Proapoptotic effects of antiestrogens, progestins and androgen in breast cancer cells ☆,☆☆

M. Kandouz[a], A. Lombet[b], J-Y. Perrot[c], D. Jacob[d], S. Carvajal[b], A. Kazem[d],
W. Rostene[b], A. Therwath[a], A. Gompel[a, b, d],*

[a]*Laboratoire d'Oncologie Moléculaire University Paris, VII, Paris, France*
[b]*INSERM U 339 Hopital St Antoine, Paris, France*
[c]*Laboratoire d'Hématologie Biologique, Paris, France*
[d]*Service de Gynécologie, Hôtel-Dieu de Paris, 1 Place du Parvis, Notre Dame, 75005, Paris, France*

Abstract

The promoting action of E2 in breast cancer cells has been, until now, mainly linked to its action on prolifieration. Because of the importance of an increase in apoptosis in breast cancer prevention, we have studied the possible effects of various antiestrogens, progestins and an androgen on its occurrence in three hormone-dependent breast cancer cell lines. The antiestrogens were, a triphenylethylene derivative, 4 hydroxytamoxifen(4OHTAM) and two steroidal antiestrogens, ICI182780 and RU58668. The progestins were Org2058, a pregnane derivative, tibolone (OrgOD14), a normethyltestosterone derivative and OrgOM38 (the Δ4 isomer of OrgOD14) and the androgen dihydrotestosterone (DHT). Apoptosis was studied in MCF-7, ZR75-1 and T47-D cells using morphological approaches and flow cytometry. The antiestrogens, the progestins and DHT were proapoptotic but to different potencies according to the cell line studied. Indeed, the 'pure' steroidal antiestrogens were more efficient than 4OHTam in increasing apoptosis. We have also studied the level of expression of some of the proteins involved in the regulation of apoptosis. Bcl-2 and bcx$_L$, two antiapoptotic members of the bcl-2 family proteins, were inhibited by the progestins and the antiestrogens. In contrast, the proapoptotic proteins, bax and bak seemed to be constitutively expressed. Thus, since the ratio of proapoptotic and antiapoptotic proteins determines apoptosis or cell survival, the hormone effects are operating by modulating the antiapoptotic regulators of the balance. These data demonstrate that antiestrogens, progestins, and androgens can promote apoptosis in breast cancer cells, an effect which could be of importance in the therapeutic prevention of breast cancer. © 1999 Elsevier Science Ltd. All rights reserved.

1. Introduction

Breast cancer is hormone dependent cancer. Until recently, the effects of hormones were mainly studied on cell proliferation with little attention being paid to cell elimination due to cell death [1,2]. Apoptosis is involved in tissue homeostasis and its deregulation can undoubtedly promote tumorigenesis as well as prolifer-ation [3]. Apoptosis has been observed in normal breast tissue with a peak at the end of the menstrual cycle [4]. In breast cancer cell lines, cell death has been reported to be induced by estrogen (E2) deprivation [5] or antiestrogen treatment [6]. However, the effects of potent antiestrogens, androgens and progestins have not been extensively studied on the occurrence of apoptosis. Apoptosis is known to be regulated by a large set of genes. Among them, the bcl-2 family pro-teins play a key role. *Bcl-2* was first described as resulting from the t(14;18) translocation that occurs in a large number of human follicular lymphomas. *Bcl-2* has been shown to prevent cells from going into apop-tosis and to prolong cell survival time without promot-

