Systems Analysis of BCL2 Protein Family Interactions Establishes a Model to Predict Responses to Chemotherapy

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Abstract

Apoptotic desensitization is a hallmark of cancer cells, but present knowledge of molecular systems controlling apoptosis has yet to provide significant prognostic insights. Here, we report findings from a systems study of the intrinsic pathway of apoptosis by BCL2 family proteins and clinical translation of its findings into a model with applications in colorectal cancer (CRC). By determining absolute protein quantities in CRC cells and patient tumor samples, we found that BAK and BAX were expressed more highly than their antiapoptotic inhibitors. This counterintuitive finding suggested that sole inhibition of effector BAX and BAK could not be sufficient for systems stability in nonstressed cells. Assuming a model of direct effector activation by BH3-only proteins, we calculated that the amount of stress-induced BH3-only proteins required to activate mitochondrial apoptosis could predict individual death responses of CRC cells to 5-fluorouracil/oxaliplatin. Applying this model predictor to protein profiles in tumor and matched normal tissue samples from 26 patients with CRCs, we found that differences in protein quantities were sufficient to model the increased tumor sensitivity to chemotherapy compared with normal tissue. In addition, these differences were sufficient to differentiate clinical responders from nonresponders with high confidence. Applications of our model, termed DR_MOMP, were used to assess the impact of apoptosis-sensitizing drugs in lowering the necessary dose of state-of-the-art chemotherapy in individual patients. Together, our findings offer a ready clinical tool with the potential to tailor chemotherapy to individual patients. Cancer Res; 73(2); 519–28. ©2012 AACR.

Introduction

Most chemotherapeutics kill cancer cells by triggering programmed cell death or apoptosis (1). Perturbations in the apoptotic machinery contribute to carcinogenesis and are a major cause of chemoresistance and radiotherapy resistance (2). Hence, interindividual heterogeneity in tumor gene expression and protein profiles often limit the efficiency of standard-of-care, one-size-fits-all chemotherapy paradigms. Therefore, patient-stratified treatment paradigms and dosage decisions are increasingly needed, requiring new tools that incorporate patient-specific, molecular data sets.

For stage II and III colorectal cancer (CRC), current treatment involves surgical resection followed by adjuvant therapy with genotoxic stress inducers oxaliplatin or irinotecan in combination with the antimetabolite 5-fluorouracil (5-FU; ref. 3). Genotoxic chemotherapeutics activate the BCL2 protein family-controlled process of mitochondrial outer membrane permeabilization (MOMP; refs. 4, 5). MOMP is executed by oligomerization of the proapoptotic BCL2 effector proteins BAK and BAX (6), which form pores in the mitochondrial outer membrane releasing apoptotic proteins that further activate caspase-dependent and -independent cell death pathways (4). In the absence of stress, BAK and BAX are inhibited by antiapoptotic BCL2 family proteins, including MCL1, BCL(X) L, and BCL2. Upon genotoxic stress and other stressors, a further apoptotic subclass of BCL2 proteins, the BH3-only proteins such as BID, BIM, and PUMA, are induced and de-repress BAK and BAX from its inhibitors. Some BH3-only proteins may also activate BAK and BAX directly (7).

Several proteins involved in executing apoptosis prior or subsequent to MOMP, including BCL2 family members, have been proposed as molecular markers for cancer diagnosis and for patient stratification (8–11). However, the complexity of the interaction of antagonizing BCL2 family proteins makes it unlikely that mutations or deregulation of single apoptosis-related proteins are sufficient to characterize apoptosis execution or its impairment in patients. Therefore, holistic approaches studying the interplay of several proteins and including quantitative protein levels and interaction kinetics may be of significant interest (12–16). However, no model of...
BCL2 interaction and MOMP execution has yet been transferred into a clinical setting.

We here provide a computational systems model of MOMP regulation that integrates data on the interaction of pro- and antiapoptotic BCL2 proteins (17). By quantifying cell- and tissue-specific protein levels, the model developed allowed an assessment of the sensitivity of CRC cell lines to genotoxic stress and to differentiate patients with CRCs into clinical responders and nonresponders.

