Auranofin Enhances Ibrutinib's Anticancer Activity in EGFR-Mutant Lung Adenocarcinoma

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Abstract

We previously found that ibrutinib has anticancer activity in EGFR-mutant non-small cell lung cancer (NSCLC). One of our recent studies showed that auranofin, a gold complex that has been used to treat rheumatoid arthritis, inhibited the PI3K/AKT/mTOR pathway and promoted apoptosis in some NSCLC cells. Because the PI3K/AKT/mTOR pathway is one of the major downstream pathways of EGFR, we hypothesized that ibrutinib’s activity might be enhanced by combination therapy with auranofin in NSCLC cells. To this end, we examined ibrutinib’s dose responses in EGFR-mutant H1975, PC9 and H1650 cells and in EGFR-wild type Calu3 and H460 cells in the presence or absence of auranofin. While low concentrations of auranofin alone demonstrated mild anticancer activities, its presence dramatically enhanced ibrutinib’s activity in H1975, PC9 and H1650 cells (IC_{50} value reduced 10 - 100 fold), but had only mild effect on Calu3 and H460 cells, demonstrating that ibrutinib’s anti-EGFR activity is enhanced when it is combined with auranofin. A mechanistic analysis revealed that ibrutinib alone induced dramatic inhibition of the MEK/ERK pathway in both H1975 and H1650 cells, while auranofin alone inhibited the AKT/mTOR pathway. The combination of ibrutinib and auranofin led to dramatically enhanced inhibition of the expression or phosphorylation of multiple key nodes in the AKT/mTOR and MEK/ERK pathways in both cell lines. In mice, the combination of ibrutinib and auranofin significantly suppressed the growth of H1975 xenografted tumors without inducing obvious toxic effects. Our results demonstrate the feasibility of improving ibrutinib’s anti-EGFR activity for NSCLC using combination therapy with auranofin.
Introduction

Epidermal growth factor receptor (EGFR) is known to be one of the major driving forces in lung tumorigenesis; it has been extensively investigated as a target for anticancer therapy. Activating EGFR mutations are detected in about 10%-17% of human lung adenocarcinomas in the United States and Europe (1-3) and in about 30%-65% of lung cancers in Asia (4-6). These mutations are also more common in women and non-smokers (1-3). Inducing the expression of human lung cancer-related mutant EGFR genes in transgenic mice causes the development of lung adenocarcinoma, whereas stopping the expression leads to tumor regression (7,8), demonstrating that EGFR activation is required and sufficient for lung cancer tumorigenesis and maintenance. The finding that EGFR-mutant lung cancer cells are highly susceptible to the reversible EGFR inhibitors gefitinib (9,10) and erlotinib (11) has made these two agents the first choice for therapy in patients whose tumors harbor EGFR mutations.

Unfortunately, despite dramatic responses to gefitinib and erlotinib in patients with EGFR-mutant lung cancer, acquired resistance inevitably occurs at a median of 10-16 months after treatment initiation (12,13). A variety of mechanisms have been identified for this acquired resistance, including a second T790M mutation at exon 20 of the EGFR gene (14,15); amplification of the MET (16,17) or HER2 (18) gene; mutations of the K-Ras gene (19); activation of AXL, c-Src kinases (20,21), and ERK (22); loss of PTEN (23); and activation of the IL-6R/STAT3 pathway (24). Among these mechanisms, the most common cause of resistance in the clinic is the T790M mutation in EGFR, which is found in about 50% of these patients (25). Substantial efforts have been made to develop EGFR kinase inhibitors that are effective against T790M mutations in EGFR (26,27). Several novel EGFR inhibitors with novel
chemical structures have been reported to inhibit EGFR activity \textit{in vitro} and \textit{in vivo} in animal models (28) and have been evaluated in clinical trials (29-31). Afatinib, an irreversible dual EGFR/HER2 inhibitor that had some activity against T790M-mutant tumors in a preclinical study (32), was approved by the U.S. Food and Drug Administration (FDA) in 2013 for the treatment of metastatic non-small cell lung cancer (NSCLC) with EGFR mutations. However, its side effects limit its application in multimodality therapy (33). More recently, the third-generation anti-EGFR agent osimertinib was approved for the treatment of NSCLC with EGFR T790M mutation. Nevertheless, acquired resistance to osimertinib was reported (34). Therefore, new therapeutic strategies or agents to overcome the resistance to conventional EGFR inhibitors are urgently required.

