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Photodamage to multiple Bcl-xL isoforms by photodynamic therapy with the phthalocyanine photosensitizer Pc 4

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The antiapoptotic oncoprotein Bcl-2 is now a recognized phototarget of photodynamic therapy (PDT) with the phthalocyanine Pc 4 and with other mitochondrion-targeting photosensitizers. Photodamage, observed on Western blots as the loss of the native 26-kDa Bcl-2 protein, is PDT dose dependent and occurs in multiple cell lines, in the cold, and immediately upon photoirradiation. In our initial study, no photochemical damage was observed to Bcl-xL, in spite of its similarity in size, sequence, location and function to Bcl-2. The original study used a commercial anti-Bcl-xS/L antibody. We have revisited this issue by examining Western blots developed using one of three epitope-specific anti-Bcl-xL antibodies from commercial sources, a polyclonal antibody generated to the entire protein, as well as the antibody used previously. All five Bcl-xL antibodies recognized bacterially expressed Bcl-xL, but not Bcl-2, whereas an anti-Bcl-2 antibody recognized Bcl-2 and not Bcl-xL. All five Bcl-xL antibodies recognized at least one protein migrating at ~30 kDa; two of the antibodies recognized an additional band, migrating at ~33 or ~24 kDa. We now observe Pc 4-PDT-induced photodamage to all Bcl-xL-related proteins, except the 33-kDa species, in several human cancer cell lines. The results indicate that, in addition to the expected quantitative differences that may reflect exposure of individual epitopes, the antibodies also detect proteins of different apparent molecular weights that may be distinct isoforms or post-translationally modified forms of Bcl-xL. No evidence for PDT-induced phosphorylation or degradation was observed. Bcl-xL localized to mitochondria was considerably more sensitive to photodamage than was Bcl-xL in the cytosol, indicating that as previously found for Bcl-2, Bcl-xL must be membrane localized to be photosensitive.
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Introduction

Bcl-2 family proteins play key roles in the regulation of apoptosis (Gross *et al*., 1999). At least 15 Bcl-2 family members have been identified in mammalian cells and several others in lower organisms (Chao and Korsmeyer, 1998). All members possess at least one of four conserved motifs termed Bcl-2 homology domains, BH1, BH2, BH3 and BH4 (Kroemer, 1997). The family is composed of both proapoptotic and antiapoptotic members, and the regulation of apoptosis may be through the formation of homo- and heterodimers of the various members. Bcl-2 and Bcl-xL, two antiapoptotic proteins, are highly homologous within all four BH domains and their transmembrane (TM) domains. The sizes of these two proteins are similar: Bcl-2 is a 26-kDa protein (239 amino acids), and Bcl-xL is reported to be slightly smaller (233 amino acids) (Boise *et al*., 1993). Overexpression of Bcl-2 can enhance cell survival by suppressing apoptosis in cells treated with various stimuli (Reed, 1995; White, 1996). Like Bcl-2, overexpressed Bcl-xL also inhibits apoptosis induced by a variety of agents (Chao *et al*., 1995).

Photodynamic therapy (PDT) is a cancer treatment that employs photosensitizers and visible light to kill cells and ablate tumors selectively (Dougherty *et al*., 1998; Oleinick *et al*., 2002). PDT with a mitochondrion-bound photosensitizer, such as the phthalocyanine Pc 4, is a potent inducer of apoptosis in many types of cells (Oleinick and Evans, 1998; Kessel and Luo, 1999; Oleinick *et al*., 2002). Protection by Bcl-2 and Bcl-xL against PDT-induced apoptosis has been observed in cells overexpressing these proteins (He *et al*., 1996; Granville *et al*., 1999). However, Kim *et al*. (1999) reported that overexpression of Bcl-2 in human breast epithelial MCF-10A cells resulted in the upregulation of Bax and a twofold increase in cell sensitivity toward PDT. Similar results were found in Bcl-2-overexpressing human epidermoid carcinoma A431 cells (Srivastava *et al*., 2001).

PDT with a variety of mitochondrion-targeting photosensitizers directly damages Bcl-2, which is detected upon Western blot analysis as the loss of the native 26-kDa protein (Kim *et al*., 1999; Kessel and Castelli, 2001; Xue *et al*., 2001). The photodamaged Bcl-2 can be found within a series of high-molecular-weight

complexes near the top of SDS–PAGE gels (Xue *et al*., 2001; Usuda *et al*., 2003b). The photochemical damage to Bcl-2 occurs in a dose-dependent manner and can be observed in cell samples irradiated in the cold and collected immediately upon Pc 4-PDT (Xue *et al*., 2001). However, no photochemical damage was observed in Bcl-xL, when a single Bcl-xS/L antibody from Santa Cruz (Cat. No. sc-634) was used for that study. In this paper, we have revisited the question of the photosensitivity of Bcl-xL using three other commercial antibodies that each recognize single epitopes and an anti-Bcl-xL antibody (YS-TB2) prepared against the full-length protein. The results suggest the presence of at least three different Bcl-xL-like proteins in human cancer cells, all but one of which are targets of Pc 4-PDT.

