Published in final edited form as:

Sci Transl Med. 2011 June 8; 3(86): 86ra50. doi:10.1126/scitranslmed.3001384.

Off-target Lapatinib Activity Sensitizes Colon Cancer Cells through TRAIL Death Receptor Up-regulation

Nathan G. Dolloff^{1,2}, Patrick A. Mayes¹, Lori S. Hart¹, David T. Dicker^{1,2}, Robin Humphreys³, and Wafik S. El-Deiry^{1,2,4}

¹Laboratory of Molecular Oncology and Cell Cycle Regulation, Department of Medicine (Hematology/Oncology), Department of Genetics, Department of Pharmacology, The Institute for Translational Medicine and Therapeutics, Abramson Comprehensive Cancer Center, University of Pennsylvania School of Medicine, Philadelphia, PA

²Laboratory of Translational Oncology and Experimental Cancer Therapeutics, Department of Medicine (Hematology/Oncology), Penn State Hershey Cancer Institute, Penn State Hershey Medical Center, Penn State College of Medicine, Hershey, PA

³Oncology Research Department, Human Genome Sciences Inc., Rockville, MD

⁴The American Cancer Society, Atlanta, GA 1- Laboratory of Molecular Oncology and Cell Cycle Regulation, Departments of Medicine (Hematology/Oncology), Genetics, and Pharmacology, the Institute for Translational Medicine and Therapeutics, and the Abramson Comprehensive Cancer Center, University of Pennsylvania School of Medicine, Philadelphia, PA, 19104 2- Laboratory of Translational Oncology and Experimental Cancer Therapeutics, Department of Medicine (Hematology/Oncology), Penn State College of Medicine, Penn State Hershey Cancer Institute, Penn State Hershey Medical Center, Hershey, PA 17033 3- The American Cancer Society, Atlanta, GA 30329

Abstract

Lapatinib, a HER2/EGFR inhibitor, is a recently approved targeted therapy for metastatic breast cancer. As lapatinib enhances the efficacy of the antimetabolite capecitabine in breast cancer patients, we lapatinib also enhance the activity of anti-cancer agents in colorectal cancer. We found that lapatinib the pro-apoptotic effects of Tumor necrosis factor-Related Apoptosis-Inducing Ligand (TRAIL) and TRAIL receptor antibodies mapatumumab and lexatutumumab. Tumors from mice treated with lapatinibTRAIL exhibited more immunostaining for cleaved caspase-8, the extrinsic cell death pathway, tumors from mice treated with lapatinib or TRAIL alone. Furthermore, combination therapy suppressed tumor growth more effectively than treatment. apatinib up-the proapoptotic TRAIL death receptors DR4 and DR5, leading to more efficient induction of apoptosis in the presence of TRAIL receptor agonistsThis activity was independent of EGFR and HER2 off-target induction of DR5 by lapatinib activation of the JNK/c-Jun signaling axis. This activity of lapatinib on TRAIL death receptor expression and signaling may confer therapeutic benefit when increased doses of lapatinib are used in combination with TRAIL-receptor-activating agents.

Introduction

Tumor necrosis factor-related apoptosis-inducing ligand (TRAIL) and agonistic antibodies targeting TRAIL receptors (mapatumumab and lexatumumab) are attractive candidate anti-cancer drugs, as they directly induce death of target cells and selectively spare normal, non-malignant cell types (1). Cancer cells, however, resist allowing them to evade the pro-apoptotic effects of TRAIL. Mechanisms include overexpression of the inhibitor of caspase-8 activation c-FLIP, hypermethylation of caspase-8, reduced cell surface TRAIL receptor expression, overexpression of anti-apoptotic Bcl-2 family members such as Bcl-XL or Mcl-1, loss of pro-apoptotic Bax, and overexpression of the inhibitor of apoptosis (IAP) family members (2–5). These molecular events in primary human malignancies a recent phase 1 trial of mapatumumab, a humanized TRAIL-R1 (DR4)-activating antibody, no objective responses were observed in patients (6). Therefore, the effectiveness of TRAIL and TRAIL-R agonistic antibodies as monotherapies may be limited by resistance, as it is for other anti-cancer agents. Drugs are commonly combined to augment treatment efficacy and suppress the emergence of resistant clones. FOLFOX-4 (infusional 5-fluorouracil/leucovorin and oxaliplatin) plus Avastin, for example, is a standard first-line therapy for colorectal cancer, as the combination of drugs produces a greater clinical response than each individual agent alone. Clinical trials combining TRAIL-targeted agents with other therapies will reveal which drugs exhibit the best synergy with TRAIL and TRAIL-R agonistic antibodies. Preclinical studies suggest that some currently available compounds enhance TRAIL. DNA damaging agents do so by inducing p53-dependent transcription of pro-apoptotic Bax and TRAIL-R2 (DR5), one of two pro-apoptotic TRAIL death receptors (7, 8). Small molecule inhibitors also effectively sensitize cancer cells to TRAIL. The multikinase inhibitor, sorafenib, resensitizes Bax-null HCT116 colon carcinoma cells to TRAIL by inhibiting NF- κ B-dependent c-IAP2 and Mcl-1 transcription (3, 9), to the genetic makeup and resistance mechanism of the tumor. For instance, p53 mutations commonly arise in colorectal cancer cells (10). The use of DNA damaging agents for TRAIL sensitization would likely be ineffective in the absence of wild-type p53, and therefore, this circumstance may require an alternative approach.

Lapatinib is a dual EGFR/HER2 tyrosine kinase inhibitor approved by the FDA for treatment of HER2-positive, metastatic breast cancer. Lapatinib is indicated for combination therapy with the antimetabolite capecitabine, a setting in which it increases progression-free survival in patients who received prior treatment with anthracycline and the anti-HER2 antibody trastuzumab (Herceptin; 11). The clinical utility of EGFR and HER2 inhibitors is attributed to overexpression of these receptors and their ability to activate oncogenic kinases such as Akt and ERK (12, 13). In this study we sought to identify therapeutic combinations of lapatinib with agents in colon cancer cells, elevated expression of EGFR and HER2 has been reported (14–16) and EGFR-targeted therapies such as the monoclonal antibody cetuximab (Erbix) are clinically effective (17).

