

The Bcl-2/Bcl-X_L/Bcl-w Inhibitor, Navitoclax, Enhances the Activity of Chemotherapeutic Agents *In Vitro* and *In Vivo*

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Abstract

The ability of a cancer cell to avoid apoptosis is crucial to tumorigenesis and can also contribute to chemoresistance. The Bcl-2 family of prosurvival proteins (Bcl-2, Bcl-X_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_L, and Bcl-w. While navitoclax exhibits single-agent activity in tumors dependent on Bcl-2 or Bcl-X_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 downregulate 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 *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 toward navitoclax, which was associated with the downmodulation of Mcl-1 and/or upregulation of Bim. These data provide a rationale to interrogate these combinations clinically. *Mol Cancer Ther*; 10(12);2340–9. ©2011 AACR.

Introduction

The Bcl-2 family comprises 2 broad categories of prosurvival (Bcl-2, Bcl-X_L, Bcl-w, Mcl-1, and A1) and proapoptotic (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 prosurvival Bcl-2 family members provides a 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), whereas both Bcl-2 and Bcl-X_L

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-X_L, and Bcl-w but not to 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, for example, rituximab, rapamycin, rituximab-cyclophosphamide-Adriamycin-vincristine-prednisone (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 used 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 showed activity in combination with erlotinib or docetaxel in lung or ovarian cancer models *in vitro* and *in vivo*.

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Note: Supplementary material for this article is available at Molecular Cancer Therapeutics Online (<http://mct.aacrjournals.org/>).

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doi: 10.1158/1535-7163.MCT-11-0415

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Subsequent mechanism of action and short interfering RNA (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 proapoptotic BH3 proteins such as Bim and Noxa.

Materials and Methods

Compounds and cell lines

Chemotherapeutic agents were purchased from Sigma or Calbiochem 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, SKNFI, HEYA8, OVCAR-3, OVCAR-4, OVCAR-5, OVCAR-8, SKOV3, MiaPaCa, PANC-1, BxPC3, PC3, 22RV1, LNCAP, HT-1080, SK-LMS-1, A375, A431, AGS (CRL-1739), N87, HCT116, DLD1, SW620, Colo-205, EJ-1, MDA-MB-231, MDA-MB-435, MCF-7, Mx-1, BT474, K562, and H1650 cell lines were purchased from American Type Culture Collection or Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH (DSMZ). 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) supplemented with 10% FBS (Invitrogen).

Cell viability assays

Five thousand cells were plated per well in a 96-well plate and incubated with each therapeutic agent with or without 1 μ mol/L navitoclax for 72 hours in a total volume of 100 μ L culture medium supplemented with 10% FBS. Cytotoxicity was assessed using the CellTiter-Glo Assay that measures the ATP content of the cells according to manufacturer's specifications (Promega). Agents that induced 20% or more reduction in combination with navitoclax below what was observed with agent alone across 2 doses were considered synergistic hits from the screen. To quantitatively assess the combination effects of navitoclax with other chemotherapeutic agents, experiments were carried out 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 was used for this analysis. Briefly, this method of analysis distinguishes between synergistic, additive, and antagonistic drug-drug interactions. A CI value of 1.0 indicates an additive effect. CI values less than 1.0 reflect a synergistic effect, whereas CI values greater than 1.0 reflect an antagonistic effect.

Immunoblotting

Twenty to thirty micrograms of whole-cell or fractionated lysates were separated by SDS-PAGE (4%–20% gels) and transferred to Immobilon-P membranes (Millipore). The membranes were incubated with primary antibodies against Bcl-2 (Epitomics), Bcl-X_L (Epitomics), Mcl-1 (Santa

Cruz Biotechnology), Bim (Epitomics), Bid (BD Biosciences), Bax (Epitomics), Puma (Sigma), Noxa (Abcam), pEGFR (Invitrogen), EGFR (Invitrogen), pERK (Cell Signaling Technology), ERK (Cell Signaling Technology), or cytochrome *c* (BD Biosciences) overnight at 4°C. Primary antibodies were detected using either a goat antimouse or goat antirabbit Alexa Fluor 680-conjugated or IRDye 800CW-conjugated secondary antibody (Molecular Probes) diluted 1:10,000 for 30 minutes. Bands were detected using the Odyssey Infrared Imaging System (LI-COR Biosciences).

