

Original Article

Duloxetine enhances the sensitivity of non-small cell lung cancer cells to EGFR inhibitors by REDD1-induced mTORC1/S6K1 suppression

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Abstract: Although epidermal growth factor receptor-tyrosine kinase inhibitors (EGFR-TKIs) have been effective targeted therapies for non-small cell lung cancer (NSCLC), most advanced NSCLC inevitably develop resistance to these therapies. Combination therapies emerge as valuable approach to preventing, delaying, or overcoming disease progression. Duloxetine, an antidepressant known as a serotonin-noradrenaline reuptake inhibitor, is commonly prescribed for the treatment of chemotherapy-induced peripheral neuropathy. In the present study, we investigated the combined effects of duloxetine and EGFR-TKIs and their possible mechanism in NSCLC cells. Compared with either monotherapy, the combination of duloxetine and EGFR-TKIs leads to synergistic cell death. Mechanistically, duloxetine suppresses 70-kDa ribosomal protein S6 kinase 1 (p70S6K1) activity through mechanistic target of rapamycin complex 1 (mTORC1), and this effect is associated with the synergistic induction of cell death of duloxetine combined with EGFR-TKIs. More importantly, activating transcription factor 4 (ATF4)-induced regulated in development and DNA damage response 1 (REDD1) is responsible for the suppression of mTORC1/S6K1 activation. Additionally, we found that the combination effect was significantly attenuated in REDD1 knockout NSCLC cells. Taken together, our findings reveal that the ATF4/REDD1/mTORC1/S6K1 signaling axis, as a novel mechanism, is responsible for the synergistic therapeutic effect of duloxetine with EGFR-TKIs. These results suggest that combining EGFR-TKIs with duloxetine appears to be a promising way to improve EGFR-TKI efficacy against NSCLC.

Keywords: ATF4, Duloxetine, EGFR-TKI, mTORC1, non-small cell lung cancer, REDD1, S6K1

Introduction

Although many efforts have been made worldwide in recent decades to combat lung cancer, the 5-year survival rate is less than 18% [1]. Non-small cell lung cancer (NSCLC) constitutes approximately 85% of all lung cancer cases [2]. Epidermal growth factor receptor (EGFR)-activating mutations have been identified as oncogenic drivers in NSCLC. While the development of EGFR-tyrosine kinase inhibitors (EGFR-TKIs) has substantially improved the prognosis for patients with EGFR-mutant NSCLC, most eventually face disease progression due to acquired resistance [3, 4]. Resistance mechanisms include acquiring EGFR kinase structural region mutations, activating EGFR downstream

signaling pathways such as PI3K/AKT/mTOR, RAS/RAF/MEK/ERK, or JAK/STAT, and engaging EGFR alternative pathways such as MET or HER2 [5]. Several studies have demonstrated that inhibiting these pathways can overcome EGFR-TKI resistance in NSCLC [6-9].

Duloxetine, an antidepressant known as a serotonin-noradrenalin reuptake inhibitor (SNRI), is commonly prescribed for the treatment of chemotherapy-induced peripheral neuropathy [10]. Although the anticancer effects of duloxetine on some cancer cell lines have recently been investigated [11-14], little is known about the mechanism(s) by which duloxetine induces anti-tumor effects. The purpose of this study was to verify the anti-cancer properties of duloxetine

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in EGFR TKI-resistant NSCLC cells and elucidate its underlying molecular mechanism.

The mechanistic target of rapamycin (mTOR), a highly conserved serine/threonine protein kinase, senses and responds to various environmental cues to regulate cellular homeostasis and plays important roles in tumorigenesis and cancer progression [15]. mTOR is activated in various human malignancies, and its signaling is dysregulated in approximately 30% of solid tumors [16, 17]. Upon the interaction of raptor with mTOR, mechanistic target of rapamycin complex 1 (mTORC1) is formed, which activates downstream targets of ribosomal S6 kinase (S6K1) and eukaryotic initiation factor-4E binding protein-1 (4E-BP1) via phosphorylation [18]. The mTORC1 signaling pathway plays a central role in regulating cell growth, proliferation, and survival by promoting protein/lipid/nucleotide synthesis [18]. Recent studies have highlighted that mTORC1 not only promotes intrinsic tumor cell growth and survival properties but may also contribute to the regulation of an unfavorable tumor microenvironment, potentially influencing treatment outcomes [19]. Therefore, the mTORC1 signaling pathway has emerged as a promising therapeutic target in many cancers, including NSCLC.

REDD1 (regulated in development and DNA damage responses), known as a negative regulator of mTORC1, is induced by various stress conditions elicited by hypoxia, reactive oxygen species (ROS), chemotherapeutic drugs such as doxorubicin, and nutrient depletion [20]. Upregulated expression of REDD1 is required to suppress the mTORC1-mediated response to these stresses; this upregulation disrupts the inhibitory interaction between tuberous sclerosis complex-2 (TSC2) and 14-3-3 protein [21]. Therefore, this information suggests that REDD1 may negatively regulate cancer cell growth and survival via suppression of mTORC1.

In the present study, we showed that duloxetine enhances the EGFR-TKI-induced death of human NSCLC cells through upregulation of REDD1 induced by ATF4 (activating transcription factor 4) activation. Furthermore, upregulation of REDD1 induces suppression of mTORC1 and subsequent downregulation of S6K1. These results suggest that duloxetine can be useful as a combination anticancer agent to sensitize NSCLC cells to the therapeutic effect of

EGFR-TKIs by targeting the REDD1/mTORC1/S6K1 axis.

Materials and methods

Cell culture

H1299, A549, H460, and H1975 NSCLC cell lines were obtained from the American Type Culture Collection (ATCC; Manassas, VA, USA). The REDD1-knockout H1299 cell line previously reported by our group was employed [22]. The cells were maintained in RPMI 1640 medium (Welgene, Gyeongsangbuk-do, Republic of Korea) with 10% fetal bovine serum (FBS; Corning, NY, USA) in an incubator at 37°C with 5% CO₂. H1299 cells that stably overexpressed vector or REDD1 [23] were maintained in RPMI 1640 medium (Welgene) supplemented with 10% FBS and 1 µg/ml puromycin (Sigma-Aldrich; Merck KGaA, Darmstadt, Germany).

Chemicals

Duloxetine (#S4798, purity: ≥ 99%) was purchased from Tocris Bioscience (Bristol, United Kingdom). Milnacipran (#S3140, purity: 99.99%), venlafaxine (#S1441, purity: 99.98%), desvenlafaxine (#S4113, purity: 99.72%), lapatinib (#S2111, purity: 99.93%), gefitinib (#S1-025, purity: 99.97%), PF-4708671 (#S2163, purity: 99.87%), LY2584702 (#S7698, purity: 98.15%), and RAD-001 (#S1120, purity: 99.77%) were obtained from Selleckchem (Houston, Texas, USA).

Transient transfection

S6K1 (#sc-36165), ATF4 (#sc-35112) and control (#sc-37007) siRNAs were purchased from Santa Cruz Biotechnology (Dallas, TX, USA). siRNA transfections in H1299 cells were performed using Lipofectamine RNAiMAX according to the manufacturer's instructions (Invitrogen; Thermo Fisher Scientific).

