The Bcl-2/Bcl-X<sub>L</sub>/Bcl-w Inhibitor, Navitoclax, Enhances the Activity of Chemotherapeutic Agents <i>in vitro</i> and <i>in vivo</i>

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**Abbreviations used:** SCLC, small cell lung cancer; EGFR, epidermal growth factor receptor; CI, combination index; i.p., intraperitoneal; p.o., oral; i.v., intravenous; q.d. once a day; %T/C, the ratio of tumor volume for treated compared to control; %TGD, tumor growth delay; %ORR, overall response rate; %CR, complete regression; %PR, partial regression; R-CHOP, rituximab cyclophosphamide doxorubicin vincristine and prednisone; PBS, phosphate buffered saline; SD, standard deviation; SEM, standard error of the mean; PEG 400, polyethylene glycol 400; SDS-PAGE sodium dodecyl sulfate polyacrylamide gel electrophoresis; Phosal 50 PG, a dispersion of 50% phosphatidylcholine in a propylene glycol/ethanol carrier.
Abstract

A cancer cell’s ability to avoid apoptosis is crucial to tumorigenesis and can also contribute to chemoresistance. The Bcl-2 family of pro-survival proteins (Bcl-2, Bcl-X\textsubscript{L}, Bcl-w, Mcl-1 and A1) plays a key role in these processes. We previously reported the discovery of ABT-263 (navitoclax), a potent small molecule inhibitor of Bcl-2, Bcl-X\textsubscript{L} and Bcl-w. While navitoclax exhibits single-agent activity in tumors dependent on Bcl-2 or Bcl-X\textsubscript{L} for survival, the expression of Mcl-1 has been shown to confer resistance to navitoclax, most notably in solid tumors. Thus, therapeutic agents that can down-regulate or neutralize Mcl-1 are predicted to synergize potently with navitoclax. Here we report the activity of navitoclax in combination with 19 clinically relevant agents across a panel of 46 human solid tumor cell lines. Navitoclax broadly enhanced the activity of multiple therapeutic agents \textit{in vitro} and enhanced efficacy of both docetaxel and erlotinib in xenograft models. The ability of navitoclax to synergize with docetaxel or erlotinib corresponded to an altered sensitivity of the mitochondria towards navitoclax, which was associated with the down-modulation of Mcl-1 and/or up-regulation of Bim. These data provide a rationale to interrogate these combinations clinically.
Introduction

The Bcl-2 family comprises two broad categories of pro-survival (Bcl-2, Bcl-XL, Bcl-w, Mcl-1, and A1) and pro-apoptotic (Bax, Bak, Bim, Bid, Puma, Bad, Noxa, Bik, Bmf, and Hrk) proteins (1-4). In general, the balance between these proteins determines whether a cell lives or dies. The overexpression of pro-survival Bcl-2 family members provides one means by which cancer cells can overcome the continual assault of cellular stresses that would cause normal cells to undergo apoptosis. Their overexpression has also been shown to correlate with poor prognosis and resistance to therapy (5). For example, overexpression of Bcl-2 has been shown to confer resistance to cytotoxic agents in lung cancer (6, 7), while both Bcl-2 and Bcl-XL are associated with the emergence of androgen independence and chemoresistance in prostate cancer (8-11). Thus, directly targeting the apoptotic machinery represents an attractive strategy for enhancing the efficacy of certain chemotherapeutics (12).

Recently, we described the discovery of ABT-263 (navitoclax), an orally bioavailable Bcl-2 family protein inhibitor with high affinity (<1 nmol/L) to Bcl-2, Bcl-XL, and Bcl-w, but not Mcl-1 or A1 (13). Navitoclax exhibits potent single agent antitumor activity in multiple murine xenograft models of small cell lung cancer (SCLC), leukemia, and lymphoma (13, 14) and, in addition, potentiates the activity of clinically relevant chemotherapeutic regimens - e.g., rituximab, rapamycin, R-CHOP, and bortezomib, in several models of hematologic malignancies (14, 15). However, the interrogation of the ability of navitoclax to enhance the activity of chemotherapeutic agents in solid tumors has been limited (16).
To address this question, we have examined navitoclax in combination with 19 chemotherapeutic agents across a panel of 46 cell lines representing 16 tumor types. These cell lines are commercially available and often utilized for drug development. Navitoclax enhanced the activity of several chemotherapeutic agents, representing diverse mechanisms of action, across a broad spectrum of tumor types. To extend these observations, potent combinations were tested in murine xenograft models. Navitoclax demonstrated activity in combination with erlotinib or docetaxel in lung or ovarian cancer models in vitro and in vivo. Subsequent mechanism of action and siRNA-mediated knockdown experiments indicated that this efficacy was associated with the neutralization of Mcl-1. Taken together, our studies provide a mechanistic rationale for the use of navitoclax in combination with standard-of-care agents that neutralize Mcl-1 or increase pro-apoptotic BH3 proteins such as Bim and Noxa.
Materials and Methods