Results

Lapatinib sensitizes human colon cancer cells to TRAIL-induced apoptosis

We set out to identify therapeutic combinations of lapatinib and cytotoxic drugs in colon carcinoma. We tested the death-inducing effects of lapatinib in combination with conventional chemotherapeutic drugs (5'-fluorouracil, adriamycin, CPT-11, etoposide, cisplatin, and gemcitabine) and the apoptosis-inducing ligand TRAIL in mutant p53-expressing, and TRAIL-resistant SW620 colon carcinoma cells. Lapatinib failed to induce cell death ($20\mu\text{M}$) and we did not detect significant increases in cell death when lapatinib was the chemotherapeutic agent (Fig. 1A). By contrast, lapatinib pretreatment (hours) substantially increased TRAIL-induced cell death and apoptosis (Fig. 1A and 1B), a finding

that was further substantiated in a panel of TRAIL-sensitive (SW480 and DLD-1) and TRAIL-resistant (SW480-R6 and HT29) colon cancer cell lines (Fig. 1D). lapatinib, TRAIL more efficiently initiated processing of caspase-8, -9, and -3 as well as cleavage of the caspase-3 substrate PARP, further lapatinib enhance TRAIL-induced apoptosis (Fig. 1C). Lapatinib sensitized colon cancer cells to TRAIL at 5 μ M (Fig.S1), a concentration near the peak plasma concentrations achieved in patients during phase clinical trials (18) and higher than required to inhibit EGFR and HER2 (19, 20). Lapatinib-induced TRAIL sensitization was an effect of chronic (hours) exposure to the drug. Short-term pretreatment (1 hour), which was sufficient to inhibit HER2 and downstream survival signaling intermediates such as Akt (Fig. S2A), did not increase TRAIL sensitivity (Fig. S2B).

Lapatinib enhances TRAIL-induced caspase-8 signaling and anti-tumor activity *in vivo*

We next tested the therapeutic combination of lapatinib and TRAIL *in vivo*. Ice bearing xenografted SW620 colon tumors were treated with oral lapatinib for 2 days at a dose of 40mg/kg, which is roughly twice the indicated dose (18.75mg/kg/day: 1,250mg for a 70kg patient). On the third day, animals were given a single intravenous dose of TRAIL (100 μ g). We utilized immunohistochemistry to detect cleaved caspase-8 in tumors from mice treated with lapatinib, TRAIL, or lapatinib/TRAIL combinations. As caspase-8 is an initiator caspase that is activated in response to TRAIL-induced death receptor oligomerization, this marker death receptor signaling activity. As single agents, lapatinib and TRAIL induced low levels of caspase-8 cleavage, whereas the combination induced significantly higher levels of cleaved caspase-8 staining ($P<0.001$; Fig. 2A and B). The combination therapy suppressed tumor growth in long-term studies, animals were treated with three cycles of the combination therapy regimen days TRAIL $P<0.05$; Fig. 2C). This demonstrates that caspase-8 activation, which we observed immediately one treatment cycle, was associated with repression of tumor growth, and that the therapeutic combination of lapatinib and TRAIL has activity *in vivo*.

Mouse models are useful for assessing TRAIL associated toxicities, as human TRAIL binds mouse TRAIL receptors and activates apoptosis in mouse cells (21). We performed mouse liver histology and found no signs of hepatotoxicity, an adverse event associated with other death-inducing ligands such as TNF and Fas, as well as some TRAIL preparations in cultured hepatocytes (Fig. S3; 22–24). We found that dose lapatinib monotherapy depleted peripheral blood mononuclear cells (PBMC) determined by flow cytometric forward and side scatter analyses. Conversely, we observed increases in a population of PBMCs (Fig. S4). animals survived treatment, and thus, two consecutive daily treatments with high dose lapatinib was a tolerable regimen in our preclinical model. A recent phase clinical trial (25) demonstrated the safety of 2 pulse of dose lapatinib prior to administration of albumin-bound paclitaxel in solid malignancies, further supporting the clinical utility of the treatment paradigm we used in this study.

Lapatinib potentiates the cytotoxicity of TRAIL receptor agonistic antibodies

We next investigated whether lapatinib sensitizes colon cancer cells to mapatumumab and lexatumumab, two agonistic TRAIL receptor antibodies that selectively target DR4 and DR5, respectively. TRAIL receptor-activating antibodies induce apoptosis by the same mechanism as TRAIL: via activation of extrinsic and intrinsic cell death pathways. However, their pharmacokinetic properties *in vivo* differ significantly from those of TRAIL. The half-life of mapatumumab approximately 9 days compared to less than 30 minutes for TRAIL (26, 27). The stability of mapatumumab and lexatumumab *in vivo* makes them attractive agents for use in humans and several clinical trials investigating mapatumumab in solid and hematological tumors have been completed or initiated. As shown in figure S6 and in agreement with previous reports, mapatumumab induced a dose-dependent appearance of

apoptotic markers (caspase-3 and PARP cleavage products) in TRAIL-sensitive SW480 cells (26). The DR5-activating antibodies, lexatumumab and AF361, induced apoptosis with similar efficacy (Fig. S6). Pretreatment with lapatinib further enhanced the apoptotic response to DR5-targeted antibodies, but only negligible sensitization was detected when lapatinib was combined with mapatumumab at a concentration of 500ng/ml (Fig. 4A). In contrast, lapatinib increased sensitivity to both lexatumumab and mapatumumab in SW620 (Fig. 4B), HCT15 (Fig. 4C) and *p53*^{-/-} HCT116 cells (Fig. 4D), although the sensitizing effects of lapatinib were more profound when combined with lexatumumab. Therefore, lapatinib enhances apoptotic signaling induced by TRAIL death receptor agonistic antibodies, DR5 **Lapatinib up-regulates death receptor expression.**

To identify the mechanism by which lapatinib sensitizes colon cancer cells to TRAIL and TRAIL agonistic antibodies, we quantified multiple extrinsic and intrinsic cell death pathway components could be affected by lapatinib lead to more efficient apoptotic signaling. We did not detect appreciable changes in anti-apoptotic Bcl-2 family members Bcl-2, Bcl-XL or Mcl-1 upon lapatinib treatment (Fig. S7). Lapatinib also did not down-regulated caspase inhibitor family members cIAP-1, cIAP-2, XIAP, or survivin (Fig. S7). Furthermore, decoy receptor (dcR1 and dcR2) and c-FLIP amounts were unchanged lapatinib exposure (Fig. 5B). By contrast, lapatinib increased the content of pro-apoptotic death receptors, DR4 and DR5, in SW620 (Fig. 5A and 5B), HT29 (Fig. S8A), HCT15 (Fig. 5C), and DLD-1 (Fig. S8B) cells. The small molecule inhibitor of EGFR, AG1478, failed to up-regulate DR4 or DR5. We observed lapatinib-induced death receptor up-regulation in cell lysates collected both Triton X-100 and SDS detergents (Fig. S8B). Changes in death receptor protein content corresponded to increased cell surface receptor density, which was determined by FACS analysis (Fig. 5A).

