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Simultaneous Evaluation of Viability and Bcl-2 in Small-Cell Lung Cancer

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Abstract

When designing molecular targeted therapeutic strategies against cancer, it is important to correlate protein expression and cell viability. However, such goal can be difficult if performed in separate assays, especially when only a fraction of cells has been efficiently transfected. Therefore, the aim of the present study was to establish a flow cytometry procedure to assess simultaneously Bcl-2 protein level and viability in small-cell lung cancer (SCLC) cells. Viability assessment was performed by staining cells with Annexin V-fluorescein isothiocyanate (FITC) and 7-aminoactinomycin D (7-AAD). Intracellular detection of Bcl-2 was carried out by immunodetection with monoclonal antibodies. Regarding viability determination, the FSC/7-AAD plot identifies the same percentage of viable cells as the FSC/Annexin V-FITC plot, although with greater sensitivity. The procedures involving cells' fixation with 1% paraformaldehyde and permeabilization with digitonin, required for intracellular Bcl-2 immunostaining did not compromise the association of 7-ADD (nor Annexin V-FITC) previously incubated with SCLC cells. It was therefore possible to simultaneously assess cell viability and Bcl-2 protein in SCLC cells. A simple, sensitive, and versatile procedure was established for the first time for the simultaneous evaluation of cell viability and intracellular detection of Bcl-2 in SCLC. © 2008 International Society for Advancement of Cytometry

Key terms

flow cytometry; Bcl-2; 7-AAD; Annexin V; SCLC; viability

ELUCIDATION of the human genome has provided a major impetus in identifying human genes implicated in diseases, namely within cancer, leading to the development of novel classes of pharmaceutical drugs, aiming at achieving higher specificity and potency of action than conventional anticancer agents. Gene silencing agents (such as antisense oligonucleotides, ODN, or siRNA) are one example of a novel class of drugs that have shown efficacy in the selective inhibition of gene expression by inhibiting the translation of the mRNA of the target gene (1–3). When designing a therapeutic anticancer strategy, tumor cell death is the major ultimate goal. In this context, the establishment of correlations between protein expression and viability are important, but can be difficult, namely if performed in separate assays. Parameters like the percentage of cells effectively transfected (4), the occurrence of unspecific effects as a consequence of the transfection procedure, the possibility of distinct differentiation states, or the regulation of protein expression and/or protein function along the cell cycle, can make the cell populations in study extremely heterogeneous. Real time PCR, Western blot, and cytotoxicity assays such as the MTT assay, are often used to evaluate the impact of a gene silencing strategy on the mRNA level, protein expression or cell viability, respectively (5,6). Nevertheless, it is important to point out that the outcome of the mentioned techniques represents the summation of the effects within the entire cell population. A true understanding of the impact of molecular targeted strategies, as gene silencing, against diseases like cancer, demands the establishment of procedures that evaluate protein expression within several subpopulations of a certain tumor type.

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Flow cytometry, whenever applicable, provides important insights on the relation between the levels of protein expression and its function, by granting the possibility to study several cell parameters at the same time, and to study heterogeneous cell populations with respect to each identifiable subpopulation. The technique has been extensively used in the field of hematology, either in the clinical or in the fundamental research contexts. However, when adapting the procedures to different applications, a careful evaluation of their suitability to the cells in study is required. Previous examples have proven this necessity, such as the one involving peripheral blood mononuclear cells (PBMC) from AIDS patients, where methods usually applied to the study of apoptosis in PBMC from healthy donors were discordant among themselves or simply inappropriate (7,8).

The *BCL2* gene is frequently overexpressed in SCLC (9–14), and has been proposed as a proto-oncogene with relevance for the survival of these cells (15). SCLC cell lines are in their majority suspension cells, and are characteristically difficult to transfect (authors' unpublished observations). The study of Bcl-2 silencing approaches in these cells, namely on their viability, would therefore benefit from a simple method like flow cytometry, capable of discriminating the Bcl-2 level at the single cell level and the viability outcome of the effectively transfected cells. Assessment of viability can be performed, for example, with Annexin V or 7-AAD. Double staining with fluorescently labeled Annexin V and propidium iodide (PI) or 7-AAD has been extensively used to detect and quantify apoptosis induction, although it should not be used to characterize the mechanism of cell death because it also stains oncotic cells (16). From previous reports, Annexin V staining is expected to be compatible with cell fixation (8) and cell permeabilization (17); however, there are no reports, to the authors' knowledge, of any procedure of staining cells concomitantly with Annexin V and an antibody against an intracellular protein. The usefulness of 7-AAD alone to stain nonviable cells or to distinguish early apoptotic cells from viable and late apoptotic/necrotic cells, upon analysis in combination with the forward side scatter (FSC) parameter, has been also reported (18). However, concomitant cell staining with 7-AAD for viability assessment and Bcl-2 immunodetection with anti-Bcl-2 antibodies has also never been reported in SCLC.

