Dual mTORC1/2 and HER2 blockade results in antitumor activity in preclinical models of breast cancer resistant to anti-HER2 therapy

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Statement of translational relevance

Despite the success of anti-HER2 therapies, patients with HER2 positive breast cancer frequently present with either primary or acquired resistance to these agents. Thus, novel therapeutic strategies that can overcome such resistance are urgently needed. Recent reports suggest that inhibitors of the PI3K/Akt/mTOR pathway enhance the antitumor activity of compounds such as trastuzumab or lapatinib. However, these possible combinations have not been extensively studied in models of breast cancer that are refractory to anti-HER2 therapy.

Our results provide evidence that concomitant inhibition of HER2 phosphorylation and PI3K/Akt/mTOR signaling is synergistic in reducing cell viability and tumor growth in a number of trastuzumab/lapatinib resistant models. Lapatinib given in combination with INK-128 is one example of a potential combinatorial treatment available in the clinical management of HER2 positive patients who fail to respond to standard therapy. Based on our results, further clinical investigation of this therapeutic regimen is warranted.
Abstract

**Purpose:** The PI3K/Akt/mTOR pathway is an attractive target in HER2 positive breast cancer that is refractory to anti-HER2 therapy. The hypothesis is that suppression of this pathway results in sensitization to anti-HER2 agents. However, this combinatorial strategy has not been comprehensively tested in models of trastuzumab and lapatinib resistance.

**Experimental Design:** We analyzed *in vitro* cell viability and induction of apoptosis in five different cell lines resistant to trastuzumab and lapatinib. Inhibition of HER2/HER3 phosphorylation, PI3K/Akt/mTOR and ERK signaling pathways was evaluated by western blot. Tumor growth inhibition following treatment with lapatinib, INK-128 or the combination of both agents was evaluated in three different animal models: two cell-based xenograft models refractory to both trastuzumab and lapatinib, and a xenograft derived from a patient who relapsed on trastuzumab-based therapy.

**Results:** The addition of lapatinib to INK-128 prevented both HER2 and HER3 phosphorylation induced by INK-128, resulting in inhibition of both PI3K/Akt/mTOR and ERK pathways. This dual blockade produced synergistic induction of cell death in five different HER2 positive cell lines resistant to trastuzumab and lapatinib. *In vivo*, both cell line-based and patient-derived xenografts showed exquisite sensitivity to the antitumor activity of the combination of lapatinib and INK-128, which resulted in durable tumor shrinkage and exhibited no signs of toxicity in these models.

**Conclusions:** The simultaneous blockade of both PI3K/Akt/mTOR and ERK pathways obtained by combining lapatinib with INK-128 acts synergistically in inducing cell death and tumor regression in breast cancer models refractory to anti-HER2 therapy.
Introduction

Amplification of the receptor tyrosine kinase (RTK) HER2 has been reported in approximately one fourth of human breast tumors, and its overexpression is associated with a more aggressive phenotype and poor clinical outcome (1, 2). There are two main therapeutic strategies employed to target HER2 positive cells: the first are monoclonal antibodies targeting the extracellular domain of the receptor (trastuzumab, pertuzumab); the second small molecule ATP competitors that inhibit the phosphorylation of the HER2 intracellular kinase domain (lapatinib, neratinib). Trastuzumab, in combination with chemotherapy, has demonstrated a robust improvement in progression-free survival and overall survival in cases of advanced disease (3, 4) as well as in the early (adjuvant) setting (5-7). Lapatinib, when given in combination with capecitabine, significantly improves time to progression in HER2-positive breast cancer patients that have progressed on trastuzumab-based therapy (8). Moreover, lapatinib as monotherapy and in combination with paclitaxel has clinical activity as first-line treatment in HER2-positive breast cancer patients (9, 10). Recently, our group and others reported that combinations of anti-HER2 agents with non-overlapping mechanisms of action, so called dual HER2 blockade, is superior to treatment with single agent trastuzumab (11-14) and is active in patients that are resistant to trastuzumab-based therapies (15, 16).

Despite the remarkable success of anti-HER2 therapies, patients with advanced HER2 positive breast cancer frequently display primary resistance and, in patients initially sensitive to these agents, acquired resistance may emerge over time.

