The new biology of estrogen-induced apoptosis applied to treat and prevent breast cancer

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Abstract

The successful use of high-dose synthetic estrogens to treat postmenopausal metastatic breast cancer is the first effective 'chemical therapy' proven in clinical trial to treat any cancer. This review documents the clinical use of estrogen for breast cancer treatment or estrogen replacement therapy (ERT) in postmenopausal hysterectomized women, which can either result in breast cancer cell growth or breast cancer regression. This has remained a paradox since the 1950s until the discovery of the new biology of estrogen-induced apoptosis at the end of the 20th century. The key to triggering apoptosis with estrogen is the selection of breast cancer cell populations that are resistant to long-term estrogen deprivation. However, estrogen-independent growth occurs through trial and error. At the cellular level, estrogen-induced apoptosis is dependent upon the presence of the estrogen receptor (ER), which can be blocked by nonsteroidal or steroidal antiestrogens. The shape of an estrogenic ligand programs the conformation of the ER complex, which, in turn, can modulate estrogen-induced apoptosis: class I planar estrogens (e.g., estradiol) trigger apoptosis after 24 h, whereas class II angular estrogens (e.g., bisphenol triphenylethylene) delay the process until after 72 h. This contrasts with paclitaxel, which causes G2 blockade with immediate apoptosis. The process is complete within 24 h. Estrogen-induced apoptosis is modulated by glucocorticoids and cSrc inhibitors, but the target mechanism for estrogen action is genomic and not through a nongenomic pathway. The process is stepwise through the creation of endoplasmic reticulum stress and inflammatory responses, which then initiate an unfolded protein response. This, in turn, initiates apoptosis through the intrinsic pathway (mitochondrial) with the subsequent recruitment of the extrinsic pathway (death receptor) to complete the process. The symmetry of the clinical and laboratory studies now permits the creation of rules for the future clinical application of ERT or phytoestrogen supplements: a 5-year gap is necessary after menopause to permit the selection of estrogen-deprived breast cancer cell populations to cause them to become vulnerable to apoptotic cell death. Earlier treatment with estrogen around menopause encourages growth of ER-positive tumor cells, as the cells are still dependent on estrogen to maintain replication within the expanding population. An awareness of the evidence that the molecular events associated with estrogen-induced apoptosis can be orchestrated in the laboratory in estrogen-deprived breast cancers now supports the clinical findings regarding the treatment of metastatic breast cancer following estrogen deprivation, decreases in mortality following

Key Words

- ▶ acquired resistance
- ▶ selective estrogen receptor modulators
- ▶ tamoxifen
- ▶ raloxifene
- ▶ aromatase inhibitors

long-term antihormonal adjuvant therapy, and the results of treatment with ERT and ERT plus progestin in the Women's Health Initiative for women over the age of 60. Principles have emerged for understanding and applying physiological estrogen therapy appropriately by targeting the correct patient populations.

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'A chapter is written but the book is not finished' (Simon Schama)

'Science is not what it appears to be. It is not objective and impartial since every observation it makes of nature is impregnated with theory' ... 'Science theory 'creates' facts and facts prove theory, the argument of science is circular'

'The unknown can only be examined by being defined in terms of the structure' ... 'The knowledge acquired through the use of any structure is selective' ... 'There are no standards or beliefs guiding the search for knowledge which are not dependent on the structure' ... 'Scientific knowledge is the artifact of each structure and its tool'

'Science merely seeks the truth – but which truth?' ... 'The answer to the question can only be – the truth defined by the contemporary structure' ... 'Discovery is invention; knowledge is man-made' (James Burke, 1985, 'The Day the Universe Changed', Published by BBC Books. Reproduced by permission of The Random House Group Ltd (UK) and Little Brown and Co. Boston (USA))

The fact that estrogen is implicated as the 'fuel for the fire' of breast cancer is ingrained in women's psyche. The Food and Drug Administration (FDA) has institutionalized the principle, by warning of the dangers of estrogen to women who have had breast cancer. This is, in the main, correct. The enormous success of the antiestrogen tamoxifen that blocks the tumor estrogen receptor (ER) and the aromatase inhibitors (AIs) that block the capacity of postmenopausal women to synthesize estrogen as adjuvant therapies proves the point (Jordan & Brodie 2007). These rigorously tested clinical strategies with 'antiestrogens' used as adjuvant therapies or as chemopreventive strategies to prevent breast cancer in high-risk women confirm the early endocrine ablation studies (oophorectomy, adrenalectomy, or hypophysectomy) that document tumor regression when hormones were removed. In modern times, millions of women with breast cancer have longer lives thanks to antiestrogenic treatments. The structure has been impregnated by theory and confirmed. Except, is this the whole truth?

Observational clinical trials with high-dose estrogen that became the standard of care for the treatment of postmenopausal metastatic breast cancer appear to be outside the structure before and after tamoxifen (Haddow *et al.* 1944, Kennedy 1965, Ingle *et al.* 1981). Tamoxifen conformed to the structure as an antiestrogen, hence estrogen therapy was discarded and the search for mechanisms abandoned. The rediscovery of the phenomenon where physiological estrogen 'melted away' tamoxifen-resistant breast cancers under laboratory conditions (Wolf & Jordan 1993, Yao *et al.* 2000) resurrected the concept but resistance to the reintroduction of estrogen to treat breast cancer patients was fierce during the 1990s.

Following rediscovery of the antitumor action of estrogen, a new dimension to the fascinating story of estrogen action was added to the multiplicity of estrogen's actions around a woman's body. However, the new biology of estrogen-induced apoptosis is dependent not upon the estrogenic steroid itself or the ER but on the irrepressible adaptability of ER-positive breast cancer cells to survive any therapeutic intervention. In this case, the cancer cell adapts to the withdrawal of 'the fuel for the fire' to evolve, through incessant replication, trial and error to find a new successful population for estrogen-independent growth. The sacrifice that the cancer makes is that the population which has now evolved has a vulnerability – estrogen-induced apoptosis.

This review of progress in understanding the new structure for selective actions of estrogen with applications in women's health is not only about estrogen and a newly found therapeutic potential in cancer, but also about the general principle of the essence of cancer that first evades therapy and then kills the host through selection pressure. The story will first be placed into historical context, as the key to success for therapy in the future is, as it has always been, selective toxicity.

Historical introduction

The selective killing of infectious diseases to cure the patient is a noble goal. At the dawn of the 20th century, Prof. Paul Ehrlich created the systematic method used

to this day, for the synthesis and testing of selectively toxic organic molecules that would kill the disease, but not the patient (Baumler 1984). He reasoned that arsenic, an accumulated and fatal poison, could be 'emasculated' through synthetic incorporation into organic molecules that would preferentially target the disease organism. The key to translation across the 'valley of death' to effective medicines in patients was the creation of appropriate animal models to predict success in patients without fatal consequences (Jordan 2014a). In the spring of 1909, Dr Sahachiro Hata from Japan joined Ehrlich's team in Frankfurt and created animal models infected with trypanosomes or spirochetes to identify test compounds for treatment of syphilis. This was a major killer, with a long and distressing course. The discovery of Salvarsan (or compound 606) first reported by Ehrlich at the Congress of Internal Medicine at Wiesbaden on April 19, 1910, its production by Hoechst, and the successful cure of syphilis changed the approach to the treatment of human disease forever. From that time until the present day, pharmacology and therapeutics became a rational and evidence-based science. Ehrlich then turned his attention to the treatment of cancer.

Cancer therapeutics did not exist in the early years of the 20th century; only surgery was available. In Germany, Schinzinger (1889) had suggested that oophorectomy might be used to treat breast cancer, but this does not seem to have been adopted. In contrast, Beatson (1896) reported the favorable response of a premenopausal case of metastatic breast cancer in 1896. Boyd (1900) assembled all known cases of oophorectomy in the UK and reported a 30% response rate. This was perhaps the first clinical trial for the treatment of breast cancer and, remarkably, a 30% response rate has remained a 'magical' biological response rate for endocrine therapy ever since. However, responses were transient and not everyone responded. A different approach was required and perhaps Ehrlich could find a drug?

The problem was that there were no suitable animal models for cancer in the early 1900s in which to test any compounds. The ovarian dependence of animal models of spontaneous mouse mammary cancer was to be described by Lathrop & Loeb (1915a,b, 1916) and Lacassagne (1936a,b) would link estrogen with carcinogenesis in the mouse mammary gland in the mid-1930s. In contrast, Ehrlich found himself at the dawn of the new science of cancer research. In the year before he died in 1916, Ehrlich declared 'I have wasted 15 years of my life in experimental cancer research' (Schrek 1959).

The situation remained static until Haddow *et al.* (1944) reported that high doses of synthetic estrogens

were able to produce a 30% response rate in women with metastatic breast cancer. High-dose estrogen therapy remained as the standard of care until the introduction of tamoxifen, a nonsteroidal antiestrogen, for the treatment of breast cancer in the 1970s (Jordan 2003a). But this has remained as a paradox. All the laboratory and clinical evidence indicated that breast cancer was dependent on estrogen for growth, but Haddow taught us that estrogen causes tumor regression!

In 1970, during the inaugural Karnofsky Lecture, Haddow (1970) expressed dismay that there were no laboratory predictive tests available to determine whether a cancer 'chemical therapy' or chemotherapy, as Ehrlich suggested, would be effective for appropriate treatment of a cancer. It was trial and error. He was also skeptical that a truly selective drug could be developed for cancer, as cancer was 'self'. He did, however, offer one positive statement:

'...the extraordinary extent of tumour regression in perhaps 1% of postmenopausal cases (with oestrogen) has always be regarded as of major theoretical importance and it is a matter for some disappointment that so much of the underlying mechanism continues to elude us...' (Haddow 1970)

In this review, the clinical facts about the historical use of high-dose estrogen therapy will first be summarized as they provide a clinical insight into the new biology of estrogen-induced apoptosis. It is perhaps to be expected that interest in the antitumor effects of estrogen should have been abandoned once tamoxifen arrived as the antihormone therapy of choice for all stages of breast cancer, ductal carcinoma *in situ*, and male breast cancer, and as a preventive measure for breast cancer in high-risk pre- and postmenopausal women (1980s–2000). Serious side effects with high-dose estrogen would not permit development as was possible with tamoxifen. It is therefore somewhat ironic that the solution to 'Haddow's Paradox' should come initially from an understanding of acquired resistance to tamoxifen (Wolf & Jordan 1993, Yao *et al.* 2000). Laboratory models have been developed from that understanding that could be used to decipher the mechanisms. For this reason, I have dedicated this article to the memory of Sir Alexander Haddow FRS.

Facts about high-dose estrogen therapy

The discovery and availability of the synthetic estrogens, diethylstilbestrol (DES; Dodds *et al.* 1938) and the longer acting triphenylethylenes (Robson 1937, Robson & Schonberg 1938, 1942), created opportunities

Figure 1

Formulae of nonsteroidal estrogens used by Haddow *et al.* (1944) (diethylstilbestrol, triphenylchlorethylene, and triphenylmethylethylene) and later used by Walpole & Paterson (1949) (diethylstilbestrol, dienestrol, and M2613) for the treatment of metastatic breast cancer in postmenopausal women.

for applications in patient care. Following studies in animal models, Haddow & Robinson (1939) noted the antitumor properties of polycyclic hydrocarbons. However, the compounds were themselves classified as carcinogens, hence Haddow examined synthetic estrogens (Fig. 1), because he reasoned that the multiple phenyl rings had structural similarities (lucky logic; frightening rationale!). The estrogens also had antitumor properties. The results of the first clinical trial published in 1944 (Haddow *et al.* 1944) are summarized in Table 1. Responses were consistent at approximately 30% but for less than a year. These preliminary results indicated that both breast and prostate cancers were responsive but no other tumor types responded. Haddow went on to organize a larger multicentric study at the Royal Society of Medicine where he was the President of the Section of Oncology. Haddow (1970) described his discovery during his 1970 Karnofsky lecture:

'When the various reports were assembled at the end of that time, it was fascinating to discover that rather general impression, not sufficiently strong from the relatively small numbers in any single group, became reinforced to the point of certainty; namely, the beneficial responses were three times

more frequent in women over the age of 60 years than in those under that age: that oestrogen may, on the contrary accelerate the course of cancer in younger women and that their therapeutic use should be restricted in cases 5 years beyond the menopause. Here was an early and satisfying example of the advantages which accrue from cooperative clinical trials' (Haddow 1970)

Walpole & Paterson (1949) (the latter had previously worked with Haddow) followed up Haddow's study at the Christie Hospital in Manchester. The goal was to understand why some patient's tumors responded but others did not. They were unsuccessful, but did confirm Haddow's observation that older patients were more likely to respond than younger patients. Subsequently, Stoll (1977) in London reviewed response rates vs time of treatment after menopause for all breast cancer patients in his practice (Table 2). The results were clear; a period of 5 years after menopause was necessary for optimal antitumor action of high-dose estrogen for breast cancer treatment. All clinical results were, therefore, consistent – a period of time after the menopause was necessary to expose the effectiveness of high-dose estrogen as an anticancer agent for metastatic breast cancer.

The reason why some breast tumors, more than 5 years after menopause, responded to high-dose estrogen therapy had to wait for the discovery of the cellular mechanism of estrogen-stimulated growth and regression. The answer would come initially from DES itself. Stilbene can be hydrogenated with tritium across the double bond to produce a high-specific-activity [^3H] hexestrol. Hexestrol is a potent estrogen and the administration of [^3H] hexestrol to sheep and goats showed selective binding in estrogen target tissues (Glascock & Hoekstra 1959). The idea that radioactive synthetic estrogens could identify an estrogen-responsive tissue was subsequently translated to a clinical trial (Folca *et al.* 1961) to identify breast tumors more likely to respond to endocrine ablation. These preliminary encouraging results were refined by Jensen & Jacobson (1962), but using the natural hormone [^3H] estradiol E_2 to demonstrate that E_2 bound to and was retained by

Table 1 Results of the first clinical trial of synthetic estrogens for treatment of cancers. Haddow *et al.* (1944)

	Responses/numbers of patients		
	Breast	Prostate	Other
Triphenylchlorethylene	10/22	2/2	0/28
Diethylstilbestrol	5/14	–	–

Table 2 Objective response rates in postmenopausal women with metastatic breast cancer using high-dose estrogen therapy. A total of 407 patients were classified based on the time from menopause (Stoll 1977)

Age since menopause	Number of patients	Percentage responding
Postmenopausal		
0–5 years	63	9
> 5 years	344	35

estrogen target tissues (i.e., uterus, vagina, and pituitary gland) of the immature rat. Gorski's group (Toft & Gorski 1966, Toft *et al.* 1967) subsequently made two important findings: the ER was identified as an extractable complex with E₂ and that the receptor protein could be extracted and then labeled with [³H] E₂ for quantitation and identification. This property for a putative receptor was unique as all other pharmacological receptors to this point were membrane-bound. Subsequently, breast cancers were found to contain various levels of ER but some had no ER (Johansson *et al.* 1970, Feherty *et al.* 1971, Maass *et al.* 1972, Leclercq *et al.* 1973).

The validation of the clinical ER assay

On July 18th and 19th, 1974, an international workshop was held in Bethesda, Maryland, to link the biochemical measurement of tumor ER with responsiveness to endocrine therapy (McGuire *et al.* 1975a). All responses were subjected to an extramural review and the simple question posed: does the ER in the breast tumor predict the response to endocrine therapy, i.e., no ER no response? Conversely, if an ER-positive tumor regressed in response to endocrine ablation therapy, then estrogen must be stimulating and maintaining tumor growth. It should be stressed that the principal goal of the conference was to validate a test to predict the responsiveness of metastatic breast cancer to endocrine ablation (hypophysectomy, adrenalectomy, or oophorectomy). This was important because a patient would then not have to go through significant life-threatening surgery, in the case of adrenalectomy and hypophysectomy, if there was little chance of a response. The extramurally reviewed clinical data indicated that endocrine ablation did not cause tumor regression in ER-negative disease (McGuire *et al.* 1975b). The goal was achieved and all women with breast cancer were subsequently mandated to have an ER assay on a biopsy of their breast tumor upon diagnosis. The ER laboratory

was born as an essential component of a woman's breast cancer care (Jordan *et al.* 1988). However, this strategy was limited.

Antiestrogens were not generally available and tamoxifen would not be available for the treatment of metastatic breast cancer until FDA approval on December 29, 1977 (Jordan 2003a). DES was the standard of care for the treatment of metastatic breast cancer in postmenopausal patients during the 1960s and early 1970s at the time of the Bethesda Conference (Kennedy 1965). Most importantly for the current topic of estrogen-induced apoptosis, DES caused tumor regression in ER-positive breast cancer at about the same frequency (Table 3) as oophorectomy in premenopausal women. Neither treatment strategy was effective if the tumor was ER-negative. Thus, inhibition of the action of estrogen through ablation to remove the circulating effects of estrogen was as effective as using high-dose estrogen therapy for more than 5 years after menopause. The ER controlled both the growth and the death of breast tumor cells. However, the development of the antiestrogen tamoxifen throughout the late 1970s–2000, would result from the definition, in the laboratory, of the initial clinical strategies to target tamoxifen to the tumor ER, using long-term adjuvant tamoxifen therapy as the appropriate strategy to treat breast cancer (Jordan 2008a, 2014b), and the use of tamoxifen as a preventive measure for breast cancer (Jordan 2008a, 2014b). Tamoxifen took precedence. High-dose estrogen therapy and the mechanism of the antitumor effects of estrogen were relegated to the history of medical oncology.

Transition to tamoxifen

Early clinical trials of the treatment of metastatic breast cancer with tamoxifen in postmenopausal women showed similar response rates and durations of responsiveness to DES, but with fewer side effects than high doses of estrogen (Cole *et al.* 1971, Ingle *et al.* 1981). However, it was noted that reanalysis of a randomized

Table 3 Objective breast cancer regression according to ER assay and the type of therapy as decided by extramural review. Oophorectomy of premenopausal women or high-dose estrogen therapy for postmenopausal women with metastatic breast cancer (McGuire *et al.* 1975b)

Therapy	ER–	ER+
Oophorectomy	5/53 (10%)	25/35 (76%)
Estrogen	5/56 (10%)	37/57 (65%)

trial of DES vs tamoxifen (Ingle *et al.* 1981) demonstrated that patients treated with DES had a more prolonged survival when compared with those treated with tamoxifen (Peethambaram *et al.* 1999). DES was different; nevertheless, this was of no significance for the future of estrogen therapy, as tamoxifen was being developed globally. Only retrospectively, was this clinical observation of biological significance. Laboratory animal model (Jordan & Dowse 1976, Jordan & Jaspan 1976, Jordan & Koerner 1976, Jordan 1976a,b, Jordan & Prestwich 1977, Jordan *et al.* 1977) and clinical studies (Jordan & Koerner 1975, Morgan *et al.* 1976, Kiang & Kennedy 1977) all yielded results indicating essential role of the tumor ER as the target for tamoxifen action as an antiestrogen. Tamoxifen became the endocrine therapy standard of care for all stages of breast cancer (Fisher *et al.* 1987, Scottish Cancer Trials Office (MRC) 1987) and was subsequently successfully tested in the 1990s as the first chemopreventive agent for breast cancer in high-risk pre- and post-menopausal women (Fisher *et al.* 1998, 2005, Cuzick *et al.* 2007, Powles *et al.* 2007).