Results

The impetus for this study was the unexplained difference in response of Bcl-2 and Bcl-xL to Pc 4-PDT. Since Bcl-xL is highly homologous to Bcl-2 and similar in function, it was curious that Bcl-2 was readily photodamaged, whereas Bcl-xL seemed resistant to similar photochemical damage by Pc 4-PDT, as judged by Western blot analysis with an anti-Bcl-xS/L polyclonal antibody obtained from Santa Cruz Biotechnology that was raised against a peptide mapping at the amino-terminus of Bcl-x of human origin (Xue *et al*., 2001). In order to gain an insight into the differential behavior of these two proteins, we reinvestigated the response of Bcl-xL to Pc 4-PDT using four other antibodies. For these experiments, cells were exposed to various Pc 4 doses and 200 mJ/cm^2 of red light. At 10 min after irradiation, total cell protein was collected and subjected to Western blot analysis. We first studied the fate of Bcl-xL in several human tumor cell lines using a monoclonal antibody directed against an unknown epitope in human Bcl-xL protein (clone 44) obtained from Transduction Laboratories. As shown in Figure 1a, two proteins, one migrating at 24 kDa and the other at 30 kDa, were detected by this antibody. For MDA-MB468, A431 and MCF-7 cells, the 24-kDa protein band was much darker than the other, whereas for PC3 cells the 30-kDa band was more intense. The positive control (human endothelial cell lysate) provided by the supplier revealed only the 24-kDa protein (data not shown). The results in Figure 1a indicate that there was a marked reduction in the amount of both proteins when MDA-MB468, A431, MCF-7 or PC3 cells were treated with Pc 4-PDT. For comparison, the blots were reprobed with an antibody to Bcl-2. It is clear that both Bcl-2 and Bcl-xL were photodamaged, as defined by loss of the native protein, and the damage was PDT dose dependent.

To determine whether Bcl-2 overexpression can affect Bcl-xL photodamage, human prostate cancer DU145 cells and DU145 cells stably overexpressing FLAG-tagged Bcl-2 (DU145-Bcl-2 cells) were analysed similarly. The results in Figure 1b show that the PDT-induced loss of Bcl-xL, as detected by the clone 44 antibody (Transduction Laboratories), is not affected by Bcl-2 overexpression, suggesting that Bcl-2 and Bcl-xL are not competing for a limited amount of PDT-generated reactive oxygen species, and the ability of Pc 4-PDT to photodamage these molecules is not easily saturated. The cells were also treated with staurosporine (STS), a strong inducer of apoptosis. In all cells tested (MDA-MB468 and DU145 ± Bcl-2), there was no effect of STS on the level of either Bcl-xL or Bcl-2 (Figure 1a and b). Thus, the loss of Bcl-2 and Bcl-xL is a unique response associated with PDT and not a generalized response to the induction of apoptosis.

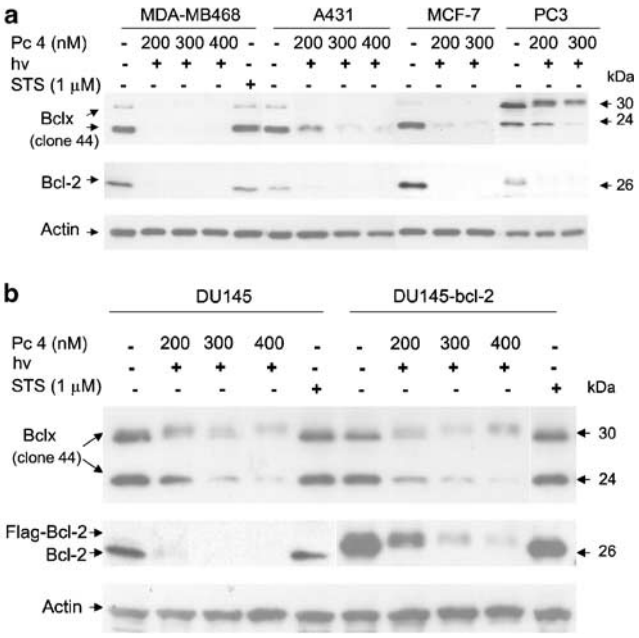

Figure 1 Bcl-xL and Bcl-2 levels in control and PDT- or STS-treated cells. The cells were untreated or treated with various doses of Pc 4 and irradiated with 200 mJ/cm^2 and then collected 10 min after PDT or treated with 1 μM STS for 6 h. The loss of Bcl-xL and Bcl-2 was analysed by Western blot using anti-Bcl-xL antibody from Transduction Laboratories (clone 44). (**a**) Dose–response for Bcl-xL and Bcl-2 loss in PDT-treated MDA-MB468, A431, MCF-7 and PC3 cells. (**b**) Bcl-xL and Bcl-2 loss in PDT-treated DU145 cells and cells overexpressing FLAG-Bcl-2 (DU145-Bcl-2)

Although both Bcl-xL and Bcl-2 could be photodamaged by Pc 4-PDT in all the cell lines studied, some differences in sensitivity were noted. For example, MDA-MB-468, A431 and MCF-7 cells appeared to lose the two proteins at lower doses than was observed in PC3 cells, with DU145 cells displaying intermediate sensitivity. We considered the possibility that these differences could be explained by differential uptake or localization of the photosensitizer in the various cells. We previously showed by confocal microscopy that Pc 4 binds to mitochondria and other organellar membranes in A431 (Lam *et al*., 2001) and DU145 cells (Usuda *et al*., 2003b). Similar results have been found for MCF-7 and PC3 cells (data not shown). The overall uptake of Pc 4 into five of the cell lines was quantified by flow

cytometry, measuring mean channel fluorescence for sufficiently low levels of Pc 4 such that aggregation of the photosensitizer is minimal and fluorescence is linearly related to Pc 4 level. When the mean channel fluorescence values for DU145, PC3 and MDA-MB468 were compared to that of MCF-7 cells, the ratios were 1.01 ± 0.42, 1.05 ± 0.25 and 1.07 ± 0.51, respectively. The ratio for A431/MCF-7 cells was 1.35 ± 0.49 for five measurements, indicating that the 35% increase in mean channel fluorescence for A431 cells was not significant. We conclude that variations in the sensitivity of Bcl-xL and Bcl-2 to PDT-induced photodamage in these cell lines are not due to large differences in uptake or localization of Pc 4 and thus not due to large differences in PDT stress.

