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ORIGINAL ARTICLE

Potential monoamine oxidase A inhibitor suppressing paclitaxel-resistant non-small cell lung cancer metastasis and growth

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Keywords
Drug resistance; heptamethine carbocyanine dye; monoamine oxidase A; non-small cell lung cancer.

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Abstract

Background: High expression of monoamine oxidase A (MAOA) in non-small cell lung cancer (NSCLC) is related to epithelial-mesenchymal transition (EMT) and the development of clinicopathological features of NSCLC. Nevertheless, the role of MAOA in drug resistance still remains unclear. Hence, the aim of this article was to evaluate a previously synthesized MAOA inhibitor (G11) on inhibiting paclitaxel-resistant NSCLC metastasis and growth.

Methods: First, MAOA expression level was evaluated in several NSCLC cell lines. An MTT assay was used to validate the inhibitory effect of G11 on NSCLC cells in vitro. Second, gene expression in G11-treated H460/PTX cells was analyzed by microarray gene expression. Third, transwell assay was performed to assess the invasion and metastasis of G11-treated A549/PTX and H460/PTX cells and western blot assay used to analyze vital protein expression level in G11-treated H460/PTX cells. Finally, the antimetastatic effect of G11 was tested in an NSCLC in vivo model.

Results: Our data revealed that G11 significantly inhibited the viability of paclitaxel (PTX)-resistant NSCLC cell lines (A549/PTX and H460/PTX). G11 dramatically reduced the expression of MAOA in A549/PTX and H460/PTX cells, which exhibited relatively high MAOA expression levels. Additionally, G11 was found to hinder A549/PTX and H460/PTX cell migration and invasion. Furthermore, the in vivo study indicated that the coadministration of G11 and paclitaxel significantly suppressed tumor metastasis in H460/PTX lung metastasis models.

Conclusions: These findings indicated G11 showed a moderate inhibitory effect on paclitaxel-resistant NSCLC metastasis and growth, and support further investigation on MAOA potentially as a promising therapeutic target for paclitaxel-resistant NSCLC treatment.

Key points

Significant findings of the study:

- Inhibition of MAOA might contribute to the suppression of metastasis and growth in PTX-resistant NSCLC cells.

What this study adds

- This study explored the potential function of MAOA in drug-resistant NSCLC and might consider MAOA as a promising target for the treatment of drug-resistant NSCLC.

Introduction

Monoamine oxidase A (MAOA) is a crucial enzyme associated with metabolism of amines in vivo in tissues and has long been investigated in the context of mental disorders.[1–5] Recently, MAOA was found to be overexpressed in prostate cancer (PCa) and is correlated with the different phases of tumor development.[6–10] Knockout of MAOA in a PCa xenograft mouse model revealed the inhibition of PCa growth and metastasis.[6] The clinical phase II trial of the MAOA inhibitor phenelzine suggests that phenelzine shows clinical activity for biochemical recurrent prostate cancer (Clinical Trials.gov Identifier: NCT02217709). In addition to the above observations, it has already been demonstrated how MAOA promotes PCa progression. MAOA not only induced epithelial-to-mesenchymal transition (EMT) through activating vascular endothelial growth factor (VEGF) and neuropilin-1, but it also stabilized the transcription factor hypoxia-inducible factor 1 alpha (HIF1α), which resulted in the elevation of reactive oxygen species (ROS). Moreover, upregulation of neuropilin-1 activated protein kinase B (AKT)/forkhead box O1 (FOXO1)/Twist family BHLH transcription factor (TWIST) signaling. Notably, high-grade PCa showed the activation of MAOA-induced HIF1α/VEGF-A/FOXO1/TWIST1 signaling.[6,11] These findings indicate that MAOA might be an effective target in cancer due to the carcinogenic role of MAOA in PCa.

Overexpression of MAOA has also been found in cholangiocarcinoma, Hodgkin's lymphoma and glioma.[12–14] A recent research result indicated that non-small cell lung cancer (NSCLC) tissues exhibited upregulation of MAOA compared with surrounding normal lung tissues.[15] Furthermore, a close relationship has been observed between increased MAOA expression in NSCLC tissues and Slug, N-cadherin and TWIST expression, EMT and the development of clinicopathological features of NSCLC such as later stage and lymph node metastasis. These findings suggest that MAOA may promote NSCLC progression by inducing EMT.