The overview of all the world's randomized adjuvant clinical trials at Oxford defined the benefits of tamoxifen in lives saved, serious side effects, and human cancer biology (Early Breast Cancer Trialists' Collaborative Group 1998, 2005). On balance, the risk-to-benefit ratio for tamoxifen was strongly in the benefit direction and the major clinical side effect of a small but significant increase in endometrial cancer was quantified and appropriate steps were taken to minimize the risks of death. Gynecologists were included in the breast cancer treatment team after 1990. The significant and sustained survival benefits of tamoxifen were, however, unanticipated and shown to increase not only with the duration of adjuvant tamoxifen therapy but also following the cessation of tamoxifen treatment. The 2011 overview analysis (Davies *et al.* 2011) effectively summarizes the state of knowledge regarding 5 years of adjuvant tamoxifen therapy (then the standard duration of treatment and of care), but the recent results of the Adjuvant Tamoxifen Longer Against Shorter (ATLAS) indicated that 10 years of tamoxifen has a greater effect on decreasing mortality than 5 years of tamoxifen (Davies *et al.* 2013). The decreases in mortality were actually found to be greater in the decade after tamoxifen treatment was stopped. This was unexpected, as an antiestrogenic drug should only control estrogen-stimulated tumor growth as long as it is taken. Some other factors were involved but elusive.

The translation from animal models of adjuvant therapy and chemoprevention (Jordan 1976b, 1983, Jordan & Allen 1980) to clinical practice was counter-intuitive, but, in reality, it turned out that the concepts found in the laboratory were correct. In the sense of the 'Ehrlich dictum' that an appropriate animal model should be used to translate findings to treat human disease (Baumler 1984), the carcinogen-induced rat mammary carcinoma model turned out to be the appropriate animal model for laboratory testing and translation to clinical trials. The value of long-term adjuvant tamoxifen clinical trials (5 or more years (Davies *et al.* 2013)) was again counterintuitive as tamoxifen only controlled metastatic breast cancer for 1–2 years (Ingle *et al.* 1981). Clearly, something was different about micrometastatic breast cancer that resulted in the control of very small cell populations, which was unlike the inability of tamoxifen to control the established bulky tumors. It is a general principle in oncology that low tumor bulk predicts therapeutic success, but this was not the real explanation for tamoxifen, a medicine classified as a cytostatic and not a cytotoxic agent (Osborne *et al.* 1987, Gottardis *et al.* 1988). Where did the cytotoxicity come from? The answer again was unanticipated.

If long-term adjuvant tamoxifen therapy was effective in clinical trials during the 1980s, it would have been naïve to believe that acquired resistance would not develop eventually. This is true for all anticancer drugs, but, at that time, there were no animal models of acquired resistance to tamoxifen. The questions to be addressed urgently in the 1980s were, 'could an appropriate animal model of human acquired resistance to tamoxifen be created, what form would resistance take, and could second line therapies be developed predictably?'

Acquired resistance to tamoxifen

The discovery of ER in some breast cancers and the general observation that there was a range of ER concentrations (femtomoles/mg cytosol protein; Jordan *et al.* 1988) meant that breast cancer could be a mix of ER-positive and -negative cells. In the 1970s, it was believed that endocrine therapy would hold the growth of ER-positive cells, but the ER-negative cells would eventually gain a growth advantage. Resistance would occur as the tumor transitioned from being ER-positive to become ER-negative by cellular population shifts. However, this conceptual model did not fit with clinical experience with 'the endocrine cascade'. Experience taught physicians that an excellent response to one endocrine therapy would herald a good

response to a second-line agent and so on until tumor bulk overwhelmed the patient. Stoll (1977) documented individual cases where the administration of high-dose estrogen in 80-year-old women could cause tumor regression, but after stopping treatment the tumor would grow back. The procedure could be repeated for years to titrate tumor bulk and enhance palliative patient care. Eventually, the tumor would grow during high-dose estrogen therapy but rapidly regress once treatment was stopped. He called this a 'withdrawal response'. None of the biology, at this time in the 1960s, was understood but the arrival of long-term adjuvant tamoxifen therapy created new laboratory priorities for the development of animal models of human disease.

The nu/nu athymic mouse model (Rygaard & Povlsen 1969) will accept heterotransplantation of human tumors for studies of cancer therapeutics (Giovannella *et al.* 1974, 1978). The MCF-7 breast cancer cell line grows into ER-positive tumors if inoculated into the axillary mammary fat pad of estrogen-treated ovariectomized athymic mice (Shafie & Grantham 1981, Huseby *et al.* 1984). Tamoxifen, despite being classified as an estrogen in the mouse (Harper & Walpole 1967, Terenius 1971), blocks estrogen-stimulated breast tumor growth. Long-term therapy can also be used for months (Osborne *et al.* 1985), hence this was viewed as a suitable model for studying acquired resistance. Osborne *et al.* (1987) first demonstrated that MCF-7 tumors would eventually grow despite long-term tamoxifen treatment. However, the unusual feature about acquired resistance to tamoxifen is that the tumors grow upon re-transplantation because of tamoxifen or physiological estrogen. The finding (Gottardis & Jordan 1988) that a tumor became dependent on the treatment was a unique observation in oncology.

The fact that tamoxifen is an estrogen in the mouse naturally raised the question of metabolic conversion of tamoxifen to estrogenic metabolites during long-term therapy in mice or indeed the development of induced enzyme systems in humans that could create estrogenic metabolites over time. Studies using the same MCF-7 tumors with acquired tamoxifen resistance developed in mice, but now implanted into athymic rats, a species where tamoxifen is predominately antiestrogenic, produced the same tamoxifen-stimulated tumor growth (Gottardis *et al.* 1989a). Additionally, studies on the metabolic stability of tamoxifen in patients treated for up to 10 years with adjuvant tamoxifen demonstrated the stability of antiestrogenic metabolite levels over the whole time period (Langan-Fahey *et al.* 1990).

The results obtained from the athymic animal/MCF-7 tumor model, which have been reported previously (Gottardis & Jordan 1988), are consistent with those obtained from human studies on acquired resistance to tamoxifen in the patient with metastatic breast cancer. The therapy for tumors failed within 1–2 years (Ingle *et al.* 1981) and a tamoxifen 'withdrawal response' has been noted (Howell *et al.* 1992). The fact that the experimental tumor with acquired resistance to tamoxifen (Gottardis & Jordan 1988) would subsequently grow with either tamoxifen or E₂ provided clues to subsequent treatment strategies. Based on the similarities of the MCF-7 breast cancer/athymic mouse model and metastatic breast cancer, results from therapeutic studies indicated that the lead compound ICI 164 384 for a new class of ER downregulators (referred to as pure antiestrogens) or no estrogen at all, i.e., an AI, was a reasonable second-line therapy to evaluate the subsequent acquired tamoxifen resistance (Gottardis *et al.* 1989b). A decade later, clinical trials confirmed that an AI and fulvestrant, the clinically available pure antiestrogen, were both acceptable second-line therapies (Howell *et al.* 2002, Osborne *et al.* 2002). Nevertheless, there was a translational flaw in the laboratory model when applied to the adjuvant therapy for breast cancer for 5–10 years. If metastatic ER-positive breast cancer cells are converted to acquired resistance to tamoxifen within 2 years, why is it that adjuvant therapy does not fail universally at the 2-year treatment mark? The answer is tumor bulk, genetic variation, and the ability to grow through trial and error.

The few micrometastatic cells exposed to tamoxifen during adjuvant therapy obviously are not recapitulated by the 10 million MCF-7 cells initially inoculated into athymic mice to create tumors that grow in the presence of estrogen or tamoxifen to become a 100 times the size. In other words, there is greater genetic diversity during selection pressure with tamoxifen for 1–2 years in the athymic mouse model.

However, transplantation of the MCF-7 tumor into subsequent generations of athymic mice for up to 5 years to maintain the phenotype of acquired tamoxifen resistance ultimately exposes a vulnerability in breast cancer through expansion and differential growth of favored populations. Long-term antiestrogen therapy with tamoxifen now creates a selected cell population that responds to physiological estrogen once tamoxifen is stopped, not as a growth signal but as an apoptotic trigger (Wolf & Jordan 1993, Yao *et al.* 2000). The evolution of cell populations to create acquired resistance to tamoxifen is illustrated in Fig. 2.

Figure 2

The evolution of drug resistance to SERMs. Acquired resistance occurs during long-term treatment with an SERM and is evidenced by SERM-stimulated breast tumor growth. Tumors also continue to exploit estrogen for growth when the SERM is stopped, so a dual-signal transduction process develops. The AIs prevent tumor growth in SERM-resistant disease and fulvestrant that destroys the ER is also effective. This phase of drug resistance is referred to as phase I resistance. Continued exposure to an SERM results in continued SERM-stimulated growth, but eventually autonomous growth (phase III) occurs that is unresponsive to fulvestrant or AIs. This is the original concept that was proposed in the mid-2000s and emphasized the switching mechanism that distinguishes phase I from phase II resistance. These distinct phases of laboratory drug resistance (Yao *et al.* 2000, Lewis *et al.* 2005b) have their clinical parallels and this new knowledge is being integrated into the treatment plan. Reproduced with permission from Jordan VC 2004 Selective estrogen receptor modulation: concept and consequences in cancer. *Cancer Cell* 5 207–213, with permission from Elsevier. Copyright 2004 Cell Press. The evolutionary concept has evolved in the past decade.

The antitumor actions of physiological estrogen *in vivo*

Cell culture models of tamoxifen resistance during the 1980s were focused on mechanistic changes and not biological changes in cellular populations. The fact that tumors with acquired resistance to tamoxifen could only be passaged into athymic mice and maintained in successive generations of animals for years was fortuitous for the chance discovery that physiological estrogen administration (Wolf & Jordan 1993) could cause tumors to undergo 'the extraordinary extent of tumor regression' (the words of Haddow (1970) about the response of a few breast tumors to high-dose estrogen treatment for metastatic breast cancer). The evolution of acquired resistance to tamoxifen *in vivo* was replicated (Yao *et al.* 2000) and the time course of the antitumor sensitivity to the antitumor action of physiological estrogen over 5 years of estrogen treatment was documented (Yao *et al.* 2000). The finding that acquired resistance to tamoxifen passes through phases of cellular sensitivity to estrogen is both intriguing and now clinically relevant. The animal transplantation studies revealed two major phases of acquired tamoxifen

resistance (Fig. 2): phase 1 occurs in about a year and the new cell population can use either E_2 or tamoxifen to stimulate growth. Phase II resistance occurs over the next 3–4 years of continuous tamoxifen treatment, but there is increasing vulnerability of the cell population to the apoptotic effects of estrogen; the process evolves or intensifies through selection pressure over a 5-year period.

The development of populations of MCF-7 cells vulnerable to estrogen-induced apoptosis is not unique to the selective ER modulator (SERM) tamoxifen. Cells incubated with raloxifene in an estrogen-free environment *in vitro* (Liu *et al.* 2003) can be inoculated into ovariectomized athymic mice and shown to grow with raloxifene. Physiological estrogen causes tumor regression once raloxifene is stopped. Similarly, long-term transplantation of MCF-7 tumors over a decade into raloxifene-treated athymic mice can replicate the cyclical sensitivity of an SERM and estrogen to shift tumor cell population sensitivity from SERMs stimulating tumor growth to SERMs inhibiting estrogen-stimulated growth (Balaburski *et al.* 2010). The cell populations seem to drift very much as Stoll (1977) had observed with DES in elderly women being titrated for tumor bulk.

Early studies of the mechanism of estrogen-induced apoptosis *in vivo* produced some interesting findings. Estrogen-induced apoptosis causes an increase in Fas receptor associated with the extrinsic pathway of apoptosis and a simultaneous decrease in NF κ B (Osipo *et al.* 2003, Lewis-Wambi & Jordan 2009). Most interesting are the observations that the pure antiestrogen fulvestrant plus physiological estrogen can reverse apoptosis and cause robust growth of tumors (Osipo *et al.* 2003). This raised the possibility that a combination of fulvestrant and AIs might be a superior therapeutic strategy for the treatment of metastatic disease. Regrettably, clinical results are conflicting (Bergh *et al.* 2012, Mehta *et al.* 2012).

The major advances in understanding estrogen-induced apoptosis, however, have come not from studies on animals, but rather mechanisms that have been systematically interrogated using estrogen-deprived cells *in vitro*. The advent of AIs as the long-term adjuvant therapies of choice for postmenopausal patients (Jordan & Brodie 2007, Dowsett *et al.* 2010) mandated a strategy for studying the mechanism of acquired resistance to estrogen deprivation. However, in the 1970s and 1980s, the understanding of estrogen-stimulated cell growth was not at all straightforward.

The few ER-positive breast cancer cell lines

Despite the fact that there are very few available ER-positive cell lines, remarkable progress has been made in understanding the basics of hormone and antihormone action that clearly translates to clinical care (Sweeney *et al.* 2012). Four ER-positive cell lines, MCF-7, T47D, ZR75-1 and BT474, are used routinely in the laboratory. But it is the ER-positive MCF-7 cell line (Brooks *et al.* 1973) that has perhaps provided the majority of data in translational research. The cells originally were derived from a plural effusion where the patient had been treated with high-dose estrogen therapy and the treatment had failed (Soule *et al.* 1973). Culture of the cells in estrogen-containing or -free conditions using charcoal stripping of serum to remove estrogenic steroids did not alter growth but tamoxifen could block spontaneous growth which could be reversed by addition of estrogen (Lippman & Bolan 1975)! Transplantation into athymic mice, however, required estrogen supplementation for growth of tumors (Soule & McGrath 1980). The hypothesis was raised that a second messenger might have to be stimulated by estrogen to cause estrogen-stimulated growth *in vivo* (Shafie 1980). The problem was resolved with the subsequent finding that the redox indicator in a culture medium, phenol red, contains a contaminant that is an estrogen (Berthois *et al.* 1986, Bindal & Katzenellenbogen 1988). Up until that time in 1987, MCF-7 cells, it seems, had always been grown in an estrogenic environment. Now it was time to see what estrogen deprivation really did to breast cancer cell populations, not unlike what could be happening at menopause.

There were two independent reports of the effects of immediate estrogen deprivation on the MCF-7 cell line (Katzenellenbogen *et al.* 1987, Welshons & Jordan 1987). Both noted a 'crisis period' at about a month after estrogen withdrawal with a catastrophic decrease in cell numbers. However, surviving cells grew back over a period of months with an elevation in ER levels and estrogen-independent growth. Several clonal populations were subsequently created; MCF-7:5C cells were refractory to the actions of a nonsteroidal antiestrogen to prevent growth or estrogen to stimulate growth or initiate PgR synthesis (Jiang *et al.* 1992). In contrast, the MCF-7:2A cells were responsive to stimulation of PgR synthesis by estrogen; antiestrogen decreased growth but estrogen did not affect growth in the 1-week growth assay (Pink *et al.* 1995). Of interest was the finding that MCF-7:2A cells also had a high-molecular-weight ER (ESR1) protein with a 6/7 exon repeat in the ligand-binding domain as well as the

WT ER (Pink *et al.* 1995, 1996a, 1997). This was a unique biological finding concerning the translation and processing of a steroid receptor protein.

A similar approach to estrogen deprivation in MCF-7 cells was taken by the Santen group but without cloning. The long-term estrogen-deprived MCF-7 cell (LTED) populations went through interesting adaptations to estrogen deprivation. Initially, the cell population experienced 'adaptive hypersensitivity' (Masamura *et al.* 1995, Jeng *et al.* 1998), i.e., the cells scavenged very low concentrations of estrogen to enhance growth. This observation was offered as an explanation for aromatase resistance, i.e., the estrogen-deprived ER-positive cells would subvert growth control by exploiting the growth potential of any ligands that could activate the enhanced concentration of ER in cells.

However, Song *et al.* (2001) reported the apoptotic role of estrogen *in vitro* and proposed this as the mechanism of high-dose estrogen therapy employed by Haddow *et al.* (1944) 60 years before to treat postmenopausal women with breast cancer. But it was clear from the concentration-response curve presented that low concentrations were able to decrease cell numbers through triggering apoptosis (Jordan *et al.* 2002); as had been noted with physiological estrogen causing tumor regression in the MCF-7 tamoxifen-resistant tumor *in vivo* (Wolf & Jordan 1993, Yao *et al.* 2000). The Song *et al.* (2001) study *in vitro* identified an increase in FAS ligand as the mechanism of estrogen-induced apoptosis (via the extrinsic or 'death receptor' pathways), but no studies up to that point, nor the subsequent animal studies with tamoxifen-resistant tumors (Liu *et al.* 2003, Osipo *et al.* 2003, 2005, 2007), identified a sequence of events that triggered estrogen-induced apoptosis.

Lewis *et al.* conducted a series of studies with MCF-7:5C cells and MCF-7:2A cells *in vitro*. The MCF-7:5C cells (Jiang *et al.* 1992) were initially noted to be ER-positive, PgR-negative, and nonresponsive to estrogen. However, alteration of the culture conditions dramatically changed estrogen responsiveness (Lewis *et al.* 2005a): the MCF-7:5C cells were now able to rapidly undergo E₂-induced apoptosis, within a few days, in a concentration-related manner. The MCF-7:5C cells (Lewis *et al.* 2005b) could also be inoculated into athymic mice and grew spontaneously, but the tumors would stop growing with fulvestrant therapy and remain static, whereas physiological E₂ administration would result in complete tumor regression. This observation was reminiscent of the effects of physiological estrogen noted a decade earlier with MCF-7 tamoxifen-resistant tumors in athymic mice (Wolf & Jordan 1993). Lewis *et al.* (2005b)

identified the intrinsic mitochondrial pathway as the primary target for estrogen-induced apoptosis with changes in pro-apoptotic markers, and leaking of cytochrome *c* through the mitochondrial membrane. In a parallel study by Song & Santen (2003) and Song *et al.* (2005), an inhibitor of bcl2 was shown to enhance estrogen-induced apoptosis in LTED MCF-7 breast cancer cells.

The MCF-7:2A cells (Pink *et al.* 1995) were initially found to be resistant to estrogen-induced apoptosis, with slow apoptotic changes occurring after 6 days of estrogen treatment (Lewis *et al.* 2004, Lewis-Wambi & Jordan 2009). These cells apparently can protect themselves from increases in reactive oxygen species (ROS) through enzymatic overproduction of glutathione. Using buthionine sulfoximine (Lewis-Wambi *et al.* 2008, 2009), which inhibits glutathione biosynthesis, estrogen-induced apoptosis was advanced to occur during the first 6 days of estrogen treatment. Recent studies have built on these original findings (Sweeney *et al.* 2014a).

With the transition from long-term adjuvant tamoxifen therapy to use of AIs and the knowledge that cell culture media contain estrogen (the 'phenol red' story (Berthois *et al.* 1986, Bindal & Katzenellenbogen 1988)), other breast cancer cell lines were investigated to document changes during estrogen deprivation. Studies using the T47D cell line were initiated 25 years ago to determine the effects of estrogen deprivation on an ER, PgR-positive breast cancer cell line that was, unlike MCF-7 cells, harboring a mutated p53. This is a key regulator in the decision network for DNA repair or apoptotic death.

