CANCER

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Synergistic action of the MCL-1 inhibitor S63845 with current therapies in preclinical models of triple-negative and HER2-amplified breast cancer

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The development of BH3 mimetics, which antagonize prosurvival proteins of the BCL-2 family, represents a potential breakthrough in cancer therapy. Targeting the prosurvival member MCL-1 has been an area of intense interest because it is frequently deregulated in cancer. In breast cancer, MCL-1 is often amplified, and high expression predicts poor patient outcome. We tested the MCL-1 inhibitor S63845 in breast cancer cell lines and patient-derived xenografts with high expression of MCL-1. S63845 displayed synergistic activity with docetaxel in triple-negative breast cancer and with trastuzumab or lapatinib in HER2-amplified breast cancer. Using S63845-resistant cells combined with CRISPR (clustered regularly interspaced short palindromic repeats)–Cas9 (CRISPR-associated 9) technology, we identified deletion of BAK and up-regulation of prosurvival proteins as potential mechanisms that confer resistance to S63845 in breast cancer. Collectively, our findings provide a strong rationale for the clinical evaluation of MCL-1 inhibitors in breast cancer.

INTRODUCTION

Breast cancer is a heterogeneous disease and can be stratified into at least six subgroups based on gene expression profiling: luminal A, luminal B [estrogen receptor–positive (ER^+)], human epidermal growth factor receptor 2 (HER2)–amplified, basal-like [predominantly triple-negative breast cancer (TNBC)], claudin-low, and normal-like (*1*, *2*). These subtypes predict clinical behavior with respect to response and resistance to therapy, patterns of metastasis, and overall survival. Multiple mechanisms contribute to tumor progression and resistance to cancer therapy, including the evasion of cell death. Cancer cells escape apoptosis through diverse strategies that include increased expression of prosurvival proteins such as BCL-2, BCL-XL, or MCL-1. Targeting these proteins with "BH3 mimetics" that mimic the function of proapoptotic proteins has emerged as a promising strategy in cancer therapy.

The first "on-target" BH3 mimetic, ABT-737, and its orally bioavailable counterpart, ABT-263 (navitoclax), exhibit broad-spectrum activity and inhibit BCL-2, BCL-XL, and BCL-W but not MCL-1 or A1. Clinical application, however, has been hampered by thrombocytopenia induced through concomitant on-target inhibition of BCL-XL in platelets (*3*, *4*). The potent BCL-2–specific inhibitor ABT-199 (venetoclax) is platelet-sparing and has demonstrated clinical efficacy as a single agent in the treatment of chronic lymphocytic leukemia (*5*, *6*). Although navitoclax and venetoclax have single-agent activity in some hematologic malignancies, combination therapy strategies are likely to be required for other cancer types (*7*).

In breast cancer, differential expression of prosurvival proteins across tumor subtypes suggests that different members of this protein class could be targeted in distinct tumor subtypes (*8*). *BCL-2*, which is an estrogen-responsive gene, is overexpressed in about 85% of ER^+ breast cancer (*9*). In preclinical models of luminal B (ER^+) breast cancer, ABT-199 was found to synergize with tamoxifen (*10*), resulting in the evaluation of this combination in the clinic (ISRCTN98335443). MCL-1 may also be a therapeutic target because *MCL-1* amplification has been observed in a large-scale high-resolution study of somatic copy number alterations across diverse cancers, including breast cancer (*11*), and MCL-1 can confer resistance to chemotherapy or targeted therapy (*12*–*14*). MCL-1 may promote metastasis (*15*), and high expression has been correlated with poor prognosis (*16*). MCL-1 appears to be the main prosurvival protein that is up-regulated in TNBC (*17*, *18*) and in *HER2*-amplified tumors, where it may stabilize HER2 and limit the efficacy of HER2-targeted therapies (*19*–*21*). Moreover, MCL-1 amplification was commonly observed in TNBC tumors that failed to achieve a complete pathological response with neoadjuvant chemotherapy (*22*).

The development of small-molecule inhibitors directed against MCL-1 has proved challenging because MCL-1 has a BH3-binding hydrophobic groove that is more rigid than in BCL-XL or BCL-2 (*23*). A number of MCL-1 inhibitors have been recently reported

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and their activity has been investigated in vitro, although their potency in vivo is less clear (*18*, *24*–*28*). Recently, a small-molecule MCL-1 inhibitor, S63845, which specifically binds with high affinity to the BH3-binding groove of MCL-1, has been developed (*29*). Although S63845 had single-agent activity in certain hematopoietic tumor cells, its activity in solid tumors is unclear. As for venetoclax, it is likely that combination therapy will be required (*7*, *30*). Here, we identify MCL-1 as a potential target in preclinical models of TNBC and HER2-amplified breast cancer, and demonstrate that S63845 enhances the action of conventional therapy in these breast cancer subtypes.

RESULTS

MCL-1 expression is prominent in TNBC and HER2-amplified tumors

To examine the expression of MCL-1 and other BCL-2 family members across the different subtypes of breast cancer, we first quantified RNA and protein in a cohort of patient-derived xenograft (PDX) models (Fig. 1, A and B; figs. S1 and S2; and tables S1 to S3). As previously reported (*9*, *10*), *BCL-2* mRNA and protein were most highly expressed in ER^+ breast cancer. Conversely, *MCL-1* expression was higher in TNBC (including *BRCA1*-mutated tumors) and HER2-amplified tumors compared to ER^+ tumors. Similar findings were observed in primary breast tumors from the Molecular Taxonomy of Breast Cancer International Consortium (METABRIC) or The Cancer Genome Atlas (TCGA) data sets (fig. S3, A and B, and table S4) (*1*, *31*). In agreement with RNA expression, the highest amount of MCL-1 protein was observed in TNBC PDXs, although MCL-1 was expressed across the entire repertoire of PDX tumors. BCL-XL protein was present in high amounts across all tumors, despite apparently higher mRNA expression in the HER2-amplified subset. The amounts of transcript of another prosurvival family member, *BCL-W*, were similar across subtypes and present at low levels. The key BH3-only proapoptotic protein, BIM, was generally expressed in lower amounts in TNBC compared to ER^+ PDXs. BCL-2 family protein expression was confirmed by immunohistochemistry in two TNBC PDX models (838 and 110) and one HER2-amplified PDX model (231) (fig. S4, A and B).