* Corresponding author. Fax: + 33-1-42-34-86-39.
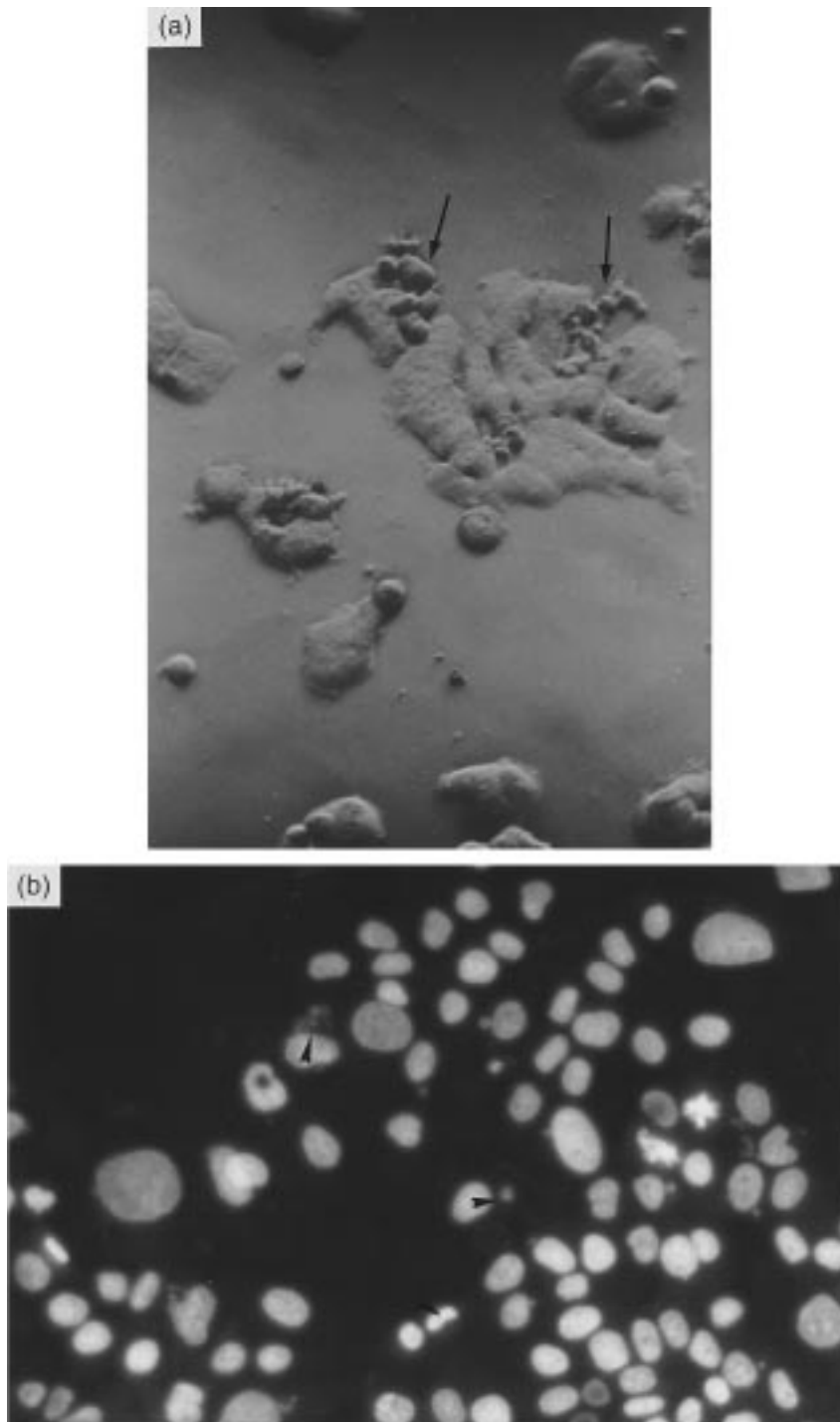*E-mail address:* anne.gompel@htd.ap-hop-paris.fr (A. Gompel)

Fig. 1. Morphological analysis of apoptosis in T47-D cells. A: aspects of the surface of T47-D cells in culture analyzed by a Hoffman modulation contrast system (×200). Arrows show the blebs of the cell surfaces. B: nuclear staining of T47-D cells using bisBenzimide. Typical apoptotic patterns can be seen (arrows) (×400).

ing cell proliferation [7]. Moreover *Bcl-2* cooperates with the *c-myc* product in promoting the proliferative activity of the latter [8]. However, transfection of *bcl-2* alone into breast epithelial cells does not confer tumorigenic capacity [9]. Other genes from the bcl-2 family are also involved in the control of apoptosis.

Among them, some promote apoptosis (Bax, Bak, Bcl-x_S) whereas others are antiapoptotic (Bcl-2, Bcl-x_L) [10–12]. These proteins are able to form heterodimers and thereby affect apoptosis. In instances of Bcl-2/Bax heterodimerization, the cellular content of Bax may remain unchanged whereas variation, if any, in Bcl-2

concentrations may affect the resultant action of the heterodimers on apoptosis [10].

In order to gain insights on the effects of the ovarian steroids or some hormonal drugs used for women, we have quantitated apoptosis in some hormone dependent breast cancer cell lines under different hormone treatments and followed the bcl-2 family proteins involved in apoptosis regulation.