We next examined Bcl-xL levels in control and PDT-treated cells using additional antibodies. Figure 2 presents results for Western blot analysis with an affinity-purified polyclonal anti-Bcl-xL antibody obtained from Cell Signaling Technology (CS-2762) raised against a peptide centered at Asp61 (Figure 2a); YS-TB2, a polyclonal antiserum raised against full-length Bcl-xL (Figure 2b); a monoclonal (2H12) antibody directed against an epitope within amino acids 3–14 of human Bcl-xL from PharMingen (Figure 2c), and the polyclonal anti-Bcl-xS/L antibody (sc-634) used in our previous study (Figure 2d). It can be seen from Figure 2a that the epitope recognized by the CS-2762 antibody was lost in response to Pc 4-PDT in all tested cell lines. For A431 and DU145 cells, Bcl-xL loss was dose dependent. In the case of MDA-MB468 cells, dose dependence was less apparent, because the lowest dose produced almost a complete loss of Bcl-xL. For MCF-7 cells, only one PDT dose is shown in this figure. More Bcl-xL loss was observed in PDT-treated MCF-7 cells than in DU145 cells. Thus, the sensitivity of this epitope of Bcl-xL to PDT-induced loss appears to be cell line dependent.

The proteins from control and PDT-treated MDA-MB468, A431, MCF-7 and DU145 cells were also probed with YS-TB2, 2H12 (PharMingen) and sc-634 (Santa Cruz). As shown in Figure 2b, the Bcl-xL protein detected by YS-TB2 was lost upon Pc 4-PDT, with a generally similar response as observed in Figures 1 and 2a. An exception appears to be with A431 cells, in which the protein band recognized by YS-TB2 and CS-2762 was shifted after PDT treatment, indicating a slower migration of the residual recognized proteins after PDT. In contrast, using the 2H12 antibody from PharMingen, strong photodamage to Bcl-xL was observed in all cells tested, but interestingly, all bands derived from PDT-treated cells that reacted with the antibody were shifted to a higher molecular weight (Figure 2c). Finally, the same samples used in Figure 2a were probed with the sc-634 antibody (Figure 2d). Two proteins, one migrating at ~ 33 kDa and the other at ~ 30 kDa, were detected by this antibody. According to the supplier (Santa Cruz), the sc-634 antibody recognizes both Bcl-xL and Bcl-xS, and it was initially assumed that they were found in the slower- and faster-migrating protein bands, respectively. The results in Figure 2d indicate that PDT has no effect

