenocarcinoma clinical trials, based on its inhibition of adenocarcinoma lines at concentrations in the 35–45  $\mu\text{M}$  range. Such concentrations are clinically attainable, albeit with significant gastrointestinal toxicity<sup>47</sup>, with ritonavir monotherapy at 600 mg twice daily<sup>48</sup>.

Study of signaling pathways affected by ritonavir by siRNA profiling revealed that survivin is an important target, whereas c-Src and STAT3 appear to be of lesser importance. Furthermore, forced over-expression of survivin confers relative resistance to ritonavir, confirming importance of survivin as a ritonavir target. Ritonavir reduces survivin, in part, by reducing its mRNA levels. Because survivin is regulated in cancer primarily through its mRNA expression<sup>49</sup> these results suggest that ritonavir is likely attacking a basic mechanism of survivin transcriptional regulation.

Ritonavir inhibits lung adenocarcinoma growth and anchorage-dependent clonogenicity, in part, by inducing G0/G1 cell cycle arrest and, in part, by inducing apoptosis. Survivin is

implicated in regulation of the G1/S<sup>8, 9, 11</sup> as well as G2/M checkpoint and therefore its reduction by ritonavir is expected to cause inhibition of the cell cycle. Ritonavir may also inhibit the G1/S checkpoint through down-regulation of CDKs, cyclin D<sub>1</sub> and associated Rb phosphorylation, as well as by induction of p27<sup>Kip</sup> and wild type p53. While reduction of survivin by ritonavir is expected to promote apoptosis in lung cancer presumably related to loss of the antiapoptotic effect of survivin<sup>50</sup>, the remaining mechanisms by which ritonavir induces apoptosis remain to be determined. These mechanisms could include induction of DNA damage, in part, through increased cleavage of PARP1.

Importantly, drug combination studies revealed that ritonavir is active at lower concentrations when combined with gemcitabine, cisplatin and the gemcitabine/cisplatin combination. In combination with gemcitabine or cisplatin, ritonavir exhibits IC<sub>50</sub> values in the range of 15–20 μM. With the gemcitabine/cisplatin combination, the ritonavir IC<sub>50</sub> is in the 8 μM range, which should be attainable with 100 mg twice daily dosing<sup>48</sup>. While the gemcitabine/cisplatin combination in advanced NSCLC resulted in the longest median time to progression compared to three other chemotherapy doublets, this was only 4.2 months<sup>45</sup>. We hypothesize, based on *in vitro* synergy, that addition of low dose ritonavir to the gemcitabine/cisplatin combination may improve time to progression, with acceptable toxicity. Furthermore, because ritonavir is not myelosuppressive and potentially could be continued through the period of gemcitabine/cisplatin treatment, ritonavir could potentially inhibit re-growth of lung adenocarcinoma between cycles of chemotherapy. Therefore, a phase I study of daily ritonavir in combination with the established gemcitabine and cisplatin schedule is an important next step. While K-ras mutation status did not affect sensitivity to ritonavir, for the H838 K-ras wild type line there was lack of synergy with gemcitabine and antagonism with cisplatin. These results suggest that K-ras mutant lung adenocarcinoma is the best candidate histology for future clinical trials.

Although the mechanisms behind cooperation between ritonavir and gemcitabine and/or cisplatin are not known, it is likely these mechanisms involve survivin effects on DNA repair pathways. Gemcitabine is a DNA strand-terminator that stalls replication forks, causes S phase arrest<sup>51</sup> and double strand breaks (DSB) while inhibiting homologous recombination repair (HRR), which is required for repairing DSB<sup>52, 53</sup>. Survivin has been reported to enhance DSB repair and we hypothesize that reduction of survivin by ritonavir may increase sensitivity to gemcitabine through this mechanism<sup>54</sup>. Survivin reduction may also explain sensitivity of lung adenocarcinoma to ritonavir in combination with cisplatin due to increased PARP1 cleavage.