Cell viability analysis

Cells were seeded in a 6-well plate and grown overnight until they reached approximately 50% cell confluence. At the end of treatment, the cells were treated with MTT solution (final concentration 0.5 mg/mL) and incubated at 37°C and 5% CO₂ for 1 h. The formazan crystals were dissolved in isopropanol, and the absorbance was measured at 570 nm. The results are

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expressed as the percent reduction in MTT, and the absorbance of the control cells was assumed to be 100%. The MTT experiments were repeated three times.

Detection of cell death

Cell death was measured with Annexin V and Propidium Iodide (PI) according to the manufacturer's instructions (#ab14085; Abcam, Cambridge, UK). H1299, H460, A549, and H1975 cells were treated with each drug for 48 h. The cells were harvested by Accutase cell detachment solution (#SCR005, Sigma) and then resuspended in binding buffer with Annexin V and PI for 10 min at room temperature in the dark. The cells were assessed immediately by CytoFLEX Flow Cytometer (Beckman Coulter, Brea, CA, USA). Cells were characterized into four groups: Live (Annexin V negative, PI negative), early apoptotic (Annexin V positive, PI negative), late apoptotic (Annexin V positive, PI positive), and necrotic (Annexin V negative, PI positive). Cell death was detected as the percentage of Annexin V and/or PI positive cells.

RNA extraction and real-time PCR analysis

RNA extraction and real-time PCR analysis were performed as described previously [24]. The following primers were used for PCR: *REDD1* (5'-GTTTGACCGCTCCACGAGCCT-3' and 5'-GCACACAAGTGTTTCATCCTCAGG-3', 156 bp product), and *β-actin* (5'-CACCATTGGCAATGAGCGG-TTC-3' and 5'-AGGTCTTTGCGGATGTCCACGT-3', 135 bp product) [25].

Western blot analysis

Western blot analysis was performed as described previously [26]. The following antibodies were used: S6K (#9202), p-S6K (Thr389) (#9205), S6 (#2217), p-S6 (Ser240/244) (#5364), and ATF4 (#11815) antibodies (all from Cell Signaling Technology); *REDD1* antibody (#10638-1-AP) (Proteintech Group; Chicago, IL, USA); and *β-actin* antibody (#A5316) (Sigma-Aldrich, Merck KGaA).

Statistical analysis

The data are expressed as the mean \pm standard deviation (SD) of three independent experiments. The significance of differences was analyzed by one-way ANOVA followed by Tukey's test using GraphPad Prism software (version

9.0, San Diego, CA, USA); differences were considered statistically significant at $P < 0.05$.

Results

Duloxetine enhanced the sensitivity of NSCLC cells to EGFR-TKIs

We investigated the sensitivity of NSCLC cells to lapatinib, gefitinib, and erlotinib, the first-generation EGFR-TKIs approved for the treatment of NSCLC. H1299, H460, and A549 cells were treated with 5 μ M lapatinib, gefitinib, or erlotinib, respectively, for 48 h, and cell viability was analyzed by MTT assay. The results showed that these EGFR-TKIs reduced the viability of NSCLC cells by less than 30% (**Figure 1A-C**). Duloxetine is an antidepressant that acts as a SNRI. Recently, duloxetine has been shown to have anticancer effects [11-14]. To investigate the sensitivity of NSCLC cells to duloxetine, we treated H1299, H460, and A549 cells with varying concentrations of duloxetine for 48 h and examined cell viability. Duloxetine reduced the viability of NSCLC cells in a dose-dependent manner (**Figure 2A-C**). To investigate whether duloxetine enhances the sensitivity of NSCLC cells to EGFR-TKIs, H1299, H460, and A549 cells were exposed to 10 μ M duloxetine in combination with 5 μ M lapatinib, gefitinib, or erlotinib for 48 h. As shown in **Figure 1A-C**, the combination of duloxetine and EGFR-TKIs suppressed cell viability more effectively than either agent alone. Next, we investigated whether other SNRIs, such as milnacipran, venlafaxine, or desvenlafaxine, enhance cell sensitivity to lapatinib, similar to the effect of duloxetine. Although the effects of duloxetine on cell viability varied among these three NSCLC cell lines, 20 μ M duloxetine reduced cell viability by more than 20% in NSCLC cells (**Figure 2A-C**). However, milnacipran, venlafaxine, or desvenlafaxine exhibited a decrease in cell viability of less than 20%, even when applied to NSCLC cells at up to 100 μ M (**Figure 2A-C**). The combination of EGFR-TKIs with milnacipran, venlafaxine, or desvenlafaxine did not further enhance NSCLC cell sensitivity to lapatinib compared to that of EGFR-TKIs combined with duloxetine (**Figure 2A-C**). Additionally, to investigate whether duloxetine enhances the cell death of NSCLC cells to lapatinib, H1299, H460, and A549 cells were exposed to 10 μ M duloxetine in combination with 5 μ M lapatinib

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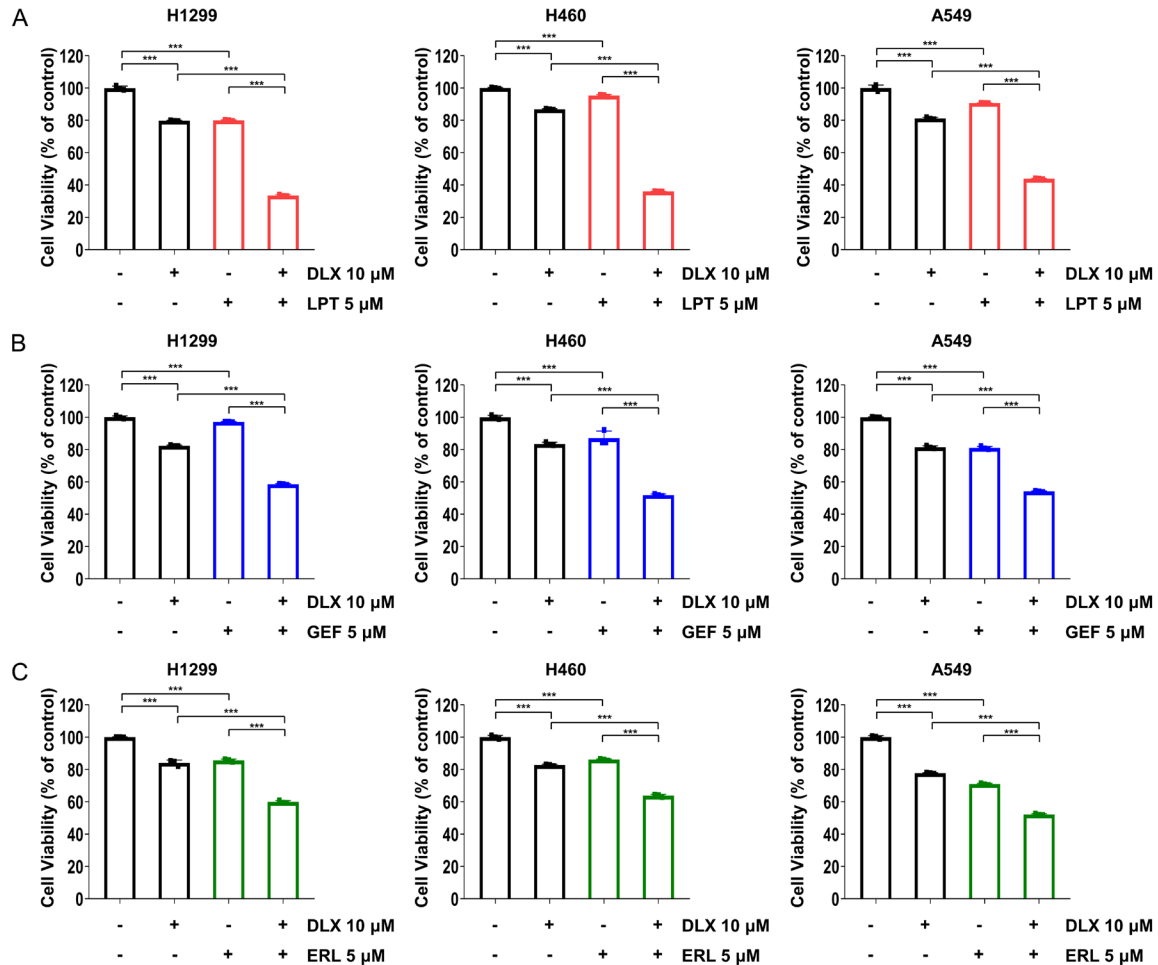