In our previous work, a nonselective small molecule MAOA inhibitor, isoniazid, was conjugated to a tumor-targeting moiety, heptamethine carbocyanine dyes, which are mediated by organic anion-transporting polypeptides (OATPs), the tumor hypoxic environment and increased mitochondrial membrane potential in cancer cells.[16–19] Several MAOA inhibitor-heptamethine carbocyanine dye conjugates were synthesized, and their antitumor effects against PC-3 cells were evaluated.[16] Among them, G11 showed an enhanced antiproliferation and MAOA inhibitory effect. However, drug resistance in NSCLC still hinders the treatment outcomes of most chemotherapeutics.[20–23] Furthermore, the mechanisms causing chemoresistance in NSCLC patients have as yet to be clearly elucidated. Would MAOA contribute to the development of drug resistance in NSCLC? To validate this hypothesis, we tested the relative expression level of MAOA in several cell types and evaluated the MAOA inhibitor G11 for paclitaxel-resistant NSCLC. Thus, the purpose of this study was to investigate the effect of G11 in drug resistant NSCLC.

Methods

Materials

G11 was prepared as described in our previous work.[16] An MTT assay kit was purchased from Sigma-Aldrich (Missouri, USA). The primary antibodies, including those against HIF1α, MMP-2, MMP-9, VEGF, AKT, p-AKT and β-actin, were purchased from Cell Signaling Technology (Danvers, MA, USA). Matrigel was obtained from Becton, Dickinson and Company (New York, NY, USA). RPMI 1640 medium, fetal bovine serum (FBS) and PBS were obtained from Gibco Corp. (California, USA). Other analytical or HPLC-grade reagents and chemicals were obtained from Sigma-Aldrich (Missouri, USA) unless specifically noted.

Cell lines and cell culture conditions

Human lung adenocarcinoma cell lines (A549, NCI-H460 and NCI-H1975) are available commercially and were purchased from American Type Culture Collection (Manassas, VA, USA). According to the instructions, the culture medium of these cells was RPMI-1640 medium containing 10% FBS, and the cells were maintained under standard conditions. The drug-resistant cells were obtained after respective treatment with paclitaxel or erlotinib (MCE, USA) at escalating concentrations (from 0.5 to 5 μM) for three months.

Cell viability assay

An MTT assay was performed to validate the effects of G11 on in vitro cell viability. A549, A549/PTX, H460, H460/PTX, H1975 and H1975/Er cells (1×10^5 cells/mL) were seeded at a density of 5000 cells per well into 96-well plates, and incubated for 24 hours. The cells were treated with different concentrations (0.1, 1, 10, 100 μM) of G11. After 72 hours, 10 μL MTT solution (2.5 mg/mL in PBS) was added to each well for further culture for another four hours at 37°C. After the liquid in the wells was removed, 100 μL DMSO was then added into each well to dissolve the formed formazan crystals. The 96-well plate was shaken for eight minutes and the optical density of the solution in each well was determined at 570 nm using a

Spectra Max Paradigm Reader (Molecular Devices, Silicon Valley, CA, USA). The IC_{50} value was calculated using Graphpad software.

MAOA enzymatic activity assay

The expression level of MAOA was measured in A549, A549/PTX, H460 and H460/PTX cell lines as previously described.[24] Briefly, cells were cultured in medium supplemented with 10% FBS in a 10 mm dish. After extracting the reaction products, MAOA activities were determined according to the instructions of the Cell MAOA Assay Kit (Shanghai Chengong Biotechnology, CHN). The level of the substrate p-tyramine determined the MAOA enzymatic activity after treatment with the MAOB inhibitor pargyline.

Analysis of microarray gene expression

A RNeasy Mini Kit (Qiagen) was used to isolate total RNA from G11-treated and DMSO-treated H460/PTX cells according to the insert product. Following the Affymetrix FS450_0002 Hybridization Protocol for gene expression, array hybridization was conducted. The Affymetrix Genechip Scanner 7G was used to scan the Affymetrix GeneChip PrimeView Human Gene Expression Arrays.