PARP1 may be involved in repair of cisplatin-induced DNA damage. PARP1 is known to recruit XRCC1 to sites of DNA damage<sup>55</sup>. XRCC1 is a scaffolding factor required for base excision repair (BER)<sup>56</sup> and recently, nucleotide excision repair (NER)<sup>57</sup>. Of interest, interference with NER interferes with repair of cisplatin-induced DNA damage<sup>53</sup>. Although PARP1 has not been implicated as a key regulator of NER, it has been recently been located at sites of cisplatin-induced DNA damage, by two photoaffinity labeling studies<sup>58, 59</sup>. This finding potentially implicates PARP1 in repair of cisplatin-mediated DNA interstrand crosslinks by NER. In addition, PARP1 reduction has also recently been demonstrated to play a critical role in chemosensitivity to the gemcitabine/cisplatin combination in triple negative breast cancer<sup>60</sup>. Future studies will determine the mechanisms by which ritonavir may enhance DNA damage by cisplatin and gemcitabine.

Based on the importance of survivin as a ritonavir target in lung adenocarcinoma, we propose that survivin may be a useful biomarker for ritonavir sensitivity. We hypothesize that among tumors expressing survivin, those exhibiting lower survivin levels will be more likely to respond to ritonavir. Our results from forced survivin over-expression are artificial



and may not reflect survivin levels in tumors occurring in patients and therefore we would not recommend excluding patients with high survivin levels from clinical trials of ritonavir. Only the analysis of data from such trials would reveal whether there is a relationship between survivin levels and ritonavir sensitivity.

## Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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## Nonstandard abbreviations used

<b>CDK</b>	cyclin dependent kinase
<b>CI</b>	combination index
<b>CM</b>	complete medium
<b>DMSO</b>	dimethyl sulfoxide
<b>EGFP</b>	enhanced green fluorescence protein
<b>GAPDH</b>	glyceraldehyde 3-phosphate dehydrogenase
<b>IC<sub>50</sub></b>	half maximal inhibitory concentration
<b>MTT</b>	3, [4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide
<b>NSCLC</b>	non small cell lung cancer
<b>PCR</b>	polymerase chain reaction
<b>PI</b>	propidium iodide
<b>STAT</b>	signal transducer and activator of transcription protein
<b>Rb</b>	retinoblastoma protein

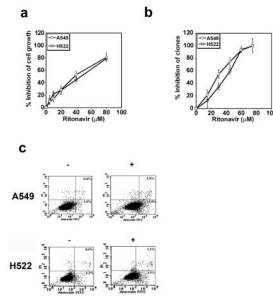
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**Figure 1.**

**Panel a. Ritonavir inhibits growth of the lung adenocarcinoma lines A549 and H522.**

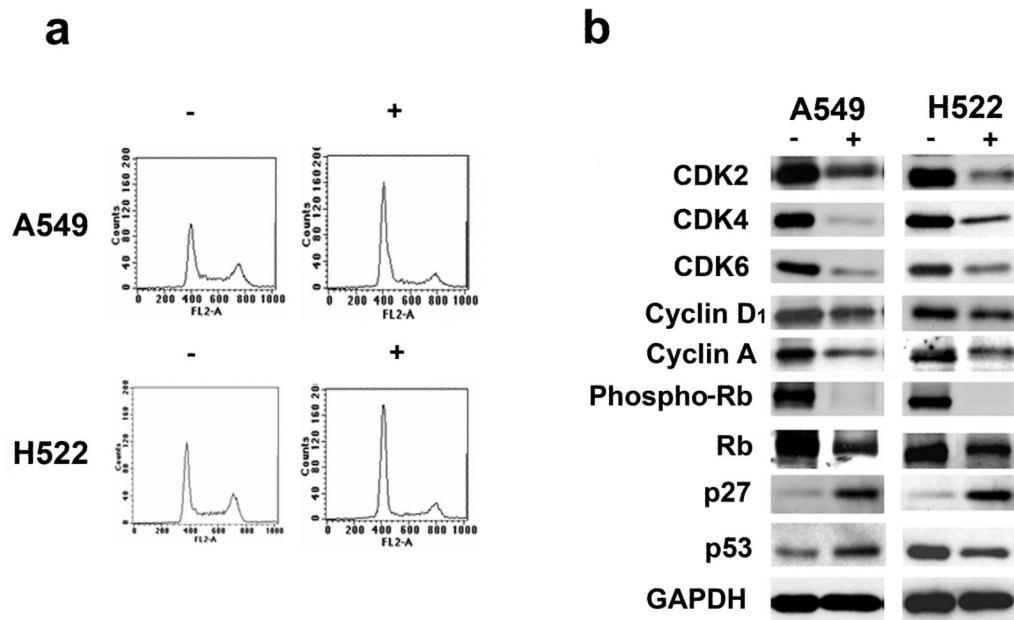
Ritonavir inhibited the growth of both lines, exhibiting an  $IC_{50}$  of  $35\mu M$  for A549 (open circles) and  $42\mu M$  for H522 (open squares). Results are representative of three consistent experiments.