Figure 1. Duloxetine, a serotonin-norepinephrine reuptake inhibitor, sensitizes non-small cell lung cancer (NSCLC) cells to EGFR inhibitors. A. H1299, H460, and A549 cells were treated with 5 μ M lapatinib and/or 10 μ M duloxetine for 48 h. B. H1299, H460, and A549 cells were treated with 5 μ M gefitinib and/or 10 μ M duloxetine for 48 h. C. H1299, H460, and A549 cells were treated with 5 μ M erlotinib and/or 10 μ M duloxetine for 48 h. A-C. Cell viability was measured by MTT assay. The data are presented as the mean percentage of control \pm SD ($n=3$; $***P<0.001$). DLX: duloxetine, ERL: erlotinib, GEF: gefitinib, LPT: lapatinib.

for 48 h, and cell death was detected as the percentage of Annexin V/PI positive cells. The combination of duloxetine and lapatinib induced cell death more effectively than either agent alone (**Figure 2D**). These results suggest that duloxetine enhances the sensitivity of NSCLC cells to EGFR-TKIs.

Treatment with duloxetine and/or lapatinib inhibits mTORC1 activity

mTORC1, which is frequently deregulated in various cancer types, has been reported to contribute to cancer cell growth and metabolism [18]. We investigated the effect of lapatinib and/or duloxetine on mTORC1 activity in

NSCLC cells. Lapatinib dose-dependently reduced the activity of mTORC1, as indicated by the decreased phosphorylation of S6K and S6 (**Figure 3A**). Duloxetine also inhibited mTORC1 activity in a dose-dependent manner, with complete inhibition observed at a concentration of 20 μ M (**Figure 3B**). In contrast, other SNRIs, including milnacipran, venlafaxine, or desvenlafaxine, did not affect mTORC1 activity, even at doses up to 100 μ M (**Figure 3C**). The suppression of mTORC1 induced by lapatinib was further enhanced when combined with duloxetine treatment (**Figure 3D**). These findings suggest that treatment with duloxetine and/or lapatinib inhibits mTORC1 activity.

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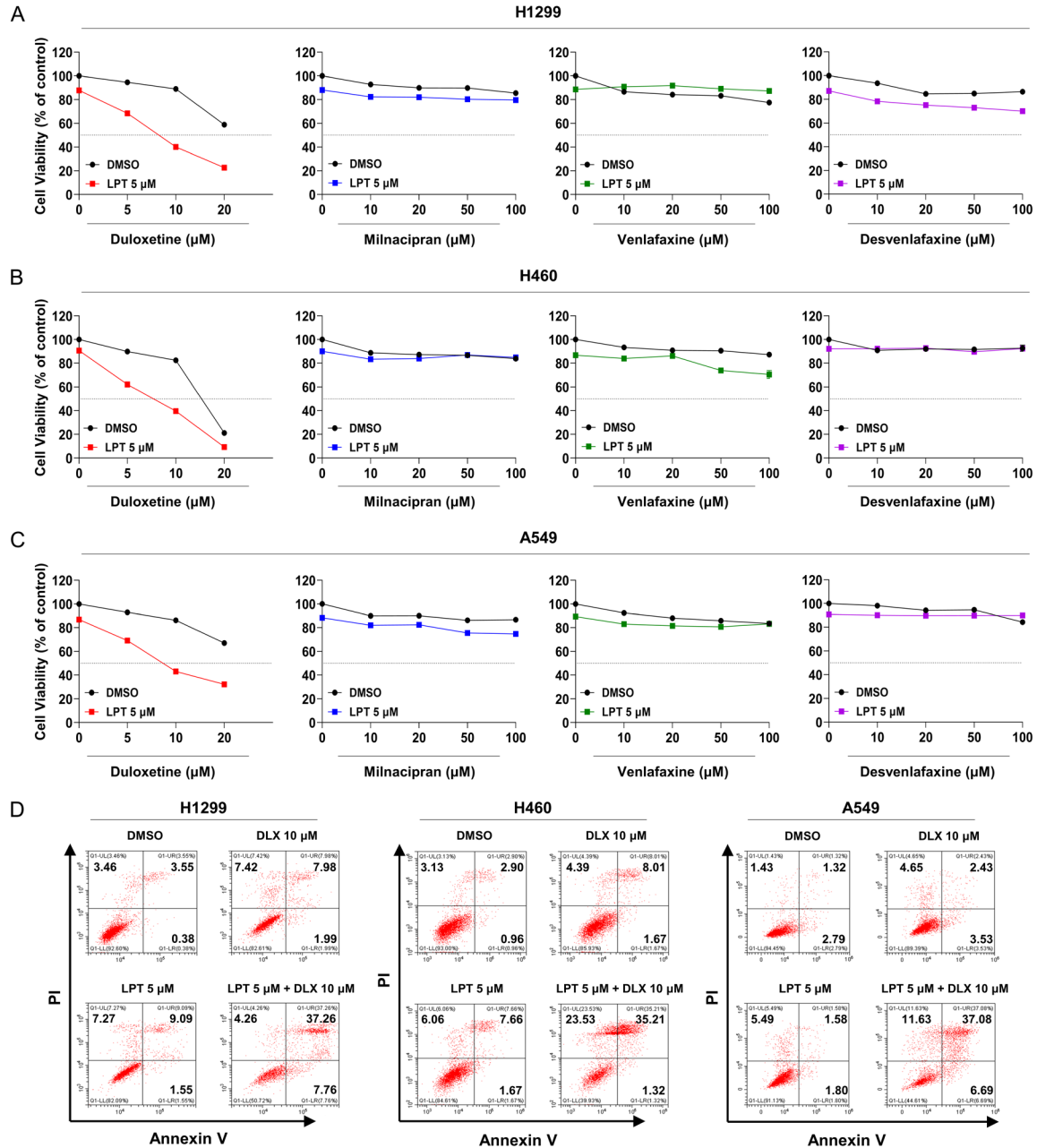


Figure 2. Duloxetine, but not milnacipran, venlafaxine, or desvenlafaxine, sensitizes NSCLC cells to lapatinib. A-C. H1299, H460, and A549 cells were treated with 5 μM lapatinib and the indicated concentrations of duloxetine, milnacipran, venlafaxine or desvenlafaxine for 48 h. D. H1299, H460, and A549 cells were treated with 5 μM lapatinib and/or 10 μM duloxetine for 48 h. A-C. Cell viability was measured by MTT assay. The data are presented as the mean percentage of control ± SD (n=3). D. Cell death was detected as the percentage of Annexin V and/or Propidium Iodide (PI) positive cells. DLX: duloxetine, LPT: lapatinib, PI: Propidium Iodide.

