Lapatinib and Obatoclax kill tumor cells through blockade of ERBB1/3/4 and through inhibition of BCL-XL and MCL-1.

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Number of text pages: 28
Number of Tables: 0
Number of Figures: 6
Words in Abstract: 195
Words in Introduction: 784
Words in Discussion: 1227

Abbreviations: ERK: extracellular regulated kinase; MEK: mitogen activated extracellular regulated kinase; JNK: c-Jun NH₂-terminal kinase; PI3K: phosphatidyl inositol 3 kinase; MAPK: mitogen activated protein kinase; ca: constitutively active; dn: dominant negative; PDGFR: platelet derived growth factor receptor; PTEN: phosphatase and tensin homologue on chromosome ten.
Abstract

Prior studies in breast cancer cells have shown that lapatinib and obatoclax interact in a greater than additive fashion to cause cell death, and do so through a toxic form of autophagy. The present studies sought to extend our analyses to CNS tumor cells, and to further define mechanisms of drug action. Lapatinib and obatoclax killed multiple CNS tumor isolates. Cells lacking PTEN function were relatively resistant to drug combination lethality: expression of PTEN in PTEN null cells restored drug sensitivity and knock down of PTEN promoted drug resistance. Based on knock down of ERBB1-4, we discovered that the inhibition of ERBB1 / 3 / 4 receptors were most important for enhancing obatoclax lethality, rather than ERBB2. In parallel we noted in CNS tumor cells that knock down of BCL-XL and MCL-1 interacted in an additive fashion to facilitate lapatinib lethality. Pre-treatment of tumor cells with obatoclax enhanced the lethality of lapatinib to a greater extent than concomitant treatment. Treatment of animals carrying orthotopic CNS tumor isolates with lapatinib and obatoclax prolonged survival. Collectively our data argues that lapatinib and obatoclax therapy could be of use in the treatment of tumors located in the CNS.
Introduction

Glioblastoma (GBM) has an abysmal 5 year survival rate and is an incurable malignancy. Even under optimal circumstances, in which essentially all of a glial tumor can be surgically removed and the patients are maximally treated with radiation and “state of the art” chemotherapeutic agents such as temozolomide (Temodar®) or BCNU (Gliadel® wafers), the median survival of this disease is only extended from ~3 months to 12–15 months (Parkin et al., 2002; Robins et al., 2007). Although in some reports up to ~80% of pediatric medulloblastoma patients can be “cured” based on a 5 year survival, linked to this is a very high degree of patient morbidity associated with the elevated levels of radio- and chemo-therapy required to treat the disease resulting in profound and sustained life altering effects on the surviving / growing child (Onvani et al., 2010; Schmidt et al., 2010). Clearly, better therapeutic approaches are required for both malignancies.

In general the effect of a single target anti-cancer kinase inhibitor in vitro most often is to elicit a cyto-static rather than a cyto-toxic effect (Mitchell et al., 2010; Martin et al., 2009, and references therein). To achieve greater effects on survival, inhibition of multiple growth factor receptors and intracellular pathways have to be targeted. It has been noted that tumors presenting with alterations in ERBB receptors are often more aggressive and frequently convey poorer clinical outcome (Hynes & Lane, 2005; Martin et al., 2008; Bigner & Vogelstein, 2008). Also, it is known that medulloblastoma and malignant glioma often exhibit expression of constitutively activated or altered ERBB receptors, therefore the use of small molecule kinase inhibitors such as lapatinib and gefitinib as anti-cancer therapeutics in CNS tumor types is logical (Miller, 2004; Hynes & Lane, 2005).

Lapatinib, a dual ERBB1/ERBB2 inhibitor, is currently clinically approved for use in ERBB2 over-expressing metastatic breast cancer combined with capecitabine (Geyer et al., 2006; Kong et al., 2008). Over time resistance to ERBB inhibitor therapeutics develops either through secondary mutations within ERBB receptors, through the initiation of alternative receptor tyrosine kinase signaling pathways or the up-regulation of pro-survival proteins in the BCL-2 family (Ware et al., 2010).
The BCL-2 family of proteins consists of protective BCL-2 family proteins (BCL-2, BCL-XL, MCL-1) and pro-apoptotic proteins in the family such as BAX, BAK, PUMA and NOXA, (Mitchell et al, 2010; Martin et al., 2009; Delft & Huang, 2009 and refs. therein). Most frequently reported is that the release of BAK and BAX from protective BCL-2 proteins results in pore formation, mitochondrial stress with ROS generation, leading to the release of cytochrome c and the activation of apoptosis. This effect can also be induced by target specific therapeutics such as obatoclax (GX15-070), that act by inhibiting the interaction between protective BCL-2 family members and toxic members of the family. Theoretically this approach would also increase the toxicity of other therapies that act to promote mitochondrial dysfunction (Martin et al., 2009). In our prior studies combining lapatinib and obatoclax, however, we demonstrated that cell killing, despite activation of BAX and BAK, was due to a toxic form of autophagy, and caspase inhibitors such as zVAD had no effect on suppressing the cell killing effect (see below, Martin et al, 2009; Mitchell et al, 2010).

The interplay between apoptosis and autophagy is still not fully understood however, many of the same pathways are implicated within both of these death processes. Autophagy is an evolutionarily conserved catabolic pathway that recycles or removes damaged or excess organelles as well as breaking down proteins into their amino acid constituents (Gewirtz et al., 2007, and refs. therein). Cancer cells often display reduced levels of autophagy, allowing continuing malignant progression and proliferation (Mathew et al., 2007), whilst also providing an anti-cancer role by limiting tumor size/growth (Hippert et al., 2006). This leaves the question as to whether autophagy in tumor cells is a cyto-protective or cyto-toxic event. As noted above, we have found that the BCL-2 inhibitor obatoclax, either alone or in combination with the ERBB1/2/4 inhibitor lapatinib, kills through a toxic form of autophagy that correlated with activation of toxic BH3 domain proteins such as BAX and BAK (Martin et al, 2009; Mitchell et al, 2010).