2. Materials and methods

2.1. Chemicals, reagents and steroids

Dulbecco Modified Essential Medium (DMEM), RPMI without phenol red, SVF, 5.6% $NaHCO_3$ solution, glutamine and 0.25% sterile trypsin solution were from Life-Technologies (Cergy-Pontoise, France). Penicillin (10000 U), streptomycin (10 mg), insulin, 17βestradiol (E_2), dihydrotestosterone (DHT), thymidine, bisBenzimide (Hoechst 33258), RNAse and propidium iodide were obtained from Sigma (St. Quentin-Fallavier, France). Org2058. OrgOD14 (Tibolone) and OrgOM38 (the Δ4-isomer of OrgOD14) were kindly provided by Dr H.J. Kloosterboer (N.V. Organon, Oss, The Netherlands). RU 38486 (mifepristone, RU486) and RU58668 (RU58), a steroidal pure antiestrogen, were kindly provided by Dr P. Van De Velde (Roussel-Uclaf, Romainville, France). 4 hydroxytamoxifen (4OHTAM) was kindly provided by Dr S. Fournier (Besins-Iscovesco, Paris, France), ICI182780 (ICI), a steroidal pure antiestrogen, was kindly provided by Dr A.E. Wakeling (Zeneca, Macclesfield, England).

2.2. Cultures procedures

MCF-7, T47-D and ZR75-1 cell lines was a gift from Dr C. Mercier-Bodard (Kremlin-Bicêtre) and originally came from the laboratories of Dr M. Lippman (Bethesda) and Dr K. Horwitz (Denver).

Cell cultures were performed in a 5% CO_2 enriched atmosphere at 37°C. MCF-7 and ZR75-1 were cultured in DMEM without phenol-red but containing 2 mM glutamine, antibiotics and 5% fetal calf serum (FCS). T47-D were cultured in RPMI without phenol-red but containing 2 mM glutamine and antibiotics. Synchronization of MCF-7, ZR75-1 and T47-D cells were conducted in their corresponding medium containing 5% steroid stripped FCS (DCC-FCS) (using dextran-coated charcoal). Thymidine, 2 mM, (final concentration) was applied during two pulses of 16 h separated by 12-h wash in DCC-FCS medium [13]. The steroid receptors content of the three cell lines used were regularly measured (Laboratory of Prof. Milgrom, Hôpital Bicêtre).

2.3. Hormonal treatments

Before adding the hormones, the basal medium was replaced for 48 h by a medium with DCC-FCS. Hormones were added in <0.1% ethanol, alone or combined with E2 (10 nM) in DCC-FCS containing medium. The medium containing fresh hormones was replaced every 2 days for 1 week. E2 and DHT were usually used at 10 nM concentration. The antiestrogens were added at 1 μM and the progestins and RU486 were used at 100 nM. The progestins consisted of a pregnane derivative, Org2058, two 19-nortestosterone derivatives, tibolone (OrgOD14) which has progestogenic, androgenic and estrogenic properties, and its Δ4-isomer, OrgOM38 which has stronger progestogenic and androgenic and no estrogenic properties in vivo [14,15]. They were added alone or combined with E2 and/or the antiprogestin RU 486. The antiestrogens 4OHTAM, RU58 and ICI were added alone or combined with E2. DHT was added alone. Quantitation of apoptosis was performed after 1 day of hormonal treatments using morphological analysis and after 3 days using flow cytometry.

2.4. Quantitation of apoptosis

2.4.1. Cytometric analysis of apoptosis

Cells (10^6) were washed with ice-cold PBS. Cells were detached using 2 ml of 0.25% trypsin and the reaction was stopped by adding 4 ml of the corresponding culture medium. The $3000 \times g$ cell pellet was obtained by centrifugation and gently washed twice with PBS before fixation in cold 90% methanol for 30 min at room temperature. The cells were then washed twice in PBS and resuspended in PBS containing 1 mg/ml RNAse. DNA was stained with 0.5 μg/ml propidium iodide. Samples were analyzed in a fluorescence-activated cell sorter (FACSORT) running a Lysis 2 software (Beckton Dickinson, Mountain View, CA) with a log-scale analysis. The subdiploid cells were considered apoptotic (Fig. 1A). Ten thousand cells were analysed in each sample. The region where the hypodiploïd DNA was quantitated was established using the position of the diploïd peak and using a threshold to eliminate the debris. We took care to verify that these positions were identical for all cell samples. The experiments were repeated at least four times each in duplicate. Results were expressed as means ± SEM of the percentage of apoptotic cells and as means ± SEM of percentage of the control cell value of the corresponding experiment.

