REVIEW

Acquired resistance to aromatase inhibitors: where we stand!

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Abstract

Aromatase inhibitors (AIs) are one of the principal therapeutic approaches for estrogen receptor-positive (ER⁺) breast cancer in postmenopausal women. They block estrogen biosynthesis through aromatase inhibition, thus preventing tumour progression. Besides the therapeutic success of the third-generation AIs, acquired resistance may develop, leading to tumour relapse. This resistance is thought to be the result of a change in the behaviour of ER in these breast cancer cells, presumably by PI3K/AKT pathway enhancement along with alterations in other signalling pathways. Nevertheless, biological mechanisms, such as apoptosis, autophagy, cell cycle modulation and activation of androgen receptor (AR), are also implicated in acquired resistance. Moreover, clinical evidence demonstrated that there is a lack of cross-resistance among AIs, although the reason is not fully understood. Thus, there is a demand to understand the mechanisms involved in endocrine resistance to each AI, since the search for new strategies to surpass breast cancer acquired resistance is of major concern.

Key Words

- ▶ estrogen receptor-positive breast cancer
- ▶ aromatase inhibitors
- ▶ exemestane
- ▶ letrozole
- ▶ anastrozole
- ▶ acquired resistance

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Estrogen-dependent breast cancer

In women, breast cancer is both the most common cancer worldwide and the second cause of cancer death (Cardoso *et al.* 2017). Approximately 60% of premenopausal and 75% of postmenopausal breast cancer patients have estrogen receptor-positive (ER⁺) carcinomas. ER expression and activation are important factors to control tumour growth and recurrence (Chen 2011). Although there are several therapeutic approaches, endocrine therapy has become the standard adjuvant treatment for postmenopausal women with ER⁺ breast cancer (Cardoso *et al.* 2017). Drugs that selectively target ER, like the selective ER downregulators (SERDs), such as fulvestrant, or the selective ER modulators (SERMs), such as tamoxifen or drugs that prevent estrogen biosynthesis, like AIs, are important therapeutic tools to block the ER signalling pathways that lead to cancer progression.

Over the last decade, AIs have been considered as first-line therapy for postmenopausal women with ER⁺ breast cancer (Cardoso *et al.* 2017). In fact, AIs therapy presents higher clinical efficacy, prolonged disease-free survival and time to recurrence, and significantly less severe side effects than tamoxifen. Nevertheless, clinical trials generally suggest that AIs therapy does not significantly improve overall survival when compared to tamoxifen therapy (Bonnetterre *et al.* 2001, Mouridsen *et al.* 2001, 2003, Milla-Santos *et al.* 2003, Paridaens *et al.* 2004, 2008, Howell *et al.* 2005, Chumsri 2015, Early Breast Cancer Trialists' Collaborative Group 2015). This unintended effect on overall survival may be associated with immature data from many studies, which report an increase in the number of non-cancer deaths in patients with prolonged AI therapy, and, consequently, this increase attenuates the

contribution of the treatment in reducing the number of breast cancer deaths (Goldvaser *et al.* 2018).

In relation to fulvestrant, it is clear that AIs are non-superior in terms of efficacy and tolerability. Clinical data suggests that, in some cases, fulvestrant may increase progression-free survival and overall survival when compared to AIs (Robertson *et al.* 2009, 2012, 2016, Ellis *et al.* 2015). Despite this, its poor solubility impairs its oral delivery to the patients, meaning that the administration must be performed by intramuscular injection. This drawback limited its clinical use as first-line therapy in the adjuvant setting. Therefore, the development of new orally available SERDs is a growing field that might strengthen the use of this class of drugs, in the future, for the adjuvant setting (Lai *et al.* 2015, Liu *et al.* 2016, Zhang *et al.* 2017). In fact, there are several oral SERDs that are being studied in clinical trials as the phase I/II non-randomised study of GDC-0810 (NCT01823835), the dose-escalation phase I trials of AZD9496 (NCT02248090) and of RAD1901 (NCT02338349) (Vries *et al.* 2016, Bardia *et al.* 2017). For these reasons, AIs remain the gold-standard treatment option in postmenopausal ER⁺ breast cancer therapy due to their improved clinical efficacy and manageable toxicity profile. Moreover, in recent years, AIs have begun to be administered in drug combinations that will be mentioned throughout this review.

Aromatase inhibitors

Human aromatase belongs to the cytochrome P450 family and is the product of the *CYP19A1* gene on chromosome 15. Aromatase is the only known enzyme in vertebrates that, through the aromatization of the A-ring of androgens, catalyses the biosynthesis of estrogens (estradiol and estrone) from the androgenic precursors (testosterone and androstenedione) (Ghosh *et al.* 2012). Ghosh *et al.* using X-ray crystallography solved the structure of aromatase and its enzyme-substrate interaction (Ghosh *et al.* 2009, 2010). The elucidation of the active site of aromatase gave new insights for the design and discovery of novel AIs (Ghosh *et al.* 2016).

AIs are a specific class of drugs that deplete the levels of circulating estrogens by inhibiting aromatase. According to AIs chemical structure, they are classified into two subtypes, steroidal (Type I) and non-steroidal (type II). Steroidal AIs compete with the natural substrate of the enzyme, binding covalently to the active site, which results in irreversible inhibition. These AIs are known as *suicidal inhibitors* because after the enzyme is inactivated, it is degraded by the proteasomes. In contrast, type II AIs