Duloxetine enhances the sensitivity of NSCLC cells to lapatinib by inhibiting mTORC1/p70S6K1

Next, we investigated whether inhibiting mTORC1/p70S6K1 enhances cell sensitivity to

lapatinib and/or duloxetine. RAD001 is a macrolide that inhibits mTORC1 and its downstream targets [27]. As shown in **Figure 4A** and **4D**, RAD001 treatment resulted in the complete inhibition of S6 phosphorylation and significantly increased cell sensitivity to lapa-

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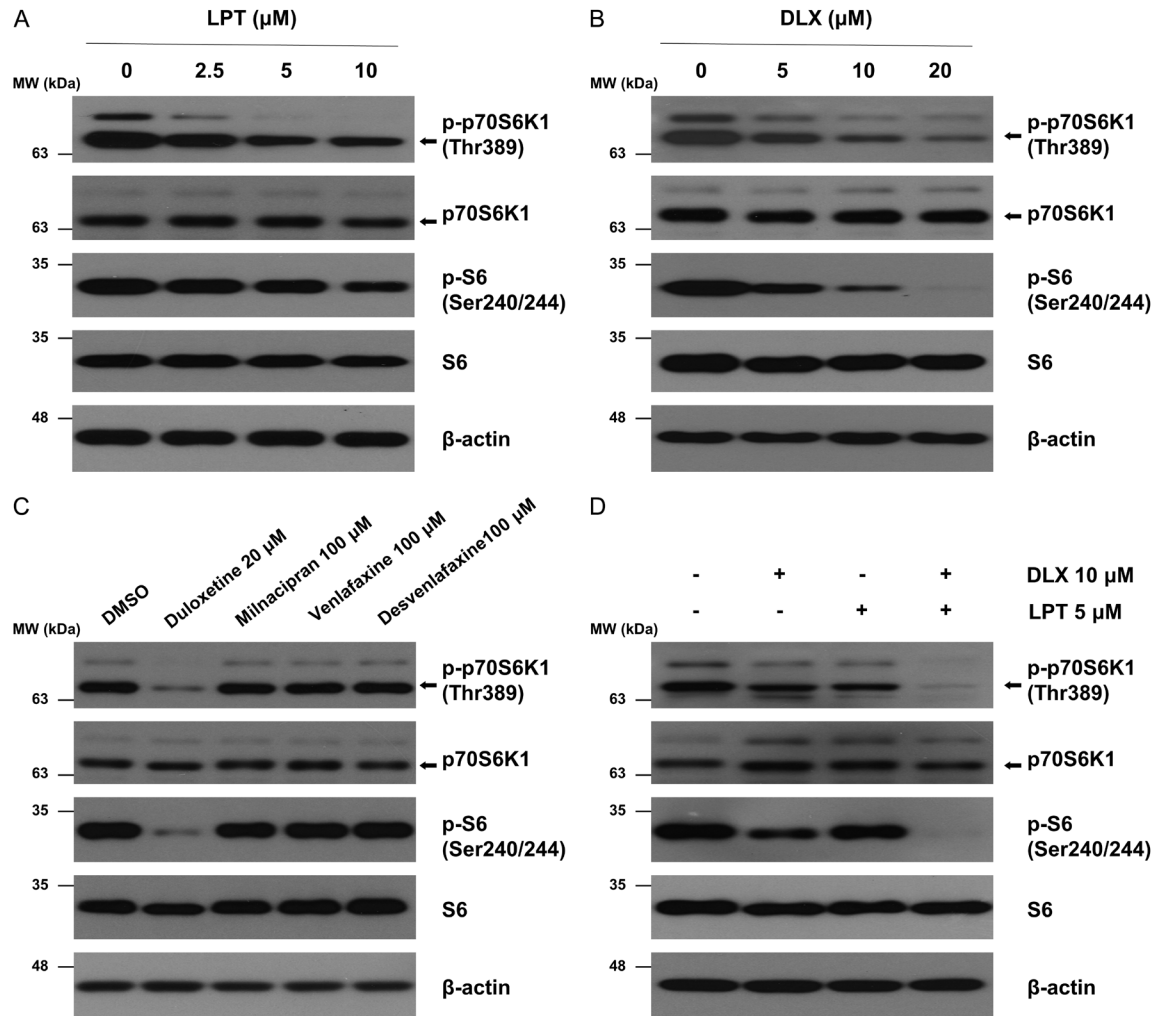


Figure 3. Duloxetine and/or lapatinib reduces 70-kDa ribosomal protein S6 kinase 1 (p70S6K1)/mechanistic target of rapamycin complex 1 (mTORC1) activity. A. H1299 cells were treated with the indicated concentrations of lapatinib for 18 h. B. H1299 cells were treated with the indicated concentrations of duloxetine for 18 h. C. H1299 cells were treated with the indicated concentrations of duloxetine, milnacipran, venlafaxine and des venlafaxine for 18 h. D. H1299 cells were treated with 5 μM lapatinib and/or 10 μM duloxetine for 18 h. A-D. The indicated protein levels were estimated by western blot analysis. Data are representative of three independent experiments. DLX: duloxetine, LPT: lapatinib.

tinib or duloxetine. Similarly, the S6K1 inhibitors PF-4708671 and LY-2584702 completely blocked S6 phosphorylation and substantially heightened cell sensitivity to lapatinib and duloxetine (Figure 4B, 4C, 4E, 4F). To further determine whether S6K1 inhibition enhances cell sensitivity to lapatinib and/or duloxetine, we transfected H1299 cells with siRNAs targeting S6K1 and subsequently treated them with lapatinib and/or duloxetine. The S6K1 siRNA effectively silenced S6K1 protein expression and significantly enhanced cell sensitivity to lapatinib and/or duloxetine, as shown in Figure

4G-I. These findings suggest that inhibiting mTORC1/S6K1 can sensitize cells to the effects of lapatinib and/or duloxetine.

