

REVIEW

Antioestrogen-mediated cell cycle arrest and apoptosis induction in breast cancer and multiple myeloma cells

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Abstract

Antioestrogens (AEs) are synthetic molecules that block proliferation and induce apoptosis in breast cancer (BC) cells, principally by competing with oestradiol for binding to oestrogen receptors. Their antiproliferative activity and their proapoptotic capacity are well documented and a small number of molecules of this class are currently used clinically for the treatment of BC. AEs also inhibit cell cycle progression and/or induce apoptosis in multiple myeloma (MM) cells. Encouraging preliminary results have been obtained with patients and on xenografts with MM, providing a rational basis for the clinical use of AEs. This review focuses on antioestrogen-mediated signalling for blocking targets involved in the cell cycle, survival and apoptosis in both BC and MM cells. Improvement in our understanding of the mechanisms underlying the relationships between these compounds and their targets should lead to more beneficial therapeutic strategies.

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Introduction

The development of hormonal treatments has led to an extensive search for highly effective and well-tolerated molecules. Antioestrogens (AEs; Fig. 1) are synthetic compounds entirely fulfilling these criteria. They inhibit the activation of two different but related ligand-induced transcription factors of the nuclear receptor superfamily: the oestrogen receptors (ER α ; Green *et al.* 1988) and ER β (Kuiper *et al.* 1996, Mosselman *et al.* 1996). ER α is the master regulator of breast cancer (BC) tumour behaviour and is much more strongly expressed in BC tumours than ER β (Dahlman-Wright *et al.* 2006). It is currently thought that ER β represses growth by inhibiting ER α -mediated transcriptional activity (Hall & McDonnell 1999, Liu *et al.* 2002, Lindberg *et al.* 2003, Faulds *et al.* 2004, Strom *et al.* 2004), and the balance between the levels of ER subtypes appears as an important regulator of the mitogenic activity of 17 beta oestradiol (E₂; Matthews *et al.* 2006, Holst *et al.* 2007). AEs block the binding of E₂ to ER α and inhibit the E₂-induced effects mediated by

the two ERs. However, this model cannot account for all the effects of AEs. Indeed, it has been shown that some ER α receptors are located in the cancer cell membrane, facilitating E₂- and DNA-independent ER activation through crosstalk with the growth factor-induced activation of cell-surface tyrosine kinase receptors (Moss *et al.* 1997, Falkenstein *et al.* 2000, Hanstein *et al.* 2004). Thus, two different types of signalling may be mediated by E₂, with nuclear (genomic) and extra-nuclear (non-genomic) effects (for reviews see (O'Malley 2005, Song *et al.* 2006)).

The ER β subtype has been identified not only in the mammary gland and both male and female reproductive tissues, but also in the colon, brain, pancreas and haematopoietic cells of the myeloid and lymphoid lineages (Enmark & Gustafsson 1999, Shim *et al.* 2003). Several groups have shown that AEs block proliferation and induce apoptosis in multiple myeloma (MM) cells, as in BC cells (Treon *et al.* 1998, Otsuki *et al.* 2000, Gauduchon *et al.* 2005, Olivier *et al.* 2006). In recent years, we have focused on deciphering the molecular mechanisms by which

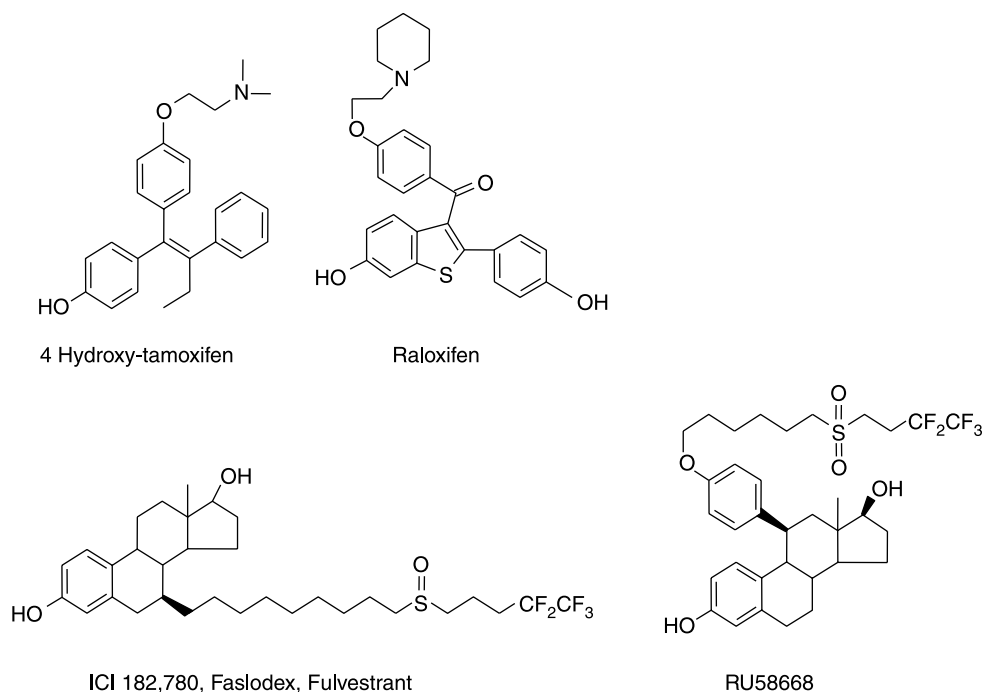


Figure 1 Chemical structures of antioestrogens. 4-hydroxy-tamoxifen (4-HT), the active metabolite of tamoxifen and raloxifen belong to the selective oestrogen receptor modulator (SERMs) family. The members of this family act either as E_2 -agonists or -antagonists depending on ER subtype and cell context. ICI 182 780 and RU58668 are selective oestrogen receptor down-regulators (SERDs), acting as pure antioestrogens.