The main goal of the present study was to establish a flow cytometry procedure to assess simultaneously Bcl-2 protein level and viability in SCLC cells. Moreover, a comparison between different viability markers (Annexin V-FITC *versus* 7-AAD) for SCLC cells was also performed.

Materials and Methods

Materials

All salts and paraformaldehyde (PFA) were from Merck (Darmstadt, Germany). 4-(2-hydroxyethyl)-1-piperazine-ethanesulfonic acid (HEPES), 2-amino-2-(hydroxymethyl)propane-1,3-diol (Tris), sodium azide (N_3Na), actinomycin D (AD), 7-AAD, bovine serum albumin (BSA), RPMI 1640 with L-glutamine and without sodium bicarbonate (RPMI 1640), penicillin–streptomycin solution (10,000 U/ml and 10 mg/ml, respectively) and digitonin were SIGMA® (Sigma-Aldrich Chemie, Steinheim, Germany). Fetal Bovine Serum (FBS) was Gibco® (Invitrogen SA, Barcelona, Spain). The desalted and full phosphorothioate form of G3139 ODN (5′-TCT CCC AGC GTG CGC CAT-3′) and the mismatch control G4126 (5′-TCT CCC AGC ATG TGC CAT-3′) were purchased from Microsynth (Balgach, Switzerland).

Cell Lines

The human variant and classic SCLC cell lines, respectively SW2 and NCI-H69, were kindly provided by Drs. U. Zangemeister-Wittke and R. Stahel (University Hospital of Zurich, Switzerland). The human variant SCLC cell line NCI-H82 SCLC cell line was purchased from the American Type Culture Collection, whereas the K562 human leukemia cell line was purchased from DMSZ GmbH. Cells were cultured in RPMI 1640 supplemented with 10% (v/v) heat-inactivated FBS, 100 U/ml penicillin, 0.1 mg/ml streptomycin, 10 mM HEPES and 0.2% $NaHCO_3$ at 37°C in a humidified atmosphere (90%), containing 5% CO_2. Cells were maintained within their exponential growth phase and periodically tested for mycoplasma contamination with MycoAlert® Mycoplasma Detection kit (Cambrex Bio Science Verviers, Liege, Belgium).

Selection of an Anti-Bcl-2 Monoclonal Antibody for Flow Cytometry

In these experiments, three SCLC cell lines, known to express different levels of Bcl-2 protein, as determined by Western blot, were used: H82 (low), SW2 (intermediate) and H69 (high levels) (19). Cells were first fixed and permeabilized and then submitted to three Bcl-2 immunostaining procedures, aiming at assaying the Bcl-2 expression level by flow cytometry. With two of the immunostaining protocols tested, 10^6 tumor cells in Dulbecco's phosphate buffer saline (PBS) containing 1% BSA and 0.1% NaN_3 were fixed and permeabilized with the Fix and Perm kit (Caltag™, Invitrogen, Barcelona, Spain) according to the manufacturer procedure. Cells were further incubated with 1 μg of fluorescein isothiocyanate (FITC)-labeled mouse anti-human Bcl-2 monoclonal antibody, Bcl-2 mAb-FITC (IgG1, clone 100, Caltag, Invitrogen, Spain), for 10 min. Alternatively, immunostaining was performed by incubation with 1.3 μg of an anti-human Bcl-2 mouse monoclonal antibody (IgG1k, clone 124, Dako, Glostrup, Denmark) for 10 min, followed by incubation with 2 μg of FITC-labeled secondary goat polyclonal anti-mouse antibody (Dako, Glostrup, Denmark) for 20 min (indirect staining). In these protocols, incubations with mAbs were performed at room temperature in the dark. A third procedure tested involved the fixation of 0.15 or 1×10^6 tumor cells in 1% PFA in PBS containing 1% BSA and 0.1% NaN_3 for 15 min at 4°C. After washing with PBS containing 1% BSA and 0.1% NaN_3, cells in 0.2 ml were incubated with 0.5 mg/ml digitonin and 20 μl/10^6 cells of phycoerythrin (PE)-conjugated mouse anti-human Bcl-2 mAb, Bcl-2 mAb-PE (IgG1k, clone

Bcl-2/100, BD Pharmingen™, BD Biosciences, Erembodegem, Belgium) or the same volume of the correspondent PE-conjugated isotype control (iso-PE) (IgG1k, clone MOPC-21, BD Pharmingen, BD Biosciences), for 10 min at 4°C. After all immunostaining procedures, cells were washed once with 1 ml of PBS containing 1% BSA and 0.1% NaN_3, resuspended in PBS, and at least 10,000 events acquired in a flow cytometer (BD FACSCalibur™, BD Biosciences, Erembodegem, Belgium). The CellQuest™ Pro software (version 0.3, BD Biosciences, Erembodegem, Belgium) was used in both acquisition and data analysis. Plotted events were gated in a region in SSC/FSC of predominantly viable cells and then the geometric mean values of the relative fluorescence units (RFU) of the FL1 or FL2 channels were considered, depending on the antibody label.