Unlike the MCF-7 cell line, the T47D cells not only take a different route for cellular survival but also were found to have a reversal of their ER regulatory mechanism (Pink & Jordan 1996). Culture of T47D in estrogen-free media results in downregulation of the ER (Murphy *et al.* 1989, 1990). Initial long-term treatment in estrogen-free media for months results in the apparent loss of ER, but this can be 'rescued' by re-culture for months in estrogen-containing media. This again is an example of shifting cellular populations with the selective pressure of an estrogen-free medium creating an apparently ER-negative cell outgrowth as a survival response, once the ER is no longer synthesized. The selection pressure of new estrogen-containing media reactivates ER synthesis in the minority of contaminating cells, and ER-positive T47D cells again dominate by overgrowth. Only with repeated dilution cloning can a pure line of ER-negative T47D cells be created that is stable when reintroduced into estrogen-containing media (Pink *et al.* 1996b). These ER-negative T47D cells are referred to as T47D C4:2 cells.

Figure 3

The diagrammatic representation of cellular estrogen receptor (ER) regulation in media with or without estradiol (E₂). This diagram is based on the general responses to estrogen illustration by western blotting and presented in detail in Pink & Jordan (1996). Model I ER regulation (MCF-7, ZR-75, and BT-474) involves upregulation of ER mRNA and protein in an estrogen-depleted environment, but ER is downregulated at the mRNA and protein levels in the presence of estrogen. Model II ER regulation (T47D) involves upregulation of ER mRNA and protein in an estrogen-containing environment, but ER is not produced in an estrogen-depleted environment. Cells lose ER to become ER-negative.

The two ER regulatory systems are illustrated in Fig. 3 and provide an excellent example of how T47D cells can respond to estrogen deprivation in order to survive. The mutant p53 cell that needs estrogen to survive chooses to lose the ER survival system and evolve to an ER-negative state. In contrast, the WT p53 cell (MCF-7) expands the ER system to survive but in so doing must sacrifice survival to the vulnerability to estrogen-induced apoptosis, should the environment again return to being estrogen-rich.

An alternate route to estrogen-induced apoptosis

There is an inverse relationship between ER status and protein kinase C alpha (PKCα) in breast cancer (Borner *et al.* 1987), breast cancer cell lines (Tonetti *et al.* 2000), and endometrial cancer (Fournier *et al.* 2001). Indeed, PKCα is associated with antiestrogen resistance (Nabha *et al.* 2005, Assender *et al.* 2007, Frankel *et al.* 2007). Tonetti *et al.* (2000) posed the question of whether the

stable transfection of the *PKC α* (*PRKCA*) gene into the ER-positive T47D:A18 estrogen-responsive breast cancer cell line would influence the responsiveness to estrogens and antiestrogens. Although the T47D:A18/*PKC α* cells are unaffected by estrogen treatment *in vitro* (Tonetti *et al.* 2000), the cells grow spontaneously into tumours when implanted into athymic mice (Chisamore *et al.* 2001, Zhang *et al.* 2009), but estrogen causes rapid tumor regression that can be blocked by fulvestrant (Zhang *et al.* 2009). Tumor regression is associated with an increase in the FAS/FASL (FASLG) proteins and a decrease in the prosurvival Akt pathway. It has been suggested that tumor regression requires the participation of ER α , the extracellular matrix, FAS/FASL, and the Akt pathway (Zhang *et al.* 2009). It is interesting to note that the T47D:A18/*PKC α* cells are resistant to tamoxifen treatment when grown into tumors in athymic mice, but raloxifene causes tumor regression via a mechanism that causes nuclear ER to translocate to extranuclear sites in response to either estrogen or raloxifene (Perez White *et al.* 2013) as well. This unusual pharmacology indicates that raloxifene derivatives may be found as unique therapeutic agents for future development in antihormone-resistant breast cancer. Those first investigations to create a new targeted group of medicines have begun (Molloy *et al.* 2014).

With the ongoing development of models to decipher mechanisms of estrogen-induced apoptosis during the first decade of the 21st century, it was also time to address translation to clinical relevance.

Estrogen salvage therapy

As noted previously, clinical experience with the 'endocrine cascade', i.e., the repeated successful use of different endocrine therapies until there is no choice but to employ combination cytotoxic chemotherapy, would evolve in the 1980s and 1990s into cycling tamoxifen, different AIs, fulvestrant, etc. Years of salvage therapy would create populations of long-term estrogen-deprived cells. High-dose estrogen was still part of the armamentarium for European medical oncologists. Lonning *et al.* (2001) examined a small series of patients to address the questions of whether estrogen salvage (an endocrine therapy) would be effective in tumors following long-term antihormone (endocrine) therapy that had become refractory to further treatment.

The interesting findings are shown in Table 4. Lonning *et al.* (2001) noted an overall 30% response rate to high-dose DES (15 mg daily, i.e., 5 mg three times per day) and one patient had a remarkable response.

Table 4 The response of 32 patients with ER-positive metastatic breast cancer to high-dose DES (15 mg daily). All patients have previously been treated exhaustively with conservative antihormone therapies and failed (Lonning *et al.* 2001)

Complete	Response	
	Partial	Stable disease
4 ^a /32	6/32	2/32

^aOne patient remained disease free 10 years and 6 months after commencing treatment (Lonning 2009).

Ellis *et al.* (2009) addressed the experimental concept of high-dose estrogen versus low-dose estrogen as a second-line treatment following recurrence during adjuvant AI treatment. Women received either 30 or 6 mg of E₂ daily (DES is not available in the USA) and a 29% clinical benefit was noted for both groups. However, the low-dose E₂ provided the same clinical benefit as high-dose therapy but with significantly fewer serious side effects.

The therapeutic use of either high- or low-dose estrogen salvage therapy provided the proof of principle that the animal and cell models had veracity in the context of exhaustive antihormone therapy by preparing a vulnerable antihormone-resistant breast cancer cell population for execution with exogenous estrogen therapy. A complementary laboratory study using transplanted MCF-7 tumors in athymic mice (Osipo *et al.* 2005) built on the original observations that low-dose estrogen could reverse exhaustive antihormone therapy (Yao *et al.* 2000) and permit the reuse of tamoxifen to control estrogen-stimulated tumor growth. The Osipo *et al.* (2005) study created four different transplantable MCF-7 tumor models: MCF-7:E₂ (WT estrogen responsive), MCF-7:TAMST (phase I resistance that is stimulated to grow with E₂ or tamoxifen), MCF-7:TAMLT (phase II resistance that is stimulated to grow with tamoxifen, but in which E₂ does not promote growth), and MCF-7:TAME (MCF-7:TAMLT that regrew after long-term E₂ treatment). The MCF-7:TAME tumors were inhibited by tamoxifen in a dose-dependent manner *in vivo*. It was interesting to note, however, that *ERBB2/NEU* and *ERBB3* mRNA in TAM-stimulated MCF-7:TAMLT tumors remained high in E₂-stimulated MCF-7:TAME tumors, thus indicating the veracity of the clinical findings that overexpression of *ERBB2/NEU* alone is insufficient to predict resistance to tamoxifen (Osipo *et al.* 2005).

However, it was the Women's Health Initiative (WHI) trials of the value of either combination synthetic progestin and conjugated equine estrogen (CEE) referred

to as hormone replacement therapy (HRT) or estrogen replacement therapy (ERT) alone to prevent coronary heart disease in postmenopausal women more than a decade after menopause, which was to provide an initial dilemma. The stop rules for the HRT trial created a predictable result with an increase in breast cancer and this trial was published first (Rossouw *et al.* 2002). In contrast, it was not deemed necessary to stop the ERT trial until several years later. Eventually, the trial was stopped due to increased strokes and not for breast cancer increases (Anderson *et al.* 2004). A rise in the incidence of breast cancer was not noted. As a supporting and important additional database, the ongoing analysis of the British Million Women's Study (Beral *et al.* 2003, 2011) also noted similar paradoxical findings with HRT and ERT. These epidemiological data and the WHI results now demand an expanded discussion. In so doing, an understanding of the essential role of timing of taking HRT/ERT can be examined, paradoxes addressed, and rules established. The questions raised are as follows: i) If estrogen causes breast cancer to grow, why does estrogen alone in these clinical studies not cause an increase in breast cancer? ii) Why does a combination of a synthetic progestin plus estrogen cause a predicted rise in breast cancer incidence?

HRT in postmenopausal women

There are two major databases from which to mine information about the role of estrogens and synthetic progestin in the life and death of breast cancer in postmenopausal women. However, based on the established laboratory data on the replication and apoptosis of breast cancer cells and the documented historical record of the actions of high-dose estrogen therapy in treatment of metastatic breast cancer in postmenopausal women, there are no real surprises (Jordan 2008b). However, one important question remains: 'why does a synthetic progestin reverse the antitumor and chemopreventive properties of ERT administered a decade after the menopause (Anderson *et al.* 2012)?

The WHI in the USA and the Million Women's Study in the UK provide the clinical databases, so that laboratory and other clinical studies can be melded to create evidenced-based principles for safer clinical care. The design and conclusions of these two studies will be summarized for completeness and interpretations made based on existing knowledge in the literature.

The WHI ERT alone trial recruited 10 739 hysterectomized postmenopausal women into a randomized trial

Figure 4
Constituents of conjugated equine estrogen.

to receive either CEE (0.625 mg daily; Fig. 4) or placebo. Women were aged between 50 and 79 years. The women's median age was in their mid 60s. The treatment phase of the trial was a median of 5.9 years, as stop rules for stroke were triggered but follow-up occurred to yield an overall study median of 11.8 years. The first clinical surprise was the finding of a lower incidence of breast cancer at the initial analysis (Anderson *et al.* 2004), which was reinforced by a second analysis (LaCroix *et al.* 2011). At the latest analysis of 11.8 years median follow-up (Anderson *et al.* 2012), there was a lower incidence of invasive breast cancer (151 cases) compared with placebo (199 cases). Fewer women died from breast cancer in the estrogen group (six deaths) compared with the placebo group (16 deaths). Indeed, few women died of any cause in the estrogen group after breast cancer diagnosis (30 deaths) than did those in the placebo group (50 deaths).

By contrast, the WHI of HRT recruited 16 608 postmenopausal women between the ages of 50 and 79 years with an intact uterus. Women were randomized to receive either CEE (0.625 mg daily) and medroxyprogesterone acetate (MPA 2.5 mg daily) (Fig. 5) or placebo. After a mean follow-up of 5.2 years, the WHI data safety-monitoring committee recommended stopping the trial based on breast cancer incidence exceeding the predefined stopping boundary (Rossouw *et al.* 2002). The HR was 1.24 for invasive breast cancer with a total of 199 cases of breast cancer vs 150 cases in placebo ($P=0.003$).

In the Million Women's study (Beral *et al.* 2003), 1 129 025 postmenopausal women were recruited to evaluate the breast cancer risk in hormone therapy users and never users. The study accrued 4.05 million women years of follow-up and 15 750 incident breast cancers with a total of 7107 breast cancer in current users of hormone therapy.

Figure 5

Steroids with the ability to bind to the progesterone receptor, estrogen receptor, glucocorticoid receptor, or multiple receptors.

The principal conclusions (Beral *et al.* 2011) for the Million Women's Study relevant to our current considerations of timing and hormone type, i.e., combination of estrogen and progestin (HRT) or estrogen alone (ERT), were as follows: the ERT current users had little increase in breast cancer if use was started more than 5 years after menopause (RR 1.05), but, if ERT was begun straight after menopause, there was an increase in breast cancer (RR 1.43). The pattern was similar for current users of HRT with an anticipated increase in breast cancer in users who start 5 years after menopause (RR 1.53), but a further elevation in risk if HRT is started immediately after menopause (RR 2.04).

Thus, both the WHI and the Million Women's Study provide evidence that combination HRT increases the risk of developing breast cancer compared with ERT, but ERT either causes a fall in breast cancer in the WHI study that persists, whereas, in the Million Women's Study, there is no major rise in breast cancer in an estrogen-deprived women, i.e., more than 5 years after menopause. Timing relative to menopause is important, i.e., a greater breast cancer risk occurs nearer to menopause. This concept (Jordan 2014c) is summarized in Fig. 6, which now establishes rules for the actions of different estrogens; estrogen deprivation for 5 years creates populations of breast cancer cells vulnerable to estrogen-induced apoptosis.

However, the second question, to be addressed in the future, is why a synthetic progestin in some way enhances breast cancer risk, whereas estrogen does not in estrogen-deprived women. Clearly, an understanding of the

mechanism and modulation of estrogen-induced apoptosis will go some way to advance clarity and create safer HRT if the rules of treatment are obeyed. The way forward is to use models to define mechanisms, so that estrogen-induced apoptosis can be modulated predictably and conclusions applied to health care.

Deciphering a mechanism of estrogen-induced apoptosis

Previously, in this review, a few tantalizing clues of the early actions of estrogen to cause apoptosis were reported from studies *in vivo* (Liu *et al.* 2003, Osipo *et al.* 2003) with acquired resistance to tamoxifen or *in vitro* in LTED cell populations or selected clones (Song *et al.* 2001, Lewis *et al.* 2005b). This 'snap shot' of molecular events is the usual method employed to decipher potential pathways either using a laboratory model or a tumor sample from heterogeneous patient populations at an arbitrary time during an important cellular process. This provides a patchwork approach that does not reproduce the most important dimension in any molecular mechanism – time. Populations of cells respond to treatment or stress with adaptation, by advancing the survival and replication of cell populations that can now thrive in a once hostile environment. Time in days can affect an individual cell's response to stress but time in months or years reconfigure the population of cells that survive and thrive. Thus, trial and error for cellular survival is the essence of cancer that kills. The original

Figure 6

Rules for the change in estrogen receptor (ER)-positive breast cancer cell populations, as they leave an estrogen-rich environment at menopause and adapt to a declining estrogen environment over a 5-year period (referred to as Gap). Estrogen-independent clones then grow out that are able to survive in an estrogen-austere environment. This is modeled in the laboratory with long-term estrogen-deprived cells that exhibit acquired hypersensitivity to estrogen for growth (Jeng *et al.* 1998) and then estrogen-induced apoptosis (Song *et al.* 2001, Lewis *et al.* 2005b).

goal of therapeutics in the time of Ehrlich was to cure infectious diseases, but, in the case of cancers we aim, in the case of solid tumors, to contain tumor growth, through an understanding of the cellular options of cancer. In this way, life can be extended by decades with dramatic decreases in mortality illustrated by adjuvant tamoxifen therapy given for 5–10 years (Davies *et al.* 2011, 2013).

To understand cancer's options, the current 'snapshot' needs to be amplified and viewed instead like a 'movie'. The long-term time course of the process to trigger apoptosis, with estrogen in this case, can then be modulated to learn more of the possible population drifts over months or years. Only in this way can true vulnerabilities be discovered. To this end, the intensive evaluation of a few well-described cellular models has been examined to provide the first 'movie' of estrogen-induced apoptosis. Unlike cytotoxic chemotherapy that kills rapidly within a few hours, the process of estrogen-induced apoptosis initially is gradual and relentless, but then is committed after days of subcellular preparation (Ariazi *et al.* 2011, Obiorah *et al.* 2014a). Based on this foundation, modulation of the mechanism is the next

Laboratory studies illustrate that the constituents of conjugated equine estrogen (CEE; Obiorah & Jordan 2013), the endocrine disruptor bisphenol A (Sengupta *et al.* 2013), and phytoestrogens (Obiorah *et al.* 2014b) can trigger cell replication or apoptosis dependent upon the cell populations and its natural estrogen-rich or -austere environment. Reproduced with permission from Jordan VC 2014a Avoiding the bad and enhancing the good of soy supplements in breast cancer. *Journal of the National Cancer Institute* 106 dju233. Copyright 2014 Oxford University Press.

essential step to define, interrogate, and validate an emerging conceptual model for clinical transition.

A 'movie' of estrogen-induced apoptosis in the cell

MCF-7:WS8, MCF-7:2A, and MCF-7:5C cells were used to identify genome-wide alterations in E_2 -regulated gene expression uniquely involved in apoptosis (Ariazi *et al.* 2011). The cells used are estrogen-stimulated MCF-7:WS8 to define growth, MCF-7:2A that is grown independently of estrogen and the cells are refractory to apoptosis during the first week of estrogen action (Pink *et al.* 1995), and MCF-7:5C that respond to E_2 -induced apoptosis in just a few days (Lewis *et al.* 2005a,b). Time courses of gene changes were compared and contrasted over a 2–96 h period with each cell line hybridized with a no treatment control using Agilent arrays. This approach, however, does not compensate for adaptive changes in the basal level of genes that must occur to permit estrogen-independent growth compared with MCF-7WS8 in the absence of estrogen. These data are currently being prepared for publication.

Examination of MCF-7:5C-specific genes reveals an attenuation of ER signaling, but enhancement of endoplasmic reticulum stress (ERS) and inflammatory stress, which heralds apoptotic gene regulation. ERS is characterized by accumulation of unfolded or misfolded proteins and the triggering of an unfolded protein response (UPR) with the function and goal of inhibiting the translation of proteins to alleviate the stress. Expression profiles of E₂-treated MCF-7:5C-specific genes related to ERS are consistent with deficiencies in the UPR, protein translation, protein folding, degradation of misfolded proteins, and fatty acid metabolism. With regard to the ERS-induced apoptosis, E₂ selectively induces the pro-apoptotic BCL2 family members BAX and BIM (Lewis *et al.* 2005b) and the inflammatory caspase CASP4. BAX, in addition to its mitochondrial outer membrane permeabilization activity, binds to, and activates ERN1 (IRE1 α), a key endoplasmic reticulum transmembrane kinase and endoribonuclease that initiates the UPR. BIM is upregulated by a variety of ERS activators and is essential in ERS-induced apoptosis in many cell types. CASP4 localizes to the endoplasmic reticulum, autoactivates in response to severe ERS, and is also required in multiple models of ERS-induced apoptosis. The functional importance of BAX, BIM, and CASP4 is demonstrated by depletion of BAX or BIM with specific siRNAs (Lewis *et al.* 2005b) or inhibition of CASP4 with z-LEVD-fmk (Ariazi *et al.* 2011, Obiorah *et al.* 2014b). Each approach blocks estrogen-induced apoptosis.

The time sequence of estrogen-induced apoptosis has been shown to commence through the intrinsic pathway targeting mitochondria and then subsequently recruiting the extrinsic pathway (death receptor) that consolidates and completes the apoptotic sequence (Ariazi *et al.* 2011, Obiorah *et al.* 2014a). However, those events are completely different from the catastrophic apoptotic response initiated almost immediately by paclitaxel. This is complete within 24 h, triggered by p53-mediated cell cycle blockade at G2 (Obiorah *et al.* 2014a). In contrast, E₂-induced apoptosis via the ER in MCF-7:5C can be rescued during the first 24 h by wash out with an excess of the high-affinity triphenylethylene antiestrogen 4-OHT. Interestingly, both E₂ (Obiorah *et al.* 2014a) and the nonsteroidal estrogen bisphenol (Sengupta *et al.* 2013) initially cause an increase in cell growth and apoptosis is not triggered by cell cycle blockade. It is clear that there is a competition between estrogen-stimulated growth for survival and estrogen-induced apoptosis that ultimately results in cell death.

The key signal transduction system for the regulation of breast cancer cell growth and cell death is the ER. As this

is a known pharmacological target and acknowledged to be the most important one in cancer therapy as a whole (Sledge *et al.* 2014), it is therefore appropriate to consider the modulation of estrogen-induced apoptosis via the ER, the role of ligand shape that programs the conformation of the ligand-ER complex, and then the regulation of ER function through the blockade of cell survival signaling via cSrc and glucocorticoids. Estrogen-induced apoptosis is a stress and inflammatory response (Ariazi *et al.* 2011); therefore, the blocking of these pathways may provide insights into future clinical applications.