AEs, both selective oestrogen receptor modulators (SERMs), such as 4-hydroxy-tamoxifen (4-HT), and selective oestrogen receptor down-regulators (SERDs), such as ICI 182 780 (ICI or Faslodex[®]) and RU58668 (RU), arrest the cell cycle and/or induce apoptosis in hormone-dependent BC and MM cells. We provide here a review of some of our data. Despite the use of different molecular partners, the signalling pathways involved in cell cycle arrest and the induction of apoptosis by these AEs are similar regardless of the type of cancer cell considered.

Oestrogens and antioestrogen effects in mammary gland cells

Growth and anti-apoptotic properties of oestrogens

ERs are the predominant target of E_2 in the mammary gland. We will not describe the mechanism of action of these transcription factors in detail here, because many reviews have covered the latest discoveries concerning nuclear and membrane ER activities and clinical studies (Osborne & Schiff 2005, Heldring *et al.* 2007, Jordan & Brodie 2007, Lonard & O'Malley 2007, Popov *et al.* 2007). In the mammary gland, E_2 promotes cell

proliferation in both normal and transformed epithelial cells by modifying the expression of hormone-responsive genes involved in the cell cycle and/or programmed cell death. In ER-positive human BC MCF-7 cells, the principal action of E_2 is the induction of proliferation through the stimulation of G1- to S-phase transition. This induction is associated with the rapid and direct up-regulation of *c-myc*, which controls cyclin D1 expression, the activation of cyclin-dependent kinase (Cdk) and the phosphorylation of retinoblastoma protein (pRb; Fig. 2; Altucci *et al.* 1996, Foster & Wimalasena 1996, Hurd *et al.* 1997, Prall *et al.* 1997, Doisneau-Sixou *et al.* 2003). Evidence for a key role of *c-myc* in E_2 action has been obtained from experiments showing that antisense oligonucleotides inhibit E_2 stimulated BC cell proliferation (Watson *et al.* 1991) and that the induction of *c-Myc* in AE-arrested cells can mimic the effects of E_2 by reinitiating cell cycle progression (Prall *et al.* 1998). The mitogenic and anti-apoptotic activities of E_2 are combined through the activation of *bcl-2* gene expression and the production of smaller amounts of p53 and caspase-3 in primary cultures of normal mammary epithelial cells (Somai *et al.* 2003). Moreover, E_2 /ER α complexes bind directly to a cAMP-response element and a more distal Sp1 site on the cyclin D1 promoter, leading to an increase in cyclin D1 mRNA levels (Altucci *et al.* 1996, Prall *et al.*

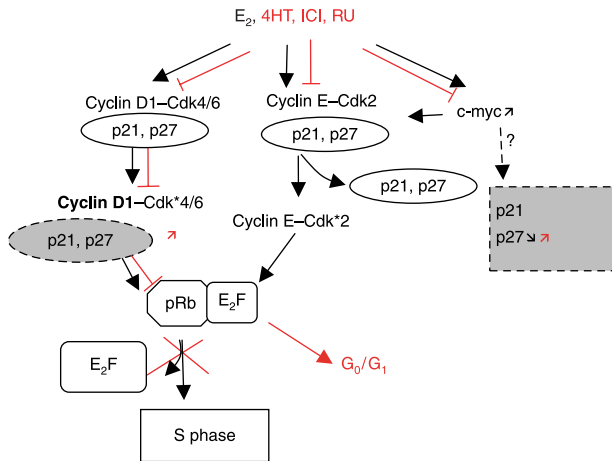


Figure 2 Schematic illustration of the activities of oestradiol and antioestrogens in MCF-7 cells. Following E₂ treatment, a rapid transcription-mediated increase (↗) in c-myc expression occurs, facilitating cyclin E–Cdk2 activation and entry into S phase. Concomitantly, E₂ down-regulates (↘) p21^{Cip1} and p27^{Kip1} and increases cyclin D1 levels. The formation of complexes containing Cdk4 and sequestering Ckis is enhanced. Ckis levels are down-regulated upon c-myc expression. All these events initiate the phosphorylation of pRb. Cyclin D1 in bold typeface: increase in cyclin D1 levels with activated cyclin–Cdk complexes; *increase in Cdk activity. In grey boxes, low levels of Cki. In black, E₂-mediated effects. In red, AE-mediated inhibition leading to cell cycle arrest in G₀/G₁. Refer to text for details and references.

1997, Sabbah *et al.* 1999, Castro-Rivera *et al.* 2001). ERβ competes with ERα in the induction of cyclin D1 transcription (Liu *et al.* 2002).

