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Erlotinib inhibits T-cell-mediated immune response via down-regulation of the c-Raf/ERK cascade and Akt signaling pathway ☆

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ABSTRACT

Erlotinib is a potent inhibitor of epidermal growth factor receptor tyrosine kinase and has been demonstrated to treat advanced or metastatic non-small cell lung cancer to prolong survival after failure of first-line or second-line chemotherapy. However, little is known about its effects on immune system. In the present study, we aimed to investigate the immunosuppressive activity of erlotinib on T lymphocytes both in vitro and in vivo, and further explore its potential molecular mechanism. Erlotinib exerted a significant inhibition on the T cell proliferation and activation induced by concanavalin A, anti-CD3 plus anti-CD28, staphylococcal enterotoxin B or phorbol myristate acetate respectively in a concentration-dependent manner and it also inhibited the secretion of the proinflammatory cytokines such as IL-2 and IFN-γ of activated T cells. Further study showed that erlotinib caused G0/G1 arrest and suppressed the phosphorylations of c-Raf, ERK and Akt in activated T cells. Moreover, erlotinib significantly ameliorated picryl chloride-induced ear contact dermatitis in a dose-dependent manner in vivo. In summary, these findings suggest that erlotinib may cause the impairment of T-cell-mediated immune response both in vitro and in vivo through inhibiting T cell proliferation and activation, which is closely associated with its potent down-regulation of the c-Raf/ERK cascade and Akt signaling pathway.

Introduction

Epidermal growth factor receptor (EGFR) is a tyrosine kinase receptor which plays an essential role in normal cell growth and differentiation, and is involved in tumor proliferation and survival. EGFR over-expression is a common feature in many human solid malignancies including non-small-cell lung cancer, and is associated with poor clinical prognosis (Onn et al., 2004; Gridelli et al., 2007). Erlotinib (Tarceva) is an oral available, selective, reversible inhibitor of EGFR tyrosine kinase (Akita and Sliwkowski, 2003; Perez-Soler, 2004). In vitro and in vivo studies show that erlotinib has activity against human colorectal, head and neck, non-small cell lung, and pancreatic tumor cells (Akita and Sliwkowski, 2003). The antitumor activity of erlotinib as a single agent and in combination with other targeted agents has been demonstrated in Phase II trials in many tumor types (Tang et al., 2006). Erlotinib has an established role in the treatment of locally advanced or metastatic non-small cell lung cancer after failure of at least one prior chemotherapy regimen (Comis, 2005; Wong et al., 2009). In a Phase III trial, the addition of erlotinib to gemcitabine improves survival in advanced pancreatic cancer (Tang et al., 2006; Bareschino et al., 2007). Encouraging indications of antitumor activity are also reported in several phase II studies including monotherapy use of erlotinib in advanced head and neck cancer, colorectal, hepatocellular, biliary, gastroesophageal, and ovarian cancer (Bareschino et al., 2007; Ciardiello and Tortora, 2008). The adverse effect of erlotinib is reported to be immune-mediated toxicity (Li et al., 2010) including drug-induced hepatitis (Liu et al., 2007b; Saif, 2008; Pellegrinotti et al., 2009), interstitial lung disease (Liu et al., 2007a; Makris et al., 2007), Stevens-Johnson syndrome and toxic epidermal necrolysis (Chou et al., 2006; Lubbe et al., 2008; Bovenschen and Alkemade, 2009), but the mechanism of toxicity has not been elucidated.

Erlotinib is ~60% absorbed after oral administration and the maximal tolerated dose on a protracted daily schedule is 150 mg/daily (Bareschino et al., 2007; Gridelli et al., 2007). Although the pharmacodynamics and pharmacokinetics of erlotinib in both healthy volunteers and adult patients with cancer have been well-characterized (Hidalgo and Bloedow, 2003; Ling et al., 2006), little is known about the effect of erlotinib on immune system. The aim of this study is to investigate whether erlotinib affects T-cell-mediated immune response. Here we showed that erlotinib inhibited T-cell-mediated immune response in vivo and in vitro, which demonstrated its

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immunosuppressive activity was proved to be associated with its inhibition of c-Raf/ERK cascade and Akt signaling in T cells.

Materials and methods

Mice. Specific pathogen-free, 8- to 10-week-old female BALB/c mice were purchased from Model Animal Genetics Research Center of Nanjing University (Nanjing, China). Animal welfare and experimental procedures were carried out strictly in accordance with the Guide for the Care and Use of Laboratory Animals (National Institutes of Health, the United States) and the related ethical regulations of our university. All efforts were made to minimize animals' suffering and to reduce the number of animals used.

Cells and reagents. Mouse T cells from splenocytes or lymph node cells were purified using magnetic beads (Miltenyi Biotec, Auburn, CA) with more than 95% purity. The cells were incubated in RPMI 1640 medium supplemented with 100 U/ml of penicillin, 100 μg/ml of streptomycin and 10% fetal calf serum under a humidified 5% (v/v) CO_2 atmosphere at 37 °C. Erlotinib (Tarceva, OSI Pharmaceuticals) was dissolved in dimethyl sulfoxide to a concentration of 15 mM as stock solution, which was stored at −20 °C until use. Concanavalin A (Con A), phorbol myristate acetate (PMA), ionomycin, 3-(4, 5-dimethyl-2-thiazyl)-2, 5-diphenyl-2 H-tetrazolium bromide (MTT), carboxyfluorescein diacetate succinimidyl ester (CFSE) and staphylococcal enterotoxin B (SEB) were purchased from Sigma Chemical Co. (St. Louis, MO). Picryl chloride was purchased from Wako Pure Chemical Industries (Osaka, Japan). Purified anti-mouse CD3 (145-2C11) and purified anti-mouse CD28 (37.51) were purchased from BD PharMingen (San Diego, CA). Annexin V-FITC/PI kit was purchased from BD Biosciences (San Jose, CA). ELISA kits for interferon-γ (IFN-γ) and interleukin-2 (IL-2) were purchased from R&D Systems (Minneapolis, MN). FITC-anti-mouse CD69 mAb and FITC-anti-mouse CD25 were purchased from Biolegend (San Diego, CA). PE-cy5 conjugated anti-mouse CD3e antibody was purchased from eBioscience (San Diego, CA). Antibodies against phospho-c-Raf (Ser259), phospho-c-Raf (Ser338), c-Raf, ERK, phospho-ERK, phospho-Akt, Akt, CDK4, phospho-retinoblastoma protein (Rb) and $p27^{kip1}$ were purchased from Cell Signal Technology (Beverly, MA). Antibody against GAPDH was purchased from Santa Cruz Biotechnology (Santa Cruz, CA). All other chemicals were purchased from Sigma Chemical Co. (St. Louis, MO).

Cell proliferation assay. Cells were cultured in 96-well plates at a density of 3×10^5 cells/well in RPMI 1640 medium (0.2 ml) and stimulated with 5 μg/ml of Con A for 72 h at 37 °C in 5% CO_2/air. Then cell growth was evaluated with modified MTT assay. In some cases, cell proliferation was also determined by CFSE assay as we previously reported (Sun et al., 2010).