The potency of the MCL-1–specific inhibitor S63845 as an inducer of cell death was first tested in a panel of six breast cancer cell lines, which expressed MCL-1 (Fig. 1, C and D). The response varied across cell lines, consistent with recent findings for other cancer types (*29*) and for the MCL-1 inhibitor A-1210477 (*18*) or MCL-1–specific small interfering RNAs (*17*). The HER2-amplified cell line SK-BR-3 was most sensitive to S63845, followed by the TNBC cell lines BT-20 and MDA-MB-468. In contrast, the ER^+ MCF-7 and BT-474 cell lines as well as the claudin-low TNBC MDA-MB-231 cell line were more resistant. It is possible that differences in BCL-XL and BCL-2 expression in these cell lines (Fig. 1D) account for their differential response. For example, SK-BR-3 cells have low expression of BCL-2 and high expression of BAK, which could have contributed to their increased sensitivity to S63845 compared to the other cell lines.

We next studied the sensitivity of three HER2-amplified and five TNBC PDX models to the BH3 mimetics ABT-737, ABT-199, WEHI-539 (a BCL-XL inhibitor) (*32*), and S63845 in short-term culture assays. In contrast to other BH3 mimetics, all tumors displayed sensitivity to 1 μM S63845, most notably the TNBC models (Fig. 1E and fig. S1C). The median inhibitory concentration (IC_{50}) for most PDX models was less than 1 μM for most models (except for PDX 951 and 45). These findings suggest that MCL-1 is an important survival factor in TNBC and HER2-amplified subtypes. Both *BRCA1*-mutated (303 and 110) and wild-type (838, 322, and 744) TNBC PDXs, which had similar MCL-1 expression, were sensitive to S63845 (Fig. 1E). S63845 appeared to be a more effective inducer of cell death than the MCL-1 compound A-1210477 in SK-BR-3 cells (fig. S1, C and D), consistent with its reported potency (*29*). Together, these findings suggest that MCL-1 could be an important survival factor and therapeutic target in TNBC and HER2-amplified tumors.

S63845 activity is dependent on BAK and is curtailed by prosurvival family members

To explore mechanisms that underpin tumor response or resistance to S63845, we performed a genome-wide CRISPR (clustered regularly interspaced short palindromic repeats)–Cas9 (CRISPR-associated 9) screen in SK-BR-3 cells, which were highly sensitive to this agent. SK-BR-3 Cas9-expressing cells were transduced with a pooled human genome-wide guide RNA lentiviral library containing 123,411 unique single-guide RNAs (sgRNAs) targeting 19,050 genes and 1864 microRNAs (miRNAs) (6 sgRNAs per gene and 4 sgRNAs per miRNA) at low multiplicity of infection in six independent infections (*33*). Next-generation sequencing (NGS) of the transduced cells confirmed high representation of the sgRNA library. Most (96%) of the library sgRNAs were present, with six sgRNAs detected for 79% of genes (fig. S5A). Each transduced cell line was treated with either S63845 (1 μM) or dimethyl sulfoxide (DMSO) (control), and genomic DNA (gDNA) of the surviving cells was subsequently isolated and sgRNAs were identified by NGS. As expected, the number of sgRNAs was reduced in the S63845-treated groups, indicating high selective pressure from S63845. This resulted in a high Gini index, denoting reduced complexity of the represented sgRNAs in the S63845-treated groups when compared to DMSO-treated controls (fig. S5B). *BAK* emerged as a key mediator of resistance, because it was the only gene for which more than one sgRNA was detected in the resistant cell clones in all replicates (Fig. 2A and table S5). This finding is consistent with those reported for A-1210477 (*18*) and suggests that S63845 likely kills through disruption of MCL-1/BAK complexes or by preventing sequestration of BAK by MCL-1 (*34*).

To further investigate the role of BCL-2 family members, we performed a focused CRISPR-Cas9 screen targeting the proapoptotic BCL-2 family members (Fig. 2B, fig. S5C, and tables S6 and S7). Cells were transduced with lentiviruses expressing sgRNAs that targeted various proapoptotic genes and then treated with increasing concentrations of S63845. Consistent with the genome-wide screen, only sgRNAs targeting *BAK* conferred resistance to apoptosis in SK-BR-3 cells treated with the MCL-1 inhibitor. Although targeting *BAX* alone did not confer resistance, *BAX/BAK* double-knockout cells were completely resistant to S63845. The dependency on BAK and BAX confirms that this small-molecule inhibitor specifically targets the intrinsic (BCL-2 family) apoptotic pathway (fig. S5D). Targeting of single BH3-only genes did not confer resistance to S63845 (Fig. 2B).

To confirm that S63845 can disrupt complexes containing MCL-1 and BH3-only proteins, we performed coimmunoprecipitation studies where human embryonic kidney 293T cells were transfected with a FLAG-tagged MCL-1 construct and hemagglutinin (HA)- or EE-tagged BH3 proteins and cultured in the presence or absence of S63845. After immunoprecipitation of FLAG-tagged MCL-1, coimmunoprecipitated HA- or EE-tagged proteins were identified by Western blot analysis. As expected from the CRISPR-Cas9 knockout studies (Fig. 2, A and B), disruption of MCL-1/BAK complexes was readily detected (fig. S5E). In addition, we found that S63845 disrupted other MCL-1 complexes

