Nelfinavir Inhibits the Growth of Small-cell Lung Cancer Cells and Patient-derived Xenograft Tumors

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Abstract. *Background/Aim:* Small-cell lung cancer (SCLC) is aggressive and confers poor prognosis. Although SCLC shows more response to chemotherapy than other types of lung cancer, it is difficult to cure because of its frequent recurrence. New drugs and molecular targets need to be identified. *Materials and Methods:* We investigated the effect of nelfinavir, an HIV protease inhibitor, on SCLC cells and in preclinical treatment studies using SCLC patient-derived xenograft (PDX) mouse models. *Results:* Nelfinavir inhibited SCLC cell proliferation and induced cell death *in vitro*, which was caused by induction of the unfolded protein response (UPR), inhibition of mammalian/mechanistic target of rapamycin (mTOR) activation, and reduction in the expression of SCLC-related molecules such as achaete-scute homolog 1 (ASCL1). *In vivo*, nelfinavir inhibited the growth of SCLC PDX tumors, which correlated with the induction of UPR and reduced expression of ASCL1. *Conclusion:* Nelfinavir is highly effective in SCLC *in vitro* and *in vivo*, suggesting possible incorporation of nelfinavir into clinical trials for patients with SCLC.

Small-cell lung cancer (SCLC) comprises 10-15% of all lung cancer cases. Because of its rarity, it is difficult to evaluate the molecular and functional characteristics of SCLC, resulting in the underdevelopment of new therapies compared with non-small cell lung cancer (NSCLC). Although SCLC shows more

response to chemotherapy than other types of lung cancer, it is difficult to cure because of its frequent recurrence (1). Recent evidence shows that critical oncogenic changes such as inactivation of tumor protein p53 (*TP53*) and retinoblastoma 1 (*RBI*) genes, are found in nearly all human SCLC tumors, and amplifications of the myelocytomatosis oncogene (*MYC*) family or fibroblast growth factor receptor (*FGFR1*) tyrosine kinase gene (2). Additionally, ASCL1 is a transcription factor that is required for the development of pulmonary neuroendocrine cells and is reported as a lineage-dependent oncogene that plays a role in tumorigenesis arising from dysregulation of genes involved in normal development (3, 4). SRY-box transcription factor 2 (*SOX2*) is a transcription factor that maintains self-renewal and pluripotency of undifferentiated embryonic stem cells, and has been identified as a gene amplified in SCLC (5). Therapeutic developments targeting these genetic alterations in SCLC are thus necessary.

Previously, we demonstrated that nelfinavir, a human immunodeficiency virus protease inhibitor, inhibited the proliferation of NSCLC cells *in vitro* and in human NSCLC xenograft tumors by inducing endoplasmic reticulum stress and apoptosis (6). Additionally, nelfinavir shows synergistic effects on NSCLC and multiple myeloma cells with a proteasome inhibitor, bortezomib, by enhancing endoplasmic reticulum stress (7). Nelfinavir has been repurposed as an anticancer drug for lung cancer. More recently, we reported the data of a phase I trial of nelfinavir in adults with solid tumors. Nelfinavir is well-tolerated and shows antitumor activity, particularly in patients with neuroendocrine carcinoma of the midgut and pancreas (8). These findings led us to hypothesize that nelfinavir shows efficacy against SCLC, especially the neuroendocrine carcinoma subtype. We assessed the efficacy of nelfinavir against SCLC cells and conducted an analysis of signal transduction to investigate new molecular therapeutic targets *in vitro* and in preclinical treatment studies using SCLC patient-derived xenograft (PDX) mouse models.