The present study aimed to establish further mechanisms of action of lapatinib and obatoclax toxicity in CNS tumor cells, and the importance of PTEN status in drug toxicity and the role of autophagy in cancer cell survival/death. We have established that cancer cells lacking PTEN are inherently resistant to drug treatment.
Our findings have also shown that in medulloblastoma and glioblastoma cells, down-regulation of ERBB1/3/4 by lapatinib and inhibition of MCL-1 by obatoclax are central to drug combination effects.
Materials and Methods.

Materials. Lapatinib was provided by Glaxo SmithKline (King of Prussia, PA). Obatoclax was provided by Geminx Pharma. (King of Prussia, PA); now owned by Cephalon (Frazer, PA). Other drugs were purchased from Selleck Chemicals LLC (Houston, TX). Trypsin-EDTA, DMEM, MEM, RPMI and penicillin-streptomycin were purchased from GIBCOBRL (GIBCOBRL Life Technologies, Grand Island, NY). All established tumor cells were purchased from the ATCC. Primary human GBM and medulloblastoma cells were from VCU and Mayo Clinic (Hamed et al, 2010; Yang et al, 2010). Short hairpin PTEN, plasmid expressing green fluorescent protein (GFP) tagged –PTEN, B-Raf, MEK, p70, mTOR and plasmid expressing Luciferase were purchased from Addgene (Cambridge, MA). Dominant negative MEK1 was purchased from Fisher Scientific (Pittsburgh, PA) and the plasmid expressing GFP – tagged human LC3 was kindly provided by Dr. S Spiegel, VCU. Commercially available validated siRNA to ErbB1, ErbB2, mTOR, Mcl-1, Bcl-XL, Beclin1, ATG5, NOXA, PUMA, AIF and BAK were purchased from Qiagen (Valencia, CA) whereas si-smart pools targeting ErbB3 and ErbB4 were purchased from Dharmacon (Lafayette, CO). Mcl-1, Bcl-XL, ErbB2, ErbB3, ErbB4, p-ErbB2, p-mTOR and ATG5 antibodies were purchased from Cell Signaling Technologies (Worcester, MA) and GAPDH, p-AKT, Beclin1, p-ERK1/2, NOXA and PUMA antibodies were purchased from Santa Cruz Biotechnologies, INC (Santa Cruz, CA). ErbB1 and p-p70 (Thr421) antibodies were purchased from Invitrogen (Carlsbad, CA) whereas p62, p-p70 (Thr389), p-ErbB1 were purchased from R&D Systems (Minneapolis, MN) and LC3 antibody was purchased from Norvus Biologicals (Littleton, CO). All secondary antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Reagents and performance of experimental procedures were described in refs. (Martin et al, 2009; Rahmani et al, 2005; Rahmani et al, 2007; Zhang et al, 2008; Park et al, 2008; Park et al, 2009; Park et al 2010; Eulitt et al, 2010).
Methods.

Culture and in vitro exposure of cells to drugs. All established cell lines were cultured at 37 °C (5% (v/v) CO₂) in vitro using RPMI (BT474/BT549), DMEM (GBMs) or MEM (DAOY and D283) supplemented with 5% (v/v) fetal calf serum and 10% (v/v) Non-essential amino acids. For short term cell killing assays, immunoblotting studies, cells were plated at ~2 x 10^5 cells per well of a 12 well plate and 48h after plating treated with various drugs, as indicated. In vitro drug treatments were from 100 mM stock solutions of each drug and the maximal concentration of Vehicle (DMSO) in media was 0.02% (v/v). Cells were not cultured in reduced serum media during any study in this manuscript.

In vitro cell treatments, microscopy, SDS-PAGE and Western blot analysis. For in vitro analyses of short-term cell death effects, cells were treated with vehicle or Lap/GX, or the combination of Lap/GX with the addition of either Rapamycin or Bez-235 for the indicated times in the Figure legends. For apoptosis assays where indicated, cells were isolated at the indicated times, and subjected to trypan blue cell viability assay by counting in a light microscope. For SDS-PAGE and immunoblotting, cells were plated at 5 x 10^5 cells / cm² and treated with drugs at the indicated concentrations and after the indicated time of treatment, lysed in whole-cell lysis buffer (0.5 M Tris-HCl, pH 6.8, 2%SDS, 10% glycerol, 1% β-mercaptoethanol, 0.02% bromophenol blue), and the samples were boiled for 30 min. The boiled samples were loaded onto 8-14% SDS-PAGE and electrophoresis was run for approximately 1.5hrs. Proteins were electrophoretically transferred onto 0.22 μm nitrocellulose, and immunoblotted with various primary antibodies against different proteins. Blots were visualized using an Odyssey infra red imaging system.

Transfection of cells with siRNA or with plasmids. For Plasmids: Cells were plated as described above and 24h after plating, transfected. For all cell types (0.5μg) plasmids expressing a specific mRNA (or siRNA) or appropriate vector control plasmid DNA was diluted in 50μl serum-free and antibiotic-free medium (1 portion for each sample). Concurrently, 2μl Lipofectamine 2000 (Invitrogen) was diluted into 50μl of serum-free and
antibiotic-free medium (1 portion for each sample). Diluted DNA was added to the diluted Lipofectamine 2000 for each sample and incubated at room temperature for 30 min. This mixture was added to each well / dish of cells containing 200μl serum-free and antibiotic-free medium for a total volume of 300 μl, and the cells were incubated for 4 h at 37 °C. An equal volume of 2x medium was then added to each well. Cells were incubated for 48h and then treated with drugs.

