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**Paclitaxel and obatoclax synergize to kill paclitaxel-resistant small cell lung cancer cells**

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Mentor: Dr. Terri B. Hunter  
Completed Fall 2010

# Paclitaxel and obatoclax synergize to kill paclitaxel-resistant small cell lung cancer cells

Neil Manimala

## ABSTRACT

Small molecule BH3 mimetic obatoclax (GX15-070) enhances the chemotherapeutic action of paclitaxel by increasing in vitro occurrence of Type I cell death (apoptosis) in selected small cell lung cancer (SCLC) lines. SCLC is historically resistant to paclitaxel treatment, yielding only single-digit response rates in second line chemotherapy. This resistance is attributed to a paclitaxel-induced upregulation of mitochondrial pro-survival protein bcl-xL. To increase SCLC response rates to taxol treatment, it is important to find a therapeutic modality that disables the bcl-xL line of defense against apoptosis. It was hypothesized that a combination of paclitaxel and obatoclax would synergize to enhance in vitro SCLC killing through a consistent mechanism of action.

Obatoclax inhibits bcl-xL, triggering an apoptosome-independent apoptotic cascade via pro-apoptotic Bak to initiate programmed cell death via unsequestration of Bak/Bax. This unsequestration is confirmed by immunoprecipitation. High throughput data for a CT Blue viability assay suggest moderate synergy in an obatoclax-paclitaxel combination against SCLC line 86M1 at optimal concentration ratios. Annexin V staining and flow cytometry further indicate synergistic SCLC apoptosis. Additional Western blot analyses confirm synergy between the two drugs and clarify signaling events upstream or downstream of Bak/Bax unsequestration in selected SCLC lines.

Subcellular fractionation reveals critical signaling translocation events as well. By combining the two drugs, it is thereby possible to maximize SCLC apoptosis while minimizing single agent toxicity. There is also clear evidence of involvement of the mTOR signaling pathway and the possibility of autophagic induction. Elucidation of these pathways can offer further possibilities of enhancing this bivalent treatment. In treating SCLC, the obatoclax-paclitaxel combination is thus a promising therapeutic modality that has clinical potential.

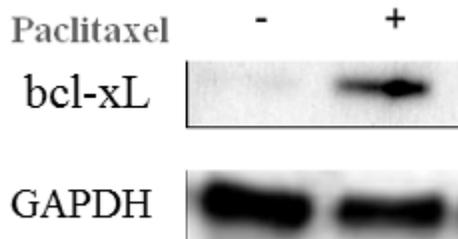
## INTRODUCTION

Small cell lung cancer (SCLC) is an aggressive form of lung cancer. Although SCLC is a highly chemosensitive disease, outcome is generally poor and the 5-year survival rate is less than 10%. Diagnosis of extensive stage (ES) comprises approximately two-thirds of new SCLC cases, and the median survival of these patients is only 2-4 months if untreated, and survival increases to 6-8 months with chemotherapy.

This disease is very responsive to first line chemotherapy with response rates of greater than 50% routinely observed. However, these patients frequently relapse. After the patients fail to respond the chemotherapy, they generally succumb to their disease within a few months [1].

Treatment of patients with relapsed SCLC is especially challenging if patients are platinum-resistant, when disease progression occurs within 3 months of completion of a platinum containing regimen. In these patients, median survival ranges from 3.7 to 4.7 months [2-7]. In SCLC, paclitaxel (PA) is considered a second line therapy after the failure of platinum-based treatment regimens [8]. Several modes of action of paclitaxel have been described. The drug is most well known as a microtubule stabilizer. That is, paclitaxel binds to tubulin and interferes with spindle formation in mitosis, ultimately arresting cells in G1 and G2/M phases of the cell cycle, leading to cell death [9-13]. In addition to stabilizing microtubules, paclitaxel may act as a molecular mop by sequestering free tubulin, effectively depleting the cells supply of tubulin [14].