Cell apoptosis assay. Cell apoptosis was determined by Annexin V-FITC (fluorescein isothiocyanate)/PI (propidium iodide) staining as previously reported (Sun et al., 2009). Samples were analyzed by FACSCalibur flow cytometer.

Measurement of CD25 and CD69 expressions with flow cytometry. The expressions of cell surface molecules in T cell cultures were evaluated by flow cytometry. Lymph node cells (5×10^5) were stimulated with 5 μg/ml Con A with the addition of erlotinib simultaneously. The surface expressions of CD69 and CD25 were assessed after 24 h of culture, respectively. At the end of the culture period, the harvested cells were washed twice with buffer. Cells were stained with specific antibodies for 30 min at 4 °C in the dark. Cells were then washed with buffer to remove the excess stains and analyzed in a FACSCalibur flow cytometer (Becton Dickinson, San Jose, CA) using CellQuest software.

Cytokine assay. Cytokines (IFN-γ, IL-2) were determined using ELISA kits from R&D systems (Minneapolis, MN).

Cell cycle assay. T cells from lymph nodes of BALB/c mice were treated with or without erlotinib for 24 h in the presence of 5 μg/ml Con A, and then collected and washed with cold PBS and fixed with 70% ethanol at 4 °C overnight. Then, the fixed cells were washed with PBS and stained with 50 μg/ml of propidium iodide (PI) containing 100 μg/ml of RNase A and 1% TritonX-100 in the dark at room temperature for 45 min. The DNA contents of the cells were analyzed with Modfit software (Becton Dickinson, San Jose, CA, USA).

Western blot. Proteins were extracted in lysis buffer (30 mmol/L Tris, pH 7.5, 150 mmol/L sodium chloride, 1 mmol/L phenylmethylsulfonyl fluoride, 1 mmol/L sodium orthovanadate, 1% Nonidet P-40, 10% glycerol, and phosphatase and protease inhibitors). The proteins were then separated by SDS-PAGE and electrophoretically transferred onto polyvinylidene fluoride membranes. The membranes were probed with antibodies overnight at 4 °C, and then incubated with a horse radish peroxidase-coupled secondary antibody. Detection was performed using a LumiGLO chemiluminescent substrate system (KPL, Guildford, UK).

Picryl chloride-induced contact hypersensitivity. Female BALB/c mice were sensitized by painting 0.1 ml of 1% picryl chloride (Wako, Japan) in ethanol on the shaved skin of their abdomens. Five days later, they were challenged by painting 30 μl of 1% picryl chloride in olive oil on right ear lobe. Eighteen hours later, ear thickness of right against left was measured with a digimatic micrometer (0.001 mm, Mitutoyo Co., Tokyo, Japan). The control animals were run parallel with other groups except for intragastric administration (i.g.) the same volume of water.

Histological analysis. Formalin-fixed, paraffin-embedded ear tissue was sectioned at 5 mm in thickness, and the sections were stained with hematoxylin and eosin. Following parameters were assessed: 1) the level of leucocyte infiltration and vascular congestion; 2) the erosion and anabrosis of epidermal cells; 3) the affection of other side of the ears. The histological scores were assessed from 1 to 4. Final data are the average scores of each animal in the same group, and the higher score means more serious inflammation.

Statistical analysis. All experiments were repeated three to five times with the similar outcome. The *P*-values between two experimental groups were tested by two-tailed Student's *t*-test. The level of significance was set at a *P*-value of 0.05. Where applicable, data were reported as the mean ± SEM.

Results

Erlotinib inhibited the proliferation of T cells in a dose-dependent manner

The maximum plasma concentration was about 1.4 μg/ml (≈3.5 μM), which was achieved approximately 2–4 h after oral administration of 150 mg/daily erlotinib (Hamilton et al., 2006; Prados et al., 2006). In the present study, we focused whether erlotinib affects T-cell-mediated immune response when it is used in clinic for a long time. And it was reported that when orally administrated 10 mg/kg of erlotinib daily for 20 days in human HN5 head and neck carcinoma xenografts athymic mice the blood drug level reached a peak at about 8 μM by a high performance liquid chromatography assay (Pollack et al., 1999). In that case we selected the range of the drug concentrations (1–20 μM) in vitro and 10, 20 and 40 mg/kg of erlotinib in vivo which just fell within the range of the drug concentrations (1–20 μM) in vitro.

Erlotinib significantly inhibited T cell proliferation induced by Con A (Fig. 1A) or anti-CD3 plus anti-CD28 (Fig. 1B) in a concentration-

