Lobaplatin Enhances Radioactive ^{125}I Seed-Induced Apoptosis and Anti-Proliferative Effect in Non-Small Cell Lung Cancer by Suppressing the AKT/mTOR Pathway

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Jia-hui Rong¹
Dong Li²
Yu-liang Li²

¹Department of Cardiology, The Second Hospital of Shandong University, Jinan, People's Republic of China; ²Department of Interventional Medicine, The Second Hospital of Shandong University, Jinan, People's Republic of China

Introduction: In recent years, radioactive ^{125}I seed implantation combined with chemotherapy has been regarded as a safe and effective treatment for advanced non-small cell lung cancer (NSCLC). However, the mechanism underlying this success is still unclear.

Methods: In this study, we investigated the apoptosis and anti-proliferative effect induced by ^{125}I in A549, H1975, and H157 cells and determined whether a sensitizing concentration of lobaplatin (LBP) could enhance these effects. We performed in vitro experiments on A549, H1975, and H157 cells; we investigated the effects of ^{125}I or lobaplatin (LBP) alone, or in combination, on cellular apoptosis and proliferation by performing flow cytometry, Bax/Bcl2 ratio, TUNEL, cell viability assay, cell cycle, and EdU. To further verify our findings, a subcutaneous tumor mouse model was established. Moreover, AKT/mTOR pathway was detected to determine whether this pathway was involved in the anti-cancer effect of ^{125}I and LBP by up-regulating or down-regulating the expression of mTOR.

Results: Based on our results, the sensitizing concentration of LBP could enhance the ^{125}I -induced apoptosis and anti-proliferation effect. Furthermore, the subcutaneous tumor mouse model obtained the consistent results. More importantly, the AKT/mTOR pathway was down-regulated after the treatment of ^{125}I and LBP, and the anti-cancer effect of ^{125}I and LBP could be compromised by up-regulating the mTOR expression.

Conclusion: Our study proved that LBP promotes the apoptotic and anti-proliferative effects of ^{125}I in NSCLC cells by inhibiting the AKT/mTOR pathway and provides a foundation for future studies and enhanced combinatorial approaches for NSCLC in the clinical setting.

Keywords: radioactive ^{125}I seed, lobaplatin, non-small cell lung cancer, apoptosis, proliferation, AKT/mTOR pathway

Introduction

Non-small cell lung cancer (NSCLC) accounts for approximately 85% of lung cancers; it is one of the most commonly diagnosed cancers and a leading cause of cancer-related deaths worldwide.^{1,2} Although early diagnosis and treatment are effective, the majority of patients with NSCLC remain inoperable due to different metastases.³ Since the 1980s, radioactive ^{125}I seed implantation has been regarded as an alternative treatment for NSCLC;⁴ it is considered a safe and effective approach for advanced NSCLC.^{5,6} Besides, combining ^{125}I with chemotherapy

Correspondence: Dong Li; Yu-liang Li
Department of Interventional Medicine,
The Second Hospital of Shandong
University, 247 Beiyuan Street, Jinan
250000, People's Republic of China
Email 714048127@qq.com;
lyl.pro@sdu.edu.cn

can significantly improve the clinical efficacy and extend the overall survival of patients with NSCLC.⁷ Notably, ¹²⁵I was reported to suppress tumor growth via Warburg effect inhibition in a dose-dependent manner in A549 xenografts.⁸ However, the mechanism by which ¹²⁵I radiotherapy suppresses NSCLC cells in vitro and in vivo is unclear.

Lobaplatin (LBP), a third-generation platinum drug, was reported to induce cellular apoptosis and cell cycle arrest in various NSCLC cells in vitro.⁹ However, which phase of the cell cycle was blocked remains debatable; Zhang et al reported that LBP may induce G1 phase cell cycle arrest in A549 cells,⁹ whereas Xie et al revealed that an increase in S phase arrest, from 25.0% to 54.4%, was induced by LBP in a dose-dependent manner in A549 cells.³ Thus, further studies ought to be carried out to verify these controversial results. In addition, drug resistance remains an important issue that affects LBP clinical efficacy. Paclitaxel, a cytoskeletal antitumor drug, was reported to increase the sensitivity of lung cancer cell line NCI-H446 to LBP via downregulating the PI3K/AKT pathway.¹⁰ Therefore, identifying whether the AKT/mTOR pathway is involved in the regulation of LBP drug resistance would be beneficial for further clinical application of LBP.