We then investigated changes in death receptor amounts in specimens from animals treated with lapatinib. We first validated the specificity of an anti-mouse/human DR5 antibody using spleen tissue from DR5 knockout and wild-type mice (Fig. S9). In tumor tissues, regions of vehicle-treated SW620 tumors were weakly positive for DR5, whereas we observed intense DR5 staining in tumors from lapatinib-treated mice (Fig. 5D). We were unable to find a suitable DR4 primary antibody for immunohistochemistry and were therefore unable to evaluate DR4 expression in tumor tissues.

Death receptor content is tightly regulated by transcriptional and post-translational mechanisms. We next asked whether lapatinib up-regulates the death receptors by promoting transcription or stabilizing death receptor proteins. To test the effects of lapatinib on death receptor protein stability, experiments were performed in the presence of cycloheximide to measure the effect of lapatinib on death receptor half-life. As expected, twenty-four hours of lapatinib exposure up-regulated DR5 protein content; however, the rate of DR5 protein turnover was not altered in cycloheximide-chase experiments (Fig. S10). This finding suggested that lapatinib up-regulated DR5 through a mechanism other than protein stabilization. We next investigated transcriptional effects of lapatinib on DR5 by measuring changes in DR5 mRNA treatment. Lapatinib increased DR5 mRNA levels 2–3 fold in SW620, HCT116 *p53*^{-/-} and DLD-1 cells (Fig. 5E and 5F), with efficacy to the introduction of ectopic wild-type *p53* coupled with CPT-11 treatment. We therefore conclude that lapatinib does not affect DR5 protein stability but rather up-regulates its mRNA, which may account, at least in part, for the increases in DR5 protein.

We then set out to determine the role of death receptors in mediating the effects of lapatinib. Although the majority of colon cancer cells were resistant to single-agent lapatinib (Fig. 1D), work by others has shown that single-agent lapatinib is toxic to HCT116 cells (27). Consistent with these previous reports, we found that concentrations of lapatinib above 5

μM significantly reduced viability of HCT116 cells (Fig. S11). We hypothesized that lapatinib-mediated death receptor up-regulation contributed to this single-agent activity. To test this hypothesis, we stably repressed DR4 expression using targeted shRNA (Fig. 5G) and found that cells became significantly less sensitive to lapatinib (EC_{50} of $28.2 \mu\text{M}$ versus $16.2 \mu\text{M}$, Fig. S11). *In vivo*, we found that a high dose of lapatinib (80 mg/kg , p.o.), given twice weekly on consecutive days, significantly reduced tumor size of control HCT116 xenografts (Fig. 5H). By contrast, there was no significant difference in tumor size between vehicle-treated and lapatinib-treated mice bearing DR4-deficient HCT116 xenografts (Fig. 5I). We also detected significant cleaved caspase-8 staining in control shRNA tumors from lapatinib-treated mice (Fig. 5J). As caspase-8 is directly activated by death receptors, this finding further suggests that death receptor-mediated apoptotic signaling contributes to an anti-tumor effect in colon tumors that are sensitive to single-agent lapatinib.

Lapatinib induces TRAIL-sensitization and death receptor up-regulation independently of EGFR and HER2 inhibition

We next investigated the molecular events initiated by lapatinib leading to up-regulation of death receptors and TRAIL sensitization. Several pieces of evidence suggested that this effect was independent of EGFR and HER2, the two primary lapatinib targets. First, lapatinib induced significant up-regulation of death receptors and enhanced TRAIL sensitivity in SW620 cells, which lack EGFR (Fig. S12) and have undetectable phosphorylation (Fig. 6A). Second, lapatinib inhibits EGFR and HER2 at concentrations of $1 \mu\text{M}$ and 100 nM (Fig. 6B), but does not alter TRAIL sensitivity or death receptor content at these doses (Fig. 1D and Fig. 6B). observations we hypothesized that EGFR/HER2 inhibition was not sufficient to induce the phenotypic changes in death receptor signaling that we observed lapatinib treatment. In support of this hypothesis, the EGFR (AG1478) and HER2 (AG825) inhibitors, at concentrations that completely inhibit phosphorylation of their respective targets, failed to up-regulate death receptors or enhance TRAIL sensitivity (Fig. 6C and 6D). Similarly, the HER2-targeted monoclonal antibody, trastuzumab (Herceptin), had no effect on death receptor content when combined with the EGFR inhibitor AG1478 (Fig. 6F and 6G). Furthermore, the combination of AG1478 and AG825 was incapable of increasing DR5 mRNA (Fig. 6E). these findings we conclude that lapatinib, in addition to inhibiting EGFR and HER2, induces death receptor up-regulation and TRAIL sensitization through an off-target mechanism.

Lapatinib-induced DR5 expression is JNK/c-Jun pathway-dependent

We next sought to identify pathways and transcription factors involved in lapatinib-induced DR5 upregulation. DR5 is a known p53 target gene; however, all the cell lines used in this study were p53-deficient, typically expressing inactivating mutations. While certain compounds can rescue p53 transcriptional responses in mutant p53-expressing cells (29–31), lapatinib up-regulated DR5 in *p53*^{-/-} HCT116 cells (Fig. H), demonstrating the p53-independent nature of this effect. We therefore investigated p53-independent mechanisms of lapatinib-induced DR5 up-regulation.

DR5 is regulated by p53-dependent as well as independent mechanisms (32, 33). Activation of the JNK/c-Jun/AP-1 pathway leads to death receptor up-regulation and increased TRAIL sensitivity in cell lines from multiple cancer types (34–36). We hypothesized that an effect of high-dose lapatinib activity could be stimulation of the JNK/c-Jun/AP-1 pathway, which is activated by various forms of cellular stress (37, 38). Indeed, we found that chronic treatment with lapatinib led to phosphorylation of JNK and its downstream substrate, c-Jun. Lapatinib also induced a modest but reproducible increase in total content of c-Jun (Fig. 7A). Activation of the JNK/c-Jun pathway by lapatinib – similar to the induction of DR5 – was only evident with high drug concentrations ($5 \mu\text{M}$; Fig. S13). The combination of

EGFR and HER2 inhibitors, AG1478 and AG825, had no effect on JNK and c-Jun phosphorylation, demonstrating the off-target nature of these events (Fig. 7B). Increases in total c-Jun were apparent in *ex vivo* tumor samples from mice treated with lapatinib. Areas of intense c-Jun staining with nuclear localization were identified by immunohistochemical staining in HCT15 (Fig. 7C) and SW620 (Fig. S14) tumors from mice treated with high-dose lapatinib. Phosphorylation of another MAP kinase, ERK1/2, was modestly increased by lapatinib; this increase was more pronounced when ERK1/2 phosphorylation was induced by the addition of HGF (Fig. S15). By comparison, lapatinib had no effect on unstimulated or HGF-stimulated Akt phosphorylation (Fig. S16). Similarly, lapatinib increased JNK phosphorylation in response to TNF- α treatment, it had no effect on TNF- α -induced I κ B degradation (Fig. S17). Taken together, these data demonstrate that lapatinib leads to the phosphorylation of JNK, c-Jun, and ERK, but has no effect on the Akt and NF- κ B pathways. To determine the relevance of increased JNK, c-Jun and ERK phosphorylation for lapatinib-induced death receptor up-regulation, we inhibited JNK2, c-Jun, and ERK1/2 production siRNA, then measured the concentration of DR5 in the presence and absence of lapatinib. While ERK1/2 knockdown had no effect on lapatinib-induced DR5 up-regulation in p53-null HCT116 cells, knockdown of JNK2 and c-Jun almost completely abrogated this effect (Fig. 7D). Similar results were observed using DLD-1 cells (Fig. 7E). These data, we propose lapatinib-mediated anti-tumor activity derives from inhibition of ErbB family members, as well as off-target induction of TRAIL death receptors and sensitization to TRAIL through activation of JNK/c-Jun signaling (Fig. 7F).