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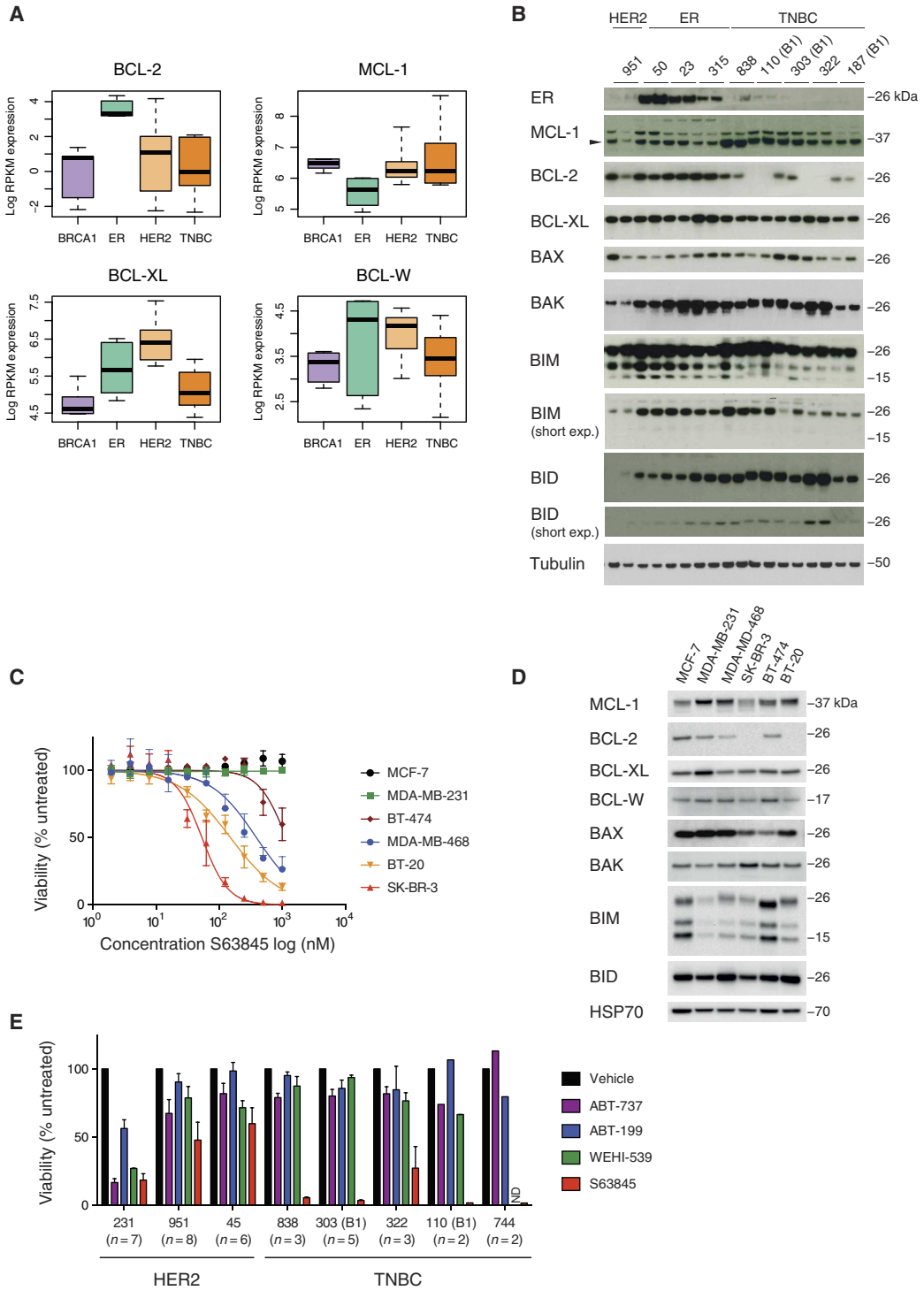

Fig. 1. MCL-1 and BCL-2 expression in PDX tumors and in vitro sensitivity to S63845 in cell lines and PDX models. (**A**) Box plots showing the relative expression (log_2 RPKM) of *BCL-2*, *MCL-1*, *BCL-XL*, and *BCL-W* across breast tumor subtypes. *BRCA1*-mutant TNBC, $n = 6$; ER, $n = 6$; HER2, $n = 8$; and TNBC (*BRCA1* wild type), $n = 10$. (**B**) Western blot analysis of ER and BH3 family member (MCL-1, BCL-2, BCL-XL, BAX, BAK, BIM, and BID) protein expression in PDX models (two independent tumors per PDX). Tubulin was used as a loading control. Arrowhead indicates MCL-1 band. B1, *BRCA1*-mutated. (**C**) Cell lines were treated at increasing concentrations of S63845 for 24 hours before assessment of viability using CellTiter-Glo. Means ± SEM for $n \geq 3$ independent experiments are shown. (**D**) Western blot showing the expression of BCL-2 family members in breast cancer cell lines. HSP70 was used as loading control. (**E**) HER2-amplified and TNBC PDX tumor cells were cultured for 24 hours in mammosphere medium in the presence of ABT-737 (1 μM), ABT-199 (1 μM), WEHI-539 (1 μM), or S63845 (1 μM), and viability was determined compared to DMSO vehicle control. Means ± SEM are shown. The number of independent experiments is indicated. ND, not determined.

containing BIM, PUMA, BID, NOXA, or BMF (fig. S5E). Together, these findings reveal the potential importance of S63845 in disrupting MCL-1 complexes containing several BH3 sensor proteins. Because deletion of a single BH3-only protein did not affect sensitivity to S63845 (Fig. 2B), these findings suggest some level of functional redundancy between MCL-1–interacting proteins. To test this hypothesis, we generated SK-BR-3 cells deficient in BIM/BID and BIM/BID/PUMA (fig. S5F). Targeting both *BIM* and *BID* induced only a partial resistance to S63845, whereas concurrent targeting of *BIM*, *BID*, and *PUMA* greatly impaired S63845-mediated cell death (Fig. 2C), indicating that these BH3-only proteins exert a redundant function in these cells.

We next generated a model of acquired resistance by continuous treatment of BT-20, MDA-MB-468, and BT-474 cell lines with S63845 at either 200 nM or 1 μM for 6 weeks (fig. S6A) and confirmed resistance by retreatment with S63845 (fig. S6B). S63845-resistant MDA-MB-468 cells were more sensitive to BCL-2 and BCL-XL inhibition than treatment-naïve cells, suggesting that concurrent targeting of other pro-survival proteins may help to trigger a response in some tumors. In keeping with this notion, the sensitivity of MDA-MB-468 and MDA-MB-231 cells to S63845 was increased by concomitant treatment with ABT-737 and, to a lesser extent, by WEHI-539 or ABT-199 (Fig. 2D and fig. S7A). In contrast, BT-474– and MDA-MB-468–resistant clones failed to be sensitized to BCL-2 and BCL-XL inhibition, despite resistant BT-474 cells containing more BCL-2 (fig. S6A). This observation suggests that other mechanisms of resistance may have an important role in these cell lines. Because most resistant clones exhibited lower amounts of BIM and BAK (fig. S6B), reduced expression of these proapoptotic proteins could have contributed to acquired