The AKT/mTOR pathway is associated with cell proliferation, cell cycle progression, apoptosis, necrosis, and drug resistance;^{11–13} AKT regulates different biological processes such as cell survival, cell proliferation, and invasion.¹⁴ Continuously activated AKT can phosphorylate mammalian target of rapamycin complex 1 (mTORC1), which is an important regulator in gene expression and translation.¹⁵ In addition, phosphorylated AKT (p-AKT) promotes radiation resistance and positively relates to recurrence in cervical cancer.¹⁶ The AKT/mTOR pathway plays an essential role in various malignant tumors, including lung cancer, breast cancer, nasopharyngeal carcinoma, and bladder cancer.^{17–19} However, the role of AKT/mTOR signaling, especially p-AKT, in ¹²⁵I irradiation and LBP treatment in NSCLC is rarely reported.

In this study, we adopted both in vivo and in vitro approaches to illustrate the potential of LBP in promoting ¹²⁵I-induced apoptosis and proliferation inhibition in A549, H1975 and H157 cells. Besides, we also verified that ¹²⁵I significantly downregulated the AKT/mTOR pathway, which was further facilitated by LBP. These

findings may lay a foundation for future combinatorial approaches based on LBP and ¹²⁵I in the clinical setting.

Materials and Methods

Mouse Subcutaneous Tumor Model Establishment

The mouse tumor model was established following the method we previously described.²⁰ A549 cells (1×10^7 /mL) were diluted in 0.9% saline solution and injected subcutaneously in the hind leg of BALB/c male nude mice (n=16, 4–6 weeks) purchased from the Animal Research Center of Shandong University (Jinan, Shandong, China). Tumor diameter was measured every other day. According to the NIH Guide for the Care and Use of Laboratory, all mice were housed under specific pathogen-free conditions and the animal experiment was approved by the Shandong University Animal Care Committee.

The humane endpoint established in this study is that the diameter of the tumor is greater than 20mm and the mice were sacrificed by cervical dislocation.

Radioactive ¹²⁵I Seed Implantation in Xenograft Mice

¹²⁵I radioactive seeds were provided by Ningbo Junan Pharmaceutical Technology Company (Ningbo, Zhejiang, China). According to our previously described method, when the volume of the tumor reached 400mm³, ¹²⁵I seeds were implanted into the mice under local anesthesia (lidocaine, 5mg/kg);²⁰ tumors were punctured using a 18G needle (Kakko, Japan) under direct vision and the injections were made in the middle of the tumors. During 27-day ¹²⁵I radioactive seed treatment, a few mice developed tumor ulceration, but no bleeding. For these mice, medical iodine volts can be used for disinfection.

Cell Culture and Transfection

The A549, H1975, and H157 cell lines were purchased from the Zhong Qiao Xin Zhou Biotechnology Company (Shanghai, China). The cells were cultured in Dulbecco's modified Eagle's medium (Corning, Inc., Corning, NY, USA) supplemented with 10% fetal calf serum (Gibco, Waltham, MA USA) and incubated in 5% CO₂ at 37°C. Transfections of short interfering RNA (NT-siRNA/mTOR-siRNA) and plasmid (pcDNA/pcmTOR) were performed using lipofectamine 2000 (Invitrogen, CA, USA). The

siRNA and plasmid were purchased from RiboBio (Guangzhou, Guangdong, China).