Transfection with siRNA: Cells were plated in 60 mm dishes from a fresh culture growing in log phase as described above, and 24h after plating transfected. Prior to transfection, the medium was aspirated and 1 ml serum-free medium was added to each plate. For transfection, 10 nM of the annealed siRNA, the positive sense control doubled stranded siRNA targeting GAPDH or the negative control (a “scrambled” sequence with no significant homology to any known gene sequences from mouse, rat or human cell lines) were used. 10 nM siRNA (scrambled or experimental) was diluted in serum-free media. 4 μl Lipofectamine (Qiagen) was added to this mixture and the solution was mixed by pipetting up and down several times. This solution was incubated at room temp for 10 min and then added dropwise to each dish. The medium in each dish was swirled gently to mix and then incubated at 37 °C for 2h. One ml of 10% (v/v) serum-containing medium was added to each plate, and cells were incubated at 37 °C for 48h before re-plating (50 x 10^3 cells each) onto 12-well plates. Cells were allowed to attach overnight, then treated with drugs (0-48h). Trypan blue exclusion / TUNEL and SDS-PAGE/immunoblotting analyses were performed at the indicated time points.

Microscopy for LC3-GFP expression. Cells were transfected with a plasmid to express an LC3-GFP fusion protein, and were then cultured for 24 h. Cells were then treated with drugs, as indicated/ LC3-GFP transfected cells were visualized at the indicated time points on the Zeiss Axiovert 200 microscope using the FITC filter.
Intra-cerebral inoculation of GBM6 and BT474 cells. Athymic female NCr-nu/nu mice (NCI-Fredrick) weighing ~20g, were used for this study. Mice were maintained under pathogen-free conditions in facilities approved by the American Association for Accreditation of Laboratory Animal Care and in accordance with current regulations and standards of the U.S. Department of Agriculture, Washington, DC, the U.S. Department of Health and Human Services, Washington, DC, and the National Institutes of Health, Bethesda, MD. The GBM6-luc and BT474 cell lines were used for these studies. Mice were anesthetized via i.p. administration of (ketamine, 40 mg/kg; xylazine, 3mg/kg) and immobilized in a stereotactic frame (KOPF). A 24-gauge needle attached to a Hamilton syringe was inserted into the right basal ganglia to a depth of 3.5-mm and then withdrawn 0.5-mm to make space for tumor cell accumulation. The entry point at the skull was 2-mm lateral and 1-mm dorsal to the bregma. Intra-cerebral injection of BT474, DAOY and GBM6-luc cells in 2 μl of PBS was performed over 10 min. The skull opening was enclosed with sterile bone wax and the skin incision was closed using sterile surgical staples. Two-four weeks after tumor cell implantation animals were segregated into treatment groups. For animal administration, Lapatinib and Obatoclax were first dissolved in DMSO and an equal volume of 50:50 Cremophor/Ethanol (Sigma) was added. After mixing, a 1:10 dilution was made with sterile PBS. Animals were treated with vehicle (PBS/Cremophor/Ethanol/DMSO), Lapatinib, Obatoclax or combination of Lapatinib and Obatoclax using oral gavage to a final concentration of 5 mg/kg body mass QD for obatoclax and 100 mg/kg BID for lapatinib. Animals were imaged as indicated. Mice were injected i.p. with luciferin at 175 mg/kg in a volume of 100 ul and were anesthetized with isoflurane before and during imaging. Animals were imaged at a peak time of 15 min post-luciferin injection via a Xenogen (IVIS) instrument.

Data analysis. Comparison of the effects of various treatments was performed using ANOVA and the Student’s t test. Differences with a p-value of < 0.05 were considered statistically significant. Experiments shown are the means of multiple individual points (± SEM).
Results.

Treatment of GBM cells (GBM6, GBM12) with lapatinib increased the toxicity of obatoclax in a time dependent fashion (Figures 1A and 1B). Similar data were observed in GBM5 and GBM15 cells (Figure 1C). In contrast to GBM5/6/12/15 cells, GBM14 cells, which lack PTEN function, were relatively resistant to drug combination toxicity (Figure 1C). Similar findings to those in short-term viability measurements were made in colony formation assays (Supplemental Figure 1). Comparable findings to those found in GBM cells were noted in pediatric medulloblastoma cell lines (Figure 1D). Of interest, even in cell lines such as DAOY, that were exhibiting ~40% killing by the drug combination via trypan blue staining, no evidence of “classic” morphological signs of apoptosis were evident. This is in agreement with our prior findings wherein the pan-caspase inhibitor zVAD did not protect cells from this drug combination and no cleavage of pro-caspase 3 or PARP was evident (Martin et al, 2009; Mitchell et al, 2010).

Loss of PTEN function has often been observed in several tumor cell types including brain, breast and prostate cancers (Depowski et al, 2001, Holand et al, 2011). Re-expression of PTEN in PTEN null GBM14 cells facilitated lapatinib and obatoclax lethality (Figure 2A; Supplemental Figure 2). In contrast knock down of PTEN in GBM6 cells inhibited drug combination lethality. Identical data were obtained in BT474 (PTEN wild type) and BT549 (PTEN null) breast cancer cell lines (data not shown). As loss of PTEN was causing resistance to the drug combination we hypothesized that inhibition of a drug-able downstream effector of PTEN, mTOR, could restore the lethal effects of lapatinib and obatoclax treatment. In agreement with our hypothesis, treatment of GBM14 cells with either rapamycin or BEZ-235 caused significant enhancements in lapatinib and obatoclax lethality compared to vehicle treated cells (Figure 2C). Knock down of mTOR expression caused a similar effect to the use of either rapamycin or BEZ-235. In colony formation assays rapamycin also enhanced lapatinib and obatoclax toxicity (Figure 2D). Based on our findings examining PTEN and mTOR we next tested whether molecular activation of signaling pathways protected against the lapatinib and obatoclax drug combination. Expression of activated forms of p70 S6K and mTOR protected tumor cells from drug lethality (Figure 2E). B-RAF V600E is a known oncogene upstream of MEK1 and can also promote,
through paracrine signaling, activation of PI3 kinase–dependent pathways (Matallanas et al., 2011).

