nature portfolio

Peer Review File

Alkaloids of Aconiti Lateralis Radix Praeparata inhibit growth of non-small cell lung cancer by regulating PI3K/Akt-mTOR signaling and glycolysis

Reviewers' comments:

Reviewer #1 (Remarks to the Author):

This study demonstrated the anti-cancer activity of the extract using in vitro, in vivo and in silico. The experimental design can support the finding. However, there are many missing points that needed clarification and experiments supported.

1. What is the rationale in studying Aconiti Lateralis as anti-cancer targeting NSCLC? Does it have a previous report of its activity or cytotoxic against NSCLC?
2. The authors need to add more detailed explanations regarding role of PI3K/AKT/mTOR in regulating metabolism in cancer
3. Why does the author choose 50 mm3 as the volume cut point before starting the treatment? Will it be too small?
4. Obviously, the authors only use the Genecards database to analyze the genes related to NSCLC. It is not enough to represent the accuracy of the data collection. Authors need to analyze at least from similar databases. For example: TTD, OMIM, etc.
5. How can authors select the concentration for in vivo study, while authors did in vitro study after in vivo?
6. Fig 1F, The images for H&E staining are not clear for identifying tumor colonization.
7. Line no 370-371 and 385, the author mentioned the result in which Figure? Fig. 3A-B or Fig. 1I? And for MTT data, which color in each line means?
8. Is there any detected metastasis of NSCLC in other organs? It should be reported.
9. The authors need to add more details, e.g., the name of each compound in Fig 2C.
10. Fig 4 and 5, the letters are not clearly seen what the author mentioned.
11. Please explain the glycolysis/gluconeogenesis in Fig 3H and 5D. It does not appear in the figure.
12. How can authors prove the pathway mechanism related to PI3K and its downstream signaling? Authors need to prove by using Knockdown or inhibitors related to PI3K.
13. How the extract inhibited PI3K/ATK-mTOR signaling pathway? Does the extract have kinase inhibitory activity? Which exact compound in the extract has this effect? The author should provide more detail.
14. How does the author control batch-to-batch of the extract? How to make sure that every batch has the same amount and compound? This is a serious point in studying the pharmacological activity of the extract rather than the pure compound, otherwise, the author includes the experiment showing which compound in the extract has this pharmacological activity.
15. From the graphic abstract, the author shows that the extract inhibits the receptor, but there are no data supported. And also the authors did not prove the downstream signaling.

Reviewer #2 (Remarks to the Author):

The research article entitled “Alkaloids of Aconiti Lateralis Radix Praeparata treated non-small cell lung cancer by regulating glycolysis via the PI3K/AKT-mTOR signaling pathway” has been found to be very well written and elaborate in its contents. The authors have framed and performed the

experiments in a very planned way. Further, their execution and representation of data are accurate.

The research provides a good overview relating the significance of exploring traditional Chinese medicine (TCM) for the treatment of non-small cell lung cancer (NSCLC) and introduces the potential use of Aconiti Lateralis Radix Praeparata (Fuzi) for this purpose. However, some modifications can be made to improve clarity and readability. Here are the suggested modifications:

Major comments:

1. Try elaborating more on multi-omics and then relating it with metabolomics in the Introduction section.
2. Discuss more the significance of Aconiti Lateralis Radix Praeparata and the various other medicinal substances mentioned as TCM. It may further be justified why Aconiti Lateralis Radix Praeparata was preferred over the others.
3. Rephrase from lines 138 to 141 for better understanding. The following extract has been added as a suggestion:
"Fuzi was mixed with eight times the amount of water and boiled for one hour. The resulting liquid was collected by passing it through a filter, and the solid residue was subjected to the same process again. The liquid obtained from the second extraction was then combined with the first one using filtration. The combined liquid was then concentrated to obtain the final extract."
4. Rephrase from lines 362 to 364 for better understanding. The following extract has been added as a suggestion:
"At the same time, it was observed that both FZA and FZP did not lead to significant weight loss and had minimal impact on the heart, spleen, lung, liver, and kidney in nude mice with A549 cell xenograft tumors when compared to the control group. This suggests that FZA and FZP have low toxicity in these mice."
5. Rephrase from lines 478 to 483 for better understanding. The following extract has been added as a suggestion:

"FZA was found to have the potential to regulate and normalize the abnormal levels of various metabolites associated with the inflammatory response. These metabolites included beta-alanine, glucose-6-phosphate, aspartic acid, proline, fumaric acid, ornithine, glycine, L-malic acid, uracil, threonine, lysine, aconitic acid, and several others. This suggests that FZA may exert its anti-NSCLC effects by regulating the expression of these metabolites.
Pathway enrichment analyses were conducted using information from the KEGG database to explore the mechanisms of action further. These analyses helped identify the specific metabolomic pathways involved in the anti-NSCLC effects of FZA."

Minor comments:

1. In line 140, replace 'with' with 'by'.
2. In line 141, replace 'to' with 'into'.
3. In line 166, write 'experiments' in place of 'experiment'.

4. In line 205, replace ‘integrating’ with ‘integrates’.
5. In line 243, replace ‘were’ with ‘was’.
6. In line 373, replace ‘exhibits’ with ‘exhibited’.
7. In line 416, ‘the’ seems unnecessary and can be omitted from the sentence.
8. In line 449, replace ‘resulted’ with ‘resulting’.
9. In line 518, rephrase the sentence to: ‘Anti-cancer effect of FZA on NSCLC was confirmed by our in vitro and in vivo experimental data.’ The use of ‘had’ has been replaced with ‘was’.
10. In line 539, replace ‘diseases’ with ‘disease’.
11. In line 541, replace ‘the ingredients’ instead of ‘ingredient’.

Dear Reviewers:

Thank you very much for the comments and suggestions from you on our manuscript entitled "*Alkaloids of Aconiti Lateralis Radix Praeparata treated non-small cell lung cancer by regulating glycolysis via the PI3K/AKT-mTOR signaling pathway*".

According to your suggestions, we have carefully checked our manuscript and revised it point by point, and have made appropriate changes in the manuscript. Additionally, we have further polished the language of our manuscript including some words, sentences, and typographical errors in the manuscript. The major revisions are indicated with **blue characters** in the revised manuscript.

We have also uploaded our revised manuscript and response to you online.

Hopefully we have addressed all of your concerns, and these revisions are acceptable.

Should you have any questions about this manuscript, please don't hesitate to let us know.

Sincerely yours,
Wen ZHANG
Email: wenzhang@njucm.edu.cn

Reviewer #1

1. What is the rationale in studying Aconiti Lateralis as anti-cancer targeting NSCLC? Does it have a previous report of its activity or cytotoxic against NSCLC?

Response:

Thank you for your question.

First of all, Aconiti Lateralis Radix Praeparata (*Fuzi*) has been frequently used in anti-cancer therapy. It was firstly recorded in *Agriculture God's Canon of Materia Medica* that *Fuzi* has the effect of treating tumors. *Fuzi* is frequently employed in clinical practice to treat lung cancer, gastric cancer, and other malignant tumors. The famous *Fuzi*-based formulas, including Sini Decoction, Mahuang Fuzi Xixin Decoction, Shenfu Injection and Compound Sansheng Injection, exert excellent therapeutic effects in anti-cancer treatment [1-3]. Meanwhile, various findings have highlighted the antineoplastic functions of *Fuzi* alkaloids such as aconitine, mesaconitine, lipomesaconitine, lipoaconitine, and neoline [4-6]. These medical practices and experimental findings collectively suggest *Fuzi*'s potential as an effective agent. Our previously published review (DOI: 10.3389/fphar.2022.870282) summarized the frontier research on the anti-tumor effects and corresponding mechanisms of *Fuzi*, including alkaloids and polysaccharides. In view of this, these previous studies provide evidences for anti-cancer effect of *Fuzi*, which has sparked increasing interest in the application of cancer therapy.

Secondly, our preliminary research published on *Phytomedicine* (DOI:

https://doi.org/10.1016/j.phymed.2023.155099) demonstrated that ***Fuzi* decoction had significant inhibitory effects on NSCLC both *in vivo* and *in vitro***, but material basis of *Fuzi*'s anti-tumor substances is not clear.