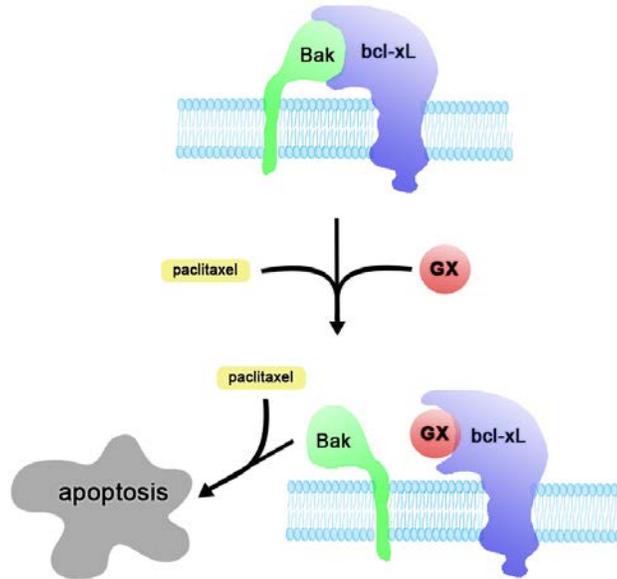
Beyond these effects on microtubules, more recent research has indicated that PA also induces classical programmed cell death, also known as apoptosis, in cancer cells by binding to the pro-survival protein Bcl-2, arresting its function [15, 16]. The pro-survival Bcl-2 family members are overexpressed in malignancies and keep these cancer cells' mitochondrial outer membranes intact to prevent release of "apoptogenic molecules" that would otherwise lyse normal cells [17, 18]. Specifically, this overexpression of pro-survival proteins prevents loss of mitochondrial membrane potential that would be the goal of normal programmed cell death pathways. Thus, paclitaxel-induced up-regulation of a pro-survival protein inhibits apoptosis and is inherently harmful to the goal of SCLC killing and tumor regression. In fact, data from the author's lab has demonstrated that SCLC cells are relatively resistant to biologically relevant doses of PA *in vitro*. Interestingly, it was demonstrated that PA induced the upregulation of anti-apoptotic bcl-xL expression, thereby promoting an active mechanism of resistance to PA (Figure 1).



**Figure 1.** Western blot data indicate bcl-xL is significantly upregulated in 86M1 treated with paclitaxel alone.

Obataclax mesylate (GX15-070, GX; Gemin X Biotechnologies Inc) is a small molecule pan-Bcl-2 family inhibitor. GX inhibits anti-apoptotic members of Bcl family. Inhibiting Bcl-2 and other anti-apoptotic members of the Bcl family (e.g. Bcl-XL) *should* induce SCLC death. GX-chemo combination already has a clinical history in Phase I/II trials for leukemia/lymphoma, Phase I/II trials for non small cell lung cancer (NSCLC), and recruiting trials for SCLC [19]. Literature suggests lung cancer is sensitive to GX; a GX-cisplatin combination effects NSCLC regression [20]. A combination of obataclax and paclitaxel thus presents a promising treatment strategy, and the proposed mechanism

of action is shown, with pro-apoptotic protein Bak being unsequestered from the bcl-xL BH3 binding domain and being free to thereby fulfill its apoptotic function (Figure 2).



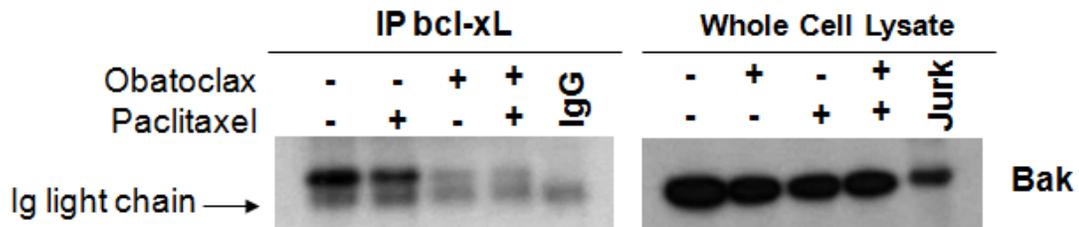
**Figure 2.** Diagram shows that GX binds bcl-xL, thus unsequestering pro-apoptotic Bak to accomplish apoptosis under paclitaxel treatment.

It is important, then, to establish that GX indeed binds bcl-xL, unsequestering Bak and effecting apoptosis in combination with paclitaxel. The extent of synergy must be noted to assess combinatorial efficacy. The mode or modes of cell death must be established, with underlying signaling events elucidated, in order to better characterize the treatment and any ways to better it.

## RESULTS

### *Immunoprecipitation of bcl-xL*

The need to confirm GX activity in paclitaxel-treated conditions is important for all successive experiments. An immunoprecipitation was performed to determine unsequestration of Bak from bcl-xL treated with GX (Figure 3).