bind non-covalently to the heme moiety of aromatase and saturate its active site. Unlike type I inhibition, type II inhibition is reversible (Chumsri 2015, Sobral *et al.* 2016). AIs can also be grouped in three generations, according to their chronological order of appearance in clinic. The first AI used for breast cancer treatment, in the late 1970s, was a former anti-epileptic drug, the non-steroidal aminoglutethimide (Griffiths *et al.* 1973). However, as aminoglutethimide inhibited not only aromatase but also another cytochrome P450 enzyme, CYP11, which is involved in the conversion of cholesterol to pregnolone, a replacement therapy with glucocorticoids was necessary (Santen 1981). Subsequently, second-generation AIs were developed during the 1980s and 1990s, with the most prominent drugs being the steroidal formestane (4-hydroxyandrostenedione, 4-OHA) (Brodie *et al.* 1977) and the non-steroidal imidazole, fadrozole (CGS16949A) (Schieweck *et al.* 1988). Both second-generation AIs were more potent than aminoglutethimide, though, their use in clinic was not attractive since fadrozole had a short half-life and also suppressed cortisol and aldosterone biosynthesis (Demers *et al.* 1990), whereas formestane had poor biological activity when administered orally and had to be delivered by intramuscular injection (Dowsett & Coombes 1994). In the current third-generation, the non-steroidal AIs are derived from imidazole (anastrozole and letrozole), while the steroidal AI (exemestane) resulted from modifications in the structure of androstenedione, the natural substrate of aromatase. This generation of AIs is used in adjuvant treatment for early and metastatic stage, as first-line treatment options for postmenopausal patients (Cardoso *et al.* 2017). Nevertheless, more recently, due to the results from clinical trials, such as Suppression of Ovarian Function Trial (SOFT) (NCT00066690), AIs are also being considered for premenopausal women with suppressed ovarian function (Pagani *et al.* 2014, Regan *et al.* 2015).

Behind its therapeutic success, different clinical trials demonstrate that prolonged therapy with AIs can cause adverse effects. Several clinical trials that compared AIs with tamoxifen therapy indicate that AIs are associated with decreased incidence of hot flushes and gynecologic and thromboembolic side effects, but increased skeletal complications, musculoskeletal pain, arthralgia, cardiovascular events and sexual dysfunction (Nabholtz 2008, Amir *et al.* 2011, Khosrow-Khavar *et al.* 2017). Nevertheless, these trials have limited power to evaluate accurately the effect of AIs on safety and tolerability, as the current knowledge is based on data that compared AIs with tamoxifen, with the toxicity profile of AIs being unclear

when compared with the no treatment group. Recently, a meta-analysis study from randomised controlled trials that compared AIs to placebo or to no treatment groups supported the fact that extended AIs therapy is associated with an increased risk of cardiovascular events and bone fractures (Goldvaser *et al.* 2018). Clinical trials that compare AIs therapy with tamoxifen also suggest that AIs lack the cardioprotective effects pointed to tamoxifen (Nabholtz 2008, Amir *et al.* 2011). Although the effect of mechanism of AIs on cardiovascular morbidity is unclear, it has been reported that AIs increase risk factors such as dyslipidemia, arterial hypertension and accelerated atherosclerosis (Nathan *et al.* 2001, Mouridsen *et al.* 2007, Nabholtz 2008, Amir *et al.* 2011, Goldvaser *et al.* 2018).

The decrease in circulating estrogen levels induced by AIs may lead to bone mineral loss and osteoporotic fractures (Morales *et al.* 1996, Williams *et al.* 1997, Reis *et al.* 2001, Aapro 2004). Several clinical trials that compare AIs with tamoxifen therapy showed a statistically significant higher risk of bone fractures in patients treated with AIs (Nabholtz 2008, van de Velde *et al.* 2011, Early Breast Cancer Trialists' Collaborative Group 2015, Sobral *et al.* 2016). Nevertheless, the impact of AIs on bone health can be attenuated, by the association of AIs with bisphosphonates, such as zoledronic acid or risedronate (Brufsky *et al.* 2012, Greenspan *et al.* 2015), or denosumab, a monoclonal antibody that inhibits Receptor Activator of Nuclear Factor κ B (RANK) and modulates osteoclast activity (Gnant *et al.* 2015). In addition, despite the general therapeutic index of AIs suggesting superiority over tamoxifen with proven improved efficacy and better toxicity profile (Nabholtz 2008, Cardoso *et al.* 2017, Goldvaser *et al.* 2018), the prolongation of treatment with AIs or with anti-estrogens may promote the occurrence of endocrine resistance, this being considered the major concern in breast cancer therapy.

Genomic and non-genomic estrogen receptor pathways

ER is a nuclear receptor involved in the regulation of many physiological processes in humans. This receptor has two isoforms, ER α and ER β , which share a 59% homology. The former is located on chromosome 6, while the latter is on chromosome 14 (Heldring *et al.* 2007, Jia *et al.* 2015). Both ERs contain a DNA-binding domain (DBD), a dimerization region (DR), a ligand-binding domain (LBD) and two transactivation domains. One of these domains is located near the N-terminus (AF-1) and is activated through phosphorylation of serine 167, by PI3K/AKT (Campbell

et al. 2001), and/or of serine 118, by RAS/MAPK (Kato *et al.* 1995, Bunone *et al.* 1996), in a ligand-independent manner and by CDK7 (Chen *et al.* 2000, 2002), in a ligand-dependent manner. The other transactivation domain is located near the C-terminus (AF-2) and is activated through binding to estradiol (E₂). Despite the high sequence homology in the DBD between the two isoforms of ER, they are not redundant genes, since they have different expression patterns and functions (Chan *et al.* 2016). ER α has been associated with growth and survival of tumour and non-tumour breast epithelial cells, while ER β is associated with growth inhibitory properties, by arresting cell cycle progression of breast cancer cells (Paruthiyil *et al.* 2004, Chan *et al.* 2016).

Within the cytosol, ER is bound through LBD (AF-2) to chaperone proteins, such as heat shock protein 90 (HSP90) and HSP70, which are essential to maintain the ER in an inactivated form (Htun *et al.* 1999, Pick *et al.* 2007, Chan *et al.* 2016). Upon binding of estrogens to ER, the receptor undergoes conformational changes, like the dissociation from the HSP and subsequent dimerization. This allows the translocation of ER to the nucleus, in order to bind estrogen-responsive elements (ERE) in the promoter region of ER-regulated genes and the recruitment of co-activators to initiate *classical* genomic transcriptional modulation. The ER can also interact with other transcription factors such as activator protein 1 (AP-1) to bind DNA indirectly, leading to the modulation of target genes located at alternative responsive elements in a process known as *non-classic* or *ERE-independent* genomic action. Besides these two different mechanisms of genomic action, a third mechanism exists. It involves the ligand-independent activation of ER through phosphorylation of the AF-1 domain by several kinases of the growth factor receptors signalling pathways, like p38, p44/42 or PI3K/AKT (Bunone *et al.* 1996, Chan *et al.* 2016). This ligand-independent activation is a process that often leads to endocrine resistance (Kato *et al.* 1995, Swaby & Jordan 2008). In addition to the genomic pathways, there are rapid effects that do not rely on transcriptional activity and are triggered through growth factor receptors (GFRs), namely fibroblast growth factor receptor 1 (FGFR1) (Turner *et al.* 2010, Andre & Cortes 2015), insulin-like growth factor 1 receptor (IGF1R) (Stephen *et al.* 2001, Fox *et al.* 2011), epidermal growth factor receptor (EGFR), human epidermal growth factor receptor 2 (HER2) (Mehta & Tripathy 2014, Flageng *et al.* 2017), other membrane receptors, like G-protein-coupled ER (GPR30) (Scaling *et al.* 2014, Kim *et al.* 2015), and ER variants (Li *et al.* 2003, Chaudhri *et al.* 2014). These are