Treatment of Tumor Cells with Etoposide or Imatinib

SW2 cells were plated at a cell density of 0.5×10^6 cells/ml in culture medium, in a 6-well plate, and treated with 0.8 μM etoposide (solution for IV injection from FARMA-APS kindly donated by the University Hospital of Coimbra) in a final volume of 1 ml, aiming at inducing cell death (including apoptosis) (20). Medium and treatment renewal was performed every 24 h. Untreated cells were equally manipulated but resuspended, when appropriate, in fresh medium. At 72 h after the first treatment, cells were collected and prepared for cytometry analysis as described in the following section. As a control for FITC-conjugated Annexin V solution staining (Annexin V-FITC), K562 cells were included in the study. To guarantee the presence of apoptotic cells, K562 cells under treatment with imatinib (kindly donated by Novartis, Basel, Switzerland) for induction of resistance, collected at a random point after the beginning of imatinib treatments, were used.

Transfection of SW2 SCLC Cells with Oligodeoxynucleotides

Cells were plated in 12-well plates (at 0.25×10^6 cells/well) in 0.4 ml of culture medium composed only by RPMI 1640 without phenol red, and 25 mM HEPES. Oligofectamine™ (Invitrogen, Barcelona, Spain), diluted 5 times in HEPES buffer saline, HBS (25 mM HEPES, 140 mM NaCl, pH 7.4), was added to ODN solutions, prepared in HBS, at a ratio of 15 μl Oligofectamine/nmol ODN, and further incubated for 20 min at room temperature. Complexes (0.1 ml) were incubated with tumor cells at 200 nM of ODN. RPMI 1640 (0.25 ml) supplemented with 30% FCS was added 4 h after the addition of complexes. Culture medium was added every day to maintain cells in exponential growth phase (2-fold dilution), and Bcl-2 and cell viability were evaluated at 72 h.

Concomitant Detection of Viability and Bcl-2 Protein Level by Flow Cytometry

In the present study, the procedure for fixation and permeabilization and further Bcl-2 immunostaining of SCLC cells (already stained with Annexin V-FITC and 7-AAD), was adapted from Gao et al. (17). Tumor cells (0.15×10^6) in 0.1 ml of binding buffer (10 mM HEPES, 140 mM NaCl, 2 mM $CaCl_2$, pH 7.4) were incubated either with 7-AAD solution (20 μg/ml), or with both 7-AAD (at the concentration just mentioned) and 2 μl of Annexin V-FITC (Pharmingen, BD Biosciences, Erembodegem, Belgium), for 15 min at 4°C. Cells were then washed once with binding buffer, and fixed in 1% PFA in binding buffer for 20 min at 4°C. Afterwards, cells were washed with 1 ml of binding buffer containing 1% BSA, 0.1% NaN_3 and 20 μg/ml AD, and incubated for 15 min in 0.2 ml of the previous washing buffer containing 0.5 mg/ml digitonin and 8 μl of the Bcl-2 mAb-PE or of the correspondent control (iso-PE). After washing once, with binding buffer containing BSA and NaN_3, cells were analyzed by flow cytometry. About 40000 events were acquired and Annexin V-FITC, PE-labeled mAbs and 7-AAD were measured in FL1, FL2, and FL3 channels, respectively. The settings and compensations were adjusted for this assay.

Results and Discussion

Characterization of the 7-AAD and Annexin V-FITC Staining of SCLC Cells for Viability Determination

Fluorescently-labeled Annexin V has proven to be a useful tool (usually in combination with propidium iodide or 7-AAD) to distinguish viable from apoptotic and oncotic cells (16,21). The usefulness of 7-AAD alone to stain non-viable cells has also been reported. This marker can only penetrate the plasma membrane when membrane integrity is compromised, as occurs in the later stages of apoptosis or necrosis, binding stoichiometrically to nuclear DNA. 7-AAD analysis performed in combination with the FSC parameter further distinguishes early apoptotic cells from viable and late apoptotic/necrotic cells. With this method, the early apoptotic subpopulation was described as the 7-AAD^{low+}/smaller size population (8).

Etoposide-treated SW2 cells, as well as untreated SW2 cells, and imatinib-treated K562 cells, were stained with 7-AAD and Annexin V-FITC as described in Materials and Methods section. Aiming at identifying, within SW2 SCLC cells, the sub-population of viable cells as well as the subset of cells in early stages of death induction, the combined analysis of 7-AAD/Annexin V-FITC was compared to the combined analysis FSC/7-AAD and FSC/Annexin V-FITC (Fig. 1). Such analysis was performed after gating the events according to their light scatter properties (SSC/FSC) to exclude part of debris (not shown).

