Lung Cancer Cell Line Sensitivity to Zoledronic Acid Is BAX-dependent

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Abstract. *Background: Zoledronate (Zol), an anti-osteoclastic and anticancer drug, is used to control bone metastasis in several cancer types, including non-small cell lung cancer (NSCLC). However, the mechanisms behind Zol resistance in NSCLC are unclear. Materials and Methods: Zol-resistant cell lines were developed by repeated treatment of A549 and H1650 NSCLC cell lines with Zol. We measured cell proliferation and apoptosis following Zol treatment and also examined the BCL2 superfamily expression. RNAi was used to confirm the role of key molecules in development of resistance. Results: Repeated Zol treatment engendered resistance, in which apoptosis induction was attenuated. From the BCL2 superfamily, BAX was commonly down-regulated in resistant cells, and silencing of BAX in parental cell lines also induced drug resistance. Conclusion: Repeated treatment of NSCLC cell lines with Zol leads to drug resistance, which is in part due to BAX down-regulation.*

Bisphosphonates (BPs) are a first-line treatment option for malignancy-induced hypercalcaemia and for bone pain caused by skeletal tumour-induced osteolysis. Zoledronate (Zol), a new generation nitrogen-containing BP, is one of the most potent inhibitors of osteolysis. It induces apoptosis in several cancer types, including myeloma, breast cancer, prostate cancer, lung cancer, and sarcomas (1, 2). Moreover, Zol-dependent expansion of gamma-delta T-cells, which perform major histocompatibility complex-unrestricted lytic activity, enhances the cytotoxic anti-tumour response (3). This has made Zol a rational treatment option for bone metastasis associated with non-small cell lung carcinomas (NSCLCs), a tumour type that frequently presents with metastasis (4, 5).

Prolonged administration of several anti-tumour agents can lead to drug resistance. This generally occurs when a tumour cell sub-population is selected due to inherent resistance. This resistance may be due to, for example, overexpression of anti-apoptotic molecules or down-regulation of pro-apoptotic proteins. Prolonged usage of anticancer compounds, including Zol, can also engender cross-resistance to a broad range of drugs that converge on a certain cellular signalling pathway (6-8). Although several groups have investigated on the mechanisms underlying the development of resistance, there have been no reports focusing on NSCLC. In the present study, we showed that drug resistance develops in an *in vitro* model following repeated Zol treatment for NSCLC. The anticancer effect of Zol is suggested to occur *via* induction of apoptosis (9-11). Therefore, we hypothesized that Zol resistance may be due to changes in the expression of the B-cell lymphoma (BCL)-2 superfamily, as these proteins are critical for apoptosis regulation.

Materials and Methods

Cell culture. The human lung cancer cell lines A549 (CCL-185; EGFR, wild-type) and H1650 (CRL-5883; EGFR, DelE746-A750) were obtained from the American Type Culture Collection (Manassas, VA, USA) and cultured at 37˚C in a humidified atmosphere containing 5% CO_2 in RPMI-1640 complete medium supplemented with foetal calf serum (10%).

Drug resistance development. Zol was kindly provided as the hydrated disodium salt (MW 401.6) by Novartis International AG (Basel, Switzerland). The Zol-resistant cell lines A549R and H1650R were derived from the parent cell lines by repeated exposure of the parent cells to sub-lethal concentrations of Zol (30-80 μM) for 96 to 120 h, followed by incubation in complete medium lacking the drug for a further 10 days.

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Cell proliferation assay. Cell proliferation was monitored as described in a previous study under some modifications (6). The cells were seeded into 96-well plates at a density of 3,000 cells/well. After 24-h incubation, the cells were treated with medium containing the indicated reagents. After 96-h incubation, the viable cells were detected using a spectrophotometric CellTiter 96 aqueous cell proliferation assay (Promega, Madison, WI).