Transwell migration and invasion assays

The migration and invasiveness of A549/PTX and H460/PTX cells were studied via transwell analysis in polycarbonate filters of 6.5 mm diameter containing membranes with an 8 μm pore size (Corning Costar, MA, USA).[25] For the migration test, Matrigel (BD Biosciences) was used to coat the lower surface of the filter was. Fresh M200 medium (1% FBS) containing 10 ng/mL VEGF was added to the lower well. After they were trypsinized and suspended in M200 containing 1% FBS, the cells (1×10^6 cells/mL) were treated with G11 (5, 10 μM for A549/PTX cells and 1, 2 μM for H460/PTX cells) for 0.5 hours at ambient temperature before seeding. Each upper well was loaded with 100 μL of cell suspension and incubated at 37°C for 12 hours. After wiping with a cotton swab to remove nonmigrating cells on the upper surface of the filter, the cells were fixed and stained with Calcein-AM. To quantify chemotaxis, the high content drug screening system ImageXpressR Micro (Molecular Devices) was used to count cells migrating to the lower surface. The protocol of the invasion assay was similar to the cell migration assay except that the upper chambers did not contain Matrigel.

Western blot analysis

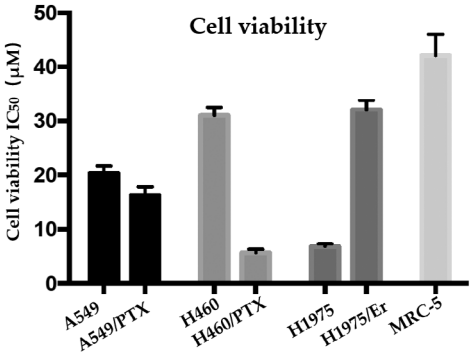
A western blot analysis was performed in accordance with that reported in a previous study.[26] Approximately 1×10^7 H460/PTX cells were collected concurrently after G11 treatment. Briefly, a bicinchoninic acid (BCA) protein assay kit was used to verify the total protein concentration of each sample, and the same amounts of protein from cultured cells were loaded on 8%–15% sodium dodecyl sulfate-polyacrylamide gels for electrophoresis (SDS-PAGE). The resulting gel was then transferred electrophoretically onto polyvinylidene difluoride (PVDF) membranes overnight. The primary and secondary antibodies were then used to blot the proteins. Finally, after incubation with an enhanced chemiluminescence reagent (ECL Plus, Amersham Pharmacia Biotech) for five minutes, targeted proteins were visualized using a chemiluminescence imaging system. Each band on western blotted film was quantified by means of measuring the integrated optical density. β-actin was used as an internal reference.

In vivo antimetastatic assay

To evaluate the tumor metastasis inhibition ability of G11 in vivo, viable H460/PTX cells (5×10^6/100 μL PBS per mouse) were stained with trypan blue and injected intravenously into the right side of each seven- to eight-week-old male BALB/c nude mouse. After one week, the mice were separated into the PTX, G11, PTX + G11 treatment groups and a control group (six mice per group) at random. The G11 single treatment group and the paclitaxel single treatment group, respectively were administered G11 (19 mg/kg/two days, i.p.) and paclitaxel (3 mg/kg/two days, i.p.). The combination group was treated with both G11 (19 mg/kg/two days, i.p.) and paclitaxel (3 mg/kg/two days, i.p.). The control group was injected with 100 μL of PBS. The mice were sacrificed after 21 days after the above mentioned treatments, the lungs were collected and fixed in Bouin's solution for one day. Lung metastatic loci were quantified by stereomicroscopy. All experimental procedures were performed strictly according to the protocol approved by the Committee on the Ethics of Animal Experiments of the Shenyang Pharmaceutical University.

Statistical analysis

Differences between experimental groups were assessed by one-way ANOVA or Student's *t*-test. Statistical significance was based on a *P*-value of 0.05 ($P < 0.05$).



Cell lines	IC_{50} (μM)
A549	20.4±1.3
A549/PTX	16.3±1.5
H460	31.0±1.5
H460/PTX	5.7±0.6
H1975	6.8±0.5
H1975/Er	32.1±1.7
MRC5	42.1±3.9

Figure 1 Cell viability studies of G11. in vitro antitumor activity of G11 against several human lung cancer cell lines and normal lung cell line (A549, A549/PTX, H460, H460/PTX, H1975 and H1975/Er, MRC-5 cells) for 72 hours. The experiments were repeated in triplicate.