Double staining with 7-AAD and Annexin V-FITC is usually analyzed in the 7-AAD/Annexin V-FITC plot by drawing quadrants (Figs. 1A–1C). Three sub-populations could be easily distinguished on the imatinib-treated K562 cells 7-AAD/Annexin V-FITC plot (Fig. 1C), corresponding to three distinct centers of density falling within different quadrants. Late apoptotic/necrotic cells stained more strongly with both 7-AAD and Annexin V-FITC (7-AAD^+/Annexin V-$FITC^+$) falling in the upper-right quadrant, whereas viable cells (7-AAD^-/Annexin V-$FITC^-$) and early apoptotic/oncotic cells (7-AAD^-/Annexin V^+) were located in the lower-left and lower-right quadrants, respectively. In contrast, with the SW2

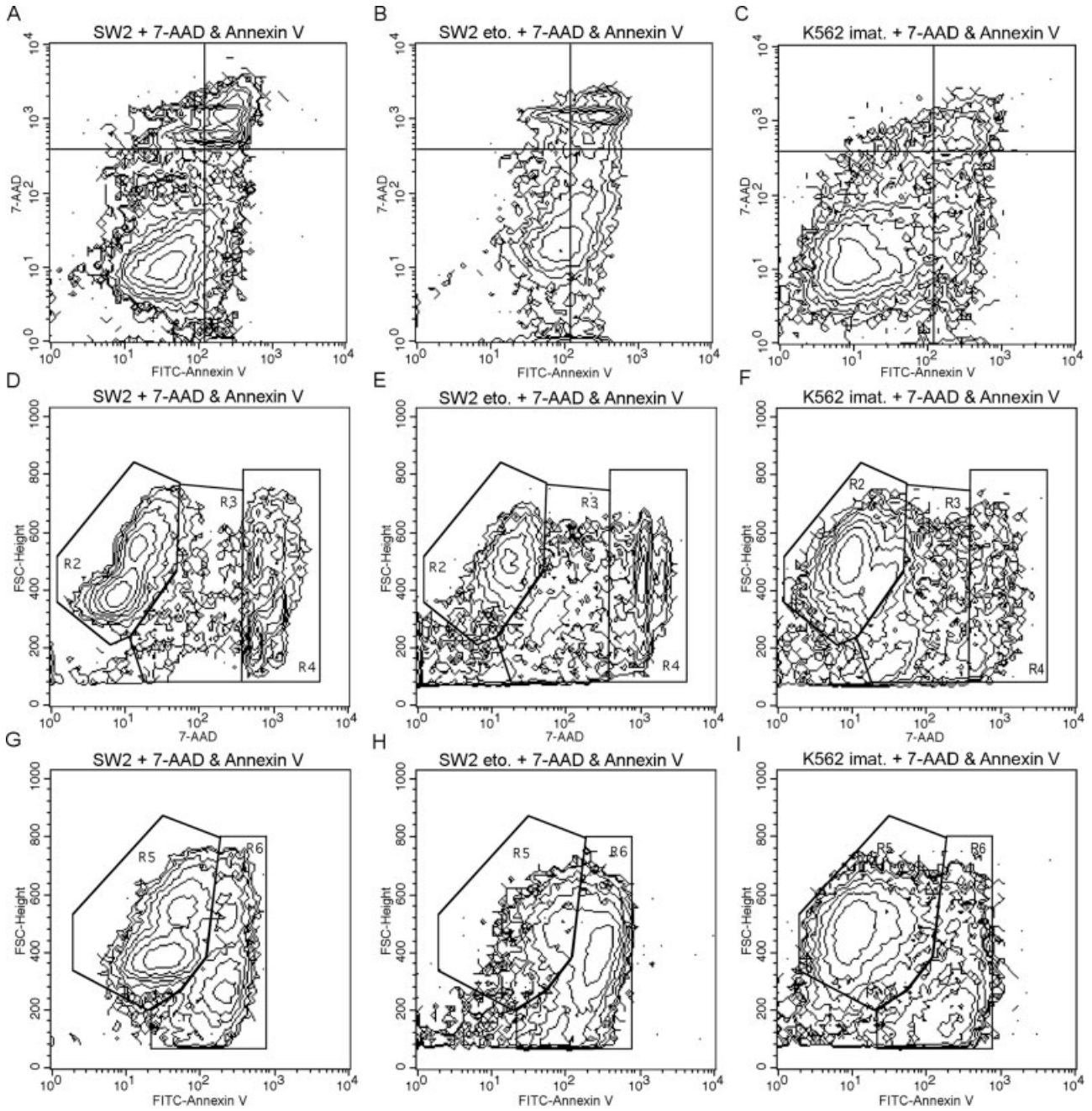

Figure 1. Assessment of viability of SCLC and leukemia cells by multiparametric flow cytometry. Untreated and etoposide-treated SW2 SCLC cells and imatinib-treated K562 leukemia cells were stained with 7-AAD and Annexin V-FITC. Annexin V-FITC and 7-AAD fluorescence were plotted in contour plots in combination with each other (**A—C**) and with the FSC parameter (**D—I**). Geometric regions labeled from R2 to R6 delimit regions containing distinct sub-populations of events, based on the distinct properties of the parameters under analysis.

