Original Research

Microarray determination of Bcl-2 family protein inhibition sensitivity in breast cancer cells

Sandra G Hudson[1], Devin R Halleran[2], Barbara Nevaldine[1], Anna Shapiro[1], Robert E Hutchison[2] and Peter J Hahn[1]

[1]Department of Radiation Oncology; [2]Department of Pathology, The State University of New York (SUNY) Upstate Medical University, 750 East Adams Street, Syracuse, NY 13210, USA
Corresponding author: Sandra G Hudson. Email: hudsons@upstate.edu

Abstract

This study tests the hypothesis that reverse transcription polymerase chain reaction (RT-PCR) microarrays can be used to predict the relative sensitivity to induction of apoptosis in breast cancer cells exposed to inhibitors of antiapoptotic Bcl-2 family proteins. Four cell lines, MDA-MB-231 (MDA-231) and MDA-MB-468 (MDA-468), BT-20 and T47-D were screened for relative expression of Bcl-2 family members A1, Mcl-1, Bcl-2, Bcl-xL and Bcl-w mRNA by RT-PCR microarrays and Western analysis. The four cell lines were treated with 1 μmol/L obatoclax (GX15-070) and/or 2 Gy radiation (RT) and monitored for apoptosis after 48 h. Cell lines showing the highest total fold-increase of Bcl-2 family member mRNA, MDA-231 and MDA-468, also showed the highest levels of apoptosis induction (approximately 70% with obatoclax alone and 82% with obatoclax plus RT). Cell lines with little or no increase in Bcl-2 family mRNA (BT-20 and T47-D) showed less apoptosis (30% following treatment with obatoclax and 42% with obatoclax plus RT). RT-PCR arrays can predict the relative apoptosis response of breast cancer cells to the pan Bcl-2 inhibitor obatoclax alone or when combined with radiation.

Keywords: personalized medicine, obatoclax, Bcl-2 (B-cell leukemia lymphoma-2), apoptosis, radiation, breast cancer

Experimental Biology and Medicine 2013; **238**: 248–256. DOI: 10.1177/1535370212474582

Introduction

In recent years, agents that target antiapoptotic Bcl-2 family proteins have been developed and are being tested as anticancer agents either alone or in combination with standard therapies.[1–15] The rationale for these drugs is that by inhibiting the antiapoptotic members of the Bcl-2 family of proteins, they disrupt a frequently utilized pathway by which many cancer cells evade and resist the normal apoptotic signals that would otherwise lead to their death. Although resistance to apoptosis is a hallmark of virtually all cancer cells,[16,17] not all cancer cells overexpress the Bcl-2 proteins and those that do, do not necessarily overexpress the same ones. A means to distinguish which tumors will potentially respond to specific Bcl-2 inhibitors is needed so that only those patients who will benefit will be treated with a particular targeted therapy.

Bcl-2 family member proteins are the primary regulators of apoptosis, and the balance between pro- and antiapoptotic Bcl-2 proteins determines the relative sensitivity of cells to induction of apoptosis.[18–21] Bcl-2 family members fall into three groups. The antiapoptotic Bcl-2 subfamily members share up to four Bcl-2 homology (BH) domains 1–4 and consist of Bcl-2, Bcl-xL, Mcl-1, A1 (Bfl-1) and Bcl-w (BCL2L2). The proapoptotic Bcl-2 members Bax and Bak have the four major BH domains and, as dimers, induce apoptosis. The remaining proapoptotic Bcl-2 family members Bid, Bik, Hrk, BNIP3, Bim, NOXA and PUMA[22] have only the BH3 domain and induce apoptosis by binding to the antiapoptotic Bcl-2 family members to prevent them from inhibiting the proapoptotic members. The BH3-only members function primarily as signaling molecules. Additionally, BH3-only Bim and Bid have been reported to be capable of inducing apoptosis by directly activating Bax and Bak.[23,24] Antiapoptotic Bcl-2 family members bind to and inhibit proapoptotic Bax and Bak to prevent apoptosis.[25] In theory, inhibiting the inhibitors of apoptosis should be tumoricidal. For example, we have previously shown that oral cancer and glioblastoma cells overexpressing Bcl-2 show improved apoptotic response to inhibition with a Bcl-2-targeted ribozyme or an antisense oligonucleotide.[26,27]

Currently, pharmacologic inhibitors of antiapoptotic Bcl-2 family member proteins are undergoing clinical trials as single agents or in combination with conventional

treatments for various types of cancer.[1–15,21,28,29] These Bcl-2 inhibitors act as small molecule BH3 mimetics and bind the hydrophobic groove of antiapoptotic Bcl-2 proteins.[30,31] For example, obatoclax (GX15-070) is a small molecule pan-Bcl-2 family-member inhibitor, which mimics proapoptotic BH3-only proteins and binds antiapoptotic Bcl-2, Bcl-xL, Mcl-1, Bcl-w and A1.[10,30,31] ABT-737 (Abbott Laboratories, Abbott Park, IL, USA) is another example of a BH3 mimetic peptide. ABT-737, however, inhibits only Bcl-2, Bcl-xL and Bcl-w. Cells expressing Mcl-1 or A1 are not sensitive to ABT-737.[30,32] An important and attractive feature of target-specific agents such as obatoclax and ABT-737 is that they are less likely to cause systemic toxicity than are non-targeted agents. Their specificity suggests, however, that specific Bcl-2 inhibitors will be effective only to the extent that specific antiapoptotic Bcl-2 family members are overexpressed.[33,34]

Breast cancer is a disease in which Bcl-2 overexpression has been recognized as an important prognostic marker. A meta-analysis published by Callagy[35] described the association between Bcl-2 expression and both disease-free survival (DFS) and overall survival in female breast cancer. Breast cancer also appears to be a good candidate for apoptosis-inducing treatments since breast cancer cells are resistant to radiation and chemotherapy-induced apoptosis. Importantly, 70% of breast cancers overexpress antiapoptotic Bcl-2.[36,37] Combining radiation with surgery reduces local recurrence of breast cancer but only in patients whose tumors do not overexpress Bcl-2.[38] In a more recent study, microarray analysis of paraffin tissue sections from early stage breast cancer patients receiving breast conservation treatment and radiotherapy revealed Bcl-2 expression to be associated with higher risk of local breast tumor recurrence.[39] Conceivably, newly developed anti-Bcl-2 agents, when properly matched with individual susceptible tumors, are likely to greatly increase the efficacy of current treatments, including radiation, analogous to the way HER2 and estrogen receptors are utilized in routine clinical practice.