the non-genomic pathways and consist of activating several kinases signalling pathways like PLC/PKC, RAS/RAF/MAPK and cAMP/PKA or even in the release of several cyclic nucleotides (cAMP, cGMP) and calcium (Chan *et al.* 2016). The activation of these signalling pathways may indirectly connect non-genomic actions of estrogens to genomic responses, since many transcription factors are regulated by protein kinases, making these transcription factors the main targets for the non-genomic actions of estrogens.

Endocrine therapy resistance

As previously referred to, estrogen deprivation has been considered an important treatment for estrogen-dependent (ER⁺) breast cancers. However, 20% of patients with early-stage disease are unresponsive to therapy (Early Breast Cancer Trialists' Collaborative Group 2011) and, despite the initial benefit, some patients with metastatic breast cancer may experience tumour progression, being in these cases, the 5-year survival rate of around 26% and the overall average survival between 2 and 3 years (Cardoso *et al.* 2017).

Resistance to AIs is divided into two main types: primary/*de novo* resistance and secondary/acquired resistance. Recently, ESO-ESMO international consensus guidelines for advanced breast cancer defined the primary resistance as a relapse during the first 2 years of adjuvant endocrine therapy or progression of disease within the first 6 months of first-line endocrine therapy for metastatic breast cancer. Secondary/acquired resistance is defined as a relapse while on adjuvant endocrine therapy but after the first 2 years, or as a relapse within 12 months of completing adjuvant endocrine therapy, or progression of disease after 6 months of initiating endocrine therapy for metastatic breast cancer (Cardoso *et al.* 2017). Although the clinical distinction between the two types of resistance is not yet well defined, the mechanisms underlying these two types of resistance are likely to overlap.

Tyson *et al.* reviewed different mathematical models that integrate the biological systems of endocrine responsiveness in ER⁺ breast cancer, leading to the identification of key molecules that may control the response of breast cancer cells to endocrine therapy (Tyson *et al.* 2011). Curiously, by analysing information about the genetic, molecular biology and physiology of cancer cells, the models allocate the key molecules that were associated to the development of AIs resistance, both *in vitro* and *in vivo*, in 'decision modules' (cell cycle and apoptosis), 'stress modules' (autophagy and unfolded

protein response) and 'signal processing modules' (ER and growth factor signalling).

As the majority of acquired endocrine resistance cases occur in ER-expressing breast cancers, it is suggested that loss of ER expression is not the main mechanism. This evidence reinforces the need to understand the molecular processes in order to design new strategies to overcome AIs-acquired resistance. In this review, only the main mechanisms associated with acquired resistance will be explored, beginning by generally describing the overall mechanisms and the specific pathways for each AI.

Estrogen-related gene mutations

The gene estrogen receptor 1 (*ESR1*) encodes ER α and its mutations have been proposed as a mechanism of AIs resistance since the early 1990s (Fuqua *et al.* 1991). This hypothesis was initially underappreciated because mutations in this gene were rare in treatment-naïve primary tumours (Roodi *et al.* 1995). In fact, it was detected that only around 3% of the primary tumours from BOLERO-2 clinical trial presented *ESR1* mutations (Toy *et al.* 2013, Jeselsohn *et al.* 2015). However, different studies demonstrated that these mutations appear with higher frequencies, ranging from 11 to 55% in metastatic tumours, especially in those that have progressed despite AI treatment (Robinson *et al.* 2013, Jeselsohn *et al.* 2015, Schiavon *et al.* 2015, Chandarlapaty *et al.* 2016, Angus *et al.* 2017). The most common point mutations in this gene occur specifically in the LBD encoding domain, like Y537S/N/C and D538G (Niu *et al.* 2015). These mutations can confer estrogen-independent ER activation, through recruitment of co-activators and stabilization of the agonist (Fanning *et al.* 2016). This process often leads to an estrogen-independent cell proliferation (Robinson *et al.* 2013, Toy *et al.* 2013). Several trials tried to assess the clinical impact of these mutations and data suggested that they may influence the clinical outcome in metastatic setting. Accordingly, in the SoFEA clinical trial and in the BOLERO-2 trial, patients who were treated with exemestane and harboured *ESR1* mutation presented a decrease in the progression-free survival when compared with wild-type *ESR1* patients treated with exemestane (Chandarlapaty *et al.* 2016, Fribbens *et al.* 2016).

ESR1 chromosomal translocation is another described mechanism where several in-frame fusion genes that preserve the first exons, including the DBD domain, are spliced in-frame into the C-terminus of other genes. Examples of these genes are Yes-associated protein 1 (*ESR1-YAPI*), DNA-polymerase η (*ESR1-POLH*), A kinase

anchor protein 12 (*ESR1-AKAP12*) and coiled-coil domain containing 170 (*ESR1-CCDC170*) (Li *et al.* 2013, Ma *et al.* 2014). *ESR1-YAP1* is the best documented translocation, and in this case, YAP1 sequences mimic the ligand-dependent transactivation domain (AF-2), inducing estradiol-independent growth and ER-regulated gene transcription (Li *et al.* 2013). *CCDC170* is only separated from *ESR1* by 69 kb. Fusion events of these two genes join the promoter regions of the *ESR1* and of the *CCDC170* genes, generating a fusion protein that corresponds to N-terminal truncated forms of *CCDC170* (Δ *CCDC170*). This fusion protein renders more aggressive luminal B breast cancers by enhancing cell migration, invasion and reducing endocrine sensibility (Veeraraghavan *et al.* 2014). However, until now, no clinical studies have been carried out in order to address the importance of these types of *ESR1* gene alterations concerning clinical outcome.