Compounds and cell lines

Chemotherapeutic agents were purchased from Sigma (St. Louis, MO) or Calbiochem (La Jolla, CA), or synthesized internally at Abbott Laboratories. The SCC25, SCC9, FADU, HN, 786-O, RENCA, HEP3B, HEPG2, A549, Calu-6, DMS-53, H196, H460, H1299, D54MG, DoHH2, SuDHL4, SKNFl, HEYA8, OVCAR-3, OVCAR-4, OVCAR-5, OVCAR-8, SKOV3, MiaPaCa, PANC-1, BxPC3, PC3, 22RV1, LNCAP, HT-1080, SK-LMS-1, A-375, A-431, AGS (CRL-1739), N87, HCT116, DLD1, SW620, Colo-205, EJ-1, MDA231, MDA435, MCF-7, Mx-1, BT474, K562, H1650 were purchased from ATCC (American Type Culture Collection) or DSMZ (Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH). These cell lines were not reauthenticated before using in this study. They are grown in medium conditions recommended by the suppliers or in RPMI medium (Invitrogen, Carlsbad, CA) supplemented with 10% fetal bovine serum (Invitrogen, Carlsbad, CA).

Cell viability assays

5,000 cells were plated per well in a 96-well plate and incubated with each therapeutic agent ± 1 μM navitoclax for 72 hr in a total volume of 100 μl culture medium supplemented with 10% fetal bovine serum. Cytotoxicity was assessed using the CellTiter-Glo Assay that measures the ATP content of the cells according to manufacturer’s specifications (Promega, Madison, WI). Agents that induced ≥ 20% reduction in combination with navitoclax below what was observed with agent alone across two doses were considered synergistic hits from the screen. To quantitatively
assess the combination effects of navitoclax with other chemotherapeutic agents, experiments were performed to determine the combination index (CI), which is a value derived from the multiple drug-effect equation of Chou-Talalay (17). CalcuSyn software from Biosoft (Cambridge, UK) was used for this analysis. Briefly, this method of analysis distinguishes between synergistic, additive, and antagonistic drug-drug interactions. A CI value = 1.0 indicates an additive effect. CI values < 1.0 reflect a synergistic effect, while CI values > 1.0 reflect an antagonistic effect.

**Immunoblotting**

20-30 μg of whole cell or fractionated lysates were separated by SDS-PAGE (4-20% gels) and transferred to Immobilon-P membranes (Millipore, Bedford, MA). The membranes were incubated with primary antibodies against Bcl-2 (Epitomics, Burlingame, CA), Bcl-Xₐ (Epitomics, Burlingame, CA), Mcl-1 (Santa Cruz Biotechnology, Santa Cruz, CA), Bim (Epitomics, Burlingame, CA), Bid (BD Biosciences, San Jose, Ca), Bax (Epitomics, Burlingame, CA), Puma (Sigma, St. Louis, MO), Noxa (Abcam, Cambridge, UK), pEGFR (Invitrogen, Carlsbad, CA), EGFR (Invitrogen, Carlsbad, CA), pERK (Cell Signaling Technology, Waltham, MA), ERK (Cell Signaling Technology, Waltham, MA) or Cytochrome c (BD Biosciences, San Jose, Ca) overnight at 4°C. Primary antibodies were detected using either a goat anti-mouse or goat anti-rabbit Alexa Fluor 680-conjugated or IRDye 800CW-conjugated secondary antibody (Molecular Probes, Carlsbad, CA) diluted 1:10000 for 30 min. Bands were detected using the Odyssey Infrared Imaging System (LI-COR Biosciences, Lincoln, NE).
Mitochondrial cytochrome c release assay

SKOV3 cells were pretreated with 1.5 nM docetaxel or 2.5 μM gemcitabine for 48 hr, washed and resuspended in cell permeability buffer (20 mM HEPES, pH 7.2, 100 mM KCl, 5 mM MgCl₂, 1 mM EDTA, 1 mM EGTA, 250 mM sucrose, 0.015% digitonin, Complete Protease Inhibitor; Roche), and incubated on ice for 3-5 min. Permeabilized cells were then incubated with 10 μM navitoclax, enantiomer, Bim-BH3 peptide or vehicle at 30°C for 75 minutes. Mitochondrial and cytosolic fractions were separated by centrifugation at 16000 x g at 4°C for 10 minutes, resolved on a 10% SDS-PAGE gel and analyzed by immunoblotting using a mouse anti-cytochrome c antibody (BD Biosciences, San Jose, CA).