Mitochondrial cytochrome *c* release assay

SKOV3 cells were pretreated with 1.5 nmol/L docetaxel or 2.5 μ mol/L gemcitabine for 48 hours, washed, and resuspended in cell permeability buffer (20 mmol/L HEPES, pH 7.2, 100 mmol/L KCl, 5 mmol/L MgCl₂, 1 mmol/L EDTA, 1 mmol/L EGTA, 250 mmol/L sucrose, 0.015% digitonin, Complete Protease Inhibitor; Roche), and incubated on ice for 3 to 5 minutes. Permeabilized cells were then incubated with 10 μ mol/L navitoclax, enantiomer, Bim-BH3 peptide, or vehicle at 30°C for 75 minutes. Mitochondrial and cytosolic fractions were separated by centrifugation at 16,000 \times *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).

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 μ L) in 96-well Collagen I-coated microplates (BD Biosciences) and incubated overnight under standard cell culture conditions. On the next day, cells were treated with docetaxel or vehicle by addition of a 50 μ L solution containing a 3 \times compound stock and incubated for an additional 48 hours. Cells were then challenged with navitoclax for the indicated times. Cells were subsequently fixed by the addition of 100 μ L of prewarmed 10% formaldehyde (Sigma) made up in PBS, pH 7.4 lacking Ca²⁺/Mg²⁺ (Invitrogen) and incubated at room temperature for 10 minutes. All subsequent reagents were made up in PBS, pH 7.4 lacking Ca²⁺/Mg²⁺. Wells were then washed twice with 200 μ L PBS and permeabilized with 0.1% Triton X-100 for 10 minutes, washed with PBS, blocked with 1% bovine serum albumin for 20 minutes and then incubated with 50 μ L of a 1:100 dilution of a rabbit antibody raised against activated caspase-3 (Cell Signaling Technology). Primary antibody was incubated overnight at 4°C, washed with 200 μ L PBS, incubated for an additional hour with a 50 μ L mixture of a 1:200 dilution of Alexa Fluor 488-conjugated goat anti-rabbit antibody and 1 mg/mL Hoechst 33342 (Invitrogen). After a 1-hour incubation in the dark, cells were washed 3 \times with 200 μ L PBS and imaged on an ArrayScan VTI HCS Reader (Thermo Scientific) with a \times 10 (0.3 numerical aperture)

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.

siRNA transfection

SKOV3 cells were plated at 1×10^4 cells/100 μ L in 96-well or 3×10^5 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. Briefly, siRNAs were prepared by incubating 1.5 μ L of a 20 μ mol/L siRNA stock with 100 μ L Opti-MEM (Invitrogen) for 10 minutes and 3 μ L Lipofectamine 2000 (Invitrogen) with 100 μ L 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 hours 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 2 to 3 times week-

ly using the following equation: volume = length \times width²/2. Percentages of tumor growth inhibition (TGI), tumor growth delay (TGD), partial regression (PR), complete regression (CR), and overall response rates (ORR) are defined as previously described (14). All studies used 6 to 10 mice per group. Statistical comparisons of tumor growth rate and TGD used the Wilcoxon rank-sum test and the Mantel-Cox log-rank test, respectively. Navitoclax was formulated in 10% ethanol, 30% polyethylene glycol 400, and 60% Phosal 50 PG (a dispersion of 50% phosphatidylcholine in a propylene glycol/ethanol carrier) and administered orally by gavage. Docetaxel was purchased from Sanofi Aventis, and gemcitabine was purchased from Eli Lilly. 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 hematologic 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 μ mol/L navitoclax against 46 human cancer cell lines representing 16 tumor types (Fig. 1A). Using a 6-point dose-response curve, positive combination activity was defined as 20% or greater reduction in survival across 2 or