Another mechanism that appears to be associated to AIs acquired resistance is the amplification of *ESR1* and *CYP19A1* genes; however, their clinical prevalence and relevance remain unclear (Holst *et al.* 2007, Adelaide *et al.* 2008, Li *et al.* 2013, Magnani *et al.* 2017). Recently, using next-generation sequencing, a rate of around 2% in both primary and metastatic tumours was described by different studies (Cancer Genome Atlas Network 2012, Jeselsohn *et al.* 2015), and it was suggested that this amplification may not play an important role in resistance development (Jeselsohn *et al.* 2015). In relation to *CYP19A1*, very rare prevalence rates have been reported (0.006%) in early-stage disease (Cancer Genome Atlas Network 2012), while in metastatic tumours, higher amplification rates (16–32%) were described in patients that received AIs (Magnani *et al.* 2017).

Aberrant growth factor receptors expression/activation

Over the last decade, several studies have described deregulated pathways and adaptive changes due to prolonged estrogen deprivation and ER signalling disruption. The deregulated activation of GFRs (Stephen *et al.* 2001, Turner *et al.* 2010, Fox *et al.* 2011, Mehta & Tripathy 2014, Andre & Cortes 2015, Flageng *et al.* 2017) and their downstream signalling components, including MAPKs (Jeng *et al.* 2000, Martin *et al.* 2003) and PI3K pathways (Miller *et al.* 2010, Fox *et al.* 2013), have been associated with AIs acquired resistance. In fact, the crosstalk between ER and GFRs allows breast cancer cells to bypass estrogen deprivation by modulating ER expression and activity. Moreover, it was demonstrated

that HER2 aberrant activation is possibly due to increased expression of neuregulin-1 (NRG1) (Flageng *et al.* 2017), a HER ligand. The increase in HER2 activation leads to a ligand-independent activation of ER through MAPK phosphorylation on S118, inducing acquired resistance to AIs (Kato *et al.* 1995, Mehta & Tripathy 2014). Recently, the importance of ER in driving the growth of AIs-resistant cells through a ligand-independent ER activation was strengthened (Hole *et al.* 2015a). This resistance is surpassed by ER downregulation through the addition of the SERD fulvestrant (Bartsch *et al.* 2007, Hole *et al.* 2015a). Furthermore, a sustained overexpression of HER2 may lead to the loss of ER expression as a mechanism of resistance, bypassing, in these cases, the beneficial effects of fulvestrant and rendering these cells ligand and ER independent (Massarweh & Schiff 2007). Similar observations were reported regarding the PI3K/AKT pathway (Sikora *et al.* 2012), with the AKT downstream effector, p70S6K, being responsible for the ER phosphorylation on S167 (Campbell *et al.* 2001), and for the loss of ER expression (Creighton *et al.* 2010). In the latter case, AKT blocks the translocation of the transcription factor forkhead box O3A (FOXO3A) to the nucleus and its binding to the *ESR1* promoter. Therefore, inhibition of PI3K induces ER expression through FOXO3A (Brunet *et al.* 1999, Guo & Sonenshein 2004). Nevertheless, the contrary is also true, since ER has the ability to bind to the regulatory subunit of PI3K, p85, activating the catalytic subunit, p110 (Simoncini *et al.* 2000). Mutations in the PI3K gene, *PIK3CA* (Perez-Tenorio *et al.* 2007, Stemke-Hale *et al.* 2008, Ellis *et al.* 2010), or the loss of phosphatase and tensin homolog (PTEN) expression (Perez-Tenorio *et al.* 2007, Saal *et al.* 2007) may activate this pathway independently of GFRs.

Several approaches to prevent or delay acquired resistance, through the inhibition of GFRs, which will be discussed later, have been tested in clinical studies. Nevertheless, more information is needed in order to fully understand the therapeutic importance of targeting the GFRs and downstream effectors in metastatic ER⁺ patients with acquired resistance to AIs.

ER hypersensitivity to low estrogen levels

Several studies have shown that tumours retaining ER expression can escape the limitations of estrogen deprivation, by increasing their hypersensitivity to residual levels of estrogens. Curiously, in these cases, high doses of estrogen are known to inhibit cell growth and induce apoptosis of resistant cells (Masamura *et al.*

1995, Ellis *et al.* 2009, Zucchini *et al.* 2015), though, recent studies have suggested that the exact mechanism of ER hypersensitivity may differ according to estrogen levels (Sikora *et al.* 2012). Santen *et al.* reported that the ER-mediated transcription of genes is different when the ER is hypersensitive (Santen *et al.* 2005). The main mechanisms, in this case, are the interactions with the GFR signalling pathways, together with non-genomic actions near or at the cell membrane (Santen *et al.* 2009). This suggested that interactions with GFR signalling pathways do not simply restore the normal ER function, but by increasing ER phosphorylation, alter and enhance ER-mediated transcription of genes. This theory is supported by the finding that elevated AKT levels can alter the genome-wide binding pattern of ER compared to the one seen in wild-type cells (Bhat-Nakshatri *et al.* 2008). Thus, these findings indicate that disrupting GFR signalling may be a more realistic approach in preventing ER hypersensitivity than to abrogate estrogen action. GFR signalling blockade in AI-resistant breast cancer is already under investigation and will be discussed later.

Androgens and androgen receptor

AR is a steroid receptor, similar to ER, involved in several physiological processes. Approximately, 85–95% of the ER⁺ breast cancers, and 77% of invasive breast cancers, express AR (Collins *et al.* 2011, Proverbs-Singh *et al.* 2015). Curiously, it is known that AR can be recruited to ERE, and that the ER can also be recruited to androgen-responsive elements (ARE), which proves the structural similarities of these receptors, especially in the DBD (Peters *et al.* 2009, Rechoum *et al.* 2014).

In AIs-sensitive breast cancer cells, the ER and AR have opposite effects. ER promotes cell growth while AR induces cell death. In these cases, AR overexpression is linked with improved disease-free survival (DFS), when compared with AR-negative cancers. Moreover, the absence of AR expression predicts an earlier treatment failure with AIs (Macedo *et al.* 2006, Elebro *et al.* 2015).

