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Original article

Anti-epidermal growth factor receptor tyrosine kinase activities of traditional Chinese medicine for cancer treatment

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Abstract

Introduction: The epidermal growth factor receptor (EGFR) is a validated target for different human malignancies. EGFR tyrosine kinase inhibitors considerably extend progression-free survival in EGFR-mutant non-small cell lung cancer. Monoclonal antibodies targeting EGFR also improve the efficacy outcomes in KRAS wild-type colorectal cancer. At present, gefitinib and erlotinib are considered the most representative of these types of drugs approved by the FDA for cancer treatment. However, only a few studies have screened the active compounds of traditional Chinese medicines.

Methods: In this study, the EGFR inhibitory activities of Chinese medicinal herb extracts traditionally prescribed to treat cancer were determined in a 384-well plate-based assay using the homogeneous time-resolved fluoroimmunoassay method. Petroleum ether, ethanol, ethyl acetate, and aqueous extracts of 49 traditional Chinese medicinal herbs (183 extracts total) were tested. The inhibitory effects were expressed as percentage of inhibition. Dose-dependent inhibitory assays were also performed for the herbal extracts that exhibited significant inhibition.

Results: The ethyl acetate extracts of *Artemisia argyi*, *Lonicera macranthoides*, *Spatholobus suberectus*, *Curcuma longa*, and *Galla chinensis*, as well as the ethanol extract of *Eriobotrya japonica* exhibited significant EGFR inhibitory activities at a concentration of 50 μg/ml. The IC_{50} values were 8.848, 2.027, 2.277, 3.621, 4.339 and 5.528 μg/mL, respectively. The EA extracts of *L. macranthoides*, *S. suberectus* had the best activities.

Conclusion: Our results indicate that screening traditional Chinese herbs for EGFR inhibitory activity may identify a large number of potential lead compounds for the development of new drugs to treat cancer.

Keywords: Cancer; Epidermal growth factor receptor; Tyrosine kinase inhibitors; Homogeneous time-resolved fluoroimmunoassay; Traditional Chinese medicine

Introduction

Cancer is the leading cause of mortality in economically developed countries and the second-leading cause of death in developing countries [1]. Because of the aging population and economic growth in developing countries, the burden of cancer is growing. Drug resistance is increasing, as are the unpleasant side effects from the cytotoxic compounds used for cancer therapy. Therefore, there is an urgent need to identify new effective drugs.

Natural products from plants have been fertile ground for the identification of novel drugs. For example, the vinca alkaloids from *Catharanthus roseus*, the DNA topoisomerase I inhibitor camptothecin from *Camptotheca acuminate*, the terpene paclitaxel from *Taxus brevifolia* and the lignan podophyllotoxin isolated from *Podophyllum peltatum* are all important antitumour drugs [2].

Several drug screening models are used to identify potential antitumour drugs. The epidermal growth factor receptor (EGFR) is the founding member of a family of four ErbB receptor tyrosine kinases (TKs), EGFR/(human epidermal growth factor receptor 1 [HER1]/ErbB1), HER2/ErbB2, HER3/ErbB3, and HER4/ErbB4. EGFR has been the focus of intense research efforts during the last two decades [3] because it can induce cells into continuous and uncontrolled division, which increases

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the production of malignant cells and increases tumour size [4]. Therefore, EGFR has become an important target for screening anti-tumour inhibitors [5–7]. These drugs, such as erlotinib and gefitinib, can inhibit the TK domain of EGFR and are becoming the most successful treatments for progression-free survival [8–10].

Traditional Chinese medicine commands a leading position among traditional medicines because of the enormous variety of drugs from plants and because it is founded on more than thousands of years of tradition. It can be concluded that searching for bioactive plant compounds in medicinal plants is a more successful strategy than screening all plant species. Therefore, herbal medicines may be a good source of EGFR inhibitors [11–13]. In this study, a total 183 traditional Chinese medicinal herb extracts, from 49 traditional Chinese medicines, were screened. The petroleum ether, ethanol, ethyl acetate, and aqueous extracts were tested for inhibitory activity using a homogeneous time-resolved fluoroimmunoassay (HTRF) [14–19].

Materials and methods

Plant materials

All herbal medicines were collected from Si chuan and Yunnan provinces by Prof. Linfang Huang. All the herbal medicines were identified by Prof. Yulin Li who majored in plant classification. The voucher specimens were deposited at the herbarium of the Institute of Medicinal Plant Development, Chinese Academy of Medical Sciences.