To date, known mechanisms of resistance to anti-HER2 agents include aberrant activation of the downstream phosphatidylinositol-3-kinase (PI3K) (17-19) and Src (20) pathways, co-expression of the truncated p95HER2 receptor (21), co-expression and dimerization with other RTKs (22, 23) and cyclin E amplification (24). These mechanisms are clinically relevant (17, 18, 20, 21, 24), therefore specific therapeutic approaches are being investigated in the patients (25).
The PI3K pathway lies directly downstream of HER2 and its aberrant activation, either due to mutations of the PIK3CA gene or through loss of phosphatase and tensin homolog (PTEN) expression, have been found to limit the sensitivity to either antibody or receptor tyrosine kinase inhibitors-based therapies (17-19, 26). Targeting the PI3K pathway in combination with anti-HER2 agents therefore appears to be a compelling therapeutic strategy.

It is well understood that PI3K signaling plays a critical role in cell growth, survival and metabolism due to the numerous effectors regulated by this pathway (27). Moreover, recent studies conducted by both our group and others have elucidated feedback mechanisms of PI3K signaling that subvert inhibition of PI3K/Akt/mTOR by reactivating upstream PI3K pathway components as well as the ERK pathway. In some breast cancer models, mTORC1/p70S6K1 activity regulates IGF1R function through negative feedback involving IRS-1 and Grb10 (28, 29). Inhibition of mTORC1 leads to disruption of this feed-back loop and, ultimately, to hyperactivation of PI3K/Akt. A second compensatory mechanism occurs through the inhibition of PI3K/Akt in HER2 positive breast cancer activating ERK1/ERK2 via increased RTKs expression and dimerization (30-32). These studies suggest that combined HER2 and PI3K/Akt/mTOR inhibition would be superior to single agent therapy. However, whether this combinatorial strategy will be effective in diverse scenarios of trastuzumab and lapatinib resistance still remains to be elucidated. One limitation of this pharmacological approach is predicted to be the increased toxicity observed in vivo (30).

In this study we investigated the antitumor activity and feasibility of dual blockade of HER2 and the PI3K/Akt/mTOR pathway in several models of resistance to anti-HER2 therapy. Since the activation of Akt requires mTORC2 (33), we chose to target both Akt and mTOR using INK-128, a potent and selective ATP competitor of both mTORC1 and mTORC2 currently in clinical development (34). To concomitantly inhibit HER receptors phosphorylation we used lapatinib.
Here we demonstrate that lapatinib in combination with INK-128 profoundly inhibits both the PI3K/Akt/mTOR and ERK pathways resulting in apoptosis in vitro and in vivo tumor shrinkage in all models tested. Importantly, no signs of toxicity were observed in our in vivo experiments.

These data indicate that it is possible to achieve durable regressive responses in tumor models refractory to anti-HER2 therapy by inhibiting both the PI3K/Akt/mTOR and HER2 pathways without signs of toxicity.
Materials and methods

Cell lines and treatments

Cell lines, except as otherwise indicated, were obtained from the American Type Culture Collection (Rockville, MD, USA). The BT474 cell line was maintained in DMEM/F12 supplemented with 10% fetal bovine serum and 2 mM L-glutamine (Live Technologies, Inc. Ltd.). SKBR3, HCC-1954 and JIMT1 cell lines were maintained, in McCoy, RPMI (Live Technologies, Inc. Ltd.) and DMEM high glucose (PAA Laboratories GmbH, Pasching, Austria) respectively, supplemented with 10% fetal bovine serum. The KPL4 cell line, kindly provided by Prof. Kurebayashi (Kawasaki Medical School, Okayama, Japan), was maintained in DMEM (Sigma-Aldrich, MO, USA) supplemented with 5% fetal bovine serum. MDA-MB361 and MDA-MB453 were maintained in L-15 (Live Technologies, Inc. Ltd.) supplemented, with 20% and 10% fetal bovine serum respectively. All the cell lines were maintained at 37 °C in 5% CO₂, except for MDA-MB361 and MDA-MB453 that were maintained at 37°C in the absence of CO₂. Mutational status of PI3K and expression of PTEN and EGFR by the cell lines have been previously described (Cosmic Database, (35, 36)). The mTORC1/2 inhibitor INK-128 was kindly provided by Intellikine Inc. (La Jolla, CA). Lapatinib (Tykerb) was kindly provided by GlaxoSmithKline (GSK, Hertfordshire, UK). Both compounds were dissolved in dimethyl sulfoxide for in vitro experiments.