One of our previous studies revealed that ibrutinib, an irreversible Bruton's tyrosine kinase (BTK) inhibitor that was recently approved by the FDA for the treatment of mantle cell lymphoma (MCL) and chronic lymphocytic leukemia (CLL), can function as an EGFR inhibitor and selectively inhibit growth and induce apoptosis in EGFR-mutant NSCLC cells \textit{in vitro} and \textit{in vivo}, including erlotinib-resistant cells that harbor a T790M mutation (35). Treatment of EGFR-mutant NSCLC cells with ibrutinib induced inhibition of phosphor-EGFR at both the Y1068 and Y1173 sites, suggesting that ibrutinib functions as an EGFR inhibitor. Moreover, ibrutinib had \textit{in vivo} anticancer activity in xenograft tumors derived from erlotinib-resistant H1975 cells. More recently, we found that auranofin, a gold complex that has been used to treat rheumatoid arthritis in the clinic and that has documented pharmacokinetics and safety in humans, also has \textit{in vitro} and \textit{in vivo} anticancer activity in some NSCLC cell lines (36). The administration of auranofin to mice with xenografted tumors derived from NSCLC cells
significantly suppressed tumor growth, suggesting that auranofin is a candidate for lung cancer treatment through drug repurposing. Because safety concerns are one of the major reasons for the discontinuation of drug development, both preclinically and clinically (37), repurposing FDA-approved drugs for new indications is expected to have a rapid effect on cancer therapy. Therefore, in this study, we tested the combined effects of ibrutinib and auranofin in three EGFR-mutant lung cancer cells (H1975, PC9 and H1650) and two EGFR wild-type NSCLC cell lines (Calu3 and H460). H1650 (mutant EGFR; del E746_A750) was resistant to erlotinib and ibrutinib in our study; PC9 (mutant EGFR; del E746_A750) was sensitive to both erlotinib and ibrutinib and H1975 (mutant EGFR; L858R, T790M) was resistant to erlotinib but susceptible to ibrutinib. Our results showed that the combination of auranofin and ibrutinib leads to enhanced anticancer activity in EGFR-mutant lung cancers.

**Materials and Methods**

**Cell lines**

Human NSCLC cell lines with EGFR mutations (H1975, PC9, H1650 and HCCC827) or with wild type EGFR (Calu3, H460 and A549) were maintained in our laboratories as previously reported (35). H1975 and PC9 were relatively sensitive to ibrutinib (IC₅₀ < 1 µM) while H1650, Calu3 and H460 cells were resistant to ibrutinib (IC₅₀ > 2 µM). Cell lines were maintained in RPMI 1640 supplemented with 5% fetal bovine serum and cultured at 37°C in a humidified atmosphere containing 5% CO₂ and 95% air. Cell line authentication was performed at our institution’s Characterized Cell Line Core using a short tandem repeat DNA fingerprint assay with a PowerPlex 16 HS kit (Promega, Madison, WI).
Cell viability assay

Both ibrutinib and acalabrutinib were obtained from Selleckchem incorporation. Auranofin was obtained from Sigma-Aldrich Corporation. *In vitro* drug sensitivity was determined using the sulforhodamine B assay, as we previously reported (38). Each experiment was carried out in quadruplicate and repeated at least three times. In brief, 3,000 cells was plated in 96-well plates and treated with the indicated doses of ibrutinib (0 - 1 µM for H1975 and 0 - 30 µM for other cell lines), auranofin (0.25 µM), or both for 72 h. A sulforhodamine B assay was conducted to determine cell viability. Cells treated with DMSO were used as controls; their value was set as 1. The median inhibitory concentration (IC$_{50}$) values were calculated using CurveExpert 1.3 software and plotted in dose-response curves.