Given the holistic and systematic nature of TCM theory, the current study aims to assess the efficacy of *Fuzi* alkaloids (FZA) and *Fuzi* polysaccharides (FZP) extracted from *Fuzi* in NSCLC and elucidate the corresponding mechanism. It is worth noting that the object of the current study is FZA rather than monomer ingredient or *Fuzi* decoction. To the best of our knowledge, **this article is the first to explore the efficacy and mechanism of FZA against NSCLC.**

2.The authors need to add more detailed explanations regarding role of PI3K/AKT/mTOR in regulating metabolism in cancer.

Response:

We sincerely appreciate the valuable comments. We have checked the literature carefully and added more explanations on PI3K/Akt/mTOR in regulating metabolism in cancer in the Introduction section in Line 97-118, Page 4 of the revised manuscript.

The phosphoinositide PI3K/Akt-mTOR signaling pathway is involved in regulating a variety of cellular physiological processes by activating the corresponding downstream effectors, which plays a key role in tumor proliferation, invasion and metastasis [7, 8]. When PI3K is activated, it catalyzes the phosphorylation of the hydroxyl group at position 3 of phosphatidylinositol 4,5-bisphosphate (PIP2) to form phosphatidylinositol 3,4,5-trisphosphate (PIP3). Then PIP3 recruits 3-phosphoinositide-dependent protein kinase-1 (PDK1) and Akt proteins to the plasma membrane, leading to phosphorylation of Akt by PDK1 [9]. Phosphorylated Akt inhibits the kinase activity of glycogen synthase kinase-3 beta (GSK-3β), preventing Cyclin D1 degradation, thereby regulating glucose metabolism. Akt activation can enhance glucose uptake in cancer cells through regulation of the major glucose transporter-1 (GLUT1) [10]. In addition to enhancing glucose uptake, Akt regulates several glycolytic enzymes such as hexokinase 2 (HK2) and phosphofructokinase (PFK) through post-translational and transcriptional mechanisms [11]. PI3K-dependent Akt activation results in enhanced PFK2 phosphorylation and production of fructose-2,6-bisphosphate, which in turn promotes PFK1 activation. The enhanced PI3K/Akt-dependent PFK1 activation and GLUT1 expression promoted the Warburg effect, tumor cell proliferation, and tumorigenesis [12]. Activation of mTORC1 downstream of Akt can promote elevated levels of hypoxia inducible factor-1 (HIF-1α) in the absence of hypoxia, leading to the induction of aerobic glycolysis in tumor cells [13]. Activated mTORC1 can also have an effect on the expression levels of glycolysis-related enzymes in tumor cells through the regulation of downstream transcription factors. These findings underscore the instrumental role of PI3K/Akt-mTOR in regulating metabolism.

3. Why does the author choose 50 mm^3 as the volume cut point before starting the treatment? Will it be too small?

Response:

Thank you for the question. Based on animal ethics and pre-testing, this trial could

be carried out with a tumor volume starting point of 50 mm^3. Selecting a tumor volume of 50 mm^3 was found to be effective in conducting subsequent experiments, which was published on *Phytomedicine* [14] (Wen Zhang, et al., Modulation of cellular metabolism and alleviation of bacterial dysbiosis by Aconiti Lateralis Radix Praeparata in non-small cell lung cancer treatment, Phytomedicine, 2023, doi: https://doi.org/10.1016/j.phymed.2023.155099).

4. Obviously, the authors only use the Genecards database to analyze the genes related to NSCLC. It is not enough to represent the accuracy of the data collection. Authors need to analyze at least from similar databases. For example: TTD, OMIM, etc.

Response:

Thank you for the suggestion. We have re-analyzed NSCLC related genes with several databases including Genecards, TTD and OMIM. The relevant methods have been modified in Line 288, Page 8. The results and figures have been added in Line 541-565, Page 21-22, which have also been attached below (Fig. R1).

Anti-cancer effect of FZA on NSCLC was confirmed by our *in vivo* and *in vitro* experimental data. Based on the complexity of TCM ingredients and the integrity of their effects, FZA might exert anti-tumor effects through multi-component, multi-target, and multi-pathway, which is consistent with the systematic view of network pharmacology. Totally 210 potential treatment targets of FZA and 1 040 target genes of NSCLC were identified, among which 34 key targets intersected (Fig. R1-A). PPI analysis concluded that FZA acted on core targets such as AKT1, EDFR, SRC, BCL2, MTOR, KDR, PI3KCA, KIT, and PARP1 NSCLC, as shown in Fig. R1-B The drug-ingredient-target-disease network was constructed with 90 nodes and 168 connecting edges (Fig. R1-C). Subsequently, GO enrichment analysis of these 34 core targets were performed to explore the potential therapeutic mechanisms of FZA in NSCLC. Moreover, KEGG pathway enrichment analysis showed the most significantly enriched 20 pathways of FZA involved in NSCLC in Fig. R1-D. The pathways in cancers showed the largest number of involved targets (17 counts), followed by the PI3K/Akt signaling pathway. The analysis revealed that FZA acted on NSCLC mainly through biological processes such as protein phosphorylation, transmembrane receptor protein tyrosine kinase signaling pathway, and PI3K signaling regulation (Fig. R1-E). Based on the above results, we speculated that FZA might treated NSCLC by regulating energy metabolism *via* PI3K/Akt signaling pathway.

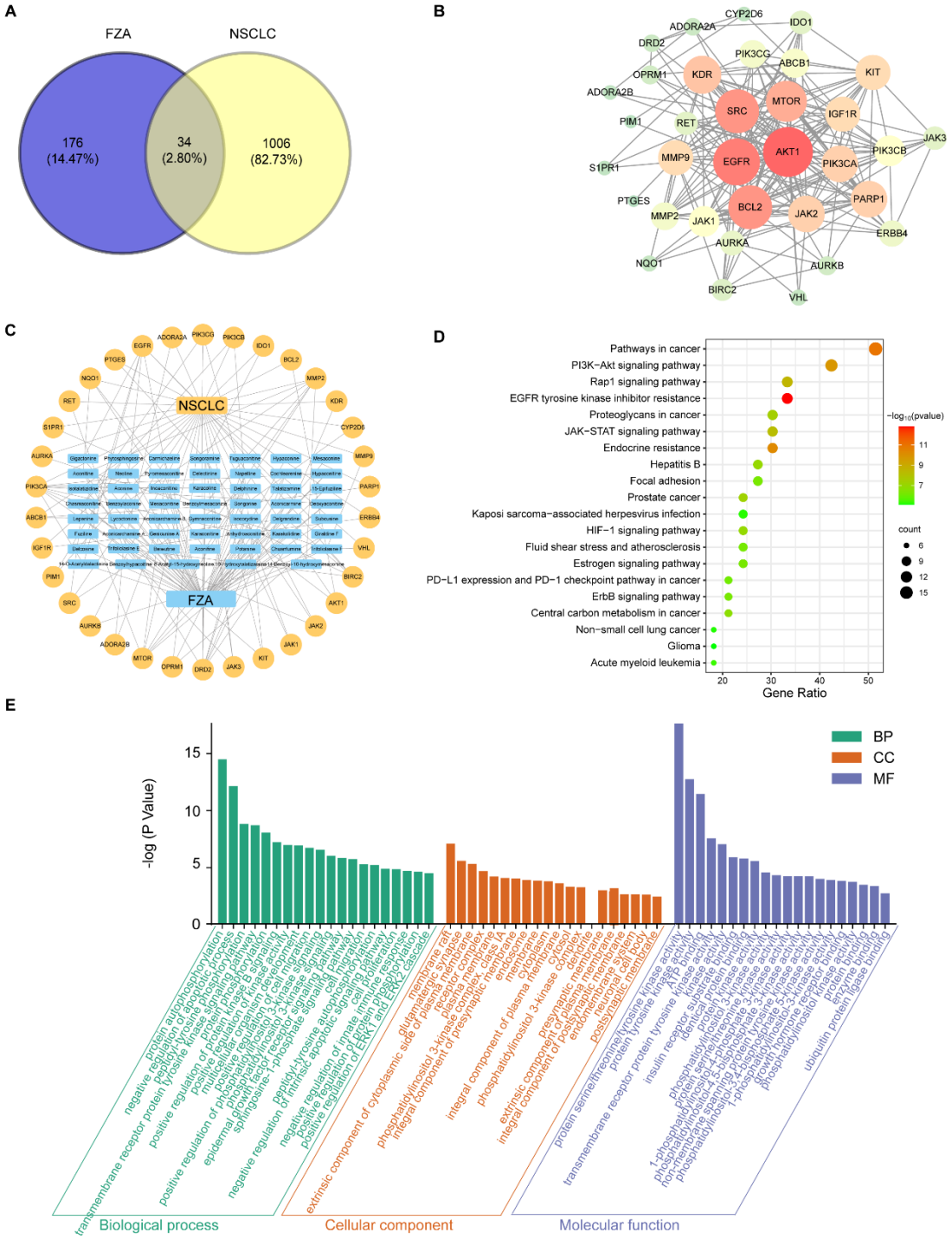

Fig. R1 Network pharmacology analysis revealed the targets and pathways regulated by FZA in NSCLC.