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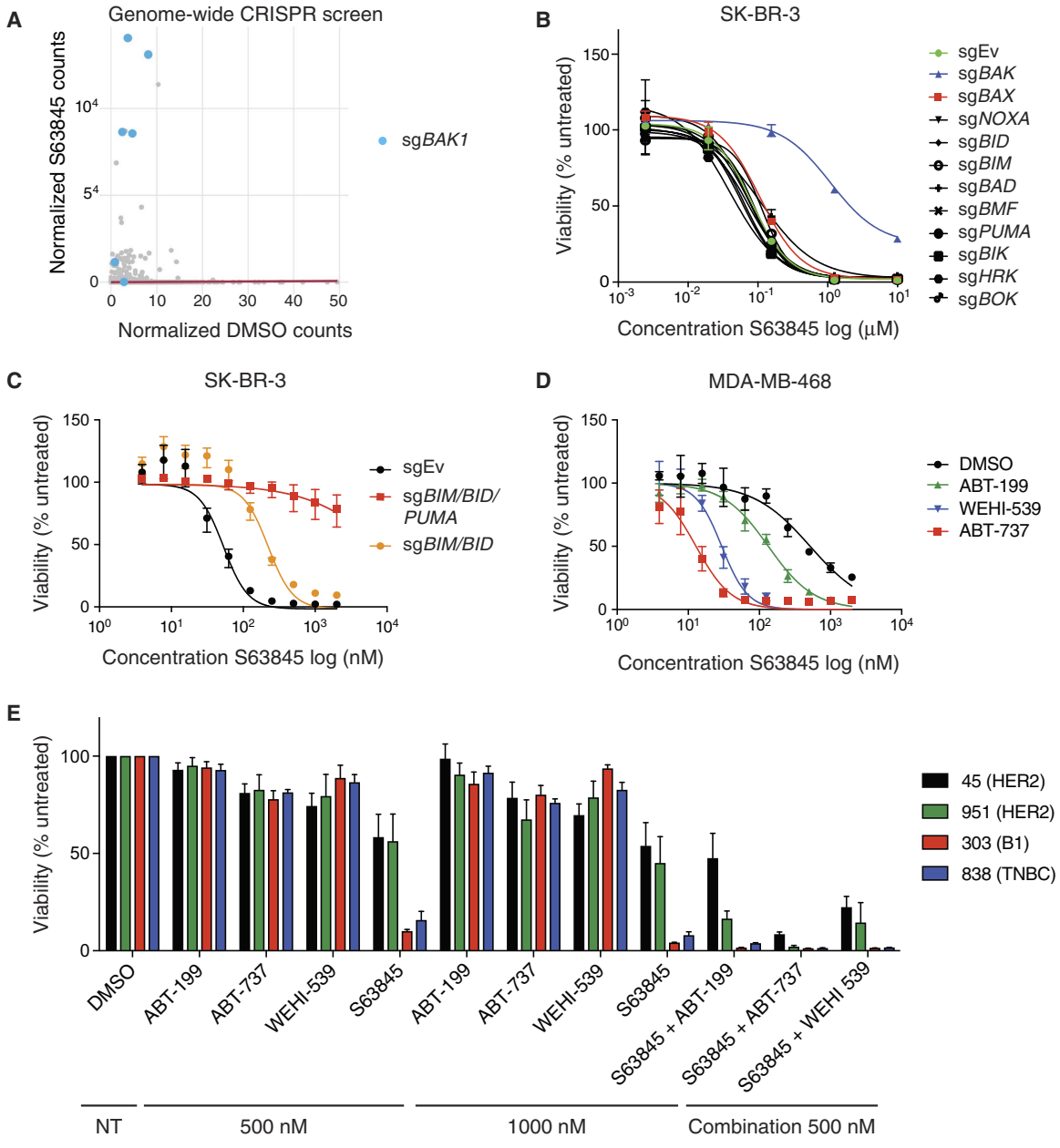

Fig. 2. Resistance to S63845-induced apoptosis through loss of BAK or elevated BCL-XL. (**A**) SK-BR-3 cells were infected with a genome-wide lentiviral sgRNA library and treated with S63845 (1 μM) or DMSO control, and then gDNA of surviving cells was isolated to identify the sgRNAs by NGS. The pooled analysis from six independent infections, displaying normalized values for S63845 or DMSO control, is shown. Solid red bar represents the regression line. The sgBAK1 hits are shown as blue dots. See table S5 for top up-regulated sgRNAs in the S63845-treated pools. (**B**) SK-BR-3 cells infected with CRISPR-Cas9 guides targeting proapoptotic proteins were treated with increasing concentrations of S63845 for 24 hours before assessment of viability using CellTiter-Glo. Ev, empty vector. (**C**) SK-BR-3 cells infected with CRISPR-Cas9 guides targeting BIM, BID, and PUMA were treated with increasing concentrations of S63845, as described above. (**D**) The MDA-MB-468 cell line was treated with increasing concentrations of S63845 in the presence of ABT-199, WEHI-539, or ABT-737 (500 nM) for 24 hours before assessment of viability using CellTiter-Glo. For (B) to (D), means ± SEM for three independent experiments are shown. (**E**) PDX tumor cells were cultured for 24 hours in mammosphere medium with ABT-199, ABT-737, WEHI-539, and S63845 (500 nM and 1 μM) or combination treatment with S63845 and other BH3 mimetics (both at 500 nM) before assessment of viability using CellTiter-Glo. Results are presented as percentages of untreated cells. Bars represent means ± SEM for at least five independent experiments per PDX. The tumor subtype for each PDX is shown in parentheses.

resistance. In addition, overexpression of MCL-1 in SK-BR-3 cells reduced the potency of the inhibitor (fig. S7, B and C), indicating that enforced expression of MCL-1 can also modulate response to S63845.

To further explore the relative contribution of other prosurvival proteins to the survival of breast cancer cells in models of innate resistance, we examined the activity of different BH3 mimetics combined with S63845 in two S63845-sensitive PDX models (303 and 838) and two less sensitive models (45 and 951) (Fig. 2E). The combination of S63845 with ABT-737 or WEHI-539 enhanced the killing of tumor cells, indicating that these cells depended on both MCL-1 and BCL-XL for survival. Addition of the BCL-2 inhibitor ABT-199 to S63845 was moderately effective in killing 951 and 303 PDX cells but had no effect on 45 PDX cells,

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consistent with its low BCL-2 expression (fig. S1B). This PDX model was intrinsically resistant to MCL-1 (S63845; IC_{50} >1 μM), although BAK was localized to the mitochondria and appeared to be functional in mitochondrial assays (fig. S7D). Thus, the relative resistance of the 45 and 951 PDX models to S63845-induced cell death is likely to be mediated by BCL-XL and/or BCL-2.