Western blots

Cells were grown in 60 mm dishes and treated with lapatinib, INK-128 or a combination of both for the indicated concentrations and times. Cells were washed with ice-cold PBS and scraped into ice-cold lysis buffer (TRIS-HCl pH 7.8 20 mM, NaCl 137 mM, EDTA pH 8.0 2 mM, NP40 1%, glycerol 10%, supplemented with NaF 10 mM, Leupeptin 10 μg/mL, Na₂VO₄ 200 μmol/L, PMSF 5 mM and Aprotinin (Sigma-Aldrich, MO, USA). Lysates were cleared by centrifugation at 13,000 rpm for 10 min at 4°C, and supernatants removed and assayed for protein concentration using the Pierce BCA
Protein Assay Kit (Thermo Scientific, IL, USA). Thirty micrograms of total lysate was resolved by SDS-PAGE, and electrophoretically transferred to nitrocellulose membranes. Membranes were then hybridized using the following primary antibodies: pAkt (Ser473), pAkt (Thr308), Akt, pS6 (Ser240/244), pS6 (Ser235/236), p4EBP1 (Thr37/46), p4EBP1 (Ser65), p4EBP1 (Thr70), 4EBP1, pFOXO1/3A (Thr24-Thr32), pPRAS40 (Thr246), PRAS40, pERK (Thr202/Tyr204), ERK, pHER3 (Tyr1289), HER3, pHER2 (Tyr1212/1222), HER2, cleaved PARP, cleaved caspase 3, cleaved caspase 7, Tubulin, (Cell Signaling, MA, USA), pan-cytokeratin (Sigma-Aldrich, MO, USA) in 5% bovine serum albumin and HER2 (BioGenex Laboratories Inc., CA, USA) and HER3 (Thermo Scientific, IL, USA), in 1% nonfat dry milk. Mouse and rabbit horseradish peroxidase–conjugated secondary antibodies (Amersham Biosciences) were used at 1:2000 in TBS-T/1% nonfat dry milk. Protein-antibody complexes were detected by chemiluminescence with the Immobilon Western HRP Substrate (Millipore, MA, USA) and images were captured with a FUJIFILM LASS-3000 camera system.

**Determination of IC50 and synergism**

Cells were seeded in 96 well plates and treated with escalating doses of lapatinib and INK-128 (100-10-1-0.1-0.01nM) as single agents or in 1:1 combinations. After 6 days of treatment, cell proliferation was analyzed with CellTiter-Glo Luminescent Cell Viability Assay (Promega, WI, USA) as described by the manufacturer. IC50s were calculated using GraphPad Prism (GraphPad Software, La Jolla, USA) and synergism was determined using CompuSyn (ComboSyn Inc., NJ, USA) (37).

**Determination of cell cycle and apoptosis**

Cell cycle and hypodiploid apoptotic cells were quantified by flow cytometry as described in (38). Briefly, cells were washed with phosphate-buffered saline (PBS), fixed in cold 70% ethanol, and then stained with propidium iodide while treating with RNase (Sigma-Aldrich, MO, USA). Quantitative analysis of sub-G1 cells was carried
Establishment of tumor xenografts in nude mice

Mice were maintained and treated in accordance with institutional guidelines of Vall d’Hebron University Hospital Care and Use Committee. Six week old female athymic nude HsdCpb:NMRI-Foxn1nu mice were purchased from Harlan Laboratories (Italy). Mice were housed in air-filtered laminar flow cabinets with a 12-h light cycle and food and water ad libitum.

MDA-MB361 or JIMT1 cells were resuspended in sterile PBS and mixed 1:1 with Matrigel (BD Biosciences, NJ, USA) before subcutaneous injection at a final concentration of 2x10^7 cells/200uL per mouse. For MDA-MB361 xenografts, a 17β-estradiol pellet (0.72 mg/pellet, 60 days of release; Innovative Research of America) was implanted subcutaneously into each mouse one day before cell injection.