High content analysis

A standard immunofluorescence protocol was used to prepare samples for high content analysis. SKOV3 cells were propagated in RPMI-1640 supplemented with 10% FBS. Cells were plated at a density of 2,000 cells per well (100 μL) in 96-well Collagen I-coated microplates (BD Biosciences, San Jose, CA) and incubated overnight under standard cell culture conditions. On the next day, cells were treated with docetaxel or vehicle by addition of a 50 μl solution containing a 3x compound stock and incubated for an additional 48 hr. Cells were then challenged with navitoclax for the indicated times. Cells were subsequently fixed by the addition of 100 μl of pre-warmed 10% formaldehyde (Sigma, St. Louis, MO) made up in PBS, pH 7.4 lacking Ca²⁺/Mg²⁺ (Invitrogen Carlsbad, CA) and incubated at room temperature for 10 minutes. All
subsequent reagents were made up in PBS, pH 7.4 lacking Ca\textsuperscript{2+}/Mg\textsuperscript{2+}. Wells were then washed twice with 200 µl PBS and permeabilized with 0.1% Triton X-100 for 10 minutes, washed with PBS, blocked with 1% BSA for 20 minutes and then incubated with 50 µl of a 1:100 dilution of a rabbit antibody raised against activated Caspase-3 (Cell Signaling Technology, Beverly, MA). Primary antibody was incubated overnight at 4 °C, washed with 200 µl PBS, incubated for an additional hour with a 50 µl mixture of a 1:200 dilution of Alexa 488-conjugated goat anti-rabbit antibody and 1 mg/ml Hoechst 33342 (Invitrogen, Carlsbad, CA). After a 1 hr incubation in the dark, cells were washed 3x with 200 µl PBS and imaged on an ArrayScan VTI HCS Reader (Thermo Scientific, Waltham, MA) with a 10x (0.3 N.A. objective). The Cell Health Profiling BioApplication was used to quantify the percentage of cells exhibiting caspase-3 activation above baseline levels determined from cells that received vehicle only.

**Small interfering RNA (siRNA) transfection**

SKOV3 cells were plated at 1 x 10\textsuperscript{4} cells/100 µl in 96-well or 3 x 10\textsuperscript{5} cells/2 ml in 6-well tissue culture plates and transfected with siRNA duplexes targeting Mcl-1 (M-004501-02) or scrambled siRNA (D-001210-01) from Dharmacon (Lafayette, CO). Briefly, siRNAs were prepared by incubating 1.5 µl of a 20 µM siRNA stock with 100 µl Opti-MEM (Invitrogen, Carlsbad, CA) for 10 minutes and 3 µl LipofectAMINE 2000 (Invitrogen, Carlsbad, CA) with 100 µl Opti-MEM. Each solution was then mixed and incubated for an additional 20 minutes at room temperature. SiRNA mixtures were then added to the cells. siRNA knockdown was confirmed by immunoblotting using whole
cell lysates of SKOV3 treated with Mcl-1 siRNA or scrambled control siRNA. Cell viability was assessed after 72 hr using the CellTiter-Glo assay as described above.
In vivo xenograft studies.

All animal studies were conducted according to guidelines established by the Abbott and Genentech Institutional Animal Care and Use Committee. Xenograft studies were conducted by implantation of cells or tumor fragments in the flanks of immunocompromised mice. Tumor-bearing mice were size matched (~235 mm³; day 0) into treatment and control groups and tumor size was monitored serially in individual animals using electronic calipers. Tumor volume was calculated two to three times weekly using the following equation: Volume=Length x Width²/2. %TGI, %TGD, %PR, %CR and %ORR are defined as previously described (14). All studies utilized 8-10 mice per group. Statistical comparisons of tumor growth rate and tumor growth delay utilized the Wilcoxon rank sum test and the Mantel-Cox log rank test, respectively. Navitoclax was formulated in 10% ethanol, 30% PEG 400, and 60% Phosal 50 PG and administered orally by gavage. Docetaxel was purchased from Sanofi Aventis (Paris, France), and gemcitabine was purchased from Eli Lilly (Indianapolis, IN). These agents were dosed intravenously and intraperitoneally, respectively, at the schedule and dose described in the table descriptions. Erlotinib was formulated in 7.5% Captisol (sulfobutylether-β-cyclodextran) and dosed daily by oral gavage.
Results