Results

Cell viability studies of G11 in parental NSCLC cells and drug-resistant NSCLC cells

Among previous conjugates, compound G11 exhibited a significant inhibitory effect on PC-3 cells. An MAOA inhibitory activity assay showed that the MAOA level was also suppressed by G11, which suggests that this compound exerts its cytotoxicity by targeting tumors and MAOA inhibition.[7] Thus, we evaluated G11 on NSCLC. The antitumor effect of G11 was tested by 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide (MTT) assay. Several NSCLC parental cell lines (A549, H460 and H1975) and their chemotherapy-resistant cell lines (A549/PTX: resistance index ≥ 20, H460/PTX: resistance index ≥ 20) and the erlotinib (Er)-resistant human lung adenocarcinoma cell line H1975/Er (resistance index ≥ 50) were employed in this assay. The viability of the above cell lines was tested after exposure to G11 for 72 hours. As shown in Fig 1, among the tested cell lines, A549/PTX and H460/PTX, treatment with G11 led to a reduction in cell viability. Evaluation of IC_{50} values indicated that G11 even had five-folds higher cytotoxicity against H460/PTX cells than against parental H460 cells but exhibited a much lower cytotoxicity against the erlotinib-resistant cell line H1975/Er, which implies that G11 may possess the ability to reverse paclitaxel resistance in NSCLC cells. Furthermore, we found that G11 showed a relatively weak inhibitory effect against normal lung cell lines MRC-5, suggesting a potential selectivity of G11 towards lung cancer cells.

Inhibitory effect of G11 on MAOA activity in parental NSCLC cells and paclitaxel-resistant NSCLC cells and investigation of its mechanism of action

In our previous work, we showed that G11 displayed moderate inhibitory action on MAOA levels in human PCa LNCaP cells.[16] In order to explore the relationship between MAOA and drug resistance in NSCLC cells, herein, we performed an MAOA activity assay in parental NSCLC cells (A549, H460) and paclitaxel-resistant NSCLC cells (A549/PTX, H460/PTX). Compared with basal levels of MAOA in A549 and H460 cells, MAOA relative activity was markedly increased in A549/PTX and H460/PTX cells, which dramatically decreased after G11 treatment (Fig 2a). Together with the potent cytotoxic efficacy of G11 in A549/PTX and H460/PTX cells, these results demonstrate that MAOA may play a crucial role in paclitaxel-resistant NSCLC cells and that G11 reverses drug resistance by suppressing MAOA activity.

The mechanism by which the above results would occur was further investigated. Gene set enrichment analysis (GSEA) revealed that AKT signaling in paclitaxel-resistant NSCLC cells was suppressed after exposure to G11 (Fig 2b). Studies by Wu *et al.* showed that MAOA-induced AKT phosphorylation and its downstream target FOXO1 were attenuated in MAOA-knockdown LNCaP cells.[6] These results verified the AKT-related signaling pathway was regulated by MAOA. As shown in Fig 2(b), the expression of VEGF-relevant genes in downstream of AKT signaling pathway was also reduced. Taken together, G11 exerted cytotoxic efficacy towards paclitaxel-resistant NSCLC cells through MAOA inhibition followed by the suppression of AKT which resulted in the downregulating of AKT downstream target genes.

Effect of G11 on the AKT signaling pathway and the HIF1α expression level

Since MAOA promotes the phosphorylation of AKT and activates downstream target genes to promote tumor development,[27] the effect of G11 on the AKT signaling pathway was determined. Due to the alterations of molecular events might be happened under non-cytotoxicity concentration, thus we treated cells with non-cytotoxicity concentrations of G11. The results shown in Fig 3a,b confirmed that the phosphorylation of AKT was suppressed