Materials and Methods

Reagents. Nelfinavir used in the *in vitro* studies was obtained through the National Institutes of Health AIDS Research and Reference

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Reagent Program, Division of AIDS, NIAID, National Institutes of Health. Nelfinavir used in the *in vivo* study was obtained from Pfizer Inc. (New York, NY, USA). Rapamycin (an inhibitor of mechanistic target of rapamycin kinase, mTOR) and tunicamycin (endoplasmic reticulum stress inducer) were obtained from LC Laboratories (Woburn, MA, USA) and Sigma (St. Louis, MO, USA), respectively. Primary antibodies for aurora B, actin, c-MYC (D84C12), SOX2 (D6D9), activating transcription factor 4 (ATF4, D4B8), CCAAT-enhancer-binding protein homologous protein (CHOP, L63F7), sestrin-2 (SES2, D1B6), phospho-5' AMP-activated protein kinase α (P-AMPK α , Thr172) (40H9), AMPK α , P-S6 ribosomal protein (Ser235/236), S6 ribosomal protein, cleaved/total poly ADP-ribose polymerase (PARP), protein kinase B (AKT), P-AKT (Ser473, D9E), and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) were from Cell Signaling Technology (Beverly, MA, USA). Anti-n-MYC (C-19), l-MYC (C-20), and ATF3 (C-19) antibodies were from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Anti-ASCL1 (MASH1, clone 24B72D11.1) and α -tubulin antibodies were purchased from BD Pharmingen™ (San Jose, CA, USA) and Sigma, respectively.

Cell cultures. SCLC (H526, H82, H146, and H69) cell lines were kindly provided by Dr. Charles M. Rudin (Memorial Sloan Kettering Cancer Center, New York, NY, USA). H146 and H69 cells express SOX2 (5). All cell lines were maintained in 75 cm² flasks in RPMI 1640 (Life Technologies, Grand Island, NY, USA) with 5% fetal bovine serum (Life Technologies) at 37°C in an incubator with 5.0% CO₂.

Cell proliferation assay. SCLC cells (15,000 live cells per well) were plated in 96-well plates and treated with the following drugs dissolved in dimethyl sulfoxide (DMSO) for 72 h at increasing concentrations of between 1.25 and 20 μ M for nelfinavir, 0.1 and 1000 nM for rapamycin, or 0.01 and 10 μ g/ml for tunicamycin. Growth inhibition was determined using the WST1 assay according to the manufacturer's protocol (Roche Diagnostics; Indianapolis, IN, USA). The percentage growth value was calculated using the absorbance values of untreated cells on day 0 (D₀), and of DMSO-treated control cells (C), and drug-treated cells (T) as follows: $[(T - D_0)/(C - D_0)] \times 100$ for concentrations for which $T \geq D_0$, or $[(T - D_0)/D_0] \times 100$ for concentrations for which $T < D_0$. Experiments were performed three times, and each drug concentration was evaluated in sextuplet wells for a given experiment.

Cell death assay. Cells (2.5×10⁵ cells per well) were plated in 12-well plates and treated with nelfinavir at 10 μ M or an equal volume of DMSO for 72 h. Following incubation, cells were harvested and resuspended in a solution of 1 mg/ml propidium iodide in phosphate-buffered saline and then immediately acquired on the FL3 channel of a flow cytometer using a Becton Dickinson FACSsort and by manual gating using CellQuest software (FACSsort, BD Biosciences, San Jose, CA, USA). The propidium iodide-positive population of cells was considered non-viable, whereas the propidium iodide-negative population was considered viable.

Immunoblotting analysis. Cells (5×10⁵ cells per well) were plated in 6-well plates and treated with nelfinavir at 10 μ M or 20 μ M for 4, 12, or 24 h; rapamycin at 100 nM for 12 h; tunicamycin at 1 μ g/ml for 12 h; or an equal volume of DMSO and then lysed in 2× lysis buffers as described previously (22). For tumor-tissue homogenates *in vivo*, frozen tumors were allowed to thaw on ice, then homogenized in