Cell cycle and cell apoptosis analysis

The cell cycle and number of apoptotic cells (sub-G$_1$) were measured using fluorescence-activated cell sorting (Becton Dickinson). Cells (2×10$^5$/mL/well) were inoculated in six-well plates. They were then treated with the indicated doses of ibrutinib (0.1 µM for H1975 and 0.3 µM for H1650), auranofin (0.25 µM), or both for 24 h and 48 h (36). After treatment, cells were harvested and washed once with phosphate-buffered saline (PBS) and then fixed with 70% ethanol overnight at 4°C. Cell pellets were harvested and resuspended with 200 µL of 50 µg/mL propidium iodide/RNase and then incubated for 30 min at 37°C in the dark. Flow cytometry and CellQuest software (BD Biosciences) were used to measure the sub-G0/G1 cellular DNA content.
Western blot analysis

Whole-cell lysates were prepared by washing the cells with PBS and subjecting them to lysis with radioimmunoprecipitation assay buffer supplemented with protease inhibitor cocktail. After the lysates had been sonicated for 15 s, the protein concentrations were quantified using the Bio-Rad protein assay kit. Equivalent amounts of each protein were loaded, separated by 10% or 12% sodium dodecyl sulfate-polyacrylamide gel electrophoresis and transferred to polyvinylidene fluoride membranes at 80 V for 2 h. The membranes were blocked for 1 h with 5% non-fat dried milk in PBS buffer containing 0.1% Tween-20 and probed with diluted primary antibody at 4°C overnight. The membranes were then washed three times in the PBS-Tween-20 buffer and probed with infrared dye-labeled secondary antibodies. The immunoreactive bands were visualized with the Odyssey Imager (Li-COR Biosciences, Lincoln, NE). H1975 and H1650 cells were treated with DMSO, 0.25 µM auranofin, 0.1 µM (H1975) or 0.3 µM (H1650) ibrutinib, or combination of auranofin and ibrutinib for 24 h or 48 h before harvest for western blot analysis. The antibodies used for the Western blot analysis included anti-STAT3 (CST, #4904), p-STAT3 (CST, #9145, at Tyr705 site), AKT (Abcam, ab179463), p-AKT (Bioworld Technology, BS4007, at Ser473 site), mTOR (Abcam, ab2732), p-mTOR (CST, #5536, at Ser2448 site), p-70S6K (Proteintech, 14485-1-AP), MEK1/2 (CST, #8727), p-MEK1/2 (CST, #9154, at Ser217/221 sites), ERK1/2 (CST, #4695), p-ERK1/2 (CST, #4370, at Thr202/Tyr204 sites), and β-actin (ZSGB-BIO, TA-09).

Animal experiments

Animal experiments were carried out according to the approved protocol by the Institutional
Animal Care of Committee of Harbin Medical University in China and in accordance with the Guidelines for the Care and Use of Laboratory Animals published by the National Institutes of Health (revised 1985). We inoculated $5 \times 10^6$ H1975 cells subcutaneously into the right dorsal flanks of 6- to 8-week-old female nude mice. Treatment started when tumors reached a mean volume of approximately 50 mm$^3$. The mice were grouped randomly into four groups, with six mice in each group: (i) ibrutinib only (25 mg/kg/d; oral), (ii) auranofin only (5 mg/kg; injected into the tail vein), (iii) both ibrutinib and auranofin as in groups 1 and 2, and (iv) solvent only (10% dimethyl sulfoxide and 10% polyethylene glycol 400). Animals were treated daily, and tumor volumes were measured blindly every 2 days. The tumor volumes were calculated using the formula $\text{length} \times \text{width}^2 \times 0.52$. Mice were euthanized with CO$_2$ when their tumor volume was larger than 2,000 mm$^3$ or animals became morbid or moribund. Mean tumor growth was analyzed using the Kruskal-Wallis test for overall significance among different treatment groups and the Mann-Whitney U test for significance between two treatment groups (two-tailed). The Kaplan-Meier method was used to analyze survival.