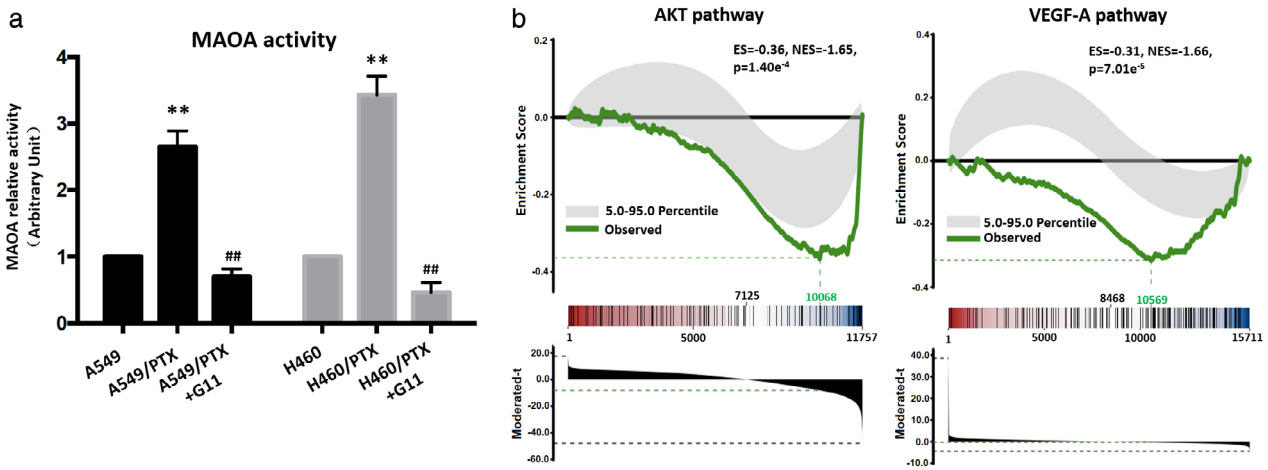


Figure 2 Inhibition of MAOA by G11. (**a**) MAOA relative activity was determined by MAOA enzymatic activity assay. G11 (1 μM) was incubated with cells for 48 hours (▒) 5.0–95.0 Percentile, and (——) Observed. (**b**) GSEA in paclitaxel-resistant cell lines (H460/PTX) after administration of G11 (▒) 5.0–95.0 Percentile, and (——) Observed. $^{**}P < 0.01$, in comparison with parental cells; $^{\#\#}P < 0.01$, in comparison with resistant cells. The experiments were performed in duplicate or triplicate.

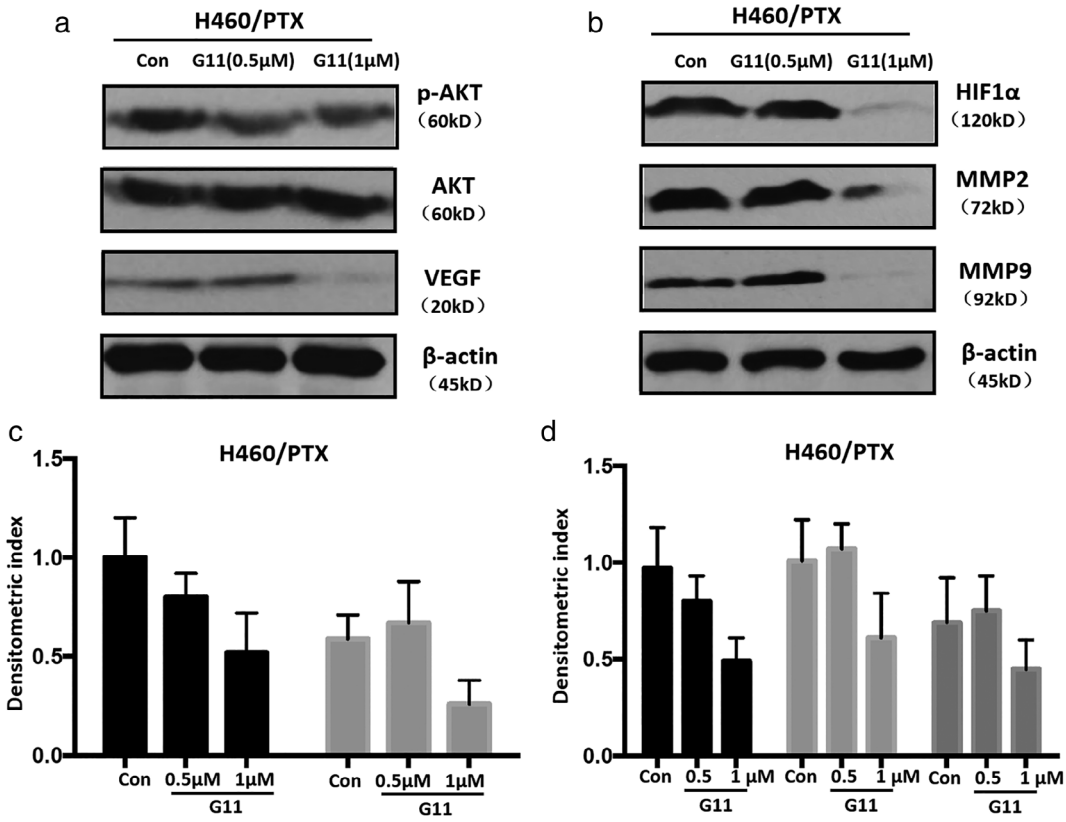


Figure 3 The expression levels of p-AKT, AKT (related to proliferation), and HIF1α, VEGF, MMP2, MMP9 (related to invasion and migration). (**a**) The p-AKT, AKT, and VEGF expression were analyzed by western blot in H460/PTX cell line after treated with G11 (0, 0.5, 1 μM) for 48 hours. (**b**) The densitometric analysis of western blot data in Fig. 3a (■) p-AKT/AKT, and (■) VEGF/Actin. (**c**) HIF1α, MMP2 and MMP9 proteins were determined by western blot in H460/PTX cell line after treated with G11 (0.5, 1 μM) for 48 hours. (**d**) The densitometric analysis of western blot data in Fig. 3c (■) H1F1α/Actin, (■) MMP2/Actin, and (■) MMP9/Actin. β-Actin expression was served as a loading control. The experiments were repeated in triplicate.