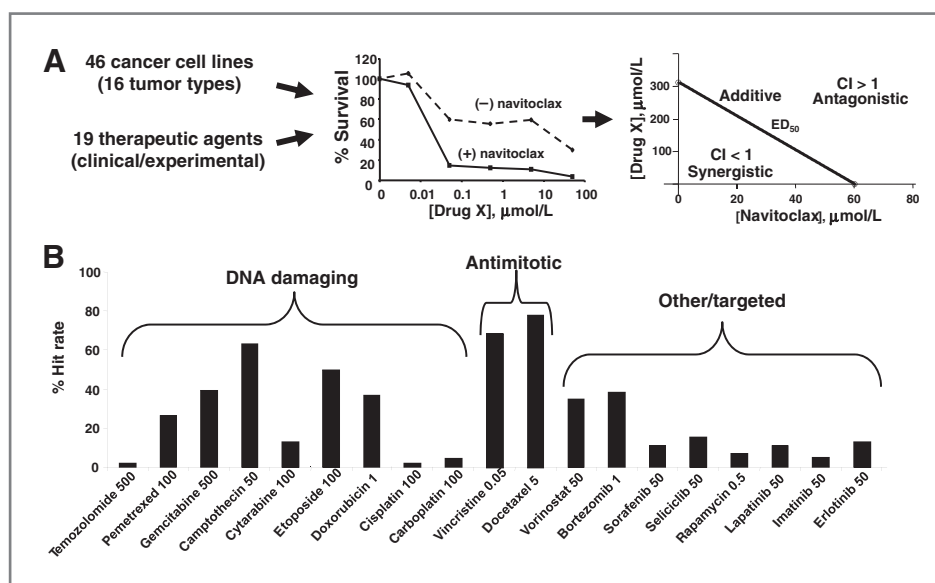


Figure 1. Navitoclax broadly potentiates the activity of multiple therapeutic agents. **A**, screening strategy used to identify combination synergies. Therapeutic agents were screened in a 6-point dose-response curve with 10-fold dilutions (except doxorubicin and vincristine, where 3-fold dilutions were used) with or without 1 μ mol/L navitoclax. Potentially synergistic combinations were further interrogated in an 8×10 dose-response matrix between navitoclax and the agent of interest. The 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 agent used in the screening is listed next to the agent on the x-axis in micromole per liter.

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 of 774 examined (~25% hit rate). Combination synergy, defined as a response that is greater than the expected additive effect, was evaluated using CI analysis according to the multiple drug-effect equation of Chou-Talalay (17). To select the best positive combination activities for further investigation *in vivo*, CI analyses were conducted 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 antimetabolic agents (e.g., docetaxel and vincristine; Fig. 1B) in a variety of tumor types *in vitro*, as confirmed by CI analyses (Table 1).

Navitoclax enhances the activity of docetaxel *in vivo*

As shown in Fig. 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 (Fig. 2). As a monotherapy, navitoclax dosed orally once a day at 100 mg/kg for 2, 14, or 21 days was not efficacious in the SKOV3 xenograft model (Fig. 2). Docetaxel administered once weekly for 3 cycles at 10 mg/kg/d 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 intravenously inhibited tumor growth by only 48% with no significant effect on time to progression. ORRs were slightly higher when cyclical dosing was administered, rather than bolus dosing (30% vs. 0%).

Navitoclax enhances the activity of docetaxel under most schedules. Chronic (14 or 21 days) daily administration of navitoclax concurrently with either docetaxel regimen yielded greater than additive inhibition of tumor growth, although this only reached statistical significance