Duloxetine enhances the sensitivity of NSCLC cells to lapatinib through REDD1-mediated mTORC1 inhibition

REDD1 is one of the best characterized suppressors of mTORC1 [28]. We investigated the role of REDD1 in the reduction in cell viability induced by lapatinib and/or duloxetine. Both lapatinib and duloxetine dose-dependently in-

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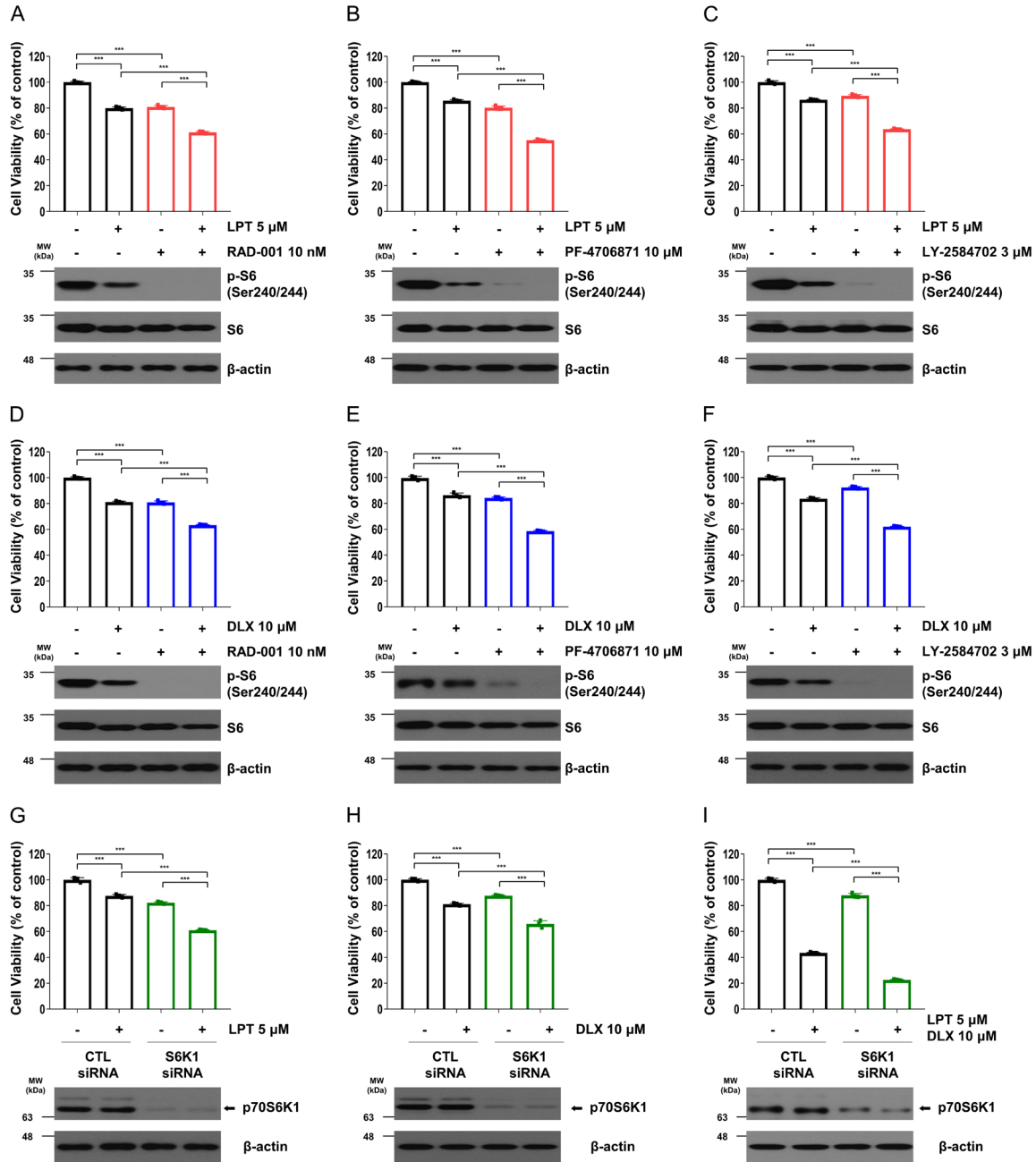


Figure 4. Duloxetine enhanced lapatinib-induced cell sensitivity by inhibiting mTORC1/S6K1. A. H1299 cells were treated with 5 μ M lapatinib and/or 10 nM RAD-001 for 48 h. B. H1299 cells were treated with 5 μ M lapatinib and/or 10 μ M PF-4708671 for 48 h. C. H1299 cells were treated with 5 μ M lapatinib and/or 3 μ M LY2584702 for 48 h. D. H1299 cells were treated with 10 μ M duloxetine and/or 10 nM RAD-001 for 48 h. E. H1299 cells were treated with 10 μ M duloxetine and/or 10 μ M PF-4708671 for 48 h. F. H1299 cells were treated with 10 μ M duloxetine and/or 3 μ M LY2584702 for 48 h. G. H1299 cells were transfected with CTL siRNA or S6K1 siRNA and then treated with 10 μ M lapatinib. H. H1299 cells were transfected with CTL siRNA or S6K1 siRNA and then treated with 10 μ M duloxetine. I. H1299 cells were transfected with CTL siRNA or S6K1 siRNA and then treated with 10 μ M duloxetine and 5 μ M lapatinib. A-I. Cell viability was measured by MTT assay. The indicated protein levels were estimated by western blot analysis. The data are presented as the mean percentage of control \pm SD (n=3; ***P<0.001). CTL: control, DLX: duloxetine, LPT: lapatinib.

creased the mRNA and protein expression of REDD1 (Figure 5A and 5B). Combined treat-

ment with lapatinib and duloxetine increased the mRNA and protein expression of REDD1