Together, these findings indicate that loss of BAK and augmented expression of other prosurvival proteins, particularly BCL-XL, are likely to be the main factors with potential to produce resistance to S63845 in TNBC and HER2-overexpressing breast cancer cells. Therefore, maximal induction of apoptosis could be achieved by targeting additional prosurvival proteins or through concomitant therapy that primes cells for apoptosis.

S63845 synergizes with docetaxel, lapatinib, or trastuzumab in vitro

MCL-1 inhibitors are most likely to be effective in breast cancer therapy when used in conjunction with a "priming" agent that delivers another apoptotic signal. We therefore investigated whether S63845 elicited synergistic activity with agents currently used in the treatment of TNBC and HER2-amplified breast cancer. SK-BR-3 cells were treated with S63845 combined with the dual receptor tyrosine kinase inhibitor lapatinib, the anti-HER2 monoclonal antibody trastuzumab, or the taxane docetaxel (Fig. 3A). Docetaxel and S63845 elicited marked synergy at very low concentrations of docetaxel (2 nM) and S63845 (30 nM) (Fig. 3, A and B). Similarly, S63845 synergy was observed with both lapatinib and trastuzumab (Fig. 3, A and C), although slightly longer cotreatment was required for trastuzumab, presumably because of its different mechanism of action. Inhibition of caspases with Q-VD-OPh efficiently blocked cell death, confirming that cell death was via apoptosis (Fig. 3, A and D).

We next compared S63845 to other BH3 mimetics as inducers of apoptosis alone or in combination with trastuzumab, docetaxel, or lapatinib (fig. S8). SK-BR-3 cells were treated with increasing concentrations of S63845, ABT-737, ABT-199, or WEHI-539 (up to 2 μM) in the presence of vehicle, trastuzumab, lapatinib, or docetaxel. Treatment provoked cell death that was markedly augmented when combined with S63845 but not the other BH3 mimetics (fig. S8, A to D), consistent with the synergy observed above between S63845 and conventional therapy.

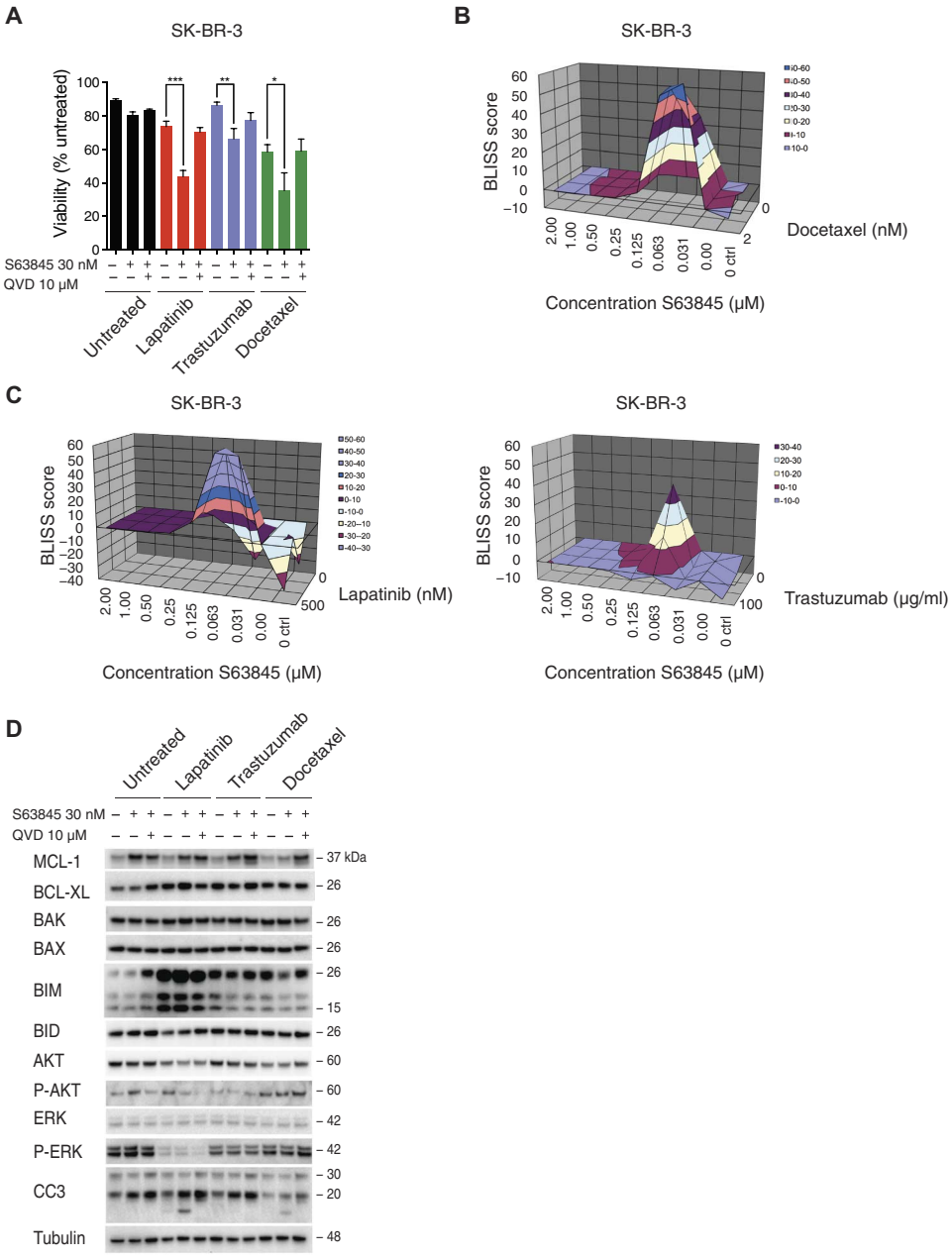

Fig. 3. Synergistic effect of S63845 with lapatinib, trastuzumab, or docetaxel. (**A**) SK-BR-3 cells were treated with lapatinib (500 nM), trastuzumab (100 μg/ml), and docetaxel (2 nM) or left untreated in the presence of S63845 (30 nM), with or without Q-VD-OPh (QVD; 10 μM), for 72 hours before viability analysis with propidium iodide staining. Results are presented as a percentage of untreated cells and represent three to five independent experiments. Means ± SEM are shown. *$P < 0.05$, **$P < 0.005$, ***$P < 0.001$. (**B** and **C**) SK-BR-3 cells were treated with increasing concentrations of S63845 and docetaxel (B), or HER2-targeted therapies lapatinib (left panel) for 72 hours or trastuzumab (right panel) for 96 hours (C), and then subjected to viability assays using CellTiter-Glo followed by BLISS score analysis. BLISS synergy values are >0.0 on the vertical axis. (**D**) Western blot analysis of lysates from (A) showing expression of MCL-1, BCL-XL, BAK, BAX, BIM, BID, AKT, P-AKT, ERK, P-ERK, and cleaved caspase 3 (CC3). Tubulin was used as a loading control.

