Involvement of bcl-2 and p21\textsuperscript{waf1} proteins in response of human breast cancer cell clones to Tomudex

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Summary Mechanisms of resistance to Tomudex include increased thymidylate synthase activity, as well as reduced intracellular drug uptake and polyglutamation. However, little is known about other mechanisms of resistance, such as a possible protection against Tomudex-induced apoptosis mediated by bcl-2. We transfected the MDA-MB-435 human breast cancer cell line, which is characterized by a mutated p53 gene, with cDNA of the bcl-2 gene and generated two clones (MDA-bcl4 and MDA-bcl7) characterized by bcl-2 expression twofold and fourfold that observed in the control cell clone (MDA\textsuperscript{neo}). A concomitant overexpression of p21\textsuperscript{waf1} was also detected in the MDA-bcl7 clone. The MDA-bcl4 clone was three times more resistant to a 24-h Tomudex exposure than the MDA\textsuperscript{neo} clone, whereas the MDA-bcl7 clone was as sensitive to Tomudex as the control cell clone. A lower sensitivity of the MDA-bcl4 clone than MDA\textsuperscript{neo} and MDA-bcl7 clones to 5-fluorouracil and gemcitabine was also observed. No significant difference was noted in the susceptibility of clones to fludarabine and methotrexate. Basal levels of thymidylate synthase activity were superimposable in the three clones. Tomudex induced a marked accumulation of cells in the S phase in all the clones. However, an apoptotic hypodiploid DNA peak and the characteristic nuclear morphology of apoptosis were observed only in the MDA-bcl7 clone after exposure to Tomudex. No difference in the treatment-induced modulation of proteins involved in cell cycle progression (cyclin A, cdk2, pRB, E2F-1) and apoptosis (bcl-2, bax) was observed in the other clones. Overall, the results indicate that bcl-2 and p21\textsuperscript{waf1} proteins concur in determining the cellular profile of sensitivity/resistance to Tomudex.

Keywords: Tomudex; resistance; bcl-2; p21\textsuperscript{waf1}; breast cancer cells

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In order to elucidate such differences, we analysed the effects of TDX on proteins involved in the control of cell cycle progression (mainly G1 to S phase) and apoptosis. In parallel, sensitivity of cell clones to other antimitabolites or drugs with different action mechanisms was also assessed.

**MATERIALS AND METHODS**

**Cell lines and transfection procedures**

The MDA-MB-435 human breast cancer cell line used in the study is characterized by a mutated p53 gene (266GGA→GAA) (O’Connor et al, 1997). The bcl-2 gene under the control of the SSFV promoter inserted into the native LTR-SV neo vector (kindly provided by Dr SJ Korsmeyer, Washington University, St Louis, MO, USA) was introduced into MDA-MB-435 cells using Lipofectamine (Gibco-BRL, Gaithersburg, MD, USA) according to the manufacturer’s protocol. MDA-MB-435 cells (4 x 10⁵) were plated in 100-mm dishes; the next day they were washed with phosphate-buffered saline (PBS) and overlaid with serum-free Dulbecco’s modified Eagle’s medium (DMEM) Ham’s F-12 (Gibco) containing 10 µg linearized bcl-2 expression or control vector DNA and 20 µl Lipofectamine. After 16 h, medium was replaced with DMEM/Ham’s F-12 containing 10% fetal bovine serum (FBS; Gibco) for 5 h, followed by incubation in DMEM/Ham’s F-12 containing 5% FBS for 24 h. Stable transfectants were selected in the presence of 900 µg ml⁻¹ G418 (Geneticine, Gibco). After 15–18 days, 40 resistant colonies containing bcl-2-expressing vector or the vector alone were harvested and tested for bcl-2 expression by Western blot analysis. Overall, three clones were selected: MDAneo (control vector), MDA-bcl4 and MDA-bcl7 (bcl-2 transfectants).