Table 1. CI of various therapeutic agents in combination with navitoclax

Tissue	Cell line	Agent	Average CI	Tissue	Cell line	Agent	Average CI
Breast	MCF-7	Camptothecin	0.75	Lung	H196	Camptothecin	0.49
Breast	MCF-7	Docetaxel	0.70	Lung	H196	Etoposide	0.46
Breast	MCF-7	Etoposide	0.57	Lung	H460	Docetaxel	0.39
Breast	MCF-7	Rapamycin	0.25	Lung	H460	Vincristine	0.50
Breast	MDA-MB-231	Docetaxel	0.19	Lung	H460-LM	Carboplatin	0.69
Breast	MDA-MB-231	Doxorubicin	0.44	Lung	H460-LM	Gemcitabine	0.40
Breast	MDA-MB-231-LC	Docetaxel	0.22	Lung	NIC-H1650	Erlotinib	0.45
Breast	MDA-MB-231-LC	Doxorubicin	0.24	Ovarian	OVCAR-3	Etoposide	0.79
Breast	MDA-MB-231-LC	Gemcitabine	0.35	Ovarian	OVCAR-4	Etoposide	0.76
Colorectal	Colo-205	Camptothecin	0.45	Ovarian	OVCAR-5	Camptothecin	0.33
Colorectal	DLD1	Camptothecin	0.64	Ovarian	OVCAR-5	Carboplatin	0.54
Colorectal	HCT116	Camptothecin	0.47	Ovarian	OVCAR-5	Docetaxel	0.44
Colorectal	HT29	Camptothecin	0.59	Ovarian	OVCAR-5	Etoposide	0.27
Colorectal	SW620	Camptothecin	0.56	Ovarian	OVCAR-5	Gemcitabine	0.14
Colorectal	SW620	Docetaxel	0.39	Ovarian	OVCAR-8	Etoposide	0.31
Colorectal	SW620	Vincristine	0.42	Ovarian	SKOV3	Carboplatin	0.62
Gastric	AGS	Etoposide	0.26	Ovarian	SKOV3	Docetaxel	0.39
Gastric	N87	Camptothecin	0.18	Ovarian	SKOV3	Gemcitabine	0.09
Gastric	N87	Docetaxel	0.18	Pancreatic	BxPC3	Camptothecin	0.21
Gastric	N87	Erlotinib	0.44	Pancreatic	BxPC3	Docetaxel	0.05
Gastric	N87	Etoposide	0.42	Pancreatic	BxPC3	Erlotinib	0.33
Gastric	N87	Selaciclib	0.38	Pancreatic	BxPC3	Etoposide	0.34
Hepatic	Hep3B	Vorinostat	0.37	Pancreatic	BxPC3	Gemcitabine	0.26
Lung	A549	Bortezomib	0.27	Pancreatic	MiaPaCa	Etoposide	0.77
Lung	A549	Carboplatin	0.40	Pancreatic	PANC-1	Camptothecin	0.31
Lung	A549	Etoposide	0.35	Pancreatic	PANC-1	Etoposide	0.96
Lung	Calu-6	Carboplatin	0.49	Prostate	DU-145	Docetaxel	0.50
Lung	Calu-6	Docetaxel	0.63	Prostate	PC3	Docetaxel	0.15
Lung	H1299	Camptothecin	0.24	Skin	A431	Erlotinib	0.46

NOTE: The combination index (CI) values were calculated by averaging the CI values at ED_{50} , ED_{75} , and ED_{90} . Shown is the mean ($n \geq 2$).