For patient-derived xenografts, patient consent for tumor use in animals was obtained under a protocol approved by the Vall d’Hebron Hospital Clinical Investigation Ethical Committee and Animal Use Committee. Tumors were subcutaneously implanted in 6 week old female HsdCpb:NMRI-Foxn1nu mice (Harlan Laboratories, Italy). Animals were supplemented with 1μM 17β-estradiol (Sigma) in their drinking water, an amount demonstrated to be sufficient to reach serum levels and uterine growth in ovariectomized female mice similar to the ones obtained with other mechanisms of 17β-estradiol supplementation (39, 40). Upon xenograft growth, tumor tissue was re-implanted into recipient mice, which were randomized upon implant growth.

In vivo treatment study

Cell line-derived xenografts: growth took approximately 2-3 weeks prior to animal randomization at a volume of 200-300 mm^3. Animals were randomized to 4 groups, consisting of 6-9 mice per group. Patient-derived xenografts: growth of subcutaneous...
implants took approximately three weeks prior to animal randomization at a volume of 100-200 mm$^3$. Animals were randomized into 5 groups, consisting of 6-8 mice per group.

Animals were treated with INK-128 6QD/W (0.3 mg/kg in 5% NMP, 15% PVP in water, oral gavage), lapatinib 6QD/W (150 mg/kg in 0.5% HPMC, 0.1% Tween 80 in water, oral gavage), or trastuzumab 2QD/W (10mg/kg, in water, intraperitoneal injection).

Tumor xenografts were measured with calipers and tumor volumes were determined using the formula: (length $\times$ width$^2$) $\times$ ($\pi/6$). At the end of the experiment, animals were euthanized using CO$_2$ inhalation. Tumor volumes are plotted as means $\pm$ SE.

For western blotting, lysates from two different tumors derived from each treatment were processed as described above.

**Statistical analysis**

Two-way ANOVA with Bonferroni post-test was performed using GraphPad Prism (GraphPad Software, La Jolla, USA). Error bars represent the SE. All experiments were repeated at least three times.
Results

*In vitro* activity of anti-HER2 and mTORC1/2 therapy in HER2 positive cell lines

In order to test the effects of dual mTORC1/2 blockade in HER2 positive cells, we screened a panel of seven HER2 positive breast cancer cell lines for both lapatinib and INK-128 sensitivity using an ATP-based cell viability assay (Fig 1A and 1B). After six days of treatment, we observed varying sensitivity to lapatinib, with SKBR-3 and BT474 cells being significantly more sensitive than were MDA-MB361, MDA-MB453, KPL4, HCC-1954 and JIMT1 cell lines (Fig 1A). Moreover, the latter five cell lines were completely refractory to the antiproliferative effects of trastuzumab (data not shown). Given these striking differences in anti-HER2 sensitivity, we defined BT474 and SKBR-3 cell lines as sensitive and MDA-MB361, MDA-MB453, KPL4, HCC1954 and JIMT1 cell lines as resistant (Fig 1A). While we acknowledge that lower lapatinib IC50s have been previously reported for MDA-MB361 cells (36, 41, 42) all five resistant cell lines tested were found to harbor activating alterations of the PI3K pathway (PI3K mutations, low/loss of expression of PTEN or both), whereas the two sensitive cell lines did not (with the exception of the non-activating K111N PIK3CA mutation (43) present in BT474 cells). A detailed biochemical analysis of the sensitive cell lines confirmed that both the PI3K/Akt/mTOR and ERK pathways were profoundly suppressed at low concentrations of lapatinib (Supplementary Fig 1). In contrast, the same molecular markers, especially the ones related to the PI3K/Akt/mTOR axis, were only moderately affected by HER2 inhibition in the resistant cells (Supplementary Fig 2). When treated with INK-128, the proliferation of all seven cell lines was significantly inhibited at low nanomolar ranges (Fig 1B).