after exposure to G11. Moreover, VEGF, an AKT downstream gene that contributes to invasion of the bloodstream by cancer cells,[28] regulates tumor angiogenesis and is closely correlated with metastasis, was also downregulated after G11 treatment.

As mentioned above, MAOA stabilizes HIF1α, which is a vital regulatory factor of hypoxia, by activating relevant downstream tumorigenic genes and leads to tumor occurrence and progression.[29] HIF1α activation promotes the expression of hundreds of target genes including matrix metalloproteinase 2 (MMP2), which has been demonstrated to be pivotal in tumor migration and invasion.[30] Additionally, matrix metalloproteinase 9 (MMP9) is also involved in metastasis, as it modifies extracellular matrix components of neoplastic and stromal cells.[25] HIF1α, MMP2 and MMP9 all participate in tumor invasion, migration and metastasis. Using H460/PTX cells, we performed a further investigation to determine if G11 would affect another MAOA-regulated HIF1α pathway that participates in tumor invasion, migration, and metastasis. As illustrated in Fig 3b, G11 suppressed the expression of HIF1α, MMP2 and MMP9 at the concentration of 1 μM. The results described above revealed that G11 suppresses tumor progression by inactivating MAOA, the downstream AKT pathway and HIF1α pathway.

Inhibitory effect of G11 on the migratory and invasive potential of paclitaxel-resistant NSCLC cells

In consideration that the GSEA and molecular mechanistic studies both revealed that G11 dramatically suppresses the expression levels of multiple invasion- and migration-related genes in NSCLC cells, the capability of G11 to suppress migration and invasion of A549/PTX and H460/PTX cells was subsequently assessed using transwell assay. The results revealed an obvious decrease in migrating A549/PTX and H460/PTX cells after treatment with G11 at non-cytotoxic concentrations (Fig 4a). These results indicate that G11 possesses antimigratory potential at a non-cytotoxic dose against drug-resistant NSCLC cells. As shown in Fig 4b, the potential invasion of both paclitaxel-resistant cells could also be remarkably suppressed by G11 in a concentration-dependent manner.

In vivo tumor metastasis inhibition of G11 in paclitaxel-resistant lung cancer metastasis models

The main factor that causes poor prognosis in cancerous patients is the occurrence of tumor metastasis. Based on the favorable in vitro migration and invasion inhibitory effect of G11 on NSCLC cells, an H460/PTX lung metastasis mouse model was established, and in vivo antimetastatic activities of G11 were further investigated. As shown in Fig 5a,b, an average of more than 30 metastatic foci with considerable sizes could be observed in the control group (phosphate-buffered saline, PBS). In contrast, mice treated with G11 or paclitaxel alone both displayed similarly obvious decreases in the number of lung metastatic foci, which indicates that the two drugs demonstrate comparable in vivo antitumor efficacy of delayed tumor progression and metastasis. It is particularly noteworthy that the biggest reduction in lung metastatic foci was observed in tumor samples that were coadministered G11 and paclitaxel (the dose of each single drug remained unchanged). The results show that combination treatment of G11 and paclitaxel markedly enhanced the suppression of paclitaxel-resistant tumor metastasis and was superior to each drug alone; this was inspiring and provided a robust pharmacological basis for future exploration.

Discussion

The mechanisms of drug resistance in NSCLC have not previously been clarified. However, recent studies have indicated that HIF1α is a key transcription factor in drug resistance in NSCLC.[31–33] EMT also regulates drug resistance in NSCLC due to its close relationship with HIF1α.[34] Hence, MAOA may hold great potential in inducing chemotherapy resistance in NSCLC, which would affect the process of EMT and HIF1α expression.

