**Metapristonesuppresses non-small cell lung cancer
proliferationand metastasis via
modulatingRAS/RAF/MEK/MAPK and PI3K/AKT
signaling pathways**

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1 ABSTRACT

2 **Background:** Metastasis is the key phase of cancer progression that characterizes a
3 more advanced stage and a poorer prognosis. The majority of cancer fatalities occur
4 as a consequence of metastasis.

5 **Objective:** Mifepristone (RU486), a chemical abortifacient, has recently been used in
6 clinical trials for psychotic depression and cancer chemotherapy. As the most
7 predominant biological active metabolite of mifepristone, metapristone is being
8 developed as a novel cancer metastasis chemopreventive agent by us. However, there
9 is no information available to address the effects of metapristone on non-small cell lung
10 cancer (NSCLC). The aim of our study was to investigate the inhibitory effect of
11 metapristone on the proliferation and metastasis of NSCLC cells.

12 **Method:** In the present study, we evaluated the efficacy of metapristone on the
13 growth, migration and invasion in different kinds of NSCLC cells (A549, H1975 and
14 H1299), and further investigated the underlying mechanism of metapristone by real
15 time PCR and western blot assay.

16 **Results:** Metapristone could significantly inhibit the proliferation, migration and
17 invasion of NSCLC cells through suppressing RAS/RAF/MEK/MAPK and PI3K/AKT
18 signaling pathways. Moreover, metapristone could effectively inhibit the formation of
19 NSCLC cells' cytoskeleton in a concentration-dependent manner, which possibly led
20 to the inhibition of NSCLC cells' migration.

21 **Conclusion:** Overall, it was preliminarily demonstrated that metapristone could be
22 developed as a useful agent to show anti-metastasis activity for NSCLC.

23
24
25 **Keywords:** *Non-small cell lung cancer, Metapristone, Proliferation, Metastasis,*
26 *Migration, Invasion*

27

INTRODUCTION

Either in China or the United States, lung cancer has been one of the four most common cancers for a long time[1-3]. Among all of lung cancer patients, non-small-cell lung carcinoma (NSCLC) accounts for approximately 75–85% of these cases. Due to its high metastatic properties, the survival rate of NSCLC in recent years is less than 15%[4]. The whole process of cancer metastasis can be divided into five major steps: invasion, intravasation, dissemination, extravasation and colonization[5]. It has been reported that about 30% to 40% of patients with advanced lung cancer will develop cancer metastasis, which is defined as lung tumor cells' migration to other organs such as bones and liver [5], and the high mortality of lung cancer resulted from postoperative recurrence and distant migration[6-8].

Mifepristone (RU486), as a contraceptive, has been used by millions of women all over the world. It shows glucocorticoid receptor antagonist activity at high concentrations, with more than three times over the binding affinity for glucocorticoid receptors than dexamethasone[9]. Glucocorticoid receptors are one of nuclear receptor superfamily which are closely related with the treatment of some essential diseases[10]. Due to this significant superiority, it has recently been used in trials for psychotic depression and anticancer treatments. Metapristone is found to be one primary metabolite of mifepristone[11]. Expect for stronger efficacy, metapristone also shows a better safety profile than mifepristone. Considering its unique metabolic stability, as well as safety and efficacy estimated from the existing information of parent drug mifepristone, metapristone was chosen by us to be developed as a novel cancer metastasis chemopreventive agent for curing NSCLC patients[11]. However, the inhibitory potential against the proliferation, migration and invasion of NSCLC cells induced by metapristone has not been investigated in vitro systems.

In this study, we investigated the growth inhibition and anti-metastasis effect of metapristone on different kinds of NSCLC cells *in vitro* and explored the underlying molecular mechanism of metapristone on the EGFR-related signaling pathway in NSCLC cells. Besides, we also examined the inhibition effect of metapristone on the

formation of NSCLC cells' cytoskeleton, which possibly led to the inhibition of NSCLC cells' migration. This is the first study to investigate the anti-proliferation and metastasis effects and clarify the possible molecular mechanisms of metapristone on NSCLC.