Patients with locally advanced breast cancer (LABC) have historically had a very poor prognosis. Until recently, and despite aggressive surgical resection or primary radiation therapy (RT), there remained a high risk of local recurrence and distant metastases. Fewer than 20% of patients survived beyond five years.[40,41] The addition of postoperative radiation to surgery has improved local control and DFS. The addition of systemic therapy has further improved prognosis and may even permit breast preservation for patients with LABC. A study of the Danish Breast Cancer Cooperative Group 82b Trial reported 48% DFS after 10 y for high-risk premenopausal breast cancer patients.[42] A more recent study, analyzing data from a 20-y follow-up of the British Columbia randomized radiation trial, also showed that systemic relapse-free survival is approximately 50%.[43] Furthermore, 30% disease recurrence is found even among early stage breast cancer patients.[44]

Microarrays have recently been employed to predict the aggressiveness and general chemosensitivity of breast cancer tumors.[45–49] These have been primarily used to place the tumors in taxonomic groupings. Microarrays measuring the levels of the Bcl-2 family member gene expression profiles may additionally be useful to predict the relative sensitivity of breast cancer cells to Bcl-2 inhibitors.

We report here that microarrays can predict the relative amount of apoptosis from obatoclax combined with 2 Gy radiation roughly in proportion to the relative amount of the antiapoptotic Bcl-2 family members overexpressed in the individual cell lines.

Methods and materials

Cell lines and reagents

The four breast cancer cell lines examined were obtained from the American Type Culture Collection (ATCC, Manassas, VA, USA). Each cell line was resuscitated for less than three months in our laboratory. The BT-20 line was maintained in Dulbecco's Modified Eagle's medium (DMEM) along with Ham's F-12 Nutrient Mixture in a 1:1 ratio (Hyclone Laboratories, Logan, UT, USA) with 10% fetal bovine serum (FBS), 2% penicillin/streptomycin, 10 mmol/L HEPES, 1% sodium pyruvate, 2% sodium bicarbonate and 1% non-essential amino acids. T47-D, MDA-MB-468 (MDA-468) and MDA-MB-231 (MDA-231) were maintained in DMEM, which contained 10% FBS, 2% penicillin/streptomycin, 10 mmol/L HEPES, 1% sodium pyruvate, 2% sodium bicarbonate and 20 mmol/L glucose. The T47-D line was derived from infiltrating ductal carcinoma whereas the other cell lines were of adenocarcinoma origin.

Drug treatment and irradiation

The small molecule pan Bcl-2 inhibitor, obatoclax mesylate (GX15-070), was purchased from Selleck Chemicals LLC (Houston, TX, USA). Obatoclax was dissolved in dimethyl sulfoxide (DMSO) at a stock concentration of 1 mmol/L and working dilutions were prepared in DMEM medium. Untreated control cells had the equivalent amount of DMSO added as the agent was diluted. Mock-irradiated cells (0 Gy) in plates were manipulated in the same way as those that received 2 Gy dose of x-irradiation. The X-ray source was an RS 2000 X-ray Biological Irradiator (Rad Source Technologies, Inc., Suwanee, GA, USA). Four hours after irradiation, cells were treated with 1 μmol/L of obatoclax in a 37°C 5% CO_2 incubator. Assays were performed at 48 h postirradiation.

Microarrays

SABiosciences apoptosis arrays were used as described by the manufacturer (SABiosciences, Qiagen Inc., Valencia, CA, USA). The plates were read on a Roche LightCycler 480 System. The values for the four samples on each plate were separated using the online software at the SABioscience website.

To control for minor DNA concentration differences between samples, all values were normalized in comparison to the average of the five housekeeping genes on the microarray. All samples were compared with normal breast

HMECs and normalized by the difference between the housekeeping gene averages relative of the HMEC controls.

Further normalization controls: If the average of the housekeeping genes differed by more than 2 (log2), that entire sample was discarded. Additionally, if any individual housekeeping gene was missing or off scale, that individual housekeeping gene was excluded, except where we had multiple samples from the same DNA sample. In that case the missing housekeeping value was replaced by the average of the others from the same DNA and same housekeeping gene. An average of three replicates was used for each cell line except for MDA-468 where there were four replicates.

Western blotting

The level of protein expression was determined by immunoblotting. Proteins were extracted from cell extracts by lysis in RIPA buffer and a Complete Mini Protease Inhibitor tablet (Roche Diagnostics, Indianapolis, IN, USA). The 7.4 pH RIPA buffer was comprised of 150 mmol/L sodium chloride, 1.7 mmol/L monobasic sodium phosphate, 9.1 mmol/L dibasic sodium phosphate, 0.5% sodium deoxycholate (Fisher Scientific Chemicals, Fair Lawn, NJ, USA), 0.1% sodium dodecyl sulfate and 1% Nonidet P-40 (Sigma Chemical Co., St. Louis, MO, USA). Proteins were separated on 4–12% polyacrylamide gels and transferred to polyvinylidene difluoride membranes, blocked with 5% milk block overnight, rinsed with TBS/Tween-20 and hybridized with antibodies specific to human: Bcl-2, Bcl-xL, Mcl-1, A1 and Bcl-w (Santa Cruz Biotechnology Inc., Santa Cruz, CA, USA) at a 1:200 dilution for 1.5 h at room temperature. SeeBlue Plus2 Prestained Standard (Life Technologies, Grand Island, NY USA). The loading control was β-actin, clone AC15 (MA1-91399, Thermo Scientific Inc., Rockford, IL, USA).

Antibodies were detected on the membranes with an ECF Western Blotting Kit (Amersham Biosciences, Piscataway, NJ, USA) and scanned on a Storm 840 Phosphorimager (Molecular Dynamics, Sunnyvale, CA, USA). Each experiment was performed at least twice and results were determined using ImageJ64 software (rsbweb.nih.gov/ij/download.html). All Western blot images were quantified using unsaturated densitometry scans. Western blot band densities were quantified with ImageJ64 software and normalized by re-probing each membrane with an antibody to β-actin as a loading control.

Statistical analysis

Error bars on graphs represent standard errors of the mean. The *t*-test and the Newman–Keuls Multiple Comparison Test were performed using GraphPad (Prism) graphing software.

Flow cytometric and fluorescent microscopic analysis of apoptosis by terminal deoxynucleotidyl transferase dUTP nick end labeling

Treated and control cells were assayed by terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) staining for late apoptosis.

All steps of the TUNEL procedure were performed at room temperature unless otherwise specified. Cell lines MDA-231 and BT-20 were seeded in a six-well plate at a concentration of 3×10^5 cells per well in 1 mL of corresponding media and the T47-D and MDA-468 cells were plated at 2×10^5 cells per well. The six-well plates were placed in a 37°C 5% CO_2 incubator for 24 h. After 24 h, the cells were irradiated at a 2 Gy dose of radiation with the RS 2000 X-ray Biological Irradiator. The plates were set up to have both irradiated samples (2 Gy) and mock irradiated samples (0 Gy) handled in the same manner. Four hours after irradiation, cells were treated with 1 μmol/L obatoclax for an additional 44 h in a 37°C 5% CO_2 incubator. Cells were collected 48 h postirradiation. Acquisition was with a BD LSRII flow cytometer with a 488 nm argon ion source and FACSDiVa software (Becton Dickinson Labware, Franklin Lakes, NJ, USA) and analysis with FlowJo software. Each apoptosis value was an average of two replicas for BT-20, three for MDA-468 and T47-D, and four for MDA-468. Immediately following flow cytometry the cells remaining in the tubes were cytospun onto glass slides and DAPI-containing vectashield (Vector Laboratories, Burlingame, CA, USA) was placed on the cytospun cells, coverslipped and visualized with an Olympus BX 60 fluorescence microscope. Four to five fields per slide with at least 100 cells total per slide were photographed at ×400 magnification and examined using MagnaFire software. The cells shown in Figure 5 are all from single fields, except for BT-20, for which a composite was made due to the low number of cells. Simple color balance and contrast adjustments were made using Adobe Photoshop and applied identically to all.