Expression of B-RAF V600E modestly suppressed drug lethality suggesting that the ERK1/2 pathway was not a key mediator of drug resistance (Supplemental Figure 3). Expression of dominant negative MEK1 partially abrogated the modest protective effects of B-RAF V600E expression.

Lapatinib can inhibit ERBB1, ERBB2 and ERBB4 kinase activities (Berezowska and Schlegel, 2011, and references therein). We next defined in several cell lines the relative contribution of ERBB1-4 in the biological actions of lapatinib. DAOY cells expressed ERBB1 and ERBB4; GBM12 cells expressed ERBB1, ERBB2, ERBB3 and ERBB4 (Figure 3A). In DAOY cells knock down of ERBB1 or ERBB4 and to a greater extent both ERBB1 and ERBB4 enhanced obatoclax lethality in a dose-dependent fashion (Figure 3B). In GBM12 cells it was apparent that a similar ERBB1 / ERBB4 protective signaling axis was present, as had been observed in DAOY cells, although knock down of ERBB3 alone in GBM12 cells permitted a strong toxic interaction with obatoclax (Figure 3C). After examining the relative roles of ERBB receptors we then examined which protective BCL-2 family proteins most influenced lapatinib sensitivity. Knock down of BCL-XL or MCL-1 increased lapatinib toxicity in DAOY and GBM12 cells (Figure 3D). Knock down of both BCL-XL and MCL-1 caused an additive increase in killing compared to knock down of the individual proteins. Finally, we compared and contrasted the abilities of various small molecule BCL-2 inhibitors to promote lapatinib toxicity. In a dose-dependent manner the BCL-2/BCL-XL/MCL-1 inhibitor obatoclax enhanced lapatinib lethality in DAOY and GBM12 cells (Figure 3E, data not shown). However, at similar mole-for-mole drug concentrations the BCL-2/BCL-XL inhibitor ABT-263 did not alter lapatinib lethality (Figure 3E, data not shown).

Many therapeutic drugs are administered in a sequential fashion to achieve their greatest ant-tumor effect in vitro and in vivo. We next tested whether pre-treatment of cells with obatoclax could enhance the overall lethal response to a subsequent lapatinib treatment. A 24h pre-treatment of DAOY cells with obatoclax caused a significant rise in the percentage of cells killed by a later exposure to lapatinib (Figure 4A). Similar data were also observed in GBM12 cells (Figure 4B). We then performed immunoblotting to define alterations in protein
expression and protein phosphorylation. Concomitant treatment of cells with lapatinib and obatoclax after 12h reduced mTOR, p70 S6K and AKT activity and increased the levels of PUMA and NOXA (Figure 4C). In cells pre-treated with obatoclax for 24h, the levels of mTOR, p70 S6K, AKT and ERK1/2 activity were further reduced; the levels of NOXA and PUMA remained elevated. We next determined whether NOXA, PUMA and other survival regulatory factors altered viability after drug exposure. Knock down of NOXA, PUMA, BAK or BAX, protected cells from the drug combination (Figures 4D and 4E).

Prior studies in breast cancer cells have shown that the induction of autophagy plays an important role in lapatinib and obatoclax lethality (Martin et al, 2009; Mitchell et al, 2010). In DAOY cells, that are particularly sensitive to the lapatinib / obatoclax drug combination we noted a rapid induction of autophagy as judged by formation of LC3-GFP punctae (Figure 5A). Loss of PUMA, NOXA or BAX prevented the drug-induced induction of LC3-GFP punctae (Figure 5B). This also correlated with a reduction in LC3-II formation by blotting cell lysates. Incubation with 3-methyl-adenine and knock down of ATG5, Beclin1 or AIF all protected cells from lapatinib and obatoclax toxicity (Figures 5C and 5D).

We next determined whether the lapatinib and obatoclax drug combination could prolong animal survival, with animals carrying intra-cranial tumors (Awada et al, 2001; Tasker et al, 2011). In GBM6 and in DAOY tumors lapatinib as a single agent enhanced animal survival (Figure 6; Supplemental Figure 4). Lapatinib combined with obatoclax extended survival further than lapatinib alone. Drug combination treatment did not alter mouse body mass (Supplemental Figure S5). In multiple normal tissues known to be targets for chemotherapy toxicity no obvious toxic effects were observed (Supplemental Figures 6-11).
Discussion.

In the present manuscript we aimed to define the importance of each ERBB protein to the lapatinib and obatoclax drug combination response; the PTEN status as an outcome for the response to drug combination treatment; to define whether MCL-1 and/or BCL-XL expression impacts the response to drug combination treatment of GBM and medulloblastoma cell lines; and to establish the involvement of autophagy in response to this drug combination in CNS tumor cells.

Lapatinib inhibits ERBB1/ERBB2, the expression of which is frequently elevated in CNS tumors however; CNS and other tumor types often demonstrate limited toxicity to lapatinib as a single agent through de novo mechanisms and the acquisition of drug resistance (Duham-Tonnelle et al, 2010). Lapatinib resistance can be attributed to further mutations, both point and splicing, within ERBB receptors, the initiation of alternative receptor tyrosine kinase signaling pathways, or the up-regulation of pro-survival proteins (BCL-2 family) (Ware et al., 2010; Martin et al, 2008). These multi-factorial resistance mechanisms demonstrate the importance of simultaneously targeting multiple proteins vital in cell survival signaling pathways in order to enhance tumor cell killing. Our prior research using a variety of drug combinations has emphasized the success of targeting multiple pro-survival signaling pathways through combinational treatment of CNS and other tumor types both in vivo and in vitro.