2.4.2. Morphological studies of apoptosis

Apoptosis is associated with specific alterations including cell shrinkage, blebbing or budding of the cell membrane, and in the nucleus the chromatin mar-

ginates and becomes pyknotic; the nuclear fragments and the chromatin are included in apoptotic bodies [15,16]. The Hoffman microscope (combining contrast and fluorescence microscopy) offered a useful method to demonstrate and quantitate such morphological alterations. Cells treated with steroids were observed using a Hoffman modulation contrast system with a Nikon Diaphot TDM inverted microscope [17]. This system provides a three-dimensional view of cells, revealing their shape and some of the morphological characteristics of apoptosis (Fig. 1B). In order to compare the effects of the various treatments, a quantitative study was performed. Cells were plated at 5×10^4 cells/20-mm well. The number of apoptotic cells displaying blebs (Fig. 1A) was counted by scanning 10 different areas at random from the bottom to the top of the well on two contiguous vertical lines at ×200 magnification. The results were expressed as the mean number of apoptotic cells per field. Following morphological examination, cells were fixed with 4% paraformaldehyde in 0.1% phosphate buffer for 1 h and washed with PBS. The vital dye Hoechst 33258 (bisBenzimide) was then added (10 μg/ml final concentration) to the preparation for 10 min, followed by washing in PBS. The cells were examined with a fluorescence Nikon Diaphot inverted microscope (Fig. 1B), which can be used indifferently for contrast and fluorescence observations, allowing cells to be viewed successively using the two techniques. The number of apoptotic (cells with DNA condensation and fragmentation) was counted. These studies were performed in MCF-7 cells and T47-D cells but were not possible in ZR75-1 due to the shape of these colonies.

The hormonal treatments were studied in at least four separate experiments each in duplicate. Results were expressed as means ± SEM of the number of apoptotic cells and as means ± SEM of apoptotic cells in the percent of the control cell value of the corresponding experiment.

2.5. Western blotting

The Western blots were performed as previously described for bcl-2 protein detection [18].

The bclx$_L$, bax and bak proteins were detected using rabbit polyclonal anti-bclx$_L$ (Transduction Lab., Mahemad Castle, UK) anti-bax and anti-bak antibodies (Santa Cruz Biotechnology Inc. CA) (1:5000) overnight, biotinylated goat anti-rabbit antibody (1:2500) (Dako). Actin was detected using a mouse monoclonal antibody (Amersham) (1:1000), incubated for 2 h. The experiments were performed at least three times in duplicate. Relative amounts of bcl-2 and bclx$_L$ proteins were quantitated using a CCD camera (ISS, Pessac, France). The level of the proteins was normalized with respect to actin and the results expressed as means ± SEM.

2.6. Statistical analysis

Nonparametric Mann–Whitney tests were used using SPSS statistical software to compare the amount of apoptosis in the hormone-treated cells compared to the control cells. After a oneway ANOVA analysis, multiple range Student–Newman–Keuls tests were performed to evaluate the relative efficiencies of the treatments.

3. Results

The two methods used to quantitate apoptosis were correlated with an $r = 0.8$ ($P < 0.01$).

The proportion of apoptotic cells varied for each cell line. The mean percentage of apoptotic cells quantitated using flow cytometry was for control MCF-7 cells, $6 \pm 1\%$, for T47-D cells, $5.9 \pm 1\%$ and for ZR75-1, $18.6 \pm 4\%$ (Tables 1 and 2) and in morphological analysis, 6.5 ± 0.5 apoptotic MCF-7 cells per field (for a mean number of 110 cells per field) and 3.8 ± 0.7

Table 1
Antiestrogens in breast cancer cells: percentage of apoptotic cells measured by flow cytometry (Means ± SEM, *P < 0.05, **P < 0.01, ***P < 0.001)

	ZR75-1	T47-D	MCF-7
C	18.6 ± 4	5.9 ± 1	6 ± 1
E2	18.93 ± 3	6.9 ± 1.5	7.8 ± 1.5
4OHTAM	24 ± 4*	6.5 ± 0.3	6.6 ± 0.3
E2 + 4OHTAM	31 ± 5**	7.5 ± 3*	7.9 ± 0.6*
RU58	28.3 ± 4**	9.5	9.6 ± 1**
E2 + RU58	35 ± 3.5***	14 ± 3.7***	11.5 ± 1.2**
ICI	35.5 ± 3.3***	9.4 ± 4**	–
E2 + ICI	32.5 ± 3.6***	12 ± 3.8**	–

Table 2
Percentage of apoptotic cells measured by flow cytometry under estradiol and progestins in breast cancer cell lines (means ± SEM, *P < 0.05, **P < 0.01, *** < 0.001)