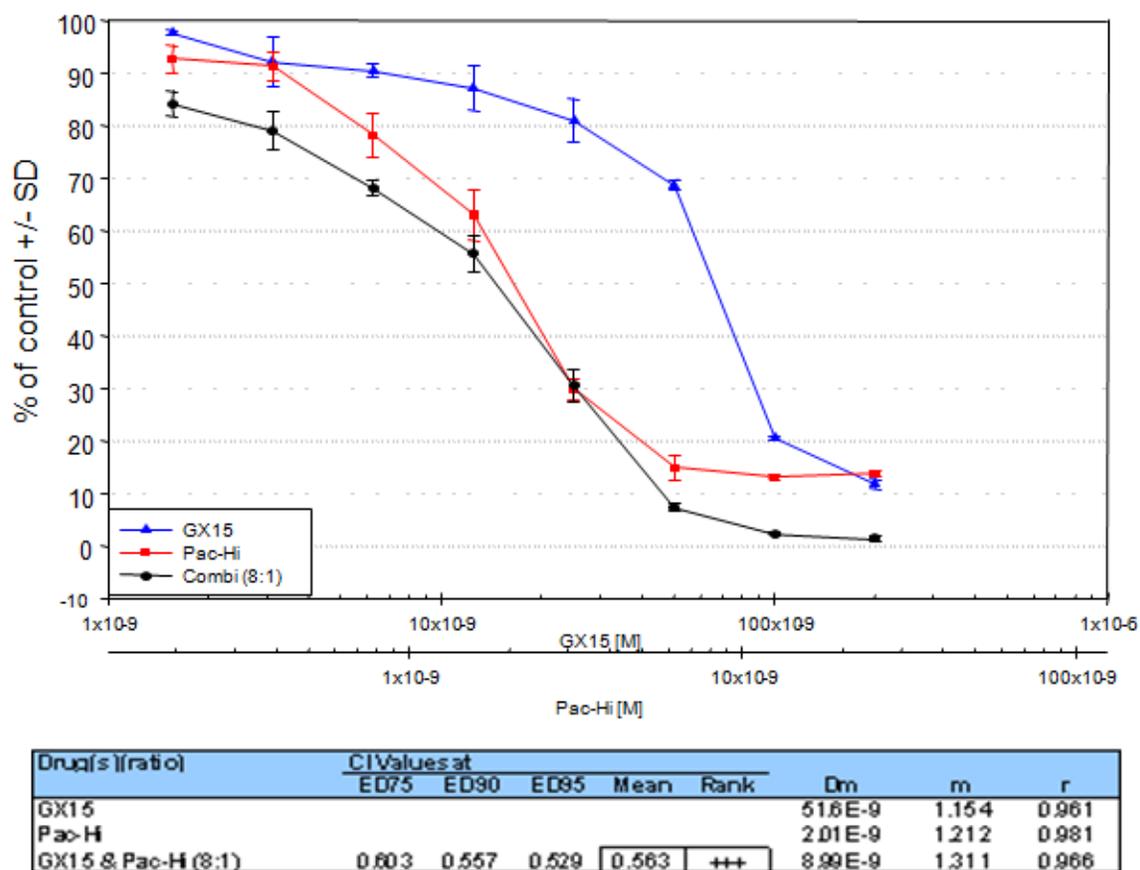


**Figure 3.** Immunoprecipitation of bcl-xL and co-immunoprecipitation of Bak confirm that GX performs its bcl-xL-inhibiting function even in a paclitaxel-treated chemical environment for

86M1 SCLC. These data provide *in vitro* evidence of drug activity for these experiments by indicating unsequestration of Bak from bcl-xL under GX treatment conditions, even in samples for which paclitaxel was also present.