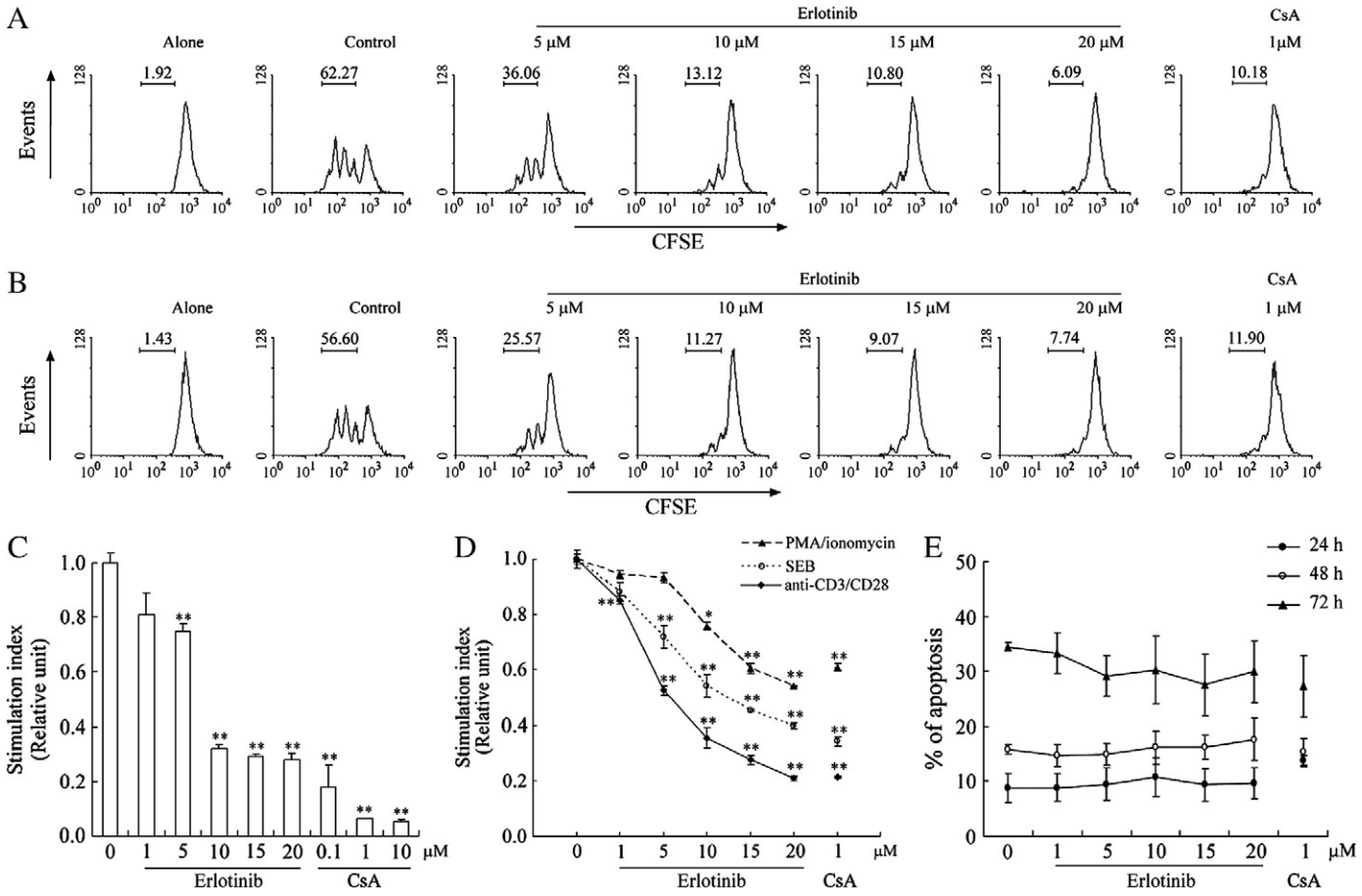

Fig. 1. Erlotinib significantly inhibited T cell proliferation. (A) Lymph node cells was labeled with 2.5 μM CFSE, and pre-incubated with or without erlotinib for 1 h. Then, they were stimulated with Con A (5 μg/ml) for 72 h. The numbers gated indicated the dividing $CD3^+$ T cell population. Data shown here are one of three different experiments with similar results. (B) Lymph node cells was labeled with 2.5 μM CFSE, and pre-incubated with or without erlotinib for 1 h. Then, they were stimulated with anti-CD3 (1 μg/ml) plus anti-CD28 (10 μg/ml) for 72 h. Data shown here are one of three different experiments with similar results. (C) Lymph node cells (5×10^5) were incubated for 72 h at 37 °C and 5% CO_2 in the presence of 5 μg/ml Con A and 1–20 μM erlotinib. Cell proliferation was measured at 540 nm by MTT uptake assay. $^*p<0.05$, $^{**}p<0.01$ versus drug-untreated group. (D) Lymph node cells were prepared from naive mice and T cells were purified using commercial enrichment columns. T cells were stimulated with anti-CD3 (1 μg/ml) plus anti-CD28 (10 μg/ml) or SEB (10 μg/ml) or PMA (50 ng/ml) plus inomycin (0.5 μg/ml) in the presence of 1–20 μM erlotinib for 72 h, then cell proliferation was measured at 540 nm by MTT uptake assay. $^*p<0.05$, $^{**}p<0.01$ versus drug-untreated group. (E) Lymph node cells (5×10^5) were incubated from 24 h to 72 h at 37 °C and 5% CO_2 in the presence of 1–20 μM erlotinib. Cell apoptosis was measured by Annexin V/PI assay. Results were represented as the mean ± SEM of three experiments. CsA, cyclosporin A; SEB, staphylococcal enterotoxin B; PMA, phorbol myristate acetate.

dependent manner by CFSE assay. The similar result was also seen in Con A-activated T cells (Fig. 1C) and anti-CD3 plus anti-CD28-, SEB- or PMA/ionomycin-activated T cells (Fig. 1D) by MTT assay. It was important to notice that erlotinib, at the concentrations mentioned above, did not affect lymphocyte's viability by MTT uptake assay (Supplemental Fig. 1) or hardly induced apoptosis of lymphocyte by Annexin V/PI binding assay (Fig. 1E). All these results indicated that the immunosuppressive activity of erlotinib observed here, at the concentrations up to 20 μM, was not due to its cytotoxicity. It should be noticed that immunosuppressant cyclosporine A (CsA) developed at the end of the 1970s, which showed selective inhibition dominantly on T lymphocytes rather than other cell types and was widely used for the treatment of various autoimmune diseases, was used as a positive control here.

Erlotinib suppressed the expressions of CD69 and CD25 in activated T cells

CD69 and CD25 induction can be triggered by Con A which acts as a T cell mitogen to interact with the T cell receptor (TCR)/CD3 complex in T cells. It was shown that CD69 and CD25 cell surface expressions were up-regulated in mouse T cells after 24 h incubation with 5 μg/ml Con A, while erlotinib-mediated a potent inhibitory effect on CD69 and CD25 expressions in Con A-treated mouse T cells in a dose-dependent manner (1–15 μM) (Fig. 2A).

Erlotinib reduced the production of proinflammatory cytokines in activated T cells

To examine the effect of erlotinib on the production of proinflammatory cytokines such as IL-2 and IFN-γ, ELISA was performed to measure the level of the cytokines in the culture supernatant of activated T cells. Our results showed that the stimulation of mouse T cells with Con A resulted in the considerable production and secretion of IL-2 and IFN-γ into the culture medium. Significant reduction of these proinflammatory cytokines was found in activated T cells with the treatment of 1–20 μM erlotinib (Fig. 2B and C).

Erlotinib caused G0/G1 phase arrest in activated T cells

As shown in Fig. 3A and B, Con A (5 μg/ml) stimulation resulted in the progression into the S and G2/M phase and notable DNA synthesis. With flow cytometry analysis, the propidium iodide-stained cells showed an increased arrest in the G0/G1 phase of the cell cycle following 1–15 μM erlotinib treatment. Con A stimulated 19.0% of T cells to S and G2/M phase and this progression was efficiently reduced by 18.1, 16.5, 14.3 and 13.8% ($p<0.05$) with to the addition of erlotinib at 1, 5, 10, 15 μM, respectively. The influence of erlotinib on the protein levels of phosphorylated Rb, CDK4 and $p27^{kip1}$ in purified T cells were further investigated. As shown in Fig. 3C, consistent with