cells, although late apoptotic/necrotic cells were easily distinguished by staining more strongly with 7-AAD, viable cells and early apoptotic/oncotic cells sub-populations could not be easily delimited from each other (Figs. 1A and 1B). As compared to imatinib-treated K562 cells (Fig. 1C), viable SW2 cells displayed a higher basal intensity of Annexin V-FITC signal. This, together with dispersion in signal from doublets or higher cell number events, makes positive and negative populations for Annexin V-FITC signal partially overlap and therefore difficult to distinguish, introducing uncertainty in the quadrants positioning and subsequent quantification of events. The same Annexin V-FITC and 7-AAD staining patterns were observed with untreated SCLC H82 and H69 cells (not shown), suggesting that the described features are generally applicable to SCLC cell lines, and are not exclusive of the SW2 cell line.

Given the previous reported limitations, the analysis of the 7-AAD fluorescence in combination with FSC was carried out (8). The lines defining the quadrants in Figures 1A–1C, were positioned more accurately after gating FSC/7-AAD and FSC/Annexin-V plots. In the FSC/7-AAD contour plot (Figs. 1D–1F), three regions corresponding to distinct cellular sub-populations can be delimited: 7-AAD$^-$ and normal sized events (R2), 7-AAD^{low+} and normal to small sized events (R3), and 7-AAD$^+$ and normal to small sized events (R4). The events within R2 and R4 correspond to viable cells and late apoptotic/necrotic cells, respectively. The nature of the events within R3 is less clear, although it might correspond to early apoptotic or oncotic cells (8), as well as cell debris. Unstained cells already present events within this region (not shown), indicating that at least a fraction of the events within the R3 region exhibit not true 7-AAD^{low+} signal but rather higher auto-fluorescence. The FSC parameter revealed to be useful in viability discrimination not only when used together with 7-AAD fluorescence but also when conjugated with the Annexin V-FITC signal. Combining the FSC information with the Annexin V-FITC signal (Figs. 1G and 1H), the region of viable cells (R5) is better discriminated from dying or late apoptotic/necrotic cells (R6) than in the 7-AAD/Annexin V-FITC plots (Figs. 1A and 1B).

At this stage, it was therefore important to compare 7-AAD and Annexin V-FITC (in conjunction with the FSC parameter) in terms of the percentage of cells in different subpopulations. The percentage of viable cells determined with 7-AAD (R2) (Figs. 1D–1F) or Annexin V-FITC (R5) (Figs. 1G–1I), was equivalent in all three groups of cells tested (Fig. 2A), indicating that both procedures are suitable to determine quantitatively the viable cell population. In addition, it might be important to assess the cells in early phases of death induction, which, as previously referred for SW2 cells, is not determined with precision through the establishment of quadrants in the 7-AAD/Annexin V-FITC plot (Figs. 1A and 1B). This quantification can be carried out by subtracting the number of events within R4 region on the FSC/7-AAD plot (corresponding to late apoptotic/necrotic cells) to the number of events within the R6 region on the FSC/Annexin V-FITC plot (corresponding to early and late apoptotic/necrotic cells), Figure 1. Alternatively, determination can be based on the R3 region in the FSC/7-AAD plot (Fig. 1). The percentage of events determined by each procedure revealed to be similar (Fig. 2B).

Despite the previous finding, the methods do not discriminate exactly overlapping populations of viable cells and cells in a phase of death induction. This was illustrated for etoposide-treated SW2 cells in Figures 2C and 2D, and was quantified for all three conditions (Figs. 2E and 2F). In an FSC/Annexin V-FITC plot of etoposide-treated cells gated in R2, the percentage of total events within R6 (10%) represents either the false positive non-viable cells found by Annexin V-FITC or the false negative non-viable cells determined in the FSC/7-AAD analysis (Fig. 2C). This type of discrepancy was negligible (2%) in untreated-SW2 and K562 cells (Fig. 2E). Furthermore, in an FSC/Annexin V-FITC plot gated in R3, the percentage of cells within R5 represents the false negative non-viable events found by Annexin V-FITC or the false positive events determined in the FSC/7-AAD analysis (Fig. 2D). This discrepancy was minimal (1–2%) in untreated and etoposide-treated SW2 cells and in the order of 6% in K562 leukemia cells (Fig. 2F). The use of complementary methods like evaluation of caspase activation and/or the terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling (TUNEL) assay are, therefore, required to warrant a clear definition of apoptosis induction, if this is a concern. Nevertheless, the percentages determined show that the putative false positives or false negatives have little impact in overall viability determination.

