Original article

Antitumor activity of F90, an epidermal growth factor receptor tyrosine kinase inhibitor, on glioblastoma cell line SHG-44

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Keywords: glioblastoma; epidermal growth factor receptor; Iressa

Background Over-expression of epidermal growth factor receptor (EGFR) is thought to be related to cell proliferation, invasion, metastasis, resistance to chemoradiotherapy and poor prognosis of various human cancers. Forty percent to fifty percent of glioblastoma multiforme (GBM) possess deregulated EGFR, which may contribute to the aggressive and refractory course of GBM. Therefore, blockade of EGFR signal transduction may be a promising treatment strategy for GBM.

Methods MTT assay, cell growth curve assay and tumor xenograft model were used to evaluate the antitumor activity of F90 against SHG-44 in vitro and in vivo. Western blot assay was applied to evaluate the expression of p-EGFR, p-ERK1, p-JNK, p-P38, Bcl2 and P53 proteins.

Results F90 inhibited the cell proliferation in a dose-dependent manner in vitro. The growth of SHG-44 tumor xenografts was suppressed by F90 at a high dose level (100 mg·kg⁻¹·d⁻¹). Phosphorylation of EGFR and activated downstream signaling proteins, such as ERK1, JNK and P38, were found to be depressed after incubation with F90 for 48 hours in vitro. Down-regulated Bcl2 protein and up-regulated P53 protein were also observed.

Conclusions The results demonstrate that F90 is effective in inhibiting the proliferation of SHG-44 cells in vitro and tumor growth in vivo, suggesting that F90 may be a new therapeutic option for treatment of GBM.

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Epidermal growth factor receptor (EGFR) is a tyrosine kinase receptor which is over-expressed in cancer cells, and has been identified as a new target of tumor therapy. The downstream signaling pathways of activated EGFR include the mitogen-activated protein kinase (MAPK) pathway, the phosphatidylinositol 3 kinase pathway and the Janus kinase signaling transducer and activator of transcription pathway. All these pathways can lead to cell proliferation, differentiation, angiogenesis, metastasis and anti-apoptosis.¹

Over-expression of EGFR has been reported in various kinds of malignancies such as head and neck tumors, colorectal cancer, ovarian cancer, breast cancer, lung cancer and bladder cancer.²⁻⁴ Glioblastoma multiforme (GBM; WHO grade IV) is the most aggressive primary brain tumor, because of its high degree of cellularity, anaplasia, vascular proliferation, and necrosis. As many other human solid tumor types, EGFR plays a pivotal role in the growth and progression of GBM. Forty percent to fifty percent of these highly malignant neoplasms show a high level of EGFR.⁵⁻⁶

Interference with growth factor receptor activation and/or with intracellular growth factor-activated signal transduction pathways represents a promising strategy for the development of novel and selective anticancer therapies.⁷⁻⁸ Iressa (Gefitinib, ZD1839) is a quinazoline derivative, an orally active, selective EGFR tyrosine kinase inhibitor (TKI) that competitively binds ATP of EGFR and blocks signal transduction processes implicated in the proliferation, metastasis and angiogenesis of cancer cells. It has shown antiproliferative and anti-invasive effects in human GBM cells with amplified or transfected EGFR.⁹

F90, obtained as white powder, MW 547, is also a quinazoline derivative. Purity of the compound used in the present study was checked by high performance liquid chromatography and found to be greater than 98%. It is a novel EGFR-TKI, synthesized by Institute of Materia Medica, Chinese Academy of Medical Sciences (CAMS), which can be transformed to Iressa by incubation with lipase.

This study was undertaken to investigate the antitumor activity of F90 against human GBM cell line SHG-44 in vitro and in vivo.

METHODS

Drugs and reagents
F90 was provided by Institute of Materia Medica, Chinese Academy of Medical Sciences and incubated with lipase for 24 hours at 37°C before in vitro use. Iressa was purchased from AstraZeneca Pharmaceutical Co. Ltd.

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concentration (IC 50) was calculated from the linear regression curve. The 50% inhibitory concentration was calculated according to the following formula: growth inhibition (%) = (mean OD of the control wells×100%. The 50% inhibitory concentration was calculated from the linear regression curve.

The primary antibodies used included goat polyclonal antibodies against phosphorylated EGFR, rabbit monoclonal antibody against phosphorylated extracellular signal-regulated kinase 1 (ERK1), mouse monoclonal antibody against phosphorylated c-Jun NH (2)-terminal kinase (JNK), mouse monoclonal antibody against phosphorylated P38, mouse monoclonal antibody against P53, and mouse monoclonal antibody against B cell lymphoma/leukemia-2 (Bcl2) (Santa Cruz Biotech, Inc, USA). Secondary antibodies used were peroxidase-labeled antibodies against mouse, rabbit and goat (Promega, USA).