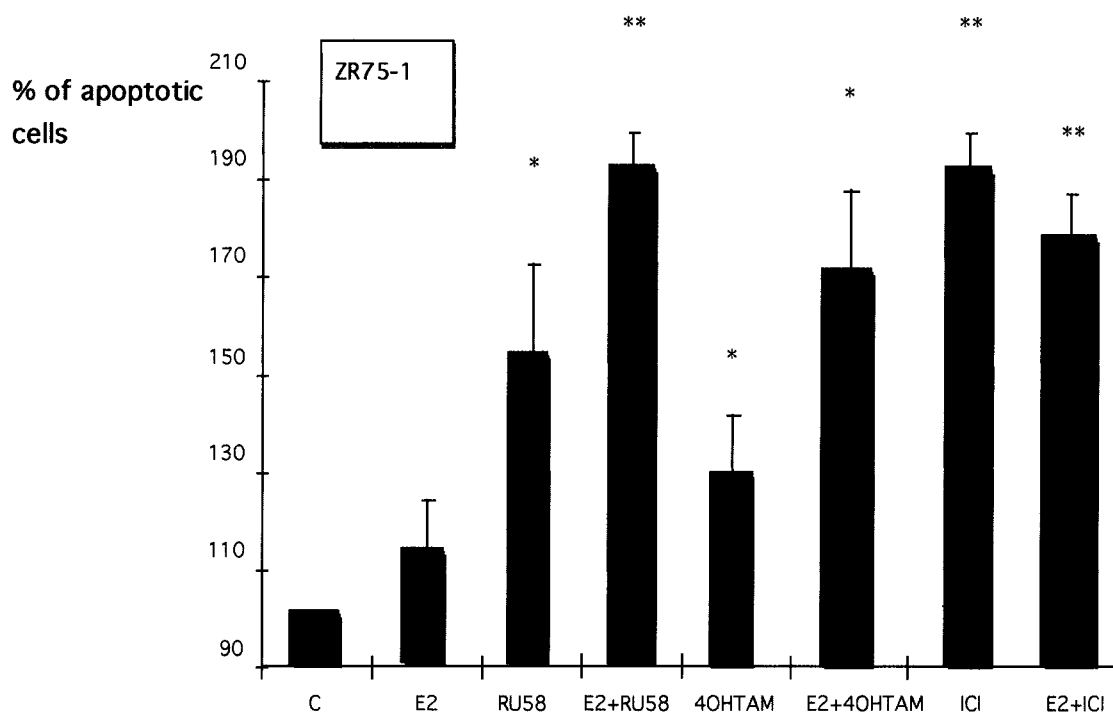|	ZR-75-1	T47-D
C	18.6 ± 4	5.9 ± 1
E2	18.9 ± 3	6.9 ± 1.55
Org2058	24 ± 6*	10 ± 0.27***
OrgOD14	37 ± 4***	8 ± 1.8*
OrgOM38	25.4 ± 2*	8 ± 2.47*
E2 + Org2058	38 ± 1***	8.5 ± 1.7*
E2 + OrgOD14	42 ± 5***	10 ± 2**
E2 + OrgOM38	28 ± 3**	9.8 ± 1.16**
RU486	31.5 ± 5*	7.5 ± 3

Fig. 2. Antiestrogens are proapoptotic in ZR75-1 cells. Percentage of apoptotic cells were quantitated by flow cytometry. 4OHTAM, RU58, ICI were used at 1 μM, E2 at 10 nM. Results are expressed as mean ± SEM of apoptotic cells as a percentage of the corresponding control after 3 days of hormonal treatment ($^*P < 0.05$, $^{**}P < 0.01$ vs control).

apoptotic T47-D cells per field (for a mean number of 85 cells per field).

3.1. Antiestrogens are proapoptotic in breast cancer cell lines

As shown in Table 1 and Fig. 2, the antiestrogens were potently proapoptotic whether in the presence or absence of E2 in the various cell lines studied, irrespective of the steroid receptor content. The steroidal derivatives were far more potent than 4OHTAM in the three cell lines ($P < 0.05$).

3.2. Progestins and DHT are proapoptotic in breast cancer cell lines

We studied the pregnane derivative and the nor-methyltestosterone derivatives in T47-D and ZR75-1 cells. As demonstrated in Table 2 and Fig. 3, the progestins were proapoptotic in the cell lines studied, whether in the presence or absence of E2. In order to compare their relative potencies, different concentrations of progestins and DHT were studied in ZR75-1 cells (Fig. 3). DHT displayed a proapoptotic effect, with a potency related to its concentration: at 10 nM, it increased the proportion of apoptotic cells to 1.5 fold compared to the control, an increase similar to what was observed with Org2058 and OrgOM38 at

100 nM and to a 2.3–2.5 fold at 100 nM and 1 μM. At these concentrations, OrgOD14 and OrgOM38 multiplied by 2–2.4 fold the proportion of apoptotic cells.

OrgOD14 and DHT were more potently proapoptotic than Org2058 or the antiestrogens in ZR75-1 cells (ANOVA analysis: $P < 0.05$) whereas, in T47-D cells, the steroidal antiestrogens were more potent than the progestins (ANOVA analysis: $P < 0.05$).

RU486 was proapoptotic only in ZR75-1 and did not alter the effect of progestins on apoptosis (not shown).