E₂ also rapidly activates cyclin E–Cdk2 complexes and, by relieving the inhibition mediated by the natural Cdk inhibitor (Cki) p21^{Cip1}, accelerates the G1 to S transition (Foster & Wimalasena 1996, Planas-Silva & Weinberg 1997, Prall *et al.* 1997, 2001). Another key element of the regulatory apparatus of the G1 phase is Cki p27^{Kip1}, which inhibits both Cdk2 activity in G0 and early G1 and acts as an assembly factor for cyclin D–Cdk4/6 complexes in early G1 (see Sherr & Roberts 1999, for a review). The functional expression of p27^{Kip1} is essential for normal proliferative responses in the mammary epithelium (Muraoka *et al.* 2001). E₂ activates cyclin E–Cdk2 complexes in MCF-7 cells, and the G1–S transition is associated with a decrease in the levels and activities of p27^{Kip1} and p21^{Cip1} (Foster & Wimalasena 1996, Prall *et al.* 1997, Foster *et al.* 2001; Fig. 2). Moreover, E₂ up-regulates Bcl-2 protein levels in MCF-7, T47-D and ZR-75-1 BC cells (Gompel *et al.* 2000).

Growth inhibition of AEs

The growth-inhibiting effects of AEs in ER-positive BC cells and normal epithelial mammalian cells result from cell cycle arrest in the G₀/G₁ phase (Musgrove *et al.* 1993, Doisneau-Sixou *et al.* 2003, Somai *et al.* 2003, Jamerson

et al. 2004). This antiproliferative activity is associated with the inhibition of Cdk activity and a decrease in pRb phosphorylation (Watts *et al.* 1995). In addition to the cell cycle regulator c-Myc (Jamerson *et al.* 2004), AEs target cyclin D1, cyclin E, p21^{Cip1} and p27^{Kip1} (Caldon *et al.* 2006). Both Cki molecules are weakly expressed in mammary cells, such as BC cells, but exposure to 4-HT, ICI or RU strongly increases their expression (Cariou *et al.* 2000, Slingerland & Pagano 2000, Foster *et al.* 2003). Inhibition of the expression of either p21^{Cip1} or p27^{Kip1} with antisense oligonucleotides maintains Cdk2 in an active state and prevents AE-mediated G1 blockade (Cariou *et al.* 2000, Carroll *et al.* 2000). AEs from different classes have different effects on ERα-positive BC cells: 4-HT blocks these cells in the G1 phase, whereas ICI and RU render these cells quiescent (Carroll *et al.* 2000, 2003; Fig. 2).

Pro-apoptotic activity of AEs

Mitochondria are known to play an integral role in apoptosis, and it was demonstrated that tamoxifen (Tam) induces increased rate of apoptosis after long-term treatment (Thiantanawat *et al.* 2003). Caspase-3 and caspase-7 are the most commonly studied effector caspases. When activated, they directly target proteins involved in cell integrity and are responsible for the final stage of cell death (Strasser *et al.* 2000). Specific caspase activation cascades are dependent on apoptotic stimuli (Riggins *et al.* 2005a). T47-D and ZR-75-1 cells contain caspase-3 that is absent from MCF-7 cells (Janicke *et al.* 1998). In MCF-7 cells, apoptosis proceeds via the sequential activation of caspase-7 and caspase-6 (Table 1). However, Tam increases the activity of caspases-3 in ER-negative BC cells (Mandlekar *et al.* 2000) and of caspase-9, caspase-6 and caspase-7 in MCF-7 cells (Thiantanawat *et al.* 2003) with concomitant down-regulation of Bcl-2 and up-regulation of Bax. In T47-D cells, Tam treatment activates caspase-3 (Ellis *et al.* 2003). It was also shown that in MCF-7 cells and rat mammary tumours, well known to resemble human BC tumours, Tam causes activation of caspase-8, a caspase normally related to membrane death receptor activation (Mandlekar *et al.* 2000). Indeed, caspase inhibitor z-VAD-fmk completely blocks Tam-induced apoptosis (Mandlekar *et al.* 2000). Although intriguing, these data suggest that AEs may affect both intrinsic (via caspase-9 activation) and extrinsic (via caspase-8 activation) apoptotic pathways.

Both SERMs like Tam and SERDs like ICI and RU induce moderate apoptosis in MCF-7 cells (Kandouz *et al.* 1999, Gompel *et al.* 2000) accompanied by poly-(ADP-ribose) polymerase (PARP) cleavage. These apoptotic properties are observed at micromolar concentrations of AEs in all BC cells, whereas cell cycle blockade occurs at nanomolar concentrations of AEs, suggesting that these two processes are independent and triggered by separated pathways. It was recently shown that

Table 1 Comparison of the apoptotic effects of selective oestrogen receptor modulators (SERMs) and selective oestrogen receptor down-regulators (SERDs) in breast cancer and multiple myeloma cells

	BC cells				MM cells			
	MCF-7		T47-D		RPMI 8226		LP-1	
	4-HT	RU	4-HT	RU	4-HT	RU	4-HT	RU
Death ligand/receptor								
TNF/TNFR	↗	↗	ND	ND	ND	ND	ND	ND
TRAIL/DR4/5	ND	ND	ND	ND	→ (a)	→	→ (a)	→
FasL/Fas	↗	↗ (a)	↗	↗ (a)	→	→ (a)	→	→ (a)
Bcl-2 family								
Bcl-2	↘	↘ (b)	↘	↘	→	→	→	→
Bcl-XL	ND	ND	ND	ND	→	→ (a)	→	→ (a)
Bax	→ (a)	→ (a)	→ (a)	→ (a)	→	→ (a)	→	→ (a)
Bik	→ (a)	↗ (b)	ND	ND	ND	ND	ND	ND
Mcl-1	→ (a)	≪ (b)	ND	≪ (b)	ND	≪	↘	↘ (a)
Caspase								
Caspase-3	ab	ab	↗	↗ (a)	↗	↗	↗	↗
Caspase-4	ND	ND	ND	ND	ND	↗	ND	→
Caspase-7	↗	↗	ND	ND	ND	ND	ND	ND
Caspase-8	↗	ND	ND	ND	↗	↗	↗	↗
Caspase-9	↗	ND	ND	ND	↗	↗	↗	↗