Although BCL-XL has been reported to be down-regulated after lapatinib treatment (*35*), the amount of BCL-XL did not appreciably change at the low doses deployed here (Fig. 3D), and BCL-2 was undetectable in this cell line (Fig. 1D). The amounts of BID, which contributed to S63845-mediated sensitivity (Fig. 2C), were also similar

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after treatment with the various agents. Moreover, knockout of *BID* using CRISPR-Cas9–mediated editing revealed that BID was not required for the synergistic effect of S63845 with docetaxel or anti-HER2 therapy (fig. S9A). In contrast, BIM protein expression increased after lapatinib treatment (Fig. 3D). This was presumably due to decreased AKT and extracellular signal–regulated kinase (ERK) activation (P-ERK and P-AKT), which phosphorylates BIM and thereby reduces BIM levels (*29*). To explore a potential role for BIM in the synergistic response, we treated BIM-deficient clones (achieved through CRISPR-Cas9–mediated editing of *BIM*) with S63845 plus trastuzumab, lapatinib, or docetaxel (fig. S9A). Although *BIM* deletion did not completely block the cytotoxic activity of any of these drugs, it significantly reduced their synergy at low concentrations ($P < 0.05$). These results are consistent with a previous study showing the potential contribution of the BIM/MCL-1 complexes in HER2-overexpressing breast cancer cells (*36*). We further confirmed that S63845 was able to disrupt BIM/MCL-1 complexes in PDX-derived cells (fig. S9B). Together, these findings suggest a key role for BIM in the synergistic action of S63845 with docetaxel or anti-HER2 therapy.

MCL-1 inhibition sensitizes PDX tumors to conventional therapy in vivo

Because in vitro assays revealed that both breast cancer cell lines and PDX-derived cells were sensitive to S63845 in combination therapy, we next determined their therapeutic effect in vivo using PDX models, including two TNBC models and one HER2-amplified model (Fig. 4 and fig. S10). S63845 alone was insufficient to inhibit tumor growth. Nonetheless, S63845 synergized with docetaxel or trastuzumab, resulting in improved survival. For the 110 and 838 PDX models, mice were treated with docetaxel (every 3 weeks) and S63845 (weekly) for two treatment cycles (Fig. 4, A and B, and fig. S10). Tumor growth was impeded by combination therapy, although tumors relapsed after treatment was stopped. Evaluation of tumor lysates from mice bearing PDX 838 tumors after short-term treatment revealed increased CC3 after combination therapy, consistent with augmented tumor cell death (fig. S11, A and B). The addition of S63845 to twice weekly trastuzumab also augmented responsiveness in the HER2-amplified 231 PDX model (Fig. 4C). Notably, S63845 therapy appeared to be well tolerated in combination therapy with either docetaxel or trastuzumab, with mice maintaining normal body weight during therapy (fig. S12A). No perturbation in urea, creatinine and liver enzymes (fig. S12B), or blood counts (fig. S12C) was observed after S63845 treatment. These results suggest that combining MCL-1 inhibitors with either chemotherapy or HER2-targeted therapy has the potential to enhance tumor response and clinical outcome.

DISCUSSION

MCL-1 is a crucial regulator of cell survival in both normal and neoplastic cells and is often responsible for resistance to anticancer therapy (*37*–*39*). The observation that MCL-1 is amplified in breast cancer (*11*), together with recent reports indicating that breast cancer cells depend on MCL-1 for survival, suggests a potential clinical role for MCL-1 inhibitors (*17*, *18*). Here, we tested the MCL-1 inhibitor S63845 in breast cancer cell lines and PDX tumor cells in vitro and observed synergistic activity with docetaxel in TNBC and with trastuzumab in HER2-amplified tumor cells. This synergy translated into improved tumor response in vivo and enhanced overall survival in PDX models. Given that Fc receptor–mediated cell death is lacking in NSG mice, it is possible that a more profound effect of anti-HER2 monoclonal antibody therapy might be observed in immunocompetent models and patients.

We previously demonstrated that inhibition of BCL-2 and BCL-XL with ABT-737 alone was insufficient to inhibit the growth of TNBC tumors (*40*). Although PDX tumor cells appeared to be more sensitive to S63845 than to BCL-2– or BCL-XL–specific inhibitors in vitro, S63845 alone did not induce a clinical response in TNBC and HER2-amplified PDX tumors. It is possible that a lower IC_{50}, similar to that recently described for leukemic cells (*29*), is required to elicit an in vivo tumor response to single-agent therapy. Moreover, because the addition of other BH3 mimetics greatly enhanced the efficacy of S63845, TNBC and HER2-amplified breast cancer cells likely deploy additional prosurvival BCL-2 family members, in contrast to certain leukemic cell types where a single prosurvival protein can have a dominant role (*41*).

S63845 notably attenuated tumor growth in combination with docetaxel in TNBC and trastuzumab in HER2-amplified PDX models. Both docetaxel and trastuzumab have been shown to reduce MCL-1 (*20*, *35*, *42*), perhaps in part accounting for the augmented response to combination therapy, although MCL-1 expression did not appear to be modulated at the doses used here. Synergism between docetaxel and ABT-737 or the BCL-XL inhibitor A-1331852 has also been observed (*40*, *42*, *43*), suggesting that direct inhibition of BCL-XL and MCL-1 with BH3 mimetics could be investigated. Dual treatment of cell lines with the BCL-XL inhibitor WEHI-539 and the MCL-1 inhibitor A-1210477 in vitro appears to be efficacious and may sensitize cells to chemotherapy (*44*). It remains to be established, however, whether there is a suitable therapeutic window for combining the potent MCL-1 inhibitor S63845 with other BH3 mimetics in vivo.