Annexin V/PI flow cytometry analysis. Apoptotic rates were determined by flow cytometry analysis using an Annexin V-FITC Apoptosis Kit (Becton Dickinson, San Jose, CA). Cells were grown to be semi-confluent overnight in flasks and then treated with different Zol concentrations for 30 h. Staining was performed according to the manufacturer's instructions, and flow cytometry was conducted on a FACScan flow cytometer (Becton Dickinson, San Jose, CA).

Apoptosis detection by ELISA. The cells were seeded into 96-well plates at a density of 3,000 cells/well and incubated for 24 h. Then medium containing the indicated reagents was added to the wells. After 48-h incubation, apoptosis was detected using the ApoStrand™ ELISA Apoptosis Detection Kit (EnZolife International. Inc., Plymouth Meeting, PA).

Immunoblot analysis. Cell lysates (10 μg of protein/sample) were resolved using 5% sodium dodecyl sulphate-polyacrylamide gel electrophoresis and transferred to a polyvinylidene difluoride membrane. Immunoblotting was performed using affinity antibodies against BCL2 (sc-7382, Santa Cruz Biotechnology, Santa Cruz, CA, USA), Bcl-XL (sc-8392, Santa Cruz Biotechnology), MCL1 (#4572, Cell Signaling Technology, Danvers, MA, USA), Bad (#9239, Cell Signaling Technology), BAX (#5023, Cell Signaling Technology), Bak (#3814, Cell Signaling Technology), BIM (#2933, Cell Signaling Technology), BID (#2002, Cell Signaling Technology), and β-actin (M177-3, Medical and Biological Laboratories, Woburn, MA). The blots were incubated with horseradish peroxidase (HRP)-conjugated immunoglobulin G and HRP-linked secondary antibody (Cell Signaling Technology).

RNA interference. RNA interference (RNAi) of *BAX* expression was induced using the Signal Silence *BAX* siRNA kit (Cell Signaling Technology). Lipofectamine2000 (Invitrogen, Carlsbad, CA) was used as the transfection agent.

Results

Repeated treatment with sub-lethal concentrations of Zol induces resistance in NSCLC cell lines. To investigate drug resistance in A549 and H1650 cells, variant cell lines, A549R and H1650R cells, were derived by exposing the parental cell lines to increasing Zol concentrations for several months. A sub-population of cells insensitive to Zol at concentrations that were lethal in the parental cell line lines was obtained. Zol responses of parental and resistant cells after cytotoxicity assays are shown in Figure 1A and B. The IC_{50} values were 28 and 270 μM for A549 and A549R and 18 and 108 μM for H1650 and H1650R, respectively. Morphological comparison of the two cell lines on the basis of microscopic examinations showed no evident differences between the two cell populations (Figures 1C-F). Furthermore, growth curve analysis showed no marked differences in the cell line growth profiles (Figure 1G and H).

Zol-induced apoptosis is reduced in the resistant cell lines. Since Zol-induced growth inhibition of cancer cells can be mediated by apoptosis (6, 12), we examined apoptosis induction in each of the paired cell lines. A significant attenuation of apoptosis was obtained in both cytometric analysis (Figure 2A-D) and the ELISA apoptosis detection system (Figure 2E and F).

Expression of BCL2 superfamily members. The above findings suggest that resistance of the derived cells was, at least partly, mediated by inhibition of apoptosis induction. The BCL2 superfamily is supposedly implicated in the regulation of Zol-induced apoptosis (13). To investigate this, we analyzed the expression of a panel of BCL2 proteins, including BCL2, Bcl-XL, MCL1, Bad, BAX, Bak, BIM, and BID (Figure 3A). BAX down-regulation was observed in the resistant cell lines, while there were no consistent changes for the other BCL2 proteins.

RNAi-mediated knockdown confirms the role of BAX in development of drug resistance. The above results suggest that resistance was, at least partly, due to reduced BAX expression. To test this hypothesis, we performed RNAi-mediated knockdown of BAX in parental cell lines that had not been exposed to Zol (Figure 3B and C). Strikingly, this conferred resistance to Zol concentrations up to 100 μM (Figure 3E and F). This observation was also supported by flow cytometric (Figure 4A-D) and ELISA (Figure 4E and F) analyses of apoptosis in control *versus* RNAi-*BAX* cells. Taken together, these data strongly implicate *BAX* as a key player in the response of NSCLC cell lines to Zol.