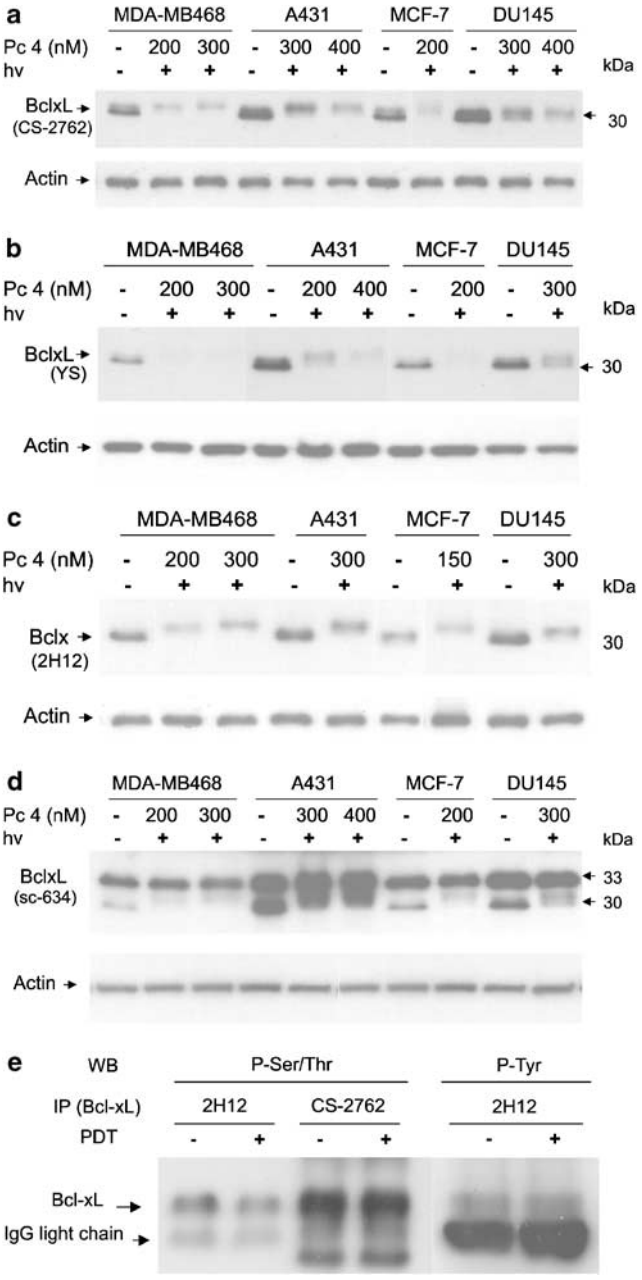

Figure 2 Bcl-xL levels and phosphorylation in control and PDT-treated cells. For Panels a–d, MDA-MB468, A431, MCF-7 and DU145 cells were untreated or treated with the indicated Pc 4 doses and irradiated with 200 mJ/cm^2 of red light. At 10 min after PDT, the cells were collected, and Western blot analysis was performed with the following anti-Bcl-xL antibodies: (**a**) Bcl-xL antibody from Cell Signaling (CS-2762). (**b**) Polyclonal full-length Bcl-xL antibody YS-TB2 (YS). (**c**) Bcl-xL antibody from PharMingen (2H12). (**d**) Bcl-xL antibody from Santa Cruz (sc-634). For Panel e, Bcl-xL from control and Pc 4-PDT-treated MDA-MB468 cells was immunoprecipitated using either the 2H12 or CS2762 antibody, and equal amounts of the recovered Bcl-xL protein were immunoblotted with antibodies to phospho-serine/threonine or phospho-tyrosine

on the more slowly migrating protein in any of the treated cells, a result consistent with our previous report (Xue *et al*., 2001); however, the more rapidly migrating

protein represented by the 30-kDa band was lost after PDT.

In summary, based on the above results, a Bcl-xL protein detected by each of the five antibodies is markedly photodamaged after Pc 4-PDT; one of the antibodies (2H12) detects a residual protein that was modified such that its electrophoretic mobility was markedly reduced. In order to determine whether the reduced mobility resulted from Bcl-xL phosphorylation, lysates from control and PDT-treated MDA-MB468 cells were immunoprecipitated with either the 2H12 or CS2762 antibody, and equal amounts of the recovered Bcl-xL protein were immunoblotted with antibodies to phospho-serine/threonine or phospho-tyrosine. The results shown in Figure 2e reveal that Bcl-xL is phosphorylated in untreated cells; however, PDT did not change the level of phosphorylation. Therefore, the reduced electrophoretic mobility of Bcl-xL from PDT-treated cells may result from as yet unidentified structural changes in the protein. We also sought evidence concerning possible protein degradation as an explanation for the PDT-induced loss of native protein. However, overexposure of the Western blot films revealed no small fragments that were recognized by the antibody, suggesting that degradation of Bcl-xL protein is unlikely to be extensive.

In addition to a 30-kDa protein recognized by each antibody, the sc-634 and clone 44 antibodies detected bands of 33 and 24 kDa, respectively. The band at 33 kDa observed with the sc-634 antibody did not appear to sustain photodamage; however, the 24-kDa band observed with the clone 44 antibody appeared to be as sensitive to PDT-induced photodamage as the ~30 kDa protein.

Our previous studies of the photosensitivity of Bcl-2 mutants revealed that deletion of the TM domain prevented binding of the protein to the mitochondrial, endoplasmic reticulum (ER) and nuclear membranes and prevented the induction of photodamage by PDT. Therefore, one possible explanation of the inability of some Bcl-xL-related proteins to sustain photodamage was localization in the cytoplasm rather than bound to a membrane. To test this concept, the effect of Bcl-xL localization on PDT-induced Bcl-xL loss was examined. For these experiments, we used MCF-7 cells that stably expressed either wild-type Bcl-xL (MCF-7wt-Bcl-xL) or Bcl-xL targeted to the cytoplasm (MCF-7cyto-Bcl-xL). The cells were exposed to PDT, after which the Bcl-xL level was detected on Western blots with the YS-TB2 antibody. The results shown in Figure 3 reveal that overexpressed wild-type Bcl-xL, which localizes to cytoplasmic membranes similar to the endogenous protein, sustained strong photodamage in PDT-treated MCF-7wt-Bcl-xL cells (Figure 3a). In contrast, when the protein is unable to bind to membranes (MCF-7cyto-Bcl-xL cells), its susceptibility to PDT-induced loss was markedly reduced. Since Pc 4 is localized in mitochondria, ER/Golgi and nuclear membranes, it appears that membrane-localized Bcl-xL is a much better target of Pc 4-PDT than is soluble Bcl-xL. The slight sensitivity of cytoplasm-targeted Bcl-xL could be due to a minor amount of membrane localization through protein : protein interactions or leakage of a minor amount of singlet oxygen from the membranes where it is formed into the cytosol.

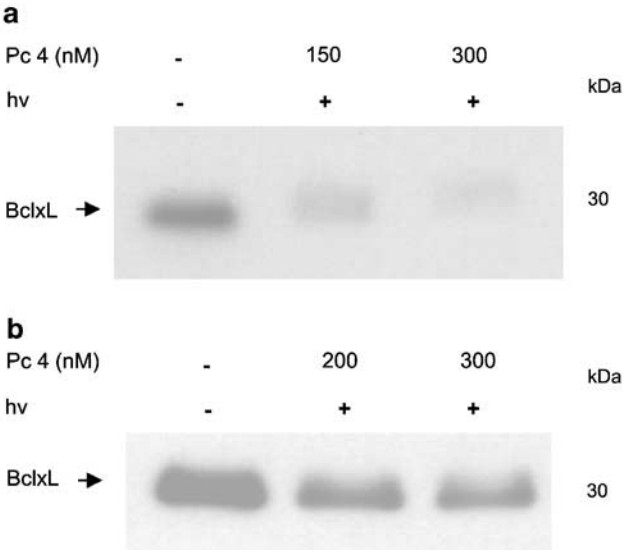

Figure 3 Bcl-xL in control and PDT-treated MCF-7 cells transfected with wild-type Bcl-xL (**a**) and cytoplasm-targeted Bcl-xL (**b**). Cells were loaded with various doses of Pc 4, irradiated with 200 mJ/cm^2 and collected for Western blot analysis. Bcl-xL was detected by YS-TB2 antibody