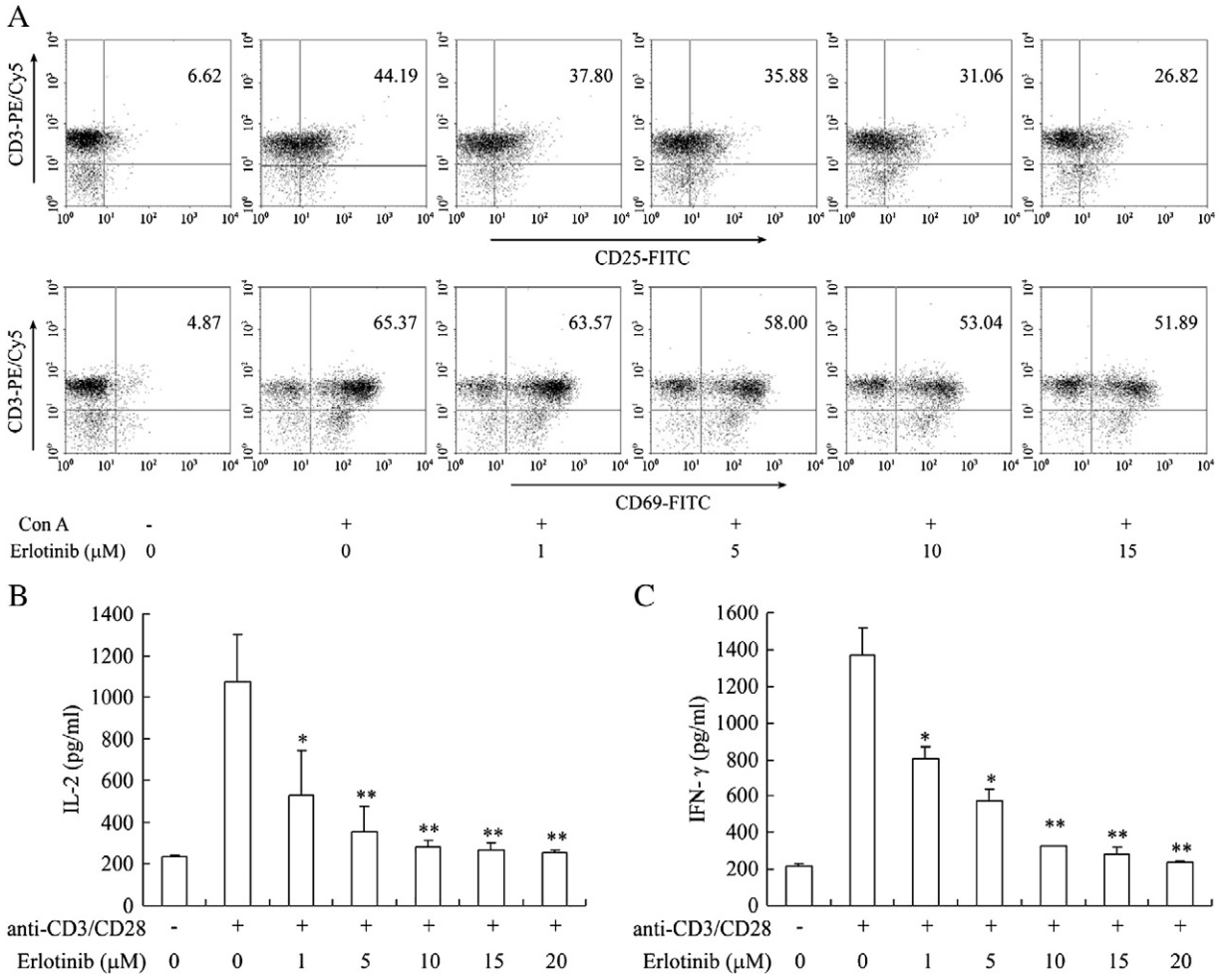

Fig. 2. Erlotinib inhibited T cell activation and proinflammatory cytokine production in T cells. (A) Lymph node cells (5×10^5) were stimulated with 5 μg/ml Con A for 24 h, then they were collected and analyzed by flow cytometry. Data shown here are one of three different experiments with similar results. (B, C) Lymph node cells were prepared from naive mice and T cells were purified using commercial enrichment columns. T cells were stimulated with anti-CD3 (1 μg/ml) plus anti-CD28 (10 μg/ml) in the presence of 1–20 μM erlotinib for 72 h, then the productions of IL-2 (B) and IFN-γ (C) were measured by ELISA assay. $^*p<0.05$, $^{**}p<0.01$ versus drug-untreated group.

the observations on cell cycle distribution, erlotinib dramatically inhibited the increase of phosphorylated Rb and CDK4. Meanwhile, 1 and 5 μM of erlotinib slightly increased the amount of $p27^{kip1}$ compared with drug-untreated group but higher concentrations of erlotinib hardly affect the expression of $p27^{kip1}$. These results suggest CDK4 but not p27 is the major target of erlotinib in cell cycle arrest of activated T cells. The results showed that erlotinib influenced the cell cycle regulatory molecules of G1 phase and blocked cell cycle progression through G1/S transition.

Erlotinib inhibited both Raf-stimulated ERK signaling and Akt signaling

It is known that the Ras/Raf/ERK (extracellular-signal-regulated kinase) pathway and PI3K/Akt pathway are at the heart of signaling networks that govern proliferation, differentiation and cell survival. To further delineate the molecular mechanisms of erlotinib against T-cell-mediated immune response, the effects on Raf/ERK and Akt signaling pathway were investigated. As shown in Fig. 4, erlotinib dose-dependently inhibited the phosphorylations of c-Raf, ERK, and Akt in Con A-activated T cells.

Erlotinib ameliorated picryl chloride-induced contact hypersensitivity in mice

To further assess the immunosuppressive property of erlotinib in vivo, we used the picryl chloride-induced contact dermatitis in BALB/c mice. Previously, Pollack et al. reported that when orally administrated 10 mg/kg of erlotinib daily for 20 days in human HN5 head and neck carcinoma xenografts athymic mice the blood drug level reached a peak at about 8 μM by a high performance liquid chromatography assay (Pollack et al., 1999), which just fell within the range of the drug concentrations (1–20 μM) in vitro. Together with the result of our pre-experiment, 10, 20 and 40 mg/kg of erlotinib were selected for in vivo experiments. Administration for 6 days after the sensitization, erlotinib significantly inhibited the ear swelling in a dose-dependent manner, and the positive control CsA also showed a strong inhibition (Fig. 5A). Fig. 5B was a representative photo of H&E staining for ear tissues from various groups of mice. The histopathological changes in the ear were mainly observed in the dermis as severe inflammatory infiltration, vascular congestion, and moderate edema in the control group. Compared with the animals in the control group, the mice treated with erlotinib only showed a mild cellular infiltration and vasodilatation without obvious edema (Fig. 5B and C).