The ER as a target for modulating estrogen-induced apoptosis

The ER is extremely promiscuous in its desire to bind with a wide spectrum of phenolic ligands either to switch off or to switch on the ER signal transduction pathway (Jordan 1984, 2003b,c). The steroidal antiestrogen fulvestrant and the triphenyl ethylene hydroxylated metabolite of tamoxifen 4-hydroxytamoxifen (4-OHT) both block estrogen-induced apoptosis (Obiorah & Jordan 2014, Obiorah *et al.* 2014b), but it is the wide variety of phenolic compounds with estrogenic properties that is of particular interest with regard to triggering estrogen-induced apoptosis.

Estrogens are classified based on their structure that programs the conformation of the estrogen-ER complex (Jordan *et al.* 2001). Planar (e.g., E₂) estrogens are referred to as class I and angular estrogens (e.g., bisphenolic triphenylethylene) as class II. The classification is based on the molecular pharmacology of a mutant ER (D351G) with impaired function, as the natural D351 in the ligand-binding domain pocket needs to communicate with amino acid L540 in helix 12 to seal the ligand-binding domain (Fig. 7). The D351G mutant ER, unlike WT ER, is unable to close helix 12 appropriately to activate transcription of an estrogen-regulated gene transforming growth factor alpha (TGF α (TGFA); Schafer *et al.* 1999), when there is steric hindrance from an angular estrogen in the ligand-binding domain. Leclercq and colleagues (Bourgoin-Voillard *et al.* 2010) and Gust and colleagues (Lubczyk *et al.* 2002) have confirmed and extended the ligand classification using other techniques. The molecular pharmacology of estrogen binding to the ER will be described briefly.

X-ray crystallography (Brzozowski *et al.* 1997, Shiau *et al.* 1998) of the ligand-binding domain of the ER liganded with a class I estrogen (i.e., E₂ or DES) or with a nonsteroidal antiestrogen (i.e., 4-OHT or raloxifene) provides precise structural data for the extremes of estrogen and antiestrogen action at one stable moment

Figure 7

These images show the binding site of ER α co-crystallized with E₂ and the H-bond network between E₂ and amino acids from the ligand-binding site, also the H-bond between the backbone of L540 and side chain of D351. It appears that this interaction adds some stability to the agonist conformation and helps to keep H12 in place.

in time. The X-ray crystallography model resolved had been predicted by structure–function relationships at the PRL target in primary cultures of immature rat pituitary gland cells regulated by the ER more than a decade earlier (Jordan 1984, Jordan *et al.* 1986). The biological assay is a dynamic analytical model that integrates receptor responses over time. Thus, structural interpretation of the extremes of estrogen/antiestrogen action and the predictable modulation of gene function by a broad range of systematic ER-binding ligands are consistent. The extremes of the ER complex in both cases predicted that closure of helix 12 sealing a class I ligand within the ligand-binding domain programs full estrogen action. In contrast, the antiestrogenic bulky side chain of nonsteroidal antiestrogens prevents the closure of helix 12 and impairs the binding of co-activator molecules essential for gene transcription. However, an engineered cell model of TGF α transcription demonstrated that there were predictable changes to be anticipated due to interactions of the antiestrogenic side chain of 4-OHT or raloxifene with D351 (Levenson & Jordan 1998, Liu *et al.* 2001, 2002). Raloxifene has a pharmacological activity at the ER that is less estrogen like than 4-OHT in general. This is also true for the TGF α transcription system: 4-OHT does not block the synthesis and transcription of TGF α but raloxifene does (Levenson *et al.* 1997, 1998). Modeling demonstrates that the dimethylaminoethoxy side chain of 4-OHT is not long enough to interact with D351 but the piperidine ring system of the antiestrogenic side chain of raloxifene both neutralizes and shields D351 from interactions with

helix 12 (Fig. 8). A D351Y mutation restored binding of Y351 with helix 12 liganded with raloxifene, thereby allowing helix 12 to close and TGF α transcription (Levenson & Jordan 1998, Liu *et al.* 2001, 2002). The basis for the use of the D351G mutation in the engineered cells at the TGF α target (MacGregor Schafer *et al.* 2000) is that class I planar estrogens still allow helix 12 closure and TGF α transcription, but class II angular estrogens (e.g., bisphenol triphenylethylene) do not allow helix 12 closure and the D351G is now exposed but unsuitable for interaction with L540 in helix 12. As a result, closure of helix 12 does not occur because of steric hindrance from the projecting phenyl group of a triphenylethylene-type angular estrogen. As illustrated in Fig. 9, the class II estrogens tend to favor an antiestrogenic conformation for the complex and therefore do not allow transcription of TGF α . With this background of the molecular pharmacology of estrogen shape that programs the conformation

Figure 8

The comparative analysis of the experimental structures of ER α -LBD co-crystallized with 4-OHT (PDB entry 3ERT) and RAL (PDB entry 1ERR). Both structures superimposed with helix 12 positioned in the same way for both proteins with the amino acids lining the binding pockets, while only the amino acids involved in H-bonds with the ligands in the 'Leu-crown'. The first noticeable difference is the orientation of H524. In RAL complex, the side chain of H524 is drawn toward the ligand, being involved in a H-bond with the hydroxyl group. This interaction is missing in the 4-OHT complex. Also, L536, L539, and L540 adopt different conformations than the ones they adopt in the 4-OHT complex, being 'pushed away' by the piperidine ring of RAL. The side chain conformations of the amino acids surrounding the ring involved in contact with H524, e.g., M343, M421, M423, and I424, are modified.

	Complex conformation	Action	Result and extrapolated interpretation	Mutant ER assay at TGF α
A. Type I estrogen Planar		Helix 12 seals LBD to AF2 coactivators bind. D351 under helix 12. TGF α gene activation	Full estrogen action Growth + apoptosis	Full estrogen action
B. Type II estrogen Angular TPE		Helix 12 pushed back AF2 reduced less coactivator binding. D351 exposed. TGF α gene active	Differential estrogen action Growth + delayed apoptosis	No estrogen action
C. Nonsteroidal antiestrogens TPE based		Helix 12 pushed back. AF2 reduced coactivator binds. D341 exposed. Tamoxifen activates TGF α gene	No estrogen action with no growth or apoptosis	No estrogen action

Figure 9

Functional test: putative conformations of the complex with ligand in LBD for type II estrogen to be 'antiestrogenic' with regard to helix 12 positioning. The assay discriminates between ligands (A), which allow helix 12 to seal the LBD or not (B and C). Sealing of helix 12 over the LBD is important for the ability of the ligands to trigger apoptosis. Reproduced

with permission from Obiorah I, Sengupta S, Curpan R & Jordan VC 2014c Defining the conformation of the estrogen receptor complex that controls estrogen-induced apoptosis in breast cancer. *Molecular Pharmacology* **85** 789–99. Copyright 2014 American Society for Pharmacology and Experimental Therapeutics.

of the ER complex, this can now be applied to understanding estrogen-induced apoptosis.

The planar class I estrogens trigger estrogen-induced apoptosis within a few days with the MCF-7:5C cells committed to apoptosis after 24 h of E₂ exposure. In contrast, the angular triphenylethylene estrogen bisphenol (class II) binds to the ER in MCF-7:5C cells, but does not trigger apoptosis in a 7-day assay. Bisphenol (class II) binds to ER and blocks E₂ (class I)-induced apoptosis (Sengupta *et al.* 2013). This supports the hypothesis that the class II angular estrogen adopts the 'antiestrogenic' conformation of the ER complex despite the fact that bisphenol is a full estrogen agonist on the growth of MCF-7 breast cancer cells (Sengupta *et al.* 2013) and triphenylethylene estrogens trigger vaginal cornification in ovariectomized mice (Robson & Schonberg 1938). This latter assay was the primary methodology used to discover and classify triphenylethylene compounds as long-acting estrogens *in vivo* more than 60 years ago.

However, despite the veracity of the *in vitro* ligand conformation assay, there is a disagreement with the results of the first successful clinical trial in metastatic breast cancer by Haddow *et al.* (1944). They used several

of the class I and II (triphenylethylene) estrogens (Fig. 1) and noted tumor regression with both classes (Table 1). Recent investigations in the laboratory demonstrated that the time courses of class I and II estrogens causing apoptosis are actually different and not static and stable over time. The altered conformation of the resulting class II estrogen–ER complex initially retards and then triggers apoptosis. There is a delay to commitment to apoptosis with the class II estrogen bisphenol of 3 days (Obiorah & Jordan 2014), whereas the class I estrogen (e.g., E₂) is committed after 24 h (Obiorah *et al.* 2014a). Thus, the *in vitro* ligand conformation ER efficacy assay does in fact comply with a clinical reality in triggering apoptosis and causing tumor regression (Haddow *et al.* 1944).

A larger screen (Obiorah *et al.* 2014c) of class I and II estrogens confirms that class II estrogens, whose structures are based on triphenylethylene, not only have a delay in apoptosis but also exhibit an accumulation of ER that is routinely downregulated by class I estrogen through ubiquitination and proteasomal destruction. However, this initial accumulation is not as pronounced as the accumulation of ER complex noted with 4-OHT and

endoxifen (Maximov *et al.* 2014a). What is particularly informative about the dimension of time is the fact that the class II angular estrogens initially accumulate the ER complex and do not downregulate ER mRNA in MCF-7:5C cells. The class II estrogen-ER complex activates downregulation of the complex and mRNA of ER over time, consistent with the delivery of coactivators to the appropriate protein synthetic machinery. This consummates a UPR response and invokes delayed apoptosis. The recent crystallization of the ER with a class II estrogen, the resolution of the structure, and the finding of a novel closed helix 12 conformation (Maximov *et al.* 2014a) are consistent with this interpretation of changes in the accumulation, evolution, and slow decrease in the level of class II estrogen-ER complex concentrations. This heralds the triggering of apoptosis by the class II estrogen-ER complex.

A summary of simple facts can now be made. The ligand shape can delay or advance estrogen-induced apoptosis in correctly configured LTED cells. The shape of the estrogen-ER complex determines the delivery of the coactivator SRC3 (NCOA3) to the relevant site within the vulnerable cell to initiate apoptosis. A previous study has shown that knock out of NCOA3 by siRNA will blunt estrogen-induced apoptosis (Hu *et al.* 2011). The question now is whether other logical treatment strategies can modify or modulate estrogen-induced apoptosis in a predictable manner.

Inhibition of cSrc signaling in LTED cells

The human homolog of the oncogene *vSrc* plays a fundamental linker role in the signal translation pathways from growth factor (GF) receptors on the cell membrane to activate nongenomic growth pathways in cancer. The essential role of *vSrc* is to act as a protein kinase that phosphorylates tyrosine (Hunter & Sefton 1980).

There is a cell-membrane-bound ER in the GF pathways, which is involved in immediate estrogen-mediated responses via a nongenomic phosphorylation cascade. However, cSrc is also critically involved in the phosphorylation of Y537 that regulates ER turnover and accumulation (Arnold *et al.* 1995, Chu *et al.* 2007).

Inhibitors of cSrc have attracted some attention as potential therapeutic agents in breast cancer; however, the unanticipated finding that a cSrc inhibitor stopped E₂-induced apoptosis created a new research opportunity (Fan *et al.* 2012).

A cSrc inhibitor stops E₂-regulated decreases in ER complex concentrations and this event (Fan *et al.* 2013), in turn, is responsible for regulating protein synthesis

within the cell. The antiestrogen-ER complex accumulates but does not facilitate transcription of mRNA for protein synthesis; despite the fact that excess ER binds to the promoter region of genes, there is little or no coactivator binding. The binding of a class I estrogen-ER complex to the promoter regional genes with coactivators is required to 'breathe' at the promoter with cyclical destruction of DNA-bound complexes to maintain mRNA transcription (Shang *et al.* 2000). This would not happen in the presence of a cSrc inhibitor, as there is receptor stagnation.

The fact that a cSrc inhibitor blocks estrogen-induced apoptosis in LTED breast cancer cells required broad mechanistic explanation as there is not only ER at the genome but at the cell membrane. The treatment of MCF-7:5C cells with E₂ increases the phosphorylation of cSrc that acts as a key adaptor protein for the activation of stress responses before triggering apoptosis. The sensors of UPR, IRE1 α , and Perk kinase (that phosphorylates eukaryotic translational initiation factor 2 α : eIF2 α) are activated by E₂ (Fan *et al.* 2013). There is also a dramatic increase in ROS and heme oxygenase HO-1 (an indicator of oxidative stress) and the central energy sensor kinase AMPK. However, a cSrc inhibitor and siRNA knockdown of cSrc both block estrogen-induced apoptosis. Despite the delay of commitment to apoptosis by 24 h (Obiorah *et al.* 2014a), the central role of cSrc raises the possibility that the nongenomic membrane ER may be involved in apoptosis. The estrogen-dendrimer conjugate (EDC) will only bind to membrane ER and is not taken up by the cell (Fig. 10) (Harrington *et al.* 2006). The treatment of cells with EDC neither initiates pS2 synthesis nor triggers apoptosis, both of which are mediated by a genomic mechanism of the nuclear ER. Interestingly enough, cell replication is increased by the EDC (Fan *et al.* 2013). It is therefore plausible that cSrc modulation can regulate estrogen-induced apoptosis, probably by reducing ER turnover.

However, all the previously discussed studies are conducted for a short term (1 week) and reflect biochemical events, which influence cell replication or death. A more clinically relevant study is to compare and contrast the actions of estrogen or a cSrc inhibitor alone or the combination over a period of 2 months (Fan *et al.* 2014a). This is used clinically to assess the success or failure of a treatment regimen for patient care. Studies (Fan *et al.* 2014a) demonstrate that blockade of cSrc is a reversible process and washes out rapidly to restore estrogen-induced apoptosis. In contrast, E₂ alone causes catastrophic cell death and then regrowth of a new cell population that is not rapidly growing but achieves

Figure 10

E₂-induced estrogen receptor signaling. (1). The genomic mechanism of ER signaling is by estrogen binding to the nuclear ER and then binding to hormone response elements in the promoters of target genes (classical) or through protein–protein tethering with nuclear DNA-binding transcription factors (nonclassical) to alter gene transcription. (2). E₂ can act through nongenomic signaling by activating cell surface membrane-localized extranuclear ER. (3). Estrogen–dendrimer conjugate (EDC) specifically activates the nongenomic signaling of ER action. Reproduced with permission from Obiorah IE, Fan P, Sengupta S, Jordan VC 2014d Selective estrogen-induced apoptosis in breast cancer. *Steroids* 90 60–70, with permission from Elsevier. Copyright 2014 Elsevier, Inc.

an equilibrium state between cell growth and cell death. However, the combination of E₂ and a cSrc inhibitor results in the outgrowth of a new cell population that grows vigorously with estrogen treatment and in which growth is stimulated by either 4-OHT or endoxifen (Fan *et al.* 2014b,c). These cells are driven through membrane-bound IGFIRβ and recapitulate phase I acquired resistance to tamoxifen noted in athymic animals (Gottardis & Jordan 1988; Fig. 11). This is not only a new model system *in vitro* to study acquired resistance to SERMs but also illustrates how selection pressure for only 8 weeks can rapidly change the form of acquired resistance to antihormones. This is the challenge for laboratory research to translate to the clinical setting and block tumor adaptation. In this case, it is the rapidity of change in such a short time.

Thus, it is the results of the cSrc inhibitor plus estrogen acting as selection pressure on MCF-7:5C aromatase-resistant cells over only a few months that ultimately governs the eventual survival of new breast cancer cell populations. This, in turn, illustrates the rapidity of antihormone resistance plasticity to different agents. These data also have important implications for the results of HRT/ERT in the WHI raising again the question as to

why MPA plus CEE increases, but CEE alone decreases, breast cancer incidence. Perhaps, the answer lies in the ability of synthetic progestins to be promiscuous and interact with other steroid hormone receptors.

Glucocorticoid activity and apoptosis

The glucocorticoid dexamethasone is known to antagonize a number of estrogen-mediated events via an AP-1 (c-fos/cJun) pathway (Uht *et al.* 1997). Simply stated, estrogens can stimulate transcription of genes through interaction at the promoter site but glucocorticoids counteract transcription. The respective actions are dependent on the concentrations of the ER and glucocorticoid receptor (GR) complexes at an AP-1 response element in the promoter (Uht *et al.* 1997). Recent studies have implicated changes in histone remodeling as a possible mechanism (Pradhan *et al.* 2012, Miranda *et al.* 2013). Earlier, estrogen-induced apoptosis has been reported to be mediated by an increase in inflammatory responses (Ariazi *et al.* 2011), hence it was appropriate to evaluate the action of glucocorticoid in model systems. Dexamethasone reduces the growth rate of spontaneously growing MCF-7:5C cells in a concentration-related manner (Obiorah *et al.* 2014b). The inhibition of estrogen-induced apoptosis by dexamethasone is reversible with RU486, an antiglucocorticoid. The antiglucocorticoid RU486 is also, actually, a much more potent antiprogesterin, in which application it is used as the ‘early abortion pill’. This fact highlights the promiscuous nature for synthetic steroids to interact with both progesterone and GR. This knowledge now opens up new opportunities to understand the results of the two trials from the WHI (Anderson *et al.* 2012).

There is considerable promiscuity of ligands between the progesterone receptor and the GR. Indeed, a high dose of MPA used as a breast cancer therapy or as a depot injection for contraception causes weight gain as a glucocorticoid-type side effect. The activation of glucocorticoid and progestin by synthetic progestins has been recognized in laboratory studies and a recent study (Sweeney *et al.* 2014b) addressed the hypothesis that the glucocorticoid properties of MPA could influence and modulate the apoptotic actions of E₂ in LTED breast cancer cells.

Although MPA can activate the GR-regulated gene, it is much less potent than dexamethasone. Similarly, MPA only weakly modifies estrogen-induced apoptosis, but is effective in reducing the activation of estrogen-induced apoptotic genes. However, these clues that MPA might modulate estrogen-induced apoptosis over time and create

Figure 11

The melding of model systems. During the past 25 years, the MCF-7 breast cancer cell line has been used to recapitulate an evolving model *in vivo* of acquired tamoxifen resistance (Levenson & Jordan 1997) observed in clinical breast cancer. In parallel, the same cell line has been used to recapitulate models *in vitro* of estrogen deprivation using either fulvestrant (Liu *et al.* 2006), that destroys the ER protein, or AIs that create a long-term estrogen-deprived state. The cells derived from estrogen deprivation with fulvestrant lose the ER, but estrogen deprivation in an estrogen-free environment *in vitro* increases the ER level. Clones grow out that are sensitive to estrogen-induced apoptosis (Lewis-Wambi &

Jordan 2009). A cSrc inhibitor blocks estrogen-induced apoptosis in the short term (Berthois *et al.* 1986), but long-term (2 months) treatment with estrogen plus a cSrc inhibitor results in a new population of cells (MCF-7:PF) (Fan *et al.* 2014a, b) that recapitulates *in vitro* phase I resistance to SERMs *in vivo*. These data, accumulated over decades, illustrate the plasticity of cell populations in adapting to a hostile environments. Reproduced with permission from Fan P & Jordan VC 2014 Acquired resistance to selective estrogen receptor modulators (SERMs) in clinical practice (tamoxifen & raloxifene) by selection pressure in breast cancer cell populations. *Steroids* 90 44–52, with permission from Elsevier. Copyright 2014 Elsevier, Inc.

new cell populations that grow into tumors are an important finding: MPA could select for surviving breast cancers by modulating estrogen-induced apoptosis over years of therapy in women who are a decade or more from menopause as documented in the WHI (Anderson *et al.* 2012). Laboratory evidence for modification of apoptosis by estrogen through the glucocorticoid action of MPA was presented by Sweeney *et al.* (2014b). This new finding, coupled with the knowledge that rapid plasticity of hormone resistance occurs as a response to selection pressure (Fan *et al.* 2013, 2014a,b,c), indicates that a fundamental principle has now emerged based on selection pressure over years in patients.