Discussion

BCL2 superfamily proteins share homology in the four common BCL2 homology domains (BH1-4). The presence of all BH4 domains distinguishes the anti-apoptotic proteins (BCL2, BCL-XL, and MCL1) from pro-apoptotic members such as BAX, which lack the BH4 domain. Under physiological conditions, BAX is localized in the cytosol, but upon apoptotic signalling, it translocates to the mitochondria. BAX oligomerization on the mitochondrial outer membrane with other pro-apoptotic members such as truncated BID leads to pore formation and cytochrome *c* release (14). In many types of cancer, overexpression of anti-apoptotic proteins such as BCL2 or down-regulation of pro-apoptotic proteins such as BAX leads to an increase in the BCL2/BAX ratio, thereby blocking apoptosis (7, 15). Consistent with this, we found that BAX down-regulation was a common feature in Zol-resistant cell lines.

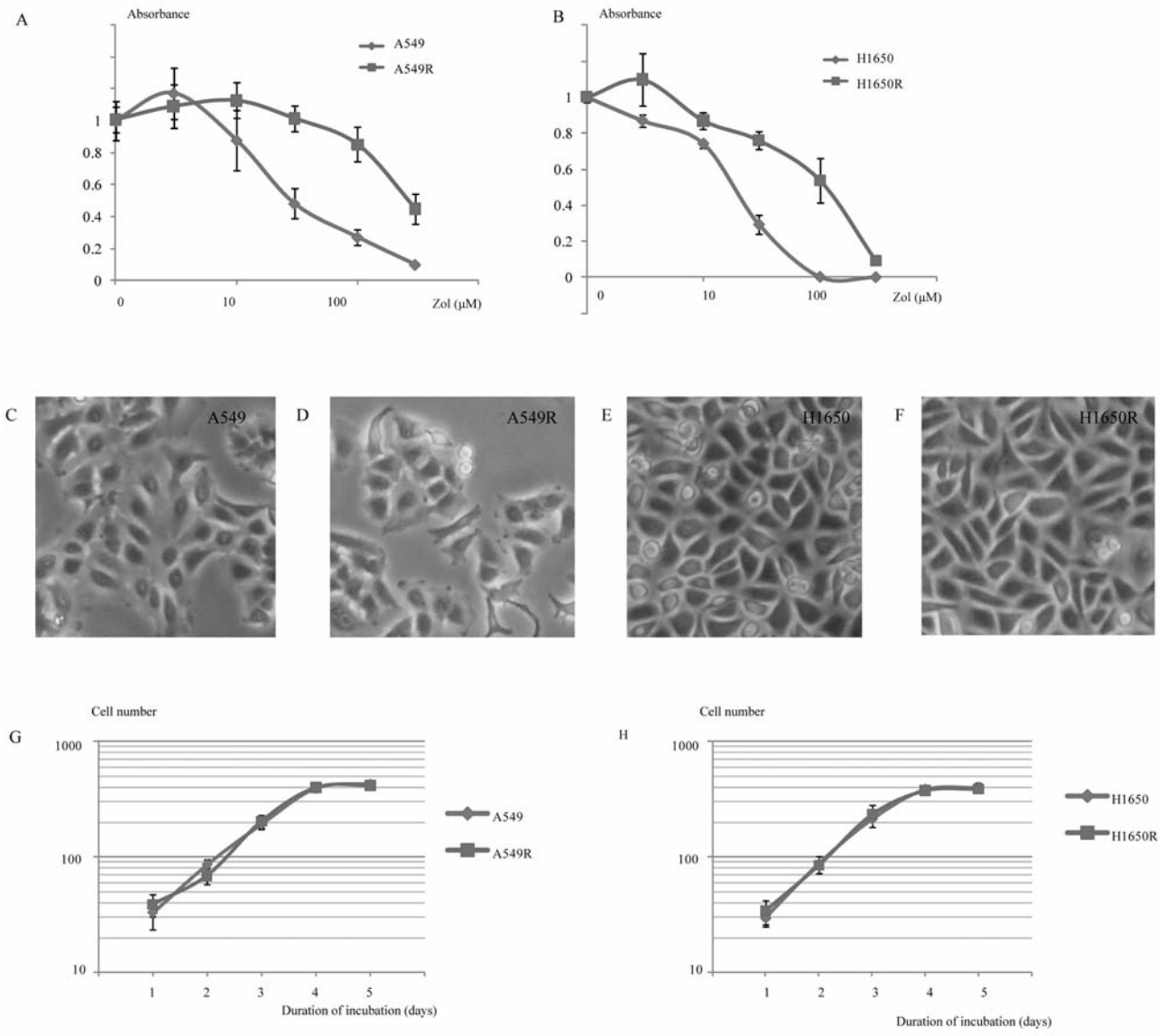

Figure 1. *Development of resistance to Zoledronate (Zol). Both parent and resistance cells were exposed to the indicated concentration of Zol for 96 h, and viable cells were quantified (A, A549; B, H1650). C-F, Morphological features of the parental (C, A549; E, H1650) and resistant (D, A549R; F, H1650R) cells (original magnification ×100). Growth curve of the parental and resistant cell lines (G, A549 and A549R; H, H1650 and H1650R). Cells (1×10^4) were seeded into 6-well plates, and cell proliferation was evaluated at 1-5 days after culturing by cell counting using a light microscope.*

In clinical studies, tumors with reduced *BAX* expression are often associated with a poor prognosis (16-18). Sun *et al*. (19) reported a wide range of *BAX* mRNA and protein expression levels in NSCLC samples. Interestingly, in the present study, a significant down-regulation of *BAX* expression compared to normal tissues, together with significant correlations between *BAX* expression and clinicopathological variables, such as the degree of differentiation and pathological stage, were confirmed. Thus, repeated and prolonged exposure to Zol could be expected to engender for selection of cells with greater drug tolerance. This may include an out-growth of tumour cells that express reduced BAX levels.

Various molecular mechanisms explaining the onset of drug resistance following Zol treatment have been suggested. For example, we showed that Hsp27 up-regulation is a contributing factor in Zol resistance in osteosarcoma cell lines, evolving in a promising novel target in cancer therapy (6). Furthermore, changes in cell-cycle regulation (8), expression of farnesyl diphosphate synthase (main molecular