Discussion

Lapatinib, a dual inhibitor of EGFR and HER2, was approved by the FDA for the treatment of metastatic breast cancer. This regimen is effective in patients who received prior therapy with taxanes and the HER2-targeted monoclonal antibody trastuzumab (Herceptin). Lapatinib is currently indicated for breast cancer patients that express HER2, it also inhibits EGFR and signaling by the ErbB family member ErbB3/HER3 (39). As ErbB kinases are expressed by many tumor types, it is possible that lapatinib has anti-tumor effects in non-breast cancers as well, and lapatinib is currently being tested in over 150 clinical trials of solid and hematological malignancies alone and in combination with other cancer therapeutics (40).

In this study we screened for potential therapeutic combinations involving lapatinib and cytotoxic agents in colon cancer cell. We found that lapatinib enhanced cell death by the apoptosis-inducing ligand TRAIL. This sensitizing effect derives from an EGFR/HER2-independent off-target effect that involves activation of the JNK/c-Jun axis and up-regulation of pro-apoptotic TRAIL death receptors. We observe TRAIL sensitizing effects of lapatinib *in vitro* at concentrations that are higher than plasma concentrations in patients the indicated dose for late stage breast cancer (1,250 mg daily/40). In our *in vivo* studies, we treated tumor-bearing mice with lapatinib doses to than twice clinical dose (40 mg/kg/day or 2,800 mg/day for a 70 kg patient), in order to model a clinical situation patients receive high of lapatinib for 2 days prior to TRAIL treatment. The feasibility and tolerability of this 2-day, high dose lapatinib pulse prior to treatment with a cytotoxic agent was recently demonstrated in a phase clinical trial, lapatinib was used as a sensitizing agent for albumin-bound paclitaxel (25). Chien and colleagues (25) reported a maximum tolerated dose of 5250mg/day, and lapatinib concentrations that exceeded 5 μ M in patient plasma. Our data suggest that this same treatment protocol may be effective for priming colon tumors to TRAIL or TRAIL death receptor agonistic antibodies, mapatumumab and lexatimumab.

The dose of lapatinib to treat mice in our preclinical studies a linear correlation between dose and subject weight. Variables (drug absorption, metabolism, *etc.*) may differ between humans

and mice, that the dose scaling may not be a direct correlation. Nevertheless, the 40 mg/kg dose that used in our study increased DR5 and c-Jun expression, two of off-target lapatinib activity, in tumors from mice treated with lapatinib. Furthermore, HCT116 tumors, which are sensitive to lapatinib as a single-agent, underwent more apoptosis and grew more slowly than controls, suggesting that the HER2/EGFR-independent off-target activity that we characterized *in vitro* was observable *in vivo*. We also noted leukopenia using this dose, which is consistent with expos to high dose lapatinib adverse event (25). the treatment of colon cancer, it is possible that local drug concentrations within the colon higher than those measured in the bloodstream, as lapatinib is an orally administered drug. Therefore, tumors within the colon may be exposed to the highest amounts of lapatinib, exceed plasma. As the TRAIL-sensitizing activity of lapatinib requires elevated concentrations of lapatinib, tumors of the colon or other malignancies originating in the gastrointestinal tract may be most susceptible to these off-target effects. We also that because the established dose of lapatinib is not limited by drug-related toxicities (18), increasing the dose of lapatinib may bring about beneficial, TRAIL-sensitizing effects without increasing the frequency of adverse events. TRAIL preferentially and directly induces apoptosis in tumor cells and is therefore a unique and promising targeted anti-cancer agent. TRAIL kills target cells by engaging death receptors, which initiate a caspase cascade that is amplified by mitochondrial permeabilization, ultimately leading to caspase-3 activation as well as membrane blebbing and DNA fragmentation (1). Tumor cells may acquire deficiencies or resistance mechanisms at multiple levels of the extrinsic and intrinsic cell death pathways thus allowing them to evade TRAIL-induced signaling and apoptosis. Critical to the clinical success of TRAIL is the development of therapeutic strategies aimed at overcoming TRAIL resistance. We show here that lapatinib, an FDA-approved treatment for metastatic breast cancer, increases recombinant TRAIL ligand and TRAIL agonistic antibody-induced apoptosis in colorectal cancer cells. We also demonstrate that lapatinib, in addition to inhibiting EGFR and HER2 oncoproteins, up-regulates TRAIL receptors through an off-target mechanism dependent on JNK and c-Jun activation. these findings, we combination of lapatinib and TRAIL targeted therapies second-line therapy for colon cancer. Previous studies reported enhance by ErbB family kinase inhibitors, as these receptors activate anti-apoptotic pathways such as Akt (42, 43). ur finding that the effects of lapatinib TRAIL sensitivity are independent of EGFR and HER2 and occur up-regulation of pro-apoptotic TRAIL death receptors. apatinib induces this up-regulation activation of the JNK/c-Jun signaling pathway. Previous studies have demonstrated that JNK signaling increases TRAIL sensitivity (35, 44, 45). Furthermore, agents that induce JNK activation and increase apoptotic signaling in response to TRAIL do so by up-regulating both DR4 and DR5 (34, 46). We observed up-regulation and phosphorylation of c-Jun, an AP-1 complex family member. c-Jun phosphorylation enhance the transcription of AP-1 target genes, and both DR4 and DR5 express functional AP-1 binding sites within their promoter regions (46), which likely accounts for the dependence of lapatinib-induced DR5 up-regulation on c-Jun and JNK. While TRAIL and antibodies that activate TRAIL receptors are attractive cancer treatments and have been well tolerated in early phase clinical trials, few objective responses have been reported (6, 48). It is possible that adjuvant therapies will be required to enhance their effectiveness, and our findings, lapatinib appears to be an outstanding candidate for such clinical studies.