Sensitizing Concentration of LBP on NSCLC Cells

Based on the pilot experiment, the A549, H1975, and H157 cells were treated with different concentrations of LBP, ranging from 0 to 80 $\mu\text{g/mL}$, for 72 h. Cell proliferation was detected by the CCK-8 assay (Dojindo, Kumamoto, Japan) and the half-maximal inhibitory concentration (IC_{50}) was calculated using Prism 7.0 software (GraphPad, San Diego, CA, USA). According to the previously published method, 10% of the IC_{50} was considered as a sensitizing concentration.²⁰

Cell Viability Assay

After being treated with a sensitizing concentration of LBP (10% of IC_{50} , 0.7867 $\mu\text{g/mL}$ for A549 cells, 0.2119 $\mu\text{g/mL}$ for H1975 cells, and 0.2907 $\mu\text{g/mL}$ for H157 cells), ^{125}I , or their combination, the A549, H1975, and H157 cells were seeded in 96-well plates at a density of 3×10^3 cells/well. After cell adherence, the CCK-8 reagent was added and incubated for 3 h to detect the optical density at 24, 48, and 72 h; measurements were performed using a microplate reader (Thermo, Waltham, MA, USA).

Flow Cytometry

Cells were seeded in 35 mm-dishes at 1×10^5 cells/dish and treated with sensitizing concentration of LBP, ^{125}I , or a combination of both. The cell cycle assay was performed per the manufacturer's protocol (BD Biosciences, Franklin Lakes, NJ, USA). As for the apoptosis analysis, cells were stained with PI and Annexin V-FITC (BD Biosciences). Both the cell cycle and apoptosis assays were performed using a flow cytometer (BD Biosciences).

Terminal Deoxynucleotidyl Transferase dUTP Nick End Labeling (TUNEL) Assay

The TUNEL assay was performed as recommended by the manufacturer (Beyotime Biotechnology, Nanjing, Jiangsu, China). Fluorescence microscopy (Olympus, Tokyo, Japan) was used to observe the apoptotic cells and obtain images (at three different fields).

5-Ethynyl-2'-Deoxyuridine (EdU) Staining

According to the manufacturer's instructions, the Cell-Light EdU Apollo 567 in vitro kit (RiboBio, Guangdong,

China) was used to examine cell proliferation. After the A549 cells were treated as indicated, the cell medium was replaced with 50 μM of EdU solution, diluted with DMEM, and incubated for 2 h. Fluorescence microscopy (Olympus, Tokyo, Japan) was used to visualize the cells and acquire images (at three different fields).

Western Blotting, Antibodies, and Other Reagents

The procedure was performed based on our previously reported method.²¹ The primary antibodies anti-Bax (ab32503), anti-Bcl-2 (ab692), anti-p-mTOR (ab109268), anti-mTOR (ab32028), anti-p-AKT (ab8805), and anti-AKT (ab105731) were purchased from Abcam (Cambridge, UK). The secondary antibodies goat anti-rabbit IgG (H&L) and goat anti-mouse IgG (H&L) were purchased from GenScript (Piscataway, NJ, USA). LBP was obtained from Hainan Changan International Pharmaceutical Co. (Haikou, Hainan, China).

Statistical Analysis

Data are presented as the mean $\pm$ SD of at least three experiments. The one-way ANOVA with Tukey's multiple comparison were used to determine statistical significance; variations having a $p < 0.05$ were considered significant.

Results

LBP Enhanced ^{125}I -Induced Apoptosis in NSCLC Cells

To investigate the effect of a sensitizing concentration of LBP, defined as 10% of the IC_{50} , on ^{125}I , we initially tested a range of LBP concentrations on NSCLC cells. Using the CCK-8 assay, we determined that the viability of the A549, H1975 and H157 cell lines was influenced by LBP in a dose-dependent manner; the IC_{50} values of LBP for A549, H1975, and H157 cells after a 72 h-treatment were 7.867 $\mu\text{g/mL}$, 2.119 $\mu\text{g/mL}$, and 2.907 $\mu\text{g/mL}$, respectively (Figure 1A).