Materials and Methods

Patient cohort

Patient tissue was collected and stored from the Departments of Surgery, Gastroenterology, and Pathology, Beaumont Hospital, Dublin, Ireland. Tumor tissue was collected from 8 patients with stage II or 18 patients with stage III CRCs along with matched adjacent normal tissue. Clinical follow-up was obtained through a review of medical records by a clinical research nurse. Patients showing 4 years of disease-free survival were classified as favorable outcome and patients whose cancer recurred or who died from CRC as unfavorable outcome. Patients with hereditary forms of CRCs were excluded. Ethical approval was obtained by the Beaumont Hospital Ethics Committee, and informed consent was obtained from all patients. Tissue was stored as snap-frozen (−80°C) or in RNAlater (Ambion;−20°C). Tables 1 and 2 details the patient clinical characteristics and treatments.

Absolute protein quantifications

Patient tissue was lysed in 500 μL ice-cold buffer [50 mmol/L HEPES (pH 7.5), 150 mmol/L NaCl, 5 mmol/L Na-EDTA] and protease inhibitor (Sigma) and homogenized on ice. Samples were centrifuged at 14,000 × g for 10 minutes, supernatant collected and stored at −80°C. HeLa and CRC cell lysates were prepared similarly. For quantitative Western blotting, standard curves were constructed with varying concentrations (0.1–10.0 ng) of recombinant BAX (a kind gift from Prof. Christoph Borner), BAK (Abnova), BCL2 (R & D Systems), BCL(X)L (Abnova), and MCL1 (a kind gift from Prof. Christoph Borner), and varying concentrations (2–20 μg) of HeLa extract.

Western Blot images were acquired using a LAS-3000 Imager (FUJIFILM UK Ltd. Systems) and densitometry was conducted using ImageJ software. The intracellular concentration of each protein was calculated as previously described (14).

Table 1. Patient clinical characteristics

<table>
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<th>Stage</th>
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<td></td>
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<td>Overall</td>
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</tr>
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</table>

Table 2. Details of the disease stage, chemotherapy treatment, and disease outcome for each of the 26 patients with CRCs within the study

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<td></td>
<td>Favorable</td>
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</table>

NOTE: Outcome was classified as favorable or unfavorable on the basis of disease recurrence/death within a 4-year period as described in Materials and Methods.

Abbreviations: DFS, disease-free survival within a 4-year period; Irin, irinotecan; Leu, leucovorin; none, no chemotherapy received; OS, overall survival within a 4-year period; Oxal, oxaliplatin.


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the investigated proteins in the lysate was obtained from the calibration curves. Finally, assuming a HeLa cell volume of 3.1 pl. (14) and the appropriate molecular weights for BAK, BAX, BCL2, BCL(X)L, and MCL1, cellular concentrations for these proteins were calculated (Fig. 3B).

Protein concentrations in the CRC cell lines and the patient samples were determined by comparison to signals from HeLa cell extracts as previously described (14, 18). Primary antibodies to MCL1 (1:250; BD Biosciences), BCL2 (1:100; Santa Cruz Biotechnology), BCL(X)L (1:250; Santa Cruz Biotechnology, Inc.), and β-actin (1:5,000; Sigma) were mouse monoclonal. Antibodies to BAK (1:250; Santa Cruz Biotechnology, Inc.) and BAX (1:1,000; Upstate Biotechnology) were rabbit polyclonal. The horseradish peroxidase (HRP)-conjugated secondary antibodies were from Jackson Immunoresearch.

Mathematical model of stress dependency of MOMP

Absolute protein levels of the investigated family proteins BAK, BAX, BCL2, BCL(X)L, and MCL1 were used as model input and, therefore, were determined in the CRC cell lines Colo-205, HCT-116 (wild-type), HCT-116 puma−/−, HCT-116 p53−/−, HT-29, and LoVo, as well as in the patients’ tumor and matched normal tissue. Expression of BCLW was not evident in any of the CRC cell lines or the patient-derived tissue. BH3-only proteins were calculated (Fig. 3B).