Navitoclax potentiates multiple therapeutic agents across multiple tumor types

The single agent cellular activity of navitoclax has only been shown in SCLC and hematological malignancies (13, 18). To evaluate the breadth of cellular activity in combination with other chemotherapeutic agents, 19 therapeutic agents were tested in the presence or absence of 1 μM navitoclax against 46 human cancer cell lines representing 16 tumor types (Figures 1A). Using a six-point dose response curve, positive combination activity was defined as ≥ 20% reduction in survival across two or more doses in the presence versus absence of navitoclax. Notably, navitoclax enhanced the activity of multiple therapeutic agents broadly across multiple tumor types, with 196 combination hits identified out of 774 examined (~25% hit rate). Combination synergy, defined as a response that is greater than the expected additive effect, was evaluated using combination index analysis according to the multiple drug-effect equation of Chou-Talalay (17). To select the best positive combination activities for further investigation in vivo, combination index analyses were performed across different cell line models to determine the CI_{50-90} for these combinations. Navitoclax displayed synergy with DNA damaging agents (e.g., gemcitabine, etoposide, camptothecin and doxorubicin) and anti-mitotic agents (e.g., docetaxel and vincristine) (Figure 1B) in a variety of tumor types in vitro, as confirmed by combination index analyses (Table 1).

Navitoclax enhances the activity of docetaxel in vivo

As shown in Figure 1B, docetaxel exhibited positive combination activities with navitoclax in a high percentage of cancer cell lines (78%). Docetaxel represents a
clinically relevant anti-microtubule agent approved for use in a variety of tumors (19). To extend these observations to *in vivo*, navitoclax was tested in combination with docetaxel in the SKOV3 ovarian cancer xenograft model using a variety of different schedules (Figure 2). As a monotherapy, navitoclax dosed orally once a day at 100 mg/kg/day for 2, 14 or 21 days was not efficacious in the SKOV3 xenograft model (Figure 2). Docetaxel administered once weekly for 3 cycles at 10 mg/kg/day produced a significant decrease in tumor burden (TGI) of 82% with a delay in regrowth (TGD) of 114% (Table 2). In contrast, bolus dosing of docetaxel dosed 30 mg/kg once i.v. inhibited tumor growth by only 48% with no significant effect on time-to-progression. Overall response rates were slightly higher when cyclical dosing was administered, rather than bolus dosing (30% v. 0%).

Navitoclax enhances the activity of docetaxel under most schedules. Chronic (14d or 21d) daily administration of navitoclax concurrently with either docetaxel regimen yielded greater than additive inhibition of tumor growth, although this only reached statistical significance in the bolus dosing group (Figure 2A & B, Table 2). Effects on delay of tumor regrowth and overall response with concurrent, chronically dosed navitoclax were significantly improved in both docetaxel regimens compared to docetaxel alone. Tumor growth delay (TGD), a measurement of durability of antitumor effect, was also significantly improved from 12% to 135% and from 114% to >175% in the bolus dosing and cyclical dosing, respectively. Overall response rates were also significantly improved from 0% to 89% and 30% to 100% in the bolus dosing and cyclical dosing, respectively. Concurrent, acute dosing of navitoclax (2d) with bolus administration of docetaxel also yielded greater than additive results, with tumor growth
inhibition from 57 to 86% and tumor growth delay from 12% to 54% (Figure 2C, Table 2). Sequential dosing of docetaxel and navitoclax was also investigated. Administration of docetaxel on day 1, followed by 14 days of navitoclax therapy induced a significant inhibition in tumor growth and a greater than additive delay in regrowth, similar to acute concurrent administration of navitoclax and docetaxel (Figure 2D). In contrast, acute navitoclax treatment following a bolus dose of docetaxel yielded only modest increases in tumor growth inhibition and delay, as did administration of a bolus dose of docetaxel following 14 days of navitoclax (Figure 2E & F).