Moreover, flow cytometry analysis demonstrated that cellular apoptosis was significantly increased when NSCLC cells were concurrently treated with a sensitization concentration LBP and ^{125}I compared with that in cells treated with either treatment alone (Figure 1B and C). The TUNEL assay results further confirmed that the combined treatment with a sensitization concentration of LBP and ^{125}I could induce a more obvious cellular apoptosis than did either treatment alone in A549 cells

Figure 1 LBP enhanced ^{125} I-induced apoptosis in NSCLC cells. **(A)** After treatment with LBP concentrations ranging from 0 to 80 μ g/mL for 72 h on A549, H1975, and H157 cells, the CCK-8 assay was used to determine cell proliferation; optical density values were detected using a microplate reader; the sensitization concentration was defined as 10% of IC_{50} . **(B and C)** After cells were treated with ^{125} I, LBP (10% of IC_{50}), or their combination on A549 and H1975 cells, Annexin V-FITC/PI was performed to analyze cellular apoptosis. The data are presented as the mean $\pm$ SD. One-way ANOVA was used for data analysis; * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$.

(Figure 2A). Consistently, the Bax/Bcl-2 ratio of combined treatment was higher than that of single treatment in A549, H1975, and H157 cells (Figure 2B). Taken together, our results show that LBP enhanced ^{125}I -induced apoptosis in NSCLC cells.

LBP Increased ^{125}I -Induced Anti-Proliferative Effect in NSCLC Cells

The cell cycle analysis showed that G2/M cell cycle arrest was detected in all three treatment groups; however, this arrest was more intense after the combined treatment (Figure 3A and B). Moreover, the results of the CCK-8 assay revealed that although the proliferation of A549, H1975, and H157 cells was suppressed by single treatment with either ^{125}I or LBP, their combination could induce a more significant cell growth inhibition (Figure 4A). Consistently, the EdU assay in A549 cells further supported the previous experiments and showed that fewer cells were considered to have a proliferative status in the combined treatment group (Figure 4B). Overall, the obtained results indicate that LBP increased ^{125}I -induced anti-proliferative effect in NSCLC cells.

LBP Facilitated ^{125}I -Induced Tumor Suppression in vivo

To investigate whether LBP could promote ^{125}I -induced tumor suppression in vivo, an A549 xenograft nude mouse model was used. As shown in Figure 5A, the combination of ^{125}I and LBP restrained tumor growth more effectively than did single ^{125}I treatment. The tumor burden results are shown in Supplementary Figure S1. In addition, the tumor volume was more significantly decreased in the combined treatment group than in the ^{125}I single treatment group (Figure 5B). Taken together, our data suggest that LBP facilitated ^{125}I -induced tumor growth inhibition in vivo.

LBP Increased the ^{125}I -Induced Down-Regulation of the AKT/mTOR Pathway

In our previous iTraQ study, we found that the AKT/mTOR pathway was significantly down-regulated in HepG2, a hepatocellular carcinoma cell line, after treatment with ^{125}I (Supplementary Figure S2). Based on our findings, we hypothesized that ^{125}I could also downregulate the AKT/mTOR pathway in the A549 cell line. As shown in Figure 6A, to verify the potential mechanism by which LBP enhanced ^{125}I -induced apoptosis and proliferation

inhibition in A549 cells, AKT/mTOR pathway expression levels were detected after combined treatment or single treatment. The results demonstrated that combined treatment induced a more significant downregulation of the AKT/mTOR pathway than did single treatment. To further investigate whether AKT/mTOR pathway is involved in the anti-cancer of ^{125}I and LBP, the Bax/Bcl2 ratio and cell viability in A549 cells were detected after the mTOR expression was up-regulated or down-regulated. The results showed that the down-regulation of mTOR could enhance the anti-cancer effect, while the up-regulation of mTOR could compromise the effect (Figure 6B and C). The original uncut Western blot bands are shown in Supplementary Figure S3–4.

Discussion

In recent years, radioactive ^{125}I seed implantation has increasingly been regarded as an alternative and efficient treatment for advanced NSCLC.⁷ Besides, combining ^{125}I with chemotherapy can significantly improve the clinical efficacy and extend the overall survival of NSCLC patients.⁶ However, the underlying mechanism of ^{125}I brachytherapy in tumors is still unclear. Our previous research indicated that ^{125}I induced apoptosis and proliferation inhibition in HCC by upregulating the PERK-eIF2 α -ATF4-CHOP pathway.²⁰ Based on our previous iTraQ results, we found that the function of ^{125}I is related to the AKT/mTOR pathway. In this study, we explored whether LBP could sensitize A549 cells to ^{125}I radiation and determined the potential links among ^{125}I , LBP, and the AKT/mTOR pathway. As indicated by our results, LBP could enhance ^{125}I -induced apoptosis and anti-proliferative effect in A549 cells through the AKT/mTOR pathway.