Materials and Methods

Cell Culture and Reagents

All cell lines were purchased from ATCC unless otherwise stated, and maintained in their appropriate growth medium at 37°C and 5% CO₂. SW620, SW480, HCT15 and DLD1 colon cancer cells were grown in DMEM supplemented with FBS and gentamicin. TRAIL-resistant SW480-R6 colon cancer cells were selected from SW480 parental cells, as

described (5) and were maintained in DMEM supplemented with FBS and gentamicin. HT29 and HCT116 colon cancer cells were grown in McCoy's 5A medium supplemented FBS and gentamicin. His-tagged, recombinant human TRAIL was produced and purified as described previously (48). The human DR5-activating antibody, AF631, was purchased from R&D Systems. AG1478 and AG825 were purchased from Sigma. JNK2 siRNA was purchased from Santa Cruz Biotechnology (Sense: GACUCAACCUUCACUGUCCUAtt, Antisense: UAGGACAGUGAAGGUUGAGUCtt). An siRNA pool targeting c-Jun was purchased from Santa Cruz Biotechnology (duplex 1: Sense: 5'-GUGACGGACUGUUCUAUGAtt, Antisense: 5'-UCAUAGAACAGUCCGUCACtt; 2: Sense: 5'-CCAGAAAGGAUAUUUAAGAtt, Antisense: 5'-UCUUAUAUCCUUCUGGtt; 3: Sense: 5'-GAUGGCCUUGCUUAUGAAtt, Antisense: 5'-UUCAUAAGCAAAGGCCAUCtt; 4: Sense: 5'-GCAUCAUCUGUAGAUACUAtt, Antisense: 5'-UAGUAUCUACAGAUGAUGCtt). ERK1/2 siRNA oligos (Sense: 5'-CCUCCAACCUGCUCAUCAAtt, Antisense: 5'-UUGAUGAGCAGGUUGGAGGtt) were purchased from Cell Signaling Biotechnology.

Cell Death, Apoptosis, and Cell Viability Assays

Cell death was quantified by propidium iodide (PI) staining and fluorescence-activated cell sorting (FACS). Floating and adherent cells were collected and fixed in 70% ethanol, followed by RNase A treatment and PI staining. For quantification of apoptosis, cells were fixed with methanol and treated with formamide to denature DNA. Single stranded DNA was detected with a mouse monoclonal antibody (Apostain, Axxora Platform), then cells were stained with PI and analyzed by FACS. For cell viability assays, cells were detached treatment, diluted 50 in standard culture media and replated. When control cells reached 90% confluence, cells were fixed, stained with Coomassie Blue and imaged. Alternatively, cell viability was measured using the Cell TiterGlo assay system (Promega) according to the manufacturer's instructions. Active caspase-3 was detected using a rabbit polyclonal antibody (BD Pharmingen) and quantified using FACS analysis after fixation and permeabilization with the Cytotfix/Cytoperm (BD Biosciences) solution according to the manufacturer's instructions.

SDS-PAGE and Western Blotting

Cells were lysed in a single detergent lysis buffer (50mM Tris, pH 8.0, 150mM NaCl, 10mM NaF, 1% TritonX-100) supplemented with protease (Sigma) and phosphatase (Calbiochem) inhibitor cocktails. Protein concentrations were measured using the BioRad Protein Assay (BioRad Laboratories) according to the manufacturer's instructions. Equal amounts of protein were separated by 4–12% SDS-PAGE and transferred to PVDF membrane (Immobilin-P, Millipore). Primary antibodies to caspase-8, caspase-9, caspase-3, PARP, HER2, phospho-Y1221/1222 HER2, phospho-Y877 HER2, phospho-Y1086 EGFR, phospho-Y1068, phospho-JNK, JNK, and c-Jun were purchased from Cell Signaling. DR5 antibodies were from Sigma and Cell Signaling and produced identical results. DR4 was detected using antibodies from BD Pharmingen. Antibodies directed to dcR1 and dcR2 were from Pharmingen and Imgenex, respectively. Anti-Ran primary antibody was purchased from BD Transduction Labs. Phospho-c-Jun (Ser73) was from Calbiochem.

Xenograft Studies

Mice were housed and maintained in accordance with the University of Pennsylvania Institutional Animal Care and Use Committee and state and federal guidelines for the humane treatment and care of laboratory animals. Four to six week old NCr nude mice were purchased from Taconic and injected subcutaneously with 1×10^6 SW620 or HCT15 cells mixed with Matrigel (v:v; BD Biosciences). For short-term studies, animals were treated with 40 mg/kg lapatinib by oral gavage for two consecutive days. On the third day, animals

were administered an i.v. dose of rhTRAIL (150 µg). After 24 hours animals were sacrificed, tumors were excised and fixed in 10% buffered formalin and subsequently processed for immunohistochemical staining of paraffin-embedded sections. For long-term tumor growth studies, treatment cycles of two daily lapatinib (40 mg/kg) doses followed by one intravenous dose of rhTRAIL (90 µg) began 7 days after subcutaneous inoculation of tumor cell suspensions, when tumors had progressively grown to a volume of 100–150 mm³. Tumor volumes were determined using caliper measurements and the formula for approximating ellipsoid volumes ($1/6 \times \text{width}^2 \times \text{length}$). Animals were treated weekly with this dosing schedule for three consecutive weeks. Tumor volumes were determined weekly by caliper measurements and using the following formula for the volume of an ellipsoid [volume (mm³) = $0.52 \times (\text{width})^2 \times (\text{length})$]. For in vivo experiments using HCT116 xenografts, 2×10^6 cells were injected subcutaneously with Matrigel (v:v, BD Biosciences). We initiated lapatinib treatment (80 mg/kg, p.o., 2 consecutive daily doses per week) after 7 days, when tumors had grown to a volume of 50–100 mm³.