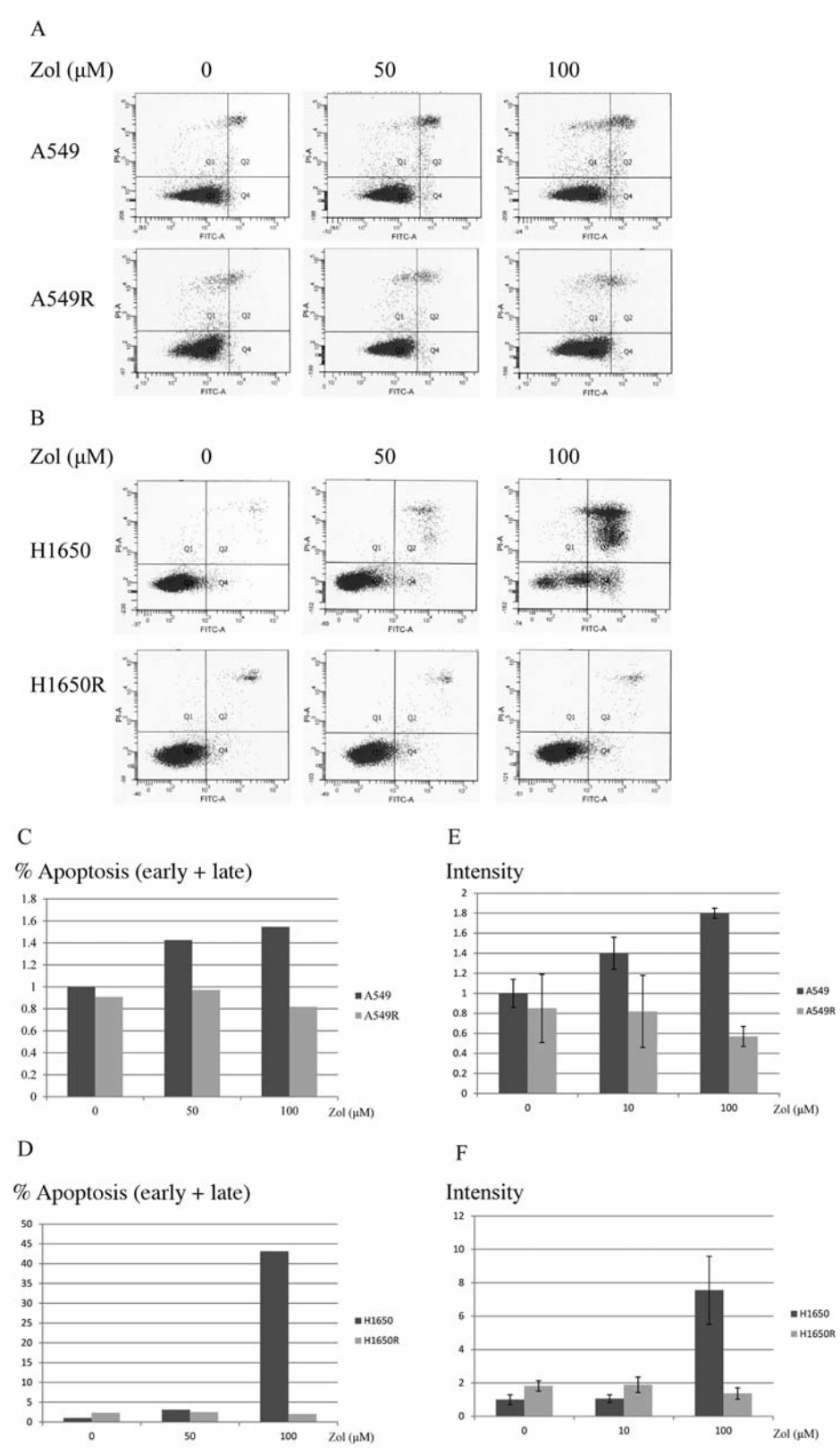

Figure 2. *Detection of apoptosis induced by the indicated concentrations of Zoledronate (Zol) in the parental and resistant cell lines by flow cytometry (A-D) and ELISA (E and F). A significant down-regulation of apoptosis induction, following Zol treatment, is observed in the resistant cell lines.*