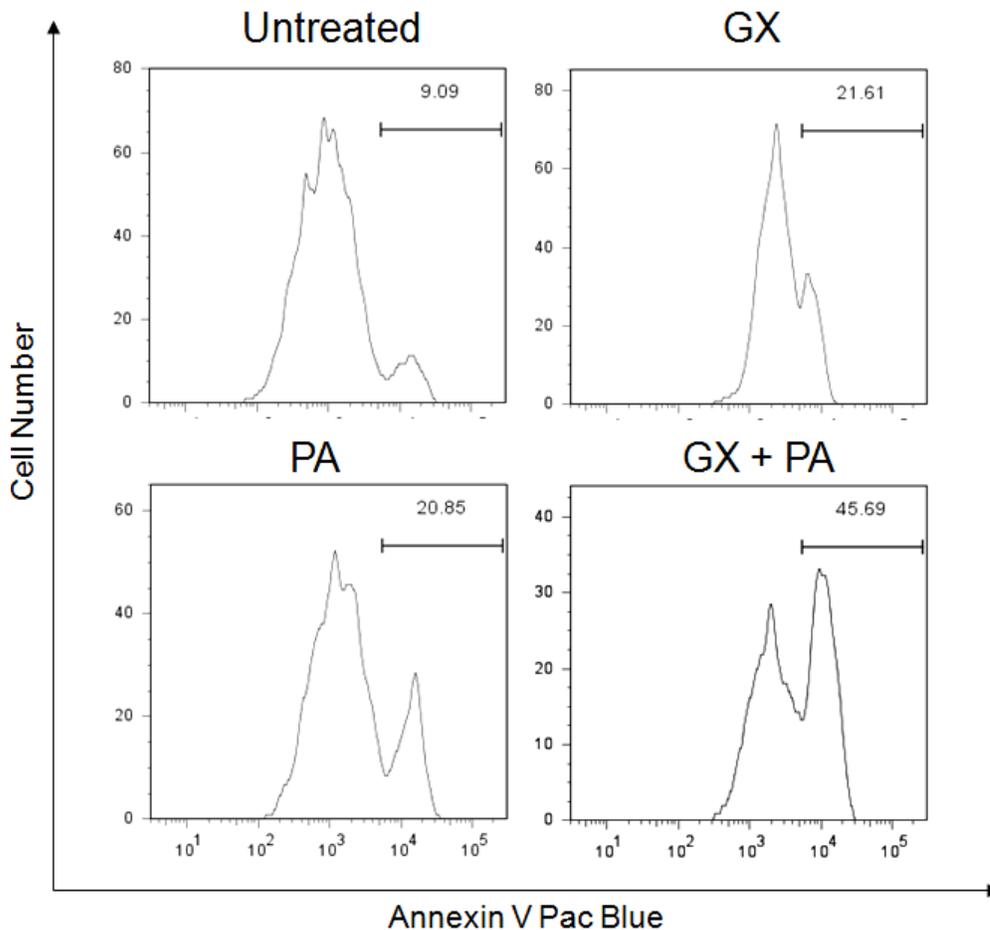
**Assessments of combination synergy**

Then, combinatorial synergy of PA and GX had to be determined with respect to killing SCLC in general, regardless of mechanism. A high throughput screening presented a straightforward and accessible means of attaining these data with a CT Blue viability assay (Figure 4).



**Figure 4.** Assessment of combination efficacy in inducing general cell death. Cell Titer Blue high throughput assay compares cell death for 86M1 treated with obatoclax alone (GX15), paclitaxel alone (Pac-Hi), or their 8:1 combination (GX:PA ratio) for 72h exposure time. Results show a higher degree of general killing by combination with lower required drug concentrations than in single treatments. Note the synergistic ranking (+++), indicating statistically significant synergy based on the high throughput software average of combination index values at selected effective dosages.  $6 \cdot 10^3$  cells were plated in each well of 384-well plates and incubated overnight at 37°C, 5% CO<sub>2</sub>. The next day, the drugs were diluted in media and 25 µl of these dilutions added to appropriate wells using an automated pipetting station. Cell viability was assessed by the ability of the remaining viable cells to bioreduce resazurin to resorufin. Resazurin is dark blue in color and has little intrinsic fluorescence until it is reduced to resorufin (579 nm E<sub>x</sub>/584nm E<sub>m</sub>).