Plasticity of populations of antihormone-resistant breast cancer cells

There are few estrogen-responsive ER-positive breast cancer cell lines (Sweeney *et al.* 2012), but the MCF-7 cell

line continues to provide invaluable information (Levenson & Jordan 1997) and approaches to disease control that can be translated to patient care. Principles emerge that can be addressed in the clinic.

The understanding of acquired hormone resistance has been approached in two ways: either the long-term retransplantation of MCF-7 tumor tissue into successive generations of tamoxifen-treated (Yao *et al.* 2000) or raloxifene-treated (Balaburski *et al.* 2010) ovariectomized athymic mice or the estrogen deprivation of MCF-7 cells grown in culture over years. Studies *in vivo* revealed changes in the populations of cells that no longer responded to tamoxifen, displaying blocked estrogen-stimulated tumor growth, but now were stimulated to grow by either tamoxifen or physiological E₂ treatment (Gottardis and Jordan 1988, Gottardis *et al.* 1989a,b). The populations evolved further during years of retransplantation into tamoxifen-treated athymic mice, with tamoxifen continuing to stimulate tumor growth but now

physiological levels of E₂ caused tumors to regress rapidly (Wolf & Jordan 1993, Yao *et al.* 2000). Results of studies with MCF-7 under estrogen-deprived conditions indicated that cells develop that are able to grow autonomously in the absence of estrogen, but physiological E₂ could now induce apoptosis in cell populations (Song *et al.* 2001) or individual clones could respond rapidly to estrogen-triggered apoptosis (MCF-7:5C) or apoptosis could be delayed by a week (MCF-7:2A) (Ariazi *et al.* 2011). However, the phenotypes of the tamoxifen-stimulated MCF-7 cells *in vivo* and the estrogen-deprived MCF-7:5C cells *in vitro* are different. The tamoxifen-stimulated tumor *in vivo* is ER- and PgR-positive (Gottardis & Jordan 1988), but the selection pressure of estrogen deprivation *in vitro* creates an autonomously growing tumor when transplanted into athymic mice (Lewis *et al.* 2005b) that is ER-positive but PgR-negative. Fulvestrant will partially reduce the growth rate of MCF-7:5C tumors, but SERMs are, in the main, ineffective, although high concentrations of bazedoxifene *in vitro* are capable of causing death of MCF-7:5C cells (Lewis-Wambi *et al.* 2011). The question therefore arises, within the same MCF-7 cell line – are these very different forms of antihormone resistance phenotypically interchangeable if the appropriate selection pressure is applied?

The finding that a cSrc inhibitor can block estrogen-induced apoptosis in the MCF-7:5C estrogen-deprived cloned cell line (Jiang *et al.* 1992, Fan *et al.* 2012) turned out to be an appropriate model of selection pressure to determine the plasticity of the cell population during an 8-week treatment period. This time of treatment was selected as it is consistent with clinical practice for determining tumor progression or regression to endocrine therapy in metastatic breast cancer.

Treatment with estrogen alone for 8 weeks resulted in a much reduced cell population with cells replicating and undergoing apoptosis in equilibrium, but PgR synthesis was restored. In contrast, the cSrc inhibitor plus estrogen increased IGF1R β levels and the new cell population grew vigorously with estrogen treatment alone with increased PgR production. The role of IGF1R β and ER in facilitating vigorous growth was demonstrated using AG1024, an inhibitor of IGF1R β , and fulvestrant to destroy ER; both strategies to block receptor-mediated growth resulted in the complete inhibition of estrogen-mediated growth (Fan *et al.* 2014a,b,c). The new cell line was designated MCF-7:PF.

The discovery was that, for the first time to this author's knowledge, a cell line was able to replicate the estrogenicity of individual SERMs to cause cell replication *in vitro* (Fan *et al.* 2014b) in much the same way as SERMs cause tumor

growth in proportion to their intrinsic estrogenicity in the athymic mouse model *in vivo* (Wardell *et al.* 2013). This new cell line was unanticipated, as the new cell population that was created subsequently grew in response to an SERM. This result created by selection pressure through cellular trial and error was not based on a predetermined hypothesis; it was a discovery outside the accepted structure. Therefore, the plasticity of the MCF-7:5C estrogen-deprived cell line, that has no responsiveness to tamoxifen (i.e., SERM resistance), can have SERM-mediated growth expressed through an inhibition of estrogen-induced apoptosis. Thus, the different results with regard to the development of antihormone resistance *in vivo* and *in vitro* can now be connected (Fig. 11), through selection pressure on cell populations.

What is, however, particularly informative is the mechanisms of SERM-stimulated breast cancer cell growth that has now been defined (Fan *et al.* 2014b,c). Previous studies *in vivo* noted a paradox; tamoxifen-stimulated tumor growth occurred despite the fact that estrogen-regulated genes that are controlled via the ER through the genomic route were all blocked during growth stimulated by tamoxifen (Osipo *et al.* 2007). Pathway analysis of MCF-7:PF cells indicates similar patterns for tamoxifen- and estrogen-stimulated growth (Fan *et al.* 2014c), but closer examination of individual genes regulated by the ER via the genomic route revealed that they are blocked. This is consistent with the results of earlier studies by Osipo *et al.* (2007) *in vivo* using tamoxifen-stimulated tumors. In contrast, IGF1R β is activated and SERM-stimulated growth is blocked by AG1024 (Fan *et al.* 2014b). Other investigators have implicated the IGF receptor system in earlier studies (Massarweh *et al.* 2006, 2008, Becker *et al.* 2011, Fagan *et al.* 2012).

Summary and future clinical perspective

The chance 'rediscovery' of estrogen-induced regression of long-term (over 5 years) estrogen-deprived breast tumors (Wolf & Jordan 1993, Yao *et al.* 2000) under laboratory conditions opened the door to deciphering mechanisms that now are the evidence-based scientific framework for future clinical applications.

The dramatic improvements in survival noted with increasing years of adjuvant tamoxifen (Early Breast Cancer Trialists' Collaborative Group 1998, 2005) and the ATLAS trial of 5 vs 10 years of adjuvant tamoxifen (Davies *et al.* 2013) were unanticipated. This is because tamoxifen is classified as a nonsteroidal antiestrogen (Jordan 1984) that blocks the tumor ER to stop tumor growth. A medicine that is cytostatic but not cytocidal

could not decrease mortality. Nevertheless, mortality decreases improved not only following the cessation of adjuvant tamoxifen (Davies *et al.* 2011, 2013) but also following 5 years of adjuvant AI therapy (Forbes *et al.* 2008, Cuzick *et al.* 2010), and a continuing effect of tamoxifen occurs during chemoprevention that is also noted after tamoxifen is stopped (Fisher *et al.* 2005, Cuzick *et al.* 2007, Powles *et al.* 2007). These consistent clinical results have now been linked to selection pressure of antihormone therapy creating a vulnerability in new populations of cells ready to undergo apoptosis if exposed to a woman's own estrogen (Jordan 2014d). Indeed, the Study of Letrozole Extension (SOLE; Fig. 12) is testing this hypothesis (Wolf & Jordan 1993, Yao *et al.* 2000) by comparing 5 years of continuous adjuvant letrozole treatment with 5 years of intermittent adjuvant letrozole treatment (a 3-month annual drug holiday) after completing an initial 5 years of adjuvant antihormone therapy. However, it is now possible to incorporate another complication to the long-term adjuvant tamoxifen and estrogen-induced apoptosis decreasing the mortality. Tamoxifen is considered not only to be a competitive inhibitor of estrogen action at the human tumor ER (Jensen & Jordan 2003) but also to be a prodrug that is converted into hydroxylated metabolites 4-hydroxytamoxifen and endoxifen. Endoxifen is formed by the CYP2D6 enzyme system in the patient (Brauch & Schwab 2014), but aberrant genotypes can create extremes of extensive metabolites (EMs) through multiple gene copies or poor metabolizers (PMs) with zero gene function. This has become an extremely controversial topic for clinical application. No prospective studies have been

completed, but retrospective studies have revealed a positive correlation, i.e., EM-genotype patients do better than PM-genotype patients. The logic is that the anti-estrogenic mix for the EM patients will serve to block the ER better in the tumor and, as a result, stop estrogen reactivating tumor growth. But there is now another way (Fig. 13) to view these data based on the results discussed previously in the section describing the drift in cell populations *in vitro*, if estrogen-induced apoptosis is blocked by an inhibitor of cSrc. The principle of improving the antiestrogenic mixture of metabolites in both premenopausal and postmenopausal patient environments has recently been modeled *in vitro* (Maximov *et al.* 2014b,c). These studies model the immediate effects of tamoxifen and metabolites of estrogen-stimulated growth and gene regulation. They do not, however, model the years of selection pressure that must occur to create vulnerable cell populations for execution with endogenous estrogen once long-term therapy stops. A challenge for the future is to produce an extensive panel of ER-positive breast cancer cell lines from patients and then model years of adjuvant therapy with tamoxifen using cell culture and animal models. In this way, the veracity of the hypothesis (Fig. 13) that endoxifen is an essential component of adjuvant tamoxifen action can be confirmed as justification to revisit clinical investigations. The examination of tumor cell biology under selection pressure must then be melded with the mechanism of estrogen-induced apoptosis in the new cell line panels. Cellular barriers to apoptosis must be identified and tumor responsiveness enhanced methodically through a multifaceted study of mechanisms.

Figure 12

Schema for the Study of Letrozole Extension (SOLE; IBCSG 35-07) conducted by the International Breast Cancer Study Group (IBCSG). Upon completion of 4–6 years of previous adjuvant endocrine therapy with SERM(s) and/or AI(s), patients were randomly assigned to continuous or intermittent letrozole (3-month drug holidays/year) for 5 years. The rationale for this approach was that the woman's own estrogen in the intermittent arm would trigger apoptosis in long-term estrogen-deprived breast cancer and

reduce recurrence rates. Adapted from International Breast Cancer Study Group – Study of Letrozole Extension (www.ibcsg.org). Reproduced with permission from Jordan VC & Ford LG 2011 Paradoxical clinical effect of estrogen on breast cancer risk: a 'new' biology of estrogen-induced apoptosis. *Cancer Prevention Research* 4 633–637. Copyright 2011 American Association for Cancer Research.

Current new concept

Figure 13

A new concept of the evolution of the breast cancer cell populations under the increasing selecting pressures of antiestrogenic environments to become vulnerable populations that are killed by estrogen when therapy is stopped. Adapted, with permission, from [Jordan VC 2014d](#) Linking estrogen-induced apoptosis with decreases in mortality following long term adjuvant tamoxifen therapy. *Journal of the National Cancer Institute* 106 dju296. Copyright 2014 Oxford University Press.

The mechanism of estrogen-induced apoptosis can be summarized as shown in [Fig. 14](#). Estrogen binds to the ER in the correctly selected breast cancer cell population derived under estrogen-deprived conditions. Growth of cells occurs through a genomic and a nongenomic pathway and the cell triggers an UPR through ERK signaling. Apoptosis occurs first through the mitochondrion (intrinsic pathway) but subsequently the death receptor (extrinsic pathway) is recruited to consolidate the pathways that execute the cell ([Obiorah et al. 2014a](#)).

The fact that estrogen-induced apoptosis does not occur in all ER-positive breast cancer cell populations at all times is important. Not only is the influence of estrogen important for creating a cell line dependent on the ER signal transduction pathway for growth, which dominates, but also estrogen-deprived cells do or do not respond to estrogen to trigger estrogen-induced apoptosis. The question for the refractory LTED cells will be, 'why not?' Based on the initial findings regarding the treatment of metastatic breast cancer with high-dose estrogen ([Haddow et al. 1944](#), [Stoll 1977](#)) discussed earlier, it was clear that a 5 year 'gap' must occur between menopause and treatment success ([Fig. 6](#)). If patients are treated too soon after menopause, the tumors grow. Today, these same rules apply for CEE (a hormone replacement), bisphenol A (an endocrine disruptor), and phytoestrogen consumption in soy products. The rules that must be applied to optimize success from estrogen-induced apoptosis in breast cancer are illustrated in [Fig. 6](#).

Laboratory studies demonstrate that the treatment of breast cancer cells cultured routinely in estrogen (estrogen rich) will always grow rapidly with CEE-derived steroids

([Obiorah & Jordan 2013](#)) bisphenol A, an endocrine disruptor ([Sengupta et al. 2013](#)) or phytoestrogen (daidzein or genistein) ([Obiorah et al. 2014b](#)), whereas some long-term estrogen-depleted cells will undergo estrogen-induced apoptosis with all three ligands that bind to ER ([Sengupta et al. 2013](#), [Obiorah & Jordan 2013](#), [Obiorah et al. 2014b](#)). Although there is no relevant clinical translation to breast cancer for bisphenol A, there is clinical translation for CEE and phytoestrogens. The administration of either ERT or soy around menopause increases tumor cell replication ([Beral et al. 2011](#), [Shike et al. 2014](#)). We have previously emphasized the fact that administration of CEE in the Million Women's Study straight after menopause during the gap period increases breast cancer risk ([Beral et al. 2011](#)), but, after the gap period, there is no increase in breast cancer risk ([Beral et al. 2011](#)). With regard to soy supplements and phytoestrogens, the situation is less clear because the appropriate studies have not been completed. This is an opportunity for the future.

The recent study by [Shike et al. \(2014\)](#) is the first, to our knowledge, to examine gene activations in mixed population of patients (premenopausal and early postmenopausal women with breast cancer) who elected to take a soy supplement for 7–30 days. Blood levels of daidzein and genistein broadly range from 0 to 400 mg/ml. However, a genistein signature is identified that contains activated replication genes. This illustrates that soy products are not safe to take to protect against breast cancer in the 'gap' period to ameliorate menopausal symptoms. The question for the future is whether soy administration in a woman's 60s would protect some of them against breast cancer in the same way as CEE ([Anderson et al. 2012](#)). The hope is that there would be fewer strokes with soy products. This was the reason that the WHI CEE alone study was stopped ([Anderson et al. 2004](#)).

Thus, the rules ([Fig. 6](#)) to provide selection pressure through estrogen deprivation after menopause have veracity in the laboratory and at least with CEE therapy in the clinical setting. Another approach to controlling menopausal symptoms may be bazedoxifene/conjugated estrogen combination that has been recently approved for reducing menopausal symptoms through estrogen action in the CNS but with an SERM, bazedoxifene, to prevent uterine carcinoma or breast cancer growth from unopposed estrogen action ([Komm & Mirkin 2012](#)). A recent publication ([Jordan 2014e](#)) has raised the proposal a clinical trial of the 5-year use of bazedoxifene/conjugated estrogen around menopause to drive occult breast cancer cell selection, but followed by a 'purge' for a few months with estrogen alone to execute prepared and vulnerable

Figure 14

The mechanism of E_2 -induced apoptosis. (1), activation of ER by E_2 induces activation of AP-1 complex; (2), endoplasmic reticulum stress caused accumulation of unfolded proteins that stimulate a UPR signal; (3), failure to combat ERS induces apoptosis via induction of the mitochondrial

pathway; (4), the subsequent activation of the extrinsic pathway of apoptosis occurs through the TNF family of proapoptotic genes; (5), apoptosis can occur independent of the intrinsic and extrinsic pathways through activation of caspase 4.

LTED breast cancer cells. However, the question remains: 'If estrogen triggers apoptosis in ERT given to women in their 60's, and the administration CEE with MPA a synthetic progestin (HRT) increases the risk of breast cancer, because it has associated glucocorticoid activity, can a safer HRT be developed?'

It is known that 19-nortestosterone derivatives that are progestogenic also have estrogenic activity and trigger breast cancer cell proliferation (Catherino *et al.* 1993, Jeng *et al.* 1992, Jeng *et al.* 1993). However, MPA is not a 19-nortestosterone derivative and does not stimulate breast cancer cell proliferation (Catherino *et al.* 1993). But MPA does have an associated glucocorticoid activity (Koubovec *et al.* 2005, Courtin *et al.* 2012), so that there is the potential, during years of therapy, to modify and protect cells from estrogen-induced apoptosis, thereby modulating breast cancer growth. If this were true, then it would seem to be more reasonable to select a 19-nortestosterone derivative such as norethindrone acetate to reinforce estrogen-induced apoptosis but still provide uterine protection from the growth of endometrial cancer.

Thus, the administration of not only CEE at the appropriate time to trigger apoptosis, but also a 19-nor synthetic progestin to protect the uterus and provide backup sustained estrogen action may be a double application of the new science of estrogen-induced apoptosis. This could benefit millions of women who wish to take a safer HRT preparation. The solution for applications in women's health may lie in a closer look at the synthetic progestin in HRT.

The search for the mechanism of estrogen-induced apoptosis started with the clinical observations of Haddow *et al.* (1944) FRS who went on to create the database for the first chemical therapy for any cancer validated in a clinical trial. The clinical findings have stood the test of time and through laboratory, clinical, and epidemiological studies have now created a principle based on the response of some breast cancer cell populations to longer term estrogen deprivation rules that can now be applied (Fig. 6). Some surviving cells become vulnerable to estrogen-induced apoptosis through a unique molecular mechanism targeted by the ER. This mechanism is now validated and has the potential to expose future targets,

even in ER-negative cells, for drug discovery. Following the ER in the estrogen-dependent cell has shown us where to look. Cancer in general is ER-negative, so perhaps it is more vulnerable than we think.

The story of the evolution of some ER-positive breast cancer cell populations in an estrogen-deprived environment to become hormone-independent for growth in response to selection pressure may in fact be a general principle in cancer cell therapeutics. The plasticity of cell populations ebbs and flows rapidly, but results from clinical trials indicate (Anderson *et al.* 2012, Davies *et al.* 2013) that decreases in mortality are possible with low tumor burdens in an adjuvant setting and patient population survival is enhanced. This is a potential start for a new structure to seek the vulnerabilities in not only breast cancer, but also in other solid tumors that respond and subsequently adapt to selection pressure by succeeding in developing new growth mechanisms. This is not a new idea as Darwinian adaptation teaches that each species or variety of a species must adapt to a hostile environment and can only survive if characteristics favor growth of the population. Population growth is only possible if limited resources are only acquired by the fittest by competition. Survival of population with favorable characteristics was described as 'survival of the fittest'. But the population shifts of species adapting to change took hundreds of millions of years. For cancer, in the human host, no more than a decade or two is required to create a change in adaptive survival; the random power of continuous replication and mutations creates the 'survival of the fittest' by mindless trial and error that ends by killing the host. This is the renegade cell usurping the controlled process for short-term gain now as a parasite that will eventually destroy the host.