Genotoxic stress was modeled to be p53-dependent and to involve the upregulation of the proapoptotic BH3-only proteins PUMA and NOXA (22). In addition, we modeled a FOXO3- (or E2F1-) dependent upregulation of BIM (23, 24). Production rates of all 3 proteins were assumed to be identical and modeled by a step function. Unless otherwise specified, production of BIM, PUMA, and NOXA was modeled to proceed at a constant rate until termination after 12 hours. The entire protein production over 12 hours was defined as (protein) dose η and used throughout the study as means to model stress severity. We considered an increased rate of MCL1 degradation upon genotoxic stress as shown previously (25) by modeling a drop of the MCL1 half-life from 45 to 17 minutes (Supplementary Table S2).

Protein production, degradation, and interactions were modeled by a pseudo-reaction network using mass action kinetics, translated into a set of ordinary differential equations (ODE) and solved using the MATLAB 7.3 (MathWorks, R2007b, 7.5.0.342) function ode15s. The detailed signaling pathways of the BCL2 family proteins are described in the Supplementary Modeling Details and Supplementary Tables S1–S7. Briefly, the antiapoptotic proteins BCL2, BCL(X)L, and MCL1 were modeled to bind to the proapoptotic proteins BIM, PUMA, and NOXA (and tBID wherever indicated) and to replace them with hexamers or effector homomers larger or equal to hexamers were considered as mitochondrial pores (26). MOMP was assumed to occur once 10% of the total effectors have formed pores as previously experimentally deduced (27). BH3-mimetics ART-737 (28) and ApoG2 (29) were assumed to bind to and inhibit antiapoptotic proteins (Supplementary Table S6A), thereby facilitating apoptosis execution. To determine the minimal BH3-only stress to induce MOMP (Fig. 3–5), an iterative procedure was used. This procedure varied the protein dose in the model until the minimal dose η for which MOMP was achieved was determined within a range of 1.2 × 10−2 nmol/L. To test correlation of model predictions with survival of cell populations subsequent to administration of 5-FU/oxaliplatin, Pearson correlation analysis and Wilcoxon rank-sum were conducted using the MATLAB routines corr and ranksum. For the statistical analysis of the predicted stress dose η for the patients’ tissue, as well as for analysis of patients, the Wilcoxon signed an unsigned rank-sum test were used to test statistical independence between different Kaplan–Meier curves.

Qualitative analysis of activator and effector inhibition

As described in Llambi and colleagues (30), 2 tBID chimeras, tBIDBAK and tBIDBAX, were considered where the BH3 domains of tBID were replaced by the BH3 domains of either BAK or BAX. tBIDBAK and tBIDBAX binding affinities to the antiapoptotic protein were modeled to be the same as for the protein (BAK or BAX) from where the BH3 domain was taken (Supplementary Table S7A). Activation kinetics of BAK and BAX by each chimera were taken from those of native tBID (Supplementary Table S4AB and S7B). A protein dose η of 1 μmol/L of either tBIDBAK (B) and tBIDBAX (C) was assumed with 500 nmol/L of BAX and no BAX (B) or cells with 500 nmol/L of BAX and no BAK (C). BCL2 or MCL1 were administered at specified doses. The maximum amount of BAK or BAX pores was calculated. The amount of pores in the area plots were normalized to 500 nmol/L.