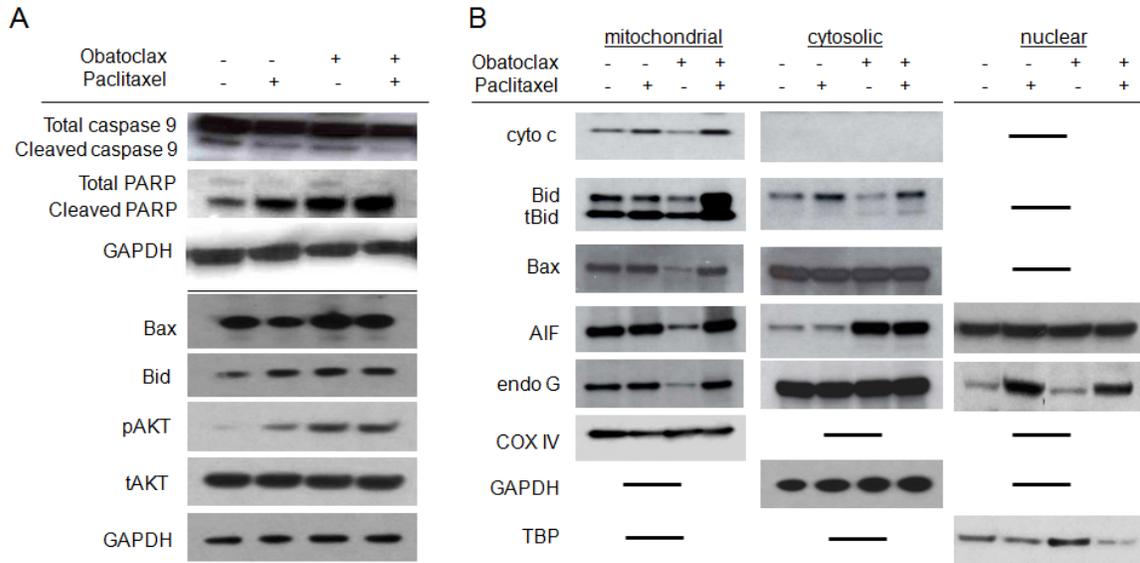
The specific mode of cell death synergized had to be determined as well. That is, if Bak is unsequestered and fulfills apoptosis, then combinatorial enhancement of apoptosis is expected to be observed. Annexin staining and flow cytometry allowed the degree of apoptosis to be determined, despite experimental obstacles from GX fluorescence (Figure 5).



**Figure 5.** Assessment of combination efficacy in inducing apoptosis. Annexin-staining apoptosis assay suggests combination synergy in inducing apoptosis. 86M1 were stained with Annexin V Pacific Blue fluorochrome-conjugate label and analyzed by flow cytometry. Each graph has a left peak, indicating non-apoptotic cells, and a right peak, indicating apoptotic cells. Note the combination right peak is higher than the addition of the single treatment right peaks.

### ***Elucidation of apoptotic mechanisms***

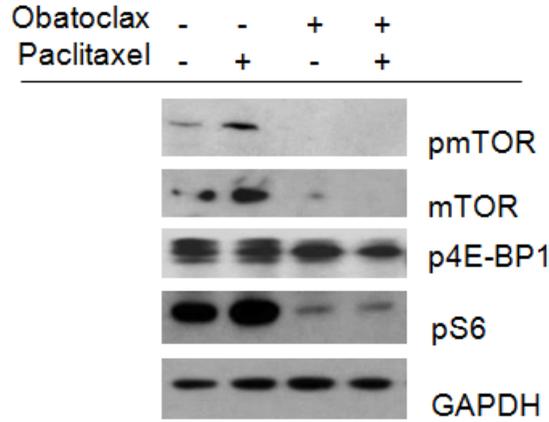
Also, with respect to the numerous types of cell death, especially in regards to the subtypes of apoptosis, it is necessary to determine protein-mediated signaling events to better characterize the type of apoptosis occurring. Numerous Western blots of apoptosis-related proteins were performed on whole cell lysates and some organelle fractions (Figure 6).