The ERBB1-4 receptors can homo- or hetero-dimerize with each other. Lapatinib, a known ErbB1/2/4 receptor inhibitor, can thus affect multiple ERBB receptor combinations dependent on cell type, and by implication the downstream pathways controlled by a combination of ERBB1/2 and ERBB3/4 could also be regulated by lapatinib treatment. Our findings showed that knockdown of ERBB1/4 in DAOY and ERBB1/3/4 in GBM12 cells were responsible for cell killing in combination with obatoclax. Accompanying this, knockdown of MCL-1 and subsequent treatment with lapatinib greatly enhanced or restored lapatinib toxicity in CNS tumor cells, suggesting MCL-1 as a key target of obatoclax. Obatoclax acts to inhibit protective BCL-2 family proteins, MCL-1, BCL-2 and BCL-XL thereby releasing pro-apoptotic proteins such as NOXA, BAX and BAK.
Inhibition or down-regulation of MCL-1 results in the release of these toxic BH3 domain proteins, which form pores, increasing mitochondrial permeability (Supplemental Figure 12).

Obatoclax has recently completed its Phase 2 trial evaluation with some clinical responses evident, but has yet to be tested for anti-tumor effects in CNS tumor types. Combinational treatment of lapatinib with another BCL-2 family inhibitor, ABT263 (inhibitor of BCL-2 and BCL-XL), did not demonstrate the same enhanced level of killing as observed with obatoclax. This emphasizes the importance of subverting the protective effect of MCL-1, the main target of obatoclax, to induce cell death. This was confirmed using molecular approaches. Pretreatment of CNS tumor cells with obatoclax prior to treatment with lapatinib and obatoclax further enhanced cell killing. In vitro, using molecular tools, we attributed the sequence dependent effect due to lower activity levels of p70 S6K and mTOR and enhanced levels of the toxic BH3 domain proteins BAK, PUMA and NOXA (Mitchell et al, 2010) (Supplemental Figure 12).

Although combination treatment with lapatinib and obatoclax shows promise, we noted that certain cell lines, e.g. GBM14 and BT549, remained relatively resistant to the drugs. PTEN, a tumor suppressor that negatively regulates the PI3K/AKT pathway, is often lost or mutated in cancer brain and breast cancer cells, resulting in the constitutive activation of the PI3 kinase pathway and its downstream cell survival effectors (Seminario et al., 2003). Restoration of PTEN in PTEN null cancer cell lines restored drug combination sensitivity and in parallel, silencing of PTEN, in lapatinib sensitive cells, decreased drug lethality demonstrating the importance of PTEN in the therapeutic response. The involvement of this signaling pathway was further confirmed by targeting downstream effectors such as mTOR and p70 S6K (see below).

The kinase mTOR acts to balance nutrient availability and cell growth through phosphorylation of p70 S6K and autophagy regulatory proteins, ensuring regulation of protein synthesis, degradation and survival. Two mTOR specific inhibitors, rapamycin and BEZ-235, can block the PI3K pathway downstream of PTEN causing growth arrest and in some cells, cell death (Alva et al., 2004). Both mTOR inhibitors enhanced cell killing by lapatinib
and obatoclax in PTEN null cells. Knocking down the expression of mTOR in PTEN null cells also increased cell death, replicating results seen with rapamycin and BEZ-235. Furthermore, lapatinib and obatoclax mediated cell death was reduced by expression of activated forms of mTOR or p70 S6K, validating that inactivation of PI3 kinase pathway is essential in lapatinib and obatoclax lethality. However, expression of mutated active B-RAF (V600E), that caused further activation of the MEK/ERK1/2 pathway, only slightly decreased sensitivity to lapatinib and obatoclax, suggesting that signaling by ERK1/2 was a peripheral pathway to the drug combination effects.

Autophagy occurs at a basal rate in all cells and involves the sequestration of cytoplasmic constituents, such as damaged or excess organelles, which are then removed, degraded and recycled to supply the cell with nutrients; this enables cancer cells to survive under extreme stress (Hippert et al., 2006). Autophagy involves the initiation, nucleation, cycling and expansion of an autophagosome (Wang & Klionsky, 2003; Mehpour et al., 2010; Jia et al., 2010; Pattingre et al., 2008; Yoshimori & Noda, 2008). The autophagosome fuses with a lysosome, forming an autolysosome, which acts to degrade and recycle the vesicle contents (Graf et al., 2009). Geimsa staining of lapatinib and obatoclax treated CNS tumor cells revealed a relative lack of morphological changes e.g. profound nuclei fragmentation normally associated with apoptosis (Alva et al., 2004).

Increased levels of BCL-2 family members in lapatinib resistant cells can prevent autophagy through the sequestration and inhibition of Beclin-1 (Hippert et al., 2006; Martin et al., 2009). Pre-treatment of CNS tumor cells with 3-methyladenine (3-MA), which inhibits the class III PI3K Vps34, resulted in reduced sensitivity of tumor cells to lapatinib and obatoclax treatment. Furthermore, knockdown of Beclin-1 and ATG5 using siRNA abrogated lapatinib and obatoclax -induced cell death. These findings, collectively, suggest that autophagic flux is significant in lapatinib and obatoclax induced cell death.

Medulloblastoma and glioblastoma are tumor types that use high doses of chemotherapy and radiotherapy to achieve a therapeutic response. Approaches to use less toxic drug and radiation regimens would be of benefit to
patients. In orthotopic glioblastoma tumors lapatinib and obatoclax treatment prolonged animal survival to a greater extent than either drug individually. Collectively these data argue that the lapatinib & obatoclax drug combination has efficacy in vivo using multiple tumor models.