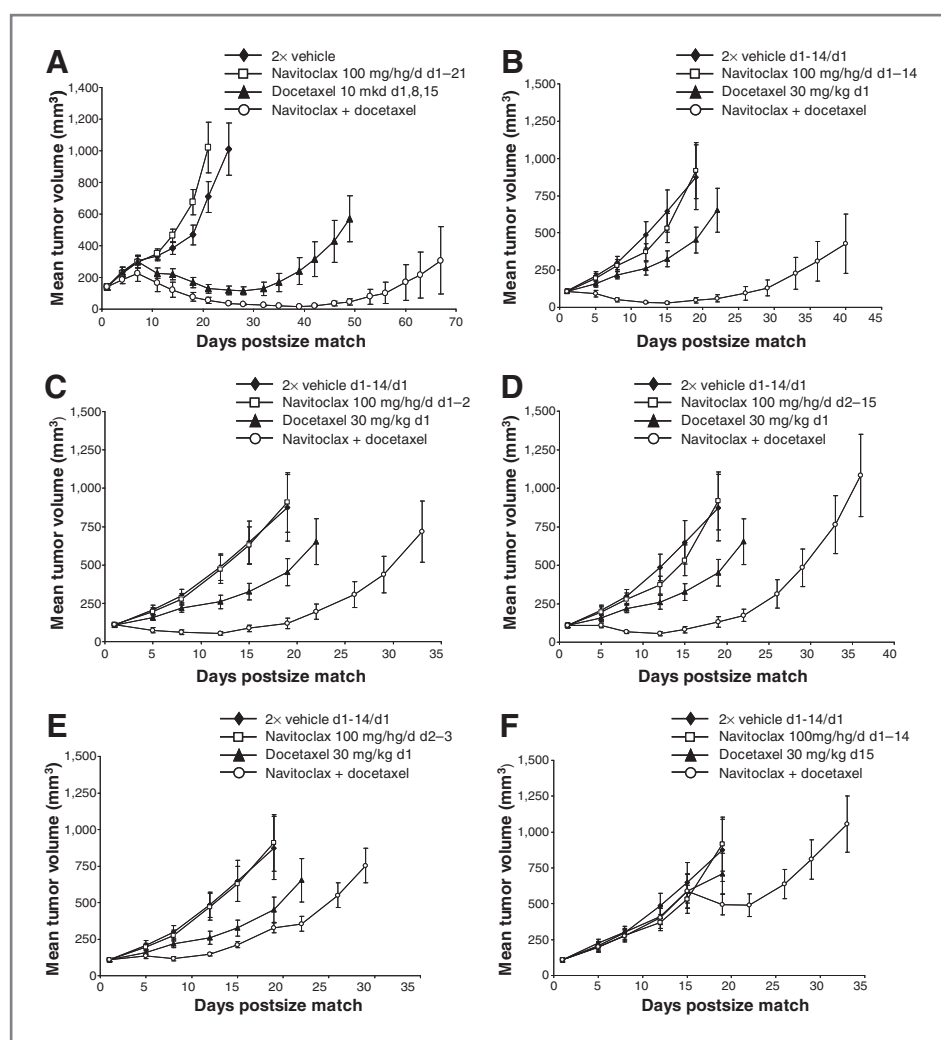


Figure 2. *In vivo* antitumor effect of navitoclax in combination with docetaxel in SKOV3 xenograft models with different scheduling. Tumor volume was plotted over days posttumor staging with SKOV3 xenografts. Each treatment group consisted of 8 to 10 mice. Points, mean; bars, SEM.

Navitoclax and docetaxel were dosed as described in legends. 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.

in the bolus dosing group (Fig. 2A and 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 with docetaxel alone. TGD, a measurement of durability of antitumor effect, was also significantly improved from 12% to 135% and from 114% to more than 175% in the bolus dosing and cyclical dosing, respectively. ORRs 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 (2 days) with bolus administration of docetaxel also yielded greater than additive results, with TGI from 57% to 86% and TGD from 12% to 54% (Fig. 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 concurrent, acute administration of navitoclax and docetaxel (Fig. 2D). In contrast, acute navitoclax treatment following a bolus dose of docetaxel

yielded only modest increases in TGI and TGD, as did administration of a bolus dose of docetaxel following 14 days of navitoclax (Fig. 2E and F).

Docetaxel increases mitochondrial sensitivity to Bcl-2/Bcl-X_L

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 (Fig. 3A). In contrast, 48 hours of pretreatment with 1.5 nmol/L 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 conducted. As shown in Fig. 3B, no increase in activated caspase-3 is detected in cells treated with navitoclax alone. However, pretreatment of SKOV3 cells