In AIs-resistant breast cancer cells, several studies suggested a different role for androgens and AR in cell fate (Macedo *et al.* 2006, Chia *et al.* 2015). One of the proposed mechanisms for AIs-acquired resistance involves AR overexpression, as a response to decreased ER activity (Fujii *et al.* 2014, Ali *et al.* 2015). To enhance ER transcriptional activity, a cooperation between AR and ER, via PI3K pathway, have been described (Rechoum *et al.* 2014). Another proposed mechanism involves 5 α -androstane-3 β ,17 β -diol (3 β -diol), an androgen metabolite with

androgenic and estrogenic activities, that induces ER activation (Hanamura *et al.* 2013). Curiously, despite the pro-survival effects in the resistance onset, 3 β -diol inhibits the growth of MCF-7 cells due to its agonistic effects on ER β (Lattrich *et al.* 2013). The production of this androgen metabolite causes a decrease in AR signalling, since androgens are converted into 3 β -diol, by the estrogen deprivation-induced enzyme 3 β -HSD (Sikora *et al.* 2009, Hanamura *et al.* 2013).

Based on these findings, several clinical trials are ongoing in order to assess the viability of modulating AR, both positively and negatively, in ER⁺/AR⁺ breast cancer cases. The efficacy and safety of a selective androgen receptor modulator, enobosarm, for the treatment of ER⁺/AR⁺ patients (NCT02463032) is being assessed, and the evaluation of the effects of enzalutamide, an AR antagonist, alone or in combination with exemestane is ongoing in a phase II clinical trial (NCT02007512). Recently, the latter clinical trial was supported by a phase I/Ib study with the combination of enzalutamide with anastrozole, exemestane or fulvestrant (Schwartzberg *et al.* 2017). Moreover, the combination of the AR antagonist, bicalutamide and an aromatase inhibitor is also being studied in a clinical trial (NCT02910050).

Thus, although AR is considered as a favourable prognostic marker, the exact role and importance of targeting AR in AIs-acquired resistance is not well defined, and further studies need to be performed. Nevertheless, the recent discovery of the importance of AR in breast cancer and its dual role in AR-sensitive and resistant cells, praise its significance as an attractive therapeutic target.

Cell cycle related mechanisms

Aberrant expression/activation of cell cycle regulators has also been associated with AIs-acquired resistance. Ormandy *et al.* had previously shown that the cyclin D1 encoding gene, *CCND1*, is commonly amplified in breast cancer (Ormandy *et al.* 2003). This overexpression may inhibit CDK interacting protein/kinase inhibitory protein (CIP/KIP) molecules that are pivotal for cell cycle regulation (Hui *et al.* 2002, Chu *et al.* 2008). Several survival pathways, such as MAPK, NF- κ B or ER α , promote the expression of cyclin D1 (Altucci *et al.* 1996), which can directly activate ER α in a cyclin-CDK complex-independent way (Zwijsen *et al.* 1997). This cyclin is responsible for the progression of G1-to-S phases, by forming a complex with cyclin-dependent kinase 4/6 (CDK4/6), inactivating the retinoblastoma protein and inducing the synthesis of cyclin E. The joint action of

cyclin D1-CDK4/6 and cyclin E-CDK2 contributes to the progression of cell cycle.

Following the results of the PALOMA-1/TRIO-18 (NCT00721409) and MONALEESA-2 (NCT01958021) clinical trials, the combinations of two CDK 4/6 inhibitors, palbociclib and ribociclib, with letrozole were approved by FDA as first-line treatment in ER⁺ postmenopausal women. These combinations demonstrated an increase in progression-free survival when compared to letrozole alone (Finn *et al.* 2014, 2015, Beaver *et al.* 2015, Hortobagyi *et al.* 2016). The combination of palbociclib with fulvestrant was also approved since according to PALOMA-3 phase 3 randomised controlled clinical trial (NCT01942135), an improvement on progression-free survival was observed when compared with fulvestrant alone (Cristofanilli *et al.* 2016). Furthermore, the CDK 4/6 inhibitors, abemaciclib and ribociclib, are also being studied in combination with anti-estrogens (Hortobagyi *et al.* 2016). In addition, an overexpression of Aurora kinases in models of resistance to third-generation AIs was recently reported (Hole *et al.* 2015b). Aurora kinases are Ser/Thr kinases involved in cell proliferation through the control of chromatid segregation. These results support the idea that targeting cell cycle regulators, in particular CDK4/6, is a promising therapeutic option. Indeed, modulators of the cell cycle have gained reputation in the last years as attractive strategies to overcome resistance.

Apoptosis, autophagy and endoplasmic reticulum stress

In addition to cell cycle regulators, apoptosis resistance and/or pro-survival autophagy are also described mechanisms associated to AIs-acquired resistance. In fact, an upregulation of the anti-apoptotic molecules, Bcl-2 and Bcl-xL, and a downregulation of the pro-apoptotic molecules, Bad and Bik, blocked anti-estrogen therapy-induced apoptosis (Musgrove & Sutherland 2009, Dalby *et al.* 2010, Giuliano *et al.* 2011). Moreover, the downregulation of programmed cell death 4 (PDCD4), an apoptosis-induced tumour suppressor that inhibits protein translation, is associated with poor prognosis in AIs-resistant cells (Chen *et al.* 2015b).

Autophagy has also been associated with endocrine resistance to anti-estrogen therapies (Cook *et al.* 2011, Amaral *et al.* 2012, 2013, Cook & Clarke 2014). Autophagy is regulated by the anti-apoptotic protein Bcl-2 and by PI3K pathway, through mammalian target of rapamycin (mTOR) activation, which inhibits autophagy initiation (He & Klionsky 2009). A recent *in vitro* study suggested

that one of the reasons for the insensitivity to everolimus, an mTORC1 inhibitor, is the autophagic activation. In this study, autophagic inhibition by chloroquine restored sensitivity to everolimus in AIs-resistant breast cancer cells derived from MCF7 cells, suggesting a protective role of this cellular process in AIs-acquired resistance (Lui *et al.* 2016).

c-MYC gene expression has also been shown to be upregulated in AIs-resistant cells. This upregulation was due to a crosstalk between HER2 and ER (Miller *et al.* 2011, Chen *et al.* 2015a) and was associated with everolimus resistance in AIs-resistant breast cancer cells (Bihani *et al.* 2015). As c-MYC regulates the autophagic process through changes in Bcl-2 phosphorylation in cancer cells (Toh *et al.* 2013), the study of c-MYC involvement in resistance to everolimus demonstrated that autophagy was associated with everolimus insensitivity to AIs-resistant breast cancer, as discussed earlier (Lui *et al.* 2016).