In conclusion, the drug combination of lapatinib and obatoclax kills multiple CNS and mammary tumor types. The death promoting effects of lapatinib in this combination, in two of the cell lines tested, were dependent on inhibition of ERBB1 / 3 / 4 and not on ERBB2. Based on molecular as well as drug interactions, MCL-1 was shown to be the key protective BCL-2 family protein that was inhibited by obatoclax. Downstream of the receptors loss of PTEN function reduced drug combination toxicity. Downstream of PTEN, both p70 S6K and mTOR were shown to play an essential regulatory role in the actions of the drug combination. And, in agreement with reduced mTOR activity, the drug combination killed tumor cells through a form of toxic autophagy and that was also dependent on the actions of NOXA, PUMA and BAK. As both lapatinib and obatoclax are clinically relevant agents, translation into the clinic of this drug combination appears warranted.
Authorship Contribution.

Participated in research design: Dent, Fisher, Poklepovic, Grant

Conducted experiments: Bareford, Hamed, Cruickshanks

Contributed new reagents: n/a

Performed data analysis: Cruickshanks, Dent

Wrote or contributed writing of the manuscript: Dent, Cruickshanks.
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MOL #77586


Support for the present study was funded from PHS grants from the National Institutes of Health [R01-CA141704, R01-CA150214, R01-DK52825] and the Department of Defense [DAMD17-03-1-0262].
Figure Legends

Figure 1. Lapatinib and obatoclax interact to kill multiple CNS tumor cells but not those cells lacking PTEN function / expression. (A) GBM12 cells were treated with vehicle (DMSO), lapatinib (lap, 1 μM) and/or obatoclax (GX, 50 nM) as indicated. Cells were isolated 24-96h after exposure and viability determined by trypan blue exclusion (n = 3, +/- SEM) * p < 0.05 greater than vehicle control. Upper blot: The drug combination causes inactivation of ERBB2, p70 S6K and mTOR. (B) GBM6 cells were treated with vehicle (DMSO), lapatinib (lap, 1 μM) and/or obatoclax (GX, 50 nM) as indicated. Cells were isolated 24-96h after exposure and viability determined by trypan blue exclusion (n = 3, +/- SEM) * p < 0.05 greater than vehicle control. (C) GBM5, GBM14 and GBM15 cells were treated with vehicle (DMSO), lapatinib (1 μM) and/or obatoclax (GX, 50 nM) as indicated. Cells were isolated 48h after exposure and viability determined by trypan blue exclusion (n = 3, +/- SEM) * p < 0.05 greater than vehicle control. Upper blot: the expression of ERBB1, ERBB4, MCL-1 and BCL-XL in GBM5 and GBM12 cells. (D) VC312, DAOY and D283 pediatric CNS tumor cells were treated with vehicle (DMSO), lapatinib (1 μM) and/or obatoclax (GX, 50 nM) as indicated. Cells were isolated 24-48h after exposure and viability determined by trypan blue exclusion (n = 3, +/- SEM) * p < 0.05 greater than vehicle control. Upper: Geimsa staining of DAOY cells 24h after drug treatment.

Figure 2. Loss of PTEN function renders tumor cells resistant to lapatinib and obatoclax treatment. (A) GBM14 cells were transfected with either control (GFP) or with a plasmid to express PTEN (GFP-PTEN). GBM6 cells were transfected with either a control plasmid shRNA or a plasmid to express a shRNA to knock down PTEN. Twenty four h after transfection cells were treated with vehicle (DMSO) or with lapatinib (lap, 1 μM) and obatoclax (GX, 50 nM). Cells were isolated 24h after exposure and viability determined by trypan blue exclusion (n = 3, +/- SEM). (B) GBM14 cells were treated with vehicle (DMSO) or with lapatinib (1 μM) and obatoclax (GX, 50 nM) in the presence or absence of rapamycin (rap, 10 nM) or BEZ-235 (BEZ, 50 nM). Parallel sets of cells were transfected to knock down mTOR. Cells were isolated
24 after exposure and viability determined by trypan blue exclusion (n = 3, +/- SEM). (C) GBM14 cells were plated as single cells in sextuplicate and were treated for 48h with vehicle, lapatinib (1 μM) + obatoclax (GX, 50 nM), rapamycin (10 nM) or the drug combination as indicated. Colony formation 14 days later was determined (n = 3, +/- SEM). (D) DAOY and GBM12 cells were transfected with empty vector (CMV) or with plasmids to express activated p70 S6K and activated mTOR. Cells were treated with vehicle (DMSO) or with lapatinib (1 μM) and obatoclax (GX, 50 nM). Cells were isolated 24h after exposure and viability determined by trypan blue exclusion (n = 3, +/- SEM).

Figure 3. Dissecting the roles of ERBB receptors and protective BCL-2 family members in the toxic interaction between lapatinib and obatoclax. (A) Expression of ERBB1-4, BCL-XL and MCL-1 in CNS tumor cells and the knock down of these proteins by siRNA. (B) DAOY and (C) GBM12 cells had expression of the indicated receptors knocked down. Cells were treated with vehicle (DMSO) or with obatoclax (GX, 10-100 nM). Cells were isolated 24h after exposure and viability determined by trypan blue exclusion (n = 3, +/- SEM): the values shown indicate the true percentage of cell death above its matched vehicle control * p < 0.05 greater than vehicle control; ** p < 0.05 greater than the same value under parallel other conditions. (D) DAOY and GBM12 cells had expression of the indicated protective BCL-2 family proteins (MCL-1, BCL-XL) knocked down. Cells were treated with vehicle (DMSO) or with lapatinib (100-3000 nM). Cells were isolated 24h after exposure and viability determined by trypan blue exclusion (n = 3, +/- SEM): the values shown indicate the true percentage of cell death above its matched vehicle control. (E) DAOY cells were treated with 1 μM lapatinib in the presence of: obatoclax (GX, 10-100 nM); ABT-263 (ABT, 10-100 nM). Cells were isolated 24h after exposure and viability determined by trypan blue exclusion (n = 3, +/- SEM). * p < 0.05 greater than vehicle control.
Figure 4. Pre-treatment of cells with obatoclax promotes greater drug combination toxicity than concomitant drug treatment. (A) DAOY cells were pre-treated with either vehicle (DMSO) or with obatoclax (GX, 50 nM). After twenty four h, as indicated, cells were treated with lapatinib (1 μM) and/or obatoclax (GX, 50 nM). Cells were isolated after 12h and viability determined by trypan blue exclusion (n = 3, +/- SEM) * p < 0.05 greater than vehicle control; ** p < 0.05 greater than parallel value in un-pretreated cells. (B) GBM12 cells were pre-treated with either vehicle (DMSO) or with obatoclax (50 nM). After twenty four h, as indicated, cells were treated with lapatinib (1 μM) and/or obatoclax (GX, 50 nM). Cells were isolated after 24-48h and viability determined by trypan blue exclusion (n = 3, +/- SEM) * p < 0.05 greater than vehicle control; ** p < 0.05 greater than parallel value in un-pretreated cells. (C) DAOY cells (see Panel A) were isolated and immunoblotting performed on cell lysates. (D) and (E) DAOY and GBM12 cells were transfected to knock down PUMA and/or NOXA expression or to knock down BAK or BAX expression. Twenty four h later cells were treated with vehicle (DMSO) or with lapatinib (1 μM) and obatoclax (GX, 50 nM). Cells were isolated 24h later and viability determined by trypan blue exclusion (n = 3, +/- SEM) # p < 0.05 less than vehicle control value.

