# Dual ErbB1 and ErbB2 receptor tyrosine kinase inhibition exerts synergistic effect with conventional chemotherapy in pancreatic cancer

SMIT SINGLA<sup>1</sup>, JAMES A. PIPPIN<sup>2</sup> and JEFFREY A. DREBIN<sup>2</sup>

<sup>1</sup>Department of Surgery, Temple University Hospital, and <sup>2</sup>Department of Surgery, Hospital of the University of Pennsylvania, 4 Silverstein Pavilion, Philadelphia, PA 19104, USA

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Abstract. Patient survival in pancreatic cancer remains poor with gemcitabine (GEM)-based regimens. The target specific molecular agent lapatinib, a dual ErbB1 and ErbB2 receptor tyrosine kinase inhibitor, has shown significant activity against ErbB1 and ErbB2-expressing tumors. Since pancreatic tumors frequently overexpress these proteins, we investigated its effects, both alone and in conjunction with 5-FU or GEM. The pancreatic cancer cell lines PANC-1 and AsPC were treated with varying doses of lapatinib in vitro. The effects on ErbB1/ ErbB2 protein phosphorylation and on the cell survival protein survivin were determined by western blotting. Cytotoxicity was determined by MTT assay and apoptosis was measured using the caspase-3 colorimetric assay. Similar dose-response lapatinib experiments were conducted with varying concentrations of 5-FU or GEM and isobolograms were constructed to evaluate therapeutic synergy. Lapatinib inhibited protein phosphorylation in the range of 4-16  $\mu$ M, a clinically achievable concentration. The lapatinib-treated cells showed a dose-dependent inhibition of cell proliferation and induction of apoptosis at the same concentrations that blocked ErbB1/ ErbB2 phosphorylation. The addition of 5-FU or GEM to these cells resulted in synergistic effects. The lapatinib-treated cells also demonstrated downregulation of survivin. Simultaneous dual ErbB1 and ErbB2 receptor tyrosine kinase inhibition with lapatinib results in significant reduction of pancreatic cancer cell growth and proliferation. These effects occur at clinically achievable concentrations and are synergistic with the effects of 5-FU or GEM. These findings support the potential role of lapatinib in the treatment of pancreatic cancer.

*Correspondence to:* Dr Smit Singla, Department of Surgery, Temple University Hospital, 3401 N. Broad Street, 4th Floor Parkinson Pavilion, Philadelphia, PA 19140, USA E-mail: smit.singla@tuhs.temple.edu

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## Introduction

Pancreatic cancer is the fourth leading cause of cancer death in both men and women in the United States. It has amongst the worst prognoses of all human malignancies. A majority of patients are symptomatic at the time of diagnosis and have developed advanced metastatic disease (1). Most patients die within a year of diagnosis and the 5-year survival rate is <5% (2). Despite many multi-modal treatment strategies, the survival rates have not improved significantly and mortality remains high (3).

Gemcitabine (GEM) has remained the mainstay primary chemotherapeutic agent for systemic treatment in advanced pancreatic adenocarcinoma. Multiple studies have shown survival benefits of GEM chemotherapy, both alone as well as in combination with other drugs (4-6). However, despite significant advances and improvement in therapy, the 5-year survival remains quite low. As a result, there is now a shift towards target specific agents, in addition to conventional cytotoxic drugs, with an aim towards increasing overall survival (7-11).

ErbB1 and ErbB2 are members of the epidermal growth factor (EGF) receptor family and are known to play an essential role in regulation of cell proliferation and differentiation. The formation of homodimers and heterodimers between different EGF receptors is believed to regulate complex signal transduction pathways via activation of intrinsic protein tyrosine kinase activity. This leads to recruitment and phosphorylation of several intracellular substrates leading to various cellular activities including mitogenic signaling and cell growth (12,13).

Some studies have also suggested that overexpression of ErbB1 and ErbB2 in certain cancers is associated with increased tumor aggression and poor prognosis (12,14,15). Targeted downregulation of these receptors has been shown to cause apoptosis and cell death in cancers that overexpress these receptors (12). Since a significant percentage of pancreatic tumors overexpress both ErbB1 and ErbB2 (14-16), targeting these receptors may be a viable strategy in patients diagnosed with such tumors.