1. MATERIALS AND METHODS

1.1 Materials and Reagents

Reference standard of metapristone (98.0% purity) was synthesized and purified in our laboratory; NSCLC cell lines (A549, H1299 and H1975) were purchased from the Food Industry Research and Development Institute (Hsin-Chu, Taiwan); And all other chemicals and reagents used in our experiment were of analytical grade.

1.2 Methods

1.2.1 Cell Culture

Cells (A549, H1299 and H1975 cells) were maintained in RPMI 1640 medium containing 10% fetal bovine serum, 100 U/mL penicillin, and 100 mg/L streptomycin. All of these cultures with cancer cells were maintained at 37°C in a humidified atmosphere of 5% CO₂ and 95% air.

1.2.2 Cytotoxicity Assay

The cytotoxicity of metapristone was determined by the MTT assay as previously described [12, 13]. Cells were plated in 96-well plates and treated with various concentrations (0, 10, 20, 30, 40, 60 and 80 µM) of metapristone for 24 h, 48 h and 72 h, respectively. After incubation for established times at 37 °C in a humidified chamber, MTT reagent (5 mg/mL in PBS) was added to each well and incubated for another 4 h. Then, the medium was discarded and the formazan formed by metabolically viable cells was dissolved in 150 µL dimethyl sulfoxide (DMSO). The absorbance was measured by an infinite M200 Pro microplate reader at the test wavelength of 570 nm.

1.2.3 Apoptosis Assay

Annexin V-FITC/PI double staining kit was performed by us as described

previously[14]. Cells were treated with different concentrations (0, 5, 10 and 20 μ M) of metapristone for 24 h. Then the cells were harvested by centrifugation at 1500 rpm for 5 min, then suspended with 500 μ L of 1 X binding buffer and stained with 10 μ L of the solution containing Annexin V-FITC (5 μ L) and PI (5 μ L) for 15 min in the dark according to the manufacture of instructions. Annexin V has been one of the most sensitive index which could be used to determine the apoptotic conditions of cells. Cells were then analyzed by using flow cytometry (BD Bioscience, FACS Aria III).

1.2.4 Wound Healing and Transwell Assay

The wound healing assay was carried as reported[15-17] with few modifications. Cells were separately plated in 12-well plates. It is essential that the cells must be attached to the substrate of plate. Then cells in the individual well were wounded to form three parallel lines by scratching with a 10 μ L pipette (to remove the cells but both sides still have original cells). The monolayer was then washed twice with PBS to remove debris or detached cells, and then cells were treated with various concentrations of metapristone (0, 5, 10, and 20 μ M). Photographic images were taken from each parallel line at special times.

Cancer cells' ability of passing through matrigel-coated filters was measured by the Boyden chamber invasion assay. Cells were kept for 24 h in the medium without serum and then were trypsinized and resuspended in the medium without phenol red and serum (containing 10% FBS). Then cells were placed in the upper chamber of the transwell insert (5×10^4 cells/well) and incubated with various concentrations (0, 5, 10 and 20 μ M) of metapristone. The medium containing 10% FBS was added to the lower chamber. The plates were incubated in a humidified atmosphere with 95% air and 5% CO₂ at 37°C for 24 h. Then cells in the upper surface of the membrane were carefully removed with a cotton swab and cells that had invaded across the matrigel to the lower surface of the membrane were fixed with 4% formaldehyde in PBS and were stained with 0.1% crystal violet. The picture of cells in the lower surface of the filter which penetrated through the matrigel was caught under a light microscope at 200 $\times$ [13].