Results

In these experiments we tested the hypothesis that the relative mRNA expression levels of antiapoptotic Bcl-2 family proteins present in pretreatment samples predict the relative apoptosis response of breast cancer cells to the pan-Bcl-2 inhibitor, obatoclax, and obatoclax combined with radiation.

To test this hypothesis, we selected four breast cancer cell lines, T47-D, BT-20, MDA-231 and MDA-468, which had been previously reported to overexpress antiapoptotic Bcl-2 family members. Based on immunohistochemistry, T47-D and BT-20 overexpressed the Bcl-2 gene by a small amount, and MDA-231 and MDA-468 by an intermediate amount.[50] Our strategy was to quantify the mRNA expression levels for the Bcl-2 family members in the four cell lines, to treat each cell line with the pan-Bcl-2 inhibitor and/or ionizing radiation (RT) and to compare the resulting apoptosis to the mRNA levels.

Microarrays

Reverse transcription polymerase chain reaction (RT-PCR) microarrays have been used to quantify mRNA from clinical samples. To determine whether RT-PCR microarrays could be used to determine relative sensitivity to

Table 1 RT-PCR microarray quantification of Bcl-2 family member mRNA overexpression levels

	MDA-468 Ct (log2)	SD	MDA-231 Ct (log2)	SD	T47-D Ct (log2)	SD	BT-20 Ct (log2)	SD
Bcl-2	**3.62**	0.3	**2.6**	0.6	**2.4**	0.9	−0.8	0.3
A1	**3.25**	0.5	0.3	0.9	1.2	0.9	0.6	2.9
Bcl-xL	0.22	0.6	**3.3**	0.9	0.4	0.3	−2.1	2.7
Bcl-w	1.19	0.4	0.1	0.5	−0.6	2.3	−0.6	0.4
Mcl-1	0.6	0.5	−0.4	0.1	0.5	0.7	−0.8	0.8
Family total	6.90	0.7	5.9	1.0	2.4	0.9	0.0	0.0
Fold increase	21.9		17.4		5.2		0.0	
Bak	0.76	0.4	0.9	0.8	0.0	2.9	−1.9	1.1
Bax	**2.72**	0.6	**3.0**	1.1	0.9	1.8	−0.2	0.8
Bim	**3.99**	0.5	**2.0**	1.1	0.6	1.4	**2.7**	0.9
Family total	6.70	0.7	5.0	2.2	0.0	0.0	2.7	0.9
Fold increase	22.5		12		0		0	
NAIP	−2.85	0.8	**2.9**	0.3	1.5	0.8	0.9	0.4
BIRC2	**2.22**	0.6	**1.2**	0.2	0.8	0.6	−0.7	0.7
BIRC3	**4.47**	1.9	**3.7**	0.7	−0.2	1.5	1.4	1.1
XIAP	**2.12**	0.3	0.7	0.1	−0.3	1.3	1.5	1.0
BIRC6	**2.36**	0.2	0.6	0.8	0.6	0.4	1.7	0.9
Family total	8.10	1.0	6.6	0.8	0.0	0.0	0.0	0.0
Fold increase	29.1		20.1		0		0	

Values, one standard deviation above one doubling of mRNA expression, are in bold. Family totals are the sum of the doublings for the bold values. Fold increase for each family is calculated as the sum of the individual fold increases, equivalent to a single gene being overexpressed. Multiple replicas (at least 3) of RT-PCR amplifications of the apoptotic gene expression levels from the four cell lines were quantified and compared with similarly determined normal mRNA levels from breast HMECs. RT-PCR, reverse transcription polymerase chain reaction

obatoclax-induced apoptosis, we used the SABiosciences RT-PCR apoptosis microarray kit. This array contained primers to detect all the Bcl-2 family members, both pro- and antiapoptotic, as well as most of the XIAP family of genes that act downstream of the Bcl-2 family. The reader for the RT-PCR quantified doubling cycles (Ct) to reach a maximum rate of increase for each primer pair. We compared the normalized Cts (measured as $\log_2$) for each of the apoptotic genes from the cell lines with similar normalized values for breast HMECs as a point of comparison.

The results are shown in Table 1. Highlighted in bold are the values where the average is at least one standard deviation above a single doubling of the expression level relative to the normal HMECs. The total as well as the equivalent family fold-overexpression increase is also shown. The total Bcl-2 family member's fold-increase was determined by adding the increases due to the individual Bcl-2 family member overexpressed mRNAs to give a number equivalent to the fold-increase that would be measured if a single gene were being overexpressed. Values less than a single doubling were ignored because these numbers mostly reflect noise.

Table 1 shows that both MDA-468 and MDA-231 each overexpressed two of the five antiapoptotic Bcl-2 family members, but not the same two. Both overexpressed Bcl-2 itself. Additionally, MDA-468 also overexpressed A1 whereas MDA-231 overexpressed Bcl-xL. T47-D overexpressed only Bcl-2 and to a lesser extent than MDA-468 (although at a similar level as MDA-231). BT-20 did not overexpress any of the antiapoptotic Bcl-2 family proteins.

In general, Bax and Bim, the proapoptotic Bcl-2 family members, were overexpressed to the same levels as the antiapoptotic Bcl-2 family members. Bax and Bim were significantly overexpressed by MDA-468 and MDA-231, whereas BT-20 overexpressed only Bim. T47-D overexpressed no single proapoptotic Bcl-2 family member significantly, but the sum total was significantly overexpressed. The antiapoptotic XIAP family members were also overexpressed in cell lines MDA-231 and MDA-468 in proportion to the overexpressed antiapoptotic Bcl-2 family members.

Western blots

The RT-PCR microarrays quantified mRNA levels, but not protein concentrations. To confirm that the overexpressed mRNAs resulted in elevated levels of their protein products, we used Western blots (Figure 1) to quantify the relative

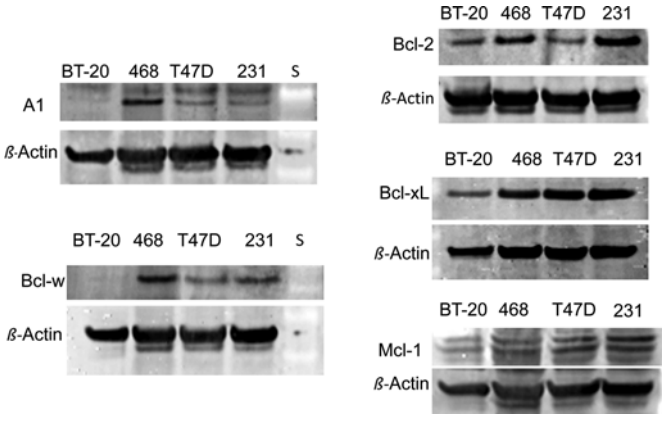

Figure 1 Western analysis of Bcl-2 family member proteins A1, Bcl-2, Mcl-1, Bcl-w and Bcl-xL. Total protein extracts from four different breast cancer cell lines were analyzed by Western blotting, with antibodies to each of five antiapoptotic Bcl-2 family proteins. β-Actin was used to control for loading differences ($n = 2$). Molecular weight standards are in lane s