**Docetaxel increases mitochondrial sensitivity to Bcl-2/Bcl-X<sub>L</sub>**

To gain a better mechanistic understanding of how docetaxel synergizes with navitoclax, we examined the effect of single agent and combination treatments on apoptotic signaling in SKOV3 in vitro. Neither docetaxel nor navitoclax elicited cytochrome c release in permeabilized SKOV3 cells (Figure 3A). In contrast, 48 hours pretreatment with 1.5 nM docetaxel followed by 75 minutes of navitoclax treatment led to cytochrome c release similar to that observed by treatment with Bim BH3 peptide. The enantiomer of navitoclax with much lower target affinity is unable to elicit this effect. To examine effects on apoptosis in intact cells, high content analysis was performed. As shown in Figure 3B, no increase in activated caspase-3 is detected in cells treated with navitoclax alone. However, pretreatment of SKOV3 cells with 1.5 nM docetaxel and subsequent incubation with navitoclax induced a concentration-dependent increase in activated caspase-3 within 2 hours. Taken together, these data indicate that docetaxel acts
synergistically with navitoclax by increasing mitochondrial sensitivity to Bcl-2 and/or Bcl-XL inhibition.

**Docetaxel increases mitochondrial sensitivity to navitoclax by neutralizing Mcl-1 function**

In order to determine which events might be responsible for the combination synergy observed with navitoclax, the expression levels of several key Bcl-2 family proteins were examined in response to docetaxel treatment. Treatment of SKOV3 cells with docetaxel resulted in a decrease in Mcl-1 levels (Figure 3C). No other significant changes were observed with the exception of a modest increase in BH3-only protein Noxa levels at 12 nM docetaxel. Because Mcl-1 is a well known resistance factor for ABT-737 (20, 21), we next evaluated whether targeted knockdown of Mcl-1 would enhance navitoclax activity in these cells. While knockdown of Mcl-1 on its own had no effect on cell viability (Figure 3D), it enhanced the cytotoxicity of 1 μM and 5 μM navitoclax by 35% and 25%, respectively. The p values are <0.0002 and <0.002 for 1 μM and 5 μM navitoclax treatment respectively. These data suggest that the combination synergy observed between docetaxel with navitoclax is due, at least in part, to the neutralization of Mcl-1.

**Navitoclax enhances the activity of erlotinib in vitro**

In addition to the broad synergy observed with cytotoxic agents, navitoclax also enhanced the activity of erlotinib, an epidermal growth factor receptor (EGFR)-specific kinase inhibitor. Positive combination activity between navitoclax and erlotinib was
observed in 5 of the 46 lines examined (5 out of 774 combinations examined; Figures 4A). Interestingly, in all five of these lines (A431, BxPC3, N87, OVCAR3, and SKOV3) EGFR has been reported either amplified (A431) or overexpressed (22-25). Clinically, tumors with activation mutations such as the EGFR deletion 746-750 have been most responsive to erlotinib therapy (26). We next investigated the combination in a non-small cell lung cancer line (NCI-H1650) with this genetic alteration to assess the ability of navitoclax to enhance apoptotic potential and anti-tumor efficacy of erlotinib. Combination index analysis of the navitoclax-erlotinib combination in NCI-H1650 cells confirmed synergistic activity with an average combination index of 0.44 across the EC$_{50}$ to EC$_{90}$ (CI$_{50-90}$) (Figure 4B). Examination of Bcl-2 family proteins indicated the coordinated down-modulation of Mcl-1 and up-regulation of Bim$_{EL}$ after 6 hr treatment with erlotinib in vitro. These data are consistent with previous findings demonstrating synergy between erlotinib (or gefitinib) and ABT-737, an earlier generation Bcl-2 family protein inhibitor (27, 28).

**Navitoclax enhances the activity of erlotinib in vivo**

To confirm the synergistic effects of navitoclax and erlotinib on tumor cell proliferation observed in vitro in the NCI-H1650 model, we tested the combination of these two targeted agents in vivo. As a single agent 100 mg/kg navitoclax alone dosed daily had no significant anti-tumor activity, while daily dosing of erlotinib at 50 mg/kg resulted in significant tumor stasis (%TGI = 52) during a 21 day treatment period (Figure 4C). Notably, the combination of navitoclax and erlotinib dosed daily for 21 consecutive days resulted in 98% TGI and durable tumor regressions in 100% of tumor bearing mice.
treated (Figure 4C). The latter was sustained for 25 days even after the cessation of dosing (Figure 4C). Additionally the combination of navitoclax and erlotinib was well tolerated, resulting in less than 5% body weight loss during the treatment period.