Histology, Immunohistochemistry, and Fluorescence Microscopy

Excised tumors were collected and fixed in 10% buffered formalin prior to embedding in paraffin, sectioning and immuno-staining. Antibodies detecting cleaved caspase-8 and DR5 were purchased from Cell Signaling Technology and Abcam, respectively. C-Jun was detected using a rabbit polyclonal antibody from Cell Signaling. For detection of lapatinib fluorescence in ex vivo colonic tissue, mice were treated for 2 days with 40 mg/kg of oral lapatinib and then sacrificed. Descending colons were snap-frozen in OCT compound and sectioned using a microtome. Sections were analyzed using the Nuance multispectral imaging system (CRi) with UV excitation (360 nm) and long pass emission. The lowest wavelength of emitted light detected by the imaging system was 500 nm.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

References

1. Ashkenazi. Herbs To kill a tumor cell: the potential of proapoptotic receptor agonists. *J. Clin. Invest.* 118:1979–1990. [PubMed: 18523647]
2. Chawla-Sarkar. Downregulation of Bcl-2, FLIP or IAPs (XIAP and survivin) by siRNAs sensitizes resistant melanoma cells to Apo2L/TRAIL-induced apoptosis. *Cell Death Differ.* 11:915–923. [PubMed: 15118763]
3. Kim, Ricc, El-Deiry. Mcl-1: a gateway to TRAIL sensitization. *Cancer Res.* 68:2062–2064. [PubMed: 18381408]
4. Fulda. Sensitization for death receptor- or drug-induced apoptosis by re-expression of caspase-8 through demethylation or gene transfer. *Oncogene.*
5. Jin, McDonald, Dicker, El-Deiry. Deficient tumor necrosis factor-related apoptosis-inducing ligand (TRAIL) death receptor transport to the cell surface in human colon cancer cells selected for resistance to TRAIL-induced apoptosis. *J. Biol. Chem.* 279:35829–35839. [PubMed: 15155747]
6. Hotte. A phase 1 study of mapatumumab (fully human monoclonal antibody to TRAIL-R1) in patients with advanced solid malignancies. *Clin. Cancer Res.* 14:3450–3455. [PubMed: 18519776]
7. Wu. KILLER/DR5 is a DNA damage-inducible p53-regulated death receptor gene. *Nat. Genet.* 17:141–143. [PubMed: 9326928]
8. Miyashita. Tumor suppressor p53 is a direct transcriptional activator of the human bax gene. *Cell.* 80:293–299. [PubMed: 7834749]
9. Ricci. Reduction of TRAIL-induced Mcl-1 and cIAP2 by c-Myc or sorafenib sensitizes resistant human cancer cells to TRAIL-induced death. *Cancer Cell.* 12:66–80. [PubMed: 17613437]

10. Levine. p53, the cellular gatekeeper for growth and division. *Cell*. 88:323–331. [PubMed: 9039259]
11. Geyer. Lapatinib plus capecitabine for HER2-positive advanced breast cancer. *N. Engl. J. Med*. 355:2733–2743. [PubMed: 17192538]
12. Ono. Sensitivity to gefitinib (Iressa, ZD1839) in non-small cell lung cancer cell lines correlates with dependence on the epidermal growth factor (EGF) receptor/extracellular signal-regulated kinase 1/2 and EGF receptor/Akt pathway for proliferation. *Mol. Cancer Ther*. 3:465–472. [PubMed: 15078990]
13. Niu, Carter. Human epidermal growth factor receptor 2 regulates angiopoietin-2 expression in breast cancer via AKT and mitogen-activated protein kinase pathways. *Cancer Res*. 67:1487–1493. [PubMed: 17308086]
14. Radsinski, Risin, Fan, Dong, Bielenberg, Bucana, Fidler. Level and function of epidermal growth factor receptor predict the metastatic potential of human colon carcinoma cells. *Clin. Cancer Res*. 1:19–31.
15. Spano, Lagorce, Atlan, Milano, Domont, Benamouzig, Attar, Benichou, Martin, Morere, Raphael, Penault-Llorca, Breau, Fagard, Khayat. Wind Impact of EGFR expression on colorectal cancer patient prognosis and survival. *Ann. Oncol*. 16:102–108. [PubMed: 15598946]
16. Ochs. Expression of vascular endothelial growth factor and HER2/neu in stage II colon cancer and correlation with survival. *Clin. Colorectal Cancer*. 4:262–267. [PubMed: 15555209]
17. Sobrero. EPIC: Phase III trial of cetuximab plus irinotecan after fluoropyrimidine and oxaliplatin failure in patients with metastatic colorectal cancer. *J. Clin. Oncol*. 26:2311–2319. [PubMed: 18390971]
18. Burris. Phase I safety, pharmacokinetics, and clinical activity study of lapatinib (GW572016), a reversible dual inhibitor of epidermal growth factor receptor tyrosine kinases, in heavily pretreated patients with metastatic carcinomas. *J. Clinical Oncol*. 23:5305–5313. [PubMed: 15955900]
19. Xia. Anti-tumor activity of GW572016: a dual tyrosine kinase inhibitor blocks EGF activation of EGFR/erbB2 and downstream Erk1/2 and AKT pathways. *Oncogene*. 21:6255–6263. [PubMed: 12214266]
20. Zhou. Blockade of EGFR and ErbB2 by the novel dual EGFR and ErbB2 tyrosine kinase inhibitor GW572016 sensitizes human colon carcinoma GEO cells to apoptosis. *Cancer Res*. 66:404–411. [PubMed: 16397255]
21. Pitti. Induction of apoptosis by Apo-2 ligand, a new member of the tumor necrosis factor cytokine family. *J. Biol. Chem*. 271:12687–12690. [PubMed: 8663110]
22. Jo, Kim, Seol, Esplen, Dorko, Billiar, Strom. Apoptosis induced in normal human hepatocytes by tumor necrosis factor-related apoptosis-inducing ligand. *Nat. Med*. 6:564–567. [PubMed: 10802713]
23. Schilling, Murray, Markowitz. Novel tumor necrosis factor toxic effects. Pulmonary hemorrhage and severe hepatic dysfunction. *Cancer*. 69:256–260. [PubMed: 1309306]
24. Ogasawara. Lethal effect of the anti-Fas antibody in mice. *Nature*. 364:806–809. [PubMed: 7689176]
25. Chien. A phase I study of a 2-day lapatinib chemosensitization pulse preceding nonopartical albumin-bound paclitaxel for advanced solid malignancies. *Clin Cancer Res*. 15:5569–5575. [PubMed: 19706807]
26. Pukac. HGS-ETR1, a fully human TRAIL-receptor 1 monoclonal antibody, induces cell death in multiple tumour types *in vitro* and *in vivo*. *Br. J. Cancer*. 92:1430–1441. [PubMed: 15846298]
27. Martin. Lapatinib resistance in HCT116 cells is mediated by elevated MCL-1 expression and decreased BAK activation and not by ERBB receptor kinase mutation. *Mol. Pharmacol*. 74:807–822. [PubMed: 18544666]
28. Kelly. Preclinical studies to predict the disposition of Apo2L/tumor necrosis factor-related apoptosis-inducing ligand in humans: characterization of *in vivo* efficacy, pharmacokinetics, and safety. *J. Pharmacol. Exp. Ther*. 299:31–38.
29. Foster, Coffey, Morin, Rastinejad. Pharmacological rescue of mutant p53 conformation and function. *Science*. 286:2507–2510. [PubMed: 10617466]