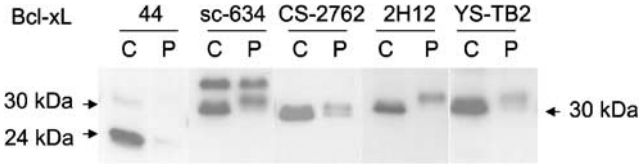

Figure 4 Comparison of the size and level of Bcl-xL detected by five antibodies in control and PDT-treated MCF-7 cells. The cells were untreated or treated with 200 nM Pc 4 and 200 mJ/cm^2 red light. At 10 min after irradiation, cells were collected. Samples from control (C) and PDT-treated (P) cells and protein size markers were loaded onto an SDS–PAGE gel in sets of three lanes. After electrophoresis, the protein was transferred to a PVDF membrane. The membrane was then cut into five pieces, so that each piece contained the same control and treated samples and size markers. Each piece was probed with a different anti-Bcl-xL antibody, as indicated. The results of a single gel representative of three gels are shown

As the number of proteins and the pattern of photodamage detected by Western blotting varied when different antibodies were used, we sought to differentiate between different epitopes recognized on the same protein from different isoforms of Bcl-xL. Accordingly, we compared directly the apparent migration of the major species detected by each of the five antibodies. As shown in Figure 4 and summarized in Table 1, YS-TB2, 2H12 and CS-2762 all detect a single protein band migrating at 30 kDa on SDS–PAGE analysis. In contrast, clone 44 detects proteins of both 24 and 30 kDa, while sc-634 detects proteins of ~33 and 30 kDa. It should also be noted that although all the

Table 1 Properties of Bcl-xL as revealed by five antibodies. See text for details

Source of antibody (clone or catalog number)	*Molecular mass of protein (kDa)*		*Recognizes bacterially expressed Bcl-xL*	*Photodamage in cells*
	Catalog[a]	*Our data*		
Santa Cruz (sc-634)	34	33	Yes	No
		30		Yes
Andrews (YS-TB2)	—	30	Yes	Yes
PharMingen (2H12)	25–29	30	Yes	Yes
Cell Signaling (2762)	30	30	Yes	Yes
Transduction (44)	26	30	Yes	Yes
		24		Yes

[a]Data provided by commercial supplier

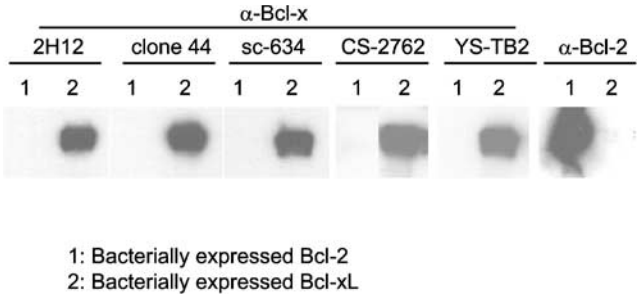

Figure 5 Specificity of Bcl-xL antibodies for bacterially expressed Bcl-xL protein. Bacterially expressed Bcl-2 (lane 1) and Bcl-xL (lane 2) proteins were separated on SDS–PAGE gels, then Western blotted with 2H12 anti-Bcl-xL antibodies from PharMingen, clone 44 from Transduction Laboratories, sc-632 from Santa Cruz, CS-2762 from Cell Signaling, and YS-TB2 (YS), as well as with the anti-Bcl-2 antibody

antibodies recognize proteins migrating at ~30 kDa, they may not all recognize the same protein, because in the case of sc-634, 2H12 and YS-TB2, but not in the case of clone 44 and CS-2762, the electrophoretic migration of the residual protein from PDT-treated cells was slower than observed for the protein from untreated cells. Thus, the data are consistent with the presence in the cells of at least three, and possibly four, different Bcl-xL-related proteins detected by these antibodies.

Although Bcl-xL is most often observed to migrate as though it is larger than Bcl-2, in one case (clone 44, Transduction Laboratories), the 24-kDa protein was only slightly different in size from Bcl-2. Therefore, to ensure that the observation of a protein in this size range was not due to reactivity of the anti-Bcl-xL antibody with Bcl-2, we tested the specificity of the antibodies against bacterially expressed Bcl-2 and Bcl-xL proteins. The results in Figure 5 indicate that all five anti-Bcl-xL antibodies recognize Bcl-xL, but not Bcl-2, whereas the anti-Bcl-2 antibody recognizes only Bcl-2 and not Bcl-xL. The results imply that although the anti-Bcl-xL antibodies detect proteins of different apparent size, all the proteins are Bcl-xL related and may be different variants of the same protein.

Since the antibodies may detect differential exposure of specific epitopes after partial refolding on blots or different isoforms of Bcl-xL, we sought to distinguish them further by estimating the relative levels of the presumed isoforms in a series of human cancer cell lines.