↗, enhanced or activated for caspase; ↘, decreased; →, identical; ≪, cleaved; ND, not determined; (a), our unpublished results; (b), obtained with ICI; ab, absent.

micromolar concentrations of ICI, 4-HT and raloxifen but also E₂ induce cell death due to the formation of reactive oxygen species (ROS), triggering classical caspase-dependent apoptosis (Mandlekar *et al.* 2000, Obrero *et al.* 2002, Kallio *et al.* 2005). Tam-induced production of ROS associated with release mitochondrial cytochrome *c* appears responsible for the early apoptotic effects of Tam. In addition, Tam-induced rapid death of MCF-7 cells is mediated by extracellular signal-regulated kinase signalling that can be abrogated by E₂ but not by ICI in an ER-independent mechanism (Zheng *et al.* 2007). E₂-induced apoptosis also occurs in BC cells resistant to long-term oestrogen deprivation (aromatase inhibitors) and to AEs (Gottardis *et al.* 1988, Lewis *et al.* 2005) and E₂ treatment may constitute a new clinical strategy following exhaustive antihormone therapy (Jordan *et al.* 2005).

The E₂-responsive cell lines MCF-7, T47-D and ZR75.1 all express the surface tumour necrosis factor (TNF), TRAIL and Fas-L receptors, the key mediators of the extrinsic apoptotic pathway the ligand-dependent activation of which generally results in rapid cell death. Unlike E₂, Tam up-regulates Fas-L expression (Nagarkatti & Davis 2003). However, in MCF-7 cells subjected to long periods of E₂ deprivation, E₂-induced apoptosis is correlated with Fas-L expression (Song & Santen 2003). TNF-mediated apoptosis in MCF-7 cells can be abolished by E₂ treatment, via an increase in production of the anti-apoptotic mediator Bcl-2 (Burow *et al.* 2001). Both ICI and Tam enhance TNF-induced cell death, by triggering TNF receptor production and expression at the cell surface (Smolnikar *et al.* 2000). Thus, significant crosstalk

occurs between the TNF- and AE-induced apoptosis pathways (Table 1).

Contradictory data have been published regarding the effects of AEs on the members of the Bcl-2 family. E₂ has been shown to up-regulate the anti-apoptotic Bcl-2 protein in oestrogen-dependent BC cells (Gompel *et al.* 2000), an effect counteracted by both Tam and pure AEs (Kandouz *et al.* 1999, Zhang *et al.* 1999, Ameller *et al.* 2003, Somai *et al.* 2003, Thiantanawat *et al.* 2003). In MCF-7 cells, E₂ has been shown to decrease levels of the pro-apoptotic Bak protein, whereas aromatase inhibitors or AEs have been shown to increase Bak levels (Leung *et al.* 1998, Thiantanawat *et al.* 2003). However, other studies have reported no effects of oestrogens or AEs on levels of Bax or Bak protein (Kandouz *et al.* 1999, Zhang *et al.* 1999, Gompel *et al.* 2000). There is therefore no clear consensus concerning the roles of these molecules in AE-mediated apoptosis. Bcl-XL is produced in small amounts, unaffected by AEs in BC cells. The pro-apoptotic protein Bik is down-regulated by E₂ and up-regulated by ICI, but not by the SERMs 4-HT and raloxifen (Frasor *et al.* 2003, Hur *et al.* 2004, Frasor *et al.* 2006).

Thus, in BC cells, the pro-apoptotic activity of AEs is triggered through multiple pathways that are whether dependent or independent on ER signalling.

p53

The tumour suppressor p53 is inactivated in 30% of all BCs. Together with p63 and p73, two other proteins from

the same family, p53 is a global regulator of cell cycle checkpoints and apoptosis. MCF-7 and ZR.75.1 cells contain wild-type p53, whereas T47-D cells produce a mutated form unable to bind DNA. As p53-regulated target genes, such as those encoding p21^{Cip1} and the pro-apoptotic Bax protein, are activated in response to various stimuli, and since ER α binds directly to p53 (Liu *et al.* 2006, Sayeed *et al.* 2007), it was suggested that p53 is involved in the response to AEs in BC cells. However, the effects of AEs on p53 remain unclear. It has been reported that the depletion of Tam and E₂ decreases p53 levels and that Faslodex can reverse the E₂-mediated increase in p53 levels in T47D cells (Dinda *et al.* 2002). The group responsible for this study also showed that E₂ and Tam induce the transcriptional activity of the P1 promoter of p53 in MCF-7 cells, whereas Faslodex does not (Hurd *et al.* 1997). These data contrast with data from other studies reporting no change in p53 levels in response to Tam, despite the significant induction of apoptosis and pRb dephosphorylation (Fattman *et al.* 1998, Zhang *et al.* 1999). These differences may be due to the subcellular distribution of p53 within cells (Molinari *et al.* 2000, Lilling *et al.* 2002). Furthermore, the product of the *Mdm2* oncogene, which is overexpressed in various cancers, and encodes an E3 ubiquitin ligase, regulates the stability of ER α by forming a ternary complex with p53 (Duong *et al.* 2007). In turn, p53 and Mdm2 regulate the E₂-dependent down-regulation of ER α and, probably, susceptibility to AE (Duong *et al.* 2007). Indeed, since ER α inhibits p53-mediated transcriptional repression (Sayeed *et al.* 2007), it can be emphasised that AE induces apoptosis in BC cells by, at least in part, relieving the inhibitory activity of ER α on p53 activity.