As the final goal is to analyze Bcl-2 levels in correlation with viability, the compatibility of both Annexin V-FITC and 7-AAD with a PE-labeled antibody is, as well, of interest for the discussion of viability analysis methods, and will be addressed in the last section of Results and Discussion.

Selection of an Anti-Human Bcl-2 Monoclonal Antibody to Evaluate Bcl-2 Levels and Heterogeneity of Protein Expression in SCLC Cells

To establish a sensitive procedure to detect Bcl-2 in SCLC, with good discrimination between different Bcl-2 levels, aiming at providing a correlation between fluorescence signal and Bcl-2 levels, three different procedures were evaluated. H82, SW2, and H69 SCLC cell lines, expressing low, intermediate and high Bcl-2 levels, respectively, were used (19). The Bcl-2 mAb-FITC did not discriminate between different Bcl-2 levels, namely within the range of intermediate and high levels of Bcl-2 (Fig. 3). In contrast, the indirect staining procedure (unlabeled anti-Bcl-2 plus FITC-labeled secondary Ab) and the direct staining with Bcl-2 mAb-PE provided a good discrimination (positive correlation between fluorescent signal and Bcl-2 levels). The correspondent unspecific iso-PE signal was very low and equivalent for the three cell lines, demonstrating the specificity of the signal (Fig. 3). A careful antibody selection is crucial for a correct evaluation of any gene silencing strategy involving the downregulation of Bcl-2 in SCLC and likely in any type of tumor. For example, the downregulation of Bcl-2 from a high level to an intermediate level of expression (Fig. 3) would not be detected with the Bcl-2 mAb-FITC, leading to misinterpretation of results. The simplicity, sensitivity and specificity of the use of the Bcl-2 mAb-PE led us to select this mAb for further studies.

The evaluation of the Bcl-2 levels by flow cytometry evaluates not only the mean protein levels, as shown in Figure 3 and as provided by other quantification methods like Western blot and enzyme-linked immunosorbent assays, but, in addition, it also gives information about the heterogeneity of the protein expression. It was possible to identify in Bcl-2-low expressing H82 cells a small (around 2%), but reproducible, percentage of viable cells with high Bcl-2 expression. The staining with the isotype control mAb does not detect any events within this region proving its specificity (data not shown). This is the type of information that would not be detected by Western blot, emphasizing the advantage of flow cytometry in detecting intracellular proteins in cases of heterogeneous protein expression, as a result, for example, of a incomplete cell transfection with a gene silencing molecule (4).