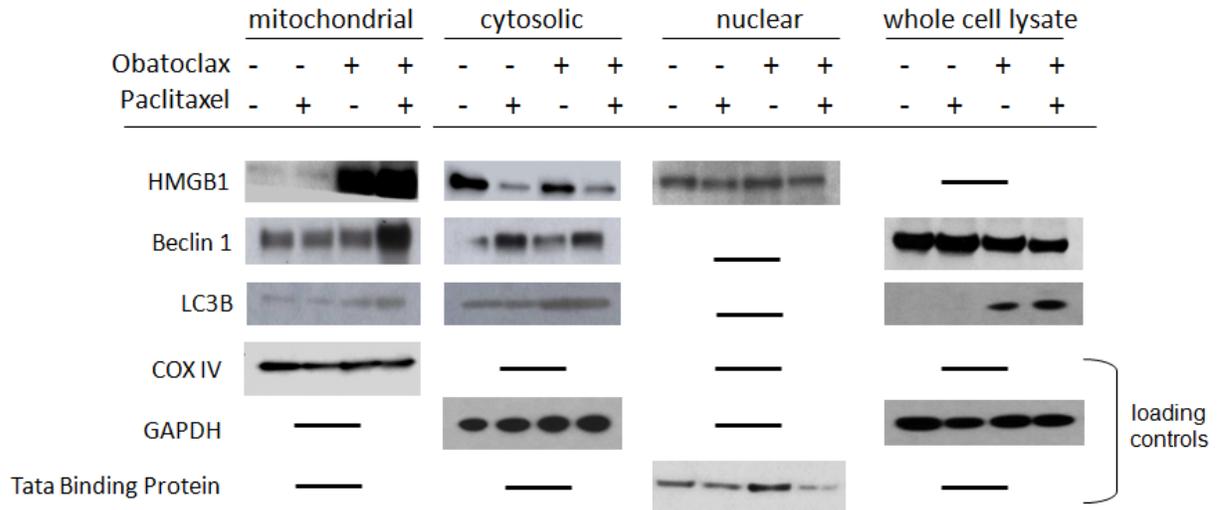
**Figure 6.** Western blots suggest a possible apoptosome-independent apoptotic pathway for this combination (a) Western blots of whole cell lysates suggest caspase 9 expression remains constant, hinting at a lack of apoptosome activity that would be proven with lack of cytochrome c involvement. pAKT appears to be more active in treated samples, which is optimal for SCLC killing. (b) Mitochondrial and cytosolic fractions were immunoblotted for apoptotic proteins. There is a differential increase in AIF release from the mitochondria to the cytosol in the GX-treated lysates. There is also a corresponding increase in truncated Bid in the mitochondria. Note the lack of cytochrome c release from the mitochondria, suggesting a cytochrome c-independent apoptosis mediated by AIF. COX IV confirmed mitochondrial fractionation success, and GAPDH was used as a loading control. There is clear synergy in pro-apoptotic Bid involvement with the combination.

***Involvement of non-apoptotic signaling pathways***

While apoptosis is an important means of cell death, other cell death-related pathways were investigated to determine a more widespread applicability of the combination. Involvement of the mTOR pathway was assessed (Figure 7). Involvement of the autophagic pathway or a pathway related to autophagy was also determined by probing for some key autophagic proteins (Figure 8). The autophagic pathway could lead to cell death.



**Figure 7** Data suggest mTOR pathway involvement. There is a decrease in mTOR activity via GX treatment, and there is an increase in phosphorylation of mTOR in singly treated paclitaxel samples. There is also a decrease in phosphorylation of pS6, which is downstream of mTOR, which confirms decreased mTOR activity.



**Figure 8.** Western blots were performed on mitochondrial and cytosolic fractions in addition to whole cell lysate. The most notable whole cell lysate finding is the involvement of downstream autophagic protein LC3B in GX-treated samples with possible slight synergy in an autophagy-related pathway. IB HMGB1 suggests increased autophagy-promoting translocation of this protein from the cytosol to the mitochondria with the GX-treated samples. There was a differential increase in pro-autophagy Beclin 1 in the mitochondria for the combination.

## MATERIALS & METHODS

### *Culture of cell line*

86M1 SCLC cells were maintained in complete RPMI (RPMI 1640 media, supplemented with 10% FBS, 100U/ml penicillin, 100µg/ml streptomycin, and 2mM L-

glutamine and penicillin-streptomycin, Invitrogen). Cells were enumerated, and live cells counted, using a hemacytometer and trypan blue exclusion.

### ***Drugs***

Paclitaxel was obtained as a 7 mM stock from Cardinal Health through the Moffitt Cancer Center pharmacy. Obatoclox was obtained from Gemin X Biotechnologies Inc.

### ***Immunoprecipitation***

This relatively unique immunoprecipitation technique was based on a Maxisorp adsorbent substrate from an ELISA plate (Nunc). Cells were treated with drugs and incubated overnight. After harvesting, they were suspended in a PBSCM mild buffer. The ELISA plate was coated with primary bcl-xL capture antibody and incubated overnight. The plate was blocked with 1% BSA, washed, and incubated with whole cell lysate for capture. After washing, the plate was denatured with heat and SDS to capture immunoprecipitate for Western blot analysis with whole cell lysate and immunoglobulin G controls.