Survival proteins

The deregulation and inappropriate activation of phosphoinositol-3-kinase (PI3K) signalling and its downstream targets, Akt and nuclear factor- κ B (NF- κ B) have been linked to cancers (Luo *et al.* 2003). The transcriptional activity of ER α is activated by constitutively activated Akt independently on the presence of E₂ but in a PI3K-dependent manner since the PI3K inhibitor wortmanin inhibits both phosphorylation (Ser-167) and activation of ER α (Sun *et al.* 2001). Such activity is abolished by ICI but not completely by Tam, implicating that Akt-activated ER α contributes to Tam resistance (Michalides *et al.* 2004, Riggins *et al.* 2005*a,b*). Moreover, the p85 subunit of PI3K binds to E₂-free ER α (Sun *et al.* 2001) and the overexpression of Akt protects against Tam-induced apoptosis (Campbell *et al.* 2001). However, the role of Akt in BC cells is still unclear: for example, in MCF-7 and MDA-MB-231 cells, E₂ induces PI3K/Akt activity via an ER-independent signalling pathway that is not affected by AEs (Tsai *et al.*

2001). However, Akt must be considered as a target of choice in BC therapy since its suppression prevents endocrine therapy resistance (Glaros *et al.* 2006).

The NF- κ B family of transcription factors controls various aspects of the immune system. Among the NF- κ B proteins, some possess a Rel homology domain (RHD), such as RelA (also named p65), cRel and RelB, whereas others such as the p105/p50 and the p100/p52 do not. They form hetero-complex proteins by associating with inhibitors (I κ B) of NF- κ B, which bind to and inhibit DNA binding of the RHDs (Hayden & Ghosh 2004). These complexes are activated through the activation of an externally activated I κ B kinase that phosphorylates I κ B, leading to its proteasome-mediated degradation and enabling NF- κ B-mediated transcription following nuclear translocation. As shown previously, NF- κ B binds ER (Ray *et al.* 1994) and ER inhibits NF- κ B pathway by interacting at various steps of the activation cascade (see Kalaitzidis & Gilmore 2005 and references herein). NF- κ B activity is repressed by E₂ and AEs (Nakshatri *et al.* 1997, Pratt *et al.* 2003). Members of the NF- κ B/Rel family form dimers that regulate the transcription of genes encoding regulators of proliferation (*c-myc* and cyclin D1) and inhibitors of apoptosis (Chen & Greene 2004). According to several observations, NF- κ B activity is important for mammary carcinogenesis. Several reports have indicated that SERMs (like Tam and raloxifen) as well as SERDs (like ICI) inhibit the NF- κ B activity in various cell types (see Biswas *et al.* 2005 for a review). Thus, most results suggest that inhibiting the NF- κ B pathway in BC could have therapeutic effects principally in Tam-resistant tumours treated with E₂ (Jordan 2004) or ICI (Riggins *et al.* 2005*a,b*).

Effects of oestrogens and AEs in MM

Expression of oestrogen receptors

Oestrogens influence the differentiation, proliferation and survival of haematopoietic cells of the B lineage (Smithson *et al.* 1998, Medina *et al.* 2000, Grimaldi *et al.* 2002) and increase the number of plasma cells and the capacity of these cells to synthesise immunoglobulins (Molina *et al.* 1999). It is therefore not surprising that normal plasma cells and plasma cells from tumours both produce oestrogen receptor mRNA and protein. Transcripts for both ER subtypes are present in MM cell lines (Fig. 3; Otsuki *et al.* 2000), together with protein isoforms (Treon *et al.* 1998, Gauduchon *et al.* 2005, Maillard *et al.* 2006, Olivier *et al.* 2006). However, these proteins are expressed in small amounts and are difficult to detect by immunoblotting. This is particularly true for ER β , for which the quality of commercially available antibodies is low. In contrast to previous