Cell line and animals
The human GBM SHG-44 cells were kindly provided by Pharmacology Department, Institute of Material Medical, Chinese Academy of Medical Sciences. Cells were cultured in DMEM supplemented with 10% heat-inactivated fetal bovine serum, penicillin (100 U/ml) and streptomycin (100 mg/ml) in a humidified atmosphere of 95% air and 5% CO2 at 37°C. Balb/c-nu mice (8 weeks old, weighing 18–22 g) were purchased from Institute of Laboratory Animal Science, Chinese Academy of Medical Sciences. The certificate number is SCXK Peking 2004-0001.

MTT assay
Briefly, cells were seeded on 96-well plates at a density of 1000 cells/well and allowed to attach to the plate overnight. Triplicate wells were treated with media or various concentrations of reagents (0.78–12.5 µmol/L). After another 96 hours incubated, the medium was removed and replaced by 100 µl of fresh medium containing 0.5 mg/ml MTT. After 4-hour incubation, the medium was discarded and the purple blue sediment was dissolved in 150 µl DMSO. The relative optical density (OD)/well was determined at a test wavelength of 570 nm in a WELLSCAN MK3 ELISA (Labsystems, Dragon, Finland) using a 450 nm reference filter. The growth inhibition was calculated according to the following formula: growth inhibition (%) = (mean OD of the control wells – mean OD of the treated wells)/mean OD of the control wells×100%. The 50% inhibitory concentration (IC50) was calculated from the linear equation, which was deduced using concentration versus growth inhibition regression curve.

Cell growth curve assay
SHG-44 cells were seeded on 6-well plates at a density of 1×10^4 cells/well with complete culture medium and allowed to adhere to the plate overnight. The cells were then incubated with various concentrations of drugs (1 and 10 µmol/L) for another 6 days at 37°C in 5% CO2 and performed in triplicate. Cell numbers per well were counted every day.

Tumor xenograft model
Nude mice (4–6 weeks old, BALB/c) were introduced to establish xenograft tumor models of SHG-44. Studies about the animal were conducted according to protocols approved by the Animal Ethics Committees of the Institute of Materia Medica, Chinese Academy of Medical Sciences. All in vivo animal studies described here were carried out in compliance with the standards for use of laboratory animals.

Exponentially growing 1×10^6 SHG-44 cells were suspended in 0.2 ml physiological saline and injected subcutaneously into the axilla of the upper limb of a nude mouse. After two passages, tumors were chopped into 3 mm × 3 mm × 3 mm pieces and implanted into axillary region of the other 40 new mice. When the diameter of the tumor reached between 8 mm and 10 mm, mice were randomized into 5 groups and drug treatment was initiated. F90 (25, 50, 100 mg·kg⁻¹·d⁻¹) and Iressa (50 mg·kg⁻¹·d⁻¹) were orally administrated for 6 days every week for 3 weeks. The control group received the vehicle in the same way. Tumor volume (length × width²/2) was measured twice a week with a caliper for up to the endpoint days. The relative tumor volume at day n (RTVn) versus day 0 was expressed according to the following formula: RTVn = TVn/TV0. Tumor regression (T/C (%)) in treated versus control mice was calculated using: T/C (%) = (mean RTV of treated group)/(mean RTV of control group) × 100%.

Western blot assay
Briefly, after treatment with various concentrations of drugs (F90 2.5, 5 and 10 µmol/L, Iressa 5 µmol/L) for 48 hours at 37°C in 5% CO2, cells were harvested and lysed on ice with lysis buffer. Protein concentrations were determined using the Bradford assay (Bio Rad, Hercules, CA, USA). The protein was denatured in boiled water for 5 minutes. Equal amounts of protein were separated on 8% or 10% SDS-polyacrylamide gel by electrophoresis and blotted onto polyvinylidene fluoride membrane using the SemiPhor Semi-Dry transfer unit (Hoefer Scientific Instruments, San Francisco, CA, USA). Membranes were blocked overnight at 4°C in blocking buffer and then immunoblotted with primary antibodies overnight at 4°C. The blots were then incubated with the appropriate secondary antibodies conjugated with horseradish peroxidase for 2 hours at room temperature. Detection was performed using the enhanced chemiluminescence plus Western blotting detection system (ECL Plus; Amersham Pharmacia Biotech, USA).

Statistical analysis
All experiments were carried out in triplicate. Pictures of the Western blot assay were analyzed using software.
Inhibitory effect of F90 on SHG-44 cell growth

MTT is a measure of mitochondrial dehydrogenase activity within the cell and thereby provides an indication of cellular proliferation. Cells were incubated with various concentrations of F90 or Iressa for 96 hours. Both drugs inhibited cellular proliferation in a dose-dependent manner. The percentage of growth inhibition was from 10% to 80% at concentrations of 0.78, 1.56, 3.13, 6.25 and 12.5 µmol/L of F90. The values of IC50 of F90 and Iressa were (5.08±1.21) and (3.32±0.82) µmol/L, respectively. The cell growth curve assay also showed that F90 inhibited cell growth in a dose-dependent manner. Compared with the positive drug Iressa, F90 had a similar inhibitory effect on SHG-44 cells and significantly inhibited the proliferation of the cells at a concentration of 10 µmol/L (Figure 1).