**Statistical analysis**

Each experiment or assay was conducted at least three times, and representative examples are shown. Data are reported as the mean ±SD. The statistical significance of the differences between treated samples was determined using the two-tailed Student t test and one-way ANOVA analysis. Differences were considered statistically significant at $p<0.05$. The significance of the in vivo animal study data was determined using the Kruskal-Wallis test for overall significance among different treatment groups and the Mann-Whitney U test for
significance between two treatment groups (two-tailed). The Kaplan-Meier method was used to analyze animal survival.

**Results**

*Auranofin enhanced ibrutinib’s anticancer activity in EGFR-mutant NSCLC cells.*

To determine whether ibrutinib’s activity is enhanced by being combined with auranofin, we examined ibrutinib dose responses in EGFR-mutant NSCLC cell lines H1975, PC9 and H1650 and in EGFR-wild type NSCLC cell lines Calu3 and H460 in the presence or absence of low-dose auranofin (0.25 µM). Our previous study showed that auranofin had narrow dose response ranges for most lung cancer cell lines, ranging from 0.25 µM to 2.5 µM (36). Therefore, in this study, we used 0.25 µM for testing auranofin’s role in the combination.

Auranofin at 0.25 µM alone induced a 20%-30% reduction in cell viability in H1975, PC9, H1650, and Calu3 cells, but had not obvious effect on H460 cells. It also dramatically enhanced ibrutinib’s activity in H1975, PC9 and H1650 cells (Fig. 1A, 1B, 1C). The IC$_{50}$ dropped from 0.20 µM (ibrutinib alone) to 0.001 µM (with the presence of auranofin) in H1975 cells, from 0.69 µM to 0.06 µM in PC9 cells and from 8.20 µM to 0.23 µM in H1650 cells (Fig. 1F). In contrast, the presence of auranofin had only minor effect on responses to ibrutinib in Calu3 and H460 cells (IC$_{50}$ value reduced only about 2 fold) (Fig. 1D, 1E, 1F). We also tested whether acalabrutinib, a second generation of Bruton’s tyrosine kinase inhibitor (39), had the similar effects on NSCLC cells as the ibrutinib. The results showed the results showed that acalabrutinib did not induce any growth inhibition in all cell lines tested (Supplement Fig.1).

This result suggested that presence of auranofin can enhance ibrutinib’s activity in EGFR
mutant NSCLC cells \textit{in vitro}.

\textbf{Auranofin and ibrutinib combination therapy enhanced apoptosis induction.}

To determine whether the enhanced anticancer activity of ibrutinib and auranofin combination therapy was caused by cytostatic or cytotoxic effects, we treated H1975 and H1650 cells with 0.25 µM auranofin, 0.1 µM (H1975) or 0.3 µM (H1650) ibrutinib, or both. The cells were harvested 24 h and 48 h after treatment. Cell apoptosis was determined by the number of sub-G1 cells in a fluorescence-activated cell sorting analysis. The results showed that treatment with ibrutinib alone induced substantial apoptosis in H1975 cells but not in H1650 cells. Treatment with auranofin alone induced apoptosis in both H1975 and H1650 cells; this effect was more dramatic in H1975 cells than in H1650 cells. Nevertheless, significantly higher apoptosis induction was found in the combination therapy group than in the single-agent therapy groups at 48 h in H1975 cells ($p<0.05$, Fig. 2A, 2B). In H1650 cells, the combination treatment also induced a time-dependent increase in apoptosis at 24 h and 48 h ($p<0.05$, Fig. 2A, 2C). A Western blot analysis of PARP cleavage confirmed enhanced apoptosis induction with combination treatment compared with single-agent treatment as described above (Fig. 3) (Supplement Fig 2). Together, these results indicate that the combination of ibrutinib and auranofin induces at least additive cytotoxic effects of both agents.