radioimmunoprecipitation assay buffer [150 mmol/l NaCl, 1% Igepal CA-630, 0.5% sodium deoxycholate, 0.1% sodium dodecyl sulphate, 50 mmol/l Tris (pH 8.0)] containing 2.5 mol/l β -glycerol phosphate, 0.2 mol/l sodium orthovanadate, 1.25 mol/l sodium fluoride, and 1 × protease inhibitor cocktail (Roche Diagnostics) using a hand-held Tissue-Tearor homogenizer (Biospec Products, Bartlesville, OK, USA). Cell lysates or tumor-tissue homogenates with equal amounts of protein were separated using sodium dodecyl sulphate-polyacrylamide gel electrophoresis and then transferred to nitrocellulose membranes. The membranes were blocked for 1 h in blocking buffer (1× TBS, 5% milk, 0.1% Tween 20) and placed in primary antibodies described above, which were diluted in 1× TBS, 5% bovine serum albumin, 0.1% Tween 20, overnight at 4°C. On the following day, membranes were washed three times in wash buffer (0.1% Tween 20, 1× TBS). Primary antibodies were detected using horseradish peroxidase-linked secondary antibodies and visualized with the enhanced chemiluminescent detection system (Amersham Biosciences, Pittsburgh, PA, USA). Immunoblot experiments were performed at least three times.

Drug treatment SCLC PDX models. LX48 and LX44 PDX tumor cells were isolated from patients with extended-stage SCLC and were maintained solely in immunocompromised mice by serial passaging as previously described (9-11). For nelfinavir treatment studies, 6-week-old female NOD/SCID mice (Charles River Labs, Frederick, MD, USA) were injected subcutaneously in both rear flanks with 2×10⁶ LX48 or LX44 PDX tumor cells in 50 μ l phosphate-buffered saline and 50 μ l BD Matrigel Basement Membrane Matrix (BD Biosciences). When the transplanted tumors reached a volume of 50-100 mm³, mice were divided into the following two groups (10 mice per group): intraperitoneal injection of either vehicle (4% DMSO, 5% polyethylene glycol, 5% Tween 80 in saline) once daily for 13 or 15 days, or 100 mg/kg nelfinavir once daily for 13 or 15 days. Animal weights and tumor size were measured every other day. In all studies, tumor volume was calculated using the formula $v=(ab^2)/2$, where a was the long axis and b was the short axis of the tumor. We conducted *in vivo* experiments using a protocol approved by the Animal Care and Use Committee of the Johns Hopkins University, Baltimore, Maryland (Protocol Number: MO12M148). The primary study endpoint was the efficacy of tumor growth inhibition, and a secondary endpoint was the evaluation of the biomarkers.

Statistical analysis. Statistical significance of differences observed in drug-treated and untreated cells was analyzed using one-way ANOVA, and multiple comparisons were then performed using the Bonferroni test. All analyses were performed using GraphPad Prism software version 9 (GraphPad Software, Inc., San Diego, CA, USA). Statistical significance was set at $p < 0.05$.

Results

Nelfinavir inhibits proliferation of SCLC cells. To assess the effects on cellular proliferation, nelfinavir was tested at different concentrations in a series of four SCLC cell lines. Nelfinavir showed cytostatic effect at 10 μ M nelfinavir for 72 h (Figure 1A) and cytotoxicity at 20 μ M (Figure 1A and B). The efficacy of nelfinavir against SCLC cells was confirmed by measuring total cell death (Figure 1C). Treatment with 10 μ M

Figure 1. Nelfinavir (NFV) inhibits proliferation of small-cell lung cancer (SCLC) cells. A: Growth inhibition by NFV. SCLC cells were treated with NFV at the indicated concentrations for 72 h. B: SCLC cells were treated with either dimethyl sulfoxide (DMSO) or 20 μ M NFV for 72 h. Optical microscopy images are shown (40 $\times$ magnification). C: For total cell death assay, SCLC cells were treated with either 0.1% DMSO or 10 μ M NFV for 72 h, and then harvested and analyzed using cell death assays as described in the Materials and Methods. Data are the mean $\pm$ SD of at least three separate experiments. Significantly different at: *** p <0.001 and **** p <0.0001 compared with vehicle treatment.

nelfinavir for 72 h inhibited cell viability in SCLC cells, except for H69 cells. To confirm the efficacy of nelfinavir against H69 cells, we assessed apoptosis by quantifying sub-2N DNA by flow cytometry. Treatment with 20 μ M nelfinavir for 48 h increased the proportion of apoptotic H69 cells (data not shown). These data suggest that nelfinavir is highly effective against SCLC cells *in vitro*.