LBP has been reported as the first-line treatment for NSCLC with minimal side effects and high specificity.^{9,20,22} Moreover, combining LBP with radiotherapy resulted in a better clinical efficacy than did single treatment alone.⁷ However, drug resistance is an important factor that affects LBP clinical efficacy. Thus, many studies have aimed at improving the sensitivity of LBP; paclitaxel, an antitumor drug that targets tubulin, was reported to increase the sensitivity of the lung cancer cell line NCI-H446 to LBP via the PI3K/AKT pathway.¹⁰ Furthermore, LBP could arrest the cell cycle at the G1 phase and induce apoptosis in A549 cells via the p53/ROS/p38MAPK signaling pathway.⁹ In contrast, another study demonstrated that LBP induce cell cycle arrest at

Figure 2 LBP enhanced ^{125}I -induced apoptosis in NSCLC cells. (**A** and **B**) After NSCLC cells were treated with ^{125}I , LBP (10% of IC_{50}), or their combination, TUNEL assay in A549 cells was performed and Bax/Bcl-2 expression level in A549, H1975, and H157 cells was detected to analyze cellular apoptosis. The data are presented as the mean $\pm$ SD. One-way ANOVA was used for data analysis; * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$.

Figure 3 LBP increased ^{125}I -induced anti-proliferative effect in NSCLC cells. **(A and B)** After treatment with ^{125}I , LBP (10% of IC_{50}), or their combination, cell cycle analysis was performed to verify the anti-proliferative effect of LBP and ^{125}I on A549 and H1975 cells. The data are presented as the mean $\pm$ SD. One-way ANOVA was used for data analysis; * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$.

G2/M phase.²³ In our study, we also verified that LBP could induce apoptosis and inhibit the proliferation of A549 cells. And we found that LBP induced cell cycle arrest at the G2/M phase; we believe that these opposing results may be attributed to the difference in LBP concentration, considering that we used a sensitizing concentration (10% of the IC_{50}). Moreover, our study suggested that

LBP could enhance ^{125}I -induced apoptosis and proliferation inhibition in A549 cells, which verified that the combination of LBP chemotherapy and ^{125}I radiotherapy leads to a better outcome than either treatment alone.

Since the 1980s, radioactive ^{125}I seed implantation has been widely used as an alternative treatment for NSCLC,⁴ radioactive ^{125}I seeds could locally control tumors by

Figure 4 LBP increased ^{125}I -induced anti-proliferative effect in NSCLC cells. **(A)** After treatment with ^{125}I , LBP (10% of IC_{50}), or their combination, the proliferation of A549, H1975, and H157 cells was detected by CCK-8 assay at 0, 24, 48, and 72 h. **(B)** The EdU assay were performed to verify the anti-proliferative effect of LBP and ^{125}I on A549 cells. The data are presented as the mean $\pm$ SD. One-way ANOVA was used for data analysis; * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$.

continuously releasing a low dose of radiation to surrounding lesions for a long period of time.⁶ However, the underlying mechanism of ^{125}I in NSCLC is still obscure; ^{125}I was reported to suppress tumor growth via Warburg effect inhibition in a dose-dependent manner in A549 xenografts.⁸ Although the Warburg effect may be related to mTOR, c-Myc, HIF-1 α , and GLUT1 downregulation, the exact mechanism remains unclear. From our previous study, we saw that ^{125}I could induce cellular apoptosis and cell cycle arrest in PANC-1 cells;²¹ moreover, another recent study on HCC demonstrated that LBP promoted ^{125}I -induced apoptosis and anti-proliferative effect via the PERK-eIF2 α -ATF4-CHOP pathway, which we identified

by iTraQ.²⁰ In our present study, we verified that ^{125}I could significantly inhibit tumor growth in vivo and decrease proliferation with G2/M cell cycle arrest in vitro; interestingly, these anticancer effects could be enhanced by LBP.