Discussion

Recently, molecular targeted therapeutics provides a different mechanism of action from chemotherapy and can be much more specific in their approach to cancer treatment (Comis, 2005). This therapeutics has the potential to maximize therapeutic benefit while minimizing toxicity to normal cells. Some protein tyrosine kinases are such targets, which are involved in different cellular functions including

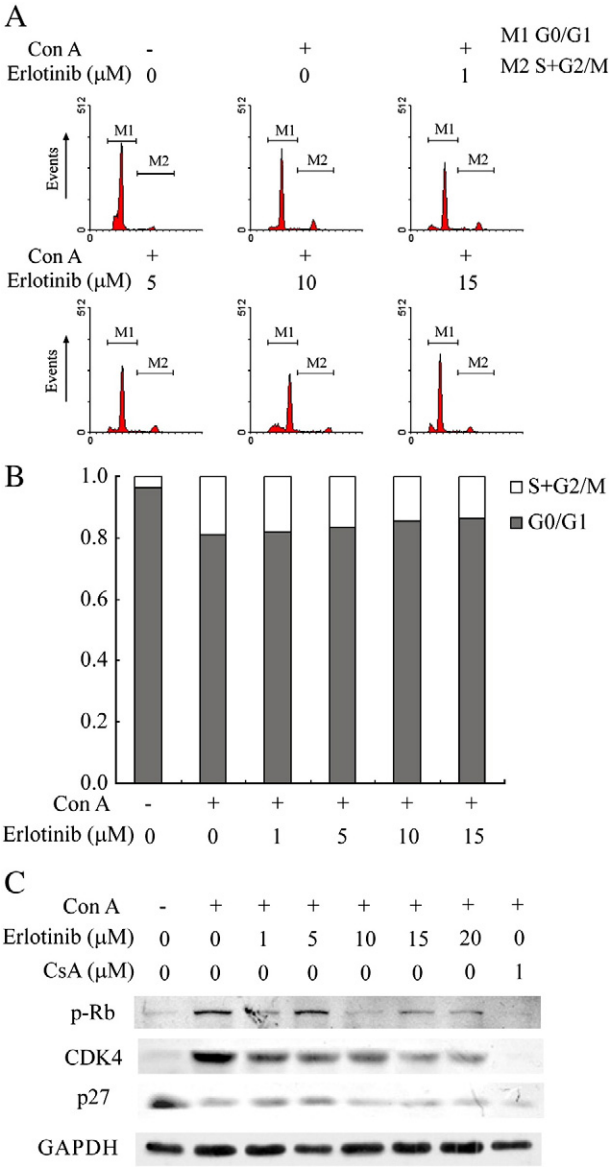

Fig. 3. Erlotinib caused T cell arrest in G0/G1 phase of the cell cycle. (A) Lymph node cells were prepared from naive mice and T cells were purified using commercial enrichment columns. T cells were stimulated with 5 μg/ml Con A for 24 h in the absence or presence of 1–15 μM erlotinib, then the cells were stained with propidium iodide (PI) for cell cycle distribution analysis in flow cytometry. (B) Data shown here are one of three different experiments with similar results. (C) T cells were stimulated by 5 μg/ml Con A with or without erlotinib for 24 h. Whole cell extracts were obtained and the expressions of cell cycle regulators were analyzed by Western blot. Data shown here are one of three different experiments with similar results.

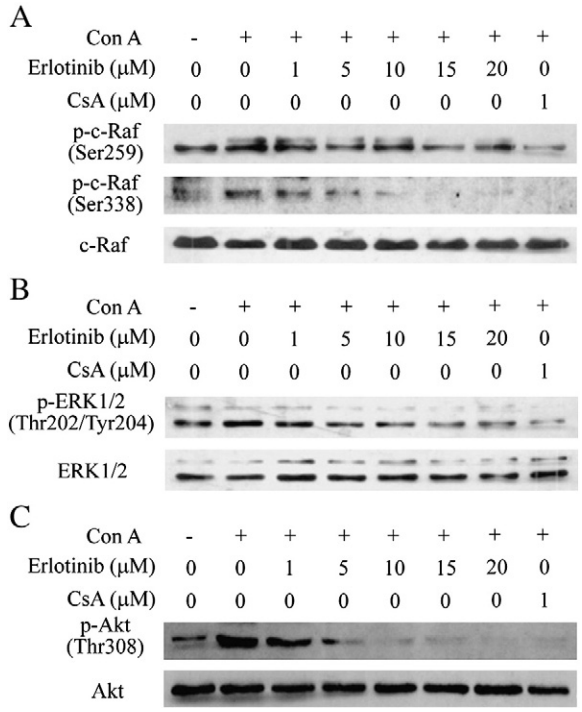

Fig. 4. Erlotinib inhibited the activation of ERK and Akt in Con A-activated T cells. Lymph node cells were prepared from naive mice and T cells were purified using commercial enrichment columns. T cells were stimulated with 5 μg/ml Con A in the absence or presence of erlotinib for 24 h. Then cells were harvested and the whole cell extracts were analyzed by Western blot for the phosphorylations of c-Raf (A), ERK1/2 (B) and Akt (C). Data shown here are one of three different experiments with similar results.

extracellular signaling, intracellular communication, proliferation, cell cycle or apoptosis/survival regulation (Ocana et al., 2009). As most of them manage physiologic functions, their dysregulation causes an oncogenic phenotype (Blume-Jensen and Hunter, 2001; Hynes and Lane, 2005). A clear example is the oncogenic role of the EGFR family in different tumor types (Hynes and Lane, 2005). The EGFR family is part of a complex signal-transduction network that is central to several critical cellular processes. Since EGFR is often found in non-small cell lung cancer cells, it has been considered as a target for new drug candidates. Erlotinib (Tarceva) is one of the most important anti-cancer drugs developed to target EGFR tyrosine kinase (Shepherd et al., 2005; Siegel-Lakhai et al., 2005). Erlotinib has been demonstrated to be efficient in the treatment of patients with the advanced or metastatic non-small cell lung cancer (Tiseo et al., 2009), to prolong survival after first-line or second-line chemotherapy.

Despite this large amount of data, little is known about the effect of erlotinib on immune system. Usually, anti-cancer drugs are known to cause severe adverse effects such as immune system damage, which constrains their use in clinic. Therefore, it is urgent to investigate whether erlotinib affects immune system especially T cells. In the present study, we examined the effect of erlotinib on three critical parameters of T cell response: proliferation, activation and cytokine production. As the results, all of these events were inhibited by erlotinib, suggesting that erlotinib may induce impairment of T cell response and cause some adverse effects in its clinical practice. Importantly, when examined the relationship between the erlotinib-induced inhibition of T cell proliferation and the induction of apoptosis, we found the high dose of erlotinib (20 μM), which almost completely inhibited T cell proliferation, could not induce apoptosis. These findings suggest the impairment of T cell response by erlotinib is not caused through inducing apoptosis.