Recent studies have indicated that MAOA has been linked to the promotion of PCa and other types of carcinomas.[12,35] Advanced PCa exhibits the features of increased MAOA expression and metastasis.[6,11] Study results suggested that MAOA-induced EMT would be the prime motivator to promote the progression of PCa. However, contradictory results were obtained in hepatocellular carcinoma (HCC) and cholangiocarcinoma.[12,35] Thus, the role of MAOA in various types of cancers is worthy of further exploration.

The report that MAOA promoted NSCLC progression by mediating EMT raised an intriguing question of whether MAOA could serve as a therapeutic target in NSCLC treatment.[15] In this study, we used a previously synthesized compound, G11, whose in vitro antitumor efficacy and MAOA inhibitory ability have been confirmed in human prostate cancer cells. Through cytotoxicity evaluation and MAOA activity assay, we found that MAOA activities were relatively higher in paclitaxel-resistant NSCLC cells than in parental cells. G11 inhibited paclitaxel-resistant NSCLC growth more effectively than in parental cells. G11 treatment remarkably decreased MAOA activities in PTX-resistant cells. Taken together, these data revealed for the first time the association between MAOA

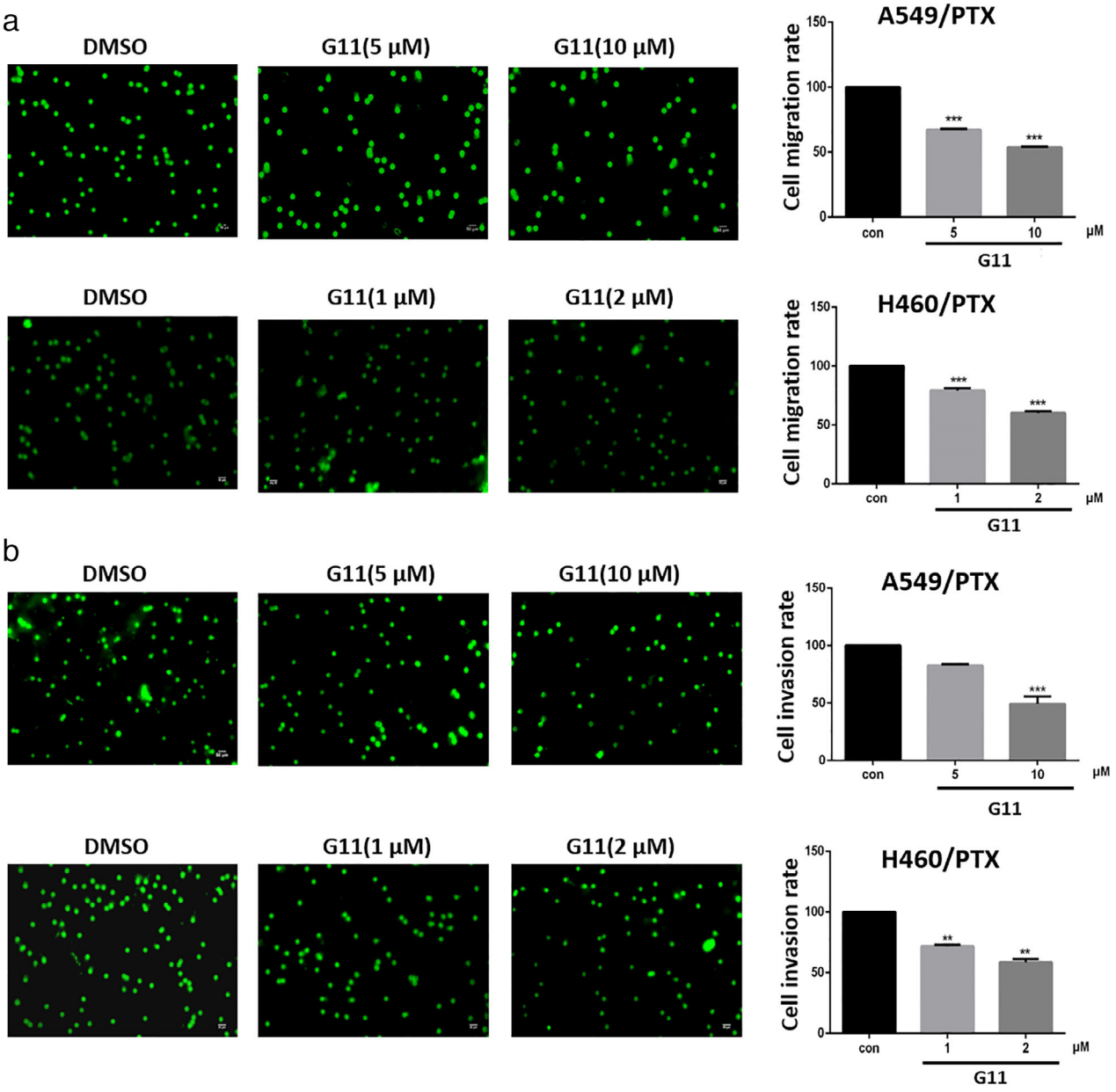


Figure 4 Inhibition of A549/PTX and H460/PTX cells migration and invasiveness upon treatment with G11. (**a**) A549/PTX and H460/PTX cell migration was investigated by transwell assay at various concentrations of G11 (0, 5, 10 μM; 0, 1, 2 μM, respectively) for 24 hours (■) con, (■) G11 (5 μM), and (■) G11 (10 μM); (■) con, (■) G11 (1 μM), and (■) G11 (2 μM). (**b**) A549/PTX and H460/PTX cell invasion was examined by Transwell assay at various concentrations of G11 (0, 5, 10 μM; 0, 1, 2 μM, respectively) for 24 hours (■) con, (■) G11 (5 μM), and (■) G11 (10 μM); (■) con, (■) G11 (1 μM), and (■) G11 (2 μM). The images on the left are microscopic images and the pictures on the right show the quantitative analysis. All error bars are the s.e.m. *Indicates a marked difference between the test group and the control group, $P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$. The experiments were repeated in triplicate.