Table 2 Bcl-2 family protein levels quantified by Western blotting

Westerns	MDA-468	SE	MDA-231	SE	T47-D	SE	BT-20
Bcl-2	**1.53**	0.38	**2.03**	0.40	**1.35**	0.31	1.00
A1	**1.44**	0.11	1.40	0.71	1.25	0.47	1.00
Bcl-xL	**1.32**	0.12	**2.00**	0.16	**1.85**	0.12	1.00
Bcl-w	1.31	0.31	1.1	0.04	1.11	0.13	1.00
Mcl-1	0.82	0.82	1.27	0.51	1.27	0.51	1.00

Values, one standard error above BT-20, are in bold. The results of two separate Western analyses for each Bcl-2 family member and each cell line were averaged and the standard error of the mean calculated. Note: In this table, the amounts are presented relative to BT-20, which had the lowest level in the Western blots, and also did not overexpress in the microarray analysis of the mRNAs

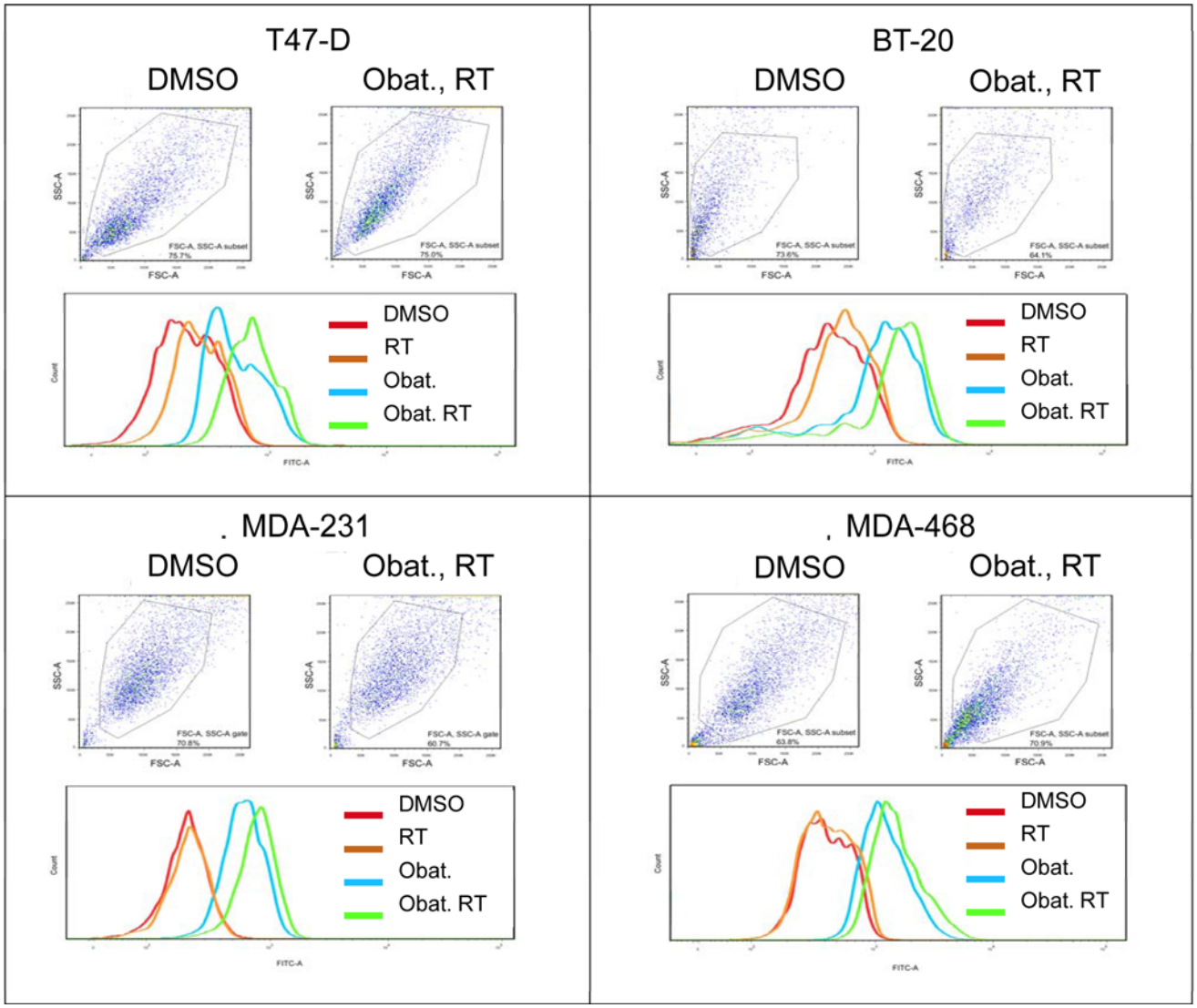

Figure 2 Flow cytometry measurements of terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) stained cells. Representative TUNEL flow cytometry apoptosis assay: cell lines T47-D, BT-20, MDA-MB-231 and MDA-MB-468 were treated with dimethyl sulfoxide (DMSO) control, obatoclax, RT or RT plus obatoclax. (DMSO was added as control to all as in Figures 1 and 2.) The cells were stained with labeled TUNEL antibody and analyzed by flow cytometry 48 h later. Gating was performed as shown to remove small debris from the analysis

amount of the five commonly overexpressed antiapoptotic Bcl-2 family members: A1, Bcl-2, Bcl-xL, Mcl-1 (both bands), and Bcl-w in each of the cell lines. Labeled antibodies were used to quantify the amounts of the antiapoptotic Bcl-2 family members on the Western blots, which were then normalized by determining the amount of β-actin present in each sample. We compared the protein concentrations to BT-20 since it had the lowest level of mRNA expression.