**Chemicals**

Doxorubicin (Pharmacia-Upjohn, Uppsala, Sweden) was dissolved in normal saline solution. Taxol (Sigma Chemical Co., St Louis, MO, USA) was stored as a 200 µmol ml⁻¹ stock solution in dimethyl sulphoxide and then reconstituted and diluted in sterile water to obtain a solvent concentration of less than 0.25%. Fludarabine (kindly supplied by Zeneca, Macclesfield, UK), was dissolved in normal saline solution. Taxol (Sigma Chemical Co., St Louis, MO, USA) was stored as a 200 µmol ml⁻¹ stock solution in dimethyl sulphoxide and then reconstituted and diluted in sterile water to obtain a solvent concentration of less than 0.25%. Fludarabine (kindly supplied by Zeneca, Macclesfield, UK), was dissolved in normal saline solution. Taxol (Sigma Chemical Co., St Louis, MO, USA) was stored as a 200 µmol ml⁻¹ stock solution in dimethyl sulphoxide and then reconstituted and diluted in sterile water to obtain a solvent concentration of less than 0.25%. Fludarabine (kindly supplied by Zeneca, Macclesfield, UK), was dissolved in normal saline solution. Taxol (Sigma Chemical Co., St Louis, MO, USA) was stored as a 200 µmol ml⁻¹ stock solution in dimethyl sulphoxide and then reconstituted and diluted in sterile water to obtain a solvent concentration of less than 0.25%. Fludarabine (kindly supplied by Zeneca, Macclesfield, UK), was dissolved in normal saline solution. Taxol (Sigma Chemical Co., St Louis, MO, USA) was stored as a 200 µmol ml⁻¹ stock solution in dimethyl sulphoxide and then reconstituted and diluted in sterile water to obtain a solvent concentration of less than 0.25%. Fludarabine (kindly supplied by Zeneca, Macclesfield, UK), was dissolved in normal saline solution. Taxol (Sigma Chemical Co., St Louis, MO, USA) was stored as a 200 µmol ml⁻¹ stock solution in dimethyl sulphoxide and then reconstituted and diluted in sterile water to obtain a solvent concentration of less than 0.25%. Fludarabine (kindly supplied by Zeneca, Macclesfield, UK), was dissolved in normal saline solution. Taxol (Sigma Chemical Co., St Louis, MO, USA) was stored as a 200 µmol ml⁻¹ stock solution in dimethyl sulphoxide and then reconstituted and diluted in sterile water to obtain a solvent concentration of less than 0.25%. Fludarabine (kindly supplied by Zeneca, Macclesfield, UK), was dissolved in normal saline solution. Taxol (Sigma Chemical Co., St Louis, MO, USA) was stored as a 200 µmol ml⁻¹ stock solution in dimethyl sulphoxide and then reconstituted and diluted in sterile water to obtain a solvent concentration of less than 0.25%. Fludarabine (kindly supplied by Zeneca, Macclesfield, UK), was dissolved in normal saline solution. Taxol (Sigma Chemical Co., St Louis, MO, USA) was stored as a 200 µmol ml⁻¹ stock solution in dimethyl sulphoxide and then reconstituted and diluted in sterile water to obtain a solvent concentration of less than 0.25%. Fludarabine (kindly supplied by Zeneca, Macclesfield, UK), was dissolved in normal saline solution. Taxol (Sigma Chemical Co., St Louis, MO, USA) was stored as a 200 µmol ml⁻¹ stock solution in dimethyl sulphoxide and then reconstituted and diluted in sterile water to obtain a solvent concentration of less than 0.25%. Fludarabine (kindly supplied by Zeneca, Macclesfield, UK), was dissolved in normal saline solution. Taxol (Sigma Chemical Co., St Louis, MO, USA) was stored as a 200 µmol ml⁻¹ stock solution in dimethyl sulphoxide and then reconstituted and diluted in sterile water to obtain a solvent concentration of less than 0.25%. Fludarabine (kindly supplied by Zeneca, Macclesfield, UK), was dissolved in normal saline solution. Taxol (Sigma Chemical Co., St Louis, MO, USA) was stored as a 200 µmol ml⁻¹ stock solution in dimethyl sulphoxide and then reconstituted and diluted in sterile water to obtain a solvent concentration of less than 0.25%.