1.2.5 Western Blot Assay

Western blotting assay was performed as described previously[18]. Cells in RPMI 1640 medium were plated in 6-well plates for 24 h. Then the cells were treated with various concentrations of metapristone for 24 h and then cells were incubated with 200 μ L RIPA on the ice for 30 min and centrifuged in 1500 rpm to take supernatant upper sample and prepare the total protein. Protein concentrations were determined by bicinchoninic acid (BCA) Protein Assay Kit. The denatured samples (20 μ g purified protein) were resolved on 10% SDS-PAGE gels. Proteins were then transferred onto poly-vinylidene (PVDF). The PVDF was blocked with tris-buffered saline (TBST) containing 5% (w/v) nonfat dry milk for more than 2 h. Then the PVDF was incubated with an appropriate dilution of specific primary antibodies in TBST overnight at 4 °C and then incubated with an appropriate secondary antibody (horseradish peroxidase-conjugated goat anti-mouse or anti-rabbit IgG) for 2 h. Subsequently, the particular protein was visualized with the ECL kit. Band detection was revealed by using Image J.

1.2.6 Real-time PCR assay

RT-PCR assay was performed as described previously[18]. Total RNA was extracted using TRIzol (Invitrogen, Carlsbad, CA, USA) and concentrations of RNA were determined by using a NanoDROP 2000 Spectrophotometer (Thermo Scientific, USA). Reverse transcription for synthesizing cDNA was carried out through using PrimerScript™ First Strand cDNA Synthesis Kit. PCR amplification conditions: 40 cycles of 95°C for 30 sec, 95°C for 5 sec and 60°C for 30 sec. Use SYBR Premix Ex Taq to detect the expressive levels of pivotal genes in the RAS/RAF/MEK/MAPK and PI3K/AKT signaling pathways. The primers used for PCR amplification were shown in table 1.

1.2.7 Immunofluorescence (IF) assay

Cells were planted onto 24-well plates and then were treated with various concentrations (0, 5, 10 and 20 μ M) of metapristone for 24 h. The MitoTracker® Red CMXRos (the final concentration was 250 nm) was added into each well after cells were incubated for 30 min at 37°C. Cells were cultured through formaldehyde-fixed form for 10 min and then were treated with 1% Triton X-100 in G-PBS for 5 minutes. Through the whole process, the cytoskeleton of A549 cells were observed under the confocal

laserscanning microscopy.

2.Results

2.1 Effect of metapristone on cell viability

The cytotoxic effect of metapristone was evaluated by MTT assay. A549, H1299 and H1975 cells were separately treated with a range of concentrations of metapristone (0, 10, 20, 30, 40, 60 and 80 μ M) for established time (24, 48 and 72 h). Metapristone had caused a dose-dependent decrease in the viability of these three cells (Fig.1A, Fig.1B and Fig.1C). When cancer cells incubated with low concentration of metapristone ($<10 \mu$ M) for 24 h~72 h, the integral decreasing tendency of cell viability induced by metapristone showed no significant change. When the concentration of metapristone was up to 20 μ M, the viability of A549 and H1299 cells were still higher than 40% when cells were treated with metapristone for 72 h. However, under the same concentration, the viability of H1975 cells was only approximately 20% after 72 h, showing that metapristone could have higher cytotoxicity upon H1975 cells than A549 and H1299 cells. Meanwhile, either in A549 or H1299 cells, when the concentration of metapristone was higher than 20 μ M, the viability showed a significant decrease. For the purpose of ensuring a reliable result of cell experiment, we chose A549 and H1299 cells to investigate the effect of metapristone with the dose range lower than 20 μ M.

2.2 Effect of metapristone on the apoptosis of A549 and H1299 cells

To investigate the effects of metapristone on cell apoptosis, we treated A549 and H1299 cells with different concentrations of each preparation, and then quantified apoptosis by flow cytometry using an annexin V-FITC/PI apoptosis assay detection kit. As shown in Fig.1D, very few necrotic or apoptotic cells were detected in control A549 or H1299 cells (untreated group). The percentage of cells undergoing early apoptosis or late apoptosis showed a concentration-dependent increase within the established concentration range. However, when the concentration of treated metapristone was 20 μ M, the whole percentage of apoptotic cells was still lower than 13%, much lower in lower concentrations. The results indicated that low concentrations of metapristone didn't cause evident apoptosis of A549 or H1299 cells,

which provided the possible theoretical basis for applying metapristone in inhibiting the metastasis of cancer cells.