\textbf{Enhanced inhibition of AKT/mTOR and MEK/ERK signaling with ibrutinib and auranofin combination therapy.}

Our previous study showed that auranofin-mediated anticancer activity in NSCLC cells was associated with inhibition of the PI3K/AKT/mTOR axis (36). To determine the underlying
mechanisms of ibrutinib and auranofin combination therapy, we analyzed the status of EGFR downstream pathways in H1975 and H1650 cells after treating them with DMSO, 0.25 μM auranofin, 0.1 μM (H1975) to 0.3 μM (H1650) ibrutinib or both for 24 h or 48 h. Cell lysates were subjected to Western blot analyses for the STAT3, AKT/mTOR, and MEK/ERK pathways. The results showed that treatment with ibrutinib alone induced dramatic inhibition of the MEK/ERK pathway in both H1975 and H1650 cells, consistent with our previous report that ibrutinib functions as an EGFR inhibitor in EGFR-mutant NSCLC (40). However, treatment with ibrutinib inhibited the phosphorylation of STAT3 (Tyr705), AKT (Ser-473), and mTOR (Ser2448) in H1975 cells, particularly at 48 h, but not in H1650 cells (Fig. 4). Treatment with auranofin alone inhibited the phosphorylation of AKT and mTOR, particularly at 48 h, but did not have an obvious effect on phosphorylation of MEK/ERK and STAT3 and in both H1975 and H1650 cells. The combination therapy led to dramatically enhanced inhibition of the AKT/mTOR, and MEK/ERK pathways in both cell lines, particularly at 48 h (Fig. 4) (Supplement Fig. 3), suggesting that this combination inhibited major EGFR downstream pathways.

**Auranofin enhanced ibrutinib-mediated anti-lung cancer activity in vivo.**

To determine whether ibrutinib and auranofin combination therapy can induce enhanced antitumor activity in EGFR-mutant NSCLC tumors in vivo, we established xenograft tumors from H1975 cells in nude mice. After the tumors had grown to about 50 mm$^3$ in diameter, the mice were grouped randomly into 4 groups (n=6) and treated daily with various agents as described in the Materials and Methods. The result showed that treatment with ibrutinib or auranofin alone led to 62% and 57% inhibition of tumor growth, respectively, compared with
control. The combination of ibrutinib with auranofin led to 82% tumor growth suppression compared with control group, and was significantly different from that of ibrutinib and auranofin alone ($p<0.05$). While the mean survival durations of the control and auranofin-treated mice were 26 days and 28 days, the mean survival durations of the ibrutinib- and combination therapy-treated mice were 31 days and 38 days ($p=0.016$ and $p=0.005$), demonstrating the in vivo efficacy of ibrutinib in EGFR-mutant cancer (Fig. 5). No obvious weight loss was detected in any of the treatment groups. These results suggest that combination therapy with ibrutinib and auranofin has enhanced in vivo anticancer activity and is well tolerated.

**Discussion**

Our previous study revealed that ibrutinib can function as an EGFR inhibitor; it selectively inhibited growth and induced apoptosis in EGFR-mutant NSCLC cells in vitro and in vivo, including erlotinib-resistant cells that harbored a T790M mutation (35). We also found that thioredoxin reductase 1 inhibitor auranofin, a gold complex that has been used in the clinic for the treatment of rheumatoid arthritis, has anticancer activity in NSCLC cells in vitro and in vivo (36). Thus, both ibrutinib and auranofin might be repurposed for the treatment of lung cancer. In this study, we found that the combination of ibrutinib and auranofin had enhanced in vivo activity in an EGFR-mutant NSCLC model; the combination was well tolerated. We previously found that ibrutinib can function as an EGFR inhibitor and selectively inhibit growth and induce apoptosis in EGFR-mutant NSCLC cells in vitro and in vivo, including erlotinib-resistant cells that harbor a T790M mutation (35). Treatment of EGFR-mutant NSCLC cells with ibrutinib induced inhibition of phosphor-EGFR at both the Y1068 and Y1173 sites, suggesting that
ibrutinib functions as an EGFR inhibitor. A recent study by other researchers demonstrated that ibrutinib directly targets mutant EGFR via irreversible binding with Cys797 (41). In this study, we found that ibrutinib inhibited MEK/ERK phosphorylation in both H1975 and H1650 cells. Because MEK/ERK is one of the major EGFR downstream pathways, this result further supports the reports on ibrutinib-mediated EGFR inhibition (35,41).