Figure 5. Lapatinib and obatoclax interact to kill CNS tumor cells through a toxic form of autophagy. (A) DAOY cells were transfected with a plasmid to express LC3-GFP. Cells were treated with lapatinib (1 μM) and/or obatoclax (GX, 50 nM) and the number of LC3-GFP punctae determined over 6-24h (n = 3 +/- SEM) * p < 0.05 greater than vehicle control. (B) DAOY cells were transfected to knock down PUMA and/or NOXA expression or to knock down BAX expression in parallel with a plasmid to express LC3-GFP. Cells were treated with lapatinib (1 μM) and/or obatoclax (GX, 50 nM) and the number of LC3-GFP punctae determined over after 24h (n = 3 +/- SEM) # p < 0.05 less than vehicle control. (C) DAOY and GBM12 cells were transfected with a scrambled siRNA (siSCR) or siRNA molecules to knock down Beclin1 or ATG5. Twenty four h after transfection cells were treated with vehicle (DMSO) or with lapatinib (1 μM) and obatoclax (GX, 50 nM). Cells were isolated 24h later and viability determined by trypan blue
exclusion (n = 3, +/- SEM) # p < 0.05 less than vehicle control. (D) DAOY and GBM12 cells were transfected with a scrambled siRNA (siSCR) or a siRNA molecule to knock down apoptosis inducing factor (AIF). Twenty four h after transfection cells were treated vehicle (PBS) followed as indicated by 3-methyl adenine (3MA, 5 mM) followed by with vehicle (DMSO) or with lapatinib (1 μM) and obatoclax (GX, 50 nM). Cells were isolated 24h later and viability determined by trypan blue exclusion (n = 3, +/- SEM) # p < 0.05 less than vehicle control.

Figure 6. Lapatinib and obatoclax prolong survival of animals with intra-cranial tumors.

GBM6-luciferase cells (1 x 10^6) were implanted into the brains of mice. Eight days after implantation animals were treated with vehicle (cremophore); lapatinib (100 mg/kg BID); obatoclax (5 mg/kg QD) or the drug combination for 5 days. Upper Panel: Animals carrying tumors show regression 14 days after initiation of drug combination exposure as judged by bio-luminescent imaging. Lower Graph: Animals exposed to lapatinib show elevated survival compared to vehicle control (Two studies, n = 6 +/- SEM) * p < 0.05 greater than vehicle treatment; ** p < 0.05 exposure to lapatinib and obatoclax causes greater survival than lapatinib alone.
Figure 2

A

Percentage cell death

GBM14

GFP

GFP

GFP

GFP

PTEN

PTEN

VEH

VEH

VEH

VEH

LapX+

LapX+

LapX+

LapX+

de-

de-

de-

de-

GBM6

siSCR

siSCR

siPTEN

siPTEN

B

Percentage cell death

GBM14

24h

4.1

12.5

26.7

20.5

VEH

LapX+

LapX+

LapX+

LapX+

de-

de-

de-

de-

RAP, 10 nM

BEZ, 50 nM

siSCR

si-mTOR
**Figure 2**

**C**

![Bar chart showing plating efficiency](image)

**D**

![Western blot analysis](image)
Figure 3

C

Percentage cell death

GBM12

VEH 10 nm VEH 100 nm VEH 10 nm VEH 100 nm VEH 10 nm VEH 100 nm VEH 10 nm VEH 100 nm
siSCR siERBB1 siERBB4 siERBB3 siERBB1 siERBB4 siERBB3 siERBB2 siERBB2

* 15.0 * 17.0 * 16.2 7.1 11.3

D

GBM12

DAOY

BCL-XL
GAPDH
MCL-1
GAPDH

siSCR siMCL-1 siMCL-1+ siMCL-1 siBCL-XL siBCL-XL siBCL-XL siBCL-XL

48h

24h

Percentage cell death

[ Lapatinib], nM

[ Lapatinib], nM
Figure 3

Percentage cell death

DAOY

VEH, GX, 10 nM, GX, 30 nM, GX, 100 nM, VEH, GX, 10 nM, GX, 30 nM, GX, 100 nM, VEH, ABT, 10 nM, ABT, 30 nM, ABT, 100 nM, VEH, ABT, 10 nM, ABT, 30 nM, ABT, 100 nM
Figure 4

C

DAOV
24hr pre-GX, 12hr LAP+GX
GX, (24h pre)