**Cell proliferation studies**

The MDA-MB-435 parental cell line, the MDAneo clone (transfected with LTR-SV neo vector alone) and bcl-2 transfectants (MDA-bcl4 and MDA-bcl7) were cultured in DMEM/Ham’s F-12 medium lacking G418, supplemented with 5% FBS. After harvesting in the logarithmic growth phase, cells were seeded in 6-well plates and treated with varying doses of taxol, fludarabine, gemcitabine, 5-FU, methotrexate and TDX for 24 h, or doxorubicin for 1 h. Exposure times to different drugs were chosen according to the treatment times used in previous studies (Silvestrini et al, 1993). At the end of treatment, adherent cells were washed with PBS and incubated at 37°C in a 5% carbon dioxide humidified atmosphere for 3 days. Cells were then trypsinized and counted in a particle counter (Coulter Counter, Coulter Electronics, Luton, UK). The percentages of adherent viable cells were determined by the Trypan blue dye exclusion test. Viability always exceeded 95%. Each experimental sample was run in triplicate. The results were expressed as the total number of adherent cells in treated samples compared with control samples. In vitro activities of different drugs were expressed in terms of concentrations able to inhibit cell proliferation by 50% (IC₅₀).

**TS catalytic activity assay**

The assay proposed by Van der Wilt et al (1992) was used with some modifications. Briefly, 10 × 10⁶ cells were suspended in 0.8 ml of ice-cold assay buffer containing 200 mM Tris–HCl, 20 mM β-mercaptoethanol, 100 mM NaF and 15 mM cytidine-5'-monophosphate (pH 7.4). Cells were then sonicated and centrifuged at 12 000 rpm. The supernatants containing the enzyme (enzyme suspensions) were split into two parts for protein content determination and TS catalytic activity assay. The TS catalytic activity assay was carried out with 10 µl of enzyme suspensions (in different dilutions), 5 µl N₃,N₁⁰-methylene-tetrahydrofolate and 10 µl of [5-³H]-2'-deoxyuridine-5'-monophosphate (10-µM final concentration). Reaction mixtures were incubated for 30 min at 37°C and stopped by addition of 50 µl ice-cold 35% trichloroacetic acid and 250 µl 10% neutral activated charcoal. After centrifugation, radioactivity present in 150 µl of supernatant was determined by liquid scintillation counting. TS activity was expressed as ³H release (pmol h⁻¹ 10⁻⁶ cells) produced during the conversion of [³H]deoxyuridine monophosphate to ³H₂O.

**Flow cytometric analysis**

Immediately after a 24-h TDX exposure, or after an additional 8 and 24 h in drug-free medium, samples of 1 × 10⁶ cells were fixed in 70% ethanol. Before analysis, cells were washed in PBS and stained with a solution containing 50 µg ml⁻¹ propidium iodide, 50 mg ml⁻¹ RNAase, and 0.05% NP40 for 30 min at 4°C and then analysed with a FACScan flow cytometer (Becton Dickinson, Sunnyvale, CA, USA). An aliquot of each sample was also analysed under fluorescence microscopy for evaluation of nuclear morphology. The cell cycle distribution was evaluated on DNA plots by CellFit software according to the SOBR model (Becton Dickinson).

**Immunohistochemistry**

After trypsinization of exponentially growing clones, samples of cell suspensions were seeded onto glass slides, air-dried and immediately fixed in formalin for 3 min. Fixed cells were rehydrated with PBS and labelled with the monoclonal antibody anti-p21wafl (clone DCS 60.2; Neomarkers, Freemont, CA, USA). Antibody binding to cells was evidenced by the biotin–streptavidin alkaline phosphatase technique.
Cell lysis and immunoblotting