To investigate potential mechanisms of action underlying the enhanced combination activity of navitoclax and erlotinib in the NCI-H1650 xenograft model in vivo, tumors were harvested post-treatment and analyzed for EGFR and Bcl-2 pathway markers. Treatment with erlotinib resulted in decreased pERK levels and induced significant down-modulation of Mcl-1 and up-regulation of Bim (Figure 4D), which is consistent with previous in vitro findings (Figure 4B). Thus, the enhanced anti-tumor activity of erlotinib in combination with navitoclax in vivo is due to simultaneous inhibition of the EGFR and Bcl-2 pro-survival pathways.
Discussion

Defects in the apoptotic cascade are a hallmark of cancer and are often associated with chemoresistance (29). Thus, agents that restore the ability of cancer cells to undergo apoptosis may enhance the activity of chemotherapies when used in combination. We recently reported the discovery of navitoclax, an orally bioavailable small molecule Bcl-2 family protein inhibitor currently in Phase 1/2 clinical development. Preclinically, navitoclax exhibits single-agent activity in small cell lung cancer and leukemia and lymphoma cell lines but has limited activity in the majority of solid tumor cell lines examined (13-15, 30). In combination, navitoclax has been shown to enhance the efficacy of rapamycin, rituximab, R-CHOP and bortezomib in various leukemia and lymphoma models (13, 15). In non-small cell lung cancer xenograft tumor models, navitoclax enhances the efficacy of taxanes (16). However, investigation of the ability of navitoclax to enhance the efficacy of clinically relevant therapeutics in other solid tumor models has been limited. To address this, we conducted a screen of navitoclax in combination with 19 therapeutically relevant agents across 46 solid tumor cell lines to identify potential combinations for clinical interrogation.

In vitro, navitoclax enhanced the activity of several chemotherapeutic agents across multiple cell lines. Notably, the greatest synergy was observed in combination with therapeutics that act as anti-mitotics (docetaxel and vincristine) or induce DNA-damage (camptothecin, gemcitabine, etoposide and doxorubicin). These in vitro findings were confirmed in vivo. Navitoclax treatment in the SKOV3 xenograft model significantly enhanced the anti-tumor activity of the anti-mitotic agent docetaxel with significantly
improved overall response rates compared to docetaxel treatment alone. Additionally, combination treatment resulted in more durable anti-tumor responses, as evidenced by the significant increase of %TGD. In that it has been noted that the scheduling of docetaxel dosing can have a significant effect on antitumor activity (16, 31), we investigated a variety of doses and schedules in our combination studies. Addition of navitoclax to docetaxel treatment resulted in enhanced efficacy under a variety of dose schedules. We find that navitoclax is effective when administered concurrent with or immediately after dosing of docetaxel, while pre-dosing with navitoclax prior to docetaxel administration did not significantly enhance efficacy. The combinations of navitoclax-docetaxel and navitoclax-gemcitabine were evaluated once in N87 (gastric) and OVCAR5 (ovarian) xenograft models (Supplemental Figure 1). Consistent with our in vitro findings, these combinations demonstrated enhanced anti-tumor activity in vivo over that of the chemotherapeutic treatment alone, as evidenced by a significant increase in %ORR (from 50% to 100%) for navitoclax-docetaxel in N87 and significant improvement in %TDG (from 39% to 76%) for navitoclax-gemcitabine in OVCAR-5 (Supplemental Table 1).

Mechanistically, we observed that navitoclax induces cytochrome c release and caspase-3 activation upon pretreatment with docetaxel. As such, we hypothesized that docetaxel may alter the sensitivity of the mitochondria (and hence the cell viability) in part by neutralization of Mcl-1 via a down-regulation of Mcl-1 protein levels. The role of Mcl-1 as a resistance factor to navitoclax and ABT-737 has been heavily investigated in multiple cellular systems (13, 20, 21, 32, 33). Indeed, siRNA knockdown of Mcl-1 in SKOV3 cells sensitized these cells to navitoclax, further indicating that neutralization of Mcl-1 may, in part, underlie the observed combination synergy. Our data indicate that
chemotherapies that indirectly modulate Mcl-1 levels or function may exhibit *in vivo* combination synergy with navitoclax. If so, this criterion may be used as a rational approach in the selection of combination agents.