Nelfinavir induces unfolded protein response (UPR), inhibits activation of mTOR, and reduces the expression of SCLC-related molecules. Because nelfinavir induced UPR and increased the expression of the endogenous mTOR inhibitor SESN2 in breast and ovarian cancer cells and cervical adenocarcinoma cells (12), we performed biomarker analysis of the SCLC cells treated with nelfinavir in a time- and dose-dependent manner. As shown in Figure 2, nelfinavir increased expression of the markers of UPR, such as ATF4, ATF3 and CHOP, as well as the expression of SESN2, which was associated with mTOR inhibition in SCLC cell lines, except for H69, which has a PIK3CA activating mutation (13). Interestingly, AMPK α was activated within 4 h, which was independent of the induction of SESN2. In terms of SCLC-related molecules, nelfinavir reduced the expression of n-MYC, c-MYC, and aurora B in SCLC cells in a dose-dependent manner (Figure 2). Although n-MYC in H69 cells was slightly inhibited by treatment with 20 μ M nelfinavir for 12 h, we confirmed that nelfinavir at the same dose reduced the expression of n-MYC within 4 h in the early phase (data not shown). ASCL1 was clearly inhibited in H526, H82 and H146 cells, as was SOX2 in H146 cells; however, there was less inhibition of both in H69 cells. Taken together, these findings indicate that nelfinavir has pleiotropic mechanisms of action for the inhibition of growth of SCLC cells.

Pharmacological manipulation was performed to evaluate inhibition of mTOR and induction of UPR. To assess whether mTOR inhibition and UPR induction by nelfinavir are involved in the growth inhibition of SCLC cells, we treated these cells with either rapamycin or tunicamycin. Although H82 cells were resistant to rapamycin, the other cells showed growth inhibition by nelfinavir, which was associated with mTOR inhibition, but no alteration in UPR markers and SCLC-related molecules (Figure 3A). In contrast, tunicamycin showed cytotoxicity in H146 cells and a cytostatic effect in the other cell lines, which was associated with UPR induction, as indicated by the increased expression of ATF4, ATF3 or CHOP. Interestingly, tunicamycin inhibited mTOR in SCLC cell lines, except for H146 (Figure 3B). Collectively, these findings indicate that the efficacy of nelfinavir in H526 cells might be based on mTOR inhibition as well as UPR induction, whereas those of nelfinavir in H82 and H146 cells are due to UPR induction rather than mTOR inhibition. The pleiotropic mechanisms of action of nelfinavir against SCLC are cell line-dependent.

Nelfinavir inhibits the growth of SCLC PDX tumors. To confirm the efficacy of nelfinavir *in vivo*, NOD/SCID mice bearing established LX48 (Figure 4A) and LX44 (Figure 4B) PDX tumors were treated with either a vehicle or 100 mg/kg nelfinavir. The treatment was well-tolerated and reduced LX48 PDX tumor growth by nearly 60% ($p<0.001$, Figure 4A, left), in association with increased expression of ATF3 and the inhibition of aurora B, ASCL1, and mTOR ($p<0.05$ and $p<0.01$, Figure 4A, right). In LX44 PDX tumors, nelfinavir was also effective, decreasing growth by nearly 80% ($p<0.0001$, Figure 4B, left), which was associated with increased expression of ATF4 and the inhibition of n-MYC, l-MYC, and aurora B ($p<0.05$ and $p<0.01$, Figure 4B, right). Collectively, these findings show that nelfinavir is highly effective against SCLC *in vivo* as well as *in vitro*.