The unfolded protein response (UPR) has also been linked to the regulation of autophagy-apoptosis switch. The UPR regulator, glucose-regulated protein (GRP78), is overexpressed in ER⁺ acquired resistant cells and has been shown to balance the pro-survival autophagy and apoptosis, conferring resistance to the hormonal therapy *in vitro* and *in vivo* (Cook *et al.* 2012, 2014). The GRP78 stimulates autophagy through mTOR inhibition, and its knockdown re-sensitised breast cancer cells through apoptosis induction (Cook *et al.* 2012).

Recently, the relevance of endoplasmic reticulum homeostasis in AIs-acquired resistance was demonstrated by a study showing that inhibition of the serum/glucocorticoid-regulated kinase 3 (SGK3) induced a reduction in AIs-resistant cells survival. SGK3 is upregulated in AIs-resistant cells, maintaining the endoplasmic reticulum calcium levels, by preserving endoplasmic reticulum calcium ATPase 2b (SERCA2b) activity, involved in endoplasmic reticulum homeostasis (Wang *et al.* 2017).

Other mechanisms

The role of miRNAs (miR) in endocrine resistance is currently being explored (Muluhngwi & Klinge 2015). Vilquin *et al.* reported a deregulation of miR-125b, miR-205 and miR-424 in acquired letrozole- and Anastrozole-resistant cell models, when compared to a hormone-sensitive breast cancer cell line that overexpresses aromatase (MCF-7aro). Expression levels of miR-125b and miR-205 were upregulated, whereas miR-424 was downregulated. This deregulation was sufficient to confer resistance to letrozole and anastrozole

by the activation of the PI3K/AKT pathway, in MCF-7-a cells transfected with miR-125b, miR-205 or treated with inhibitors of miR-424 (Vilquin *et al.* 2015). Other studies also correlated miR-155 and miR-128a with a poor response to anastrozole and letrozole, respectively (Masri *et al.* 2010a, Bacci *et al.* 2016).

Molecular alterations in resistance to third-generation AIs

The third-generation AIs are very effective, potent and specific; but tumours still relapse in many patients because of acquired resistance. The lack of cross-resistance among AIs however suggests different resistance mechanisms (Lonning 2008, 2009). In this section, a global overview of molecular alterations described for each third-generation AIs resistance model will be accomplished (Table 1).

Letrozole resistance

Several mechanisms that involve MAPK and PI3K pathways and cell cycle regulators are linked to letrozole acquired resistance. Letrozole-resistant cells exhibit a dependency on the MAPKs pathways, mainly through HER2 and EGFR overexpression (Tilghman *et al.* 2013), which highlights the role of GFRs on the ligand-independent activation

of ER (Fig. 1). Co-targeting HER2, via trastuzumab, and ER signalling, in long-term letrozole-treated tumour (LTLT-Ca) cells, a resistant cell model, restored letrozole sensitivity, inducing tumour regression as a consequence of a re-expression of ER (Jelovac *et al.* 2005, Sabnis *et al.* 2009, 2010, Sabnis & Brodie 2010). Nevertheless, recent data also described that letrozole-resistant cells present a higher expression/activation of the PI3K/AKT/mTOR pathway. In a reported phase I trial (NCT01248494), the inhibitor buparlisib (BKM120) showed to be safe and to have anti-tumour efficacy in combination with letrozole (Mayer *et al.* 2014). Different PI3K inhibitors, such as pilaralisib and voxalisib, are currently being tested in phase I/II studies (Blackwell *et al.* 2015). Moreover, in letrozole-resistant cells, taselisib, also a PI3K inhibitor, demonstrated anti-tumour efficacy in combination with letrozole (Hoeflich *et al.* 2016).

Recent studies have also described an association with cell cycle regulation and letrozole resistance. FDA recently approved as first-line treatment in ER⁺ postmenopausal women the inhibition of CDK 4/6 with palbociclib in combination with letrozole. This approach showed high efficacy, as previously described by the PALOMA-1/TRIO-18 (NCT00721409) clinical trial, and the ability to prevent letrozole resistance (Finn *et al.* 2014, 2015, Beaver *et al.*

Table 1 Summary of the main mechanisms of acquired resistance to third-generation AIs.

Alteration	Mechanism of resistance	References
Letrozole resistance		
HER2 overexpression	Decrease expression and ligand-independent activation of ER	Jelovac <i>et al.</i> (2005), Sabnis <i>et al.</i> (2009), Sabnis & Brodie (2010)
PI3K/AKT/mTOR overexpression	Decrease expression and ligand-independent activation of ER	Cavazzoni <i>et al.</i> (2012), Mayer <i>et al.</i> (2014), Hoeflich <i>et al.</i> (2016)
CDK4/6 activity	Promotion of cell cycle progression	Finn <i>et al.</i> (2014, 2015), Beaver <i>et al.</i> (2015), Hortobagyi <i>et al.</i> (2016), (American Association for Cancer Research 2017)
Cyclin E overexpression	Promotion of cell cycle progression	Akli <i>et al.</i> (2010)
Aurora kinase A/B upregulation	Promotion of cell cycle progression	Hole <i>et al.</i> (2015b)
HDAC aberrant activity	HER2 modulation	Sabnis <i>et al.</i> (2013a), Schech <i>et al.</i> (2015)
Anastrozole resistance		
IGFR1 overexpression	Decrease expression and ligand-independent activation of ER	Macedo <i>et al.</i> (2008), Rechoum <i>et al.</i> (2014)
PI3K/AKT/mTOR overexpression	Decrease expression and ligand-independent activation of ER	Vilquin <i>et al.</i> (2013), Schmid <i>et al.</i> (2016)
MAPK overexpression	Decrease expression and ligand-independent activation of ER	Sabnis <i>et al.</i> (2013b)
Androgen receptor overexpression	Increase IGF1R and PI3K/AKT/mTOR signalling	Rechoum <i>et al.</i> (2014)
CCND1 amplification	Promotion of cell cycle progression	Lundgren <i>et al.</i> (2012)
Exemestane resistance		
AREG overexpression	Increase MAPK pathway activity	Wang <i>et al.</i> (2008)
PI3K/AKT/mTOR overexpression	Decrease expression of ER	Baselga <i>et al.</i> (2012), Steger <i>et al.</i> (2017)
Aurora kinase A/B upregulation	Promotion of cell cycle progression	Hole <i>et al.</i> (2015b)
HDAC aberrant activity	NF-κB expression	Kubo <i>et al.</i> (2013), Yardley <i>et al.</i> (2013)
Autophagy	Pro-survival cellular mechanism	Amaral <i>et al.</i> (2012, 2013)