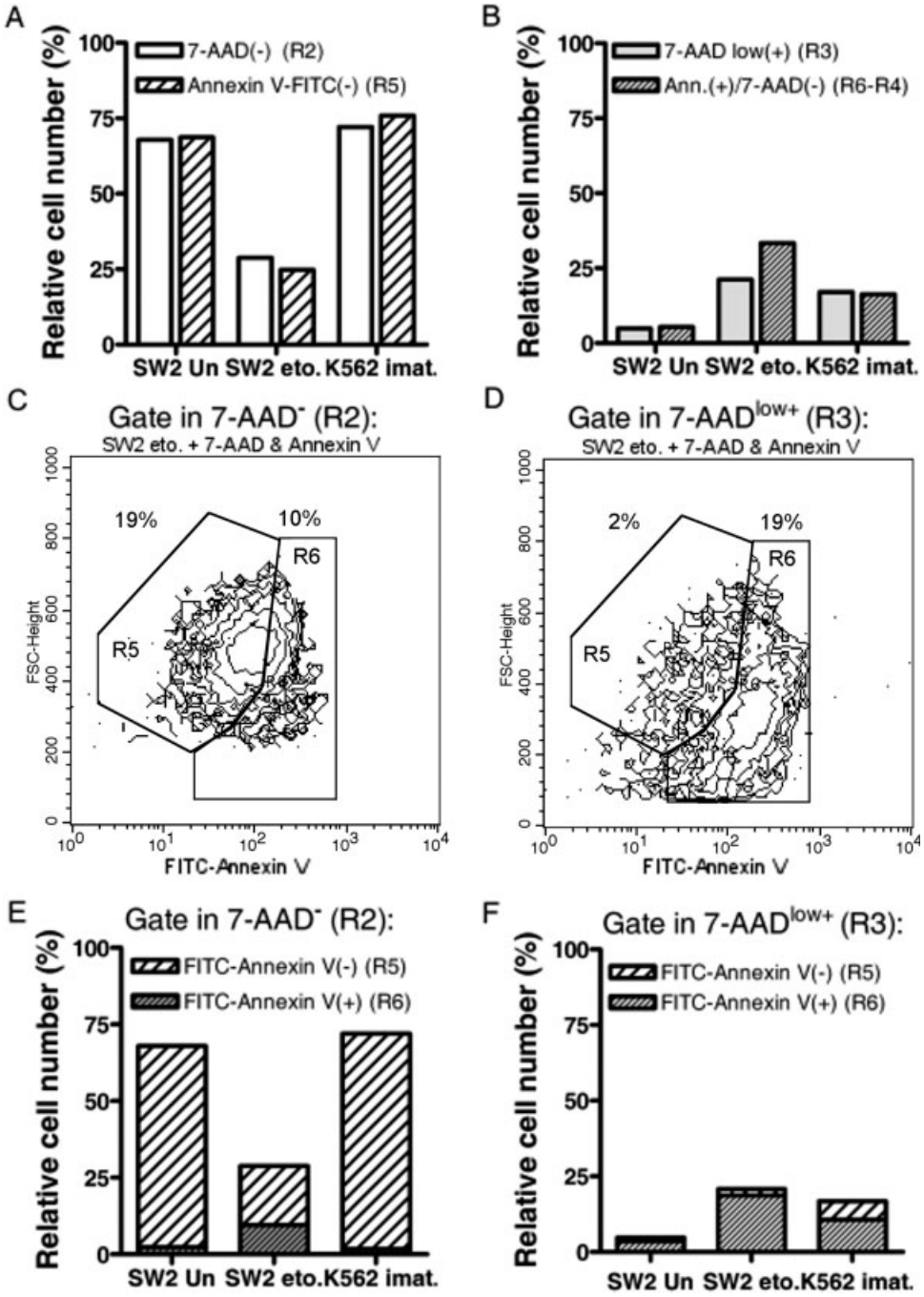

Figure 2. Comparison of Annexin V-FITC and 7-AAD methods analyzed each one in combination with the FSC parameter. In all three groups of cells studied, the percentage of viability (**A**) and the percentage of events in early phases of death induction (**B**) were quantified. Events were also displayed in FSC/Annexin V-FITC contour plots gated in the R2 or R3 regions of FSC/7-AAD plots, as exemplified for etoposide-treated SW2 cells (**C** and **D**, respectively). The percentage of total events gated in R2 or R3 falling into each of the gated regions of the Annexin V-FITC plot is presented for all three studied conditions (**E** and **F**). This percentage of events corresponds to the fraction of false positives or false negatives with each stain.

Concomitant Measurement of Cell Viability and Bcl-2 Protein Level by Flow Cytometry

The simultaneous evaluation of cell viability and Bcl-2 protein level requires the establishment of procedures that guarantee an accurate correlation. Bcl-2 being an intracellular protein, its immunodetection requires cell fixation and permeabilization. The selection among the several available methods to fix and permeabilize cells should consider their impact on cell viability assessment. In the present study, cells fixed with 1% PFA and permeabilized with digitonin had the same Annexin V-FITC and 7-AAD staining patterns and FCS distribution than cells acquired in flow cytometry immediately after

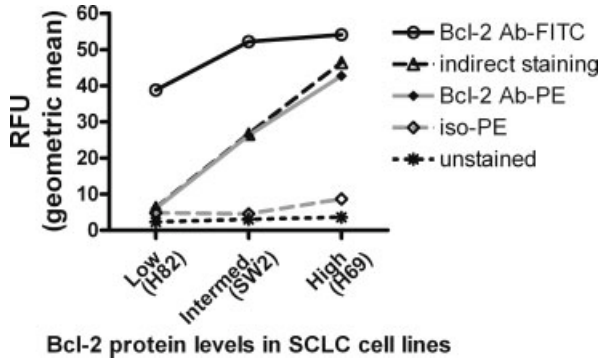

Figure 3. Correlation between fluorescence signal and Bcl-2 protein of levels in SCLC cell lines. Several SCLC cell lines expressing different levels of Bcl-2 protein were submitted to three immunostaining procedures, corresponding to the incubation with different monoclonal antibodies. The immunodetection comparison among the mAb tested was based on the geometric mean values of fluorescence.

staining (not shown). Therefore, the selected fixation and permeabilization procedures revealed to be compatible with the selected markers of viability, without compromising the morphology of the cells, namely in respect to size.