3.3. Bcl-2 family proteins

We had previously shown that in MCF-7 cells, E2 increased the expression of bcl-2 over short and long term periods of treatment [18]. Its effect was reversed in the presence of the antiestrogens RU58 and 4OHTAM [18]. The antiestrogens alone did not have any effect. The level of the proapoptotic protein, bax, did not vary.

In contrast, Org2058 inhibited bcl-2 expression in T47-D cells over short and long periods of treatment without any effect on bax [18]. We have confirmed the same effects of E2 on ZR75-1 (Fig. 4). Moreover we observed also a potent inhibiting effects of OrgOD14 and OrgOM38 in T47-D and ZR75-1 cells on bcl-2 ex-
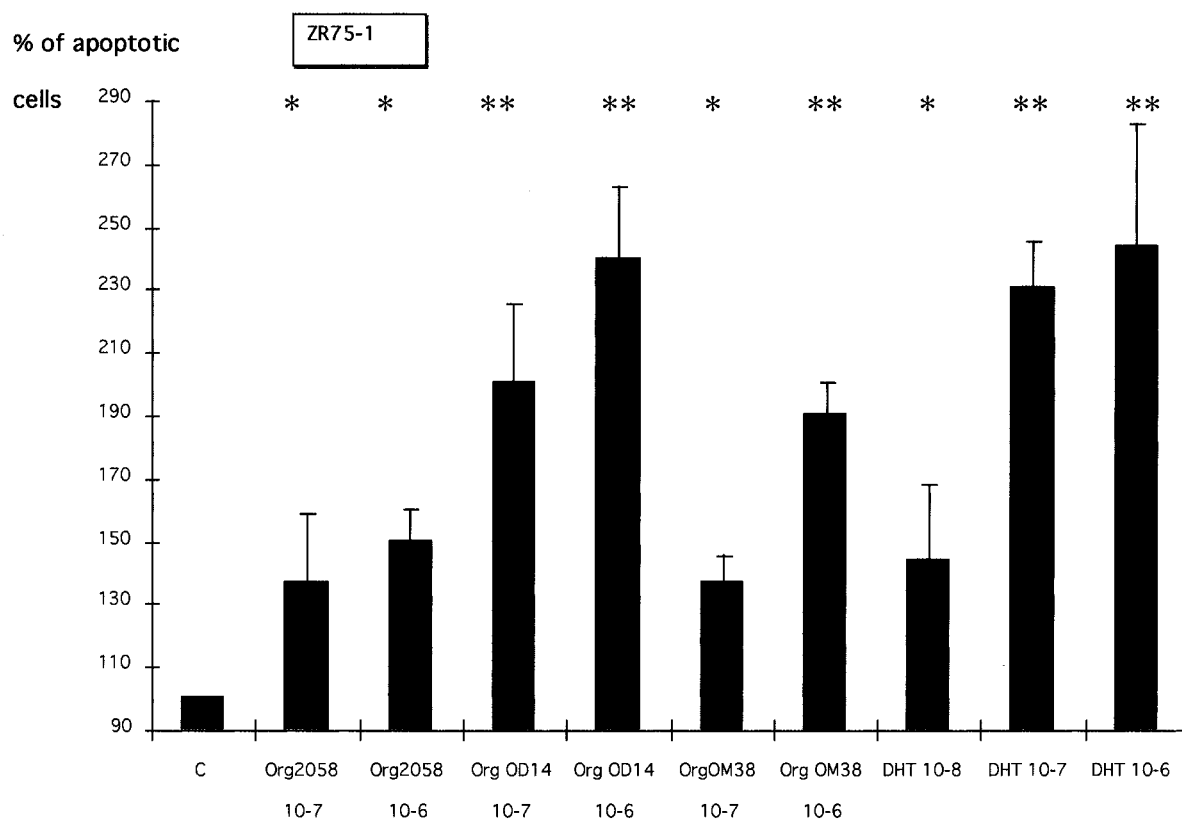
Fig. 3. Dose-dependent proapoptotic effects of the progestins and the androgen (DHT) in ZR75-1 cells. Results are expressed as means $\pm$ SEM of apoptotic cells as a percentage of the corresponding control after 3 days of hormonal treatment ($^*P < 0.05$, $^{**}P < 0.01$ vs control).

pression (Fig. 4). The addition of E2 to the progestins did not alter their inhibitory effects on bcl-2 expression (Fig. 4A). The antiestrogens had also an inhibiting effect on bcl-2 expression in ZR75-1. The antiestrogen, RU58 was, again, more potent than 4OHTAM (Fig. 4).

We have also studied the antiapoptotic protein, $bclx_L$. Its expression was slightly diminished by progestins and antiestrogens (Fig. 4) and increased by E2 (130%), whereas the level of the proapoptotic proteins, bak, did not vary (Fig. 4). RU486 had no clear effect on the expression of the bcl-2 family proteins (Fig. 4).