To elucidate some of the underlying mechanisms of erlotinib against T cell proliferation, we further examined the effect of erlotinib on cell cycle of T cells. PI staining data indicated that erlotinib inhibited T cell cycle progression, and we found that T cell proliferation was generally controlled by erlotinib at the G0/G1 phases. Cyclin-dependent kinase (CDK) and CDK inhibitors are the key regulators of cell cycle transitions. In mammalian cells, CDK4 and associated CDK inhibitor $p27^{kip1}$ control the G1 to S phase transition (Lea et al., 2003). A requirement of entry into the S phase is the phosphorylation of Rb, which is regulated by the cyclin D3/CDK4/CDK6 complex. Cell progression through G1 to S transition was markedly inhibited by erlotinib, which was correlated with an erlotinib-mediated reduction of phosphorylations of CDK4 and Rb. Previously, Ling et al. had reported that induction of $p27^{kip1}$ was required for G1 arrest and cell growth inhibition by erlotinib in human non-small cell lung cancer cells (Ling et al., 2007). In this study, we also found erlotinib increased the expression of CDK inhibitor $p27^{kip1}$ in activated T cells. These

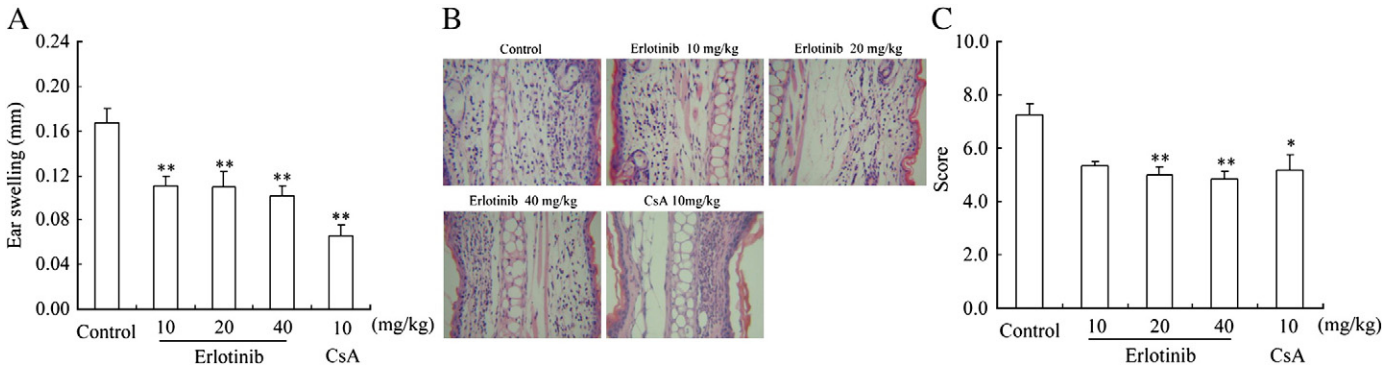

Fig. 5. Erlotinib suppressed picryl chloride-induced contact hypersensitivity in mice. (A) Ear swelling was evaluated by the difference in thickness between the right and left ears 24 h after challenge. Mice were administered i.g. with erlotinib every day during the experiment. Each column represents the mean ± SEM of 10 animals. $^{*}p<0.05$, $^{**}p<0.01$ versus Control. (B) Ear tissues were stained with hematoxylin and eosin (original magnification × 200). (C) The pathological scoring was carried out by using a range from 0 (no change) to 4 (most severe) to evaluate congestion, edema and inflammatory cell infiltration and so on. $^{*}p<0.05$, $^{**}p<0.01$ versus Control.

findings suggested that erlotinib remarkably inhibited T cell cycle progression in vitro.

The epidermal growth factor receptor (EGFR) is a member of the ErbB family of receptors, a subfamily of four closely related receptor tyrosine kinases (RTKs). On binding ligands, RTKs dimerize, activate Ras-GTPase and phosphatidylinositol-3-OH kinase (PI3K). Each of these proteins then activates a number of downstream effectors such as Raf and Akt (McCubrey et al., 2006). The Raf/MEK/ERK signaling pathway is an important mediator of tumor cell proliferation (Gollob et al., 2006). And this protein kinase cascade is also essential for T cell receptor function, cell cycle progression and cytokine production in T lymphocytes (Aktas et al., 1997; Czyzyk et al., 2008; Li et al., 2008). Ras and its effectors, particularly the Raf-ERK pathway, are activated within minutes after stimulation of T cell receptor and continue to be essential for T cell activation (Li et al., 2008). Similarly, the phosphoinositide 3-kinases/Akt pathway can mediate diverse biological responses and is crucial for optimal immune responses and lymphocyte development (Xue et al., 2008; Zhang et al., 2008). The effects of erlotinib on cell activation might be explained by the inhibition of early signaling events, mediated by the receptor tyrosine kinase. In fact, we found that erlotinib markedly reduced the phosphorylations of c-Raf, ERK and Akt in activated T cells, suggesting the inhibition of c-Raf/ERK cascade and Akt signaling pathway are involved in the impairment of T cell response by erlotinib. Using flow cytometric assay, we found that EGFR was hardly expressed on the surface of naïve or activated T lymphocytes (Supplemental Fig. 2), indicating that erlotinib inhibits T lymphocyte proliferation and activation in an EGFR-independent manner.

Delayed-type hypersensitivity is typically used for the evaluation of in vivo immunocompetency because it has been shown to be entirely dependent on the effects of T cells. In the experiment of contact dermatitis in mice as a delayed-type hypersensitivity model, erlotinib showed a significant inhibition of the ear swelling in response to picryl chloride (Fig. 5). These findings indicate that the inhibition of c-Raf/ERK cascade and Akt signaling pathway by erlotinib blocks the proliferation and activation of T lymphocytes and finally contributes to the alleviation of the ear swelling and inflammation. Our present study, which was focused on the c-Raf/ERK cascade and Akt signaling, did not exclude other possible molecular pathways from being influenced by erlotinib. For instance, it had been reported that erlotinib inhibited tyrosine kinase JAK2 for the treatment of patients with polycythemia vera (Li et al., 2007). It is well known that JAK2/STAT1 signaling is also involved in T cell activation and can induce various genes expression, which in turn, lead to T cell proliferation and cytokine