**Panel b. Ritonavir reduces the clonogenic efficiency of the lung adenocarcinoma lines A549 and H522.**

Ritonavir was efficient in reducing the clonogenic efficiency in both the A549 (open circles) and H522 (open squares) lines, exhibiting an  $IC_{50}$  of 30 and  $40\mu M$ , respectively.

**Panel c. Ritonavir induces apoptosis in the lung adenocarcinoma lines A549 and H522.**

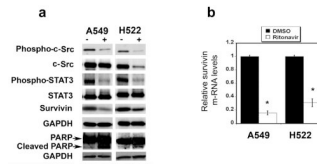
Ritonavir significantly increased early and late apoptotic events in both lines. The results are representative of two consistent experiments.



**Figure 2.**

**Panel a. Ritonavir causes G0/G1 cell cycle arrest in the A549 and H522 lung adenocarcinoma lines.** Ritonavir was able to induce a G0/G1 block as demonstrated by increase of the G0/G1 population and by a corresponding reduction of the S and G2/M populations. Cells growth with ritonavir or vehicle are indicated with (+) and (-), respectively. For quantitation of the flow cytometry analysis see Table 2S).

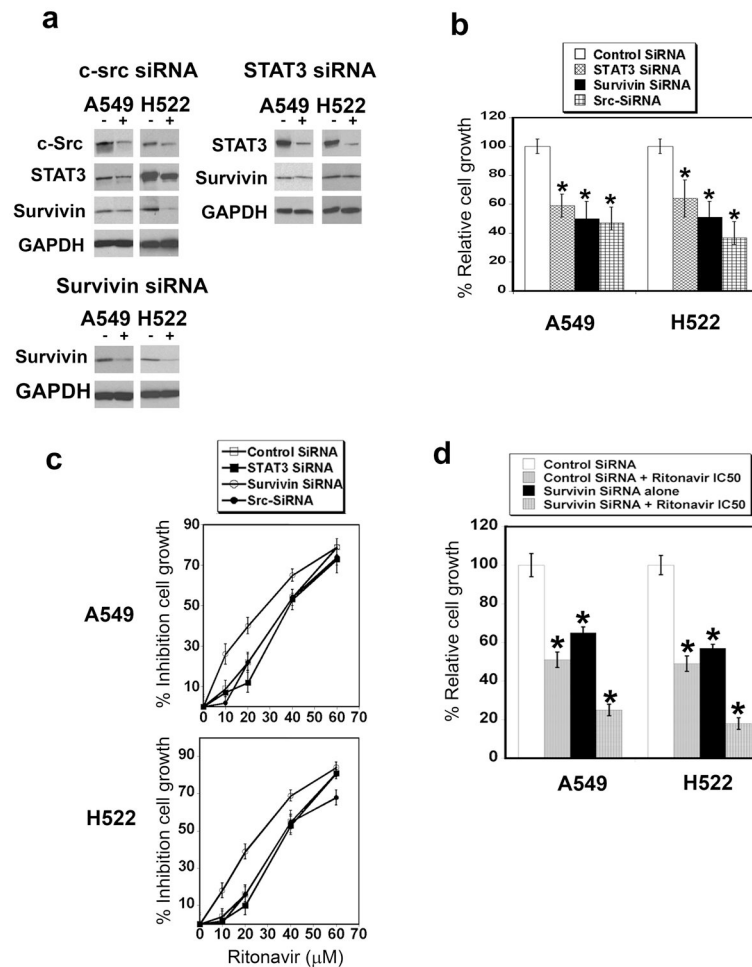
**Panel b. Ritonavir down-regulates the expression of numerous cell cycle-associated proteins in the lung adenocarcinoma lines A549 and H522.** CDK2, CDK4, CDK6, cyclin D<sub>1</sub>, cyclin A, phospho-Rb, and Rb levels were reduced in both lines treated with ritonavir. Levels of p53 were reduced in the H522 line but increased in the A549 line. The levels of p27<sup>Kip1</sup> were increased in both lines. For quantitation of the Western blots see Table S3.