In analyzing these data, we reasoned that the use of lapatinib in combination with a dual mTORC1/2 inhibitor, such as INK-128, would result in enhanced antiproliferative activity. Possible synergism between lapatinib and INK-128 in lapatinib-resistant cell lines was evaluated using the combination index (CI) (37). In all five cell lines tested the combination of INK-128 and lapatinib demonstrated synergistic activity (CI lower
than 1) (Table 1 and Supplementary Fig 3A). In particular, the best efficacy of this combination was observed at concentrations between 0.1 and 10nM, depending on the cell lines (Supplementary Fig 3B).

**Dual PI3K/mTOR and ERK suppression combining INK-128 with lapatinib**

It has been previously described by our group and others that PI3K/Akt/mTOR pathway inhibition may result in the activation of compensatory pathways that could reduce the antiproliferative activity of these inhibitors (28, 31, 44-46). As expected, we observed reproducible increases in HER2/HER3 phosphorylation following INK-128 treatment in all the tested cell lines (Fig 2 and data not shown). This phenomenon coincided with ERK activation, which was more evident at the 2h timepoint.

The combination of lapatinib with INK-128, tested in three lapatinib/trastuzumab-resistant cell lines, was demonstrated to effectively prevent HERs phosphorylation, to suppress PI3K/Akt/mTOR signaling, and to limit ERK phosphorylation consequent to INK-128 treatment (Fig 2).

**Cell cycle analysis and apoptosis in trastuzumab/lapatinib resistant cell lines treated with lapatinib and INK-128**

The in vitro synergistic effects summarized in Table 1 prompted us to investigate both cell cycle progression and apoptosis following treatments with lapatinib, INK-128 and the combination of both. Cell cycle progression of HCC-1954, KPL4 and MDA-MB453 cell lines was affected by both lapatinib and INK-128 (Supplementary Fig 4A, 4B and 4C). Each inhibitor alone was sufficient to delay the cell cycle (increased G1 phase) of these cells and the combination of both treatments further enhanced these effects. No major changes in cell cycle progression were observed in the other two resistant cell lines tested (Supplementary Fig 4D and 4E).

We then analyzed the proapoptotic effects of these compounds, as single agents and in combination. Treatments with either lapatinib or INK-128 variably increased the
percentage of apoptotic cells in lapatinib resistant cells (Fig 3A and Supplementary Fig 5 and 6). The combination of both agents, however, exacerbated the induction of cell death. In Fig 3B we confirm that this biological effect is accompanied by higher activation of both caspase 3 and 7 along with increased PARP cleavage. Therefore, whereas cell-cycle arrest is observed in some models treated with lapatinib and INK-128, enhanced cell death is a common feature of this combination in vitro.

**Antitumor activity of lapatinib and INK-128 in cell-based trastuzumab/lapatinib resistant xenograft models**

To expand our findings in vivo we determined the activity of INK-128, lapatinib or the combination in different animal models. We first analyzed the tumor growth of MDA-MB361 xenografts under the therapeutic conditions shown in Fig 4A. As expected from the in vitro findings, MDA-MB361 xenografts scarcely responded to lapatinib. While an initial response to INK-128 was observed, the xenografts eventually gained resistance after 20 days of treatment. In contrast, the combination of lapatinib and INK-128 induced long-lasting tumor regression. Specifically, 40% of the mice in this combination arm experienced complete tumor shrinkage. To evaluate the degree of signaling inhibition in these tumors we conducted pharmacodynamic studies, treating the tumors for 3 days with lapatinib, INK-128 and the combination of both agents. In Fig 4B we show by western blot that lapatinib resulted in marked inhibition of ERK phosphorylation but only moderately affected the PI3K/mTOR pathway. Conversely, treatment with INK-128 resulted in a reduction of pAkt and downstream pS6 and p4EBP1 compared to lapatinib, with no demonstrable effects on the ERK pathway. Importantly, significant inhibition of both pathways was observed in the samples treated with the combination.

Previous experiments combining lapatinib with other inhibitors of the PI3K/mTOR pathway were run for shorter times or were limited by in vivo toxicity (31, 46, 47). In our hands, the antitumor activity of INK-128 plus lapatinib was accompanied by an
absence of toxicity (defined as weight loss or lethargy) after 80 days of continuous administration with the drugs (Fig 4C).