5. How can authors select the concentration for in vivo study, while authors did in vitro study after in vivo?

Response:

Thank you for the question. Our previous studies published on *Phytomedicine* (Wen Zhang, et al., Modulation of cellular metabolism and alleviation of bacterial dysbiosis by Aconiti Lateralis Radix Praeparata in non-small cell lung cancer treatment,

Phytomedicine, 2023, doi: https://doi.org/10.1016/j.phymed.2023.155099) found that 10, 15 and 20 g/kg *Fuzi* decoction has significant anti-NSCLC effects achieving inhibition rates of 60.02 %, 68.75 %, and 71.68 %, respectively (Figure R2). According to these results, we chose 15 g/kg as the dosages of *Fuzi decoction* in this article. Subsequently, *Fuzi* alkaloids (FZA) and *Fuzi* polysaccharides (FZP) were extracted and purified. The preparation description for FZA and FZP is available inline 156-177, page 5 of the revised manuscript. Based on the alkaloid and polysaccharide contents in the *Fuzi* decoction in our previous study, the dosages of FZA were determined to be 2 g/kg FZA and 7.5 g/kg FZP.

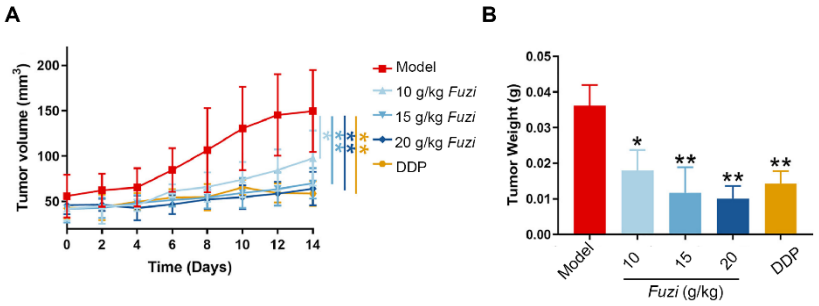

Fig. R2 *Fuzi* decoction inhibited the growth of NSCLC in xenograft mice. (Wen Zhang, et al., Modulation of cellular metabolism and alleviation of bacterial dysbiosis by Aconiti Lateralis Radix Praeparata in non-small cell lung cancer treatment, Phytomedicine, 2023, doi: https://doi.org/10.1016/j.phymed.2023.155099)

6. Fig 1F, The images for H&E staining are not clear for identifying tumor colonization.

Response:

Many thanks for your suggestion. We are sorry that the photo combination reduced the clarity. The images for H&E staining of tumor sections have been rearranged and enlarged to be clear, which have been attached below (Fig. R3) and added in the revised manuscript (Fig. 1D).

H&E staining showed that the tumor cells in the tumor tissue were densely arranged. The H&E staining results of tumor tissues showed that the control group had a dense arrangement of tumor cells, regular morphology and large volume, with high nucleoplasm ratio and less division, accompanied by a small number of lymphocytes and neutrophil infiltration, which verified the tumor formation.

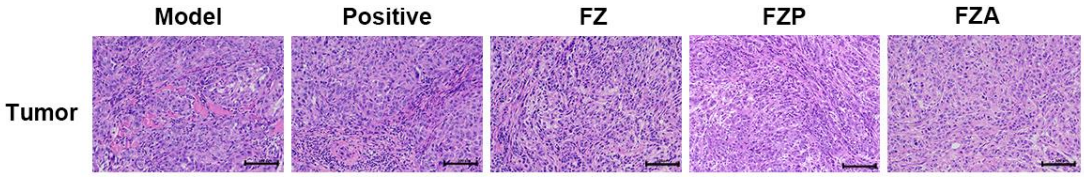

Fig. R3 H&E staining of tumor tissues

7. Line no 370-371 and 385, the author mentioned the result in which Figure? Fig. 3A-B or Fig. 1I? And for MTT data, which color in each line means?

Response:

Thank you for your question. Sorry for the misunderstanding. We have rectified the result which mentioned in Fig. 1G.

Fig. 1G showed that we replicated the MTT experiment for three times to assess the cell viability of NSCLC cells treated with FZA, where the red line, green line, and black line respectively represent the first, second, and third time of MTT results, which have been added in Line 394-395, Page 12.

8. Is there any detected metastasis of NSCLC in other organs? It should be reported.

Response:

Thank you for your question. The pathological section observation of various organs (including heart, liver, spleen, lungs, and kidneys) showed that there was no organ metastasis occurred from the subcutaneous tumor grafts in this experiment which have been attached below (Fig. R4) and added in the revised manuscript (Fig 1E).

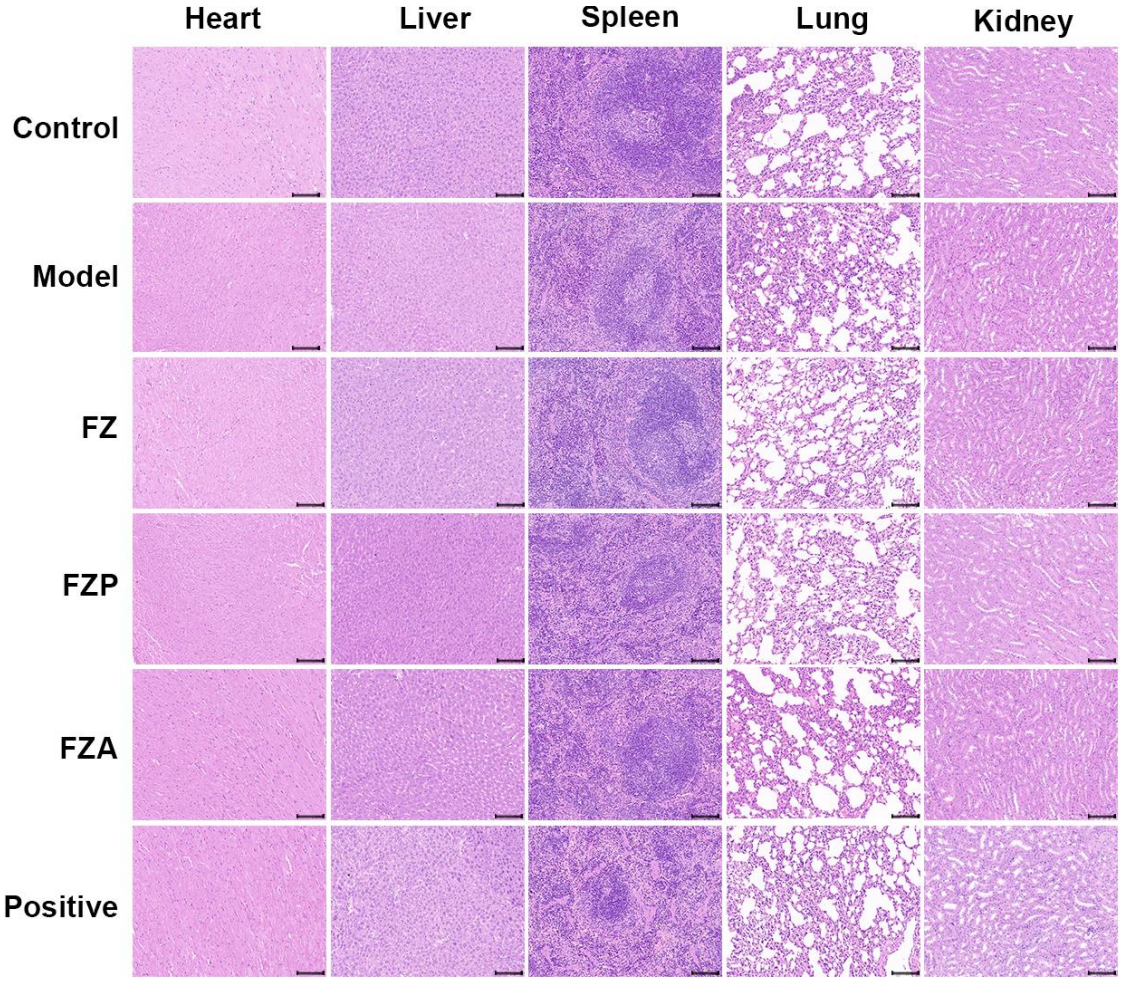

Fig. R4 Histological analysis of the major organs slices after treatments.