Interestingly, a Western blot analysis revealed that ibrutinib induced similar or stronger MEK/ERK inhibition in H1650 cells as in H1975 cells, even though H1975 cells are more sensitive to ibrutinib than are H1650 cells. It is possible that ibrutinib inhibited other EGFR downstream pathways, such as STAT3 and AKT, in H1975 cells but not in H1650 cells. Both the STAT3 and PI3K/AKT/mTOR pathways are major downstream signaling pathways of EGFR and other receptor tyrosine kinases that regulate cell growth, proliferation, metabolism, and survival; these pathways are commonly deregulated in cancer. Activated by a number of growth factor receptors through interactions with their ligands, the STAT3 and PI3K/AKT/mTOR pathways play critical roles in resistance to various therapeutic agents that target the receptor tyrosine kinases, including EGFR inhibitors (17,42,43). Transient inhibition of the STAT3 or PI3K/ATK/mTOR pathways was reported to induce apoptosis and overcome resistance to EGFR-TKIs in EGFR-mutant lung cancer (44,45).

In our previous study, we found that auranofin, a gold complex that has been used to treat rheumatoid arthritis in the clinic and has documented pharmacokinetics and safety in humans, has in vitro and in vivo anticancer activity in some NSCLC cell lines (36). Treatment of NSCLC cells with auranofin resulted in inhibition of the expression and activity of multiple key nodes in the PI3K/AKT/mTOR pathway (17,43,45). In the current study, we confirmed the roles of
auranofin in inhibiting the phosphorylation of AKT/mTOR in both H1975 and H1650 cells. We also found that auranofin had no obvious effects on phosphorylation of the STAT3 and MEK/ERK pathways. Interestingly, the combination of ibrutinib with auranofin drastically inhibited the signaling of MEK/ERK, AKT/mTOR, and STAT3, the three major pathways of EGFR and other receptor tyrosine kinases.

As the first-in-human BTK inhibitor, ibrutinib can affect the B-cell antigen receptor signaling pathway and has promising anticancer activity, especially in MCL (46), Waldenstrom macroglobulinemia, the ABC subset of diffuse large B cell lymphoma (DLBCL) (47), and CLL (40). Ibrutinib inactivates BTK through covalent binding to the active site (Cys-481) in the ATP-binding domain of BTK with an IC50 of 0.5 nM (48). Currently, ibrutinib is under late-stage clinical development for patients with CLL, MCL, Waldenstrom macroglobulinemia, DLBCL, and multiple myeloma.

Several reports have demonstrated that the clinical activity of ibrutinib on B-cell malignancies is favorable in patients with CLL, MCL, and DLBCL (38,40,46). A phase II study in relapsed MCL (111 patients, 48 of whom had been treated with bortezomib) showed a response rate of 68% (75 patients) and a complete response rate of 21%; after an estimated median follow-up of 15.3 months, the estimated median response duration was 17.5 months (95% confidence interval [CI], 15.8 to not reached), the estimated median progression-free survival was 13.9 months (95% CI, 7.0 to not reached) (46). Moreover, clinical trials of ibrutinib in lymphoma and CLL patients revealed that it has an excellent safety profile at wide-ranging doses (420-840 mg/day). Side effects were less common and less severe in patients treated with ibrutinib than in patients treated with other EGFR inhibitors (38,49). Long-term therapy with ibrutinib was...
associated with modest toxicity, and most adverse events were grade 1 or 2 (46,50). The excellent safety profile observed in clinical trials of ibrutinib in combination therapy suggests that ibrutinib is a good candidate for combined therapies (51). Moreover, a clinical study demonstrated that ibrutinib treatment markedly increased T cell number and functions in CLL patients (52), suggesting that it is beneficial for multi-modality immunotherapy. Nevertheless, this study had some limitations. We only evaluated body weight as a parameter for the toxicity of the drug combination. Whether the combination will cause other toxicity needs to be determined. In addition, in vivo activity was evaluated in one tumor model in immunocompromised mouse model. Whether the mechanism of action observed in vitro can also be observed in vivo and whether tumor immune microenvironment will have any impact on the combination therapy remains untested. Since auranofin is also reported to have effect on T cell activation (53), further investigation of ibrutinib and auranofin combination therapy in immunocompetent mouse tumor model may reveal possible interactions of ibrutinib and auranofin on tumor immune microenvironment in treatment of lung cancers.