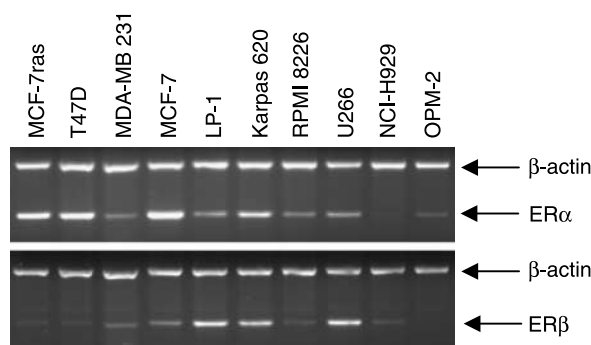


Figure 3 Oestradiol receptors in breast cancer and multiple myeloma cells. BC (MCF-7ras, T47D, MDA-MB231, MCF-7) and MM (LP-1, Karpas 620, RPMI 8226, U266, NCI-H929, OPM-2) cells were switched to phenol red-free media containing 10% charcoal-dextran stripped fetal calf serum 3 days before total RNA isolation with Trizol reagent (Invitrogen). Purified RNA was reverse transcribed with M-MuLV reverse transcriptase (Invitrogen), according to the manufacturer's instructions. PCR was carried out with *Platinum Taq* polymerase (Invitrogen). The primer sequences were as follows: β -actin (forward: 5'-tgacggggtcaccacactgtgccatcta-3', reverse: 5'-ctagaagcatttgcgggtgacgatggagg-3'), ER α (forward: 5'-gtggcctcgttttagtc-3', reverse: 5'-tcacctgtgagagaa-cag-3'), ER β (forward: 5'-tcacatctgtatgcggaacc-3', reverse: 5'-cctaaccactccgaagtcgg-3').

reports (Olivier *et al.* 2006), we found that the levels of ER α and β in MM cells never reached those in MCF-7 cells (Gauduchon *et al.* 2003, 2005). As a result, hormone-binding detection assays based on the use of tritiated high-affinity oestrogens have always been unsuccessful in our hands (unpublished data).

Effects of oestrogens and AEs on MM cell proliferation

E₂ and AEs are anti-proliferative in MM cells (Treon *et al.* 1998, Otsuki *et al.* 2000, Gauduchon *et al.* 2005, Olivier *et al.* 2006). At high concentrations (0.5 to 50 μ M), AEs arrest the cell cycle in MM cells at the G1 (Gauduchon *et al.* 2005, Table 2) or the G2 phase (Olivier *et al.* 2006) depending on the type of AE used. However, in all cases,

Table 2 Antioestrogen-mediated cell cycle arrests and induction of apoptosis in multiple myeloma cells

	4-HT	RU	ICI
U266	G1 arrest + apoptosis	No response	nd
RPMI 8226	G1 arrest + apoptosis	Apoptosis	Apoptosis
NCI-H929	G1 arrest + apoptosis	Apoptosis	Apoptosis
OPM-2	No response	G1 arrest	G1 arrest
Karpas 620	No response	No response	nd
LP-1	G1 arrest + apoptosis	G1 arrest	G1 arrest

nd, not determined.

cell cycle arrest is mediated by the down-regulation of c-myc followed by the up-regulation and redistribution of p21^{Cip1} and p27^{Kip1} within the cyclin D/E/Cdk complexes, leading to the hypophosphorylation of pRb (Gauduchon *et al.* 2005, Olivier *et al.* 2006, Table 2). A similar situation is observed in BC cells, except that the mediators of AE-mediated cell cycle arrest appear to be cell specific (Sola & Renoir 2007; Fig. 4).

Effects of AEs on the apoptosis of MM cells

In addition to their anti-proliferative activity, AEs also trigger MM cell apoptosis at micromolar concentrations (Table 2; Treon *et al.* 1998, Otsuki *et al.* 2000, Gauduchon *et al.* 2005, Olivier *et al.* 2006). The fact that U266 and Karpas 620 cell lines are listed as not responsive to RU in the face of being ER positive (Fig. 3) may be due to an insufficient (or no) ER protein expression despite the presence of the mRNAs. In agreement with that, using western blot analysis, we did not see the ER protein in Karpas 620 cells (Gauduchon *et al.* 2005). The lack of AE response could be also due to the absence of co-activators or overexpression of co-repressors as suggested previously (Shim *et al.* 2003, Shim *et al.* 2006, Sola & Renoir 2006). Moreover, the response to 4-HT may be attributable to the production of ROS at micromolar 4-HT concentration.

Cell cycle arrest and apoptosis induction are independent phenomena occurring simultaneously following the treatment with 4-HT and RU or ICI (Gauduchon *et al.* 2005). AEs activate the mitochondrial intrinsic death pathway (Treon *et al.* 1998, Otsuki *et al.* 2000, Gauduchon *et al.* 2005, Olivier *et al.* 2006) and may also activate the endoplasmic reticulum death pathway, according to plasma cell physiology. Following the AE-induced apoptotic signal, cytochrome *c* is released from the mitochondria and activates caspase-9 or caspase-4 and then caspase-3 (Fig. 5). Apoptosis signalling is amplified by the recruitment of caspase-8 and the cleavage of Bid (Maillard *et al.* 2006), but does not require the extrinsic death receptor pathways mediated by Fas/FasL and DR4/5/TRAIL (our unpublished data).

Effects of AEs on MM survival signalling

MM cells display constitutive activation of survival signalling pathways, including the Ras/mitogen-activated protein kinase (MAPK), Janus kinase (Jak)/signal transducer and activator of transcription (STAT), NF- κ B, phosphoinositol-3-kinase (PI3K)/Akt pathways (Bommert *et al.* 2006). To be effective, anti-myeloma agents must therefore target one or several of these pathways. Moreover, the ability to inhibit survival pathways seems to be essential for the treatment of MM resistant to conventional drugs (Hideshima *et al.* 2007).