**Figure 3.**

**Panel a. Ritonavir down-regulates the expression of phospho-c-Src, Src, phospho-STAT3, survivin, and increases cleaved PARP in the lung adenocarcinoma lines A549 and the H522.** Both in the A549 and H522 line, ritonavir reduced phospho-c-Src, phospho-STAT3, and survivin levels while increased the cleaved PARP levels; it did not affect total STAT3 levels and it down-regulated total c-Src only in the H522 line. Cells growth with ritonavir or vehicle are indicated with (+) and (-), respectively. For quantitation of the Western blots see Table S4a.

**Panel b. Ritonavir down-regulates survivin mRNA in the lung adenocarcinoma lines A549 and H522.** After 48 h treatment, ritonavir drastically reduced the levels of survivin mRNA in both lines.



**Figure 4.**

**Panel a. c-Src, STAT3, and survivin siRNA reduce the levels of their target proteins.**

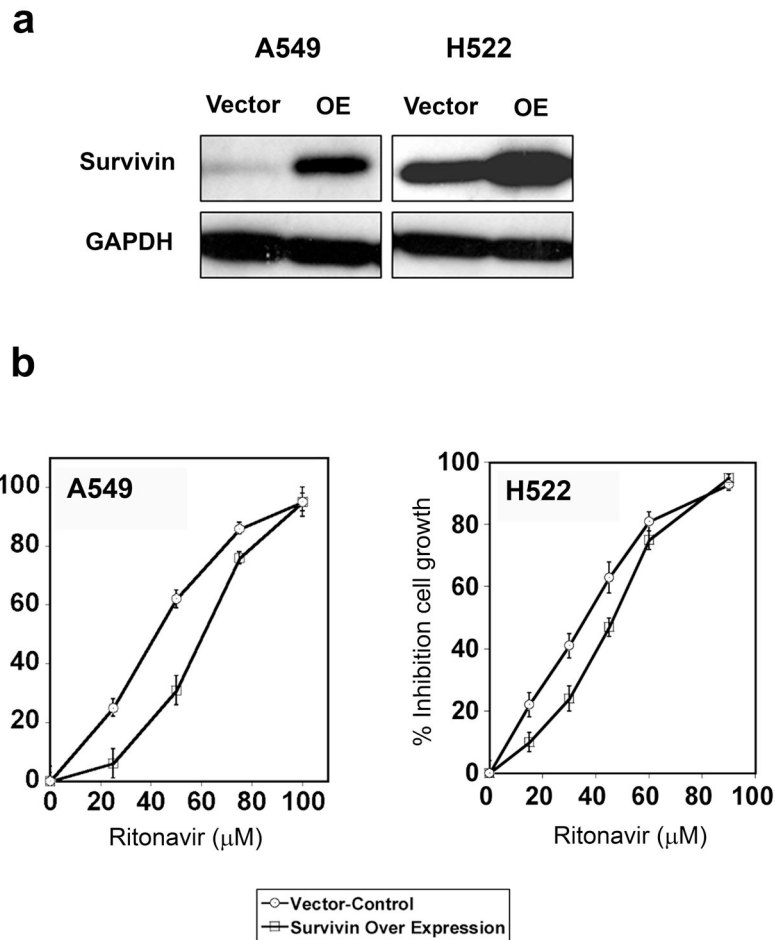
The (–) column indicates non-target siRNA, while the (+) column indicates specific siRNA as labeled. The siRNA to c-Src, STAT3 and survivin reduced the level of the corresponding protein in all three lines. For quantitation of the Western blots see Table S4b.