30. Bykov. Restoration of the tumor suppressor function to mutant p53 by a low-molecular-weight compound. *Nat. Med.* 8:282–288. [PubMed: 11875500]
31. Wang, Kim, El-Deiry. Small-molecule modulators of p53 family signaling and antitumor effects in p53-deficient human colon tumor xenografts. *Proc. Natl. Acad. Sci.* 103:11003–11008. [PubMed: 16835297]
32. Sheikh. P53-dependent and -independent regulation of the death receptor KILLER/DR5 gene expression in response to genotoxic stress and tumor necrosis factor alpha. *Cancer Res.* 58:1593–1598. [PubMed: 9563466]
33. Ravi. Regulation of death receptor expression and TRAIL/Apo2L-induced apoptosis by NF-kappaB. *Nat. Cell Biol.* 3:409–416. [PubMed: 11283615]
34. Zou. c-Jun NH2-terminal kinase-mediated up-regulation of death receptor 5 contributes to induction of apoptosis by the novel synthetic triterpenoid methyl-2-cyano-3,12-dioxooleana-1,9-dien-28-oate in human lung cancer cells. *Cancer Res.* 64:7570–7578. [PubMed: 15492284]
35. Hetschko. Upregulation of DR5 by proteasome inhibitors potentially sensitizes glioma cells to TRAIL-induced apoptosis. *FEBS J.* 275:1925–1936. [PubMed: 18341587]
36. Zhang, Li, Olumi. Low-dose 12-O-tetradecanoylphorbol-13-acetate enhances tumor necrosis factor related apoptosis-inducing ligand induced apoptosis in prostate cancer cells. *Clin. Cancer Res.* 13:7181–7190. [PubMed: 18056199]
37. Adler, Schaffer, Kim, Dolan, Ronai. UV irradiation and heat shock mediate JNK activation via alternate pathways. *J. Biol. Chem.* 270:26071–26077. [PubMed: 7592807]
38. Hertan, Koumenis. Piling up the JNK: drug synergy through ER stress. *Cancer Biol Ther.* 8:808–819. [PubMed: 19270531]
39. Ritter, Perez-Torres, Rinehart, Guix, Dugger, Engelman, Arteaga. Human breast cancer cells selected for resistance to trastuzumab *in vivo* overexpress epidermal growth factor receptor and ErbB ligands and remain dependent on the ErbB receptor network. *Clin Cancer Res.* 13:4909–4919. [PubMed: 17699871]
40. www.clinicaltrials.gov
41. Burris, Taylor, Jones, Koch, Versola, Arya, Fleming, Smith, Pandite, Spector, Wilding. A phase I and pharmacokinetic study of oral lapatinib administered once or twice daily in patients with solid malignancies. *Clin. Cancer Res.* 15:6702–6708. [PubMed: 19825948]
42. Shrader. Gefitinib reverses TRAIL resistance in human bladder cancer cell lines via inhibition of AKT-mediated X-linked inhibitor of apoptosis protein expression. *Cancer Res.* 67:1430–1435. [PubMed: 17308080]
43. Cuello. Down-regulation of the erbB-2 receptor by trastuzumab (herceptin) enhances tumor necrosis factor-related apoptosis-inducing ligand-mediated apoptosis in breast and ovarian cancer cell lines that overexpress erbB-2. *Cancer Res.* 61:4892–4900. [PubMed: 11406568]
44. Sah, Munshi, Kurland, McDonnell, Su, Meyn. Translation inhibitors sensitize prostate cancer cells to apoptosis induced by tumor necrosis factor-related apoptosis-inducing ligand (TRAIL) by activating c-Jun N-terminal kinase. *J. Biol. Chem.* 278:20593–20602. [PubMed: 12663665]
45. Shenoy, Wu, Pervaiz. LY303511 enhances TRAIL sensitivity of SHEP-1 neuroblastoma cells via hydrogen peroxide-mediated mitogen-activated protein kinase activation and up-regulation of death receptors. *Cancer Res.* 69:1941–1950. [PubMed: 19223550]
46. Chen, Chan, Waxman, Jing. Buthionine sulfoximine enhancement of arsenic trioxide-induced apoptosis in leukemia and lymphoma cells is mediated via activation of c-Jun NH2-terminal kinase and up-regulation of death receptors. *Cancer Res.* 66:11416–11423. [PubMed: 17145888]
47. Guan, Yue, Lotan, Sun. Evidence that the human death receptor 4 is regulated by activator protein 1. *Oncogene.* 21:3121–3129. [PubMed: 12082627]
48. Plummer. Phase 1 and pharmacokinetic study of lexatumumab in patients with advanced cancers. *Clin. Cancer Res.* 13:6187–6194. [PubMed: 17947486]
49. Kim. Death induction by recombinant native TRAIL and its prevention by a caspase 9 inhibitor in primary human esophageal epithelial cells. *J. Biol. Chem.* 279:40044–40052. [PubMed: 15226295]

Figure 1. Lapatinib enhances TRAIL-induced apoptosis in colon cancer cell lines

(A) Cell death (propidium iodide staining and sub-G1 DNA content) was quantified by FACS in SW620 cells treated with lapatinib for 48 hours and then an additional 24 hours in the presence of TNF (10 ng/ml), etoposide (20 μM), CPT-11 (30 μM), TRAIL (50 ng/ml), Adriamycin (0.2 μg/ml), cisplatin (20 μM), gemcitabine (20 μM), and 5'-fluorouracil (5-FU; 50 μM).

(B) Cell death (sub-G1 DNA content) and apoptosis (caspase-3) were quantified by FACS in SW620 colon carcinoma cells after pretreatment with lapatinib (10 μM) for 48 hours and exposure to soluble human TRAIL (50 ng/ml; 8 hours).

(C) Western blot processing of caspases and the caspase-3 substrate PARP in SW480-R6 cells treated with lapatinib, TRAIL, or the combination. Ran protein loading.

(D) Cell death (sub-G1 DNA content) was measured by FACS in SW620, SW480, SW480-R6, HT29, and DLD-1 human colon cancer cell lines lapatinib (1, 10, or 20 μ M; 48 hours) and TRAIL (50 ng/ml; 24 hours) treatment (mean $\pm$ S.E.; $*P < 0.05$ compared to DMSO + TRAIL group).

Figure 2. Lapatinib enhances TRAIL sensitivity *in vivo*

(A) SW620 xenograft tumors were established in nude mice by subcutaneous injection of 1×10^6 cells. When tumors reached a diameter of 10mm, mice were treated with oral lapatinib (40 mg/kg; 2 days), then given an intravenous dose of TRAIL (100 μ g). Twenty-four hours after TRAIL administration, animals were sacrificed and tumors were processed and stained with an antibody cleaved caspase-8.

(B) Quantification of cleaved caspase-8 positive cells per field. Data represent mean $\pm$ S.E. for 5 200X fields randomly chosen from one tumor (* $P < 0.05$; ** $P < 0.001$). Quantification was performed in 3 additional tumors with similar results.