The results of the Western analysis of protein concentrations (Table 2) were very similar to the microarray data for the mRNA expression levels. Breast cancer cell line MDA-468 had an elevated level of the proteins A1 and Bcl-2, and cell line MDA-231 had an elevated level of

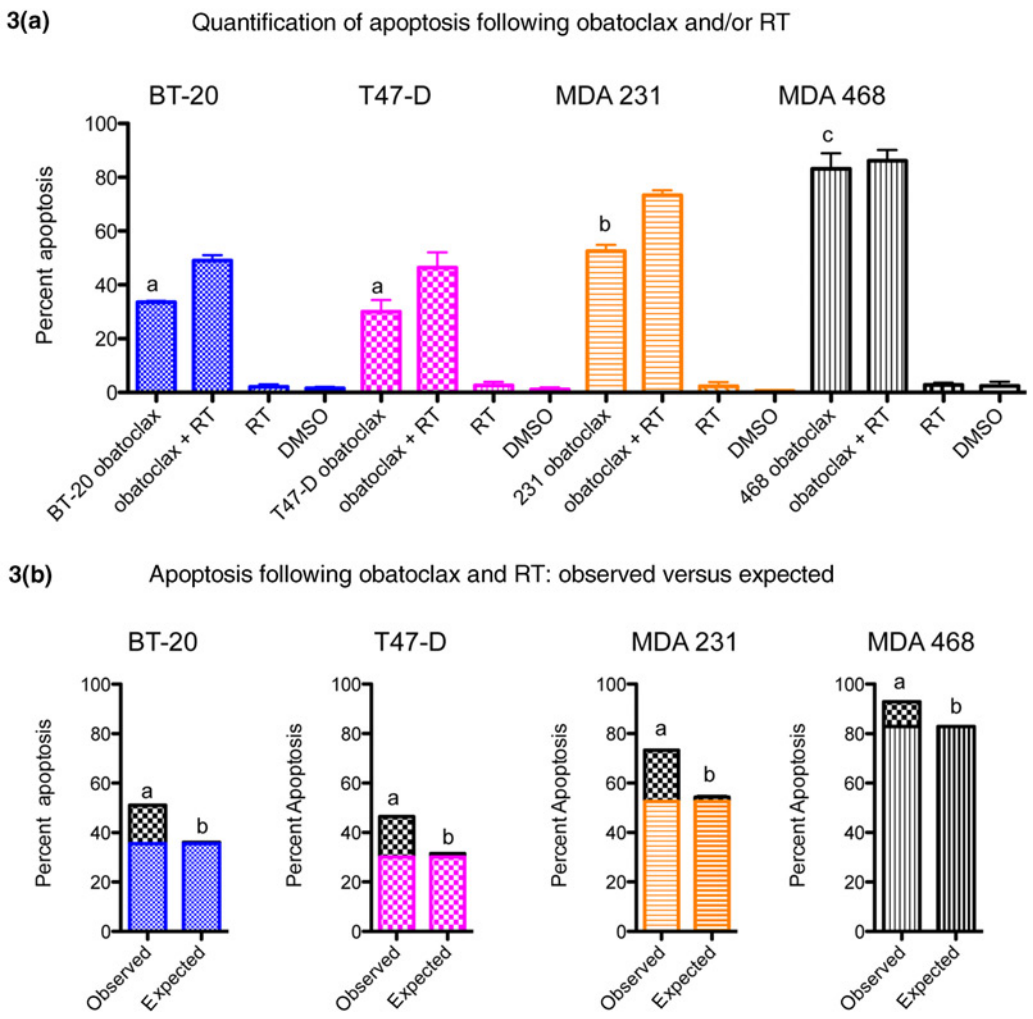

Figure 3 Quantification of apoptosis induced by either obatoclax or radiation therapy alone or in combination with obatoclax. (a) The values from three repetitions of data as in Figure 1a were averaged for each cell line. The differences between columns labeled a, b or c are statistically significant ($P < 0.001$) by the Newman–Keuls multiple comparison test. The error bars show the standard errors. Note that the amount of apoptosis induced by RT alone is approximately the same as the amount in the dimethyl sulfoxide controls. (b) The observed RT-plus-obatoclax apoptosis is significantly increased ($P < 0.05$) relative to what would be expected by simply adding the amount of apoptosis induced by obatoclax alone to the very small amount induced by RT alone. The apoptosis in the combined treatment is separated into the component induced by obatoclax alone (lower section) and the component induced by RT (A color version of this figure is available in the online journal)

Bcl-2 and Bcl-xL. T47-D had high levels of Bcl-2 but relatively low levels of the other proteins, and BT-20 had the lowest levels of all the five proteins. The major difference between the microarray data and the Western blot data was that levels of Bcl-xL were higher in MDA-468, MDA-231 and T47-D compared with BT-20. Note that from the Western blots, we cannot rule out that some Bcl-2 family members were present at elevated levels (e.g. Mcl-1 in MDA-468).

Apoptosis

To quantitatively determine the induction of apoptotic cell death by treatments with obatoclax and/or RT, we treated each cell line with obatoclax and/or RT, and quantified the apoptosis response by flow cytometric measurements of TUNEL-stained cells (Figures 2 and 3a). Obatoclax induced apoptosis in all four cell lines, but much more so in MDA-231 and MDA-468 than in BT-20 or T47-D cells (Figure 3a). In contrast (and as expected), RT alone (Figure 3a) induced almost no apoptosis in any of the cell lines, indicating that all four cell lines were resistant to RT-induced apoptosis. Figure 3b also showed the amount of apoptosis attributable to RT in the combined obatoclax-RT treatment as compared with what would be expected by simply adding the amounts of apoptosis produced by either treatment alone. When the obatoclax-treated cells were also treated with RT (Figure 3b), the level of apoptosis attributable to RT increased in all cell lines more than would be expected by a simple additive effect. Thus obatoclax sensitized cells to RT-induced apoptosis.

The values in Figure 3b, however, underestimated the interaction between obatoclax and RT. After the obatoclax treatment, a much lower percentage of MDA-231 and MDA-468 cells survived, compared with T47-D and BT-20.

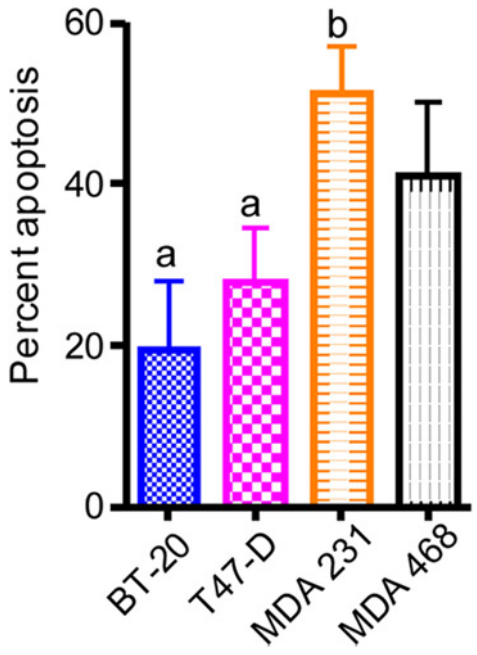

Figure 4 The apoptosis resulting from the combined treatment is also quantified as the percentage of apoptosis induced by RT in the non-apoptotic cells remaining after obatoclax treatment (e.g. in MDA-231, it would be the percentage of the 50% non -apoptotic cells remaining after the obatoclax-only treatment (Figure 3a) that subsequently became apoptotic after RT). Note: in the percent remaining data, one of the three repetitions of the MDA-468 data was omitted from the average because well over 90% of the cells were apoptotic following obatoclax alone treatment in that repetition, and the addition of radiation did not increase apoptosis further. Statistics: a and b are significantly different by the Student's *t*-test ($P < 0.05$). Error bars are the standard errors (A color version of this figure is available in the online journal)

When we determined the percentage of cells induced to undergo apoptosis by RT in the population remaining after obatoclax treatment (Figure 4), a much greater percentage of the surviving MDA-231 and MDA-468 were apoptotic compared with the percentage of surviving BT-20 or T47-D cells. This was consistent with the results from the obatoclax alone treatment.