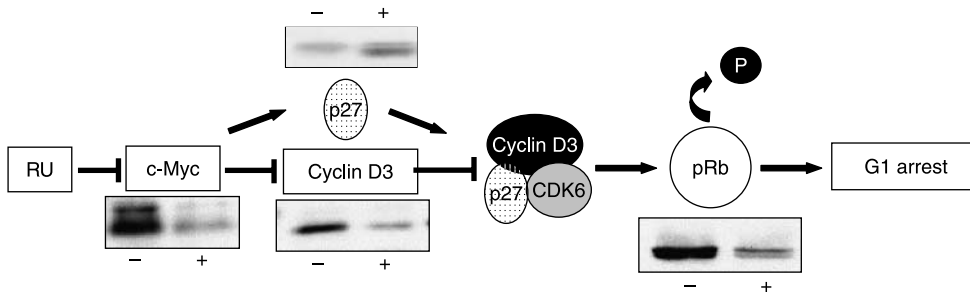


Figure 4 Mechanism of cell cycle arrest in multiple myeloma cells. Cultured LP-1 MM cells were treated with 10 μ M RU (+) for 24 h or left untreated (-) and harvested. Total proteins were extracted, separated by SDS-PAGE, transferred onto membranes then incubated with various antibodies (anti-c-Myc, anti-p27^{Kip1}, anti-cyclin D3, anti-phospho-pRb antibodies). In MM cells, RU down-regulates c-Myc, its primary target, and consequently cyclin D3. In the mean time, there is an up-regulation of the cki p27^{Kip1} that binds the cyclin D3/CDK6 complexes. Finally, the inhibition of cdk activity leads to the hypophosphorylation of pRb and the cell cycle arrest at the G1 phase.

As in some BC cells, it has been shown that the NF- κ B p65 subunit is associated with ER α in the nucleus of MM cell lines although weakly. Upon raloxifen treatment, there is a transient increase in the p65/NF- κ B interaction and simultaneously a loss of p65 binding on specific gene promoters such as mip-1 α (Olivier *et al.* 2006). We do not have evidence of any interaction between ER α and NF- κ B subunits in MM cells after RU treatment. Nevertheless,

RU induces an inhibition of I κ B kinase activity in RPMI 8226 cells that undergo apoptosis (Fig. 6) and inhibits p65 binding on its DNA target (data not shown). RU also interferes with the STAT3 and Akt pathways by modulating the phosphorylation of signal transducers (Fig. 6). However, the demonstration that the inhibition of these survival pathways is necessary for apoptosis triggering needs some additional work.

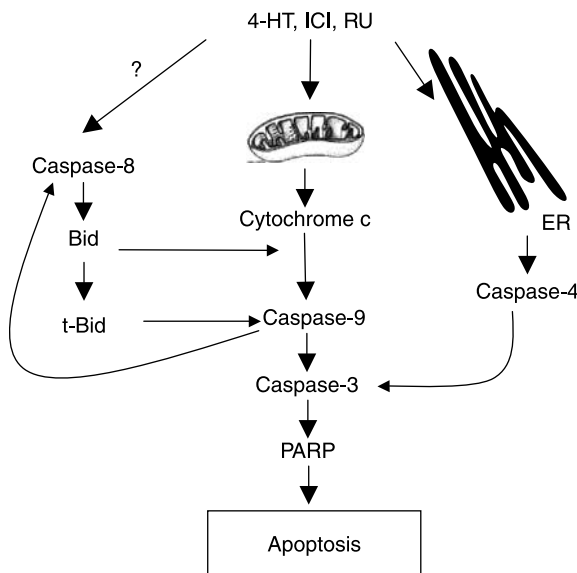


Figure 5 Schematic illustration of the apoptotic pathways activated by antioestrogens in breast cancer and multiple myeloma cells. At micromolar concentrations, AEs activate the intrinsic mitochondrial pathway by inducing the release of cytochrome c and the successive activation of caspase-9 and caspase-3 in MM cells (replaced by caspase-6/7 in MCF-7 cells), leading to the cleavage of PARP. In MM cells, RU and ICI also induce apoptosis by activating caspase-4, which, in turn activates caspase-3. Finally, the activation of caspase-8 leads to the cleavage of Bid, resulting in the amplification of caspase-9 activation, further increasing the level of apoptosis. ER, endoplasmic reticulum.

Effects of AEs in MM xenografts

Although not reproducing the exact human pathology, a commonly used model using s.c. injection of RPMI 8226 cells in nude mice was developed (Chauhan *et al.* 2002, Cuendet *et al.* 2004). This type of tumour preserves neo-angiogenic characteristics necessary for the development of the disease. Using a stealth liposomal delivery system, we have demonstrated that both RU (Maillard *et al.* 2005, 2006) and 4-HT (manuscript in preparation) induced a decrease of RPMI 8226 tumour growth. In addition, AE-targeted to solid tumours by delivery systems such as those generated for RPMI 8226 (Maillard *et al.* 2006) and MCF-7 xenografts induce strong anti-angiogenesis, potentiating the anti-proliferative and apoptotic activities of AEs (Reinor *et al.* 2006).