The conversation between laboratory and clinical research is a two-way conversation. The translational research by Haddow in the 1940s (Haddow & Robinson 1939, Haddow *et al.* 1944) posed a question that lingered for 60 years, 'how does estrogen kill breast tumor cells and why does this occur?' This review article describes the successes so far in documenting the mechanism of how apoptosis is triggered to kill breast cancer. I also offer herein an explanation as to why cell populations become vulnerable to estrogen-induced apoptosis. The mechanism and rules presented do not provide the understanding of all cancers; that is a naïve goal. The goal is more success in our understanding than failures. The fact that there are failures in our comprehension must be viewed as an opportunity for future generations of investigators to

add more to the expanding mosaic in the quest to control the relentless adaptation of cancer to selection pressure.

'Success is not final. Failure is not final. It is the courage to continue that counts'

Declaration of interest

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References

- Anderson GL, Limacher M, Assaf AR, Bassford T, Beresford SA, Black H, Bonds D, Brunner R, Brzyski R, Caan B *et al.* 2004 Effects of conjugated equine estrogen in postmenopausal women with hysterectomy: the Women's Health Initiative randomized controlled trial. *Journal of the American Medical Association* **291** 1701–1712. (doi:10.1001/jama.291.14.1701)
- Anderson GL, Chlebowski RT, Aragaki AK, Kuller LH, Manson JE, Gass M, Bluhm E, Connelly S, Hubbell FA, Lane D *et al.* 2012 Conjugated equine oestrogen and breast cancer incidence and mortality in postmenopausal women with hysterectomy: extended follow-up of the Women's Health Initiative randomised placebo-controlled trial. *Lancet Oncology* **13** 476–486. (doi:10.1016/S1470-2045(12)70075-X)
- Ariazi EA, Cunliffe HE, Lewis-Wambi JS, Slifker MJ, Willis AL, Ramos P, Tapia C, Kima HR, Yerrum S, Sharma CGN *et al.* 2011 Estrogen induces apoptosis in estrogen deprivation-resistant breast cancer through stress responses as identified by global gene expression across time. *PNAS* **108** 18879–18886. (doi:10.1073/pnas.1115188108)
- Arnold SF, Obourn JD, Jaffe H & Notides AC 1995 Phosphorylation of the human estrogen receptor on tyrosine 537 *in vivo* and by src family tyrosine kinases *in vitro*. *Molecular Endocrinology* **9** 24–33. (doi:10.1210/mend.9.1.7539106)
- Assender JW, Gee JM, Lewis I, Ellis IO, Robertson JF & Nicholson RI 2007 Protein kinase C isoform expression as a predictor of disease outcome on endocrine therapy in breast cancer. *Journal of Clinical Pathology* **60** 1216–1221. (doi:10.1136/jcp.2006.041616)

- Balaburski GM, Dardes RC, Johnson M, Haddad B, Zhu F, Ross EA, Sengupta S, Klein-Szanto A, Liu H, Lee ES *et al.* 2010 Raloxifene-stimulated experimental breast cancer with the paradoxical actions of estrogen to promote or prevent tumor growth: a unifying concept in anti-hormone resistance. *International Journal of Oncology* **37** 387–398. (doi:10.3892/ijo_00000687)
- Baumler E 1984 *Paul Ehrlich: Scientist for Life*. First Edition. New York: Holmes & Meier.
- Beatson GT 1896 On the treatment of inoperable cases of carcinoma of the mamma: suggestions for a new method of treatment, with illustrative cases. *Lancet* **2** 104–107. (doi:10.1016/S0140-6736(01)72307-0)
- Becker MA, Ibrahim YH, Cui X, Lee AV & Yee D 2011 The IGF pathway regulates ER α through a S6K1-dependent mechanism in breast cancer cells. *Molecular Endocrinology* **25** 516–528. (doi:10.1210/me.2010-0373)
- Beral V, Banks E, Reeves G & Bull D 2003 Breast cancer and hormone-replacement therapy in the Million Women Study. *Lancet* **362** 419–427. (doi:10.1016/S0140-6736(03)14596-5)
- Beral V, Reeves G, Bull D *et al.* 2011 Breast cancer risk in relation to the interval between menopause and starting hormone therapy. *Journal of the National Cancer Institute* **103** 296–305. (doi:10.1093/jnci/djq527)
- Bergh J, Jonsson PE, Lidbrink EK, Trudeau M, Eiermann W, Brattström D, Lindemann JP, Wiklund F & Henriksson R 2012 FACT: an open-label randomized phase III study of fulvestrant and anastrozole in combination compared with anastrozole alone as first-line therapy for patients with receptor-positive postmenopausal breast cancer. *Journal of Clinical Oncology* **30** 1919–1925. (doi:10.1200/JCO.2011.38.1095)
- Berthois Y, Katzenellenbogen JA & Katzenellenbogen BS 1986 Phenol red in tissue culture media is a weak estrogen: implications concerning the study of estrogen-responsive cells in culture. *PNAS* **83** 2496–2500. (doi:10.1073/pnas.83.8.2496)
- Bindal RD & Katzenellenbogen JA 1988 Bis(4-hydroxyphenyl)[2-(phenoxysulfonyl)phenyl]methane: isolation and structure elucidation of a novel estrogen from commercial preparations of phenol red (phenolsulfonphthalein). *Journal of Medicinal Chemistry* **31** 1978–1983. (doi:10.1021/jm00118a020)
- Borner C, Wyss R, Regazzi R, Eppenberger U & Fabbro D 1987 Immunological quantitation of phospholipid/Ca²⁺-dependent protein kinase of human mammary carcinoma cells: inverse relationship to estrogen receptors. *International Journal of Cancer* **40** 344–348. (doi:10.1002/ijc.2910400310)
- Bourgoin-Voillard S, Gallo D, Laios I, Cleeren A, Bali LE, Jacquot Y, Nonclercq D, Laurent G, Tabet JC & Leclercq G 2010 Capacity of type I and II ligands to confer to estrogen receptor α an appropriate conformation for the recruitment of coactivators containing a LxxLL motif-relationship with the regulation of receptor level and ERE-dependent transcription in MCF-7 cells. *Biochemical Pharmacology* **79** 746–757. (doi:10.1016/j.bcp.2009.10.015)
- Boyd S 1900 On oophorectomy in cancer of the breast. *BMJ* **2** 1161–1187.
- Brauch H & Schwab M 2014 Prediction of tamoxifen outcome by genetic variation of CYP2D6 in post-menopausal women with early breast cancer. *British Journal of Clinical Pharmacology* **77** 695–703. (doi:10.1111/bcp.12229)
- Brooks SC, Locke ER & Soule HD 1973 Estrogen receptor in a human cell line (MCF-7) from breast carcinoma. *Journal of Biological Chemistry* **248** 6251–6253.
- Brzozowski AM, Pike ACW, Dauter Z, Hubbard RE, Bonn T, Engström O, Ohman L, Greene GL, Gustafsson JA & Carlquist M 1997 Molecular basis of agonism and antagonism in the oestrogen receptor. *Nature* **389** 753–758. (doi:10.1038/39645)
- Catherino WH, Jeng MH & Jordan VC 1993 Norgestrel and gestodene stimulate breast cancer cell growth through an oestrogen receptor mediated mechanism. *British Journal of Cancer* **67** 945–952. (doi:10.1038/bjc.1993.175)
- Chisamore MJ, Ahmed Y, Bentrem DJ, Jordan VC & Tonetti DA 2001 Novel antitumor effect of estradiol in athymic mice injected with a T47D breast cancer cell line overexpressing protein kinase C α . *Clinical Cancer Research* **7** 3156–3165.
- Chu I, Arnaout A, Loiseau S, Sun J, Seth A, McMahon C, Chun K, Hennessy B, Mills GB, Nawaz Z *et al.* 2007 Src promotes estrogen-dependent estrogen receptor α proteolysis in human breast cancer. *Journal of Clinical Investigation* **117** 2205–2215. (doi:10.1172/JCI21739)
- Cole MP, Jones CT & Todd ID 1971 A new anti-oestrogenic agent in late breast cancer. An early clinical appraisal of ICI46474. *British Journal of Cancer* **25** 270–275. (doi:10.1038/bjc.1971.33)
- Courtin A, Communal L, Vilasco M, Cimino D, Mourra N, de Bortoli M, Taverna D, Faussat AM, Chaouat M, Forgez P *et al.* 2012 Glucocorticoid receptor activity discriminates between progesterone and medroxyprogesterone acetate effects in breast cells. *Breast Cancer Research and Treatment* **131** 49–63. (doi:10.1007/s10549-011-1394-5)
- Cuzick J, Forbes JF, Sestak I, Cawthorn S, Hamed H, Holli K & Howell A 2007 Long-term results of tamoxifen prophylaxis for breast cancer—96-month follow-up of the randomized IBIS-I trial. *Journal of the National Cancer Institute* **99** 272–282. (doi:10.1093/jnci/djk049)
- Cuzick J, Sestak I, Baum M, Buzdar A, Howell A, Dowsett M & Forbes JF 2010 ATAC/LATTE investigators. Effect of anastrozole and tamoxifen as adjuvant treatment for early-stage breast cancer: 10-year analysis of the ATAC trial. *Lancet Oncology* **11** 1135–1141. (doi:10.1016/S1470-2045(10)70257-6)
- Davies C, Godwin J, Gray R, Clarke M, Cutter D, Darby S, McGale P, Pan HC, Taylor C, Wang YC *et al.* 2011 Relevance of breast cancer hormone receptors and other factors to the efficacy of adjuvant tamoxifen: patient-level meta-analysis of randomised trials. *Lancet* **378** 771–784. (doi:10.1016/S0140-6736(11)60993-8)
- Davies C, Pan H, Godwin J, Gray R, Arriagada R, Raina V, Abraham M, Medeiros Alencar VH, Badran A, Bonfill X *et al.* 2013 Long-term effects of continuing adjuvant tamoxifen to 10 years versus stopping at 5 years after diagnosis of oestrogen receptor-positive breast cancer: ATLAS, a randomised trial. *Lancet* **381** 805–816. (doi:10.1016/S0140-6736(12)61963-1)
- Dodds EC, Lawson W & Noble RL 1938 Biological effects of the synthetic oestrogenic substance 4:4'-dihydroxy- α : β . *Lancet* **232** 1389–1391. (doi:10.1016/S0140-6736(00)89468-4)
- Dowsett M, Cuzick J, Ingle J, Coates A, Forbes J, Bliss J, Buyse M, Baum M, Buzdar A, Colleoni M *et al.* 2010 Meta-analysis of breast cancer outcomes in adjuvant trials of aromatase inhibitors versus tamoxifen. *Journal of Clinical Oncology* **28** 509–518. (doi:10.1200/JCO.2009.23.1274)
- Early Breast Cancer Trialists' Collaborative Group 1998 Tamoxifen for early breast cancer: an overview of the randomised trials. *Lancet* **351** 1451–1467. (doi:10.1016/S0140-6736(97)11423-4)
- Early Breast Cancer Trialists' Collaborative Group 2005 Effects of chemotherapy and hormonal therapy for early breast cancer on recurrence and 15-year survival: an overview of the randomised trials. *Lancet* **365** 1687–1717. (doi:10.1016/S0140-6736(05)66544-0)
- Ellis MJ, Gao F, Dehdashti F, Jeffe DB, Marcom PK, Carey LA, Dickler MN, Silverman P, Fleming GF, Kommareddy A *et al.* 2009 Lower-dose vs high-dose oral estradiol therapy of hormone receptor-positive, aromatase inhibitor-resistant advanced breast cancer: a phase 2 randomized study. *Journal of the American Medical Association* **302** 774–780. (doi:10.1001/jama.2009.1204)
- Fagan DH, Uselman RR, Sachdev D & Yee D 2012 Acquired resistance to tamoxifen is associated with loss of the type I insulin-like growth factor receptor: implications for breast cancer treatment. *Cancer Research* **72** 3372–3380. (doi:10.1158/0008-5472.CAN-12-0684)
- Fan P & Jordan VC 2014 Acquired resistance to selective estrogen receptor modulators (SERMs) in clinical practice (tamoxifen & raloxifene) by selection pressure in breast cancer cell populations. *Steroids* **90** 44–52. (doi:10.1016/j.steroids.2014.06.002)
- Fan P, McDaniel RE, Kim HR, Clagett D, Haddad B & Jordan VC 2012 Modulating therapeutic effects of the c-Src inhibitor via oestrogen receptor and human epidermal growth factor receptor 2 in breast

- cancer cell lines. *European Journal of Cancer* **48** 3488–3498. (doi:10.1016/j.ejca.2012.04.020)
- Fan P, Griffith OL, Agboke FA, Anur P, Zou X, McDaniel RE, Creswell K, Kim SH, Katzenellenbogen JA, Gray JW *et al.* 2013 c-Src modulates estrogen-induced stress and apoptosis in estrogen-deprived breast cancer cells. *Cancer Research* **73** 4510–4520. (doi:10.1158/0008-5472.CAN-12-4152)
- Fan P, Agboke FA, McDaniel RE, Sweeney EE, Zou X, Creswell K & Jordan VC 2014a Inhibition of c-Src blocks oestrogen-induced apoptosis and restores oestrogen-stimulated growth in long-term oestrogen-deprived breast cancer cells. *European Journal of Cancer* **50** 457–468. (doi:10.1016/j.ejca.2013.10.001)
- Fan P, Agboke FA, Cunliffe H, Ramos P & Jordan VC 2014b A molecular model for the mechanism of acquired tamoxifen resistance in breast cancer. *European Journal of Cancer* **50** 2866–2876. (doi:10.1016/j.ejca.2014.08.011)
- Fan P, Cunliffe H, Griffith OL, Agboke FA, Ramos P, Gray JW & Jordan VC 2014c Identification of gene regulation patterns underlying both E₂- and tamoxifen-stimulated cell growth through global gene expression profiling in breast cancer cells. *European Journal of Cancer* **50** 2877–2886. (doi:10.1016/j.ejca.2014.08.010)
- Feherty P, Farrer-Brown G & Kellie AE 1971 Oestradiol receptors in carcinoma and benign disease of the breast: an *in vitro* assay. *British Journal of Cancer* **25** 697–710. (doi:10.1038/bjc.1971.85)
- Fisher B, Brown A, Wolmark N, Redmond C, Wickerham DL, Wittliff J, Dimitrov N, Poisson SL, Schipper H & Prager D 1987 Prolonging tamoxifen therapy for primary breast cancer. Findings from the National Surgical Adjuvant Breast and Bowel Project clinical trial. *Annals of Internal Medicine* **106** 649–654. (doi:10.7326/0003-4819-106-5-649)
- Fisher B, Costantino JP, Wickerham DL, Redmond CK, Kavanah M, Cronin WM, Vogel V, Robidoux A, Dimitrov N, Atkins J *et al.* 1998 Tamoxifen for prevention of breast cancer: report of the National Surgical Adjuvant Breast and Bowel Project P-1 Study. *Journal of the National Cancer Institute* **90** 1371–1388. (doi:10.1093/jnci/90.18.1371)
- Fisher B, Costantino JP, Wickerham DL, Cecchini RS, Cronin WM, Robidoux A, Bevers TB, Kavanah MT, Atkins JN, Margolese RG *et al.* 2005 Tamoxifen for the prevention of breast cancer: current status of the National Surgical Adjuvant Breast and Bowel Project P-1 study. *Journal of the National Cancer Institute* **97** 1652–1662. (doi:10.1093/jnci/dji372)
- Folca PJ, Glascock RF & Irvine WT 1961 Studies with tritium-labelled hexoestrol in advanced breast cancer. Comparison of tissue accumulation of hexoestrol with response to bilateral adrenalectomy and oophorectomy. *Lancet* **2** 796–798. (doi:10.1016/S0140-6736(61)91088-1)
- Forbes JF, Cuzick J, Buzdar A, Howell A, Tobias JS & Baum M 2008 Effect of anastrozole and tamoxifen as adjuvant treatment for early-stage breast cancer: 100-month analysis of the ATAC trial. *Lancet Oncology* **9** 45–53. (doi:10.1016/S1470-2045(07)70385-6)
- Fournier DB, Chisamore M, Lurain JR, Rademaker AW, Jordan VC & Tonetti DA 2001 Protein kinase C α expression is inversely related to ER status in endometrial carcinoma: possible role in AP-1-mediated proliferation of ER-negative endometrial cancer. *Gynecologic Oncology* **81** 366–372. (doi:10.1006/gyno.2001.6164)
- Frankel LB, Lykkesfeldt AE, Hansen JB & Stenvang J 2007 Protein Kinase C α is a marker for antiestrogen resistance and is involved in the growth of tamoxifen resistant human breast cancer cells. *Breast Cancer Research and Treatment* **104** 165–179. (doi:10.1007/s10549-006-9399-1)
- Giovannella BC, Stehlin JS & Williams LJ Jr 1974 Heterotransplantation of human malignant tumors in “nude” thymusless mice. II. Malignant tumors induced by injection of cell cultures derived from human solid tumors. *Journal of the National Cancer Institute* **52** 921–930. (doi:10.1093/jnci/52.3.921)
- Giovannella BC, Stehlin JS Jr, Williams LJ Jr, Lee SS & Shepard RC 1978 Heterotransplantation of human cancers into nude mice: a model system for human cancer chemotherapy. *Cancer* **42** 2269–2281. (doi:10.1002/1097-0142(197811)42:5<2269::AID-CNCR2820420527>3.0.CO;2-F)
- Glascock RF & Hoekstra WG 1959 Selective accumulation of tritium-labelled hexoestrol by the reproductive organs of immature female goats and sheep. *Biochemical Journal* **72** 673–682.
- Gottardis MM & Jordan VC 1988 Development of tamoxifen-stimulated growth of MCF-7 tumors in athymic mice after long-term antiestrogen administration. *Cancer Research* **48** 5183–5187.
- Gottardis MM, Robinson SP & Jordan VC 1988 Estradiol-stimulated growth of MCF-7 tumors implanted in athymic mice: a model to study the tumorigenic action of tamoxifen. *Journal of Steroid Biochemistry* **30** 311–314. (doi:10.1016/0022-4731(88)90113-6)
- Gottardis MM, Wagner RJ, Borden EC & Jordan VC 1989a Differential ability of antiestrogens to stimulate breast cancer cell (MCF-7) growth *in vivo* and *in vitro*. *Cancer Research* **49** 4765–4769.
- Gottardis MM, Jiang SY, Jeng MH & Jordan VC 1989b Inhibition of tamoxifen-stimulated growth of an MCF-7 tumor variant in athymic mice by novel steroidal antiestrogens. *Cancer Research* **49** 4090–4093.
- Haddow A 1970 David A Karnofsky memorial lecture. Thoughts on chemical therapy. *Cancer* **26** 737–754. (doi:10.1002/1097-0142(197010)26:4<737::AID-CNCR2820260402>3.0.CO;2-T)
- Haddow A & Robinson AM 1939 The association of carcinogenicity and growth-inhibitory power in the polycyclic hydrocarbons and other substances. *Proceedings of the Royal Society of London. Series B, Biological Sciences* **127** 277–287. (doi:10.1098/rspb.1939.0022)
- Haddow A, Watkinson JM, Paterson E & Koller PC 1944 Influence of synthetic oestrogens on advanced malignant disease. *BMJ* **2** 393–398. (doi:10.1136/bmj.2.4368.393)
- Harper MJ & Walpole AL 1967 A new derivative of triphenylethylene: effect on implantation and mode of action in rats. *Journal of Reproduction and Fertility* **13** 101–119. (doi:10.1530/jrf.0.0130101)
- Harrington WR, Kim SH, Funk CC, Madak-Erdogan Z, Schiff R, Katzenellenbogen JA & Katzenellenbogen BS 2006 Estrogen dendrimer conjugates that preferentially activate extranuclear, nongenomic versus genomic pathways of estrogen action. *Molecular Endocrinology* **20** 491–502. (doi:10.1210/me.2005-0186)
- Howell A, Dodwell DJ, Anderson H & Redford J 1992 Response after withdrawal of tamoxifen and progestogens in advanced breast cancer. *Annals of Oncology* **3** 611–617.
- Howell A, Robertson JF, Quaresma Albano J, Aschermannova A, Mauriac L, Kleeberg UR, Vergote I, Erikstein B, Webster A & Morris C 2002 Fulvestrant, formerly ICI 162,780, is as effective as anastrozole in postmenopausal women with advanced breast cancer progressing after prior endocrine treatment. *Journal of Clinical Oncology* **20** 3396–3403. (doi:10.1200/JCO.2002.10.057)
- Hu ZZ, Kagan BL, Ariazi EA *et al.* 2011 Proteomic analysis of pathways involved in estrogen-induced growth and apoptosis of breast cancer cells. *PLoS ONE* **6** e20410. (doi:10.1371/journal.pone.0020410)
- Hunter T & Sefton BM 1980 Transforming gene product of Rous sarcoma virus phosphorylates tyrosine. *PNAS* **77** 1311–1315. (doi:10.1073/pnas.77.3.1311)
- Huseby RA, Maloney TM & McGrath CM 1984 Evidence for a direct growth-stimulating effect of estradiol on human MCF-7 cells *in vivo*. *Cancer Research* **44** 2654–2659.
- Ingle JN, Ahmann DL, Green SJ, Edmonson JH, Bisel HF, Kvols LK, Nichols WC, Creagan ET, Hahn RG, Rubin J *et al.* 1981 Randomized clinical trial of diethylstilbestrol versus tamoxifen in postmenopausal women with advanced breast cancer. *New England Journal of Medicine* **304** 16–21. (doi:10.1056/NEJM198101013040104)
- Jeng MH, Parker CJ & Jordan VC 1992 Estrogenic potential of progestins in oral contraceptives to stimulate human breast cancer cell proliferation. *Cancer Research* **52** 6539–6546.
- Jeng MH, Langan-Fahey SM & Jordan VC 1993 Estrogenic actions of RU486 in hormone-responsive MCF-7 human breast cancer cells. *Endocrinology* **132** 2622–2630. (doi:10.1210/endo.132.6.8504763)