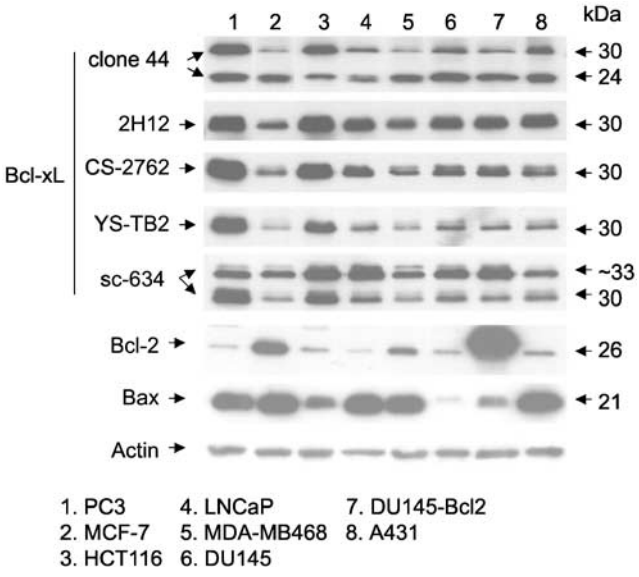

Figure 6 Expression of Bcl-xL, Bcl-2 and Bax in various cell lines. Whole-cell lysates from PC3 (lane 1), MCF-7 (lane 2), HCT116 (lane 3), LNCaP (lane 4), MDA-MB468 (lane 5), DU145 (lane 6), DU145-Bcl-2 (lane 7) and A431 (lane 8) cells were separated on SDS–PAGE gels, transferred to PVDF membranes and probed with Bcl-xL antibodies, as indicated: clone 44 from Transduction Laboratories, 2H12 from PharMingen, CS-2762 from Cell Signaling, YS-TB2 (YS) and sc-632 from Santa Cruz. Western blots were also probed with anti-Bcl-2, anti-Bax and anti-actin (as a loading control)

Equal amounts of protein from whole-cell lysates prepared from eight human cancer cell lines were loaded onto a series of polyacrylamide gels and analysed by Western blot with the five anti-Bcl-xL antibodies. The same samples were also probed with antibodies against Bcl-2, Bax and actin. As shown in the top panel of Figure 6, the clone 44 anti-Bcl-xL antibody (Transduction Laboratories) detected two proteins (30 and 24 kDa) with higher expression of the 24-kDa protein, but little of the 30-kDa protein, in MCF-7, MDA-MB468 and DU145 cells. In contrast, higher expression of the 30-kDa protein was observed in PC3 and HCT116 cells. For A431 cells, the expression of the two proteins differed only slightly. The 2H12 antibody from PharMingen revealed light bands in MCF-7 and MDA-MB468 cells, but there was high expression in the other cell lines. Similar results were found when the blot

was probed with the CS-2762 (Cell Signaling) antibody. YS-TB2 detected Bcl-xL protein in all the cell lines, with the highest expression in PC3 and HCT-116 cells and lowest in MCF-7 and MDA-MB468 cells. Finally, two Bcl-x proteins were detected in all cells with the sc-634 antibody. For the 30-kDa species, the pattern is similar to that detected by YS-TB2.

The level of Bcl-2 and Bax varied across these cell lines as well, but the level of actin was similar for all cell lines, confirming approximately equal loading of protein in each of the lanes. Thus, for each of the antibodies, a unique pattern of expression of Bcl-xL was found, arguing against differential exposure of epitopes on the Western blots, and providing additional evidence for the presence of different isoforms of this protein.

Discussion

The two primary results from this study are that (a) Bcl-xL, like Bcl-2, can be photodamaged by Pc 4-PDT and (b) Bcl-xL exists in at least three isoforms in human cancer cells, each of which can be identified by reaction with a different antibody.

Evidence for multiple Bcl-xL-related proteins in human cancer cells

All the antibodies employed recognize the Bcl-xL protein, but not the Bcl-2 protein expressed in *Escherichia coli*, giving confidence that the mammalian proteins detected by them are all Bcl-xL-related. However, the relationship among the various isoforms is not clear. The migration variations revealed on Western blots (Figures 1, 2, 4 and 6; summarized in Table 1) and the differential expression levels in various human cancer cells (Figure 6) are consistent with a series of related proteins, which could be processing intermediates of a common translation product, may result from splice variants of a common transcription product, or may result from differential post-translational modifications, such as phosphorylation or deamidation or even protein folding. Indeed, several splice variants of Bcl-x have been identified in human or mouse cells in addition to the major protein, Bcl-xL. Bcl-xS is a proapoptotic protein 63 amino acids smaller than Bcl-xL (Boise *et al*., 1993). Bcl-x delta TM is a soluble protein deleted in the TM domain that has been described in mouse cells (Fang *et al*., 1994). Bcl-x gamma is expressed only in T cells upon T-cell receptor and CD28 ligation (Ye *et al*., 2002), and would not be expected in the epithelial-derived cell lines used in the present study. Bcl-x beta, which results from the unspliced mRNA, is expressed in a variety of embryonic and adult mouse tissues (Gonzalez-Garcia *et al*., 1994), but has not yet been described in humans. Which of the above isoforms, if any, is represented by the 24- and 33-kDa proteins we have observed is not known at present. Whatever the explanation for the different forms of Bcl-xL, it is clear that no single antibody is detecting all of them, so that any study of Bcl-xL levels in mammalian cells that relies upon only one of the antibodies may produce incomplete results, depending upon the levels of the various isoforms in the cell lines under study. Furthermore, there is the potential for additional isoforms not recognized by any of the antibodies used in this study.