9. The authors need to add more details, e.g., the name of each compound in Fig 2C.

Response:

Thank you for your suggestion. We have added the name of each compound in Fig 2C.

10. Fig 4 and 5, the letters are not clearly seen what the author mentioned.

Response:

Many thanks for your suggestion. Fig 6-7 and Fig 8 in the current version of the manuscript have been rearranged and enlarged to be clear, which have added on page 18,20 and 22 respectively.

11. Please explain the glycolysis/gluconeogenesis in Fig 3H and 5D. It does not appear in the figure.

Response:

Many thanks for your suggestion. Based on the enrichment pathway analysis of proteomics (Fig. 4D in the current version of the manuscript) and network pharmacology (Fig. 7D in the current version of the manuscript), PI3K/Akt-mTOR signaling pathway was enriched. The glycolysis/gluconeogenesis pathway is essentially a bidirectional conversion between glucose and pyruvate, which is regulated by PI3K/Akt-mTOR. Therefore, we speculated that FZA might treat NSCLC by regulating glycolysis *via* PI3K/Akt-mTOR signaling pathway. In the following experiment, the mechanism was verified by assay kits, GC-MS analysis and western blotting.

12. How can authors prove the pathway mechanism related to PI3K and its downstream signaling? Authors need to prove by using Knockdown or inhibitors related to PI3K.

Response:

Many thanks for your suggestion. Based on the results of quantitative proteomics and network pharmacology, we hypothesized that FZA might treat NSCLC *via* PI3K/Akt-mTOR signaling pathway. In order to ascertain this hypothesis, we performed western blot assays and used PI3K inhibitor.

Firstly, NSCLC cells were treated with various concentrations of FZA for 24 h. Western blotting assay verified that FZA dose-dependently reduced the protein expression of p-PI3K, p-Akt and p-mTOR without influencing the protein levels of PI3K, Akt and mTOR, which have been attached below (Fig. 5R-A) and added in the revised manuscript (Fig.8D).

Secondly, we employed PI3K inhibitor LY294002 to measure cell viability by using the MTT assay. A549 cells were divided into control group, FZA group, LY294002 group and combination group. Control group were incubated with vehicle, while FZA group was treated with 200 μg/mL FZA for 24 h and LY294002 group was treated with vehicle after 2 h of 20μM LY294002 treatment. The combination group was administered with 20μM LY294002 for 2 h prior to 24 h of FZA treatment. As shown in Fig. 5R-B, compared with the control group, the cell viability decreased to 60.8%, 65.5%, or 56.8% respectively. The cell viability of the 2-hour pre-treatment group was lower than FZA treatment group. Compared with the FZA group, the cell viability of the combination group was significantly reduced ($P<0.05$). The MTT assay showed that inactivation of PI3K/Akt-mTOR enhances the inhibitory effect of FZA on A549 cell proliferation.

Based on above-mentioned results, we speculated that FZA possibly regulated the PI3K/Akt-mTOR signaling pathway to exert anticancer effects.

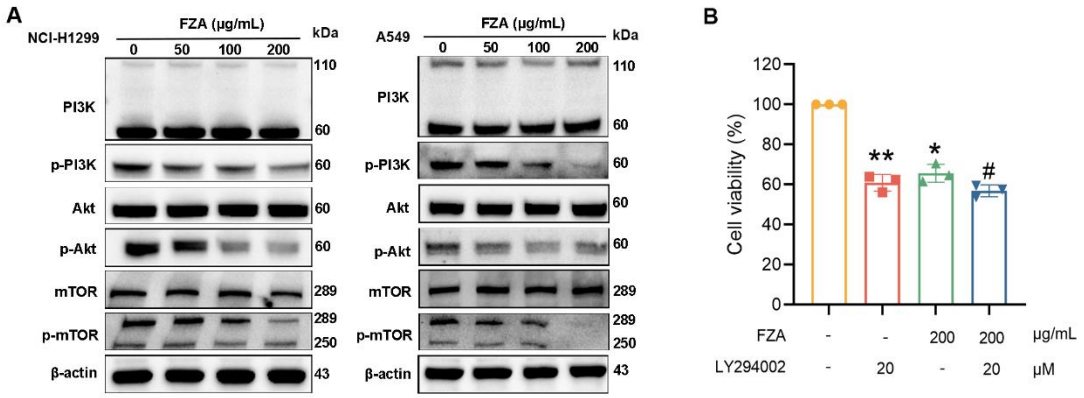

Fig. 5R FZA treated NSCLC *via* PI3K/Akt-mTOR signaling pathway. (A) FZA inhibited phosphorylation of PI3K, Akt and mTOR. (B)The effect of FZA combined with LY294002 on the proliferation of A549 cells. $^{**}p < 0.001$ VS. control group; $^{*}p < 0.05$ VS control group; $^{\#}p < 0.05$ VS FZA group.

13. How the extract inhibited PI3K/ATK-mTOR signaling pathway? Does the extract have kinase inhibitory activity? Which exact compound in the extract has this effect? The author should provide more detail.

Response:

Many thanks for your suggestion. To determine how the extract inhibits the PI3K/Akt-mTOR signaling pathway, we measured PIP3 levels in A549 cells after various concentrations of FZA treatment.

A549 cells were plated in 6-well plates with a density of 5×10^5 cells/well, treated with 0, 50, 100, 200 μg/mL FZA or 20 μM LY294002 (PI3K inhibitor, TargetMol Chemicals Inc., Shanghai, China) for 3 h. Cells were lysed and the contents of PIP3 were evaluated using an ELISA kit (Jiangsu Jingmei Biotechnology Co., Ltd, Yancheng, China) according to the manufacturer's instructions. Compared with the control group, the content of PIP3 decreased significantly after 100 and 200 μg/mL FZA treatment. The results reveal that FZA inhibited the PI3K enzyme in a concentration dependent manner (Fig.6R-A).

Furthermore, to predict the potential interaction of FZA and the PI3K protein. We performed the molecular docking. Based on literatures and HPLC content determination of FZA, the main active ingredients of FZA were selected, which have been reported to have anticancer activity, such as benzoylmesaconitine, benzoylaconitine, benzoylhypaconitine, mesaconitine, hypaconitine and aconitine [15-19]. The results showed that the six compounds could bind to PIK3CA and may affect its functions (Fig.6R-B). Among them, benzoylaconitine and aconitine had a strong affinity for the PIK3CA protein. benzoylaconitine forms five hydrogen bonds with the amino acid residues Ile459, Asn457, Asp454, Glu453 and Gln1014 of PIK3CA, whereas aconitine bonds with Asp538 and Lys1030. Therefore, benzoylaconitine or aconitine and PIK3CA likely form a stable complex, which can prevent Asp from binding with phosphoric acid and phosphorylate, thus inactivating PIK3CA (Fig. 6R-C). Therefore, FZA may treat NSCLC through its active components by targeting PI3K/Akt-mTOR pathways.