and paclitaxel resistance in NSCLC. G11 reversed drug resistance in NSCLC by suppressing MAOA activity. Phosphorylation of AKT was upregulated by MAOA overexpression and MAOA stabilized HIF1α. Western blot results showed that the expression levels of proliferation-related p-AKT, invasion- and migration-associated VFGF, HIF1α and its target genes MMP2 and MMP9 were all downregulated by G11. Using a transwell assay, we saw that G11 inhibited the migration and invasiveness of A549/PTX and H460/PTX cells under cytotoxic concentrations.

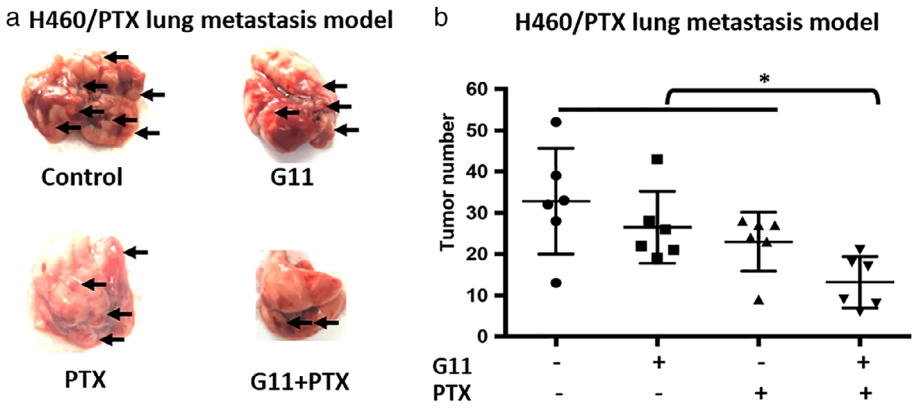


Figure 5 The effects of G11 in a paclitaxel-resistant lung cancer metastasis mouse model. (**a**, **b**) show the lung metastatic foci number in different treatment groups. The dissected and harvested tumor-bearing lung metastatic foci after treatment with G11 (8 mg/kg/two days, i.p.) and paclitaxel (3 mg/kg/two days, i.p.).

These results mechanistically indicated that G11 inhibited tumor cell angiogenesis, migration and invasion by suppressing of the AKT pathway and HIF1α pathway of MAOA downstream. The in vivo study further confirmed that G11 possessed the ability to suppress paclitaxel-resistant NSCLC metastasis.

Based on the above results, we concluded that the potent in vitro and in vivo antitumor efficacy of G11 against paclitaxel-resistant NSCLC was closely correlated with MAOA inhibition. The potential MAOA inhibitor G11 may inhibit paclitaxel-resistant NSCLC metastasis and growth by impacting on p-AKT, VEGF, HIF1α and MMP2 or MMP9. These findings have established MAOA as a promising therapeutic target in drug-resistant NSCLC and provide a feasible method of MAOA-targeted combined therapeutics.

Disclosure

The authors declare that they have no conflicts of interest.

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