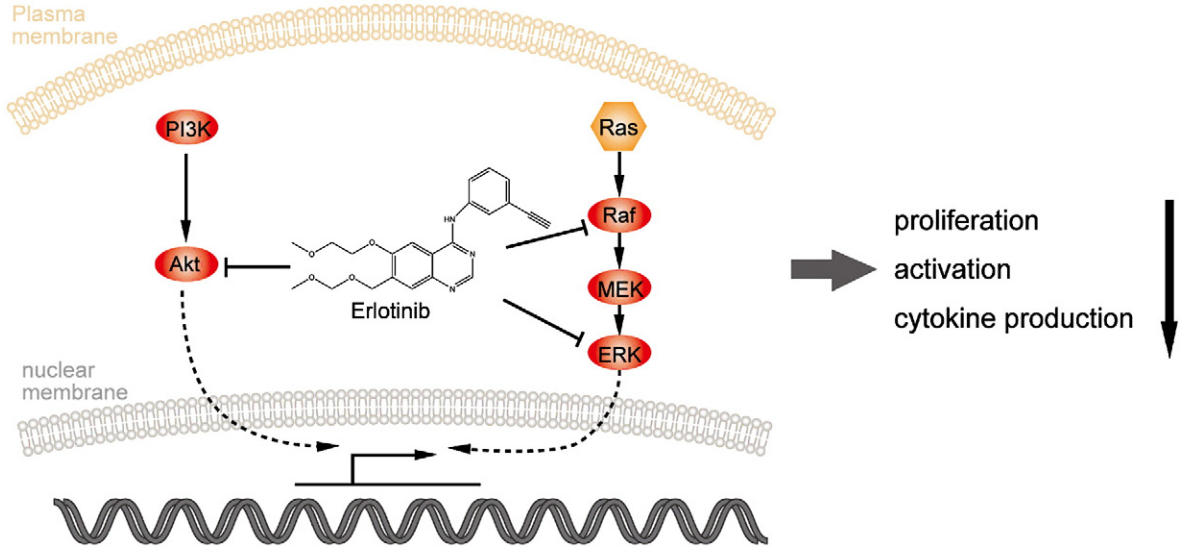

Fig. 6. The hypothetical model of erlotinib-induced impairment of T-cell-mediated immune response. Erlotinib may cause the impairment of T-cell-mediated immune response both in vitro and in vivo through inhibiting T cell proliferation and activation, which is closely associated with its potent down-regulation of the c-Raf/ERK cascade and Akt signaling pathway.

production (O'Shea et al., 2004). The results of this study suggest that the immunosuppressive effect of erlotinib on T cells is at least partly mediated through c-Raf/ERK cascade and Akt signaling pathway.

Taken together, our study demonstrated the potential immunosuppressive effect of erlotinib on T lymphocytes, which is closely associated with its potent down-regulation of the c-Raf/ERK cascade and Akt signaling pathway (summarized in Fig. 6). The balance between the immunosuppression of erlotinib and its anti-cancer efficacy should be investigated in future clinical trials.

Supplementary materials related to this article can be found online at doi:10.1016/j.taap.2010.12.011.

Conflict of interest

The authors declared no conflict of interest.