The *in vivo* efficacy of dual HER2 and PI3K/Akt/mTOR inhibition was also confirmed in xenografts derived from JIMT1 cells (Supplementary Fig7). Despite the absence of complete tumor shrinkage in this trastuzumab/lapatinib resistant model, we reproducibly observed superior efficacy of the combination of lapatinib and INK-128 as compared to treatment with a single agent.

**Antitumor activity of lapatinib and INK-128 in a trastuzumab resistant patient-derived xenograft**

To better recapitulate the clinical setting, we tested the activity of lapatinib and INK-128 in a HER2 positive, patient-derived-xenograft (PDX) model. This was derived from a biopsy of a patient who relapsed after trastuzumab-based therapy (see Materials and Methods). We have previously shown that both morphologic and gene expression signatures of the original surgical samples are maintained in our PDX models (48). Deriving from a patient who became refractory to trastuzumab-based therapy, we first confirmed that this PDX model was unresponsive to trastuzumab therapy (Supplementary Fig 8). We then tested the tumor activity of lapatinib, INK-128 and the combination of both. As shown in Fig 5A, patient derived-tumors showed initial response to INK-128, but became partially resistant after approximately 20 days of treatment, recapitulating the results obtained with the MDA-MB361 xenograft model (Fig 4A). Conversely, tumor regression induced by lapatinib was maintained over time, although this regimen never achieved complete tumor shrinkage. Despite the strong antitumor effects exhibited by lapatinib when used as single agent, its activity was enhanced by the addition of INK-128. Superior (and more rapid) tumor regression was achieved by dual HER2 and PI3K/Akt/mTOR blockade, compared with lapatinib as a single agent, and complete tumor shrinkage was observed in two out of six mice. By western blot analysis we confirmed that only tumor samples exposed to the
combination therapy demonstrated significant inhibition of both PI3K/Akt/mTOR and ERK pathways (Fig 5B).
Discussion

Hyperactivation of the PI3K/Akt/mTOR pathway has previously been reported to negatively correlate with sensitivity to anti-HER2 therapy in breast cancer (17, 18, 49). Direct inhibition of PI3K/AKT/mTOR signaling is therefore an attractive clinical strategy for this disease. Insufficient target inhibition and/or activation of compensatory pathways that diminish the efficacy of these agents are, however, predictable limitations to the clinical development of PI3K/Akt/mTOR inhibitors. Combination therapies in which both the PI3K/Akt/mTOR pathway and key reactivated targets are inhibited together may result in improved efficacy. Rapalogs, for example, lead to the hyperactivation of Akt by relieving p70S6 kinase driven suppression of the IGF-IR signaling (28). Dual mTORC1 and IGF-IR receptor blockade has been tested in estrogen receptor (ER) positive breast cancer with encouraging results (50). Some light has also been shed recently on cross-talk between the PI3K/Akt/mTOR pathway and ER signaling. Supplementation of estradiol seems to protect from the proapoptotic effects of PI3K/Akt/mTOR inhibition (51) whereas mTORC1 was described to activate ER in a ligand-independent fashion (52). Moreover, hyperactivation of the PI3K/Akt/mTOR pathway was observed in endocrine resistant breast cancer cells (53). These findings provided the rationale to test combined mTORC1 and ER blockade in the clinic, with significant improvement of progression-free survival in ER positive patients who recurred following anti-estrogen therapy (54). In addition, inhibition of mTORC1 and/or Akt has been described to increase the phosphorylation of both EGFR and HER3 in both trastuzumab sensitive and resistant cell lines (55). In their work, Miller et al showed that a combined HER2 and mTORC1 blockade was more effective than single agents at inducing tumor regression and inhibiting cell growth. In three independent studies, trastuzumab and the mTORC1 inhibitor everolimus showed encouraging antitumor activity in heavily pretreated trastuzumab resistant patients (56-58). In a similar fashion, an RTK-dependent activation of ERK1 and ERK2 in response to PI3K/Akt/mTOR inhibition has also been described in preclinical models of HER2
positive breast tumors (30-32). In these cases, the combination of PI3K/Akt/mTOR inhibitors with either an anti-HER2 agent or a MEK inhibitor was superior to single agents.