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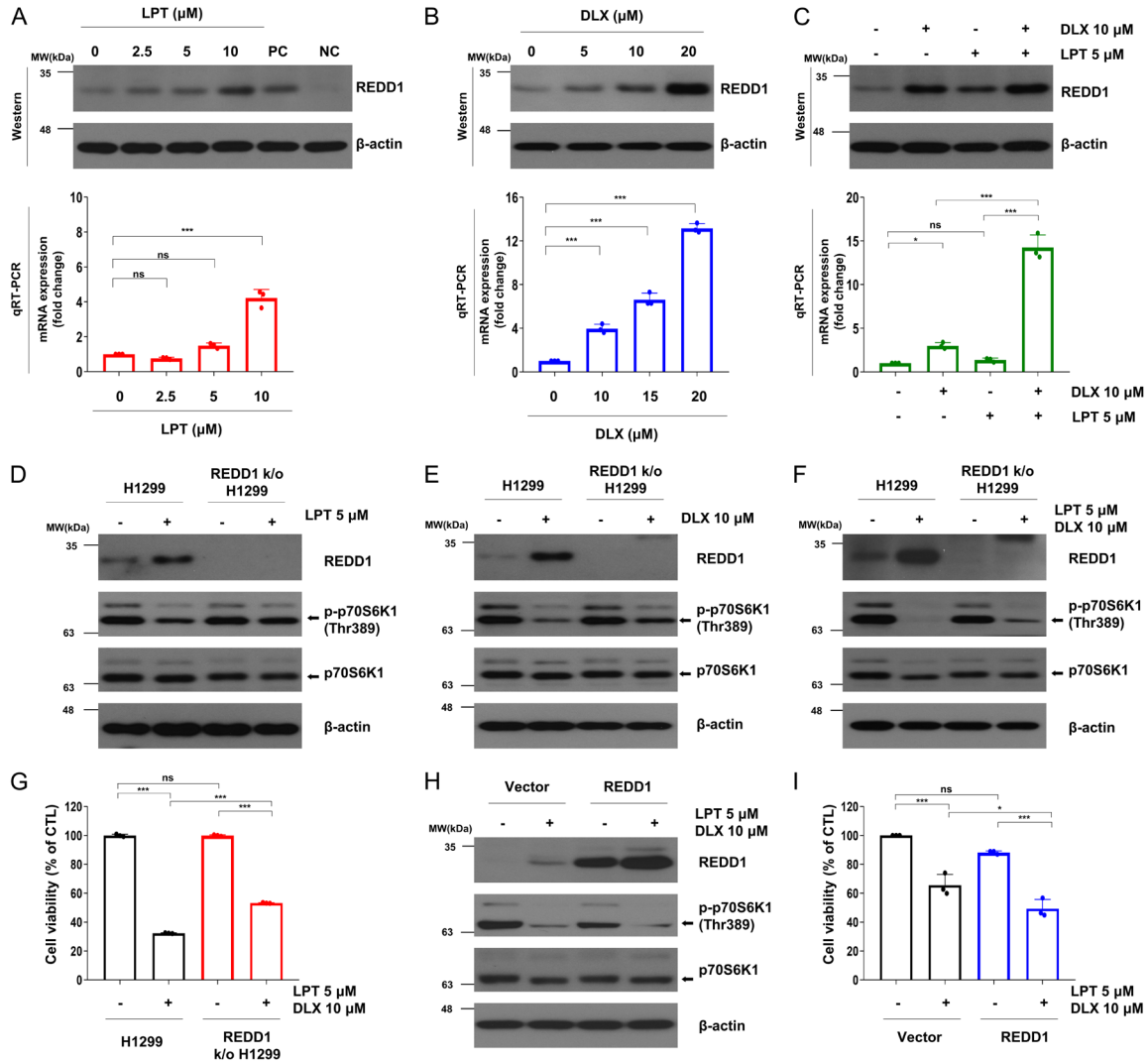


Figure 5. Duloxetine enhanced the lapatinib-induced inhibition of p70S6K1 phosphorylation via induction of regulated in development and DNA damage response 1 (REDD1). A. H1299 cells were treated with the indicated concentrations of lapatinib for 18 h. PC: H1299 cells were transiently transfected with control siRNA for 18 h and subsequently incubated in lysine-free medium for 6 h. NC: H1299 cells were transiently transfected with REDD1 siRNA for 18 h and subsequently incubated in lysine-free medium for 6 h. B. H1299 cells were treated with the indicated concentrations of duloxetine for 18 h. H1299 cells were deprived with lysine as a positive control. C. H1299 cells were treated with 10 μM duloxetine and/or 5 μM lapatinib for 18 h. D. H1299 cells or REDD1 k/o H1299 cells were treated with 5 μM lapatinib for 18 h. E. H1299 cells or REDD1 k/o H1299 cells were treated with 10 μM duloxetine for 18 h. F, G. H1299 cells or REDD1 k/o H1299 cells were treated with 10 μM duloxetine and 5 μM lapatinib for 18 h (F) or 48 h (G). H, I. H1299 cells that stably overexpressed vector and REDD1 were treated with 10 μM duloxetine and 5 μM lapatinib for 12 h (H) or 24 h (I). A-C. The indicated mRNA levels were estimated by real-time PCR analysis. (A-F, H) The indicated protein levels were estimated by western blot analysis. G-I. Cell viability was measured by MTT assay. The data are presented as the mean percentage of control ± SD (n=3; *P<0.05, ***P<0.001, ns: not significantly different). DLX: duloxetine, LPT: lapatinib, PC: positive control, NC: negative control.

compared to that with treatment with either drug alone (Figure 5C). H1299 cells with REDD1 knocked out showed less reduction in p70S6K1 phosphorylation by lapatinib and/or duloxetine than H1299 cells (Figure 5D-F). REDD1 knock-out prevented the inhibitory effect on cell viability

induced by lapatinib and duloxetine at 48 h (Figure 5G). Furthermore, we investigated the effects of lapatinib and/or duloxetine on the viability of stably overexpressing vector or REDD1 cells. In our previously report, we established a cell line that stably overexpressed

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REDD1 [23]. REDD1 stably overexpressing cells showed slightly reduced expression of p70S6K1 phosphorylation and greater inhibition of p70S6K1 phosphorylation by lapatinib and/or duloxetine compared to control vector cells (**Figure 5H**). Overexpression of REDD1 reduced the growth rate by approximately 15% compared to control vector cells and enhanced the inhibitory effect on cell viability induced by lapatinib and duloxetine (**Figure 5I**). These data suggest that duloxetine enhances the sensitivity of NSCLC cells to lapatinib through REDD1-mediated mTORC1 inhibition.

Duloxetine enhances the sensitivity of NSCLC cells to lapatinib through ATF4/REDD1-mediated mTORC1 inhibition

ATF4 is an essential transcription factor that mediates the expression of the mTORC1 upstream regulatory protein REDD1 [29]. We investigated whether lapatinib and/or duloxetine increase the expression of the ATF4 protein. Both lapatinib and duloxetine increased the protein expression of ATF4 (**Figure 6A and 6B**). Combined treatment with lapatinib and duloxetine resulted in a greater increase in the protein expression of ATF4 than that upon treatment with either drug alone (**Figure 6C**). Knocking down ATF4 by siRNA in H1299 cells abrogated ATF4 induction in cells treated with lapatinib and/or duloxetine (**Figure 6D-F**). ATF4 depletion blocked REDD1 induction and restored p70S6K1 phosphorylation reduction upon lapatinib and/or duloxetine treatment (**Figure 6D-F**). Disruption of ATF4 restored cell viability, which had been reduced by lapatinib and duloxetine (**Figure 6G**). These data suggest that duloxetine enhances the sensitivity of NSCLC cells to lapatinib through ATF4/REDD1-mediated mTORC1 inhibition.

Duloxetine enhanced the sensitivity of H1975 NSCLC cells to EGFR-TKIs

We further investigated the effects of EGFR inhibitors and/or SNRIs on the viability of H1975 NSCLC cells bearing EGFR gene mutations and amplification [30]. When H1975 cells were treated with 5 μ M each of the EGFR TKIs lapatinib, gefitinib, or erlotinib for 48 h, the cell viability inhibition rate was less than 20% (**Figure 7A**). Duloxetine treatment further enhanced the inhibition of cell viability by the EGFR-TKIs lapatinib, gefitinib, and erlotinib

(**Figure 7A**). Duloxetine reduced cell viability by more than 60% at 20 μ M in H1975 cells, whereas milnacipran, venlafaxine, or desvenlafaxine reduced H1975 cell viability to less than 20%, even when applied at up to 100 μ M (**Figure 7B**). The combination of milnacipran, venlafaxine, or desvenlafaxine did not further enhance H1975 cell sensitivity to lapatinib compared to duloxetine (**Figure 7B**). Further, we investigated whether other SNRIs, such as milnacipran, venlafaxine, or desvenlafaxine, induce cell death to lapatinib, similar to the effect of duloxetine. The combination of duloxetine and lapatinib induced cell death approximately 50% more effectively than either drug alone (**Figure 7C**). However, milnacipran, venlafaxine, or desvenlafaxine, even in combination with lapatinib, resulted in less than 20% cell death in H1975 cells when applied at up to 100 μ M (**Figure 7C**). These results suggest that duloxetine enhances the sensitivity of H1975 NSCLC cells to EGFR-TKIs.