- Jeng MH, Shupnik MA, Bender TP, Westin EH, Bandyopadhyay D, Kumar R, Masamura S & Santen RJ 1998 Estrogen receptor expression and function in long-term estrogen-deprived human breast cancer cells. *Endocrinology* **139** 4164–4174. (doi:10.1210/endo.139.10.6229)
- Jensen EV & Jacobson HI 1962 Basic guides to the mechanism of estrogen action. *Recent Progress in Hormone Research* **18** 387–414.
- Jensen EV & Jordan VC 2003 The estrogen receptor: a model for molecular medicine. *Clinical Cancer Research* **9** 1980–1989.
- Jiang SY, Wolf DM, Yingling JM, Chang C & Jordan VC 1992 An estrogen receptor positive MCF-7 clone that is resistant to antiestrogens and estradiol. *Molecular and Cellular Endocrinology* **90** 77–86. (doi:10.1016/0303-7207(92)90104-E)
- Johansson H, Terenius L & Thoren L 1970 The binding of estradiol-17 β to human breast cancers and other tissues *in vitro*. *Cancer Research* **30** 692–698.
- Jordan VC 1976a Antiestrogenic and antitumor properties of tamoxifen in laboratory animals. *Cancer Treatment Reports* **60** 1409–1419.
- Jordan VC 1976b Effect of tamoxifen (ICI 46,474) on initiation and growth of DMBA-induced rat mammary carcinomata. *European Journal of Cancer* **12** 419–424. (doi:10.1016/0014-2964(76)90030-X)
- Jordan VC 1983 Laboratory studies to develop general principles for the adjuvant treatment of breast cancer with antiestrogens: problems and potential for future clinical applications. *Breast Cancer Research and Treatment* **3** (Suppl) S73–S86. (doi:10.1007/BF01855131)
- Jordan VC 1984 Biochemical pharmacology of antiestrogen action. *Pharmacological Reviews* **36** 245–276.
- Jordan VC 2003a Tamoxifen: a most unlikely pioneering medicine. *Nature Reviews. Drug Discovery* **2** 205–213. (doi:10.1038/nrd1031)
- Jordan VC 2003b Antiestrogens and selective estrogen receptor modulators as multifunctional medicines. 1. Receptor interactions. *Journal of Medicinal Chemistry* **46** 883–908. (doi:10.1021/jm020449y)
- Jordan VC 2003c Antiestrogens and selective estrogen receptor modulators as multifunctional medicines. 2. Clinical considerations and new agents. *Journal of Medicinal Chemistry* **46** 1081–1111. (doi:10.1021/jm020450x)
- Jordan VC 2004 Selective estrogen receptor modulation: concept and consequences in cancer. *Cancer Cell* **5** 207–213. (doi:10.1016/S1535-6108(04)00059-5)
- Jordan VC 2008a Tamoxifen: catalyst for the change to targeted therapy. *European Journal of Cancer* **44** 30–38. (doi:10.1016/j.ejca.2007.11.002)
- Jordan VC 2008b The 38th David A. Karnofsky lecture: the paradoxical actions of estrogen in breast cancer – survival or death? *Journal of Clinical Oncology* **26** 3073–3082. (doi:10.1200/JCO.2008.17.5190)
- Jordan VC 2014a Proven value of translational research with appropriate animal models to advance breast cancer treatment and save lives: the Tamoxifen Tale. *British Journal of Clinical Pharmacology* [in press]. (doi:10.1111/bcp.12440)
- Jordan C 2014b Tamoxifen as the first targeted long term adjuvant therapy for breast cancer. *Endocrine-Related Cancer* **21** R235–R246. (doi:10.1530/ERC-14-0092)
- Jordan VC 2014c Avoiding the bad and enhancing the good of soy supplements in breast cancer. *Journal of the National Cancer Institute* **106** dju233. (doi:10.1093/jnci/dju233)
- Jordan VC 2014d Linking estrogen-induced apoptosis with decreases in mortality following long term adjuvant tamoxifen therapy. *Journal of the National Cancer Institute* **106** dju296. (doi:10.1093/jnci/dju296)
- Jordan VC 2014e A(nother) scientific strategy to prevent breast cancer in postmenopausal women by enhancing estrogen-induced apoptosis? *Menopause* **21** 1160–1164. (doi:10.1097/GME.0000000000000220)
- Jordan VC & Allen KE 1980 Evaluation of the antitumour activity of the non-steroidal antioestrogen monohydroxytamoxifen in the DMBA-induced rat mammary carcinoma model. *European Journal of Cancer* **16** 239–251. (doi:10.1016/0014-2964(80)90156-5)
- Jordan VC & Brodie AM 2007 Development and evolution of therapies targeted to the estrogen receptor for the treatment and prevention of breast cancer. *Steroids* **72** 7–25. (doi:10.1016/j.steroids.2006.10.009)
- Jordan VC & Dowse LJ 1976 Tamoxifen as an anti-tumour agent: effect on oestrogen binding. *Journal of Endocrinology* **68** 297–303. (doi:10.1677/joe.0.0680297)
- Jordan VC & Ford LG 2011 Paradoxical clinical effect of estrogen on breast cancer risk: a ‘new’ biology of estrogen-induced apoptosis. *Cancer Prevention Research* **4** 633–637. (doi:10.1158/1940-6207.CAPR-11-018)
- Jordan VC & Jaspan T 1976 Tamoxifen as an anti-tumour agent: oestrogen binding as a predictive test for tumour response. *Journal of Endocrinology* **68** 453–460. (doi:10.1677/joe.0.0680453)
- Jordan VC & Koerner S 1975 Tamoxifen (ICI 46,474) and the human carcinoma 8S oestrogen receptor. *European Journal of Cancer* **11** 205–206. (doi:10.1016/0014-2964(75)90119-X)
- Jordan VC & Koerner S 1976 Tamoxifen as an anti-tumour agent: role of oestradiol and prolactin. *Journal of Endocrinology* **68** 305–311. (doi:10.1677/joe.0.0680305)
- Jordan VC & Prestwich G 1977 Binding of [³H]tamoxifen in rat uterine cytosols: a comparison of swinging bucket and vertical tube rotor sucrose density gradient analysis. *Molecular and Cellular Endocrinology* **8** 179–188. (doi:10.1016/0303-7207(77)90090-9)
- Jordan VC, Dix CJ, Rowsby L & Prestwich G 1977 Studies on the mechanism of action of the nonsteroidal antioestrogen tamoxifen (I.C.I. 46,474) in the rat. *Molecular and Cellular Endocrinology* **7** 177–192. (doi:10.1016/0303-7207(77)90066-1)
- Jordan VC, Koch R, Mittal S & Schneider MR 1986 Oestrogenic and antioestrogenic actions in a series of triphenylbut-1-enes: modulation of prolactin synthesis *in vitro*. *British Journal of Pharmacology* **87** 217–223. (doi:10.1111/j.1476-5381.1986.tb10174.x)
- Jordan VC, Wolf M, Mirecki DM, Whitford DA & Welshons WV 1988 Hormone receptor assays: clinical usefulness in the management of carcinoma of the breast. *CRC Critical Reviews in Clinical Laboratory Sciences* **26** 97–152. (doi:10.3109/10408368809106860)
- Jordan VC, Schafer JM, Levenson AS, Liu H, Pease KM, Simons LA & Zapf JW 2001 Molecular classification of estrogens. *Cancer Research* **61** 6619–6623.
- Jordan VC, Liu H & Dardes R 2002 Re: Effect of long-term estrogen deprivation on apoptotic responses of breast cancer cells to 17 β -estradiol and the two faces of Janus: sex steroids as mediators of both cell proliferation and cell death. *Journal of the National Cancer Institute* **94** 1173–1175 author reply 1173–1175. (doi:10.1093/jnci/94.15.1173)
- Katzenellenbogen BS, Kendra KL, Norman MJ & Berthois Y 1987 Proliferation, hormonal responsiveness, and estrogen receptor content of MCF-7 human breast cancer cells grown in the short-term and long-term absence of estrogens. *Cancer Research* **47** 4355–4360.
- Kennedy BJ 1965 Hormone therapy for advanced breast cancer. *Cancer* **18** 1551–1557. (doi:10.1002/1097-0142(196512)18:12<1551::AID-CNCR2820181206>3.0.CO;2-1)
- Kiang DT & Kennedy BJ 1977 Tamoxifen (antiestrogen) therapy in advanced breast cancer. *Annals of Internal Medicine* **87** 687–690. (doi:10.7326/0003-4819-87-6-687)
- Komm BS & Mirkin S 2012 Incorporating bazedoxifene/conjugated estrogens into the current paradigm of menopausal therapy. *International Journal of Women's Health* **4** 129–140. (doi:10.2147/IJWH.S29346)
- Koubouec D, Ronacher K, Stubbsrud E, Louw A & Hapgood JP 2005 Synthetic progestins used in HRT have different glucocorticoid agonist properties. *Molecular and Cellular Endocrinology* **242** 23–32. (doi:10.1016/j.mce.2005.07.001)
- Lacassagne A 1936a A comparative study of the carcinogenic action of certain oestrogenic hormones. *American Journal of Cancer* **28** 735–740.
- Lacassagne A 1936b Hormonal pathogenesis of adenocarcinoma of the breast. *American Journal of Cancer* **27** 217–228. (doi:10.1158/ajc.1936.217)
- LaCroix AZ, Chlebowski RT, Manson JE, Aragaki AK, Johnson KC, Martin L, Margolis KL, Stefanick ML, Brzyski R, Curb JD *et al.* 2011 Health outcomes after stopping conjugated equine estrogens among postmenopausal women with prior hysterectomy: a randomized controlled

- trial. *Journal of the American Medical Association* **305** 1305–1314. (doi:10.1001/jama.2011.382)
- Langan-Fahey SM, Tormey DC & Jordan VC 1990 Tamoxifen metabolites in patients on long-term adjuvant therapy for breast cancer. *European Journal of Cancer* **26** 883–888. (doi:10.1016/0277-5379(90)90191-U)
- Lathrop AE & Loeb L 1915a Further investigations on the origin of tumors in mice: I. Tumor incidence and tumor age in various strains of mice. *Journal of Experimental Medicine* **22** 646–673. (doi:10.1084/jem.22.5.646)
- Lathrop AE & Loeb L 1915b Further investigations on the origin of tumors in mice: II. Tumor incidence and tumor age in hybrids. *Journal of Experimental Medicine* **22** 713–731. (doi:10.1084/jem.22.6.713)
- Lathrop AE & Loeb L 1916 Further investigations on the origin of tumors in mice. III. On the part played by internal secretion in the spontaneous development of tumors. *Journal of Cancer Research* **1** 1–19. (doi:10.1158/jcr.1916.1)
- Leclercq G, Heuson JC, Schoenfeld R *et al.* 1973 Estrogen receptors in human breast cancer. *European Journal of Cancer* **9** 665–673. (doi:10.1016/0014-2964(73)90009-1)
- Levenson AS & Jordan VC 1997 MCF-7: the first hormone-responsive breast cancer cell line. *Cancer Research* **57** 3071–3078.
- Levenson AS & Jordan VC 1998 The key to the antiestrogenic mechanism of raloxifene is amino acid 351 (aspartate) in the estrogen receptor. *Cancer Research* **58** 1872–1875.
- Levenson AS, Catherino WH & Jordan VC 1997 Estrogenic activity is increased for an antiestrogen by a natural mutation of the estrogen receptor. *Journal of Steroid Biochemistry and Molecular Biology* **60** 261–268. (doi:10.1016/S0960-0760(96)00184-7)
- Levenson AS, Kwaan HC, Svoboda KM, Weiss IM, Sakurai S & Jordan VC 1998 Oestradiol regulation of the components of the plasminogen-plasmin system in MDA-MB-231 human breast cancer cells stably expressing the oestrogen receptor. *British Journal of Cancer* **78** 88–95. (doi:10.1038/bjc.1998.447)
- Lewis JS, Cheng D & Jordan VC 2004 Targeting oestrogen to kill the cancer but not the patient. *British Journal of Cancer* **90** 944–949. (doi:10.1038/sj.bjc.6601627)
- Lewis JS, Osipo C, Meeke K & Jordan VC 2005a Estrogen-induced apoptosis in a breast cancer model resistant to long-term estrogen withdrawal. *Journal of Steroid Biochemistry and Molecular Biology* **94** 131–141. (doi:10.1016/j.jsmb.2004.12.032)
- Lewis JS, Meeke K, Osipo C, Ross EA, Kidawi N, Li T, Bell E, Chandel NS & Jordan VC 2005b Intrinsic mechanism of estradiol-induced apoptosis in breast cancer cells resistant to estrogen deprivation. *Journal of the National Cancer Institute* **97** 1746–1759. (doi:10.1093/jnci/dji400)
- Lewis-Wambi JS & Jordan VC 2009 Estrogen regulation of apoptosis: how can one hormone stimulate and inhibit? *Breast Cancer Research* **11** 206. (doi:10.1186/bcr2255)
- Lewis-Wambi JS, Kim HR, Wambi C, Patel R, Pyle JR, Klein-Szanto AJ & Jordan VC 2008 Buthionine sulfoximine sensitizes antihormone-resistant human breast cancer cells to estrogen-induced apoptosis. *Breast Cancer Research* **10** R104. (doi:10.1186/bcr2208)
- Lewis-Wambi JS, Swaby R, Kim H & Jordan VC 2009 Potential of L-buthionine sulfoximine to enhance the apoptotic action of estradiol to reverse acquired antihormonal resistance in metastatic breast cancer. *Journal of Steroid Biochemistry and Molecular Biology* **114** 33–39. (doi:10.1016/j.jsmb.2008.12.016)
- Lewis-Wambi JS, Kim H, Curpan R, Grigg R, Sarker MA & Jordan VC 2011 The selective estrogen receptor modulator bazedoxifene inhibits hormone-independent breast cancer cell growth and down-regulates estrogen receptor α and cyclin D1. *Molecular Pharmacology* **80** 610–620. (doi:10.1124/mol.111.072249)
- Lippman ME & Bolan G 1975 Oestrogen-responsive human breast cancer in long term tissue culture. *Nature* **256** 592–593. (doi:10.1038/256592a0)
- Liu H, Lee ES, Deb Los Reyes A, Zapf JW & Jordan VC 2001 Silencing and reactivation of the selective estrogen receptor modulator-estrogen receptor α complex. *Cancer Research* **61** 3632–3639.
- Liu H, Park WC, Bentrem DJ, McKian KP, Reyes Ade L, Loweth JA, Schafer JM, Zapf JW & Jordan VC 2002 Structure–function relationships of the raloxifene-estrogen receptor- α complex for regulating transforming growth factor- α expression in breast cancer cells. *Journal of Biological Chemistry* **277** 9189–9198. (doi:10.1074/jbc.M108335200)
- Liu H, Lee ES, Gajdos C, Pearce ST, Chen B, Osipo C, Loweth J, McKian K, De Los Reyes A, Wing L *et al.* 2003 Apoptotic action of 17 β -estradiol in raloxifene-resistant MCF-7 cells *in vitro* and *in vivo*. *Journal of the National Cancer Institute* **95** 1586–1597. (doi:10.1093/jnci/djg080)
- Liu H, Cheng D, Weichel AK, Osipo C, Wing LK, Chen B, Louis TE & Jordan VC 2006 Cooperative effect of gefitinib and fumitremorgin c on cell growth and chemosensitivity in estrogen receptor alpha negative fulvestrant-resistant MCF-7 cells. *International Journal of Oncology* **29** 1237–1246. (doi:10.3892/ijo.29.5.1237)
- Lonning PE 2009 Additive endocrine therapy for advanced breast cancer – back to the future. *Acta Oncologica* **48** 1092–1101. (doi:10.3109/02841860903117816)
- Lonning PE, Taylor PD, Anker G, Iddon J, Wie L, Jørgensen LM, Mella O & Howell A 2001 High-dose estrogen treatment in postmenopausal breast cancer patients heavily exposed to endocrine therapy. *Breast Cancer Research and Treatment* **67** 111–116. (doi:10.1023/A:1010619225209)
- Lubczyk V, Bachmann H & Gust R 2002 Investigations on estrogen receptor binding. The estrogenic, antiestrogenic, and cytotoxic properties of C2-alkyl-substituted 1,1-bis(4-hydroxyphenyl)-2-phenylethenes. *Journal of Medicinal Chemistry* **45** 5358–5364. (doi:10.1021/jm0209230)
- Maass H, Engel B, Hohmeister H, Lehmann F & Trams G 1972 Estrogen receptors in human breast cancer tissue. *American Journal of Obstetrics and Gynecology* **113** 377–382.
- MacGregor Schafer J, Liu H, Bentrem DJ, Zapf JW & Jordan VC 2000 Allosteric silencing of activating function 1 in the 4-hydroxytamoxifen estrogen receptor complex is induced by substituting glycine for aspartate at amino acid 351. *Cancer Research* **60** 5097–5105.
- Masamura S, Santner SJ, Heitjan DF & Santen RJ 1995 Estrogen deprivation causes estradiol hypersensitivity in human breast cancer cells. *Journal of Clinical Endocrinology and Metabolism* **80** 2918–2925. (doi:10.1210/jcem.80.10.7559875)
- Massarweh S, Osborne CK, Jiang S, Wakeling AE, Rimawi M, Mohsin SK, Hilsenbeck S & Schiff R 2006 Mechanisms of tumor regression and resistance to estrogen deprivation and fulvestrant in a model of estrogen receptor-positive, HER-2/*neu*-positive breast cancer. *Cancer Research* **66** 8266–8273. (doi:10.1158/0008-5472.CAN-05-4045)
- Massarweh S, Osborne CK, Creighton CJ, Qin L, Tsimelzon A, Huang S, Weiss H, Rimawi M & Schiff R 2008 Tamoxifen resistance in breast tumors is driven by growth factor receptor signaling with repression of classic estrogen receptor genomic function. *Cancer Research* **68** 826–833. (doi:10.1158/0008-5472.CAN-07-2707)
- Maximov PY, Fernandes DJ, McDaniel RE, Myers CB, Curpan RF & Jordan VC 2014a Influence of the length and positioning of the antiestrogenic side chain of endoxifen and 4-hydroxytamoxifen on gene activation and growth of estrogen receptor positive cancer cells. *Journal of Medicinal Chemistry* **57** 4569–4583. (doi:10.1021/jm500569h)
- Maximov PY, McDaniel RE, Fernandes DJ, Bhatta P, Korostyshevskiy VR, Curpan RF & Jordan VC 2014b Pharmacological relevance of endoxifen in a laboratory simulation of breast cancer in postmenopausal patients. *Journal of the National Cancer Institute* **106** dju283. (doi:10.1093/jnci/dju283)
- Maximov PY, McDaniel RE, Fernandes DJ *et al.* 2014c Simulation with cells *in vitro* of tamoxifen treatment in premenopausal breast cancer patients with different CYP2D6 genotypes. *British Journal of Pharmacology* **171** 5624–5635. (doi:10.1111/bph.12864)
- McGuire WL, Carbone PP & Vollmer EP (Eds) 1975a *Estrogen Receptors in Human Breast Cancer*. New York: Raven Press.
- McGuire WL, Carbone PP, Sears ME *et al.* 1975b Estrogen receptors in human breast cancer: an overview. In *Estrogen Receptors and Human Breast Cancer*, pp 1–15. Eds WL McGuire, PP Carbone & EP Vollmer. New York: Raven Press.