Figure 1

Letrozole-acquired resistance mechanisms.

Letrozole presents several resistance mechanisms that are estrogen-independent and related to the overexpression of survival/proliferation pathways, such as PI3K/AKT and MAPK/ERK. Some strategies are being studied, like the use of HDAC inhibitors, such as entinostat, HER2 blockade by Trastuzumab, PI3K inhibition by buparlisib or cell cycle regulation by Aurora kinase A/B inhibitors or by palbociclib. AKT, serine/threonine specific-protein kinase family; EGFR, epidermal growth factor receptor; ER, estrogen receptor; ERE, estrogen responsive element; ERK, extracellular signal-regulated kinases; HDAC, histone deacetylase; HER2, human epidermal growth factor receptor 2; HSP, heat shock protein; MAPK, mitogen-activated protein kinase; PI3K, phosphoinositide 3-kinase. A full colour version of this figure is available at <https://doi.org/10.1530/ERC-17-0425>.

2015). Moreover, the MONALEESA-2 (NCT01958021) clinical trial demonstrated that, in postmenopausal women with ER⁺/HER2⁻ advanced breast cancer without prior therapy, the combination of letrozole with a different CDK 4/6 inhibitor, ribociclib, extended progression-free survival when compared to letrozole alone. FDA also approved this combination (Hortobagyi *et al.* 2016, American Association for Cancer Research 2017).

An upregulation of Aurora kinase A and B also seems to be involved in letrozole acquired resistance. Their inhibition resulted in tumour growth suppression, indicating that these kinases might be new potential therapeutic targets (Hole *et al.* 2015b). Moreover, studies involving the histone deacetylase (HDAC) inhibitor, entinostat, suggested a beneficial effect, through HER2 modulation, on LTLT-Ca cells and on Letrozole-resistant MCF-7Ca xenografts (Sabnis *et al.* 2013a, Schech *et al.* 2015).

Anastrozole resistance

Different mechanisms were described to be associated with anastrozole resistance, and most of these seem to share the same growth factor receptor aberration. Anastrozole-resistant cells do not exhibit an upregulation in HER2 like in letrozole resistance (Fig. 2). Instead, they present an increase in IGF1R and a reduction in ER expression and aromatase activity, with an upregulation of the PI3K/AKT pathway (Macedo *et al.* 2008, Rechoum *et al.* 2014). In fact, a study conducted by Rechoum *et al.* showed that overexpression of AR and a

cooperation between AR and ER led to anastrozole resistance of MCF-7 cells that overexpress aromatase and AR, through the activation of IGF1R and PI3K/AKT pathways. Consistently, the use of an IGF1R inhibitor, AG1024, or a dual kinase AKT inhibitor, Akti 1/2, restored sensitivity to anastrozole (Rechoum *et al.* 2014). Similar results were found using the AKT/mTOR inhibitor MK-2206, on anastrozole-resistant cells derived from aromatase-overexpressing MCF-7 cells (Vilquin *et al.* 2013), and the PI3K inhibitor pictilisib on a phase II randomised trial (Schmid *et al.* 2016). Therefore, it can be concluded that targeting the PI3K/AKT/mTOR pathway in anastrozole resistance is of major importance. This was strengthened by the use of a MAPK inhibitor, selumetinib, which caused a downregulation of activated MAPK and phosphorylated mTOR, reverting anastrozole resistance (Sabnis *et al.* 2013b).

Moreover, in a TransATAC study, it was observed that amplifications of the *CCND1*, a gene that encodes cyclin D1, were associated with an increased risk of tumour recurrence in response to anastrozole (Lundgren *et al.* 2012). In fact, the biological effects of the combination of a CDK 4/6 inhibitor, abemaciclib, plus anastrozole vs anastrozole or abemaciclib alone, are being evaluated by the ongoing neoMONARCH clinical trial (NCT02441946).

Exemestane resistance

Exemestane, the only steroidal AI of the third-generation group, has a different resistance mechanism when

Figure 2

Anastrozole-acquired resistance mechanisms. Anastrozole presents several resistance mechanisms that are estrogen-independent and related to the overexpression of survival/proliferation pathways, such as PI3K/AKT and MAPK/ERK and to the amplification of a cell cycle regulator gene, *CCND1*. Some strategies are being studied, including IGFR1 blockade by AG1024, PI3K inhibition by pictilisib, AKT inhibition by MK-2206 or Akti 1/2 and MEK inhibition by selumetinib. AKT, serine/threonine specific-protein kinase family; AR, androgen receptor; ER, estrogen receptor; ERE, estrogen responsive element; ERK, extracellular signal-regulated kinases; HSP, heat shock protein; IGFR, insulin-like growth factor receptor; MAPK, mitogen-activated protein kinase; PI3K, phosphoinositide 3-kinase. A full colour version of this figure is available at <https://doi.org/10.1530/ERC-17-0425>.

compared to the two non-steroidal AIs (Fig. 3). Contrary to non-steroidal AI-resistant cells, Exemestane-resistant cancer cells continue to present a hormone-dependent behaviour (Masri *et al.* 2010b, Chen 2011). In the last years, different mechanisms have been associated with exemestane acquired resistance, with some of them being common to non-steroidal AIs while others are apparently specific for this steroidal AI.