Antitumor activity of F90 on SHG-44 tumor in vivo

The antitumor effect of F90 in vivo was evaluated using the model of nude mice bearing SHG-44 tumor xenografts. Compared with the control group, the tumors growth was significantly inhibited when treated with 100 mg·kg-1·d-1 of F90 (P <0.05) with a percent tumor regression (T/C) of 48%. Iressa, at a dose level of 50 mg·kg-1·d-1, showed similar results with a T/C of 59% compared with controls (P <0.05). But F90, at low (25 mg·kg-1·d-1) and middle dose levels (50 mg·kg-1·d-1), did not show significant inhibitory effect against tumor growth (Figure 2).

F90 inhibited EGFR phosphorylation and downstream signaling activation of MAPK pathway, decreased protein expression of Bcl2 and increased the protein expression of P53

Effects of F90 on the phosphorylated EGFR and downstream phosphorylated proteins of MAPK pathway was examined by Western blot in our study. The phosphorylation of EGFR was suppressed in SHG-44 cells when incubated with 2.5, 5 and 10 µmol/L of F90 in vitro for 48 hours. The lower phosphorylated MAPK family including ERK, JNK and P38 were observed simultaneously. F90 showed similar effects with Iressa in the same condition. F90 also down-regulated the level of Bcl2 protein and up-regulated that of P53 protein in SHG-44 cells when treated for 48 hours (Figure 3).

DISCUSSION

EGFR gene amplification is frequently observed in highly malignant gliomas, especially in glioblastomas. EGFR amplification is frequently observed in glioblastomas. Elevated EGFR signaling has been correlated with poor prognosis, shorter interval to relapse, chemoresistance, and radioresistance. Therefore, inhibiting the EGFR-dependent signaling pathways appears to be a promising therapeutic approach for GBM. F90 is a new compound that targets the EGFR signaling pathway. Our study suggests that F90 is a potential inhibitor of GBM that is driven by EGFR.

In our study, a dramatic inhibitory effect of F90 on SHG-44 cell in vitro was observed by MTT assay and growth curve assay. Moreover, the nude mice xenografts model demonstrated that F90 at a high dose level (100 mg·kg-1·d-1) significantly inhibited the growth of tumors. But we also observed that F90, at both the low and middle dose levels, had no effect and this provides a guideline for us that the dosage should be paid more attention in further research and clinical treatment.

A major signal transduction pathway of EGFR is the MAPK pathway. It is correlated with proliferation in gliomas, and thus has a poor prognosis. An elevated level of MAPK activation in tumors compared with their corresponding non-neoplastic tissue has been reported in several human studies.

The downstream signaling members of the MAPK family, including ERK, JNK and P38, also play important roles in cell proliferation and death. Some evidence suggests that activation of the ERK-MAPK pathway increases the cell death threshold. Activation of the JNK and P38-
MAPK cascades has a cooperatively inducing anti-apoptotic effect. Iressa inhibits autophosphorylation of the tyrosine kinase thereby inhibiting the MAPK pathway, resulting in inhibition of cellular proliferation, angiogenesis, tumor invasion and metastasis. It also appears to increase apoptosis.

Western blot analysis was used to evaluate phosphorylated EGFR and downstream signaling proteins. Both F90 and Iressa are quinazoline derivatives and have similar mechanism to inhibit phosphorylation of EGFR selectively. After incubation with F90 in vitro for 48 hours, the phosphorylation of EGFR was suppressed and this blockade inhibited the expression and/or the activation of intracellular proteins that are involved in downstream signaling transduction of the MAPK pathway. Therefore, reduced phosphorylated ERK1, JNK, and P38 were observed in vitro. These observations correlated with the reduction in growth rate in SHG-44 cells.

Antiapoptotic protein Bcl2 and proapoptotic protein Bax regulate release of cytchrome C, which has a proapoptotic influence. Bax has been shown to contain p53-binding sites in its promoter site and is up-regulated in response to DNA damage. Reactive oxygen species (ROS) are powerful activators of mitochondrial damage and apoptosis. A number of genes that increase production of ROS have been found to be induced by p53. Huang et al.20 have reported that 125I can induce SHG-44 cells apoptosis in vitro and in vitro, which may be related to the inhibition of Bcl2 expression and promotion of p53. To further evaluate the inhibitory effects of F90, Bcl2 and p53 protein were analyzed in our study. In our Western blot assay, F90 down-regulated the level of Bcl2 protein and up-regulated that of P53 protein, which may be related with antitumor activity.

In conclusion, our study demonstrates that F90 inhibits the growth of SHG-44 cell and tumors xenografts in nude mice. These inhibitory effects may be related to the down-regulated phosphorylated EGFR and the activated downstream signaling proteins of the MAPK pathway. Induction of apoptosis may also be involved. F90 may become one of the new therapeutic options for treatment of GBM and future research into this potential clinical application is planned.

REFERENCES


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