2.3 Effect of metapristone on the migration and invasion of A549/H1299 cells

The effect on the migration of A549/H1299 cells caused by metapristone was evaluated by wound healing assay. As shown in Fig.2A, compared with the control group, there was a significant decrease in the distance of migration in medication administration groups. As shown in Fig.2B, metapristone could effectively inhibit the migration of A549/H1299 cells in a concentration-dependent manner. However, the migration rates in A549 cells were comparatively lower than in H1299 cells when cells were treated with same concentration, indicating that metapristone could inhibit the migration of A549 cells more effectively as compares with H1299 cells.

The effect on the invasion of A549/H1299 cells induced by metapristone were examined by using transwell assays. As shown in Fig.2A, the number of cells in medication administration groups which could go through the microporous membrane was significantly lower than that of control group. As shown in Fig.2C, the migration rates of cells were lower and lower with the concentration increasing, indicating that higher concentrations of metapristone led to stronger inhibitory activity against cell migration either in A549 or H1975 cells. Meanwhile, this dose-dependent effect was comparatively stronger in A549 cells.

2.4 Metapristone suppressed the expressions of pivotal proteins in the RAS/RAF/MEK/MAPK and PI3K/AKT signaling pathways in A549 cells

To determine how metapristone could suppress the metastasis of NSCLC cells, we detected several pivotal proteins in RAS/RAF/MEK/MAPK and PI3K/AKT signaling pathways by western blot assay. As shown in Fig.3A and Fig.3C, metapristone could effectively inhibit the expressive levels of PI3K and Akt with a dose-dependent manner. However, as shown in Fig.3B and Fig.3D, with the concentration of metapristone increasing from 5 μ M to 20 μ M, the expressions of PI3K and Akt showed no significant changes in H1299 cells. The expressions of PI3K and Akt in A549 cells decreased more significantly than in H1299 cells when cells were treated with same concentration of metapristone.

As shown in Fig.4A and Fig.4C, with the concentration of metapristone increasing, the expressive levels of EGFR, Ras, Raf, MEK and MAPK decreased in different rates in A549 cells. However, in H1299 cells, the expressions of these proteins also decreased in a comparably lower rate correspondingly (Fig.4B and Fig.4D). Our results revealed that metapristone could inhibit RAS/RAF/MEK/MAPK and PI3K/AKT signaling pathways through down-regulating the expression of proteins associated with cancer metastasis.

2.5 Metapristone inhibited the expressions of pivotal genes in the RAS/RAF/MEK/MAPK and PI3K/AKT signaling pathways in A549 cells

The effect of metapristone on the expressive levels of pivotal genes in the RAS/RAF/MEK/MAPK and PI3K/AKT signaling pathways in A549 and H1299 cells was evaluated by Real-time PCR assay. As shown in Fig.3E and Fig.3F, metapristone could significantly degrade the expressive levels of PI3K, EGFR, Akt and MEK in both A549 and H1299 cells, especially when cells were treated with concentrations of metapristone higher than 5 μ M. However, this decrease tendency in A549 cells was comparably significant than that in H1299 cells. This result was fundamentally consistent with that of western blot assay.

2.6 Metapristone inhibited the cytoskeleton of A549 cells in vitro.

The effect of metapristone on the cytoskeleton of A549 cells could be determined by using confocal laser scanning microscopy. A549 cells were exposed to various concentrations of metapristone for 24 h. As shown in Fig.5, fluorescence intensity significantly decreased with concentrations of metapristone increasing. Moreover, some A549 cells' outline even disappeared. Our results indicated that metapristone could effectively inhibit the formation of A549 cells' cytoskeleton in a dose-dependent manner, indicating that metapristone could indirectly inhibit A549 cells' migration by inhibiting the formation of A549 cells' cytoskeleton.