Plant extraction

The medicinal plant material was crushed into a coarse powder, 500 g of which was soaked in petroleum ether for 24 h. Percolation extraction was performed to extract the light colour. The filter was reclaimed and the petroleum ether extract was obtained by evaporation. The residue was washed with 80% ethanol and subjected twice to reflux extraction with three times the amount of 80% ethanol. The extract solutions were then combined and the ethanol was reclaimed at reduced pressure until no alcohol was detected. Extraction was performed two times using an equal volume of ethyl acetate, and then the upper solution was separated and concentrated to obtain the ethyl acetate extract. The lower solution was concentrated to dryness to yield the ethanol extract. The residue was evaporated to dryness and extracted two times with water with three times the amount of the materials. The aqueous extract solutions were combined and concentrated to dryness, and the water extract was then obtained.

Chemicals and reagents

TK Substrate-biotin, Streptavidin-XL665, TK Antibody-Cryptate, Supplement. Enzymatic buffer (SEB reagent), 5× Enzymatic buffer (HEPES 250 mM (pH 7.0), NaN3 0.1%, BSA 0.05%, Orthovanadate 0.5 mM), HTRF® Detection buffer (HEPES 50 mM (pH 7.0) with additives.

EGFR (Invitrogen, USA); HTRF KinEASE-TK (Cisbio, USA); ATP (Sigma, #A7699), $MgCl_2$ (Sigma, #M1028), $MnCl_2$ (Sigma, #M1787), DTT (Sigma, #D0632), positive drugs: Non-selective inhibitior of EGFR-Staurosporine (Shanghai YantuoBiotechnology Co., Ltd.).

HTRF assay and compound screening for EGFR inhibitory activity

All extracts were dissolved in kinase buffer and tested for EGFR inhibitory activity using HTRF. The HTRF was performed in 384-well plates (Corning, USA). Briefly, the extract solution was added to a well, and initially, 10 μL EGFR was preactivated by incubation with EGF for 10 min at room temperature in 60 mM Tris-Mes (pH 7.4) buffer immediately before use. Then, 10 μL biotinylated poly-Glu-Ala-Tyr (final concentration 1 μM) and 10 μL Tris-Mes buffer containing 6 mM Mn^2+ and 30 mM Mg^2+ was added. Finally, after an incubation of 30 min at room temperature, 50 μL phosphate buffer (pH 7.0) containing XL665-labeled streptavidin (2 mg), EuK labelled antiphosphotyrosine antibody (EuK–Ab; 25 ng) and 400 mM KF was added. The fluorescence was measured with a plate reader for one second per well after a1 h incubation at room temperature. The KF was added to the HTRF assays to increase the fluorescence signal by reducing Eu^{3+} non-radiative deactivation. The fluorescence was measured at 620 nm (Cryptate) and 665 nm (XL665). The 665/620 ratio was calculated for each well [20–22], and the optical density was inversely proportional to the inhibitory activity. The percentage inhibitions were calculated relative to a DMSO control. The data analysis was performed using the Beckman Coulter detection platforms. The inhibitory effects are expressed as the percentage of inhibition. The screening experiments were repeated at least twice. For the herbal extracts that exhibited significant inhibition, dose-dependent inhibitory assays were then performed.

$$\text{Inh}\% = \frac{100\% \quad \text{Activity} - \text{Sample}}{100\% \text{ Activity} - 0\% \text{ Activity}} \times 100\%$$

Analysis the main active components of the six extracts

Chromatography was performed on an ACQUITY UPLC system (Waters Corp., Milford, MA, USA) with a conditioned autosampler at 4 °C. The separation was carried out on an ACQUITY UPLC™ BEH C_{18} column (100 mm × 2.1 mm i.d, 1. 7 μm; Waters Corp., Milford, MA, USA). The column temperature was maintained at 34 °C. The analysis was achieved with gradient elution using (A) water (containing 0. 04% formic acid) and (B) acetonitrile as the mobile phase. The injection volume was 4 μL.

For the UPLC–ESI-MS analysis, the optimal conditions were as follows: capillary voltage of 2400 V, desolvation temperature of 240 °C, sample cone voltage of 34 V, extraction cone voltage of 4.0 V, microchannel plate voltage of 1600 V, collision energy of 4 eV, source temperature of 120 °C, cone gas flow of 40 L/h and desolvation gas flow of 800 L/h for positive ion mode; A "purge-wash-purge" cycle was employed on the