Cell lines and culture

HCT-116 wt, p53−/−, and puma−/− cells were obtained from Prof. Bert Vogelstein (Johns Hopkins University, Baltimore, MD). Colo-205, HT-29, and LoVo cells were obtained from Dr. Ken Nally (University College Cork, Cork, Ireland). The HeLa cells were purchased from the American Type Culture Collection (LGC Standards). The authenticity of the cell lines was confirmed by DNA STR profiling conducted by the DSMZ in October 2012. The genotype of the HCT-116 cells was verified by Western blotting. Colo-205, HeLa, and HCT-116 cells were maintained in RPMI-1640 supplemented with 10% fetal calf serum (FCS), 2 mmol/L L-glutamine, 100 U/mL penicillin, and 100 mg/ml streptomycin at 37°C in 5% CO2. HT-29 and LoVo cells were grown in Dulbecco’s Modified Eagle’s Media (DMEM) supplemented with 10% FCS, 100 U/mL penicillin, and 100 mg/ml streptomycin.

Flow cytometry

Following treatments, cells were stained with Annexin V- FITC (Biovision) and propidium iodide (PI; Sigma) for 20
minutes at room temperature and analyzed using a CyFlow ML (Partec) flow cytometer and FloMax software. A minimum of 10,000 events were recorded for each sample.

Results

An in silico model for studying MOMP in response to genotoxic stress

We devised a computational model to study cellular BCL2 protein interaction and the process of MOMP in response to genotoxic stress (Fig. 1). We modeled genotoxic stress to induce the BH3-only proteins BIM, PUMA, and NOXA (31–33), whose expression levels we considered as surrogate of the chemotherapeutic dose and that lead to an interaction of pro- and antiapoptotic proteins resulting in effector (BAK and BAX) homo-oligomerization. MOMP was assumed to occur when homo-oligomers larger than or equal to hexamers (further denoted as pores; ref. 26) bound more than 10% of total effectors (12). The antiapoptotic proteins BCL2, BCL(X)L, and MCL1 were modeled to attenuate MOMP by engaging the BH3-only proteins and effectors. We modeled effector activation to require an activation step by PUMA or BIM. In the following section, we calculated the amount of BH3-only proteins (dose $\eta$) for 10% effector homo-oligomerization (indicating MOMP) was subsequently calculated.

Systems modeling resembles experimental findings of two-step repression of MOMP

Every newly devised in silico model should be consistent with established qualitative and quantitative experimental data (12). Therefore, we investigated whether our model was able to reproduce the experimental findings of Llambi and colleagues (30). In this study, tBID chimeras, tBIDBAK and tBIDBAX, were constructed whereby the BH3-domain of tBID was replaced with either those of BAK or BAX. Using the above model, we assumed an artificial cell line as described in Materials and Methods and resembled settings where

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Figure 1. Model of BCL2 protein interaction during genotoxic stress. Systems biology graphical notation (SBGN) scheme of the computational model. The box labeled antiapoptotic BCL2 represents BCL2, BCL(X)L, and MCL1; the box labeled BH3-only indicates the genotoxic stress induced BH3-only proteins PUMA, NOXA, and BIM, whereas their heterodimers are described by the box labeled inhibited BH3-only. Active proteins are indicated with an additional dashed border. BH3-mimetic represents ABT-737 or ApoG2, which mimic the function of BH3-only proteins. The minimal amount of BH3-only proteins (dose $\eta$) for 10% effector homo-oligomerization (indicating MOMP) was subsequently calculated.
antia apoptotic proteins only inhibited the activator and not the effector (Fig. 2A, denoted as I). We implemented this by modeling BAX<sup>−/−</sup> cells (expressing BAX) to be exposed only to tBid<sup>BAX</sup> and BCL2 (binding to the tBid<sup>BAX</sup> and not to BAX) and by modeling BAK<sup>−/−</sup> cells (expressing BAX) to be exposed to tBid<sup>BAX</sup> and MCL1 (binding to tBid<sup>BAX</sup> and not BAX). In turn, effector repression (II) was investigated by assuming either BAX-deficient cells to be exposed to tBid<sup>BAX</sup> and MCL1 or BAX-deficient cells to be exposed to tBid<sup>BAK</sup> and BCL2. For all simulations, BCL(X)L was disregarded.