Discussion

Epidermal growth factor receptor-tyrosine kinase inhibitor (EGFR-TKI) therapy has become the standard first-line treatment for non-small cell lung cancer (NSCLC) patients with EGFR mutations [31]. Unfortunately, EGFR-dependent and EGFR-independent mechanisms of drug resistance to EGFR-TKIs have emerged [32]. Drug combination approaches have been investigated to overcome this drug resistance. EGFR-TKI treatment combined with chemotherapy significantly improves progression-free survival compared to EGFR-TKI monotherapy for advanced NSCLC patients with EGFR mutations, with acceptable toxicity [33].

Duloxetine, a SNRI, functions as an antidepressant by inhibiting the reuptake of both norepinephrine and serotonin. Its clinical application includes treating depression, especially major depressive disorder, and relieving somatic and neuropathic pain [34]. Duloxetine is recommended for the management of peripheral neuropathic pain induced by chemotherapy in adult cancer survivors [35]. Recently, duloxetine has been shown to have anticancer effects [11-14]. Duloxetine exhibits an anti-proliferative effect in pancreatic adenocarcinoma cells through regulating immune and inflammatory conditions [12, 13]. In our study, we found that the exposure of NSCLC cells to duloxetine dose-

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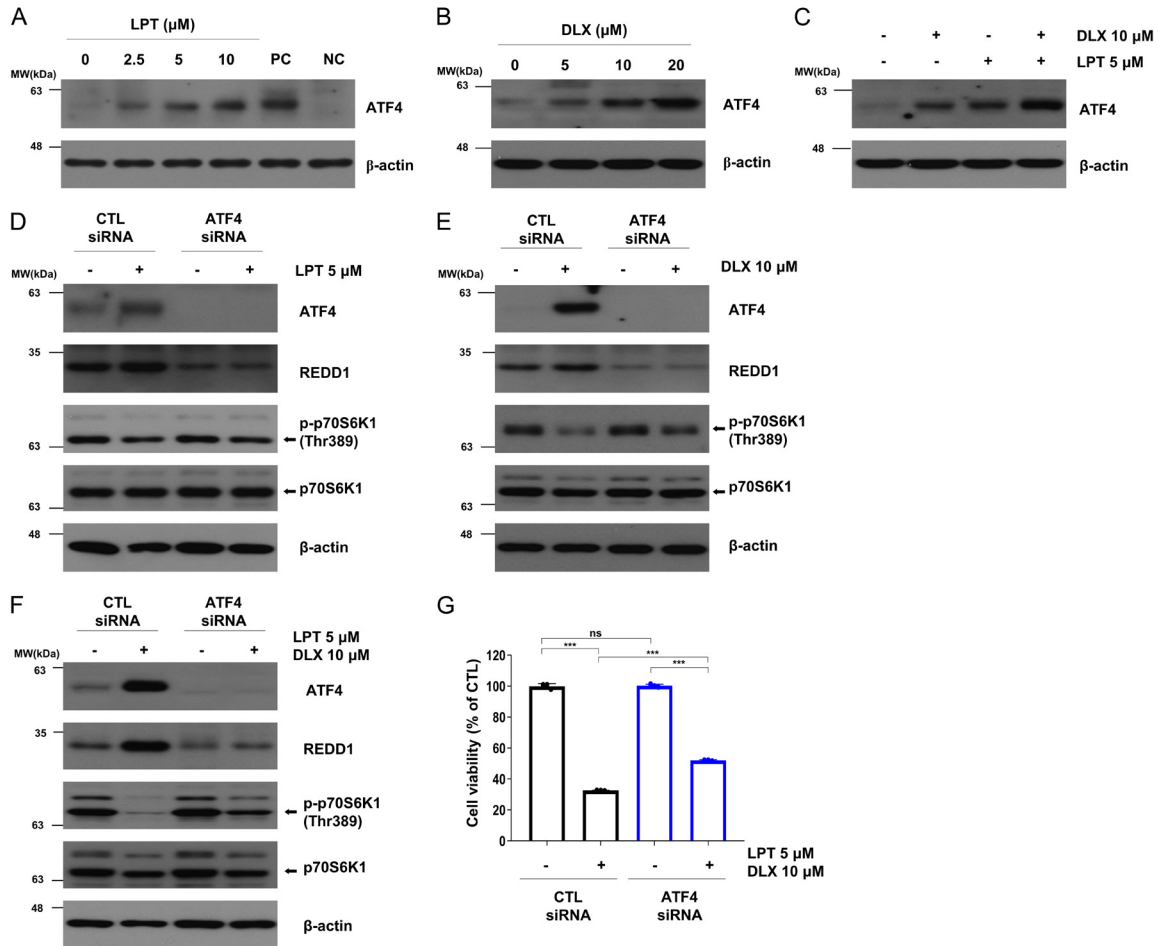


Figure 6. Duloxetine enhanced the lapatinib-induced inhibition of p70S6K1 phosphorylation via induction of REDD1 in an activating transcription factor 4 (ATF4)-dependent manner. A. H1299 cells were treated with the indicated concentrations of lapatinib for 18 h. PC: H1299 cells were transiently transfected with control siRNA for 18 h and subsequently incubated in lysine-free medium for 6 h. NC: H1299 cells were transiently transfected with REDD1 siRNA for 18 h and subsequently incubated in lysine-free medium for 6 h. B. H1299 cells were treated with the indicated concentrations of duloxetine for 18 h. C. H1299 cells were treated with 10 μ M duloxetine and/or 5 μ M lapatinib for 18 h. D. H1299 cells were transfected with control or ATF4 siRNA for 12 h followed by treatment with 10 μ M duloxetine for 18 h. E. H1299 cells were transfected with control or ATF4 siRNA for 12 h followed by treatment with 10 μ M duloxetine and 5 μ M lapatinib for 18 h (F) or 48 h (G). The data are presented as the mean percentage of control \pm SD (n=3; ***P<0.001, ns: not significantly different). CTL: control, DLX: duloxetine, LPT: lapatinib, PC: positive control, NC: negative control.

independently inhibited cell viability. However, other SNRIs, such as milnacipran, venlafaxine, or desvenlafaxine, did not exhibit a significant inhibitory effect on NSCLC cells, even at a treatment concentration of 100 μ M. These results are consistent with the finding that only duloxetine, not milnacipran, reduces cell viability in hepatocellular carcinoma cells. In our study, we observed that duloxetine increased the sensitivity of NSCLC cells to EGFR-TKIs such as lapatinib, gefitinib, or erlotinib to a greater degree

than other SNRIs. These data suggest that duloxetine exhibits more potent inhibition of viability in NSCLC cells than other SNRIs when used alone and when used in combination with EGFR-TKIs. Previous studies have shown that duloxetine overcomes resistance in TRAIL-resistant cell lines by enhancing TRAIL-mediated apoptosis [14]. Consistent with our findings, this suggests that duloxetine could potentially be used as a combination therapy in the treatment of drug-resistant lung cancer cells.