The results presented in Figure 4, A and B, confirm the applicability of the method here presented, aiming at the simultaneous assessment of SCLC viability and Bcl-2 protein. Untreated SW2 cells were stained with 7-AAD and Bcl-2 mAb-PE, and viability regions (R2, R3, and R4) selected as previously described. The treatment with the sequence-specific G3139 ODN induces a reduction of the mean Bcl-2 signal in both viable and dead cells sub-populations (30 and 41 RFU, respectively, Fig. 4A) as compared with the treatment with G4126 ODN (49 and 65 RFU, respectively, Fig. 4B). The percentage of viable and dead cells was not significantly different among the tested treatments. Moreover, it was possible to observe that Bcl-2 silencing was heterogeneous, as evidenced by the broader Bcl-2 signal frequency histograms of the G3139 ODN-treated SW2 (viable) cells (Fig. 4C, thick black line) as compared with the histogram of untreated cells (Fig. 4C, dark gray histogram). In addition, the histogram subtraction of the untreated SW2 (viable) cells to the G3139-treated SW2 (viable) cells turned possible the determination of the percentage of Bcl-2-silenced cells as well as their mean Bcl-2 signal (Fig. 4C, light gray histogram).

To our knowledge, the present results demonstrate for the first time that both 7-AAD and Annexin V-FITC stains are compatible with Bcl-2 intracellular protein immunostaining using a PE-labeled antibody in SCLC cells. Moreover, when analyzed in combination with the FSC parameter, this approach provides a helpful and convenient method for the simultaneous analysis of Bcl-2 level and cell viability in SCLC cells. Surprisingly, results demonstrated that Annexin V-FITC staining is less sensitive than 7-AAD alone in SCLC cells, as well as than Annexin V-FITC staining in other cell lines (like leukemia cells). It is therefore clear that 7-AAD is the best choice to determine viability in SCLC cells. Furthermore, the FSC/7-AAD method will allow the simultaneous study of

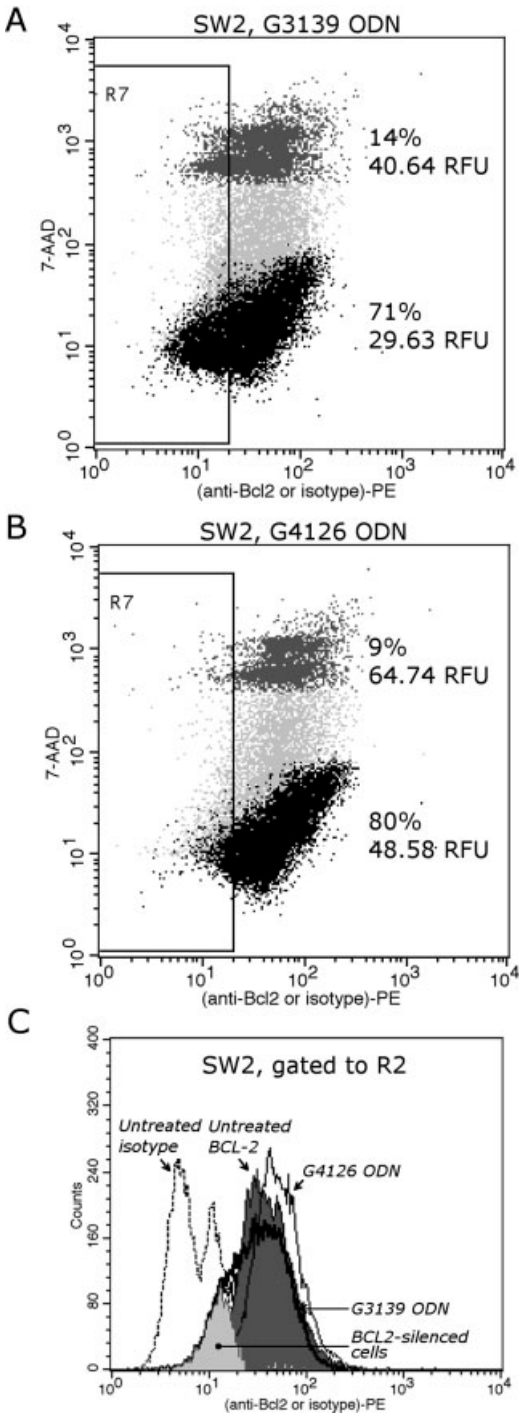

Figure 4. Simultaneous evaluation of viability and Bcl-2 protein in SW2 SCLC cells. Regions were created as in Figure 1D and then the events were plotted in 7-AAD/Bcl-2-PE dot plots representative of cells treated with G3139 ODN (**A**) or G4126 ODN (**B**). Events corresponding to R2, R3, or R4 regions are in black, light gray, or in dark gray, respectively. Next to R2 or R4 regions, it is indicated the percentage of gated events and the geometric mean of Bcl-2 signal intensity (RFU) in the correspondent region. The correspondent overlayed frequency histograms of the viable populations of different treatment conditions are presented.

other parameters, besides viability and Bcl-2 expression, even in the simplest three-color flow cytometer. We have recently observed the usefulness of this procedure to the evaluation of Bcl-2 silencing in breast cancer cell lines.

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