Two groups have reported considerably higher expression of Bcl-xL in PC3 cells than in LNCaP cells: one (Li *et al*., 2001) used an unspecified antibody from Transduction Laboratories, whereas the other (Liu and Stein, 1997), using an antibody from Santa Cruz, found threefold more Bcl-xL protein in PC3 cells than in LNCaP cells. In our study, the clone 44, YS-TB2, 2H12 and CS-2762 antibodies all detected more Bcl-xL in PC3 cells than in LNCaP cells. However, the extent of the differential expression between these two cell lines was dependent on the antibody. Clone 44 and YS-TB2 antibodies detected $>$threefold more Bcl-xL in PC3 cells than in LNCaP cells, consistent with the previous results (Liu and Stein, 1997; Li *et al*., 2001), but the difference was $\leqslant$twofold when the 2H12 and CS-2762 antibodies were used. On the other hand, the expression of the 30-kDa protein detected by the sc-634 antibody in PC3 cells was higher than in LNCaP cells, whereas higher expression of the $\sim$33 kDa protein was found in LNCaP cells. Together these antibodies recognize epitopes throughout Bcl-xL as they were made from the full-length protein and from peptides at the amino-terminus of and internal to Bcl-xL.

Bcl-xL is photodamaged by Pc 4-PDT

The present study reveals that at least one Bcl-xL protein detected on Western blots by each of the five tested antibodies was photodamaged by Pc 4-PDT in a variety of human cancer cells. Like Bcl-2, the photodamage to Bcl-xL was dose dependent and occurred immediately upon photoirradiation of Pc 4-loaded cells. Pc 4 is found in several intracellular membranes, including mitochondria, ER and the nuclear periphery (Trivedi *et al*., 2000; Lam *et al*., 2001; Usuda *et al*., 2003b). Bcl-2 has been demonstrated to localize to the nuclear envelope, ER and mitochondrial membranes (Hockenbery *et al*., 1990; Krajewski *et al*., 1993). Bcl-xL, a close homolog of Bcl-2, is also found in mitochondria (Gonzalez-Garcia *et al*., 1994) as well as in the cytosol (Hsu and Youle, 1997; Wang *et al*., 2001). Thus, Pc 4 localizes to the same membrane systems housing Bcl-2 and Bcl-xL. Since the primary damaging species generated by PDT, singlet oxygen ($^1O_2$), reacts within $<$100 nm of its site of formation (Moan and Berg, 1991), Pc 4 must reside in the membranes near Bcl-2 and Bcl-xL. Numerous other photosensitizers are known to bind to the same intracellular membrane systems as Pc 4 (Dougherty *et al*., 1998; Oleinick *et al*., 2002), so it is conceivable that some of them will act like Pc 4 in causing photodamage to both Bcl-2 and Bcl-xL. However, the present results are limited to PDT with a single photosensitizer.

Consistent with the immediate photochemical destruction of the native forms of these antiapoptotic

proteins, overexpression of Bcl-2 or Bcl-xL has limited or no ability to protect human cancer cells from apoptosis induced by Pc 4-PDT (Kim *et al*., 1999) (Xue *et al.,* data not shown). However, overexpression of very high levels of greenfluorescent protein-tagged Bcl-2 protected MCF-7 cells from apoptosis and from overall cell death (Usuda *et al*., 2003a).

Although both Bcl-2 and Bcl-xL were photodamaged, as shown in Figure 1, in most cases, Bcl-2 appeared to be more sensitive than Bcl-xL, as revealed by the extent of the loss of these proteins as the PDT dose increased (e.g., Figure 1b). The different response of the two proteins to PDT may reflect their differential localization in the membranes or structural differences between the proteins. Whereas Bcl-2 is found exclusively in membranes of the mitochondria, ER and nucleus, a significant portion of Bcl-xL can be in the cytosol (Wang *et al*., 2001). Bcl-xL targeted to mitochondria was markedly more sensitive to photodamage than was Bcl-xL targeted to the cytosol (Figure 3). Our study of Bcl-2 mutants has revealed that membrane binding is critical to the ability of Pc 4-PDT to photodamage the protein (Usuda *et al*., 2003b). Deletion of much of the N-terminal half of Bcl-2 did not reduce the ability of Bcl-2 to be photodamaged, eliminating the major structural differences between Bcl-2 and Bcl-xL in that region as determinants of photosensitivity. The study of deletion mutants also showed that as long as the protein retained at least a portion of the TM domain and a portion of the region between the BH1 and BH2 domains, the protein would localize to intracellular membranes and be photodamaged by Pc 4-PDT. Thus, it would appear that Bcl-xL that is not membrane bound may not be a target for Pc 4-PDT, and the variable response of the different Bcl-xL isoforms may reflect the ratio of each protein in mitochondria *vs* cytosol of each cell line.

Materials and methods

Cell culture

The human breast cancer MCF-7 cells were transfected with PRcCMV plasmids containing cDNAs encoding Bcl-xL (a kind gift from Gabriel Nunez) or a variant of Bcl-xL lacking the carboxyl-terminal hydrophobic domain. After selection in G418, stable cell lines were assayed for correct localization by cell fractionation and immunofluorescence microscopy. MCF-7 cells, human breast epithelial MDA-MB-468 cells, human colon cancer HCT116 and human prostate LNCaP and PC3 cells were cultured in RPMI-1640 medium containing 10% fetal bovine serum (FBS). Human epidermoid carcinoma A431 cells, human prostate DU145 cells and DU145 cells transfected with FLAG-Bcl-2 cDNA (DU145-Bcl-2) were grown in DMEM containing 10% (A431 cells) or 5% (DU145 cells) FBS. All cultures were maintained in a humidified atmosphere at 37°C with 5% CO_2/95% air. Cultures were in mid-log phase of growth at the time of treatment.