Several studies have suggested that the AKT/mTOR signaling pathway plays an essential role in cell apoptosis, cell proliferation, cell cycle progression, necrosis, and drug resistance. A recent study revealed that xeroderma pigmentosum complementation group C (XPC) inhibition could reverse cisplatin resistance in A549 cells via down-regulating the AKT/mTOR pathway.²⁴ In addition, the inhibition of mTOR by rapamycin increased curcumin-induced apoptosis and autophagy, thereby inhibiting cell

A **B**

Figure 5 LBP facilitated ¹²⁵I-induced tumor growth inhibition in vivo. To further verify the combined effect of LBP and ¹²⁵I in vivo, an A549 xenograft tumor nude mouse model was established. (A) After single or combined treatment for 27 days, the mice were sacrificed and tumors were excised. (B) The tumor weight was measured every other day for 27 days. The data are presented as the mean $\pm$ SD. One-way ANOVA was used for data analysis; * $P < 0.05$, ** $P < 0.01$.

proliferation.²⁵ Furthermore, the sensitivity of LBP was notably enhanced by paclitaxel via inhibiting the PI3K/AKT pathway.¹⁰ Another study demonstrated that casticin

could cause anti-cancer effect in several human cancer, such as breast cancer, gastric cancer, and myeloma, through AKT/mTOR pathway.²⁶ Moreover, the inhibitor

Figure 6 LBP increased the ^{125} I-induced down-regulation of the AKT/mTOR pathway. **(A)** After treatment with ^{125} I, LBP (10% of IC_{50}), or their combination on A549 cells, the expression level of AKT/mTOR signaling was detected by Western blot. **(B and C)** The Bax/Bcl2 ratio and cell viability in A549 cells were detected after the expression of mTOR was down-regulated or up-regulated by siRNA or pcDNA. The data are presented as the mean $\pm$ SD. One-way ANOVA was used for data analysis; * $p < 0.05$, ** $p < 0.01$.

of mTOR pathway combined with standard chemotherapy illustrated a positive effect in prevent and treatment of oral cancer.²⁷ As for the resistance of radiotherapy, a review had concluded that the high expression level of mTOR is involved in antioxidant-related radioresistance of solid tumor, which indicated that mTOR pathway may be a target to promote tumor sensitivity to radiation.²⁸ In our previous study, through the results of iTraQ and preliminary data from our previous experiments, we demonstrated that ¹²⁵I treatment significantly downregulated mTOR-related pathways in the HepG2 cell line;²⁰ thus, we suggested a relationship between the expression level of AKT/mTOR signaling and ¹²⁵I irradiation. In this study, we showed that ¹²⁵I treatment could indeed significantly downregulate AKT/mTOR expression.

There are several limitations in our study, which need to be addressed in future investigations. We initially hypothesized the existence of a link between ¹²⁵I irradiation and AKT/mTOR signaling based on the results of iTraQ on HepG2. Thus, it is only fair that iTraQ should be performed for the A549 cell line to identify the expression level of AKT/mTOR pathway after ¹²⁵I treatment. Although we demonstrated that the combined treatment of LBP and ¹²⁵I could induce cell cycle arrest at G2/M phase, the analysis of cyclin and CDK was not performed.

In conclusion, our results demonstrated that LBP enhanced ¹²⁵I-induced apoptosis and proliferation inhibition in NSCLC cells and that these effects were closely related to the downregulation of the AKT/mTOR signaling pathway. Besides, we believe that our study lays a foundation for follow-up research and might have great clinical implications in the future.

Data Sharing Statement

The analyzed data sets generated during the study are available from the corresponding author on reasonable request.

Ethics Approval

The study was approved by the Shandong University Animal Care Committee.

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Author Contributions

All authors made substantial contributions to conception and design, acquisition of data, or analysis and interpretation of data; took part in drafting the article or revising it critically for

important intellectual content; agreed to submit to the current journal; gave final approval of the version to be published; and agree to be accountable for all aspects of the work.

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Disclosure

The authors declare that they have no conflicts of interest for this work.

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