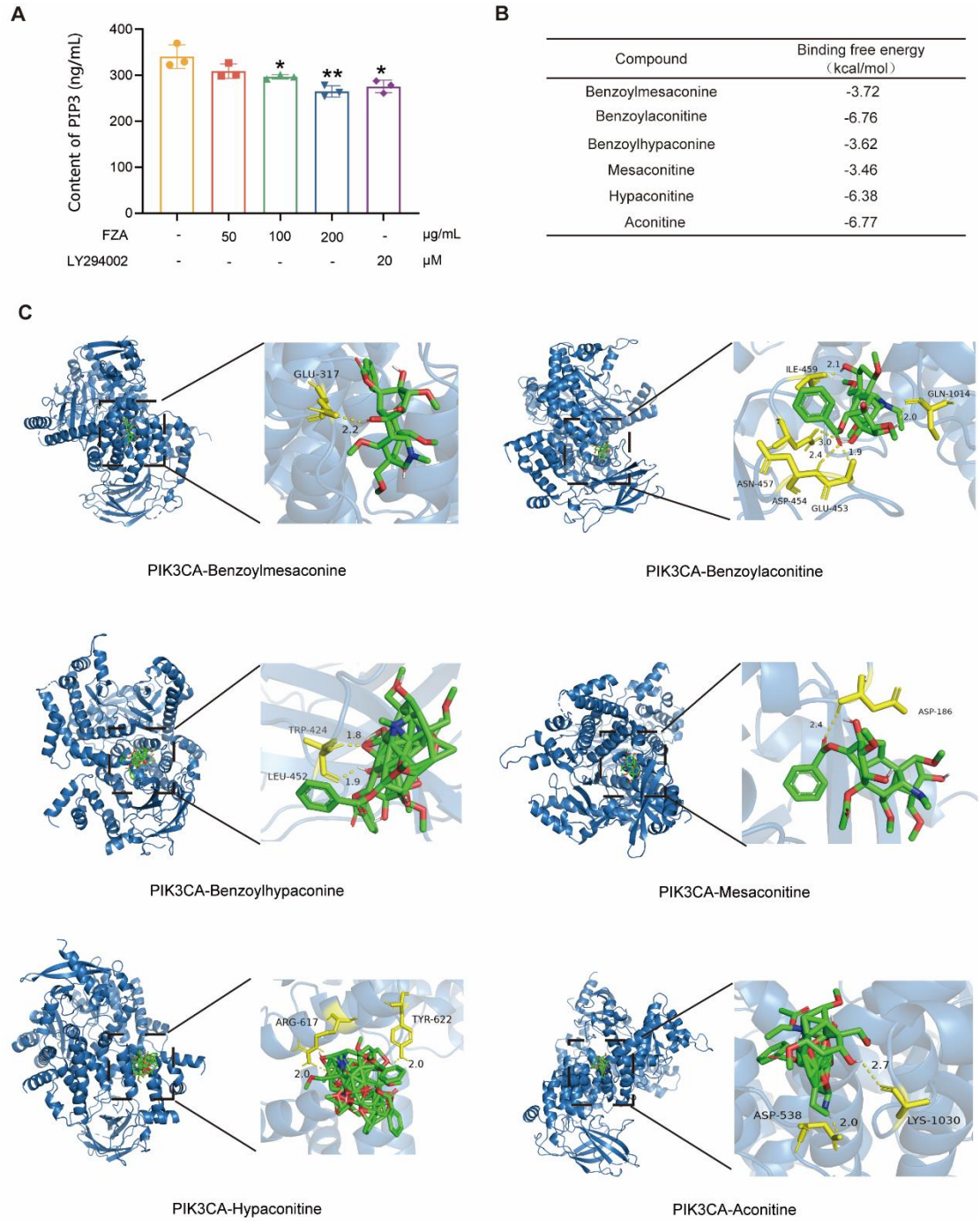

Fig. R6 The influence of FZA on PI3K kinase. (A) The effect of FZA combined with LY294002 on the content of PIP3 in A549 cells (B) The binding energy of molecular docking between FZA components and PIK3CA (C) Molecular docking simulation diagrams of FZA components and PIK3CA.

14. How does the author control batch-to-batch of the extract? How to make sure that every batch has the same amount and compound? This is a serious point in studying the pharmacological activity of the extract rather than the pure compound, otherwise, the

author includes the experiment showing which compound in the extract has this pharmacological activity.

Response:

Many thanks for your suggestion. The extraction and purification method of FZA was optimized by central composite design-response surface methodology which was reported in our previous publication [20]. Six batches of FZA were prepared and the content of main alkaloids were measured. The chromatogram is shown below. The contents of main alkaloids of FZA including benzoylmesaconitine (BMA), benzoylaconitine (BA), benzoylhypaconitine (BHA), mesaconitine (MA), and hypaconitine (HA) in six batches of FZA are shown in the table, with RSDs of 3.49%, 4.44%, 4.05%, 8.45%, and 7.52%, respectively. The results indicate that the preparation method of FZA is stable and every batch of FZA has the same amount and compound.

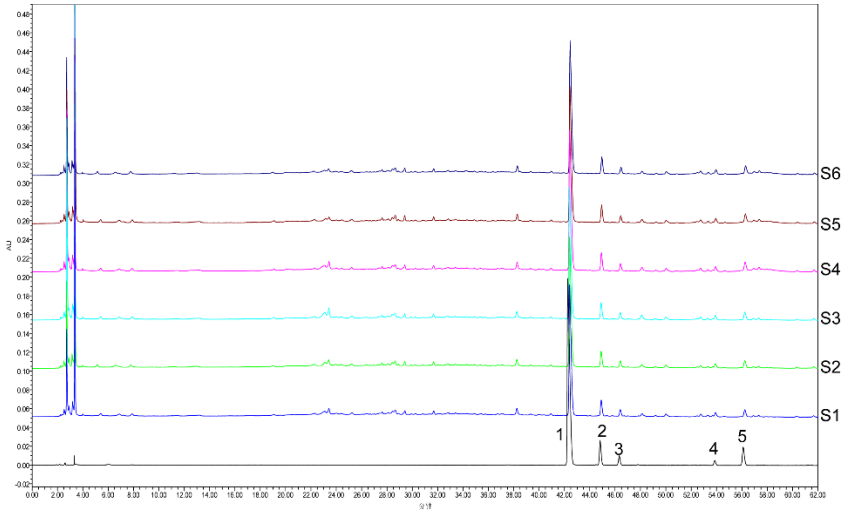

Figure R7 HPLC chromatograms of different batches of FZA and reference solutions.
1. benzoylmesaconitine, 2. benzoylaconitine, 3. benzoylhypaconitine, 4. mesaconitine 5. hypaconitine

Tab. R1 Content of main alkaloids in different preparation batches of FZA

No	BMA (μg/g)	BA (μg/g)	BHA (μg/g)	MA (μg/g)	HA (μg/g)
1	814.42	76.31	17.95	29.53	48.49
2	801.42	75.94	18.42	31.86	49.12
3	824.48	79.34	19.21	35.13	56.37
4	886.73	85.86	20.33	36.82	59.30
5	863.54	83.34	19.46	38.10	57.17
6	834.86	80.48	19.52	35.12	53.09
Mean	837.58	80.21	19.15	34.43	53.92
RSD (%)	3.49	4.44	4.05	8.45	7.52

15. From the graphic abstract, the author shows that the extract inhibits the receptor, but there are no data supported. And also the authors did not prove the downstream signaling.

Response:

Many thanks for your suggestion. We have revised the graphic extract to remove the inhibitory receptors and downstream signals of the extract.