(C) Mice bearing SW620 tumors were treated with three rounds of lapatinib (40 mg/kg) plus TRAIL (100 µg) the regimen described in (A). Tumor volumes were determined by weekly caliper measurements (* $P < 0.05$).

Figure 3. Lapatinib increases colon cancer cell sensitivity to TRAIL receptor-activating antibodies

(A) SDS-PAGE and blotting showing caspase-3 and PARP cleavage in SW480 cells pretreated with lapatinib (10 μ M) or vehicle (DMSO) for 48 hours, then exposed to TRAIL-R-activating antibodies (0.5 μ g/ml) for an additional 8 hours. As a reference, cells were treated with TRAIL (50 ng/ml) in the absence of lapatinib (lane 3). We observed cleaved caspase-3 fragments (CFs) at the predicted molecular weights of 17 and 19 kDa a smaller fragment of approximately 13 kDa.

(B) Cell viability was measured with a luciferase-based assay system in SW620 cells after treatment with 10 μ M lapatinib for 48 hours followed by TRAIL (50 ng/ml), mapatumumab

(0.5 µg/ml) or lexatumumab (0.5 µg/ml) for an additional 8 hours.(C). (*Top*) A representative bioluminescence image corresponding to cell viability is shown from HCT15 cells that were pretreated with 5 µM lapatinib for 24 hours and exposed to a range of mapatumumab and lexatumumab concentrations. (*Bottom*) Quantitative analysis of cell viability from 3 experiments is shown. (**P*<0.001)

(D) The same experiments and analyses as in (C) were conducted p53^{-/-} HCT116 cells. (**P*<0.001)

Figure 4. Lapatinib up-regulates death receptor content *in vitro* and *in vivo*

(A) DR4 and DR5 cell surface content was determined by FACS analysis in DMSO- and lapatinib-treated (10 μ M; 24 hrs) SW620 cells.

(B) Concentrations of death receptors, decoy receptors, and c-FLIP were examined by SDS-PAGE and blotting in lapatinib-treated SW620 cells (*FLIP_L*, long form; *FLIP_S*, short form). The small molecule inhibitor of EGFR (AG1478300 nM) was used as a control. Cells were treated for 48 hours.

(C) HCT15 cells were treated with lapatinib at the given concentrations for 24 hours. Western blots are shown.

(D) DR5 immunostaining SW620 colon cancer xenograft tumors from mice treated with vehicle (0 mg/kg), 40, 80, or 160 mg/kg lapatinib per day for 2 days. (E) SW620 cells were treated with 10 μ M lapatinib for 16 hours. DR5 and GAPDH mRNA were measured by RT-PCR.

(F) SW620 and *p53*^{-/-} HCT116 cells were treated with lapatinib (10 μ M) for 16 hours and DR5 mRNA was measured by qPCR. DR5 content was normalized to actin and presented relative to control (DMSO). As a positive control, cells were treated with a wild-type p53 adenovirus for 8 hours and 15 μ M CPT-11 for an additional 16 hours. (**P*<0.05)

(G) DR4 shRNA. Western blots are shown.

(H) Mice bearing HCT116 xenograft tumors were treated with lapatinib (80 mg/kg, p.o.) twice weekly on consecutive days. Images from representative micewere taken after 3 weeks of treatment.

(I) Mice bearing tumor xenografts from HCT116 cells expressing control or DR4-targeted shRNA were treated with lapatinib (80 mg/kg, p.o.) twice weekly on consecutive days. Tumor growth was monitored by caliper measurements. (**P*<0.05)

(J) Cleaved caspase-8 immunostaining in control or DR4 shRNA HCT116 xenografted tumors that were treated as in (H). Tumors were analyzed after the 3rd week of treatment.

Figure 5. Lapatinib up-regulates death receptor expression and enhances TRAIL sensitivity independently of EGFR and HER2 inhibition

(A) Western blotting analysis of phosphorylated/active HER2 and EGFR in SW620 and HCT116 cells. The concentrations of EGFR (AG1478; 500 nM) and HER2 (AG825; 5 μ M) inhibitors completely inhibit EGF (60 ng/ml) stimulated HER2 and EGFR phosphorylation in HCT116 cells.

(B) (Left) SW480 cells were pretreated with varying concentrations of lapatinib for 24 hours followed by EGF (60 ng/ml) stimulation for 15 minutes. Western blots are shown. (Right) SW480 cells were treated with varying concentrations of lapatinib for 48 hours. Western blots are shown. AG1478 (500 nM) was included as a control compound.

(C) SW620 cells were treated with lapatinib (10 μ M) for varying time points, or with AG1478 (500 nM) and AG825 (5 μ M), alone and in combination for 48 hours. Western blots are shown. HER2 serves as the loading control.

(D) Cell viability, as determined by Coomassie Blue staining, was assessed in SW620 cells treated with lapatinib (10 μ M) or the combination of AG1478 (500 nM) and AG825 (5 μ M), in the presence and absence of TRAIL (50 ng/ml).

(E) SW620 cells were treated with lapatinib (10 μ M) or the combination of AG1478 and AG825 for 16 hours. Quantitative RT-PCR for DR5 is shown. (* $P < 0.05$)

(F) HCT15 cells were treated with lapatinib (10 μ M) or the combinations of AG1478 and AG825 or AG1478 and trastuzumab (Herceptin; 40 μ g/ml). Western blots are shown.

(G) HCT15 cells were pretreated with lapatinib (10 μ M) or the combinations of AG1478 and AG825 or AG1478 and trastuzumab (Herceptin) for 24 hours, followed by EGF (60 ng/ml) stimulation for 30 minutes. Western blots are shown.

Figure 6. Lapatinib induces JNK/c-Jun pathway signaling, which is required for DR5 up-regulation

(A) SW620 cells were treated with 10 μ M lapatinib for 24 hrs. Western blots are shown.

(B) SW620 cells were treated for 24 hrs with lapatinib or the combination of AG1478 and AG825 (500 nM; 5 μ M). Western blots are shown.

(C) Mice bearing HCT15 xenografted tumors were treated with 40 mg/kg oral lapatinib for 2 consecutive days. Tumors were analyzed for c-Jun expression (inset of blue box; V: blood vessel lumen).

(D) HCT116 p53^{-/-} cells were treated with siRNA targeted to ERK1/2, JNK2 or c-Jun followed by treatment with lapatinib (10 μ M; 24 hrs). Western blots are shown.

(E) DLD-1 cells were treated with control or c-Jun-directed siRNA, then exposed to lapatinib for 24 hrs. Western blots are shown (top) along with quantification of DR5 protein densitometry from 3 experiments (bottom). (* $P < 0.05$)

(F) Schematic of lapatinib activity. Red text and arrows denote off-target effects.