Although there is evidence that both HER2 and PI3K/Akt/mTOR signaling need to be suppressed to achieve optimal therapeutic activity, this combinatorial strategy has not been extensively tested in tumors resistant to anti-HER2 therapy. In the present study we demonstrate the validity of this approach, clearly showing that the combination of lapatinib with INK-128 results in synergistic reduction of cell viability in 5 trastuzumab/lapatinib resistant cell lines, along with strong antitumor activity in 3 different in vivo models of breast cancer refractory to anti-HER2 therapy. Despite the previously described lack of correlation between PIK3CA mutations or loss of PTEN and sensitivity to lapatinib (36), we observe a trend in this direction. However, due to the small cohort of cell lines and the lower threshold to define lapatinib resistance (250nM versus 1µM) we used in this work, no definitive conclusions can be made regarding this issue.

Concomitant inhibition of the PI3K/Akt/mTOR and ERK pathways may function as a synthetic lethality-like effect in this setting, depriving the cells/tumors of the main survival stimuli downstream of HER2.

Intriguingly, our data also indicate that in tumors bearing wild type PIK3CA and normal PTEN expression, the combination of INK-128 and lapatinib is superior to single agent treatment. Despite retaining sensitivity to lapatinib, our available HER2 positive PDX model benefited from the addition of INK-128, both in terms of rapidity and intensity of response. This phenomenon could have relevant clinical implications in those patients who respond to anti-HER2 therapy, but without achieving significant tumor shrinkage.

Another important issue frequently raised in the clinic is the toxicity related to single or combined targeted therapy. In previous preclinical studies conducted in nude mice, the combination of lapatinib with inhibitors of the PI3K/Akt/mTOR pathway proved to be difficult to manage (or impracticable) due to skin toxicity and excessive weight loss (30,
31). Conversely in our hands, the combination of lapatinib and INK-128 in the same animal models did not lead to any sign of toxicity even after 80 days of treatment. It is still unclear whether this significant improvement was due to the high activity of INK-128 despite the low dosage used in our experiments (0.3mg/kg), or to the optimal pharmacokinetic characteristics of this compound.

There are currently three clinical trials of dose escalation studies with INK-128 recruiting patients. One of them is being conducted in HER2 positive breast cancer patients evaluating the safety and preliminary anti-tumor activity of the combination of INK-128, paclitaxel, and weekly trastuzumab (http://clinicaltrials.gov/ct2/show/NCT01351350?term=INK128&rank=1). It would be interesting to know whether the same patient population, who eventually relapse when treated with trastuzumab-based therapy, would benefit by combining lapatinib with INK-128.

In conclusion, we propose that the simultaneous blockade of the PI3K/Akt/mTOR and ERK pathways achieved by combining lapatinib with INK-128 acts synergistically in inducing cell death and tumor regression in breast cancer refractory to anti-HER2 therapy.
References


Tables
Table 1

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<tr>
<th>Cell line</th>
<th>CI</th>
<th>Description</th>
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<tr>
<td>MDA-MB361</td>
<td>.052 ± .018</td>
<td>Strong synergism</td>
</tr>
<tr>
<td>MDA-MB453</td>
<td>.183 ± .187</td>
<td>Synergism</td>
</tr>
<tr>
<td>KPL-4</td>
<td>.090 ± .073</td>
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</tr>
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<td>HCC-1954</td>
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<tr>
<td>JIMT1</td>
<td>.224 ± .054</td>
<td>Strong synergism</td>
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Tables and figures legends

Table 1: Combination index (CI) of the combination of lapatinib and INK-128 in resistant cell lines

Trastuzumab/lapatinib resistant HER2 positive breast cancer cell lines were treated with escalating doses of lapatinib, INK-128 (INK) or the combination. Viability was measured after 6 days of treatment with a cell viability assay (Cell Titler-Glo, Promega), and combination index (CI) was calculated using Compusyn computer program (Fa=0.9).