It has been suggested that exemestane resistance results from its weak estrogen-like activity, through the involvement of amphiregulin (AREG) expression (Wang *et al.* 2008). Exemestane may induce AREG upregulation in a similar way to estrogens (Frasor *et al.* 2004), through an ER-dependent manner (Wang *et al.* 2008). AREG is an epidermal-like growth factor that binds to and activates EGFR, leading to tumour proliferation through the MAPK

Figure 3

Exemestane-acquired resistance mechanisms. Exemestane presents several resistance mechanisms that are estrogen-dependent and related to the overexpression of survival/proliferation pathways, such as PI3K/AKT and MAPK/ERK. Some strategies are being studied, like HDAC inhibition by panobinostat or entinostat, mTORC1 inhibition by everolimus, autophagic inhibition by 3-MA and cell cycle regulation by Aurora kinase A/B inhibition. 3-MA, 3-methyladenine; AKT, serine/threonine specific-protein kinase family; AREG, amphiregulin; EGFR, epidermal growth factor receptor; ER, estrogen receptor; ERE, estrogen responsive element; ERK, extracellular signal-regulated kinases; HDAC, histone deacetylase; HSP, heat shock protein; MAPK, mitogen-activated protein kinase; mTORC1, mammalian target of rapamycin complex 1; PI3K, phosphoinositide 3-kinase. A full colour version of this figure is available at <https://doi.org/10.1530/ERC-17-0425>.

pathway (Wang *et al.* 2008, Masri *et al.* 2010b). As expected, AREG or MAPK inhibition suppresses proliferation of exemestane-resistant cancer cell lines.

The inhibition of PI3K/AKT/mTOR pathway, through everolimus, when combined with exemestane, seems to be very effective in ER⁺ patients with advanced disease after non-steroidal AI therapy, as shown by the clinical trials STEPAUT and BOLERO-2 (Baselga *et al.* 2012, Steger *et al.* 2017). mTOR inhibition exhibits high anti-proliferative effects, through reduced translation of cyclin D1, inducing cell cycle arrest at the G1/S phase (Dowling *et al.* 2010, Lui *et al.* 2016). However, some details need to be taken into account in relation to the positive outcomes of the BOLERO-2 trial. In this clinical trial, there is a lack of statistically significant survival benefit, despite the improvement in progression-free survival (Piccart *et al.* 2014). One of the reasons may reside in the complex negative feedback loop between mammalian target of rapamycin complex 1 (mTORC1) and insulin-like growth factor-1 (IGF-1), as inhibition of mTORC1 leads, paradoxically, to the activation of AKT (Wan *et al.* 2007, LoRusso 2013). Nevertheless, this therapy was approved by FDA (Cook *et al.* 2012), suggesting that targeting PI3K pathway can have clinical benefits.

Hanamura *et al.* have demonstrated that, besides inducing estrogen deprivation, exemestane therapy increases intratumoural androgen levels (Hanamura *et al.* 2013). In fact, ongoing clinical trials are studying the combination of the AR antagonist, enzalutamide, with exemestane (NCT02007512). Nevertheless, in postmenopausal patients pre-treated with non-steroidal AIs (NCT01381874), the inhibition of androgen biosynthesis, by abiraterone acetate (AA) plus prednisone, in combination with exemestane, did not improve progression-free survival when compared to exemestane treatment (O'Shaughnessy *et al.* 2016).

Another described mechanism associated with exemestane resistance, is the upregulation of the cell cycle regulators, Aurora kinase A and B, in resistant cells derived from MCF-7 cells (Hole *et al.* 2015b). Moreover, a recent phase III study is currently evaluating the efficacy of palbociclib, in combination with Exemestane or fulvestrant, vs capecitabine (NCT02028507) (Martín *et al.* 2017).

Genetic and epigenetic alterations are also known to play a role in exemestane resistance. In fact, HDAC inhibitors also seem to reverse exemestane resistance in resistant cells derived from MCF-7-aro cells (Kubo *et al.* 2013). In this study, the HDAC inhibitor, panobinostat, inhibited exemestane-resistant cancer cell proliferation, through cell cycle arrest and apoptosis. Similarly, the

use of the HDAC inhibitor, entinostat, has also shown promising results in a randomised phase II, double-blind, placebo-controlled study (NCT00676663) (Yardley *et al.* 2013). For this reason, this combination is now being studied in a phase III clinical trial (NCT02115282).

Finally, autophagy is also another potential mechanism associated with exemestane resistance, since it appears to act as a pro-survival mechanism. Our group described that the combination of the autophagic inhibitor, 3-methyladenine (3-MA), with exemestane re-sensitised resistant breast cancer cells (Amaral *et al.* 2012, 2013). A recent analysis, in ER⁺ carcinoma cells of patients following adjuvant exemestane treatment, demonstrated an increase in the immunoreactivity of autophagic markers, LC3 and beclin-1, and a correlation between beclin-1 levels in pre-treated stromal cells and poor clinical response to endocrine therapy (Ueno *et al.* 2016). Thus, this highlights the autophagic process as an important player to overcome resistance, in order to achieve therapeutic success.

Conclusions

The third-generation AIs are very effective, potent and specific; however, the development of AIs acquired resistance may lead to the relapse of ER⁺ breast tumours, this being the major concern in breast cancer therapy. The lack of cross-resistance among the different AIs proposes the existence of different resistance mechanisms. Besides the evidence provided by basic and translational research, the underlying mechanisms are not fully elucidated since some are based on data provided by various cellular models, producing confusing and complex findings. Nevertheless, some of them appear to be common to all AIs, while others seem to be AI specific. Targeting these aberrant pathways and molecules, according to the results obtained in *in vitro* studies and in clinical trials, suggests a beneficial effect, though no expansion on overall survival is observed in clinical setting. Overall, this shows the complexity of the mechanisms involved in breast cancer resistance, which emphasises the need to elucidate the underlying mechanisms in order to find new promising targets to improve AIs breast cancer therapy.

Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of this review.

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Author contribution statement

T V A and C A wrote the paper. G C d S, N T and C A revised the manuscript. T V A, G C d S, C M P R, N T and C A read and approved the manuscript for publication.

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