Many studies have looked at the response of pancreatic neoplasms to ErbB1 or ErbB2 targeted therapy. Transtuzumab



Figure 1. Immunoblotting assay, ErbB1 and ErbB2 protein expression in AsPC and PANC-1 cell lines.

(ErbB2) (11), erlotinib (ErbB1) (10), cetuximab (ErbB1) (8) and gefitinib (ErbB1) (9) have been studied in the last few years; however, none of these drugs has shown any significant improvement in mortality when compared to treatment with conventional cytotoxic drugs. It was observed that most patients eventually developed drug resistance. This negated any benefits that these agents potentially offered. Since, all these studies targeted either ErbB1 or ErbB2 separately, we hypothesized that simultaneous inhibition of both ErbB1 and ErbB2 receptors might suppress tumor growth better than targeting them individually, and may help overcome the development of drug resistance. We selected lapatinib (Tykerb), a dual ErbB1 and ErbB2 tyrosine kinase enzyme inhibitor, for our study since lapatinib has been shown in multiple studies to benefit patients who have developed drug resistance to prior targeted therapies (17-19).

## Materials and methods

*Pancreatic cell lines and culture*. Human pancreatic cell lines AsPC-1 and PANC-1 were selected for our experiments due to their expression of ErbB1 and ErbB2 receptors (Fig. 1). All cell lines were purchased from ATCC (Manassas, VA) and maintained in recommended media supplemented with 10% fetal bovine serum (Mediatech, Manassas, VA). All cells were cultured at 37°C in 5% CO<sub>2</sub> with 100% humidity.

*Chemicals*. Lapatinib (Tykerb, Genentech Inc, South San Francisco, CA) was kindly provided by Genentech Inc. (South San Francisco, CA) and gemcitabine and 5-fluorouracil (5-FU) were obtained from Sigma/Aldrich, St. Louis, MO.

In vitro proliferation assay (MTT assay). Cell growth was assessed using a 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay (MTT assay). Briefly, 5,000 viable cells were seeded into flat-bottomed 96-well plates in triplicate and allowed to adhere overnight. Cells were treated with the addition of the intended doses of lapatinib (0-16  $\mu$ M), gemcitabine (0-400 nM) and/or 5-FU (0-16  $\mu$ M). After 3-4 days of incubation, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide was added to a final concentration of 0.5 mg/ml and cells were incubated an additional 4 h. Cells were lysed with the addition of isopropanol. Absorbance was measured at 595 and 655 nm in a 96-well plate reader (Bio-Rad Model 680, Bio-Rad Laboratories, Hercules, CA) and growth curves calculated. In vitro apoptosis assay. Apoptosis activity was assessed using the ApoAlert Caspase-3 Colorimetric Assay kit (Clontech, Mountain View, CA). Briefly,  $10^6$  viable cells were seeded in 100-mm tissue culture plates and allowed to adhere overnight. Cells were treated with the addition of the intended doses of lapatinib (0-16  $\mu$ M), gemcitabine (0-400 nM) and/or 5-FU (0-16  $\mu$ M). Cells were harvested from plates using cell scrapers 48 h after the addition of drugs and lysed for analysis of caspase-3 activity according to the manufacturer's instructions. Readings were taken at 405 nM in disposable cuvettes in a spectrophotometer (Thermogenesis 6, Hopkinton, MA) and caspase-3 activity was calculated.

In vitro immunoblot analyses (western blot analysis). Cells were plated and treated in the same manner as described for the apoptosis assay. After incubating for 48 h, cells were harvested using cell scrapers and washed with DPBS. Cells were lysed on ice in HEPES lysis buffer containing 50 mM HEPES, 100 mM NaCl, 1% Triton X-100, 10% Glycerol, 1 mM Na<sub>3</sub>VO<sub>4</sub>, 5 mM NaFl, 1:50 dilution of Complete Protease Inhibitor Cocktail tablets (Roche, Indianapolis, IN). Protein concentrations were determined via Coomassie Protein Assay Reagent (Thermo Scientific, Rockford, IL) and samples were standardized to 10  $\mu$ g in SDS-PAGE loading buffer. Samples were loaded on a 7.5% SDS-PAGE acrylamide gel and run under reducing conditions for 2 h at 100 V. The proteins were transferred to Immobilon-P membranes (Millipore Co., Billerica, MA) using Towbins transfer buffer (25 mM Tris, 192 mM glycine, 10% methanol). Membranes were blocked in TBS (20 mM Tris, 150 mM NaCl) containing 0.1% Tween-20 and 5% non-fat dry milk or BSA and incubated with antibodies against target proteins. Antibodies include ErbB1 (Chemicon, Temecula, CA), ErbB2 (AbCam, Cambridge, MA), Phos-ERBB1 (AbCam), Phos-ERBB2 (Cell Signaling, Beverly, MA), α-tubulin (Santa Cruz Biotechnology, Santa Cruz, CA).