With these specific settings, we calculated the amount of BAK (or BAX) pores in BAX<sup>−/−</sup> (or BAX<sup>−/−</sup>) cells in the presence of either only BCL2 or MCL1 and under different initial concentrations ranging from 0 to 2 µmol/L. Results for BAX<sup>−/−</sup> (Fig. 2B) and BAX<sup>−/−</sup> (Fig. 2C) cells are depicted by in silico area plots (in analogy to Fig. 4A; ref. 30) where the area of the bar is proportional to the calculated maximum amount of pores that is present during a period of 96 hours. In both cells types, less antia apoptotic protein is required to prevent pore formation when they bind to the activator (I) than when they bind to the effector (II), as shown by Llambi and colleagues (30).

The model predicted BH3-only stress dose η required for MOMP correlates with sensitivity of CRC cell lines to chemotherapeutic agents

Initial model calculations for HCT-116 and HeLa cells suggested that whether or not MOMP is induced upon the same genotoxic stress may depend on expression levels of BCL2 family members (Supplementary Fig. S1 and S2). We therefore determined absolute levels of BAK, BAX, BCL2, BCL(X)L, and MCL1 in the CRC cell lines Colo-205, HCT-116, HCT-116 p53<sup>−/−</sup>, HCT-116 puma<sup>−/−</sup>, HT-29, and LoVo (Fig. 3A–C and Supplementary Table S8). The expression of BCLW was not detectable in any of the cell lines investigated (data not shown; ref. 34). Likewise, expression of BFL-1/A1/BCL2A1 is mainly limited to cells of hematologic origin (35). Strikingly, despite cell line specific differences, most cell lines showed higher expression levels of BAK and BAX than total levels of the combined levels of the quantified antia apoptotic proteins (Fig. 3C). This striking result challenges the notion that apoptosis can be prevented by sole effector repression.

We investigated whether predictions of our model correlated to the susceptibility of different CRC cell populations to genotoxic stress. Apoptosis activation was quantified after treatment with 30 µg/ml of 5-FU and 10 µg/ml of oxaliplatin for 48 hours. Cell death in the p53-mutated or -deficient cell lines was low (40%, 23%, and 20% for Colo-205, HCT-116, p53<sup>−/−</sup>, and HT-29 cells), whereas 70% and 42% cell death was observed in the p53-competent cell lines, HCT-116 and LoVo, respectively. Cell death in HCT-116 puma<sup>−/−</sup> cells was 60%.

Next, we addressed whether our model was sufficient to account for the differential sensitivity to the chemotherapeutic agents. To identify a model predictor for cell death, we assumed that stress due to 5-FU/oxaliplatin treatment would induce expression of BIM, PUMA, and NOXA in p53- and PUMA-competent cells. Expression of BIM in p53-mutated or...
-deficient cells, and expression of BIM and NOXA in HCT-116 puma<sup>−/−</sup> cells. We next assumed that the model predicted BH3-only stress that is required to induce MOMP (dose η) in response to genotoxic stress in a typical cell would be a good predictor for the apoptosis susceptibility of a population. Results of our analysis are depicted in Fig. 3D. Indeed, dose η showed a positive correlation with cell survival, suggesting that a more sensitive cell population required less genotoxic stress in the model ($R^2 = 0.70$; Pearson coefficient $= 0.84$; $P = 0.04$).

The minimal stress-induced BH3-only protein dose η that is required for MOMP is a model predictor for tumor sensitivity and clinical outcome in CRC patients

As the model predicted dose η may represent a surrogate of the stress in a cell required for MOMP, we reasoned that it may also estimate the sensitivity of cells to chemotherapeutics. We obtained primary tumor and matched normal tissue for 8 patients with stage II and 18 patients with stage III CRCs with known clinical outcome (Tables I and 2). Patients with favorable outcome were defined as showing 4 years of disease-free survival, whereas unfavorable outcome were defined as patients showing recurrence or death from disease within 4 years.