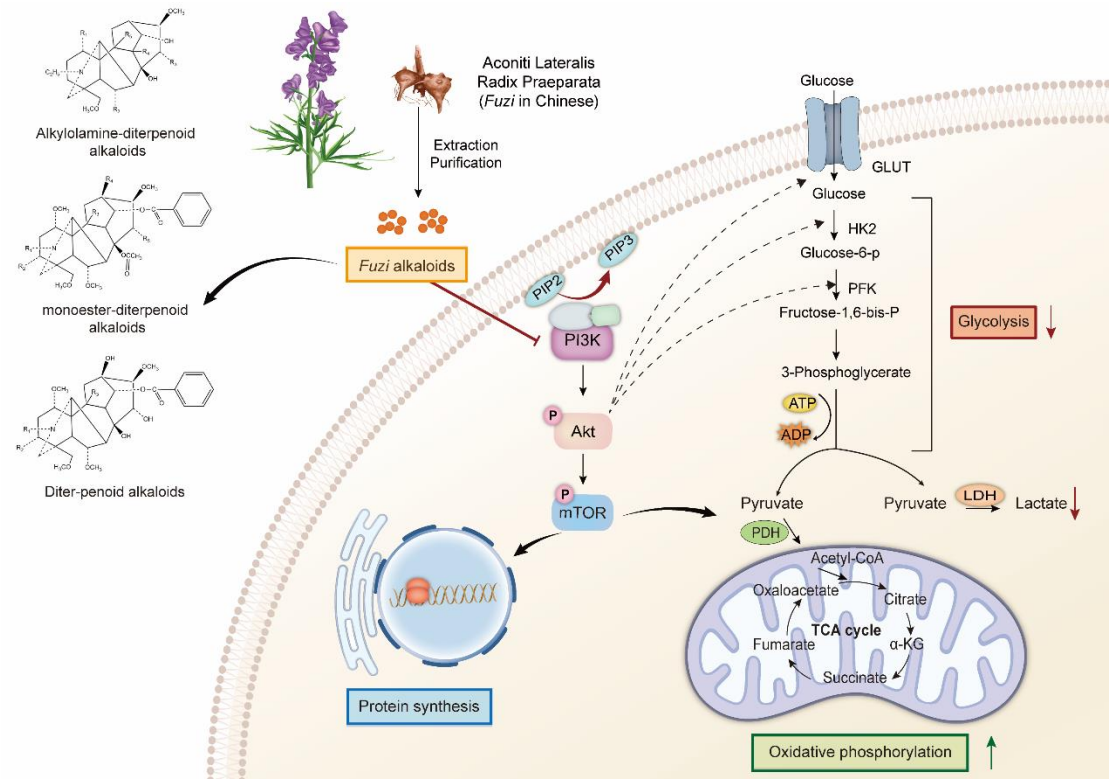

Figure R8 Graphic Abstract

Reviewer #2:

1. Try elaborating more on multi-omics and then relating it with metabolomics in the Introduction section.

Response:

We sincerely appreciate the valuable comments. We have checked the literature carefully and added more references on the relationship between multi-omics and metabolomics in the Introduction section in the revised manuscript.

Metabolomics is widely regarded as the omics discipline closest to phenotype with the identification and quantification of small-molecule metabolites, by comparing the systematic differences in metabolites between different experimental groups and revealing their inherent laws. Metabolomics is not only a descriptive indicator of phenotype, but can also affect the physiological function by regulating genomics, transcriptomics, proteomics, and other multiple omics [21].

The joint analysis of differential proteins with metabolic pathways were conducted in order to find their relevance and study of upstream and downstream regulatory pathways of proteins and metabolites through proteomics screening of differential proteins, metabolomics identification of differentially expressed metabolites and metabolic pathways [22].

This discussion has been added in Line 76-85, Page 3.

2. Discuss more the significance of Aconiti Lateralis Radix Praeparata and the various other medicinal substances mentioned as TCM. It may further be justified why Aconiti Lateralis Radix Praeparata was preferred over the others.

Response:

Many thanks for your suggestion. Aconiti Lateralis Radix Praeparata (*Fuzi* in Chinese) is the processed daughter or lateral root of Aconitum carmichaelii Debx. [23]. It was firstly recorded in *Agriculture God's Canon of Materia Medica* that *Fuzi* has the effect of treating tumors. *Fuzi* is frequently employed in clinical practice to treat lung cancer, gastric cancer, and other malignant tumors [24-26]. The famous *Fuzi*-based formulas, including Sini Decoction, Mahuang Fuzi Xixin Decoction, Shenfu Injection and Compound Sansheng Injection [1, 3, 27], exert excellent therapeutic effects in anti-cancer treatment. Modern studies indicated that the components in *Fuzi*, such as aconitine, hypaconitine, mesaconitne and Higenamine, exhibited anticancer functions [4, 17, 19, 28, 29]. However, these studies on *Fuzi* are mostly focused on monomer components. Our previous research reported that *Fuzi* decoction had a significant inhibitory effect on tumor growth [14]. However, the basis of its anti-tumor substances is unclear, so we conducted this study.

This part of description about Aconiti Lateralis Radix Praeparata has been added in the Introduction part in Line 58-69, Page 3 of the manuscript.

3. Rephrase from lines 138 to 141 for better understanding. The following extract has been added as a suggestion:

"Fuzi was mixed with eight times the amount of water and boiled for one hour. The resulting liquid was collected by passing it through a filter, and the solid residue was subjected to the same process again. The liquid obtained from the second extraction was then combined with the first one using filtration. The combined liquid was then concentrated to obtain the final extract."

Response:

Many thanks for your suggestion. We have rephrased this part according to your suggestion in Line 150-154, Page 5 of the revised manuscript.

4. Rephrase from lines 362 to 364 for better understanding. The following extract has been added as a suggestion:

"At the same time, it was observed that both FZA and FZP did not lead to significant weight loss and had minimal impact on the heart, spleen, lung, liver, and kidney in nude mice with A549 cell xenograft tumors when compared to the control group. This suggests that FZA and FZP have low toxicity in these mice."

Response:

Many thanks for your suggestion. We have rephrased this part according to your suggestion in Line 376-379, Page 10 of the revised manuscript.

5. Rephrase from lines 478 to 483 for better understanding. The following extract has been added as a suggestion:

"FZA was found to have the potential to regulate and normalize the abnormal levels of various metabolites associated with the inflammatory response. These metabolites included beta-alanine, glucose-6-phosphate, aspartic acid, proline, fumaric acid, ornithine, glycine, L-malic acid, uracil, threonine, lysine, aconitic acid, and several

others. This suggests that FZA may exert its anti-NSCLC effects by regulating the expression of these metabolites.
Pathway enrichment analyses were conducted using information from the KEGG database to explore the mechanisms of action further. These analyses helped identify the specific metabolomic pathways involved in the anti-NSCLC effects of FZA."

Response:

Many thanks for your suggestion. We have rephrased this part according to your suggestion in Line 483-490, Page 17 of the revised manuscript.

Minor comments:

1. In line 140, replace 'with' with 'by'.

Response:

We sincerely thank the reviewer for careful reading. As suggested by the reviewer, we have rephrased the sentence in the revised manuscript.

2. In line 141, replace 'to' with 'into'.

Response:

Many thanks for your suggestion. As suggested by the reviewer, we have rephrased the sentence in the revised manuscript.

3. In line 166, write 'experiments' in place of 'experiment'.

Response:

Many thanks for your suggestion. As suggested by the reviewer, we have corrected the "experiments" into "experiments".

4. In line 205, replace 'integrating' with 'integrates'.

Response:

Many thanks for your suggestion. As suggested by the reviewer, we have corrected the "integrating" into "integrates".

5. In line 243, replace 'were' with 'was'.

Response:

Many thanks for your suggestion. As suggested by the reviewer, we have rephrased the sentence in the revised manuscript.

6. In line 373, replace 'exhibits' with 'exhibited'.

Response:

Many thanks for your suggestion. As suggested by the reviewer, we have corrected the "exhibits" into "exhibited".

7. In line 416, 'the' seems unnecessary and can be omitted from the sentence.

Response:

Many thanks for your suggestion. We have deleted "the".

8. In line 449, replace ‘resulted’ with ‘resulting’.

Response:

Many thanks for your suggestion. As suggested by the reviewer, we have corrected the “resulted” into “resulting”.

9. In line 518, rephrase the sentence to: ‘Anti-cancer effect of FZA on NSCLC was confirmed by our in vitro and in vivo experimental data.’ The use of ‘had’ has been replaced with ‘was’.

Response:

Many thanks for your suggestion. As suggested by the reviewer, we have rephrased the sentence in the revised manuscript.

10. In line 539, replace ‘diseases’ with ‘disease’.

Response:

Many thanks for your suggestion. As suggested by the reviewer, we have corrected the “diseases” into “disease”.

11. In line 541, replace ‘the ingredients’ instead of ‘ingredient’.

Response:

Many thanks for your suggestion. As suggested by the reviewer, we have corrected the “the ingredients” into “ingredient”.

Reference

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10.3389/fphar.2022.870282

Reviewers' comments:

Reviewer #1 (Remarks to the Author):

The authors have clarified most of my concerns. The remained issue is that in Fig 8, the reduction of viable cells in combination treatment was not that convincing. The concentration of LY used is not sufficient to suppress Akt activation? The p-Akt blot and the downstream signaling of combination treatment should be included to confirm.

Reviewer #2 (Remarks to the Author):

The manuscript is thoroughly revised as per the suggestions, hence, can be accepted for the publication.

Dear Reviewers:

We feel great thanks for your professional review work on our manuscript entitled "*Alkaloids of Aconiti Lateralis Radix Praeparata treated non-small cell lung cancer by regulating glycolysis via the PI3K/Akt-mTOR signaling pathway*".