Photodynamic treatment

The phthalocyanine photosensitizer Pc 4, HOSiPcO-$Si(CH_3)_2(CH_2)_3N(CH_3)_2$, was provided by Dr Malcolm E Kenney, Case Western Reserve University Department of Chemistry (Oleinick *et al*., 1993). It was dissolved in dimethyl formamide to 0.5 mM. Cells were loaded with Pc 4 by addition of an aliquot of the stock solution to the culture medium ~18 h before irradiation. The light source was an EFOS LED array (EFOS, Mississauga, ONT, Canada) delivering red light ($\lambda_{max} \approx 675$ nm; band-width at half-maximum = 24 nm; fluence rate at the level of the cell monolayer = 6–7 mW/cm^2). All irradiations were performed at room temperature.

Antibodies

YS-TB2 anti-Bcl-xL antibody was produced by us. Full-length Bcl-xL was expressed in *E. coli* and purified as a fusion protein using the IMPACT system (New England Biolabs). Bcl-xL was released from the fusion protein bound to the chitin column using hydroxylamine as the nucleophile to trigger cleavage. The Bcl-xL protein obtained was greater than 90% pure as judged by visual inspection of Coomassie-stained SDS–PAGE gels. Antisera were generated against full-length protein in specific pathogen-free New Zealand White rabbits obtained from Riemans. Antisera were characterized for specificity using cell lines transfected with the human Bcl-xL gene. The best of the sera obtained, YS-TB2, recognized human Bcl-xL on Western blots and by immunoprecipitation. YS-TB2 serum was used without further purification. It was used at 1 : 10 000 dilution.

Antibodies against Bcl-xL were also obtained from the following four companies: monoclonal (clone 44) anti-Bcl-xL from Transduction Laboratories (Lexington, KY, USA, Cat. No. 610746; used at 1 : 500 dilution), monoclonal (2H12) anti-Bcl-xL from Pharmingen (San Diego, CA, USA, Cat. No. 66461A; used at 1 : 250 dilution), polyclonal IgG fraction of anti-Bcl-xL anti-peptide antiserum (S-18) from Santa Cruz Biotechnology (Santa Cruz, CA, USA, Cat. No. sc-634; used at 1 : 1000 dilution) and polyclonal anti-Bcl-xL affinity purified anti-peptide antiserum from Cell Signaling (Beverly, MA, USA, Cat. No. 2762; used at 1 : 1000 dilution). Anti-Bax antibody (N-20) was obtained from Santa Cruz Biotechnology (Santa Cruz, CA, USA, Cat. No. sc-493; used at 1 : 200 dilution) and anti-actin antibody was obtained from Amersham (Arlington Heights, IL, USA, Cat. No. N350; used at 1 : 1000 dilution). Monoclonal hamster anti-human Bcl-2 antibody (6C8) was obtained from PharMingen (San Diego, CA, USA, Cat. No. 15131A; used at 1 : 1000 dilution). For second antibodies, goat anti-hamster antibody was obtained from Jackson ImmunoResearch Laboratories (West Grove, PA, USA, Code No. 127-035-099). Both anti-mouse and anti-rabbit second antibodies were obtained from Calbiochem (San Diego, CA, USA, Cat. No. 401253 and 401352, respectively).

Western blot analysis

After treatment, cells were lysed and sonicated as described previously (Xue *et al*., 2001). An equal volume of 2 × SDS sample buffer (125 mM Tris, pH 6.8, 4% SDS, 10% mercaptoethanol and 20% glycerol) was added to the whole-cell lysate. Equivalent amounts of protein (10 μg) were loaded onto polyacrylamide gels, subjected to electrophoresis, transferred to a PVDF membrane and incubated with antibodies. The immune complexes were detected by ECL system (Amersham, Arlington Heights, IL, USA).

Immunoprecipitation

Control and PDT-treated cells were lysed in modified RIPA buffer (50 mM Tris, pH 7.4, 1% NP-40, 0.25% sodium deoxycholate, 150 mM NaCl, 1 mM EGTA, 1 mM sodium

orthovanadate, 1 μg/ml each of aprotinin, leupeptin and pepstatin, 1 mM PMSF and 1 mM NaF) at 4°C for 20 min. The lysates were centrifuged and the supernatant was mixed with 15 μl of protein-G agarose (Sigma, St Louis, MO, USA) and Bcl-xL antibodies, then rotated overnight at 4°C. The immune complexes were washed three times with ice-cold RIPA buffer. The bound proteins were eluted from the beads in SDS sample buffer and separated on a 12% SDS–PAGE gel, then transferred and Western blotted, as described above.

Production and purification of recombinant Bcl-2 and Bcl-xL protein

Full-length human Bcl-2 or Bcl-xL cDNA was cloned into the pProex-1 expression vector (Life Technologies, Inc., Grand Island, NY, USA) and expressed as a His6-tagged protein in *E. coli* (The bacteria harboring these expression vectors were generously provided by Dr Clark W Distelhorst, Departments of Medicine and Pharmacology, Case Western Reserve University). The induction of expression with IPTG (isopropyl-β-D-thiogalactopyranoside) and the purification of recombinant proteins on Ni-NTA (nitrilotriacetate)–agarose columns (Qiagen, Inc., Valencia, CA, USA) were performed according to the supplier's protocol.

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