- Mehta RS, Barlow WE, Albain KS, Vandenberg TA, Dakhil SR, Tirumali NR, Lew DL, Hayes DF, Gralow JR, Livingston RB *et al.* 2012 Combination anastrozole and fulvestrant in metastatic breast cancer. *New England Journal of Medicine* **367** 435–444. (doi:10.1056/NEJMoa1201622)
- Miranda TB, Voss TC, Sung MH, Baek S, John S, Hawkins M, Grøntved L, Schiltz RL & Hager GL 2013 Reprogramming the chromatin landscape: interplay of the estrogen and glucocorticoid receptors at the genomic level. *Cancer Research* **73** 5130–5139. (doi:10.1158/0008-5472.CAN-13-0742)
- Molloy ME, Perez White BE, Gherezghiher T, Michalsen BT, Xiong R, Patel H, Zhao H, Maximov PY, Jordan VC, Thatcher GR *et al.* 2014 Novel selective estrogen mimics for the treatment of tamoxifen-resistant breast cancer. *Molecular Cancer Therapeutics* **13** 2515–2526. (doi:10.1158/1535-7163.MCT-14-0319)
- Morgan LR Jr, Schein PS, Woolley PV, Hoth D, Macdonald J, Lippman M, Posey LE & Beazley RW 1976 Therapeutic use of tamoxifen in advanced breast cancer: correlation with biochemical parameters. *Cancer Treatment Reports* **60** 1437–1443.
- Murphy CS, Meisner LF, Wu SQ & Jordan VC 1989 Short- and long-term estrogen deprivation of T47D human breast cancer cells in culture. *European Journal of Cancer and Clinical Oncology* **25** 1777–1788. (doi:10.1016/0277-5379(89)90348-9)
- Murphy CS, Pink JJ & Jordan VC 1990 Characterization of a receptor-negative, hormone-nonresponsive clone derived from a T47D human breast cancer cell line kept under estrogen-free conditions. *Cancer Research* **50** 7285–7292.
- Nabha SM, Glaros S, Hong M, Lykkesfeldt AE, Schiff R, Osborne K & Reddy KB 2005 Upregulation of PKC- δ contributes to antiestrogen resistance in mammary tumor cells. *Oncogene* **24** 3166–3176. (doi:10.1038/sj.onc.1208502)
- Obiorah I & Jordan VC 2013 2012 NAMS/PFIZER-Wulf H. Utian endowed lecture. The scientific rationale for a delay after menopause in the use of conjugated equine estrogens in postmenopausal women that causes a reduction in breast cancer incidence and mortality. *Menopause* **20** 372–382. (doi:10.1097/GME.0b013e31828865a5)
- Obiorah IE & Jordan VC 2014 Differences in the rate of oestrogen-induced apoptosis in breast cancer by estradiol and the triphenylethylene bisphenol. *British Journal of Pharmacology* **17** 4062–4072. (doi:10.1111/bph.12762)
- Obiorah I, Sengupta S, Fan P & Jordan VC 2014a Delayed triggering of oestrogen induced apoptosis that contrasts with rapid paclitaxel-induced breast cancer cell death. *British Journal of Cancer* **110** 1488–1496. (doi:10.1038/bjc.2014.50)
- Obiorah IE, Fan P & Jordan VC 2014b Breast cancer cell apoptosis with phytoestrogens is dependent on an estrogen deprived state. *Cancer Prevention Research* **7** 939–949. (doi:10.1158/1940-6207.CAPR-14-0061)
- Obiorah I, Sengupta S, Curpan R & Jordan VC 2014c Defining the conformation of the estrogen receptor complex that controls estrogen-induced apoptosis in breast cancer. *Molecular Pharmacology* **85** 789–799. (doi:10.1124/mol.113.089250)
- Obiorah IE, Fan P, Sengupta S & Jordan VC 2014d Selective estrogen-induced apoptosis in breast cancer. *Steroids* **90C** 60–70. (doi:10.1016/j.steroids.2014.06.003)
- Osborne CK, Hobbs K & Clark GM 1985 Effect of estrogens and antiestrogens on growth of human breast cancer cells in athymic nude mice. *Cancer Research* **45** 584–590.
- Osborne CK, Coronado EB & Robinson JP 1987 Human breast cancer in the athymic nude mouse: cytostatic effects of long-term antiestrogen therapy. *European Journal of Cancer and Clinical Oncology* **23** 1189–1196. (doi:10.1016/0277-5379(87)90154-4)
- Osborne CK, Pippen J, Jones SE, Parker LM, Ellis M, Come S, Gertler SZ, May JT, Burton G, Dimery I *et al.* 2002 Double-blind, randomized trial comparing the efficacy and tolerability of fulvestrant versus anastrozole in postmenopausal women with advanced breast cancer progressing on prior endocrine therapy: results of a North American trial. *Journal of Clinical Oncology* **20** 3386–3395. (doi:10.1200/JCO.2002.10.058)
- Osipo C, Gajdos C, Liu H, Chen B & Jordan VC 2003 Paradoxical action of fulvestrant in estradiol-induced regression of tamoxifen-stimulated breast cancer. *Journal of the National Cancer Institute* **95** 1597–1608. (doi:10.1093/jnci/djg079)
- Osipo C, Gajdos C, Cheng D & Jordan VC 2005 Reversal of tamoxifen resistant breast cancer by low dose estrogen therapy. *Journal of Steroid Biochemistry and Molecular Biology* **93** 249–256. (doi:10.1016/j.jsmb.2004.12.005)
- Osipo C, Meeke K, Cheng D, Weichel A, Bertucci A, Liu H & Jordan VC 2007 Role for HER2/neu and HER3 in fulvestrant-resistant breast cancer. *International Journal of Oncology* **30** 509–520. (doi:10.3892/ijo.30.2.509)
- Peethambaram PP, Ingle JN, Suman VJ, Hartmann LC & Loprinzi CL 1999 Randomized trial of diethylstilbestrol vs. tamoxifen in postmenopausal women with metastatic breast cancer. An updated analysis. *Breast Cancer Research and Treatment* **54** 117–122. (doi:10.1023/A:1006185805079)
- Perez White B, Molloy ME, Zhao H *et al.* 2013 Extranuclear ER α is associated with regression of T47D PKC α -overexpressing, tamoxifen-resistant breast cancer. *Molecular Cancer* **12** 34. (doi:10.1186/1476-4598-12-34)
- Pink JJ & Jordan VC 1996 Models of estrogen receptor regulation by estrogens and antiestrogens in breast cancer cell lines. *Cancer Research* **56** 2321–2330.
- Pink JJ, Jiang SY, Fritsch M & Jordan VC 1995 An estrogen-independent MCF-7 breast cancer cell line which contains a novel 80-kilodalton estrogen receptor-related protein. *Cancer Research* **55** 2583–2590.
- Pink JJ, Wu SQ, Wolf DM, Bilimoria MM & Jordan VC 1996a A novel 80 kDa human estrogen receptor containing a duplication of exons 6 and 7. *Nucleic Acids Research* **24** 962–969. (doi:10.1093/nar/24.5.962)
- Pink JJ, Bilimoria MM, Assikis J & Jordan VC 1996b Irreversible loss of the oestrogen receptor in T47D breast cancer cells following prolonged oestrogen deprivation. *British Journal of Cancer* **74** 1227–1236. (doi:10.1038/bjc.1996.521)
- Pink JJ, Fritsch M, Bilimoria MM, Assikis VJ & Jordan VC 1997 Cloning and characterization of a 77-kDa estrogen receptor isolated from a human breast cancer cell line. *British Journal of Cancer* **75** 17–27. (doi:10.1038/bjc.1997.4)
- Powles TJ, Ashley S, Tidy A, Smith IE & Dowsett M 2007 Twenty-year follow-up of the Royal Marsden randomized, double-blinded tamoxifen breast cancer prevention trial. *Journal of the National Cancer Institute* **99** 283–290. (doi:10.1093/jnci/djk050)
- Pradhan M, Baumgarten SC, Bembins LA & Frasor J 2012 CBP mediates NF- κ B-dependent histone acetylation and estrogen receptor recruitment to an estrogen response element in the *BIRC3* promoter. *Molecular and Cellular Biology* **32** 569–575. (doi:10.1128/MCB.05869-11)
- Robson JM 1937 Oestrous reactions, including mating, produced by triphenyl ethylene. *Nature* **140** 196. (doi:10.1038/140196b0)
- Robson JM & Schonberg JM 1938 Duration of action of natural and synthetic oestrogens. *Nature* **142** 292–293. (doi:10.1038/142292a0)
- Robson J & Schonberg A 1942 A new synthetic oestrogen with prolonged action when given orally. *Nature* **150** 22–23. (doi:10.1038/150022a0)
- Rossouw JE, Anderson GL, Prentice RL, LaCroix AZ, Kooperberg C, Stefanick ML, Jackson RD, Beresford SA, Howard BV, Johnson KC *et al.* 2002 Risks and benefits of estrogen plus progestin in healthy postmenopausal women: principal results From the Women's Health Initiative randomized controlled trial. *Journal of the American Medical Association* **288** 321–333. (doi:10.1001/jama.288.3.321)
- Rygaard J & Povlsen CO 1969 Heterotransplantation of a human malignant tumour to "Nude" mice. *Acta Pathologica et Microbiologica Scandinavica* **77** 758–760. (doi:10.1111/j.1699-0463.1969.tb04520.x)
- Schafer JL, Liu H, Tonetti DA & Jordan VC 1999 The interaction of raloxifene and the active metabolite of the antiestrogen EM-800 (SC 5705) with the human estrogen receptor. *Cancer Research* **59** 4308–4313.
- Schinzinger A 1889 Ueber carcinoma mammae [abstract]. Beilage zum Centralblatt für Chirurgie. *18th Congress of the German Society for Surgery*. **16** 55–56.

- Schrek R 1959 Fashions in cancer research. In *The Year Book of Pathology and Clinical Pathology*, pp 26–39. Chicago: The Year Book Publishers.
- Scottish Cancer Trials Office (MRC) 1987 Adjuvant tamoxifen in the management of operable breast cancer: the Scottish Trial. Report from the Breast Cancer Trials Committee, Edinburgh. *Lancet* **2** 171–175. (doi:10.1016/S0140-6736(87)90762-8)
- Sengupta S, Obiorah I, Maximov PY, Curpan R & Jordan VC 2013 Molecular mechanism of action of bisphenol and bisphenol A mediated by oestrogen receptor alpha in growth and apoptosis of breast cancer cells. *British Journal of Pharmacology* **169** 167–178. (doi:10.1111/bph.12122)
- Shafie SM 1980 Estrogen and the growth of breast cancer: new evidence suggests indirect action. *Science* **209** 701–702. (doi:10.1126/science.6994231)
- Shafie SM & Grantham FH 1981 Role of hormones in the growth and regression of human breast cancer cells (MCF-7) transplanted into athymic nude mice. *Journal of the National Cancer Institute* **67** 51–56. (doi:10.1093/jnci/67.1.51)
- Shang Y, Hu X, DiRenzo J, Lazar MA & Brown M 2000 Cofactor dynamics and sufficiency in estrogen receptor-regulated transcription. *Cell* **103** 843–852. (doi:10.1016/S0092-8674(00)00188-4)
- Shiau AK, Barstad D, Loria P, Cheng L, Kushner PJ, Agard DA & Greene GL 1998 The structural basis of estrogen receptor/coactivator recognition and the antagonism of this interaction by tamoxifen. *Cell* **95** 927–937. (doi:10.1016/S0092-8674(00)81717-1)
- Shike M, Doane A, Russo L *et al.* 2014 The effects of soy supplementation on gene expression in breast cancer: a randomized placebo-controlled study. *Journal of the National Cancer Institute* **106** dju189. (doi:10.1093/jnci/dju189)
- Sledge GW, Mamounas EP, Hortobagyi GN, Burstein HJ, Goodwin PJ & Wolff AC 2014 Past, present, and future challenges in breast cancer treatment. *Journal of Clinical Oncology* **32** 1979–1986. (doi:10.1200/JCO.2014.55.4139)
- Song RX & Santen RJ 2003 Apoptotic action of estrogen. *Apoptosis* **8** 55–60. (doi:10.1023/A:1021649019025)
- Song RX, Mor G, Naftolin F, McPherson RA, Song J, Zhang Z, Yue W, Wang J & Santen RJ 2001 Effect of long-term estrogen deprivation on apoptotic responses of breast cancer cells to 17 β -estradiol. *Journal of the National Cancer Institute* **93** 1714–1723. (doi:10.1093/jnci/93.22.1714)
- Song RX, Zhang Z, Mor G & Santen RJ 2005 Down-regulation of Bcl-2 enhances estrogen apoptotic action in long-term estradiol-depleted ER⁺ breast cancer cells. *Apoptosis* **10** 667–678. (doi:10.1007/s10495-005-1903-2)
- Soule HD & McGrath CM 1980 Estrogen responsive proliferation of clonal human breast carcinoma cells in athymic mice. *Cancer Letters* **10** 177–189. (doi:10.1016/0304-3835(80)90042-7)
- Soule HD, Vazquez J, Long A, Albert S & Brennan M 1973 A human cell line from a pleural effusion derived from a breast carcinoma. *Journal of the National Cancer Institute* **51** 1409–1416. (doi:10.1093/jnci/51.5.1409)
- Stoll B 1977 Palliation by castration or hormone ablation. In *Breast Cancer Management Early and Late*, pp 135–149. Ed BA Stoll. London: William Herman Medical Books Ltd.
- Sweeney EE, McDaniel RE, Maximov PY, Fan P & Jordan VC 2012 Models and mechanisms of acquired antihormone resistance in breast cancer: significant clinical progress despite limitations. *Hormone Molecular Biology and Clinical Investigation* **9** 143–163. (doi:10.1515/hmbci-2011-0004)
- Sweeney EE, Fan P & Jordan VC 2014a Mechanisms underlying differential response to estrogen-induced apoptosis in long-term estrogen-deprived breast cancer cells. *International Journal of Oncology* **44** 1529–1538. (doi:10.3892/ijo.2014.2329)
- Sweeney EE, Fan P & Jordan VC 2014b Molecular modulation of estrogen-induced apoptosis by synthetic progestins in hormone replacement therapy: an insight into the Women's Health Initiative study. *Cancer Research* **74** 7060–7068. (doi:10.1158/0008-5472.CAN-14-1784)
- Terenius L 1971 Structure–activity relationships of anti-oestrogens with regard to interaction with 17- β -oestradiol in the mouse uterus and vagina. *Acta Endocrinologica* **66** 431–447. (doi:10.1530/acta.0.0660431)
- Toft D & Gorski J 1966 A receptor molecule for estrogens: isolation from the rat uterus and preliminary characterization. *PNAS* **55** 1574–1581. (doi:10.1073/pnas.55.6.1574)
- Toft D, Shyamala G & Gorski J 1967 A receptor molecule for estrogens: studies using a cell-free system. *PNAS* **57** 1740–1743.
- Tonetti DA, Chisamore MJ, Grdina W, Schurz H & Jordan VC 2000 Stable transfection of protein kinase C alpha cDNA in hormone-dependent breast cancer cell lines. *British Journal of Cancer* **83** 782–791. (doi:10.1054/bjoc.2000.1326)
- Uht RM, Anderson CM, Webb P & Kushner PJ 1997 Transcriptional activities of estrogen and glucocorticoid receptors are functionally integrated at the AP-1 response element. *Endocrinology* **138** 2900–2908. (doi:10.1210/endo.138.7.5244)
- Walpole AL & Paterson E 1949 Synthetic oestrogens in mammary cancer. *Lancet* **2** 783–786. (doi:10.1016/S0140-6736(49)91370-7)
- Wardell SE, Nelson ER, Chao CA & McDonnell DP 2013 Bazedoxifene exhibits antiestrogenic activity in animal models of tamoxifen-resistant breast cancer: implications for treatment of advanced disease. *Clinical Cancer Research* **19** 2420–2431. (doi:10.1158/1078-0432.CCR-12-3771)
- Welshons WV & Jordan VC 1987 Adaptation of estrogen-dependent MCF-7 cells to low estrogen (phenol red-free) culture. *European Journal of Cancer and Clinical Oncology* **23** 1935–1939. (doi:10.1016/0277-5379(87)90062-9)
- Wolf DM & Jordan VC 1993 A laboratory model to explain the survival advantage observed in patients taking adjuvant tamoxifen therapy. *Recent Results in Cancer Research* **127** 23–33.
- Yao K, Lee ES, Bentrem DJ, England G, Schafer JI, O'Regan RM & Jordan VC 2000 Antitumor action of physiological estradiol on tamoxifen-stimulated breast tumors grown in athymic mice. *Clinical Cancer Research* **6** 2028–2036.
- Zhang Y, Zhao H, Asztalos S, Chisamore M, Sitabkhan Y & Tonetti DA 2009 Estradiol-induced regression in T47D:A18/PKC α tumors requires the estrogen receptor and interaction with the extracellular matrix. *Molecular Cancer Research* **7** 498–510. (doi:10.1158/1541-7786.MCR-08-0415)

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