## Results

The effects of lapatinib on cell proliferation. Initial experiments studied the effects of lapatinib, both alone and in combination with 5-FU or gemcitabine, on proliferation of AsPC and PANC-1 cells *in vitro*. Lapatinib inhibited cell proliferation in a dose-dependent manner. At lower doses (0.25-1  $\mu$ M) lapatinib had no effect, but at higher doses (4-16  $\mu$ M), inhibition was significant. There was a 50% inhibition in cell proliferation at a dose of 12  $\mu$ M, but the effects plateaued at higher doses (Fig. 2).

The inhibitory effects of lapatinib were potentiated in the presence of 5-FU or GEM, with a dose-dependent shift in the proliferation curve (Figs. 3 and 4). This suggests that lapatinib may work synergistically with these other drugs, increasing their effect *in vitro*. The combination of drugs allowed similar inhibition at much lower doses. This may allow for reducing the dosage of each drug *in vivo* and thus the incidence of adverse side effects often noticed in current recommended dosages (20,21).

*The effect of lapatinib on ErbB1 and ErbB2 protein levels.* AsPC and PANC-1 cells were treated with lapatinib at dosages



Figure 2. MTT assay. Effect of lapatinib on cytotoxicity. Treatment of cells with lapatinib *in vitro* caused a dose-dependent increase in cellular toxicity.



Figure 3. MTT assay. Effect of combining lapatinib with 5-FU on cytotoxicity.



While the levels of total ErbB1 and ErbB2 proteins remained unchanged, both phosphorylated ErbB1 and ErbB2 (pErbB1-Tyr1173 and ErbB2-Tyr1248) protein levels decreased with lapatinib treatment. Again, lower doses showed no effects; however, at higher doses (12-16  $\mu$ M) decrease in phosphorylated protein was detectable. There was an estimated 50% reduction in phosphorylated protein at 12  $\mu$ M, which corresponds to the dose needed to inhibit proliferation by the same amount (Fig. 5). These sites (Tyr1173 and Tyr1248) have been found to be involved in regulation of tyrosine kinase activity and phosphorylation of these sites couples ErbB1 and ErbB2 to downstream kinase signal transduction pathways (22-24). Therefore, lapatinib downregulation of phosphorylated ErbB1 and ErbB2 may decrease tyrosine kinase activity and subsequently decrease proliferation through these downstream pathways.



Figure 4. MTT assay. Effect of combining lapatinib with gemcitabine on cytotoxicity.



Figure 5. Immunoblotting assay. Effects of lapatinib on ErbB protein phosphorylation and survivin expression. Treatment of cells with lapatinib *in vitro* caused a dose-dependent downregulation of phospho-proteins and survivin.

The effect of lapatinib on cell apoptosis. AsPC and PANC-1 cells were treated in the same manner as the proliferation assays. Forty-eight hours after treatment, apoptosis activity was evaluated using a caspase-3 and -8 colorimetric assay kit. Both cell lines exhibited an increase in cleaved caspase-3 activity indicating apoptosis. This activity corresponded with doses showing decreased proliferation with the maximal activity in the range of 4-16  $\mu$ M (Fig. 6). This would suggest that lapatinib is causing apoptosis in addition to decreasing proliferation.

The effect of lapatinib on survivin protein level. Based on the apoptosis results, we decided to test survivin levels under our treatment conditions. Survivin has been shown to inhibit apoptosis by binding to caspase-3 (25) and has also been shown to play a role in pancreatic cancer (26-28). In order to determine if lapatinib affects the survivin pathway, the expression of survivin protein was assessed in both AsPC and PANC-1 cells



Figure 6. Caspase-3 colorimetric assay. Treatment of AsPC cells with lapatinib (dose in  $\mu$ M) in vitro caused a dose-dependent increase in cell apoptosis.

using immunoblotting. Again, there was a dose-dependent decrease in expression of survivin protein levels with lapatinib treatment in both cell lines. AsPC cells showed a complete reduction of expression at 12  $\mu$ M, while PANC-1 cells showed only a 50% reduction at this same dose (Fig. 5). Assuming survivin is inhibiting apoptosis in these cells; lapatinib could play a key role restoring apoptosis in pancreatic cancer cells.