Despite the observation that MCL-1 deletion is lethal in knockout mice (*45*) and previous reports pointing to a crucial physiological role for MCL-1 in many cell types including cardiomyocytes (*46*, *47*), we found that the administration of S63845 was well tolerated, in agreement with a recent report (*29*). This may be due to partial inhibition of MCL-1 in the adult rather than complete deletion during critical developmental time points. In addition, the selectivity of the compound in cancer cells at the doses used in our preclinical models may be explained through tumor priming (*41*) or through the drug's greater binding affinity for human compared to mouse MCL-1. Finally, MCL-1 also plays an important role in mitochondrial respiration (*48*). S63845 may not interfere with this function because BH3 mimetics compete for binding to the hydrophobic groove, a conformational pocket on the surface of prosurvival proteins that is specifically involved in binding BH3-only proteins (*49*). It will be important to investigate the safety of MCL-1 inhibitors and combination therapy in the clinic.

Innate and acquired resistance to therapy remains a major challenge for patients with breast cancer. Our results predict that loss of BAK may be a potential mechanism of acquired resistance to S63845-induced cell death. Sequestration of BAK by MCL-1 might represent the primary anti-apoptotic function in these cells. This mechanism has been described as "Mode 2" in the unified model (*50*). It is also possible that BH3-only proteins (sensitizers and/or activators) are required after S63845 treatment ("Mode 1"). Our results using BIM/PUMA/BID-deficient SK-BR-3 cells support this latter model, because these BH3-only proteins were required for BAX/BAK activation in this cell line. It is noteworthy, however, that the CRISPR-Cas9 screen did not identify any single BH3-only protein directly involved in S63845-mediated cell death despite the ability of the compound to displace most BH3-only proteins from MCL-1. These results suggest a great degree of functional redundancy among BH3-only proteins in breast cancer. However, BIM

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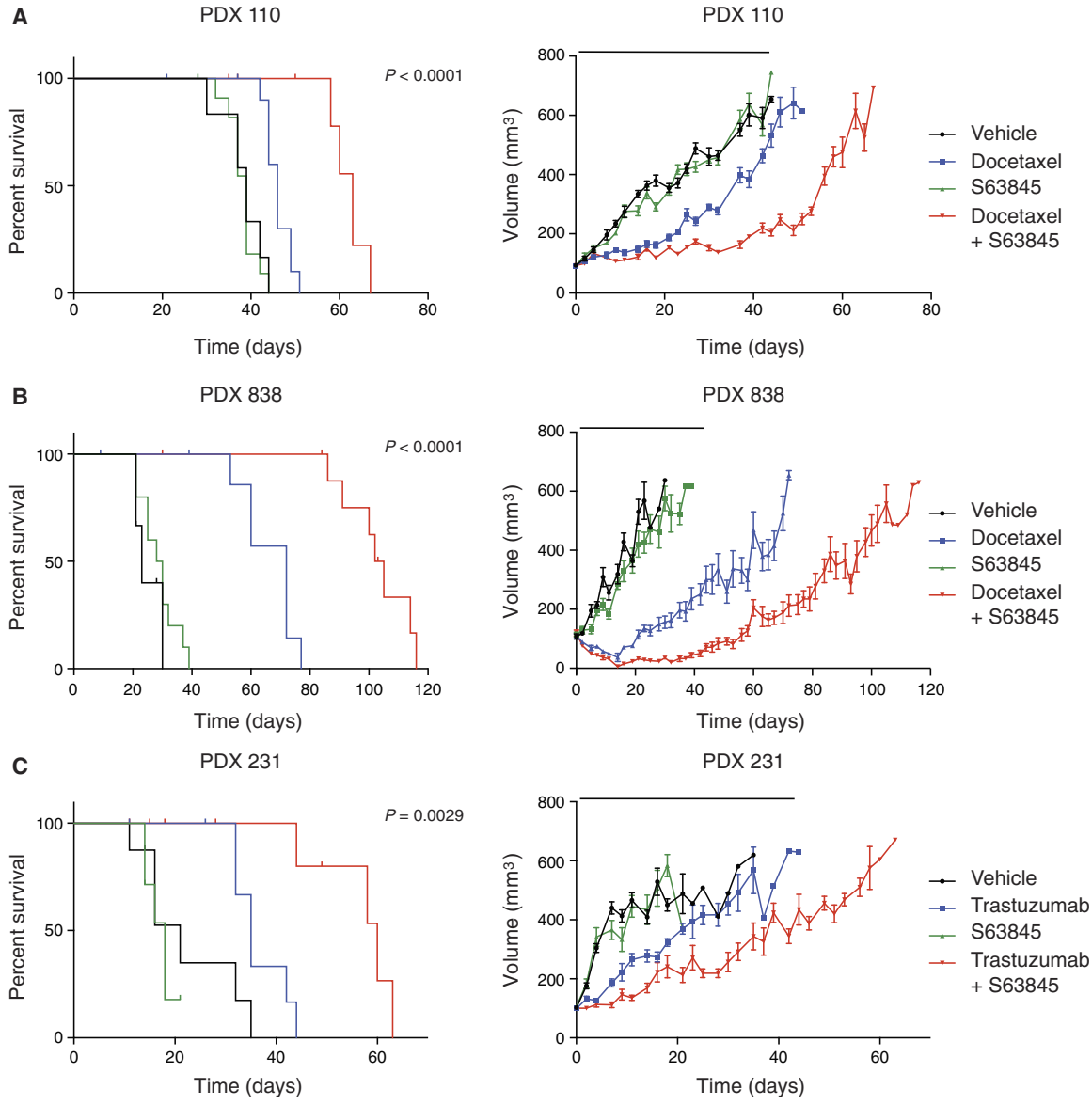