To qualitatively confirm the flow cytometry measurements, we also prepared TUNEL-stained cells for visualization by fluorescent microscopy (Figure 5). The microscopic examination analyzed fewer total cells, but without gating out dead cells or other anomalies. Additionally, microscopic examination permitted the analysis of the untreated controls, which in the flow cytometry data were considered to be near zero. In general, the microscopic observations were consistent with the flow cytometry data. Two of the cell lines, BT-20 and MDA-468, showed higher levels of apoptosis in the untreated controls than were expected from the flow cytometry data.

Discussion

This study was designed to test the hypothesis that microarrays can predict the relative ability of newly developed anti-Bcl-2 agents to induce apoptosis in breast cancer cells. We found that breast cancer cell lines with high levels of Bcl-2 family member expression, as determined by the microarrays, responded to obatoclax and radiation with greater levels of apoptosis than those cell lines with lower expression levels. These results suggest that it may be

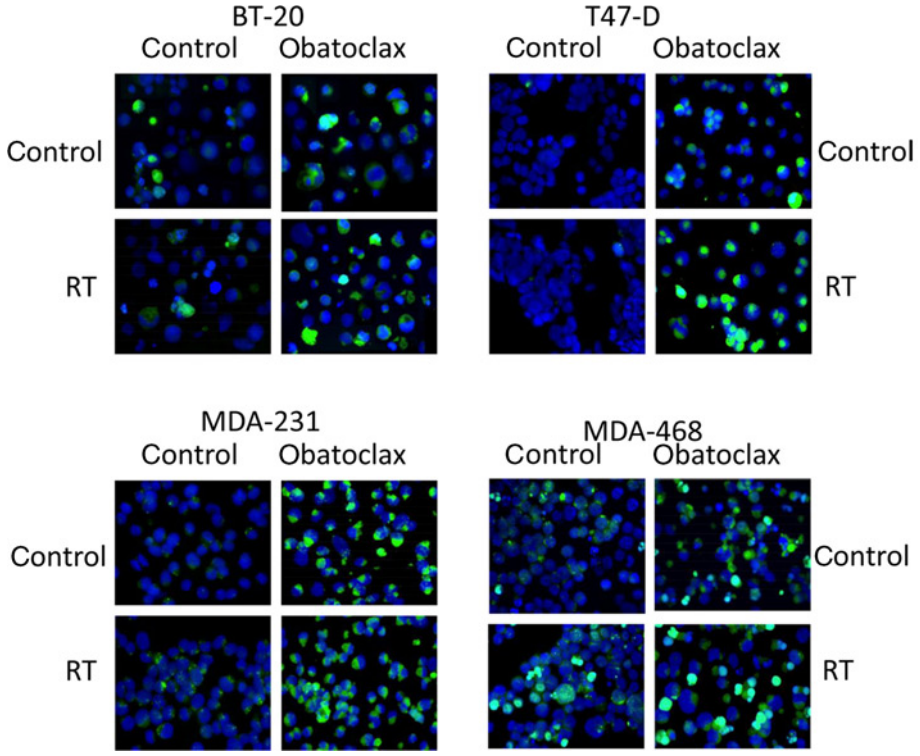

Figure 5 Fluorescent microscopy of terminal deoxynucleotidyl transferase dUTP nick end labeling stained cells prepared similarly to the ones used for flow cytometry in Figure 3

practical to test individual patient tumors for specific Bcl-2 family member levels to aid in selection of biologic treatments.

In practice, the most likely role for drugs such as obatoclax will be in combination with standard therapies to treat tumors such as LABC. We have shown here that obatoclax combined with radiation induced much more apoptosis than the sum of what would have been induced individually and roughly in proportion to the relative amount of Bcl-2 family member overexpression in the cell lines.

One of the striking observations based on the microarray data was that either multiple pro- and antiapoptotic Bcl-2 family members were upregulated along with overexpression of downstream XIAP family members, or alternatively, none of the Bcl-2 family members or the downstream XIAP family members were overexpressed. For example, in the case of MDA-468, not only were the antiapoptotic proteins Bcl-2 and A1 overexpressed, but the proapoptotic Bax and Bim were as well, and roughly to the same degree as Bcl-2 and A1. Additionally, the downstream antiapoptotic XIAP family proteins were also overexpressed. In contrast, BT-20 and T47-D apparently managed to achieve the inhibition of apoptosis primarily by suppressing Bax and Bim.

The model that seems to best explain our results is that activation of progrowth pathways leads to increased expression of proapoptotic proteins, Bax and Bim, which would normally make the cells more sensitive to proapoptotic signals as part of a feedback inhibition mechanism. Resistance to apoptosis arises secondarily to selection for resistance to stresses such as hypoxia, which would otherwise induce apoptosis.[51,52] In the case of the cell lines in this study, the selection during progression was likely for either high levels of antiapoptotic Bcl-2 and XIAP family members (MDA-231 and MDA-468) or alternatively, disabling the overexpression of the proapoptotic proteins (T47-D and BT-20). The Bcl-2 inhibitors such as obatoclax induce apoptosis in proportion to the amount of released and unsequestered Bax, Bak and Bim following treatment.

Recently Al-Harbi *et al*.[53] presented a simple RT-PCR-based system to quantify sensitivity to the inhibitor ABT-737, which inhibited Bcl-2, Bcl-xL and Bcl-w, but not Mcl-1 or A1. They showed that cells are sensitive only when the ratio of (Mcl-1 plus A1) to Bcl-2 was low. This occurred only when Bcl-2 levels were high and Mcl-1 and /or A1 were low. If both groups were low or both groups high, the cells were resistant. The advantage of the model we have described is that it can determine whether any of the Bcl-2 family proteins are overexpressed, and if so which ones and to what extent.

Determining which tumors overexpress which (if any) Bcl-2 family members will likely become increasingly important because Bcl-2 family members play important roles in breast cancer pathobiology and resistance to therapy. RT-PCR arrays capable of quantifying mRNA transcripts from multiple Bcl-2 family members and related pathways may provide a means to tailor specific treatments to individual tumors.

Author contributions: All authors participated in design, interpretation of the studies and analysis of the data and review of the manuscript; SGH, DRH and BN conducted the experiments. REH designed, interpreted and analyzed the flow data. SGH and PJH wrote the manuscript.

ACKNOWLEDGEMENTS

The authors wish to thank Kimberly Cripp for expert technical work (SUNY Upstate Medical University, Syracuse, NY, USA.) and the Carol Baldwin Breast Cancer Foundation (Syracuse, NY, USA.) for financial support. This work was supported by the Carol Baldwin Breast Cancer Awards 42573 to SGH and 37419 to PJH.