Discussion

We investigated the efficacy of nelfinavir repurposed as an anticancer drug in the treatment of SCLC and demonstrated that nelfinavir showed antiproliferative effects, with mTOR inhibition and UPR induction as the mechanisms of action *in vitro* (Figure 5). Furthermore, nelfinavir inhibited the growth of SCLC PDX tumors, which was associated with mTOR inhibition and UPR induction. Additionally, SCLC-related molecules such as MYC, aurora B, ASCL1, and SOX2 were inhibited by nelfinavir both *in vitro* and *in vivo* (Figure 5). Aurora B is a serine/threonine kinase that is associated with microtubules during chromosome movement in mitosis, and the inhibition of aurora B kinase is involved in a synthetic lethal interaction for cells that overexpress MYC by inhibiting cytokinesis through failure of both the G₁/S and G₂/M checkpoints (14). Additionally, Sos *et al.* reported that knockdown of aurora B kinase resulted in a reduction of cell viability in MYC-amplified SCLC cells (15). Although the mechanisms of action by which nelfinavir reduces the expression of MYC, aurora B, ASCL1, and SOX2 remain to be determined, nelfinavir is a potent multi-targeted inhibitor for SCLC treatment.

We found that nelfinavir inhibited mTOR activation, as S6 phosphorylation decreased in SCLC cell lines, except for H69 (Figure 2). Brüning *et al.* reported that nelfinavir induced ATF4, which regulated the expression of SESN2, resulting in mTOR inhibition (12), which supports the results presented here. In contrast, we found that mTOR was inhibited within 4 h before inducing the expression of SESN2 by nelfinavir. SESN2-independent mTOR inhibition in the early phase might be caused by the activation of AMPK α during treatment with nelfinavir because AMPK α is a negative regulator of mTOR through the activation of tuberous sclerosis 2 (16). Nelfinavir causes mitochondrial depolarization in lung cancer cells (data not shown), which activates AMPK α (17). In H69 cells (Figure 2), nelfinavir

Figure 2. Nelfinavir (NFV) induces the unfolded protein response (UPR), inhibits mammalian/mechanistic target of rapamycin (mTOR) activation, and reduces the expression of small-cell lung cancer (SCLC)-related molecules. SCLC cell lines H526, H82, H146 and H69 were treated with 0.1% dimethyl sulfoxide (D), 10 μ M, or 20 μ M nelfinavir for 4, 12 and 24 h. SCLC-related markers were assessed using immunoblotting analysis. ATF4: Activating transcription factor 4; ATF3: activating transcription factor 3; CHOP: CCAAT-enhancer-binding protein homologous protein; SESN2: sestrin-2; AMPK: 5' AMP-activated protein kinase; S6: S6 ribosomal protein; PARP: poly ADP-ribose polymerase; MYC: myelocytomatosis oncogene; ASCL1: achaete-scute homolog 1; GAPDH: glyceraldehyde-3-phosphate dehydrogenase; and SOX2: SRY-box transcription factor 2.

Figure 3. Pharmacological manipulation for inhibition of mammalian/mechanistic target of rapamycin (mTOR) and induction of the unfolded protein response in small-cell lung cancer (SCLC) cell lines H526, H82, H146 and H69. Inhibition of mTOR by rapamycin (Ra) (A) and induction of the unfolded protein response by tunicamycin (TM) (B). Left panel: SCLC cells were treated with rapamycin or tunicamycin at the indicated concentrations for 72 h. Growth inhibition was assessed using a cell proliferation assay as described in the Materials and Methods. Data are the mean±SD of at least three separate experiments; note concentration axes are logarithmic. Right panel: SCLC cells were treated with 100 nM rapamycin or 1 µg/ml tunicamycin for 12 h and biomarker analyses were then performed using immunoblotting analysis. MYC: Myelocytomatosis oncogene; ASCL1: achaete-scute homolog 1; SOX2: SRY-box transcription factor 2; ATF4: activating transcription factor 4; ATF3: activating transcription factor 3; CHOP: CCAAT-enhancer-binding protein homologous protein; S6: S6 ribosomal protein.