Cells were lysed in 1% Nonidet P-40, prepared in PBS containing 10 μg ml⁻¹ leupeptin, 10 μg ml⁻¹ aprotinin, 1 mM AEBSF, 1 mM Na₃VO₄, 1 mM NaPPO₄ and 10 mM NaF. Cell lysates were clarified (15 min, 15,000 rpm), and the resultant supernatants were used for protein analysis. Total cellular lysate (100 μg) was dissolved in 2 x sample loading buffer (2% sodium dodecyl sulphate (SDS), 5% 2-mercaptoethanol, 20% glycerol, 60 mM Tris, pH 6.8, and 0.0025% bromophenol), separated on 12% SDS-polyacrylamide gel and transferred to nitrocellulose. Filters were blocked in PBS with 5% skim milk and then incubated overnight with the primary monoclonal antibody anti-pRb (retinoblastoma protein), anti-Bax, anti-Bcl-2, anti E2F-1, anti-cyclin A and anti-cdk2 (Santa Cruz Biotechnology, Santa Cruz, CA, USA), anti-p21wafl (Oncogene Science, Cambridge, MA, USA) or anti-TS (kindly supplied by Dr PG Johnston, Queen’s University, Belfast, Ireland). Filters were then incubated with the secondary antibody antimouse or antirabbit Ig horseradish peroxidase-linked whole antibody (Amersham, Buckinghamshire, UK). Bound antibody was detected using the enhanced chemiluminescence Western blotting detection system (Amersham). An anti-PCNA (proliferating cell nuclear antigen) monoclonal antibody (Santa Cruz Biotechnology) was used on each blot to ensure equal loading of protein on the gel.

RESULTS

Cell clone characteristics and sensitivity to drugs

The parental MDA-MB-435 cell line and the control transfecant MDAneo clone showed very low and similar levels of bcl-2 expression, whereas MDA-bcl4 and MDA-bcl7 clones were characterized by bcl-2 expression twofold and fourfold (as evaluated by densitometric analysis of immunoblotting) that observed in MDAneo and MDA-MB-435 cells (Figure 1). No difference was observed in any of the three clones and the parental cell line as regards the expression of p53 and bax proteins. Conversely, an overexpression of p21wafl protein was observed in MDA-bcl7, whereas MDA-bcl4 and MDA-bcl7 clones were characterized by bcl-2 expression twofold and fourfold (as evaluated by densitometric analysis of immunoblotting) that observed in MDAneo and MDA-MB-435 cells (Figure 1). No difference was observed in any of the three clones and the parental cell line as regards the expression of p53 and bax proteins. Conversely, an overexpression of p21wafl protein was observed in MDA-bcl7 (Figure 1). Specially, the level of p21wafl in the MDA-bcl7 clone was twofold that observed in MDAneo cells. p21wafl results obtained by Western blotting were also confirmed by immunohistochemistry (Figure 2). Analysis of the logarithmic growth rate of bcl-2-transfected clones and of the clone transfected with the control vector indicated a doubling time of approximately 20 h for all the cell lines (Table 1). Moreover, similar basal catalytic activities of TS were observed in MDAneo, MDA-bcl4 and MDA-bcl7.

As regards sensitivity to different anticancer agents (Table 2), the three clones showed similar susceptibility to the antimitabolite fludarabine and drugs with other mechanisms of action such as doxorubicin and taxol, as indicated by the superimposable IC₅₀ values. The MDA-bcl4 clone was slightly, although not significantly, less sensitive than MDAneo and MDA-bcl7 clones to methotrexate. Moreover, the MDA-bcl4 clone was significantly more resistant to 5-FU, gemcitabine (P < 0.025) and TDX (P < 0.01) than the other two clones.

Effects of TDX on the cell cycle

DNA flow cytometric analysis was performed to determine whether cell cycle perturbations could explain the different cytotoxic activity of TDX in the three clones (Table 3). For MDAneo and MDA-bcl7, TDX was used at a concentration of 8 μM, which is the IC₅₀ of the two clones. For MDA-bcl4, besides the 8 μM concentration, the specific IC₅₀ of the clone (25 μM) was also used. Immediately after a 24-h treatment with 8 μM of TDX, a very slight accumulation of cells in the S phase was observed in all three clones. Such accumulations became more evident 48 h after beginning the TDX treatment, when most of the cells were arrested in the S phase. The extent of the accumulation was superimposable in the clones independently of the different cytotoxic effects induced by the drug. Moreover, a marked decrease in the G2/M cell fraction, consistent with an inhibition of dTPP synthesis induced by TDX, was observed in MDAneo and MDA-bcl4 clones.