REFERENCES

1 An J, Chervin AS, Nie A, Ducoff HS, Huang Z. Overcoming the radioresistance of prostate cancer cells with a novel Bcl-2 inhibitor. *Oncogene* 2007;**26**:652–61
2 Fulda S, Galluzzi L, Kroemer G. Targeting mitochondria for cancer therapy. *Nat Rev Drug Discov* 2010;**9**:447–64
3 Hwang JJ, Kuruvilla J, Mendelson D, Pishvaian MJ, Deeken JF, Siu LL, Berger MS, Viallet J, Marshall JL. Phase I dose finding studies of obatoclax (GX15-070), a small molecule pan-BCL-2 family antagonist, in patients with advanced solid tumors or lymphoma. *Clin Cancer Res* 2010;**16**:4038–45
4 Kang MH, Kang YH, Szymanska B, Wilczynska-Kalak U, Sheard MA, Harned TM, Lock RB, Reynolds CP. Activity of vincristine, L-ASP, and dexamethasone against acute lymphoblastic leukemia is enhanced by the BH3-mimetic ABT-737 in vitro and in vivo. *Blood* 2007;**110**:2057–66
5 Knox JJ, Chen XE, Feld R, Nematollahi M, Cheiken R, Pond G, Zwiebel JA, Gill S, Moore M. A phase I-II study of oblimersen sodium (G3139, Genasense) in combination with doxorubicin in advanced hepatocellular carcinoma (NCI # 5798). *Invest New Drug* 2008;**26**:193–4
6 Konopleva M, Watt J, Contractor R, Tsao T, Harris D, Estrov Z, Bornmann W, Kantarjian H, Viallet J, Samudio I, Andreeff M. Mechanisms of antileukemic activity of the novel Bcl-2 homology domain-3 mimetic GX15-070 (obatoclax). *Cancer Res* 2008;**68**:3413–20
7 Li J, Viallet J, Haura EB. A small molecule pan-Bcl-2 family inhibitor, GX15-070, induces apoptosis and enhances cisplatin-induced apoptosis in non-small cell lung cancer cells. *Cancer Chemother Pharm* 2008;**61**:525–34
8 Mason KD, Vandenberg CJ, Scott CL, Wei AH, Cory S, Huang DC, Roberts AW. In vivo efficacy of the Bcl-2 antagonist ABT-737 against aggressive Myc-driven lymphomas. *P Natl Acad Sci USA* 2008;**105**:17961–6
9 Paik PK, Rudin CM, Brown A, Rizvi NA, Takebe N, Travis W, James L, Ginsberg MS, Juergens R, Markus S, Tyson L, Subzwari S, Kris MG, Krug LM. A phase I study of obatoclax mesylate, a Bcl-2 antagonist, plus topotecan in solid tumor malignancies. *Cancer Chemother Pharm* 2010;**66**:1079–85
10 Schimmer AD, O'Brien S, Kantarjian H, Brandwein J, Cheson BD, Minden MD, Yee K, Ravandi F, Giles F, Schuh A, Gupta V, Andreeff M, Koller C, Chang H, Kamel-Reid S, Berger M, Viallet J, Borthakur G. A phase I study of the pan bcl-2 family inhibitor obatoclax mesylate in patients with advanced hematologic malignancies. *Clin Cancer Res* 2008;**14**:8295–301
11 Shoemaker AR, Oleksijew A, Bauch J, Belli BA, Borre T, Bruncko M, Deckwirth T, Frost DJ, Jarvis K, Joseph MK, Marsh K, McClellan W, Nellans H, Ng S, Nimmer P, O'Connor JM, Oltersdorf T, Qing W, Shen W, Stavropoulos J, Tahir SK, Wang B, Warner R, Zhang H, Fesik SW, Rosenberg SH, Elmore SW. A small-molecule inhibitor of Bcl-XL potentiates the activity of cytotoxic drugs in vitro and in vivo. *Cancer Res* 2006;**66**:8731–9
12 Tahir SK, Yang X, Anderson MG, Morgan-Lappe SE, Sarthy AV, Chen J, Warner RB, Ng SC, Fesik SW, Elmore SW, Rosenberg SH, Tse C.