3. Discussion

According to the previous studies, the majority of cancer fatalities occur as a consequence of metastasis [19, 20]. Metastasis has been an intricate process including: 1). detach from the primary tumor and invade through basement membrane to nearby

tissue (invasion); 2). go into the blood vessels (intravasation); survive in the circulation (dissemination); 3). exit the circulatory system at metastatic sites (extravasation); 4).colonize and grow at the new environment and forming a metastatic tumor (colonization)[21-24]. Upon tumor starts to transfer to other parts rapidly, it will become difficult to use chemical reagent to inhibit its tendency. So we tried to investigate the anti-metastasis potential of metapristone on NSCLC cells.

Metapristone is the most predominant biological active metabolite of mifepristone. It was reported that the blood concentration of metapristone could be equal to or even higher than that of mifepristone for at least 72 h through the same form of administration,which means that metapristone could take on effects for longer time in bodies[25-27]. Though metapristone is not a traditional anticancer drug, it may have potential to evolve into a new even novel class of cancer metastatic chemo-preventives. In this study, we investigated the possible mechanism of inhibitingthe proliferation, migration, and invasion ofNSCLCcells induced by metapristone.

In the present study, we used three NSCLC cell lines (A459, H1299 and H1975) to determine the cytotoxic effect of metapristone on different cells. The H1975 cells were the most sensitive of these three cell lines. In the concentration of metapristone below 20 μ M, it is more suitable to choose A549 and H1299 cells as our model objects without causing too much apoptosis in cancer cells. A549 and H1299 cells have been typical types of NSCLC cell lines widely used in vitro model system. According to the results of western blotting assay and Real-time PCR assay, we could find that the activities of pivotal proteins/genes inRAS/RAF/MEK/MAPKand PI3K/AKT signaling pathwaysin NSCLC cells could be inhibited by metapristone in a dose-dependent manner. All the above resultsshowed that metapristone could inhibit the proliferation, invasion and migration of NSCLC cells through down-regulating the expressions of proteins and genes involved in the metastasis cascade (Fig.6).Meanwhile, metapristone could inhibit the growth of A549 cells'cytoskeleton to prevent them transferring from one part to another in bodies. In the all assays, the concentrations of metapristone were below 20 μ M in order to ensure enough viable

cells.

In this study, we chose two representative NSCLC cell lines and demonstrated that metapristone could inhibit the proliferation, migration and invasion of NSCLC cells through down-regulating the expressions of pivotal proteins inRAS/RAF/MEK/MAPKand PI3K/AKT signaling pathways. All of these specialities were closely correlated with culture time or drug dose, which could provide a theroetical basis for clinical prescription. These findings suggested that metapristone could be recognized as a therapeutic agent tosuppress the metastasis of NSCLC cells.

However, for the purpose of inhibiting the metastasis of NSCLC cells, we just investigated two key signaling pathways, which didn't account for the molecular mechanism of metapristone to suppress the proliferation, migration and invasion of NSCLC cells. We just chose two representative NSCLC cell lines as model objects and it is not enough to say that metapristone could be used to cure NSCLC patients. Taken together, how metapristone could inhibit the metastasis of NSCLC cells still need more studies.

4. Conclusion

In this study, we demonstrated that metapristone could inhibit the proliferation, migration and invasion of NSCLC cells through down-regulating the expressions of PI3K, Akt, EGFR, Ras, Raf, MEK and MAPK. Futhermore, metapristone also exhibited the potential of affecting the formation of A549 cells' cytoskeleton. All of these specialities were closely correlated with culture time or drug dose, which could provide a theoretical basis for clinical prescription. These findings suggested that metapristone could be recognized as a therapeutic agent for inhibiting the proliferation and metastasisof NSCLC.

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Competing financial interests

The authors declare no competing financial interests.