### ***High throughput***

For analysis of suspension cells, 3.6 million 86M1 cells were used per plate. For one plate, cells were added by the high throughput pipette robot with complete RPMI media at 6000 cells/well in a 384 well plate on Day 0. After 24 hours of incubation at 37°C, the GX and PA drugs were added by this robot in 1:2 serial dilutions according to the high throughput software plan on Day 1. For the most optimal setup, the GX range was 0-1 µM, and the PA range was 0-50 nM, with a combination ratio of 8:1 GX:PA. After treatment, the plate was incubated for 72 hours at 37°C. On Day 4, after the three-day period, Cell Titer Blue (Promega Corp., Madison, WI) was added to the wells, and the plate was incubated at 37°C for 2-4 hours. The change in fluorescence was measured with a Synergy HT microplate reader (Bio-Tek Instruments, Inc., Winooski, VT), yielding raw optical data that was processed by high throughput software for synergy analysis, utilizing a combination index algorithm developed by Chou and Talalay [20].

### ***High throughput statistical analysis***

For drug combination experiments, the cell viability assays were performed as described above and the results analyzed for synergistic, additive, or antagonistic effects using the combination index (CI) method developed by Chou and Talalay. For the application of this method, the drug concentration dilutions of the drugs were used at fixed dose ratios based on the IC50s of each drug obtained from preliminary experiments. The dose-effect curve for each drug alone is determined based on experimental observations using the median-effect principle and is compared to the effect achieved with a combination of the two drugs to derive a CI value. This method involves plotting dose-effect curves, for each agent and their combination, using the median-effect equation:  $f_a/f_u = (D/D_m)^m$ , where D is the dose of the drug,  $D_m$  is the dose required for a 50% effect (equivalent to IC50),  $f_a$  and  $f_u$  are the affected and unaffected fractions, respectively ( $f_a = 1 - f_u$ ), and m is the exponent signifying the sigmoidicity of the dose-effect curve. The computer software XLfit was used to calculate the values of  $D_m$  and m.

The CI used for the analysis of the drug combinations will be determined by the “isobologram equation for mutually nonexclusive drugs that have different modes of action”:  $CI = (D)_1 / (Dx)_1 + (D)_2 / (Dx)_2 + (D)_1(D)_2 / (Dx)_1(Dx)_2$ , where  $(Dx)_1$  and  $(Dx)_2$  in the denominators are the doses (or concentrations) for  $D_1$  (drug 1) and  $D_2$  (drug 2) alone that gives certain percent inhibition, whereas  $(D)_1$  and  $(D)_2$  in the numerators are the doses of drug 1 and drug 2 in combination that also inhibited that percent inhibition – that is, it is isoeffective. Combination indices  $CI < 1$ ,  $CI = 1$ , and  $CI > 1$  indicate synergism, additive effects, and antagonism, respectively.

### ***Flow cytometric apoptosis assay***

After treatment with paclitaxel, cells were harvested and washed in cold PBS and then resuspended at a concentration of  $1-10 \times 10^6$  cells per ml in 1X Annexin V binding buffer (BD Biosciences). 100  $\mu$ l of the cells were placed in a facs tube and labelled with 10  $\mu$ l Annexin V-Pacific Blue conjugate. The cells were incubated for 15-20 minutes in the dark, after which 400  $\mu$ l 1X Annexin binding buffer was added to each tube. Cells were immediately analyzed using an LSRII cytometer (BD Biosciences). Annexin staining was analyzed using Flow Jo software (Tree Star Inc.). Apoptosis was quantified by percent positive cells following under the defined positive curve. Cycloheximide was used as a positive control.

### ***Western blot analysis***

Following the indicated treatment, cells were harvested, washed, and resuspended in 1X CHAPS buffer (Cell Signal Technologies) supplemented with 5mM DTT (Cell Signal Technologies) and 1 mM PMSF (Sigma). Protein concentration was determined using the BioRad protein assay and the lysates were diluted to equal concentrations. SDS-loading buffer (BioRad) was added to 1X and samples were boiled for 5 minutes. The proteins were resolved on 4-20 or 8-16% gradient pre-cast SDS-Hepes polyacrylamide gels (Pierce) and then transferred to PVDF membrane (Millipore). Membranes were blocked with Tris-buffered saline containing 5% non-fat milk (Carnation) and 0.5% Tween (Fisher). For phospho protein blotting, the 5% non-fat milk was replaced with 5% BSA (Sigma). The membranes were incubated in primary antibodies overnight at 4°C. The membranes were then washed and incubated for 1 hour in horseradish peroxidase-conjugated secondary goat anti-rabbit or rabbit anti-mouse antibodies (Millipore). Proteins were detected using ECL plus (Amersham) and hyperfilm (Amersham). All antibodies were purchased from Cell Signal Technologies, except AKT and pAKT, which were purchased from Santa Cruz Biotechnology Inc.