Fig. 4. Improved tumor response to docetaxel in TNBC and trastuzumab in HER2-amplified PDX models with the addition of S63845. Kaplan-Meier survival curves (left panels) and tumor volume curves (right panels) for PDX models. (**A**) TNBC PDX 110 from a *BRCA1* mutation carrier (n = 10 to 12 mice per arm) and (**B**) TNBC PDX 838 (n = 10 to 11 mice per arm). Mice were treated with vehicle alone (black line), docetaxel (10 mg/kg intraperitoneally on days 1 and 22) plus vehicle for S63845 (blue line), S63845 (25 mg/kg intravenously once weekly for 6 weeks, on days 2, 9, 16, 23, 30, and 37) plus vehicle for docetaxel (green line), or combined docetaxel and S63845 (red line). (**C**) HER2-amplified PDX 231 (n = 6 to 8 per arm). Mice were treated with vehicle (black line), trastuzumab (30 mg/kg loading dose on day 1 and then 15 mg/kg twice weekly for 6 weeks starting on day 4) plus vehicle for S63845 (blue line), S63845 (25 mg/kg once weekly for 6 weeks on days 2, 9, 16, 23, 30, and 37) plus vehicle for trastuzumab (green line), or combined trastuzumab and S63845 (red line). For tumor volume curves, black bars indicate the total duration of the treatment. Mice, which remained otherwise healthy, were sacrificed when tumor size reached the experimental ethical end point (>600 mm^3). Means ± SEM are shown. Log-rank (Mantel-Cox) P value is shown for combination therapy versus docetaxel or trastuzumab alone. Tumor growth curves for individual mice from PDX models 110, 838, and 231 are shown in fig. S10.

deletion partially impaired the synergistic effect of S63845 with docetaxel, lapatinib, and trastuzumab. In addition, the prolonged inhibition of MCL-1 can cause the up-regulation of other prosurvival proteins, similar to that seen in the case of an ABT-737–mediated increase in MCL-1 (*51*, *52*). Cumulatively, our findings suggest that either BAK inactivation or up-regulation of prosurvival proteins represents a possible strategy that could be deployed by tumor cells to acquire resistance to prolonged therapy.

The recent development of the potent MCL-1 inhibitor S63845 has boosted the prospects of targeting tumor cell dependence on this key prosurvival factor. A counterpart clinical lead compound, S64315, is now under investigation in human studies (ClinicalTrials.gov identifier NCT02992483). Here, we identify MCL-1 as an important target in TNBC and HER2-amplified breast cancer and further demonstrate that S63845 is an on-target MCL-1 inhibitor with promising activity using

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PDX models. These findings provide a strong rationale for its further investigation in the clinic.

MATERIALS AND METHODS

Study design

The study was designed to evaluate the response of breast cancer cells to the MCL-1 inhibitor S63845. We evaluated the response to S63845 alone or in combination with conventional therapy (docetaxel or anti-HER2 therapies) in triple-negative and HER2-amplified cell lines in vitro and PDX tumor models in vivo. Experiments were designed to investigate the mechanisms of tumor response. As outlined below, all mouse studies included randomization and blinding. The numbers of replicates performed for each experiment are included in the figure legends.

Statistical analysis

All statistical tests were two-sided. For the in vivo tumor studies, statistical analyses were performed in the GraphPad Prism software version 5.0a. Kaplan-Meier (log-rank test) was used to test for significant differences in the survival of mice (using the ethical end point for tumor size as a surrogate for death). Unpaired *t* tests were used to test the significance of differences in column means between treatments.

SUPPLEMENTARY MATERIALS

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Materials and Methods
Fig. S1. Expression of BCL-2 family members and sensitivity to S63845 or A-1210477.
Fig. S2. RNA-seq analysis of BCL-2 family members in PDX models.
Fig. S3. Expression of *BCL-XL*, *BCL-2*, *BCL-W*, and *MCL-1* in METABRIC and TCGA databases.
Fig. S4. Characterization of ER, PR, HER2, and BCL-2 family member protein expression in PDX models by immunohistochemistry.
Fig. S5. Deep sequencing of genome-wide lentiviral sgRNA libraries, knockdown of BH3 proteins, and S63845-mediated disruption of MCL-1 complexes containing BH3-only proteins.
Fig. S6. Generation and analysis of S63845-resistant cell lines.
Fig. S7. Exploring molecular mechanisms of resistance to S63845.
Fig. S8. Effect of concomitant treatment of SK-BR-3 cells with a BH3 mimetic and trastuzumab, lapatinib, or docetaxel.
Fig. S9. Role of BIM in the synergistic effect of S63845.
Fig. S10. Individual tumor growth curves in TNBC and HER2-amplified PDXs after combination treatment with S63845 and docetaxel or trastuzumab.
Fig. S11. Effect of combination therapy on tumor cell death.
Fig. S12. Effect of combination therapy on mouse weight, biochemistry, and blood counts.
Table S1. Clinical, histopathological, and molecular features of primary breast tumors.
Table S2. BCL-2 family mRNA expression in PDX models.
Table S3. Statistical analysis of gene expression between different molecular subtypes of breast cancer in PDX models.
Table S4. Statistical analysis of *MCL-1*, *BCL-2*, *BCL-XL*, and *BCL-W* gene expression between different molecular subtypes of breast cancer in METABRIC and TCGA data sets.
Table S5. Normalized sgRNA counts up-regulated in S63845-treated cells compared to DMSO control.
Table S6. Primers used for sequencing CRISPR clones.
Table S7. Sequencing analysis of CRISPR clones for *BAK*, *BAX*, *BMF*, and *NOXA*.
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Synergistic action of the MCL-1 inhibitor S63845 with current therapies in preclinical models of triple-negative and HER2-amplified breast cancer

Delphine Merino, James R. Whittle, François Vaillant, Antonin Serrano, Jia-Nan Gong, Goknur Giner, Ana Leticia Maragno, Maïa Chanrion, Emilie Schneider, Bhupinder Pal, Xiang Li, Grant Dewson, Julius Gräsel, Kevin Liu, Najoua Lalaoui, David Segal, Marco J. Herold, David C. S. Huang, Gordon K. Smyth, Olivier Geneste, Guillaume Lessene, Jane E. Visvader and Geoffrey J. Lindeman

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Cutting off another tumor lifeline

BH3 mimetics are drugs that inhibit the BCL-2 family of prosurvival proteins in cancer cells and thereby promote cancer cell death. Unfortunately, MCL-1, a member of this prosurvival family, can interfere with treatment because it is not sensitive to currently available BH3 mimetics. The MCL-1 inhibitor S63845 was developed to overcome this mechanism of treatment resistance, and Merino *et al*. examined the effectiveness of this drug in cell lines and xenografts derived from breast cancer patients. The authors demonstrated the drug's efficacy in combination with several drugs that are already in clinical use and also identified a protein that can promote treatment resistance, which may help predict which patients are more likely to benefit from the new treatment.

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