Figure 1: In vitro sensitivity to lapatinib and INK-128 of HER2 positive breast cancer cell lines

A. A panel of HER2 positive breast cancer cell lines was treated with escalating doses of lapatinib. B. The same panel of HER2 positive breast cancer cell lines was treated with escalating doses of INK-128. Proliferation was measured after 6 days of treatment with a cell viability assay (Cell Titler-Glo, Promega). EC50 (half maximal effective concentration) was calculated using GraphPad Prism software. Data are expressed as mean ± S.E from three independent experiments. Mutational status of PI3K and expression of PTEN and EGFR of the cell lines have been previously described (Cosmic Database, (35, 36)).
Figure 2: Biochemical analyses of trastuzumab/lapatinib resistant cells treated with lapatinib and INK-128

Trastuzumab/lapatinib resistant HER2 positive cell lines were treated with 50nM INK-128 (INK), 1μM lapatinib (L) or both (I+L) for 2 or 24 hours. Whole-cell protein extracts were analyzed with the indicated antibodies. Figures are representative of three independent experiments.

Figure 3: *In vitro* cell death induced by lapatinib and INK-128 of trastuzumab/lapatinib resistant cell lines

The indicated trastuzumab/lapatinib resistant cell lines were treated with 1μM lapatinib (Lapatinib, L), 50nM INK-128 (INK-128, INK) or the combination (INK+Lap, I+L). A. Apoptosis after 48 hours was analyzed by flow cytometry and measured as percentage of cells with subG1 DNA content. Data are expressed as mean ± S.E from three independent experiments. *** p<.001 compare with all columns. B. Whole-cell protein extracts collected after 24h were analyzed by western blot with the indicated antibodies. Tubulin antibody was used as loading control. Figures are representative of three independent experiments.

Figure 4: *In vivo* treatments of MDA-MB361 xenografts

A. Mice bearing MDA-MB361 tumors were treated with vehicle control (Control, C), lapatinib (Lapatinib, Lap, 150 mg/kg 6Q.D.), INK-128 (INK-128, INK, 0.3 mg/kg 6Q.D.) or the combination (INK+Lap, I+L) for 80 days. Measurements are displayed as mean ± S.E. *p<.05 versus Control and Lapatinib arms and ** p< .01 versus control, Lapatinib and INK-128 arms at day 23. B. MDA-MB361 tumors after 3 days of treatment were collected and analyzed by western blot with the indicated antibodies. A pan-cytokeratine antibody was used as loading control. C. Variation in body weights of the mice treated as in panel A.
Figure 5: Lapatinib and INK-128 treatments in trastuzumab-resistant patient derived-xenografts

A. Mice bearing HER2 positive patient-derived xenografts were treated with vehicle control (Control, C), lapatinib (Lapatinib, Lap, 150 mg/kg 6Q.D.), INK-128 (INK-128, INK, 0.3 mg/kg 6Q.D.) or the combination (INK+Lap, I+L) for 68 days. Measurements are displayed as mean ± S.E. * p<.05 versus INK-128 and p<.01 versus Control, ** p< 0.01 versus INK-128 and p<.001 versus Control. B. Patient-derived tumors after 3 days of treatment were collected and analyzed by western blot with the indicated antibodies. A pan-cytokeratine antibody was used as loading control.
Figure 2

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Figure 4

A

MDA-MB361 Xenografts

B

3 QD

C

MDA-MB361 Xenografts

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Figure 5

A

HER2+ PDXs

Days

Tumor volume (fold increase)

Control
Lapatinib
INK-128
INK+Lap

B

3 QD

C    LAP    INK    I+L

pHER2
pHER3
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pAKT (473)
p4EBP1 (37/46)
p4EBP1 (65)
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Cytokeratin
Clinical Cancer Research

Dual mTORC1/2 and HER2 blockade results in antitumor activity in preclinical models of breast cancer resistant to anti-HER2 therapy

Celina Garcia, Yasir H Ibrahim, Violeta Serra, et al.

Clin Cancer Res  Published OnlineFirst March 8, 2012.

Updated version  Access the most recent version of this article at: doi:10.1158/1078-0432.CCR-11-2750

Supplementary Material  Access the most recent supplemental material at: http://clincancerres.aacrjournals.org/content/suppl/2012/03/22/1078-0432.CCR-11-2750.DC1

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