Disclosure of Potential Conflict of Interest

No potential conflicts of interest were disclosed by the other authors.

Acknowledgments

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Figure Legends

Figure 1. Combination effects of ibrutinib and auranofin in EGFR-mutant or EGFR-wild type lung cancer cells in vitro. (A - E) Dose-response curves of ibrutinib with or without 0.25 µM auranofin for five NSCLC cell lines. Data are presented as the mean ± standard deviation (SD) of a quadruplet assay. Cells treated with DMSO alone were used as controls, and their values were set as 1. (F) Ibrutinib IC_{50} values (µM) in the five NSCLC cell lines, with or without auranofin. Data are presented as the mean ± standard deviation (SD) of three assays.

Figure 2. Apoptosis induction by ibrutinib and auranofin in EGFR mutant lung cancer cells. H1975 and H1650 cells were treated with DMSO, 0.25 µM auranofin, 0.1 µM (H1975) to 0.3 µM (H1650) ibrutinib, or both for 24 or 48 h. Cell apoptosis was determined by fluorescence-activated cell sorting after staining with propidium iodide. (A) Representative diagrams of the fluorescence-activated cell sorting analysis. The numbers in the upper left box of each graph represent the number of apoptotic cells (sub-G1). (B) and (C) Percentage of apoptotic cells after treatment with ibrutinib, auranofin, or both in H1975 cells (B) and H1650 cells (C) The numbers represent the mean ± standard SD of three assays. * indicates the difference is significant (p<0.05).

Figure 3. Western blot analysis of apoptosis induced by ibrutinib and auranofin. Western blot analysis of PARP levels after treatment with DMSO, 0.25 µM auranofin, 0.1 µM (H1975) to 0.3 µM (H1650) ibrutinib, or both for 24 or 48 h. β-actin was used as a loading control. C-PARP: cleaved PARP.
Figure 4. Effects of ibrutinib and auranofin on EGFR downstream signaling pathways.

H1975 and H1650 cells were treated with DMSO, auranofin, ibrutinib, or both for different times, as indicated. β-actin was used as a loading control. Ibrutinib alone inhibited the MEK/ERK pathway, and the combination with auranofin led to dramatically enhanced inhibition of the AKT/mTOR and MEK/ERK pathways in both H1975 and H1650 cells.

Figure 5. In vivo effects of ibrutinib and auranofin. Animals bearing H1975 xenograft tumors were treated with ibrutinib (25 mg/kg/d; by oral), auranofin (5 mg/kg; by intraperitoneal), or both. Animals treated with solvent were used as the control. (A) Tumor growth; (B), body weight. * indicates that the difference was significant (p<0.05). Data are presented as the mean ± standard deviation (SD) of six animals/group. (C) Cumulative survival curve. The mean survival durations of solvent- and auranofin-treated mice were 26 days and 28 days, respectively. The mean survival durations of mice treated with ibrutinib and auranofin were 31 days and 38 days (p=0.016 and p=0.005), respectively. All statistical tests were two-sided.
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**Fig 4**
Fig 5
Molecular Cancer Therapeutics

Auranofin Enhances Ibrutinib's Anticancer Activity in EGFR-Mutant Lung Adenocarcinoma

Jing Hu, Huijuan Zhang, Mengru Cao, et al.

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