Applying this criterion, we observed that erlotinib treatment was capable of down-modulating Mcl-1. As such, we investigated whether navitoclax enhanced the anti-tumor activity of erlotinib *in vivo*. Consistent with our hypothesis, the combination of erlotinib with navitoclax in a NSCLC xenograft model demonstrated durable tumor regressions and 100% ORR in mice, whereas mice treated with erlotinib alone demonstrated only a modest tumor growth inhibition during the treatment period. In addition to down-modulation of Mcl-1 in tumors isolated from mice treated with erlotinib, we also noted an up-regulation of the pro-apoptotic BH3 member Bim.

In summary, we report that navitoclax significantly enhances the activity of chemotherapeutic and targeted agents against a variety of solid tumor types *in vitro* which may in part be due restoration of the cell’s apoptotic potential. While we used CI values from these studies as a guide to select the best combinations to be tested *in vivo*, they weren’t always predictive of antitumor activity. For example, navitoclax did not consistently enhance the antitumor activity of docetaxel in BxPC3 and of gemcitabine in SKOV3 xenograft models, even though their CI values were less than 0.1 *in vitro* (data not shown). The latter demonstrates the inherent challenges in utilizing *in vitro* studies to predict *in vivo* efficacy when differences in drug exposures and microenvironment are not accounted for. Utilizing a mechanism-based approach, we identified multiple efficacious combinations that were confirmed *in vivo*. Continued exploration of mechanism-based
studies with navitoclax and other therapeutic agents should ultimately aid clinical trial design and success in the clinic.
References

Table 1.

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Navitoclax dosed p.o. 1-2h prior to docetaxel i.v. Statistical analysis for %TGI performed by Wilcoxon rank sum, for %TGD by Mantel-Cox log rank test, and for %CR and %ORR by Fisher’s exact test.

Groups 1-4 and groups 5-14 from separate experiments.

1: % complete responders.
2: % overall response rate.
3: % tumor growth inhibition.
4: % tumor growth delay.

a: P < 0.05 v. group 1
b: P < 0.05 v. group 3
c: P < 0.05 v. group 8
d: P < 0.05 v. group 11
e: P < 0.05 v. group 13
Figure Legends

Figure 1. Navitoclax broadly potentiates the activity of multiple therapeutic agents. A, Screening strategy used to identify combination synergies. Therapeutic agents were screened in a six-point dose response curve with 10-fold dilutions (except doxorubicin and vincristine, where 3-fold dilutions were used) ± 1 μM navitoclax. Potentially synergistic combinations were further interrogated in an 8 x 10 dose response matrix between navitoclax and the agent of interest. The combination index (CI) was determined as described by Chou and Talalay. B, The frequency (% hit rate) at which an agent enhanced the activity of navitoclax across the cell lines was examined. The highest dose of each therapeutic agents used in the screening were also listed next to the agent on the x-axis in μM.

Figure 2. In vivo anti-tumor effect of navitoclax in combination with docetaxel in SKOV3 xenograft models with different scheduling. Tumor volume was plotted over days post-tumor staging with SKOV3 xenografts. Each treatment group consisted of 8-10 mice. Points, mean; bars, SEM. Navitoclax and docetaxel were dosed as described in insets. A, Continuous dosing of navitoclax for 21 days + fractionated, once weekly dosing of docetaxel. B, Continuous dosing of navitoclax for 14 days + bolus dose of docetaxel. C, Abbreviated dosing of navitoclax + bolus dose of docetaxel. D, Bolus dose of docetaxel followed by continuous dosing of navitoclax for 14 days. E, Bolus dose of docetaxel followed by abbreviated dosing of navitoclax. F, Continuous dosing of navitoclax for 14 days followed by bolus dose of docetaxel.

Figure 3. Docetaxel alters mitochondrial sensitivity to navitoclax by neutralizing Mcl-1. A, Release of cytochrome c induced by 10 μM navitoclax ± pretreatment with 4 nM...
docetaxel for 48 hr. Permeabilized SKOV3 cells were fractionated into mitochondrial and cytosolic fractions and cytochrome c detected as described in the Methods section. B, High content quantification of SKOV3 cells showing caspase-3 activation in response to navitoclax ± docetaxel pretreatment. Shown is the mean ± SD (n=3). C, Immunoblots of Mcl-1 and Noxa in whole cell lysates isolated from SKOV3 cells treated with 4-12 nM docetaxel for 48 hr. D, siRNA knockdown of Mcl-1 leads to increased cellular sensitivity to navitoclax. Relative percent survival of SKOV3 ± navitoclax at the indicated concentrations in the presence of Mcl-1 siRNA or scrambled siRNA control. * p < .002, ** p <.0002 determined by Student’s T-Test.