## Discussion

Pancreatic tumors have so far shown substantial resistance to multiple chemotherapeutic drugs and regimens. It has become a major challenge for clinicians to offer substantive treatment options to patients with advanced pancreatic carcinomas. However, with recent advancements in treatment strategies directed against molecular targets, there has been a renewed interest. It has been proposed that novel molecular agents targeting ErbB1 and ErbB2 in pancreatic tumors will help overcome substantial tumor resistance against conventional cytotoxic drugs and also offer patient specific therapy depending upon the receptor and molecular subtype.

As discussed earlier, some pancreatic tumors have high levels of ErbB1 and ErbB2 cell surface receptors (14-16). These tumors, therefore, are ideal for targeted therapeutic strategy since both ErbB1 and ErbB2 receptors along with their downstream proteins have been shown to promote cell growth and survival, and mediate resistance to chemotherapy. Many studies have been published describing the effects of newer molecular agents directed against ErbB1 and ErbB2 receptors in pancreatic neoplasms (8-11), however, despite theoretical advantages; these agents have failed to yield significantly beneficial results, either alone or in combination with conventional cytotoxic drugs. Although, the exact mechanisms responsible for failure to respond are a matter of intense scrutiny, it seems that a significant number of patients develop drug resistance. Numerous studies have been published describing the potential mechanisms of resistance against these agents. Engelman *et al* (29) showed that amplification of MET caused gefitinib resistance by driving HER3-dependent activation of PI3K while Scaltriti *et al* (17) found that expression of truncated HER2 altered the ErbB2 receptor such that it impaired the access of trastuzumab to the attachment site. Furthermore, Nagy *et al* (30) described the development of resistance in some patients due to overexpression of MUC4.

While it remains to be seen what exactly causes development of resistance against these agents, there is ample evidence to suggest that ErbB subunits work in tandem as a unit and therefore, it may be possible that broad inhibition of these subunits may be necessary to overcome the development of resistance. Working on this hypothesis, we selected lapatinib, based on its mechanism of dual inhibition of ErbB1 and ErbB2 tyrosine kinase and its favorable response in patients who have developed resistance against single-receptor based molecular agents (17,31).

We found that lapatinib markedly reduced tyrosine phosphorylation of ErbB1 and ErbB2, which inhibits activation of Erk1/2 and AKT, the downstream effectors of cell proliferation and survival, respectively (32). We also found that lapatinib caused a decrease in caspase-3 activity and the expression of survivin, a protein inhibitor of the apoptosis. This is significant because the exact mechanism by which lapatinib exerts its apoptotic effect is unknown and therefore, it may be possible that the inhibition of survivin could be one of mechanisms by which lapatnib exerts its effects. Many studies have shown that survivin protects tumors from programmed cell death (33-35), however, none of the prior studies with either ErbB1 or ErbB2 inhibitors could modulate survivin.

Investigators have added lapatinib to chemotherapeutic agents such as paclitaxel (36), docetaxel (37), capecitabine (38), letrozole (39), and to combination regimens such as FOLFOX (40) and FOLFOX (41), with promising results. Although clinical trials in different tumor types are ongoing, the most mature data thus far have been in the treatment of breast cancer, particularly in trastuzumab-resistant patients (37). We also studied the effects of lapatinib in combination with 5-FU and gemcitabine. In combination, lapatinib revealed enhanced toxicity with pronounced cell death and decreased cell survival. These effects were consistent in both cell lines suggesting that its effects may be broad based in cells expressing these target proteins.

In conclusion, simultaneous dual ErbB1 and ErbB2 receptor tyrosine kinase inhibition with lapatinib inhibits pancreatic cancer cell growth and proliferation and induces apoptotic cell death. These effects occur at clinically achievable concentrations and are synergistic with the effects of conventional chemotherapy agents including 5-FU or gemcitabine. These findings support the potential role of lapatinib in the treatment of pancreatic cancer either alone or as an adjunct to conventional chemotherapy agents.

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