We subsequently obtained absolute protein expressions of BCL2 proteins (Fig. 4A–C and Supplementary Table S9) from the resected tumor tissue and matched normal tissue and calculated the model predicted dose η that is needed to induce MOMP in the respective tissue. Strikingly, on average over all patients, our model predicted that tumor tissue required a significantly lower dose η (median value of 417 nmol/L) than cells from matched normal tissues (median of 764 nmol/L; $P < 0.0001$, Wilcoxon signed rank test; Fig. 4D), in agreement with other studies suggesting higher sensitivity of tumor tissue to apoptotic agents (36, 37). This finding may be consistent with the existence of a therapeutic window, defined as the difference in the predicted dose η between tumor and matched normal tissue, which the model allowed to be calculated for each patient individually (Supplementary Fig. S3).

We then compared the model predicted dose η for all 18 patients with favorable outcome and unfavorable outcome who received adjuvant 5-FU–based chemotherapy (Fig. 4E, Tables 1 and 2), as well as for all 26 patients, irrespective of whether or not they received chemotherapy after surgery (Fig. 4F). Of note, patients with 4-year disease-free survival were predicted to be more responsive to chemotherapeutic stimuli, as, indicated by the lower amount dose η, they required for MOMP (median of 191 vs. 566 nmol/L of dose η, respectively; $P = 0.0441$, Wilcoxon rank-sum test; Fig. 4E). Moreover, patients with overall favorable outcome also tended to be less resistant to stress-induced BH3-only protein induction than those with unfavorable outcome (median of 216 vs. 581 nmol/L of dose η, respectively; $P = 0.0523$, Wilcoxon rank-sum test; Fig. 4F).

We reckoned that a protein dose of 300 nmol/L may be a good separator to distinguish between patients with favorable...
or unfavorable outcome, as this dose lies in the middle of the medians of each group and, therefore, provided the optimal trade-off between specificity and sensitivity. Using this separator, Kaplan–Meier analysis of disease-free survival showed that patients with a predicted \( \eta < 300 \text{ nmol/L} \) had a significantly longer survival than patients with \( \eta \geq 300 \text{ nmol/L} \) \((P = 0.0099\) for prediction of response to chemotherapy; Fig. 4G; \(P = 0.0169\), for overall outcome, Fig. 4H; log-rank test). Collectively, these results suggested that the increased sensitivity of tumor tissue compared with nontransformed cells may sufficiently be explained by differences in intra-individual protein profiles and that the modeled BH3-only stress dose \( \eta \) required for MOMP may be used as a prognostic marker and predictor of treatment responses to adjuvant chemotherapy. To indicate the potential of our model in aiding of dose response decisions for designing patient individual chemotherapy doses, we named the model dose response medicinal outcome model predictor (DR_MOMP).