In the MDA-bcl4 clone, exposure to 25 μM of TDX induced a slight accumulation of cells in the G0/1 phase 24 h and 32 h after beginning the TDX treatment, followed by a marked accumulation of cells in the S phase, to an extent similar to that observed with the 8-μM TDX treatment.

To investigate at a molecular level possible differences in the events that regulate progression through the S phase, we analysed the effects of 8 μM TDX treatment on pRb, E2F-1, cyclin A and cdk2 protein expression. An increase in E2F-1, cyclin A and cdk2 levels was observed in all cell clones, whereas no treatment-induced modulation of pRb expression and phosphorylation was observed (Figure 3).

Induction of apoptosis by TDX

Flow cytometric analysis of the three clones exposed to 8 μM TDX showed that 48 h after beginning the treatment, approximately 14% of MDA-bcl7 cells exhibited a hypodiploid DNA content. Such a pre-G1 apoptotic cell peak was not observed in MDA-bcl4 or in cells transfected with the control vector (Figure 4). In addition, evaluation by fluorescence microscopy of cell clones stained with propidium iodide confirmed the presence of cells with the characteristic nuclear morphology of apoptosis only in the

![Figure 1](image-url)
MDA-bcl7 clone (Figure 5). In order to elucidate the pathway of TDX-induced programmed cell death, the effect of treatment on proteins involved in the onset of apoptosis was assessed. As shown in Figure 6, no appreciable modulation of bax or bcl-2 protein expression, or in the bcl-2 phosphorylation status, was observed in any of the three clones. Conversely, an induction of p21wafl expression was found after exposure to 8 mM TDX in MDAneo and MDA-bcl7 but not in the MDA-bcl4 clone. Such an increase was maximal at 48 h after beginning the TDX treatment. In the MDA-bcl4 clone, a marked induction of the p21wafl protein became apparent when cells were treated with the highest (25 mM) TDX concentration (Figure 7).

**Effect of TDX on TS expression**

Finally, to verify whether or not TDX differentially affected the expression of its target enzyme, thus influencing cellular sensitivity to the drug, we analysed the expression in TS protein after treatment with 8 mM TDX. As shown in Figure 8, an increase of TS expression was appreciable in all cell clones to a similar extent.

**DISCUSSION**

TDX is a specific inhibitor of TS and has demonstrated its activity in many solid tumours in experimental systems. Moreover, among novel TS inhibitors, TDX is furthest along in its clinical development (Van Cutsem, 1997). Due to increasing clinical interest in the drug, it is important to identify possible mechanisms of inherent or acquired resistance, other than the well-known classical biochemical mechanisms of resistance to antimetabolites (Kinsella et al, 1997).

The present study investigated the role of bcl-2 and p21wafl proteins in the response of human breast cancer cell clones to TDX. The MDA-MB-435 human breast cancer cell line was transfected

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**Figure 2** Immunohistochemical detection of p21wafl expression in MDA-MB-435 (A), MDAneo (B), MDA-bcl4 (C) and MDA-bcl7 (D)
with cDNA of the bcl-2 gene. Two clones (MDA-bcl4 and MDA-bcl7), both characterized by bcl-2 expression respectively twofold and fourfold that observed in the control cell clone transfected with c-DNA of the neomycin-resistance gene alone (MDAneo), were generated. The three clones were similar in growth profile, basal TS activity and expression, and expression of p53 and bax, whereas in the MDA-bcl7 clone an induction of p21waf1 concomitant to bcl-2 overexpression was observed.

Analysis of the chemosensitivity profiles of the three cell clones showed a similar sensitivity towards the antimitabolite fludarabine. However, the MDA-bcl4 clone was more resistant than MDAneo and MDA-bcl7 clones to other antimitabolites such as gemcitabine, 5-FU and methotrexate, and particularly resistant (threefold) to TDX. Overall, it emerged that transfection of cells with bcl-2 resulted in a reduced activity of TS and expression of p21waf1. The results of cross-resistance studies support the possibility that the partial cross-resistance of TDX and 5-FU in the MDA-bcl4 clone is due to TS alterations, since in the clone the basal activity of TS was similar to that of MDAneo and MDA-bcl7.