**Figure 4.** Erlotinib upregulates Bim and down-regulates Mcl-1 leading to enhanced cytotoxicity in combination with navitoclax in vitro and *in vivo*. A, Relative percent survival of cells treated with 5 μM erlotinib in the presence (+) or absence (-) of navitoclax. Shown is the mean (n=3). B, CI value as a function of fractional effect (cytotoxicity) for erlotinib in combination with navitoclax in H1650 cells. Shown is a representative curve (n=3). Panel inset: protein expression levels of pERK, ERK, Mcl-1, Bim and Actin in H1650 cells treated with 0, 1 or 3 μM erlotinib for 6 hr. C, Combination of navitoclax and erlotinib in H1650 xenografts. Tumor bearing mice (n=6) were administered vehicles for navitoclax (60% phosal) and erlotinib (7.5% Captisol) simultaneously, 50 mg/kg erlotinib, 100 mg/kg of navitoclax or the combination of navitoclax and erlotinib orally (PO) for 21 consecutive days. * p < .001, ** p <.0001 determined by Student’s T-Test. D, Tumor xenografts were harvested from mice (n=3) treated with vehicle control, 50 mg/kg erlotinib, 100 mg/kg navitoclax or the combination of erlotinib and navitoclax for 21 consecutive days and analyzed for the
pEKR-1/2, Bim, or Mcl-1 by Western blotting using primary antibodies described in Materials and Methods. β-tubulin served as the protein loading control.

**Table 1.** CI of various therapeutic agents in combination with navitoclax. The CI values were calculated by averaging the CI values at ED$_{50}$, ED$_{75}$ and ED$_{90}$. Shown is the mean (n ≥ 2).

**Table 2.** Combination activity of navitoclax with docetaxel in SKOV3 tumor xenograft model.
Figure 1

A

46 cancer cell lines (16 tumor types)

19 therapeutic agents (clinical/experimental)

% Survival

[drug X], μM

0 0.01 0.1 1 10 100

(-) navitoclax

(+) navitoclax

Additive Antagonistic

CI > 1

Synergistic

CI < 1

ED₅₀

B

DNA-damaging

Anti-mitotic

Other/targeted

% Hit rate

temozolomide 500

pemetrexed 100

gemcitabine 500

cytarabine 100

etoposide 100

cisplatin 100

carboplatin 100

vincristine 0.05

docetaxel 5

vorinostat 50

bortezomib 1

sorafenib 50

seliciclib 50

rapamycin 0.5

lapatinib 50

imatinib 50

erlotinib 50
Figure 2

A

- 2x vehicle
d1-14/d1
- navitoclax 100 mkd d1-21
- docetaxel 10 mkd d1,8,15
- navitoclax + docetaxel

B

- 2x vehicle d1-14/d1
d1
- navitoclax 100 mkd d1-14
- docetaxel 30 mg/kg d1
- navitoclax + docetaxel

C

- 2x vehicle d1-14/d1
d1-14/d1
- navitoclax 100 mkd d1-2
- docetaxel 30 mg/kg d1
- navitoclax + docetaxel

D

- 2x vehicle d1-14/d1
d1
- navitoclax 100 mkd d2-15
- docetaxel 30 mg/kg d1
- navitoclax + docetaxel

E

- 2x vehicle d1-14/d1
- navitoclax 100 mkd d2-3
- docetaxel 30 mg/kg d1
- navitoclax + docetaxel

F

- 2x vehicle d1-14/d1
- navitoclax 100 mkd d1-14
- docetaxel 30 mg/kg d15
- navitoclax + docetaxel
Figure 3

A. Cytochrome c blots

B. % Cells with active Caspase-3 (t=2 h)

C. [docetaxel], nM

D. [siRNA], nM

Mcl-1

Noxa

Actin

Relative % Survival
Figure 4

A

![Graph showing relative survival of different cell lines with different drug treatments.](image)

B

![Graph showing combination index for NCI-H1650 cells with drug treatments.](image)

C

![Graph showing mean tumor volume over time for different treatment groups.](image)

D

![Western blot analysis for different cell lines and treatments.](image)
Molecular Cancer Therapeutics

The Bcl-2/Bcl-XL/Bcl-w Inhibitor, Navitoclax, Enhances the Activity of Chemotherapeutic Agents in vitro and in vivo

Jun Chen, Sha Jin, Vivek Abraham, et al.

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