Figure 4. DR_MOMP can be used as predictor of clinical success in patients with CRCs. A, representative Western blot for BAX, BCLXL, MCL1, BAK, and BCL2 in tumor and matched normal tissue from patients with CRCs alongside the HeLa control. B and C, the given BCL2 family proteins were quantified in matched normal tissue (B) and tumor tissue (C) in 26 patients with stage II and III CRCs by quantitative Western blotting. D, model predicted BH3-only stress dose \( \eta \) required for MOMP was calculated for patient matched normal and tumor tissue. Matches are indicated by solid lines. Tumor tissue (right box plot) was predicted to require less dose \( \eta \) than the matched normal tissue (left box plot), consistent with the notion of cancer cells being primed to death \((36, 37)\). Wilcoxon signed rank test confirmed significance between both groups \((P < 0.0001)\). E and F, the calculated dose \( \eta \) can distinguish patients with favorable and unfavorable clinical outcome. Tumor tissue of 18 patients (E) who received adjuvant 5-FU–based chemotherapy and all 26 patients (F) were analyzed by the model. Patients were classified on the basis of disease-free survival within 4 years after surgery. Those patients with favorable clinical outcome required a significantly lower dose \( \eta \) than patients with unfavorable outcome. G and H, Kaplan–Meier curves of disease-free survival were generated for patients where the model predicted necessary doses \( \eta < 0.3 \text{ µmol/L} \) or \( \geq 0.3 \text{ µmol/L} \). Patients who required doses \( \eta < 0.3 \text{ µmol/L} \) for MOMP (solid line) had a better outcome than those requiring doses \( \eta \geq 0.3 \text{ µmol/L} \) (dashed line). Statistical independence of Kaplan–Meier curves between both groups were tested by log-rank test for patients who received adjuvant 5-FU–based chemotherapy (G; \(P = 0.0099\)) and for all patients (H; \(P = 0.0169\)).
**Figure 5.** DR_MOMP as a tool to assess apoptosis sensitizers. Administration of either of the BH3-only mimetics ABT-737 or ApoG2 lowered the required dose \( \eta \) for chemotherapy. ApoG2 or ABT-737 was modeled to be administered over 12 hours. A, with increased ABT-737 concentrations, HT-29 cells became increasingly sensitized to treatment with 30 \( \mu \)g/mL 5-FU and 10 \( \mu \)g/mL oxaliplatin as observed by Annexin V/PI staining and subsequent flow cytometric analysis. This increased sensitivity was explained by DR_MOMP by a lower dose \( \eta \). B and C, dose \( \eta \) was calculated for tumor tissue of the patients in Fig. 4. B, total administration of 1 \( \mu \)mol/L ABT-737 or ApoG2 within 12 hours decreased the necessary median dose \( \eta \) in tumor samples from patients \( (n = 28) \) from 417 nmol/L (Fig. 4D) to 109 (with ApoG2) or 126 nmol/L (with ABT-737). Administration of a higher dose of 2.5 \( \mu \)mol/L of both drugs did not markedly overall decrease the required dose for chemotherapy for all patients but decreased the variability in required dose between patients. C, DR_MOMP allowed assessment of patients with a different response to BH3-mimetics. As an example, in tumor tissue of patient 23, ABT-737 (solid line) reduced more significantly than ApoG2 (dashed dotted line).

**DR_MOMP can be used as a tool for adjuvant and personalized therapies**

BH3-mimetics are agents that are currently in clinical trial for adjuvant chemotherapy and sensitize cancer cells to apoptosis by mimicking the BH3 domain of BH3-only proteins (38). The synthetic drug ABT-737 (28) derepresses BH3-only activators by binding to BCL2 and BCL(X)L, whereas ApoG2 (29), albeit potentially also acting on glucose metabolism (39), sensitizes cells to apoptosis by binding to BCL2 and MCL1 (40). We implemented the molecular interactions governed by these drugs into DR_MOMP and carried out flow cytometric experiments of HT-29 cells treated with 5-FU/oxaliplatin in the absence or presence of ABT-737. Our results confirmed that ABT-737 increased the susceptibility of the cell populations to cell death in a dose-dependent manner. This increased susceptibility can be explained by DR_MOMP through a decreased dose \( \eta \) required for MOMP, suggesting that a lower dose of chemotherapy is required with increased ABT-737 concentrations (Fig. 5A).

We next investigated the effects of either ABT-737 or ApoG2 in individual patients. We modeled these administrations to lead to a total concentration of 1 or of 2.5 \( \mu \)mol/L of the respective drug over 12 hours (41) in the presence of 5-FU/oxaliplatin-based chemotherapy and again calculated the dose \( \eta \) that was required for MOMP in the patient tumor tissue. The median of dose \( \eta \) was reduced when both ABT-737 (from 417 to 126 nmol/L for 1 \( \mu \)mol/L and to 58 nmol/L for 2.5 \( \mu \)mol/L) and ApoG2 (to 109 and 58 nmol/L for 1 and 2.5 \( \mu \)mol/L) were applied (Fig. 5B). In both cases, we observed not only a decreased patient variability of the dose \( \eta \) with higher drug concentrations but also interindividual difference in the predicted responses to these 2 agents (Fig. 5C).