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

	Navitoclax		Docetaxel		%CR	%ORR	%TGI	%TGD
	Dose, mg/kg/d	Schedule	Dose, mg/kg/d	Schedule				
1	0	qd d1–21	0	q7d d1, 8, 15	0	0	—	—
2	100	qd d1–21	0	q7d d1, 8, 15	0	0	–44	–11
3	0	qd d1–21	10	q7d d1, 8, 15	0	30	82 ^a	114 ^a
4	100	qd d1–21	10	q7d d1, 8, 15	33	100 ^b	92	>175
5	0	qd d1–14	0	qd d1	0	0	—	—
6	100	qd d1–14	0	qd d1	0	0	–5	0
7	100	qd d1–2	0	qd d1	0	0	–4	–27
8	0	qd d1–14	30	qd d1	0	0	48	12
9	0	qd d1–14	30	qd d15	0	0	19	12
10	100	qd d1–14	30	qd d1	56 ^c	89 ^{c-e}	95 ^{c,e}	135 ^{c-e}
11	100	qd d1–2	30	qd d1	11	22	86 ^c	54
12	100	qd d1–14	30	qd d15	0	0	43	27
13	100	qd d2–15	30	qd d1	11	22	85 ^c	38
14	100	qd d2–3	30	qd d1	0	0	63	38

NOTE: Navitoclax dosed orally daily 1 to 2 hours before docetaxel intravenous administration. Statistical analysis for %TGI conducted by Wilcoxon rank-sum, for %TGD by Mantel–Cox log-rank test, and for %CR and %ORR by the Fisher exact test. Groups 1–4 and groups 5–14 are from separate experiments.

Abbreviations: qd, once daily; q7d, once every 7 days.

^a*P* < 0.05 vs. group 1.

^b*P* < 0.05 vs. group 3.

^c*P* < 0.05 vs. group 8.

^d*P* < 0.05 vs. group 11.

^e*P* < 0.05 vs. group 13.

with 1.5 nmol/L 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-X_L inhibition.

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

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 (Fig. 3C). No other significant changes were observed with the exception of a modest increase in BH3-only protein Noxa levels at 12 nmol/L 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 (Fig. 3D), it enhanced the cytotoxicity of 1 and 5 μmol/L navitoclax by 35% and 25%, respectively. The *P* values are less than 0.0002 and 0.002 for 1 and 5 μmol/L 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 of 774 combinations examined; Fig. 4A). Interestingly, in all 5 of these lines (A431, BxPC3, N87, OVCAR-3, and SKOV3), EGFR has been reported either amplified (A431) or overexpressed (22–25). Clinically, tumors with activation mutations, such as the EGFR deletion 746 to 750, have been most responsive to erlotinib therapy (26). We next investigated the combination in a non-SCLC (NSCLC) line (NCI-H1650) with this genetic alteration to assess the ability of navitoclax to enhance apoptotic potential and antitumor efficacy of erlotinib. CI analysis of the navitoclax–erlotinib combination in NCI-H1650 cells confirmed synergistic activity with an average CI of 0.44 across the EC₅₀ to EC₉₀ (CI_{50–90}; Fig. 4B). Examination of Bcl-2 family proteins indicated the coordinated downmodulation of Mcl-1 and upregulation of Bim_{EL} after 6 hours of treatment with erlotinib *in vitro*. These data are consistent with previous findings showing