Acknowledgments

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References

Akita, R.W., Sliwkowski, M.X., 2003. Preclinical studies with erlotinib (Tarceva). Semin. Oncol. 30 (3), 15–24.
Aktas, H., Cai, H., Cooper, G.M., 1997. Ras links growth factor signaling to the cell cycle machinery via regulation of cyclin D1 and the Cdk inhibitor p27(KIP1). Mol. Cell. Biol. 17 (7), 3850–3857.
Bareschino, M.A., Schettino, C., Troiani, T., Martinelli, E., Morgillo, F., Ciardiello, F., 2007. Erlotinib in cancer treatment. Ann. Oncol. 18, 35–41.
Blume-Jensen, P., Hunter, T., 2001. Oncogenic kinase signalling. Nature 411 (6835), 355–365.
Bovenschen, H.J., Alkemade, J., 2009. Erlotinib-induced dermatologic side-effects. Int. J. Dermatol. 48 (3), 326–328.
Chou, L.S., Garey, J., Oishi, K., Kim, E., 2006. Managing dermatologic toxicities of epidermal growth factor receptor inhibitors. Clin. Lung Cancer 8, S15–S22.
Ciardiello, F., Tortora, G., 2008. Drug therapy: EGFR antagonists in cancer treatment. N. Engl. J. Med. 358 (11), 1160–1174.
Comis, R.L., 2005. The current situation: Erlotinib (Tarceva (R)) and gefitinib (Iressa (R)) in non-small cell lung cancer. Oncologist 10 (7), 467–470.
Czyzyk, J., Chen, H.C., Bottomly, K., Flavell, R.A., 2008. p21 Ras/impedes mitogenic signal propagation regulates cytokine production and migration in CD4 T cells. J. Biol. Chem. 283 (34), 23004–23015.
Gollob, J.A., Wilhelm, S., Carter, C., Kelley, S.L., 2006. Role of Raf kinase in cancer: therapeutic potential of targeting the Raf/MEK/ERK signal transduction pathway. Semin. Oncol. 33 (4), 392–406.
Gridelli, C., Rossi, A., Malone, P., Colantuoni, G., Del Gaizo, F., Ferrara, C., Nicolella, D., Guerriero, C., 2007. Erlotinib in non-small-cell lung cancer. Expert Opin. Pharmacother. 8 (15), 2579–2592.
Hamilton, M., Wolf, J.L., Rusk, J., Beard, S.E., Clark, G.M., Witt, K., Cagnoni, P.J., 2006. Effects of smoking on the pharmacokinetics of erlotinib. Clin. Cancer Res. 12 (7 Pt 1), 2166–2171.
Hidalgo, M., Bloedow, D., 2003. Pharmacokinetics and pharmacodynamics: maximizing the clinical potential of erlotinib (Tarceva). Semin. Oncol. 30 (3), 25–33.
Hynes, N.E., Lane, H.A., 2005. ERBB receptors and cancer: The complexity of targeted inhibitors. Nat. Rev. Cancer 5 (5), 341–354.
Lea, N.C., Orr, S.J., Stoeber, K., Williams, G.H., Lam, E.W., Ibrahim, M.A., Mufti, G.J., Thomas, N.S., 2003. Commitment point during G0–>G1 that controls entry into the cell cycle. Mol. Cell. Biol. 23 (7), 2351–2361.
Li, Z., Xu, M., Xing, S., Ho, W.T., Ishii, T., Li, Q., Fu, X., Zhao, Z.J., 2007. Erlotinib effectively inhibits JAK2V617F activity and polycythemia vera cell growth. J. Biol. Chem. 282 (6), 3428–3432.
Li, M., Ong, S.S., Rajwa, B., Thieu, V.T., Geahlen, R.L., Harrison, M.L., 2008. The SH3 domain of Lck modulates T cell receptor-dependent activation of extracellular signal-regulated kinase through activation of Raf-1. Mol. Cell. Biol. 28 (2), 630–641.
Li, X., Kamenecka, T.M., Cameron, M.D., 2010. Cytochrome P450-mediated bioactivation of the epidermal growth factor receptor inhibitor erlotinib to a reactive electrophile. Drug Metab. Dispos. 38 (7), 1238–1245.
Ling, J., Johnson, K.A., Miao, Z., Rakhit, A., Pantze, M.P., Hamilton, M., Lum, B.L., Prakash, C., 2006. Metabolism and excretion of erlotinib, a small molecule inhibitor of epidermal growth factor receptor tyrosine kinase, in healthy male volunteers. Drug Metab. Dispos. 34 (3), 420–426.
Ling, Y.H., Li, T., Yuan, Z., Haigentz Jr., M., Weber, T.K., Perez-Soler, R., 2007. Erlotinib, an effective epidermal growth factor receptor tyrosine kinase inhibitor, induces p27KIP1 up-regulation and nuclear translocation in association with cell growth inhibition and G1/S phase arrest in human non-small-cell lung cancer cell lines. Mol. Pharmacol. 72 (2), 248–258.
Liu, V., White, D.A., Zakowski, M.F., Travis, W., Kris, M.G., Ginsberg, M.S., Miller, V.A., Azzoli, C.G., 2007a. Pulmonary toxicity associated with erlotinib. Chest 132 (3), 1042–1044.
Liu, W.T., Makrauer, F.L., Qamar, A.A., Janne, P.A., Odze, R.D., 2007b. Fulminant hepatic failure secondary to erlotinib. Clin. Gastroenterol. Hepatol. 5 (8), 917–920.
Lubbe, J., Masouye, I., Dietrich, P.Y., 2008. Generalized xerotic dermatitis with neutrophilic spongiosis induced by erlotinib (Tarceva (R)). Dermatology 216 (3), 247–249.
Makris, D., Scherpereel, A., Copin, M.C., Colin, G., Brun, L., Lafitte, J.J., Marquette, C.H., 2007. Fatal interstitial lung disease associated with oral erlotinib therapy for lung cancer. BMC Cancer 7, 4.
McCubrey, J.A., Steelman, L.S., Abrams, S.L., Lee, J.T., Chang, F., Bertrand, F.E., Navolanic, P.M., Terrian, D.M., Franklin, R.A., D'Assoro, A.B., Salisbury, J.L., Mazzarino, M.C., Stivala, F., Libra, M., 2006. Roles of the RAF/MEK/ERK and PI3K/PTEN/AKT pathways in malignant transformation and drug resistance. Adv. Enzyme Regul. 46, 249–279.
Ocana, A., Serrano, R., Calero, R., Pandiella, A., 2009. Novel tyrosine kinase inhibitors in the treatment of cancer. Curr. Drug Targets 10 (6), 575–576.
Onn, A., Correa, A.M., Gilcrease, M., Isobe, T., Massarelli, E., Bucana, C.D., O'Reilly, M.S., Hong, W. K., Fidler, I.J., Putnam, J.B., Herbst, R.S., 2004. Synchronous overexpression of epidermal growth factor receptor and HER2-neu protein is a predictor of poor outcome in patients with stage I non-small cell lung cancer. Clin. Cancer Res. 10 (1), 136–143.
O'Shea, J.J., Pesu, M., Borie, D.C., Changelian, P.S., 2004. A new modality for immunosuppression: targeting the JAK/STAT pathway. Nat. Rev. Drug Discov. 3 (7), 555–564.
Pellegrinotti, M., Fimognari, F.L., Franco, A., Repetto, L., Pastorelli, R., 2009. Erlotinib-induced hepatitis complicated by fatal lactic acidosis in an elderly man with lung cancer. Ann. Pharmacother. 43 (3), 542–545.
Perez-Soler, R., 2004. Phase II clinical trial data with the epidermal growth factor receptor tyrosine kinase inhibitor erlotinib (OSI-774) in non-small-cell lung cancer. Clin. Lung Cancer 6 (Suppl 1), S20–S23.
Pollack, V.A., Savage, D.M., Baker, D.A., Tsaparikos, K.E., Sloan, D.E., Moyer, J.D., Barbacci, E.G., Pustilnik, L.R., Smolarek, T.A., Davis, J.A., Vaidya, M.P., Arnold, L.D., Doty, J.L., Iwata, K.K., Morin, M.J., 1999. Inhibition of epidermal growth factor receptor-associated tyrosine phosphorylation in human carcinomas with CP-358, 774: Dynamics of receptor inhibition in situ and antitumor effects in athymic mice. J. Pharmacol. Exp. Ther. 291 (2), 739–748.
Prados, M.D., Lamborn, K.R., Chang, S., Burton, E., Butowski, N., Malec, M., Kapadia, A., Rabbitt, J., Page, M.S., Fedoroff, A., Xie, D., Kelley, S.K., 2006. Phase 1 study of erlotinib HCl alone and combined with temozolomide in patients with stable or recurrent malignant glioma. Neuro Oncol. 8 (1), 67–78.
Saif, M.W., 2008. Erlotinib-induced acute hepatitis in a patient with pancreatic cancer. Clin. Adv. Hematol. Oncol. 6 (3), 191–199.
Shepherd, F.A., Pereira, J.R., Ciuleanu, T., Tan, E.H., Hirsh, V., Thongprasert, S., Campos, D., Maoleekoonpiroj, S., Smylie, M., Martins, R., van Kooten, M., Dediu, M., Findlay, B., Tu, D.S., Johnston, D., Bezjak, A., Clark, G., Santabarbara, P., Seymour, L., Natl Canc Inst Canada Clin T., 2005. Erlotinib in previously treated non-small-cell lung cancer. N. Engl. J. Med. 353 (2), 123–132.
Siegel-Lakhai, W.S., Beijnen, J.H., Schellens, J.H.M., 2005. Current knowledge and future directions of the selective epidermal growth factor receptor inhibitors erlotinib (Tarceva (R)) and gefitinib (Iressa (R)). Oncologist 10 (8), 579–589.
Sun, Y., Qin, Y., Gong, F.Y., Wu, X.F., Hua, Z.C., Chen, T., Xu, Q., 2009. Selective triggering of apoptosis of concanavalin A-activated T cells by fraxinellone for the treatment of T-cell-dependent hepatitis in mice. Biochem. Pharmacol. 77 (11), 1717–1724.
Sun, Y., Wu, X.X., Yin, Y., Gong, F.Y., Shen, Y., Cai, T.T., Zhou, X.B., Wu, X.F., Xu, Q., 2010. Novel immunomodulatory properties of cirsilineol through selective inhibition of IFN-gamma signaling in a murine model of inflammatory bowel disease. Biochem. Pharmacol. 79 (2), 229–238.
Tang, P.A., Tsao, M.S., Moore, M.J., 2006. A review of erlotinib and its clinical use. Expert Opin. Pharmacother. 7 (2), 177–193.
Tiseo, M., Gridelli, C., Cascinu, S., Crino, L., Piantedosi, F.V., Grossi, F., Brandes, A.A., Labianca, R., Siena, S., Amoroso, D., Belvedere, O., Valentino, B., Bearz, A., Venturino, P., Ardizzoni, A., 2009. An expanded access program of erlotinib (Tarceva) in patients with advanced non-small cell lung cancer (NSCLC): Data report from Italy. Lung Cancer 64 (2), 199–206.
Wong, K.Y.M., Lo, A., Lam, J., Lam, B., Ip, M.S., Ho, J.C., 2009. Erlotinib as salvage treatment after failure to first-line gefitinib in Non-Small Cell Lung Cancer. Cancer Chemother. Pharmacol. 65 (6), 1023–1028.
Xue, L., Chiang, L., Kang, C., Winoto, A., 2008. The role of the PI3K-AKT kinase pathway in T cell development beyond the beta checkpoint. Eur. J. Immunol. 38 (11), 3200–3207.
Zhang, Y., Miao, J.Y., Hanley, G., Stuart, C., Sun, X.L., Chen, T.T., Yin, D.L., 2008. Chronic restraint stress promotes immune suppression through Toll-like receptor 4-mediated phosphoinositide 3-kinase signaling. J. Neuroimmunol. 204 (1–2), 13–19.