4. Discussion

Very few other studies have dealt with apoptosis in breast cancer cells until now. The published works concerned essentially the action of tamoxifen which was shown to be proapoptotic in MCF-7 cells [6] and Kyprianou et al. [5] have reported that apoptosis was induced in MCF-7 cells following E2 deprivation. An in vivo study has quantitated the apoptotic index in biopsies from breast cancer in women treated with tamoxifen or ICI182780 [19]. The increase in apoptosis was far greater with the 'pure antiestrogen', ICI

182780. Our in vitro results fit well with these in vivo data. The 'pure' antiestrogens were more potent than the triphenylethylene derivative. This may be due to their different activities at the ER level. Indeed, it was reported that ICI inhibited the intracellular traficking of ER from the cytoplasm to the nucleus [20] and thus rapidly decreased the level of ER in contrast to TAM which decreased much more slowly the ER content of the cells. Moreover other mechanisms may be acting, such as the various efficiency of TAM and ICI for the control of transcription of the genes involved in the control of apoptosis due to differential involvement of AP-1 or ERE sites, ERα or ERβ [21].

The various progestins increased the extent of apoptosis. The concentrations used for these various compounds corresponded to the pharmacological concentrations of progestins in clinical use. Org2058 is a pregnane derivative with high affinity for PR and devoid of androgenic and estrogenic activities. Tibolone (OrgOD14) is a well-studied normethyltestosterone derivative now offered in HRT. It has three principal metabolites, two with estrogenic properties and OrgOM38, its δ4 isomer, with progestogenic and androgenic properties in vitro [15]. The metabolization of OrgOD14 into OrgOM38 has been studied in the endometrium [15]. Therefore, OrgOD14 and OrgOM38
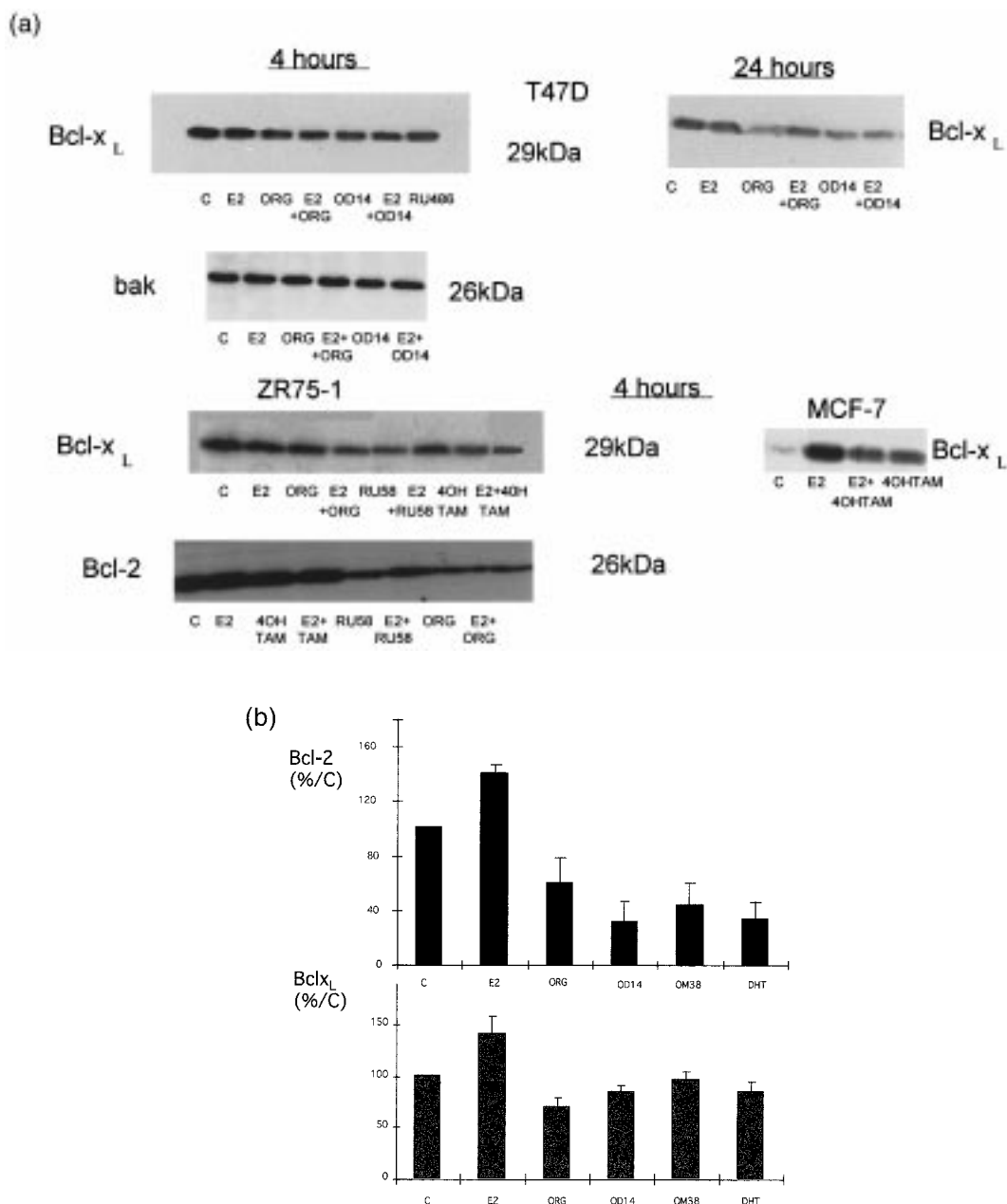
Fig. 4. Bcl-2 family proteins in breast cancer cells measured by western blots under E2, progestins and androgen treatments. A: western blots of the antiapoptotic proteins, bcl-2, bclx$_L$ and the proapototic protein, bak in breast cancer cells. B: bcl-2 and bclx$_L$ levels were quantitated in ZR75-1 cells by scanning and related to the level of actin. Results are expressed as a percentage of the control values (means $\pm$ SD).