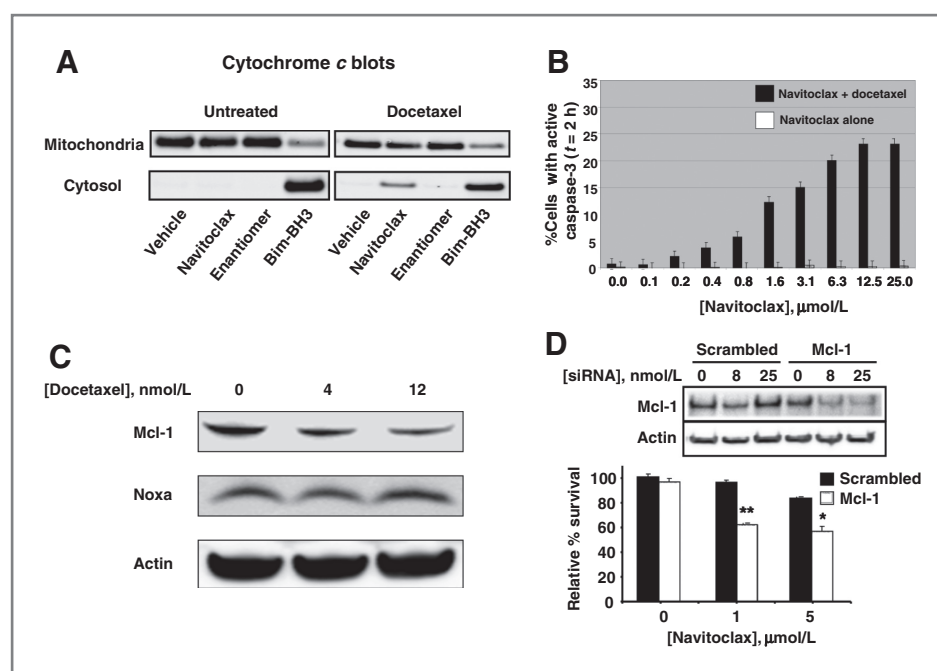


Figure 3. Docetaxel alters mitochondrial sensitivity to navitoclax by neutralizing Mcl-1. **A**, release of cytochrome c induced by 10 $\mu\text{mol/L}$ navitoclax with or without pretreatment with 4 nmol/L docetaxel for 48 hours. Permeabilized SKOV3 cells were fractionated into mitochondrial and cytosolic fractions and cytochrome c detected as described in Materials and Methods. **B**, high content quantification of SKOV3 cells showing caspase-3 activation in response to navitoclax with or without docetaxel pretreatment. Shown is the mean \pm SD ($n = 3$). **C**, immunoblots of Mcl-1 and Noxa in whole-cell lysates isolated from SKOV3 cells treated with 4 to 12 nmol/L docetaxel for 48 hours. **D**, siRNA knockdown of Mcl-1 leads to increased cellular sensitivity to navitoclax. Relative survival rate of SKOV3 with or without navitoclax at the indicated concentrations in the presence of Mcl-1 siRNA or scrambled siRNA control. *, $P < 0.002$; **, $P < 0.0002$ determined by the Student t test.

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 2 targeted agents *in vivo*. As a single agent, 100 mg/kg navitoclax alone dosed daily had no significant antitumor activity, whereas daily dosing of erlotinib at 50 mg/kg resulted in significant tumor stasis (%TGI = 52) during a 21-day treatment period (Fig. 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 treated tumor-bearing mice (Fig. 4C). The latter was sustained for 25 days even after the cessation of dosing (Fig. 4C). In addition, 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 posttreatment and analyzed for EGFR and Bcl-2 pathway markers. Treatment with erlotinib resulted in decreased phosphorylation levels of extracellular signal-regulated kinase (pERK) and induced

significant downmodulation of Mcl-1 and upregulation of Bim (Fig. 4D), which is consistent with previous *in vitro* findings (Fig. 4B). Thus, the enhanced antitumor activity of erlotinib in combination with navitoclax *in vivo* is because of simultaneous inhibition of the EGFR and Bcl-2 prosurvival 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 I and II clinical development. Preclinically, navitoclax exhibits single-agent activity in SCLC 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, rituximab-CHOP, and bortezomib in various leukemia and lymphoma models (13, 15). In NSCLC 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