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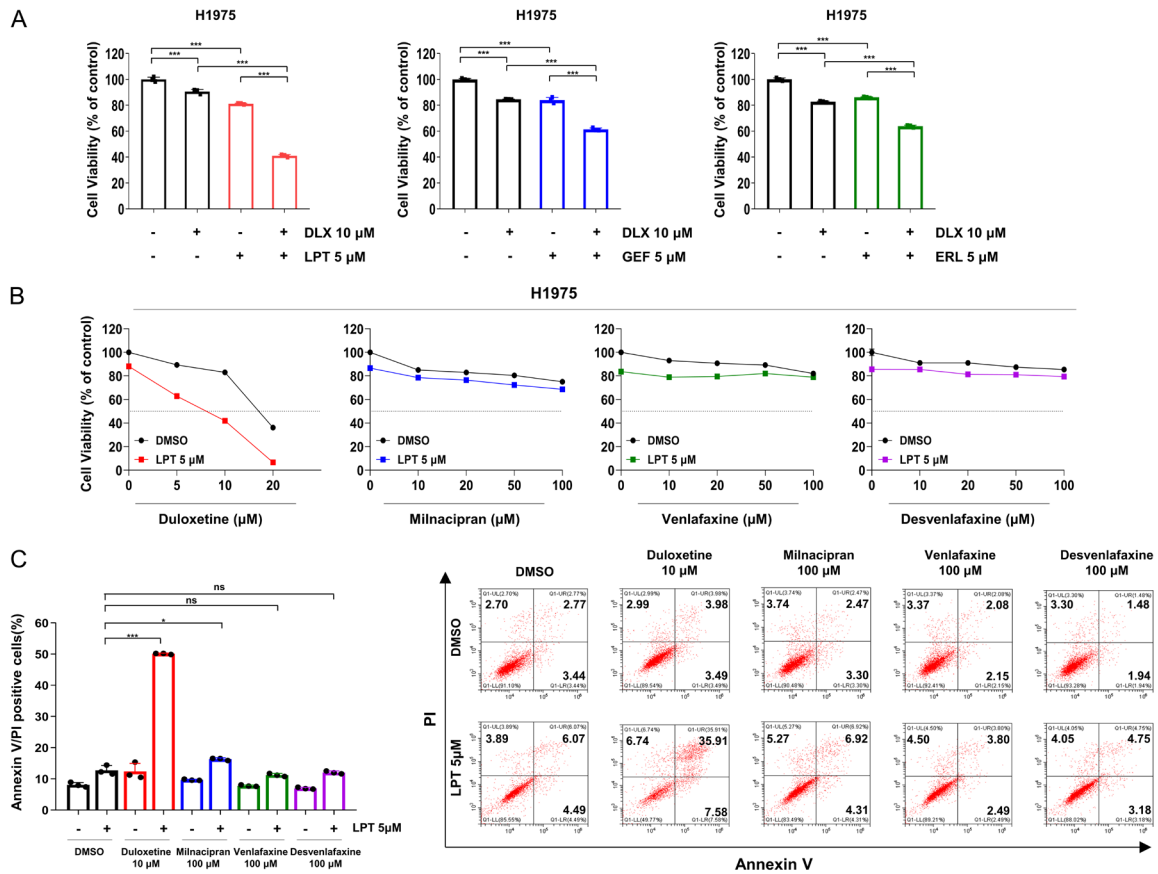


Figure 7. Duloxetine, a serotonin-norepinephrine reuptake inhibitor, sensitizes EGFR inhibitors in the EGFR-TKI-resistant lung cancer cell line H1975 (L858R/T790M-mutant EGFR). A. H1975 cells were treated with 5 μ M lapatinib, 5 μ M gefitinib or 5 μ M erlotinib with duloxetine for 48 h. B, C. H1975 cells were treated with lapatinib and duloxetine, milnacipran, venlafaxine or desvenlafaxine for 48 h. A, B. Cell viability was measured by MTT assay. The data are presented as the mean percentage of control \pm SD (n=3). C. Cell death was detected as the percentage of Annexin V and/or PI positive cells. The data are presented as the mean percentage of control \pm SD (n=3; *P<0.05, ***P<0.001, ns: not significantly different). DLX: duloxetine, ERL: erlotinib, GEF: gefitinib, LPT: lapatinib, PI: Propidium Iodide.

Given the fundamental role of the mTORC1/S6K1 pathway in NSCLC growth and survival [6, 36], mTORC1/S6K1 pathway inhibitors have emerged as a possible solution to the problem of EGFR-TKI resistance [37-39]. In our study with NSCLC cells, we found that duloxetine effectively inhibited mTORC1 activity in a dose-dependent manner and further potentiated the inhibition of mTORC1 activity when combined with lapatinib. We also found that when mTORC1/S6K1 activity was suppressed using RAD-001, PF-4708671, LY-2584702, or S6K1 siRNA, the cell sensitivity to duloxetine and/or lapatinib was enhanced. These findings suggest that inhibiting mTORC1/S6K1 can sensitize cells to the effects of lapatinib and/or duloxetine.

ATF4 (activating transcription factor 4) is an essential transcription factor that mediates the expression of the mTORC1 upstream regulatory protein REDD1 (regulated in development and DNA damage responses) [22, 40]. Thus, we investigated whether ATF4/REDD1 is involved in the inhibition of mTORC1 activity by duloxetine and/or lapatinib. We found that duloxetine dose-dependently increased ATF4 and REDD1 protein expression and further enhanced ATF4 and REDD1 protein expression when combined with lapatinib. Knocking down ATF4/REDD1 reversed the inhibition of mTORC1 activity caused by lapatinib and/or duloxetine and prevented the inhibitory effects of lapatinib and/or duloxetine on cell viability. These findings suggest that ATF4/REDD1 plays an important role

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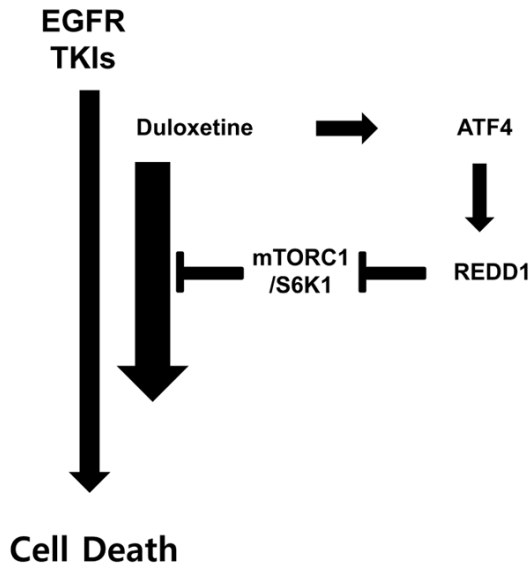


Figure 8. Proposed mechanism by which duloxetine enhances cell death to EGFR-TKIs in NSCLC cells by downregulating mTORC1/S6K1 through upregulation of REDD1 induced by ATF4 activation.

in the inhibition of mTORC1/S6K1 activity by duloxetine and lapatinib (**Figure 8**).

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Disclosure of conflict of interest

None.

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