As you are concerned, there are several problems that need to be addressed. According to your nice suggestions, we have responded and explained them one by one, which are listed below. We have also uploaded our response to you online.

If there are any other modifications we could make, we would like very much to modify them and we really appreciate your help.

We attach great importance to your feedback and thank you again for your valuable suggestions. Hopefully we have addressed all of your concerns, and these revisions are acceptable.

Should you have any questions and suggestions about this manuscript, please don't hesitate to let us know.

Sincerely yours,
Wen ZHANG
Email: wenzhang@njucm.edu.cn

Reviewer #1

1. The authors have clarified most of my concerns. The remained issue is that in Fig 8, the reduction of viable cells in combination treatment was not that convincing. The concentration of LY used is not sufficient to suppress Akt activation? The p-Akt blot and the downstream signaling of combination treatment should be included to confirm.

Response:

We sincerely appreciate the valuable suggestions.

LY294002 is a synthetic molecule known to inhibit the PI3K/Akt pathway. It demonstrates a remarkable growth-inhibitory and apoptosis-inducing effect in many cancer cell lines, with decreased expression of phosphorylated Akt (Ser473) [1, 2]. The concentration of LY294002 (20μM) was chosen based on previous studies [3-5]. Addition of LY294002 in cell cultures led to significant inhibition of proliferation in lung adenocarcinoma cell lines and MCF-7 breast cancer cell line. As shown in **Fig. 1R-A**, in HCC827 and H1975 cells but not in A549 cells treatment with 10 μM of LY294002 abrogated IFN-g-induced phosphorylation of AKT [3]. What's more, it was found that 25μM of LY294002 could prevent the phosphorylation of the PI3K signaling pathway and suppress Akt activation in A549 cells [4]. Therefore, we chose the concentration of LY294002 (20μM) for subsequent studies. Activation of PI3K catalyzes the 3-hydroxylation of PIP2 to generate PIP3, which further promotes the

downstream pathway. After PIP3 is generated, it can act as a second messenger to recruit AKT to the plasma membrane, resulting in partial activation of Akt [6].

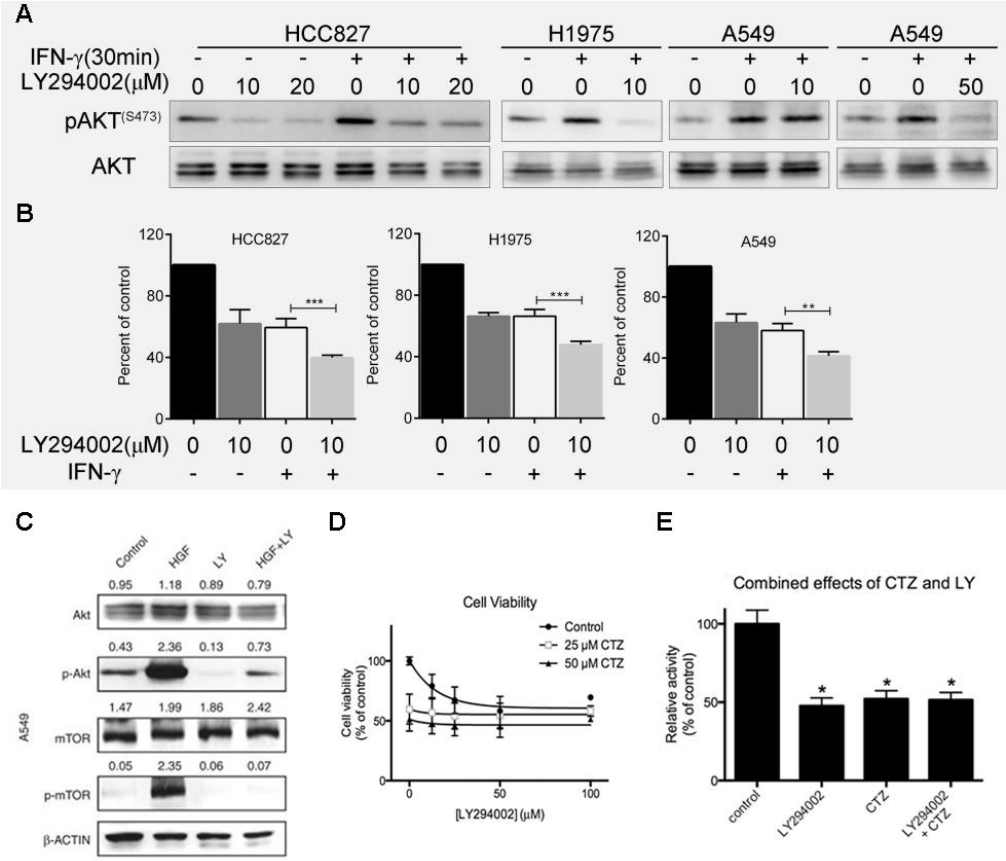

Fig. R1. Data integration of relevant literature. (A) and (B) was cited from "In vivo and in vitro ovarian carcinoma growth inhibition by a phosphatidylinositol 3-kinase inhibitor (LY294002) [3]". (C) was cited from "Transcriptome signature analysis repurposes trifluoperazine for the treatment of fragile X syndrome in mouse model [4]". (D) and (E) were cited from "Phosphatidylinositol-3-kinase as a putative target for anticancer action of clotrimazole [5]".

To determine how the FZA inhibits the PI3K/Akt-mTOR signaling pathway, we measured PIP3 levels in A549 cells after various concentrations of FZA treatment. Compared with the control group, the content of PIP3 decreased significantly after 100 and 200 μg/mL FZA treatment. The results revealed that FZA inhibited the PI3K enzyme in a concentration dependent manner (**Fig. 2R-A**, which is attached below).

Furthermore, to predict the potential interaction of FZA and the PI3K protein, we performed the molecular docking. Based on literatures and HPLC content determination of FZA, benzoylmesaconitine, benzoylaconitine, benzoylhypaconitine, mesaconitine, hypaconitine and aconitine were selected. The results showed that the six ingredients could bind to PIK3CA and might affect its functions (**Fig. 2R-B**).

What's more, it is well known that activation of PI3K/Akt pathway promotes cellular proliferation and protein synthesis in cancer cells [7]. To determine the role of FZA on PI3K/Akt signaling, A549 cells were treated with the inhibitor of PI3K, LY294002. As

shown in **Fig.2R-C**, combining FZA with LY294002 exhibited a synergistic effect on suppressing proliferation.

We believe the above results could partially explain that FZA might treat NSCLC by targeting PI3K/Akt-mTOR pathways. Recently we are on winter holiday to celebrate Spring Festival, which is the most grad holiday in China. Due to the limited time, it is difficult for us to finish the supplementary experiment of the p-Akt blot and the downstream signaling of combination treatment. Hopefully we have addressed all of your concerns, and these explanations are acceptable.