Influence of Bcl-2 family members on the cellular response of small-cell lung cancer cell lines to ABT-737. *Cancer Res* 2007;**67**:1176–83
13 Trudel S, Li ZH, Rauw J, Tiedemann RE, Wen XY, Stewart AK. Preclinical studies of the pan-Bcl inhibitor obatoclax (GX015-070) in multiple myeloma. *Blood* 2007;**109**:5430–8
14 Wei J, Stebbins JL, Kitada S, Dash R, Placzek W, Rega MF, Wu B, Cellitti J, Zhai D, Yang L, Dahl R, Fisher PB, Reed JC, Pellecchia M. BI-97C1, an optically pure Apogossypol derivative as pan-active inhibitor of antiapoptotic B-cell lymphoma/leukemia-2 (Bcl-2) family proteins. *J Med Chem* 2010;**53**:4166–76
15 Witters LM, Witkoski A, Planas-Silva MD, Berger M, Viallet J, Lipton A. Synergistic inhibition of breast cancer cell lines with a dual inhibitor of EGFR-HER-2/neu and a Bcl-2 inhibitor. *Oncol Rep* 2007;**17**:465–9
16 Hanahan D, Weinberg RA. The hallmarks of cancer. *Cell* 2000;**100**:57–70
17 Hanahan D, Weinberg RA. Hallmarks of cancer: the next generation. *Cell* 2011;**144**:646–74
18 Baguley BC. Multiple drug resistance mechanisms in cancer. *Mol Biotechnol* 2010;**46**:308–16
19 Chipuk JE, Green DR. How do BCL-2 proteins induce mitochondrial outer membrane permeabilization? *Trends Cell Biol* 2008;**18**:157–64
20 Green DR. At the gates of death. *Cancer Cell* 2006;**9**:328–30
21 Gupta S, Kass GE, Szegezdi E, Joseph B. The mitochondrial death pathway: a promising therapeutic target in diseases. *J Cell Mol Med* 2009;**13**:1004–33
22 Huang DC, Strasser A. BH3-Only proteins-essential initiators of apoptotic cell death. *Cell* 2000;**103**:839–42
23 Adams JM, Cory S. The Bcl-2 apoptotic switch in cancer development and therapy. *Oncogene* 2007;**26**:1324–37
24 Kuwana T, Bouchier-Hayes L, Chipuk JE, Bonzon C, Sullivan BA, Green DR, Newmeyer DD. BH3 domains of BH3-only proteins differentially regulate Bax-mediated mitochondrial membrane permeabilization both directly and indirectly. *Mol Cell* 2005;**17**:525–35
25 Puthalakath H, Strasser A. Keeping killers on a tight leash: transcriptional and post-translational control of the pro-apoptotic activity of BH3-only proteins. *Cell Death Differ* 2002;**9**:505–12
26 Gibson SA, Pellenz C, Hutchison RE, Davey FR, Shillitoe EJ. Induction of apoptosis in oral cancer cells by an anti-bcl-2 ribozyme delivered by an adenovirus vector. *Clin Cancer Res* 2000;**6**:213–22
27 Julien T, Frankel B, Longo S, Kyle M, Gibson S, Shillitoe E, Ryken T. Antisense-mediated inhibition of the bcl-2 gene induces apoptosis in human malignant glioma. *S*urg Neurol 2000;**53**:360–8; discussion 8–9
28 Bayes M, Rabasseda X, Prous JR. Gateways to clinical trials. *Method Find Exp Clin* 2007;**29**:427–37
29 Moulder SL, Symmans WF, Booser DJ, Madden TL, Lipsanen C, Yuan L, Brewster AM, Cristofanilli M, Hunt KK, Buchholz TA, Zwiebel J, Valero V, Hortobagyi GN, Esteva FJ. Phase I/II study of G3139 (Bcl-2 antisense oligonucleotide) in combination with doxorubicin and docetaxel in breast cancer. *Clin Cancer Res* 2008;**14**:7909–16
30 Kang MH, Reynolds CP. Bcl-2 inhibitors: targeting mitochondrial apoptotic pathways in cancer therapy. *Clin Cancer Res* 2009;**15**:1126–32
31 Warr MR, Shore GC. Small-molecule Bcl-2 antagonists as targeted therapy in oncology. *Curr Oncol* 2008;**15**:256–61
32 Huang S, Okumura K, Sinicrope FA. BH3 mimetic obatoclax enhances TRAIL-mediated apoptosis in human pancreatic cancer cells. *Clin Cancer Res* 2009;**15**:150–9
33 Borner MM, Brousset P, Pfanner-Meyer B, Bacchi M, Vonlanthen S, Hotz MA, Altermatt HJ, Schlaifer D, Reed JC, Betticher DC. Expression of apoptosis regulatory proteins of the Bcl-2 family and p53 in primary resected non-small-cell lung cancer. *Brit J Cancer* 1999;**79**:952–8
34 Yang TM, Barbone D, Fennell DA, Broaddus VC. Bcl-2 family proteins contribute to apoptotic resistance in lung cancer multicellular spheroids. *Am J Respir Cell Mol Biol* 2009;**41**:14–23
35 Callagy GM, Webber MJ, Pharoah PD, Caldas C. Meta-analysis confirms BCL2 is an independent prognostic marker in breast cancer. *BMC Cancer* 2008;**8**:153
36 Alsabeh R, Wilson CS, Ahn CW, Vasef MA, Battifora H. Expression of bcl-2 by breast cancer: a possible diagnostic application. *Mod Pathol* 1996;**9**:439–44
37 Buolamwini JK. Novel anticancer drug discovery. *Curr Opin Chem Biol* 1999;**3**:500–9
38 Daidone MG, Luisi A, Veneroni S, Benini E, Silvestrini R. Clinical studies of Bcl-2 and treatment benefit in breast cancer patients. *Endocr-Relat Cancer* 1999;**6**:61–8
39 Yang Q, Moran MS, Haffty BG. Bcl-2 expression predicts local relapse for early-stage breast cancer receiving conserving surgery and radiotherapy. *Breast Cancer Res Tr* 2009;**115**:343–8
40 Bruckman JE, Harris JR, Levene MB, Chaffey JT, Hellman S. Results of treating stage III carcinoma of the breast by primary radiation therapy. *Cancer* 1979;**43**:985–93
41 Rubens RD. Systemic therapy combined with radiotherapy for primary inoperable carcinoma of the breast. *Antibiot Chemother* 1978;**24**:205–12
42 Overgaard M, Hansen PS, Overgaard J, Rose C, Andersson M, Bach F, Kjaer M, Gadeberg CC, Mouridsen HT, Jensen MB, Zedeler K. Postoperative radiotherapy in high-risk premenopausal women with breast cancer who receive adjuvant chemotherapy. Danish Breast Cancer Cooperative Group 82b Trial. *New Engl J Med* 1997;**337**:949–55
43 Ragaz J, Olivotto IA, Spinelli JJ, Phillips N, Jackson SM, Wilson KS, Knowling MA, Coppin CM, Weir L, Gelmon K, Le N, Durand R, Coldman AJ, Manji M. Locoregional radiation therapy in patients with high-risk breast cancer receiving adjuvant chemotherapy: 20-year results of the British Columbia randomized trial. *J Natl Cancer I* 2005;**97**:116–26
44 Gonzalez-Angulo AM, Morales-Vasquez F, Hortobagyi GN. Overview of resistance to systemic therapy in patients with breast cancer. *Adv Exp Med Biol* 2007;**608**:1–22
45 Cleator S, Tsimelzon A, Ashworth A, Dowsett M, Dexter T, Powles T, Hilsenbeck S, Wong H, Osborne CK, O'Connell P, Chang JC. Gene expression patterns for doxorubicin (Adriamycin) and cyclophosphamide (cytoxan) (AC) response and resistance. *Breast Cancer Res Treat* 2006;**95**:229–33
46 Ellis M, Davis N, Coop A, Liu M, Schumaker L, Lee RY, Srikanchana R, Russell CG, Singh B, Miller WR, Stearns V, Pennanen M, Tsangaris T, Gallagher A, Liu A, Zwart A, Hayes DF, Lippman ME, Wang Y, Clarke R. Development and validation of a method for using breast core needle biopsies for gene expression microarray analyses. *Clin Cancer Res* 2002;**8**:1155–66
47 Glas AM, Floore A, Delahaye LJ, Witteveen AT, Pover RC, Bakx N, Lahti-Domenici JS, Bruinsma TJ, Warmoes MO, Bernards R, Wessels LF, Van't Veer LJ. Converting a breast cancer microarray signature into a high-throughput diagnostic test. *BMC Genomics* 2006;**7**:278
48 Korkola JE, DeVries S, Fridlyand J, Hwang ES, Estep AL, Chen YY, Chew KL, Dairkee SH, Jensen RM, Waldman FM. Differentiation of lobular versus ductal breast carcinomas by expression microarray analysis. *Cancer Res* 2003;**63**:7167–75
49 Shen R, Ghosh D, Chinnaiyan AM. Prognostic meta-signature of breast cancer developed by two-stage mixture modeling of microarray data. *BMC Genomics* 2004;**5**:94
50 Kumar R, Mandal M, Lipton A, Harvey H, Thompson CB. Overexpression of HER2 modulates bcl-2, bcl-XL, and tamoxifen-induced apoptosis in human MCF-7 breast cancer cells. *Clin Cancer Res* 1996;**2**:1215–9
51 Graeber TG, Osmanian C, Jacks T, Housman DE, Koch CJ, Lowe SW, Giaccia AJ. Hypoxia-mediated selection of cells with diminished apoptotic potential in solid tumours. *Nature* 1996;**379**:88–91
52 Hockel M, Schlenger K, Hockel S, Vaupel P. Hypoxic cervical cancers with low apoptotic index are highly aggressive. *Cancer Res* 1999;**59**:4525–8
53 Al-Harbi S, Hill BT, Mazumder S, Singh K, Devecchio J, Choudhary G, Rybicki LA, Kalaycio M, Maciejewski JP, Houghton JA, Almasan A. An antiapoptotic BCL-2 family expression index predicts the response of chronic lymphocytic leukemia to ABT-737. *Blood* 2011;**118**:3579–90

(Received April 2, 2012, Accepted November 16, 2012)