**Panel b. siRNA targeting c-Src, STAT3, and survivin reduces growth of the lung adenocarcinoma lines A549 and H522.** Cells treated with siRNA targeting c-Src, STAT3, and survivin exhibited reduced growth when compared to cells treated with non-targeting siRNA. P value of <0.05 is indicated with an asterisk.

**Panel c. siRNA targeting c-Src or STAT3 do not reduce ritonavir IC<sub>50</sub> for the lung adenocarcinoma lines A549 and H522.** A549 and H522 cells transfected with siRNA targeting c-Src, STAT3, survivin, or non-targeting siRNA were grown in the presence of increasing concentrations of ritonavir. Silencing of survivin mRNA expression increased the cell sensitivity to ritonavir, reducing its IC<sub>50</sub> to 25 μM for the A549 line and 27 μM for the H522 line.

**Panel d. siRNA targeting survivin increases the sensitivity to ritonavir of the lung adenocarcinoma lines A549 and H522.** A549 and H522 cells transfected with siRNA targeting survivin were treated with ritonavir at the appropriate IC<sub>50</sub>. Survivin siRNA and ritonavir acted together to reduce cell growth in both lines. P value of <0.05 is indicated with an asterisk.





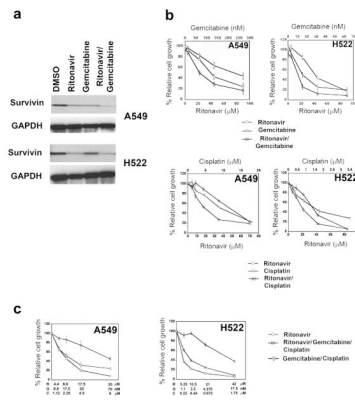
**Figure 5.**

**Panel a. Over-expression of survivin in the lung adenocarcinoma lines A549 and H522.**

Cells transiently transfected with human survivin cDNA had significantly higher levels of survivin when compared to cells transfected with the empty vector.

**Panel b. Over-expression of survivin increases resistance the lung adenocarcinoma**

**lines A549 and H522 to ritonavir.** Over-expression of survivin reduced the cell sensitivity to ritonavir, increasing its IC<sub>50</sub> to 45µM for the A549 line and 55µM for the H522 line.



**Figure 6.**

**Panel a.** Gemcitabine and ritonavir down-regulate the survivin levels of the lung adenocarcinoma lines A549, H522. Ritonavir was able to reduce the levels of survivin either when used alone or together with gemcitabine. For quantitation of the Western blots see Table S5.

**Panel b. Ritonavir and gemcitabine synergize in inhibiting the growth of the lung adenocarcinoma lines A549, H522.** The combination gemcitabine and ritonavir had a synergic effect and was significantly more effective in reducing growth compared to each drug alone. On the other hand, the combined effect of ritonavir and cisplatin was additive for the A549 and H522 lines.

**Panel c. The combination ritonavir/gemcitabine/cisplatin is synergistic in the A549 line and additive in the H522 line.** The concentrations of ritonavir (R), gemcitabine (G) and cisplatin (C) are indicated on the X axis. The combination ritonavir/gemcitabine/cisplatin acted synergistically in the A549 line and additively in the H522 line. The combination gemcitabine/cisplatin was superior to ritonavir/gemcitabine/cisplatin in the H522 line but not in the A549 line.