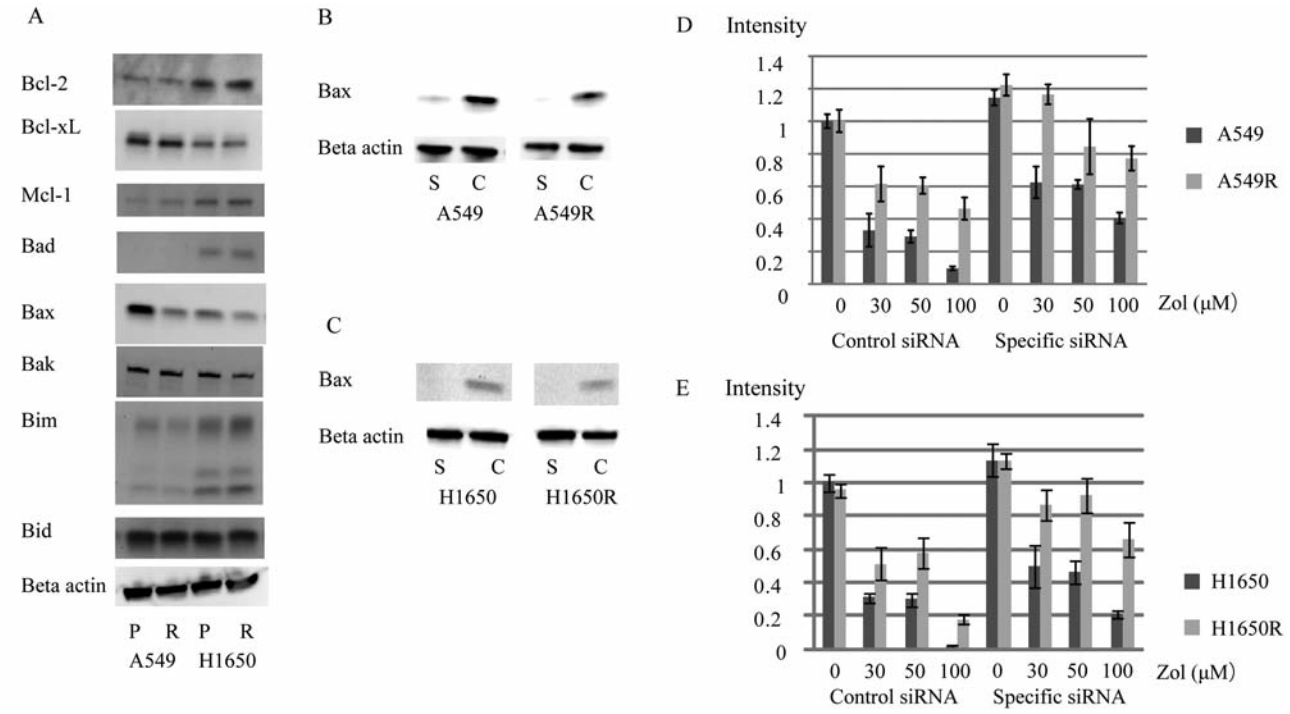

Figure 3. *Expression of a panel of BCL2 superfamily members. (A) BAX expression was significantly down-regulated both in A549R and H1650R. P: Parental cell line; R: resistant cell line. Confirmation of BAX-specific RNAi treatment on the expression of BAX protein in the cell lines (B, A549 and A549R; C, H1650 and H1650R). S; specific RNAi, C; control RNAi. BAX down-regulation in parental cells induced resistance to Zol, which attenuated the growth-inhibitory effects of the drug (D, A549; E, H1650).*

target of nitrogen-containing BPs) (8), elevated the expression of cell surface proteins such as breast cancer resistance protein and lung resistance protein (7), BCL2 overexpression and BAX down-regulation (7) are all reported to contribute to drug resistance development. Although we clearly found a critical role for BAX down-regulation in Zol resistance in our study, we underline that this may be context-dependent. For example, Biagosch *et al*. (20) reported that although doxorubicin resistance is associated with down-regulation of *BAX* mRNA expression, the phenotype cannot be re-produced solely by RNAi-mediated *BAX* knockdown. This underlines the fact that drug resistance development is multi-factorial.

Considering the genetic and molecular heterogeneity of cancer cells, multi-modal mechanisms that underpin drug resistance are to be expected. For example, the presence of cancer stem cells (a specific population of cancer cells that possess the capacity of self-renewal and differentiation) is responsible for resistance to treatments in many haematological malignancies. Specific properties of cancer stem cells include increased tumorigenic capacity, up-regulation of 'stemness' markers such as CD44, CD133, Oct 3/4, Nanog, c-myc and Sox2, and increased drug resistance (21). Although the relationship between cancer stem cells and resistance to Zol is not clear, we are currently pursuing experiments designed to evaluate this possibility.