VEH  Lap  CX  Lap+GX  CX  Lap+GX

P-ERK1/2
P-AKT (S473)
P-p70 (T389)
P-p70 (421/424)
P-mTOR (S2448)
PUMA
NOXA
BAK
GAPDH

D

Percentage cell death

GBM12
siBAK
siBAX
siPUMA
siNOXA
GAPDH

DAOV

VEH  Lap+GX  VEH  Lap+GX  VEH  Lap+GX  VEH  Lap+GX  VEH  Lap+GX

# # # #

siSCR  siKD  siSCR  siBAK  siSCR  siBAX  siSCR  siPUMA  siSCR  siNOXA  siSCR  siBAK

# # # #
Figure 5

A

B

Mean LC3-GFP puncta / cell

DAOY 6h

DAOY 12h

DAOY 24h

VEH  Lap  GX  Lap+GX

VEH  Lap  GX  Lap+GX

VEH  Lap  GX  Lap+GX

Mean LC3-GFP vesicles per cell

DAOY

VEH  Lap+GX  VEH  Lap+GX  VEH  Lap+GX

VEH  Lap+GX  VEH  Lap+GX  VEH  Lap+GX

VEH  Lap+GX  VEH  Lap+GX  VEH  Lap+GX

β-actin

LC3II
Figure 5
Figure 6

Percentage survival

Days after tumor implantation

VEH  LAPATINIB  OBATOCLAX  LAPATINIB+OBATOCLAX

Day 8

Day 22

GBM6

Vehicle
Obatoclax
Lapatinib
Lap+Obat
Molecular Pharmacology
Lapatinib and Obatoclax kill tumor cells through blockade of ERBB1 / 3 / 4 and through inhibition of BCL-XL and MCL-1.

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**Figure S1. Lapatinib and obatoclax interact to kill GBM cells.** GBM6 and GBM12 cells were plated as single cells in sextuplicate. Twelve h after plating cells were treated with vehicle control, obatoclax, or lapatinib at the indicated doses. Cells were treated with the drugs for 48h followed by culture in drug free media. Twelve days after drug treatment cells were fixed and stained and the number of colonies of > 50 cells counted. Data was placed into the CalcuSyn for windows program to determine the combination index (CI) values. The CI values are plotted in the Figure (n = 3, +/- SEM).

**Figure S2. Expression and knock down of PTEN in tumor cells.** GBM14 and GBM6 cells were transfected to either over-express GFP-PTEN or to knock down PTEN, respectively. Lysates were taken 24h after transfection followed by SDS PAGE and blotting for PTEN.

**Figure S3. Activation of B-RAF weakly protects cells from the drug combination.** DAOY cells were transfected with empty vector (CMV) or with plasmids to express activated B-RAF V600E and dominant negative MEK1, as indicated. Cells were treated with vehicle (DMSO) or with lapatinib (1 µM) and obatoclax (GX, 50 nM). Cells were isolated 24h after exposure and viability determined by trypan blue exclusion (n = 3, +/- SEM).
Figure S4. Assessment of lapatinib + obatoclax toxicity in DAOY tumors. Tumors were established in the brains of athymic mice for 14 days. Animals were then treated with vehicle diluent or with lapatinib + obatoclax as described in Methods. Tumor volumes were measured every 2-3 days as indicated in the Figure. * $p < 0.05$ greater survival compared to vehicle control.

Figure S5. Assessment of lapatinib + obatoclax normal tissue toxicity in mice: total body mass. Animals were treated with vehicle diluent or with lapatinib + obatoclax as described in Methods. The body mass after 1 week and 2 weeks of drug exposure are plotted (+/- SEM).

Figure S6. Assessment of lapatinib + obatoclax normal tissue toxicity in mice: brain. Animals were treated with vehicle diluent or with lapatinib + obatoclax as described in Methods. Organs were removed and sections and H&E stained. A representative image of 3 separate organs / animals.

Figure S7. Assessment of lapatinib + obatoclax normal tissue toxicity in mice: heart. Animals were treated with vehicle diluent or with lapatinib + obatoclax as described in Methods. Organs were removed and sections and H&E stained. A representative image of 3 separate organs / animals.

Figure S8. Assessment of lapatinib + obatoclax normal tissue toxicity in mice: kidney. Animals were treated with vehicle diluent or with lapatinib + obatoclax as described in Methods. Organs were removed and sections and H&E stained. A representative image of 3 separate organs / animals.

Figure S9. Assessment of lapatinib + obatoclax normal tissue toxicity in mice: liver. Animals were treated with vehicle diluent or with lapatinib + obatoclax as described in Methods. Organs were removed and sections and H&E stained. A representative image of 3 separate organs / animals.
Figure S10. Assessment of lapatinib + obatoclax normal tissue toxicity in mice: lung. Animals were treated with vehicle diluent or with lapatinib + obatoclax as described in Methods. Organs were removed and sections and H&E stained. A representative image of 3 separate organs / animals.

Figure S11. Assessment of lapatinib + obatoclax normal tissue toxicity in mice: spleen. Animals were treated with vehicle diluent or with lapatinib + obatoclax as described in Methods. Organs were removed and sections and H&E stained. A representative image of 3 separate organs / animals.

Figure S12. Possible signaling mechanisms by which lapatinib and obatoclax interact. Increase levels of PUMA and NOXA promote release of Beclin 1 from protective BCL-2 proteins such as MCL-1 and BCL-XL. Released Beclin 1 stimulates a toxic form of autophagy.
GBM6

- GX 25nM, Lap 0.5 μM
- GX 50nM, Lap 1.0 μM
- GX 75nM, Lap 1.5 μM

GBM12

- GX 25nM, Lap 0.5 μM
- GX 50nM, Lap 1.0 μM
- GX 75nM, Lap 1.5 μM
Percentage cell death

DAOY
24h

VEH
Lap+GX
VEH
Lap+GX
VEH
Lap+GX
VEH
Lap+GX

CMV
B-RAF
V600E
dnMEK

P-ERK
GAPDH