A finding that deserves further investigation is that TDX resistance was completely overcome in the MDA-bcl7 clone overexpressing concomitantly bcl-2 and p21waf1, which was originally identified as an inhibitor of cyclin D–cdk2, cyclin E–cdk2 kinases and complexes (Xiong et al., 1993), is a transcriptional target of p53 and is strongly induced by DNA damage in cells expressing functional p53 (El-Deiry et al., 1994). However, its activation can also occur independently of p53 (Russo et al., 1993; Sheikh, 1994). In the presence of functional pRb, p21waf1 inhibits the activity of cyclin D–cdk4, cyclin A–cdk2 and cyclin E–cdk2 complexes, thereby increasing the level of hypophosphorylated pRb, which in turn associates with E2F-1 and arrests the cells in G1 phase (Chellappan et al., 1991; Ikeda et al., 1996). Moreover, p21waf1 is involved in tumour growth suppression and cellular response to DNA damage (Chen et al., 1995; Garrenthena et al., 1995). We therefore investigated whether differences in TDX sensitivity could be supported by differences in cell cycle perturbations.
induced by TDX treatment. The TS inhibitor caused a marked accumulation of cells in the S phase, which was superimposable in all three clones. Moreover, in the MDA-bcl4 clone, TDX resistance could not be ascribed to a different interference of the drug on the cell cycle, since comparable S phase cell accumulations were observed after treatment with two drug concentrations (8 and $25\mu M$), which induced different cytotoxic effects.

At a molecular level, TDX induced increases in E2F-1, cyclin A and cdk2 levels as cells progressed and accumulated in the S phase, whereas pRb expression and phosphorylation did not show any change as a function of TDX treatment. Although a direct comparison between the data obtained in the different clones by using a non-equitoxic TDX concentration cannot be made, the superimposable patterns of protein modulation observed suggest that the levels of such proteins are not responsible for the different sensitivity to TDX but that they merely reflect a drug-induced cell cycle perturbation which is consistent in the three clones. A time-dependent increase in TS protein was similarly observed in all the three clones after TDX exposure. TS overexpression could be due to the transactivation by E2F-1 of the TS gene, which is required for entry of cells in the S phase (Sherr, 1996), or, alternatively, to a cellular effort to overcome the cytotoxic stress induced by TDX by increasing the production of its target enzyme.

Since bcl-2 (Kroemer, 1997) and p21\textsuperscript{wafl} (El-Deiry, 1994) are involved in the apoptotic process, a possibility is that a different

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**Figure 3** A representative experiment illustrating the temporal effects of TDX treatment on the expression of proteins involved in control of the S phase in MDA\textsuperscript{neo}, MDA-bcl4 and MDA-bcl7 cells. The three clones were incubated with solvent (control, CTR) or 8 $\mu M$ TDX for 24 h. At the end of the treatment, cells were washed with PBS, immediately collected (in Figure indicated as 24 h) or incubated for an additional 8 h and 24 h in drug-free medium (in Figure indicated as 32 h and 48 h from the beginning of TDX treatment). Western blots were probed with monoclonal antibodies for pRb, E2F-1, cyclin A and cdk2. PCNA was used as control for correct loading.

**Figure 4** Cell cycle analyses on MDA\textsuperscript{neo}, MDA-bcl4 and MDA-bcl7 cells 48 h after beginning a 24-h TDX treatment (8 $\mu M$ concentration) and in solvent-treated control cells. The percentage of the pre-G1 population is reported in the bottom right-hand corner for MDA-bcl7.
induction and regulation of apoptosis could be responsible for TDX resistance in the MDA-bcl4 clone and for restoring TDX sensitivity in the MDA-bcl7 clone. Flow cytometric analysis evidenced a pre-G1 apoptotic cell peak only in the MDA-bcl7 clone 48 h after beginning TDX treatment, and morphologic analysis confirmed the presence of apoptotic cells only in this clone. However, since the analysis was performed only at a constant TDX concentration, which produced different cytotoxic effects in the two cell clones, on the basis of our data it is not possible to establish that a lower susceptibility to undergo apoptosis was responsible for resistance to TDX in the MDA-bcl4 clone.