## **DISCUSSION**

To address the hypothesis that GX added to PA would permit the normal apoptotic cascade to occur, it was necessary to prove that adding GX and PA in combination works better than any single agent in killing small cell lung cancer cells of the 86M1 line. In other words, cooperativity, or synergy, of the two drugs was favorable. To determine synergy, a high volume drug assay using high throughput was performed;

specifically, it was a viability assay to see how many of the 86M1 cells were still alive after 72 hours of exposure to the drugs.

Figure 4 shows the dose response of the cells to the single agents and the optimal combination of 8:1 GX to PA. A higher curve means more cancer is alive at that concentration. There is not much single agent GX killing until the concentration is relatively high, which may lead to toxicity *in vivo*. The dose response for PA is better, but better results are achieved with the 8:1 GX-PA combination because the cancer viability is lower. High throughput software calculations indicate moderate synergy between the drugs, which, after numerous trials, is consistent.

These viability results were encouraging, but an apoptosis assay was required to show that the killing is due to apoptosis (Type I cell death) and not to the cells dying off by necrosis. Flow cytometry was used with the dye Annexin V, which stains cells that are undergoing or have already undergone apoptosis. In each of the graphs (Figure 5), the peak on the left shows live non-apoptotic cells, and the peak on the right shows apoptotic cells. One should consider the cells that died in the untreated to estimate how many cells in the other samples would have undergone apoptosis regardless of treatment. With this consideration in mind, one sees that for the samples treated with a single agent, the results are similar. In the sample treated with the combination, there is a higher effect such that apoptosis occurs more often. More cells undergo apoptosis with the combination than with the single agents, thereby confirming synergistic activity and indicating primarily apoptotic and not necrotic killing.

Some understanding the combination's mechanism of action was also favorable, so Western blots were performed on variably processed 86M1 samples (Figures 1, 6). PARP cleavage confirms treatment activity, and, more specifically, some degree of apoptotic synergy is observed (Figure 6). There does not appear to be apoptosome activity, as is indicated by the lack of cytochrome c release from the mitochondria, and the lack of a differential caspase 9 upregulation. There may also be increased Bid translocation in samples treated with the combination of GX and PA; there is clear synergy of mitochondrial Bid translocation in combination-treated samples. Results for an AIF probe indicate a GX-induced increase of cytosolic AIF that suggests DNA-interacting AIF is a key mediator of this apoptotic mechanism. Endo G, on the other hand, does not appear to share this role. Immunoprecipitation confirms obatoclax activity as well.

It is clear from immunoprecipitation of bcl-xL that Bak is indeed unsequestered upon addition of GX (Figure 1). This indicates that GX retains its activity in an *in vitro* paclitaxel-treated environment. Interestingly, the unsequestration of Bak also points to the possibility of unsequestration of other proteins that may not be directly related to apoptosis. That is, Beclin 1 is a pro-autophagy protein that is also sequestered by bcl-xL at the BH3 domain, which prevents it from fulfilling its autophagic function [22].

These findings suggest that GX may be a modulator of the process of autophagy by which stressed cells recycle macromolecular resources in autophagolysosomes, but progress to autophagic cell death (Type II cell death) under prolonged stress [23]. Further investigation of autophagic proteins indicates LC3B cleavage and generation in GX-treated samples, which is significant because LC3B is a necessary downstream marker of autophagy (Figure 8). When the other data are considered, it appears that at the very least, there is some involvement of an autophagy-related pathway. Investigation

into modulation of mTOR activity by the combination of GX and PA supplemented these data. It appears that mTOR activity is decreased in samples treated with obatoclax, since there is less mTOR phosphorylation and less phosphorylation of selected downstream substrates (Figure 7). The increased AKT phosphorylation under these treatment modalities could also relate to the mTOR pathway (Figure 6). The protein mTOR is critical to numerous metabolic and signaling events in cells but it is unclear, however, if indirect mTOR inhibition enhances cell death. There is some evidence that it may in SCLC [24].

Clearly, there is *in vitro* synergy between obatoclax and paclitaxel in the 86M1 small cell lung cancer line that is consistent after different assays and many trials. A synergistic combination is beneficial because it helps to avoid toxicity that may arise with an overdose of a single agent, which may be the only way to achieve the same effects as the combination. The involved mechanisms are not limited to only Type I cell death. The obatoclax-paclitaxel combination is thus very promising, and the project has absolute relevance to the clinical setting.

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