were studied separately. We observed that the three progestins used were able to increase the number of apoptotic cells in T47-D and ZR75-1 cells. OrgOD14 was more potent than Org2058 and OrgOM38 in ZR75-1 cells containing PR and AR. These results suggest that the proapoptotic effects of the different progestins are mediated through PR and AR which is, for the latter, further demonstrated by the proapoptotic action of DHT.

Until now, only few studies have reported on the mechanisms of action of hormones on proteins

involved in the regulation of apoptosis. Among the genes controlling the occurrence of apoptosis, the bcl-2 family proteins play a central role. Bcl-2 is a member of a larger family of proteins involved in the control of apoptosis: bax, bcl-x$_S$, bak, which promote cell death and bcl-2, bcl-x$_L$ and some viral analogs which are antiapoptotic [7–10]. Most of these bcl-2 family proteins can physically interact with each other through a network of homo- and heterodimers, the relative proportions of which ultimately control the sensitivity of resistance of cells to apoptotic stimuli.

Bcl-2 is an antiapoptotic protein which is able to counteract apoptosis induced by numerous stimuli such as: UV, gamma radiation, heat shock, chemotherapies [22]. It is thus a key protein.

We had previously reported a cyclic variation of bcl-2 expression in normal breast tissue as well as in endometrium [23,24] and we and others have confirmed its hormonal regulation in MCF-7 cells [18,25]. We had also reported an inhibitory effect of progestin on bcl-2 expression in T47-D cells [18]. The same patterns were observed herein with other progestins, DHT and in all the breast cancer cell lines studied. Moreover, even if bcl-2 seem to be very sensitive to hormonal regulation, the other antiapoptotic protein, bclx$_L$ levels were increased by E2 and mildly decreased by progestins and antiestrogens. In the same cells, the proapoptotic proteins, bax and bak did not vary under hormonal treatment under the conditions of our cultures. This is in contrast to the data from Leung et al. [26] who have recently reported that bak levels were modulated by estradiol in MCF-7 as a function of the cell density of the cultures.

Since the ratio of the pro- and antiapoptotic proteins are decisive in cell survival [10,12], the effects of the hormones on apoptosis can be at least partially explained from their actions on the bcl-2 family proteins even if other mechanisms are concurrently involved. Indeed, Hurd et al. [27] have described opposite effects of E2 and progestins on p53 levels and retinoblastoma protein phosphorylation in breast cancer cells [28]. It is very likely that many other mechanisms can operate to explain the proapoptotic effects of antiestrogens and progestins. However the data reported by Teixeira et al. demonstrated the importance of the bcl-2 pathways since an E2 treatment can reverse the effect of a chemotherapy on breast cancer cells by inducing bcl-2 expression [25].

5. Conclusions

From these results, it appears that progestins and antiestrogens are proapoptotic in breast cancer cells.

Bcl-2 is the most hormone-dependent protein, induced by E2 and inhibited by progestins and DHT in breast cancer cell lines. These steroids also moderately altered the expression of bclx$_L$ whereas, the proapototic proteins seem to be constitutively expressed.

This proapoptotic action of antiestrogens and progestins in breast cancer cells is certainly a favourable effect in terms of carcinogenesis.

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