Conclusions

These data demonstrate that, in ER-positive BC and MM cell lines, both types of AE arrest the cell cycle and/or induce apoptosis via two independent and separate but similar pathways. Indeed, AEs initially target c-myc and the cell cycle machinery, but cyclin D3 and p27^{Kip1} in MM cells replacing the cyclin D1 and p21^{Cip1} targeted in BC cells. AEs trigger the mitochondrial intrinsic death pathway in BC cells and

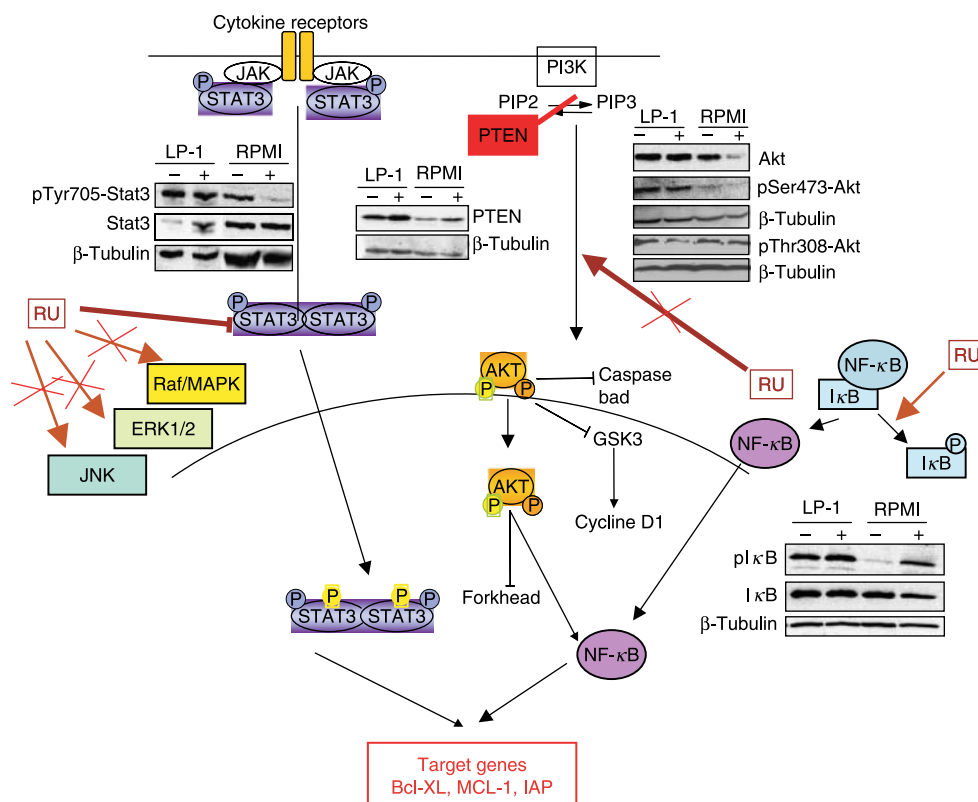


Figure 6 Effects of RU on survival pathways activated in MM cells. MM cells are characterised by the constitutive activation of pathways involved in survival signalling: PI3K/Akt, Jak/Stat3, Ras/MAPK or NF-κB. This is illustrated by the constitutive phosphorylation of proteins involved in the signalisation cascade. Using phospho-specific antibodies, we analysed the effects of RU on two MM cell lines: LP-1 and RPMI 8226. RU treatment of LP-1 cells blocks cell cycle but has no effects on cell death; by contrast, RU induces RPMI 8226 cell apoptosis but had no effects on cell cycle. The Akt pathway is activated in both cell lines. In LP-1 cells, RU has no effects on pAkt (Ser473 and Thr 308) or the phosphatase PTEN. In RPMI 8226 cells, RU treatment leads to a decrease of Akt and in turn in its phosphorylated form. RPMI 8226 and LP-1 cells display a constitutive activity of p65 NF-κB subunit binding on DNA (not shown). RU treatment has no effects on pIκB, the inhibitor of NF-κB, but up-regulates this form in RPMI 8226. RU has no effects on the MAPK, ERK1/2 and JNK pathways. On the Jak/Stat3 pathway, preliminary results indicate that in RPMI 8226 cells, RU down-regulates pTyr705-Stat3. Experiments are ongoing to determine whether the inhibition of the Jak/Stat3 is necessary for apoptosis to occur.

both the mitochondrial and endoplasmic reticulum death pathways in MM cells. ER ligands act in synergy with conventional molecules used in the treatment of cancer, such as dexamethasone, and bortezomib (Jordan 2007, Jordan & Brodie 2007), and encouraging preliminary results for MM xenografts (Maillard *et al.* 2006) and MM patients (Fassas *et al.* 2001) provide a rationale for the use of AEs in the clinical treatment of MM. AE compounds may be of greater value for the treatment than the original restriction of their use to E₂-dependent BCs might suggest. The chief unresolved question concerns the involvement of ER in mediating the anti-proliferative effects of AEs. Our working hypothesis is that in both BC and MM cells, AE-mediated cell cycle arrest is ER dependent, whereas apoptosis is ER independent. ERs require various

co-regulatory proteins for their function (Hall & McDonnell 2005), and the cell-specific expression of these molecules may account for differences in the actions of different ER antagonists. In addition, the discovery of membrane ER-triggered effects and cross-talk with growth factors, NF-κB and kinase cascade signalling (Gee *et al.* 2003, Kalaitzidis & Gilmore 2005, Martin *et al.* 2005, Nicholson *et al.* 2005) has greatly complicated our view of the mechanism of action of ER ligands. However, due to the large tissue distribution of ERs (and mainly ERβ), it is likely that AEs could have some positive or negative effects on various other cancers such as uterine, ovary, lung and colon cancers due to crosstalk with the above-mentioned pathways (AP1, SP1, EGFR, NF-κB, Akt/PKB). More work is clearly required to improve our understanding of the

activities of ER ligands regulation of the balance existing between ER α and ER β .

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