As regards the effect of TDX on the expression of proteins involved in the onset of apoptosis, no change was observed in bcl-2 and bax compared to basal levels of control, whereas an induction of p21waf1 was observed in the two clones with similar sensitivity to TDX, MDAneo and MDA-bcl7, but not in the MDA-bcl4-resistant clone. In fact, in the latter clone, a higher TDX concentration was necessary to produce an appreciable induction of p21waf1. We could hypothesize that enhanced basal expression and inducibility of p21waf1 was able to overcome the anti-apoptotic effect of bcl-2 and to restore the sensitivity to TDX in MDA-bcl7.

Our results are in agreement with the findings of Li et al (1997), who showed that in SaOs-2 osteosarcoma cells, lacking p53 and pRb, overexpression of p21waf1 increased sensitivity to TDX, methotrexate and doxorubicin by increasing the extent of apoptosis. The overexpression of p21waf1 reduced cyclin A-associated kinase activity and resulted in inhibition of phosphorylation of E2F-1 and increased E2F-1 binding activity. Such events lead to an enhanced S–G2 cell cycle delay and increased susceptibility to apoptosis. Since in our experimental models no effect of p21waf1 overexpression on the duration of the S phase block was observed, we hypothesize that the pathway described by Li et al (1997) could be different from that followed by our clones.

Overall, our results suggest that bcl-2 and p21waf1 proteins concur in determining the profile of sensitivity/resistance of MDA-MB-435 breast cancer cells to TDX. The reacquisition of TDX sensitivity in the MDA-bcl7 clone could be mediated, at least

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**Figure 5** Propidium iodide staining of MDA-bcl7 cells treated with solvent alone (A) or with 8 μM TDX (B) and collected 48 h after beginning a 24-h TDX treatment. Whole cells were collected, stained with propidium iodide and viewed under the fluorescence microscope.

| Time (h) | MDAneo | MDA-bcl4 | MDA-bcl7 |
|---------|--------|----------|----------|
| 24      | CTR    | TDX      | CTR      |
| 32      |        |          |          |
| 48      |        |          |          |

**Figure 6** A representative experiment illustrating the temporal effects of TDX treatment on the expression of proteins involved in the onset of apoptosis in MDAneo, MDA-bcl4 and MDA-bcl7 cells. Treatments and samples as indicated in Figure 3. Western blots were probed with monoclonal antibodies for bcl-2, bax and p21waf1. PCNA was used as a control for correct loading.
in part, by overexpression of p21\textsuperscript{wafl} and susceptibility of cells to undergo apoptosis. Several studies have shown that overexpression of p21\textsuperscript{wafl} alone can induce apoptosis following transfection (Sheikh et al., 1995; Prabhu et al., 1996). However, the role of p21\textsuperscript{wafl} in the apoptosis process is still controversial. Studies are warranted to elucidate the molecular mechanisms by which bcl-2 and p21\textsuperscript{wafl} are involved in TDX cytotoxic activity and apoptosis.

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Figure 7 A representative experiment illustrating the effect of TDX on p21\textsuperscript{wafl} expression in MDA-bcl4 cells. Cells were incubated with solvent (control, CTR), 8 \mu M TDX or 25 \mu M TDX for 24 h. At the end of the treatment, cells were washed with PBS, immediately collected (in Figure indicated as 24 h) or incubated for an additional 8 h and 24 h in drug-free medium (in Figure indicated as 32 h and 48 h from the beginning of TDX treatment). PCNA was used as control for correct loading.

Figure 8 Effect of TDX on TS expression in MDA\textsuperscript{neo}, MDA-bcl4 and MDA-bcl7 cells. Treatments and samples as indicated in Figure 3.
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