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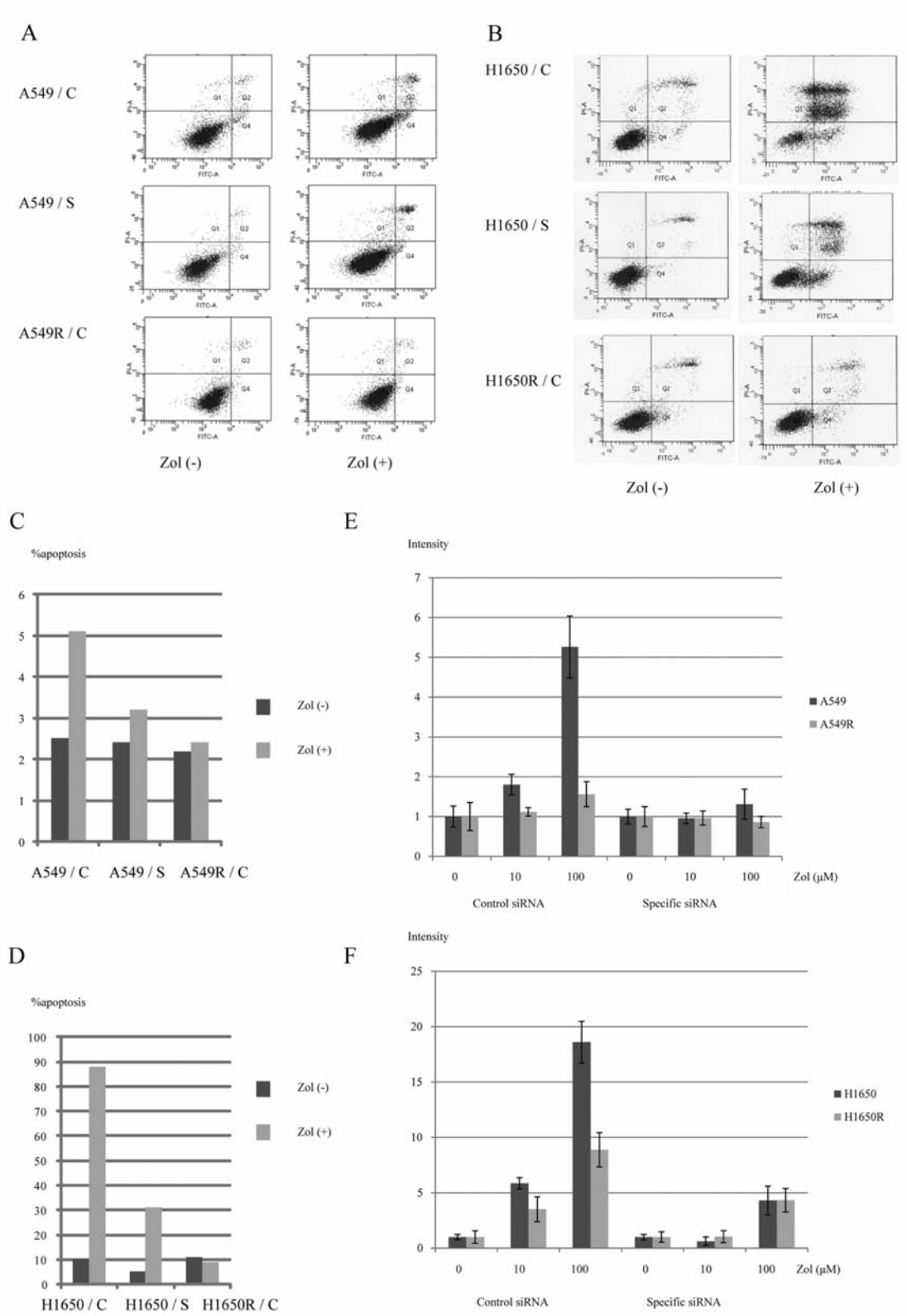

Figure 4. *Confirmation of the effect of BAX down-regulation on the response of parental cell lines to Zol. Flow cytometry analysis for detection of apoptosis (A, A549; B, H1650). S: Specific RNAi, C: control RNAi. C (A549) and D (H1650) depict the percentages of cells undergoing apoptosis, which significantly decreased in cells treated with BAX-specific RNAi. ELISA also showed decreased apoptosis in the cell lines treated with BAX-specific RNAi (E) A549, CF) H1650.*

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