296 **Author Contributions**

297 JS conceived and designed the experiments. JS, G.Z and Z.S wrote and revised the
298 manuscript. G.Z and Z.S performed most and H.C and J.L did some of the
299 experiments. J.L compounded metapristone. K.J and L.F analyzed and interpreted the
300 data. All authors discussed the results and commented on the manuscript.

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Figure Captions

Fig.1. Effects of metapristone on the cell viability of A549, H1299 and H1975 cells and the apoptosis of A549 and H1299 cells. (A, B and C) A549, H1299 and H1975 cells were treated with different concentrations of metapristone for 24 h, 48 h or 72 h **(D)** Flow cytometric analyzed cell apoptosis in A549 and H1299 cells using the Annexin V-FITC/PI dual-labeling technique. Quantified values were shown on the right. Data was shown in the mean $\pm$ SEM (n=3) as * $P < 0.05$, ** $P < 0.01$ and *** $P < 0.001$, compared with the control group.

Fig.2. Effects on the migration and invasion of A549/H1299 cells caused by metapristone. (A) The migration and invasion of A549/H1299 cells treated with various concentrations of metapristone were photographed. **(B)** Cells that invaded through the membrane were quantified under different concentrations of metapristone. **(C)** Migrated cells were quantified by manual counting under different concentrations of metapristone. Data was analysed by the mean $\pm$ SEM (n=3) as * $P < 0.05$, ** $P < 0.01$ and *** $P < 0.001$ in comparison with the control.

Fig.3. Effects on the expressive levels of PIK3 and Akt in the PI3K/AKT signaling pathway induced by metapristone and the expressive levels of pivotal genes of RAS/RAF/MEK/MAPK and PI3K/Akt signaling pathways in A549/H1299 cells caused by metapristone. (A, B, C and D) The expressions of PIK3 and Akt in A549/H1299 cells under different concentrations of metapristone were detected by western blotting. **(E and F)** The quantitative analysis of the

expressions of EGFR, Akt, MEK and PI3K in A549/H1299 cells under different concentrations of metapristone were detected by RT-PCR assay. PCR reaction solution: Dosage of SYBR® Premix Ex Taq(Tli RNaseH Plus)(2×) is 12.5 μL; Dosage of PCR Forward Primer(10 μM) is 0.5 μL; Dosage of PCR Reverse Primer(10 μM) is 0.5 μL; Dosage of DNA template (<100 ng) is 2.0 μL; Dosage of ddH₂O is 9.5 μL.

Fig.4.Effects on the expressive levels of pivotal proteins of RAS/RAF/MEK/MAPK signaling pathway in A549/H1299 cells caused by metapristone. (A, B, C and D) The expressions of EGFR, Ras, Raf, MEK and MAPK in A549/H1299 cells under different concentrations of metapristone were detected by western blotting.

Fig.5. Effects of metapristone on the cytoskeleton of A549 cells (A and B).In this assay, A549 cells were treated with 0, 5, 10 and 20 μM metapristone for 24 h. The effects of metapristone on the cytoskeleton of A549 cells was reflected from the picture taken through confocal laser scanning microscopy.

Fig.6.Proposed mechanisms diagram of signaling pathways for metapristone-regulated synergistic anti-metastasis.

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Tab 1 qRT-PCR primers

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Gene		Primer sequence (5'-3')
EGFR	Sense primer:GCTTGCATTGATAGAAATGG	
	Antisense primer: GTCGTCTATGCTGTCCTC	
MEK	Sense primer: TGACGCAGAAGCAGAAGGTG	
	Antisense primer: AGGTCGGCTATCCATTCCAT	
Akt	Sense primer:GCCGCTGCTTCTTTATCC	
	Antisense primer:GCCATTCTCCACTCCACC	
PI3K	Sense primer: AGTGGGAACCGAAGAAG	
	Antisense primer:GACGGGAAGTGAAGTGGATGAAC	

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Fig. 2.

Fig.3.

Fig.4.

Fig.5.

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Fig.7

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