Figure 4. Nelfinavir (NFV) inhibits the growth of small-cell lung cancer (SCLC) patient-derived xenograft (PDX) tumors. LX48 (A) and LX44 (B) PDX tumors were grown in NOD/SCID mice. Biomarker analyses for SCLC treatment with nelfinavir were performed using immunoblotting analysis in each right panel. At the end of the study, LX48 and LX44 PDX tumors were excised for immunoblotting analysis of the following markers: Aurora B; achaete-scute homolog 1 (ASCL1); activating transcription factor 3 (ATF3); P-S6 ribosomal protein (P-S6); n-mycelotomatosis oncogene (n-MYC) and activating transcription factor 3 (ATF4). Densitometry was performed using ImageJ version 1.52 software (25). The level of each marker was normalized to actin (A) or α -tubulin (B) for each sample. Data are the mean+SD of tumors from 10 mice. Significantly different at: * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ and **** $p < 0.0001$ compared with vehicle treatment.

activated AMPK α and increased the expression of ATF4 and SESN2; however, mTOR was not inhibited, which is explained by the fact that H69 cells have a PIK3CA activating mutation (13).

Neuroendocrine neoplasms of the lung are categorized as well-differentiated neuroendocrine tumors (NETs), otherwise termed carcinoid, and neuroendocrine carcinomas, including

SCLC and large-cell neuroendocrine carcinomas that are poorly differentiated. Everolimus, an oral mTOR inhibitor, has shown antitumor activity in adult patients with advanced gastrointestinal or lung NETs, resulting in Food and Drug Administration-approved treatment (18). In contrast, for SCLC, everolimus failed to show a significant effect on survival in patients with relapsed SCLCs (19). Lee *et al*.

Figure 5. Nelfinavir (NFV) induces the unfolded protein response (UPR) and inhibits both the mammalian/mechanistic target of rapamycin (mTOR) pathway and expression of small-cell lung cancer (SCLC)-related molecules. ATF3: Activating transcription factor 3; ATF4: activating transcription factor 4; CHOP: CCAAT-enhancer-binding protein homologous protein; SESN2: sestrin-2; AMPK: 5' AMP-activated protein kinase; MYC: myelocytomatosis oncogene; ASCL1: achaete-scute homolog 1; SOX2: SRY-box transcription factor 2.

reported mTOR activation in limited-stage SCLC to be significantly higher than in extended-stage SCLC, suggesting that i) mTOR activation might play a crucial role in the initiation of SCLC cells, and ii) inhibition of mTOR for limited-stage SCLC might be more effective than for extended-stage SCLC because of high p-mTOR expression (20). This supports our previous finding that mTOR activation is involved in NSCLC tumorigenesis (21, 22). Nelfinavir inhibits both mTOR activation and the expression of SCLC-related molecules that play a role in tumorigenesis, suggesting its efficacy in preventing SCLC development or progression.

Recently, Kern *et al.* reported that mTOR is an essential kinase in a subset of SCLC PDX tumors, including our LX48 mouse model, using functional genomics with an shRNA kinome library. Additionally, mTOR inhibition was found to reduce the growth of PDX tumors and sensitize PDX to conventional chemotherapy, such as cisplatin and etoposide (23). Moreover, nelfinavir combined with other chemotherapies or radiation can be safe and can produce a promising response, based on a phase I/II trial of nelfinavir with concurrent chemoradiation in advanced NSCLC that has been reported (24). These findings support our data that mTOR inhibition by nelfinavir is an attractive target for SCLC therapy, and nelfinavir might sensitize both limited-stage SCLC to concurrent chemoradiation and extended-stage SCLC to conventional chemotherapy.

In conclusion, we demonstrated that nelfinavir is highly effective against SCLC *in vitro* and *in vivo*. Nelfinavir is well-tolerated in patients with cancer and showed its efficacy

against NETs in a phase I trial (8). Collectively, these findings suggest a possible value of incorporating nelfinavir into clinical trials for patients with SCLC.

Conflicts of Interest

The Authors report no financial or other interests that could be construed as conflicts of interest.

Authors' Contributions

Conceived and designed the experiments: S.K. and P.A.D. Performed the experiments: S.K., N.C. and J.J.G. Analyzed the data: S.K., J.J.G. and C.L.H. Wrote the article: S.K. and P.A.D.

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