**Discussion**

Here, we investigated how the interactions of BCL2 family proteins translate different doses of chemotherapeutic stress into the all-or-none decision of MOMP. We established a model predictor acting as a surrogate for the chemotherapeutic dose required to induce cell death in tumor and matched normal tissues of individual patients. This model predictor correlated with the susceptibility of different cancer cell populations to stress, suggesting that cancer cells require less chemotherapeutic stress than nontransformed cells of the same patient (36, 37) and even was able to identify patients with favorable or unfavorable clinical outcome. Despite assuming that all tissues in all patients were unimpaired in expressing BH3-only proteins in response to chemotherapy, DR_MOMP robustly predicted patient outcome.
$\text{DR}_{\text{MOMP}}$ may aid in predicting the optimal dose of chemotherapy that preserves healthy, normal tissue while killing cancer cells and predicting which patients respond to classical chemotherapy. It may also predict which patients may benefit from the addition of BH3-mimetics such as navitoclax (ABT-263), ABT-737, ApoG2, AT-101, or obatoclax, which are currently preclinically and clinically tested (38). We applied $\text{DR}_{\text{MOMP}}$ to identify, in individual patients, how such mimetics may restore apoptosis by lowering the necessary dose of state-of-the-art chemotherapy. $\text{DR}_{\text{MOMP}}$ suggested that the predicted effects of BH3-mimetics were patient specific. As patient heterogeneity is often a reason for failure in clinical trials (42, 43), $\text{DR}_{\text{MOMP}}$ offers a possibility for in silico analysis of individual protein fingerprints from patients before administration of BH3-mimetics. As all mimetics target different protein interactions and as protein levels that mediate these interactions are patient specific, $\text{DR}_{\text{MOMP}}$ may be able to predict the most suitable co-treatment, both in terms of type and concentration and may assist as an in silico tool in future phase II and III clinical trials. Our modeling also suggested that with sufficient concentrations of BH3-mimetics, the interindividual variability in predicted responses was significantly attenuated, indicating the potential of BH3-mimetics potential as future first-line treatment paradigms.

Input to $\text{DR}_{\text{MOMP}}$ required absolute protein quantifications. We used quantitative Western blotting as opposed to alternatives such as ELISA as it allowed us to measure the signal of the protein of interest at its specific band and minimize the error due to unspecific antibody binding. However, for future clinical applications, array-based techniques such as reverse-phase protein arrays (RPPA; ref. 44) may provide a cost-effective and accurate alternative for higher numbers of proteins or specimens.

We have recently also developed a computational systems model to investigate apoptosis signaling subsequent to MOMP (14, 18) and showed that cancer cells have a decreased likelihood to undergo caspase activation than matched normal tissue (16, 18). Indeed, it is well established that caspase-inhibitory proteins such as X-linked inhibitor of apoptotic protein (XIAP) are overexpressed in cancer (45) and that caspase-activating proteins are inactivated through promoter methylation, chaperone binding, or phosphorylation (46). How can these results be reconciled with the present study? A higher $\text{DR}_{\text{MOMP}}$ may indeed be associated with higher ability of cancer cells to adapt and respond to changes in their microenvironment, such as hypoxia/ischemia, reactive oxygen species production, and oncogene signaling. Cancer cells may accumulate tonic proapoptotic stimuli without committing MOMP and therefore require less chemotherapy to be “pushed over the edge.” However, with MOMP as a potential all-or-none process, any information on dosage of genotoxic stress inducing chemotherapy and potentially any mitochondrial priming is likely to be lost after MOMP. Once a sufficient amount of stress is applied to induce MOMP, the decision whether or not apoptosis is executed may then depend on the expression levels of subsequent pro- and antiapoptotic players and potentially on bioenergetic parameters involved in this downstream process (14, 18, 47). It is therefore likely that through the analysis of both pathways independently, complementary model predictors for cancer cell survival or death can be established relating either to a dose-sensitive priming upstream to MOMP, or to a dose-independent, binary decision to evade of apoptosis execution downstream to MOMP.

Disclosure of Potential Conflicts of Interest
No potential conflicts of interest were disclosed.

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