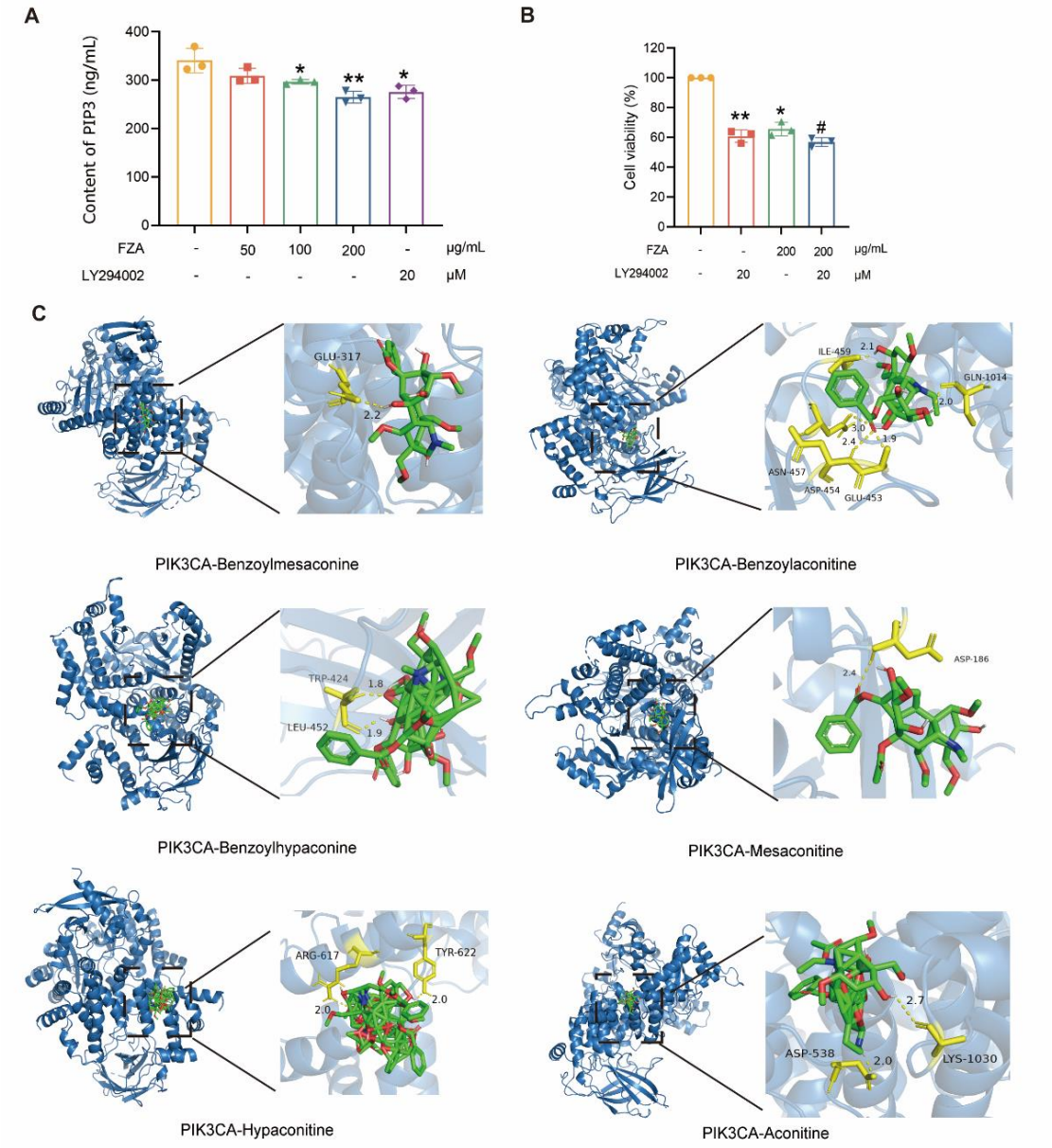

Fig. R2 The influence of FZA on PI3K kinase and proliferation of A549 cells. (A) The effect of FZA combined with LY294002 on the content of PIP3 in A549 cells (B) Molecular docking simulation diagrams of FZA components and PIK3CA (C) The effect of FZA combined with LY294002 on the proliferation of A549 cells. $^{**}p < 0.001$ VS. control group; $^{*}p < 0.05$ VS control group; # $p < 0.05$ VS FZA group.

Reference

[1] S. Semba, N. Itoh, M. Ito, M. Harada, M. Yamakawa, The in vitro and in vivo effects of 2-(4-morpholinyl)-8-phenyl-chromone (LY294002), a specific inhibitor of phosphatidylinositol 3′-kinase, in human colon cancer cells, Clin Cancer Res 8(6) (2002) 1957-1963

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Dear reviewers,

We feel great thanks for your professional review work on our manuscript entitled "*Alkaloids of Aconiti Lateralis Radix Praeparata treated non-small cell lung cancer by regulating glycolysis via the PI3K/Akt-mTOR signaling pathway*".

As you are concerned, there are several problems need to be addressed. According to your nice suggestion, we have supplemented the experiment and the results are listed below. Meanwhile, we have added the LY inhibitor experiment into the manuscript. The revisions are highlighted with **blue characters** in the revised manuscript.

We attach great importance to your feedback and thank you again for your valuable suggestions. Hopefully we have addressed all of your concerns, and these revisions are acceptable.

Should you have any questions and suggestions about this manuscript, please don't hesitate to let us know.

Sincerely yours,
Wen ZHANG
Email: wenzhang@njucm.edu.cn

1. WB of pAKT is necessary to verify the conclusions.

Response:

We sincerely appreciate the valuable suggestions.

LY294002 is a synthetic molecule known to inhibit the PI3K/Akt pathway. It demonstrates a remarkable growth-inhibitory and apoptosis-inducing effect in many cancer cell lines, with decreased expression of phosphorylated Akt (Ser473) [1, 2]. The concentration of LY294002 (20μM) was chosen based on previous studies [3-5]. A549 cells were divided into control group, FZA group, LY294002 group and combination group. Control group were incubated with vehicle, while FZA group was treated with 200 μg / mL FZA for 24 h and LY294002 group was treated with vehicle after 2 h of 20 μM LY294002 treatment. The combination group was administered with 20 μM LY294002 for 2 h prior to 24 h of FZA treatment. The treated cells were lysed with ice-cold cell lysis buffer containing protease and phosphatase inhibitor cocktail. Dissolved proteins were collected, and the debris was removed by centrifugation at 12,000 rpm for 10 min at 4 °C. The concentration of total protein was determined using the BCA protein assay kit. Equal amounts of lysate protein were electrophoresed by FuturePAGE (ACE, Nanjing, China). The separated proteins were transferred electrophoretically to PVDF membranes. The membranes were blocked with 5% BSA in tris buffer solution-tween (TBST) for 2 h and subsequently incubated overnight at 4 °C with primary antibodies. After washing with TBST, the membranes were incubated with HRP-

conjugated Affinipure Goat Anti-Rabbit lgG(H+L) for 2 h at room temperature. Thereafter, reactive bands were visualized by the Chemidoc XRS+ Imaging System (Bio-Rad; Hercules, CA) using super ECL detection reagent (Yeasen, China).

In order to further ascertain and identify the role of Akt in the FZA-modulation PI3K/Akt-mTOR signaling in A549 cells, the effect of LY294002 were detected. Compared to FZA alone, it was shown that FZA could inhibit the phosphorylation protein of PI3K and Akt expression, resulting in consistent results when FZA was co-cultured with LY294002.

We believe the above results could partially explain that FZA might treat NSCLC by targeting PI3K/Akt-mTOR pathways. Hopefully we have addressed all of your concerns, and these explanations are acceptable.

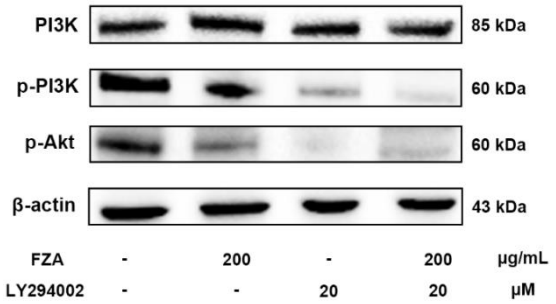

Fig. R1. PI3K/Akt-mTOR signaling related proteins were detected by western blot assay.

Reference

[1] S. Semba, N. Itoh, M. Ito, M. Harada, M. Yamakawa, The and effects of 2-(4-morpholinyl)-8-phenyl-chromone (LY294002), a specific inhibitor of phosphatidylinositol 3′-kinase, in human colon cancer cells, Clin Cancer Res 8(6) (2002) 1957-1963

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REVIEWERS' COMMENTS:

Reviewer #1 (Remarks to the Author):

The author has clarified my previos concern. Fig.R1 and their densitrometry analysis should be included in supplementary figure and mentioned it in the main text. In addition, Fig 8D there is no asteric indicating significant in the plot. Do the combination treatment has lower cell viability as compare to compound treated group alone? All the correstion are responsible by the authors. Please carefully check before resubmission.

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Reviewer #1

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Response:

We sincerely appreciate your valuable suggestions.

1) We have incorporated Fig. R1 and corresponding analysis into our revised manuscript and supplementary information. We have also mentioned the experiment and corresponding results in the main text.

2) Thank you for your careful check. There should be an asterisk in the figure. We are sorry the error. We have made the correction in Fig. 7F in the revised manuscript. Compared with the FZA group, the cell viability of the combination group was significantly reduced ($p < 0.05$). The results showed that inactivation of PI3K/Akt-mTOR enhanced the inhibitory effect of FZA on A549 cell proliferation. The relative description has been added in Line 582, Page 22 of the revised manuscript.

3) According to your suggestions, we have carefully checked our manuscript and revised it. Meanwhile, we have further polished the language of our manuscript.

Thanks again for your professional review and constructive comments. Your time and patience are highly appreciated.