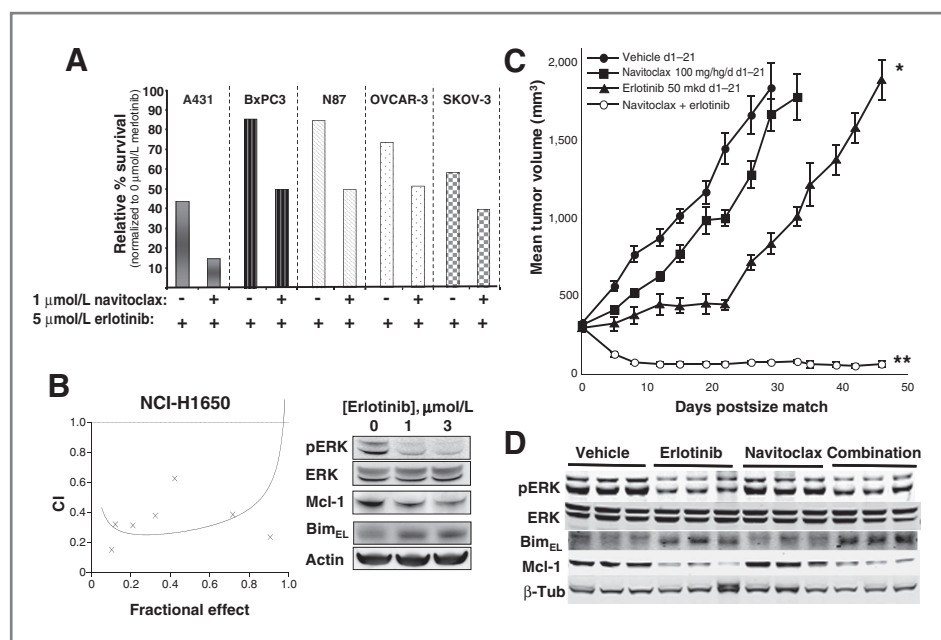


Figure 4. Erlotinib upregulates Bim and downregulates Mcl-1 leading to enhanced cytotoxicity in combination with navitoclax *in vitro* and *in vivo*. **A**, relative survival rate of cells treated with 5 $\mu\text{mol/L}$ erlotinib in the presence (+) or absence (–) of navitoclax. Shown is the mean ($n = 3$). **B**, CI value as a function of fractional effect (cytotoxicity) for erlotinib in combination with navitoclax in H1650 cells. Shown is a representative curve ($n = 3$). Inset, protein expression levels of pERK, ERK, Mcl-1, Bim, and actin in H1650 cells treated with 0, 1, or 3 $\mu\text{mol/L}$ erlotinib for 6 hours. **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 for 21 consecutive days. *, $P < 0.001$; **, $P < 0.0001$ determined by the Student *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 pERK-1/2, Bim, or Mcl-1 by Western blotting using primary antibodies described in the Materials and Methods section. β -Tubulin (β -Tub) served as the protein loading control.

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 antimetabolites (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 antitumor activity of the antimetabolite docetaxel with significantly improved ORRs compared with docetaxel treatment alone. In addition, combination treatment resulted in more durable antitumor 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, whereas predosing with navitoclax before docetaxel administration did not significantly enhance efficacy. The combinations of navitoclax–doce-

taxel and navitoclax–gemcitabine were evaluated once in N87 (gastric) and OVCAR-5 (ovarian) xenograft models (Supplementary Fig. S1). Consistent with our *in vitro* findings, these combinations showed enhanced antitumor activity *in vivo* compared with 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 %TGD (from 39% to 76%) for navitoclax–gemcitabine in OVCAR-5 (Supplementary Table S1).

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 downregulation 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 downmodulating Mcl-1. As such, we investigated whether navitoclax enhanced the antitumor activity of erlotinib *in vivo*. Consistent with our hypothesis, the combination of erlotinib with navitoclax in an NSCLC xenograft model showed durable tumor regressions and 100% ORRs in mice, whereas mice treated with erlotinib alone showed only a modest TGI during the treatment period. In addition to downmodulation of Mcl-1 in tumors isolated from mice treated with erlotinib, we also noted an upregulation of the proapoptotic 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 to restoration of the apoptotic potential of the cell. While we used CI values from these studies as a guide to select the best combinations to be tested *in vivo*, they were not always predictive of antitumor activity. For example, navitoclax did not consistently enhance the antitumor activity of docetaxel in BxPC3 and of gem-

citabine in SKOV3 xenograft models, even though their CI values were less than 0.1 *in vitro* (data not shown). The latter shows the inherent challenges in using *in vitro* studies to predict *in vivo* efficacy when differences in drug exposures and microenvironment are not accounted for. Using 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.

Disclosure of Potential Conflicts of Interest

All the authors are employees of Abbott Laboratories or Genentech whose potential product was studied in the present work.

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Received June 8, 2011; revised September 1, 2011; accepted September 2, 2011; published OnlineFirst September 13, 2011.

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