TURUN YLIOPISTON JULKAISUJA
ANNALES UNIVERSITATIS TURKUENSIS

SARJA - SER. D OSA - TOM. 795

MEDICA - ODONTOLOGICA

THE EFFECTS OF SELECTIVE ESTROGEN RECEPTOR MODULATORS ON THE DEATH OF BREAST CANCER CELLS AND OSTEOBLASTIC CELLS

by

Anu Kallio

TURUN YLIOPISTO
Turku 2008

From the Department of Anatomy, University of Turku, Finland
and Turku Graduate School of Biomedical Sciences (TuBS), Turku, Finland

Supervised by
Professor Pirkko Härkönen
Department of Laboratory Medicine,
Tumor Biology
Lund University
Malmö, Sweden and
Department of Anatomy
University of Turku
Turku, Finland

Reviewed by
Docent Juha Klefström
Biomedicum Helsinki
University of Helsinki
Helsinki, Finland

and

Docent Paula Martikainen
Department of Pathology
University of Tampere
Tampere, Finland

Dissertation opponent
Professor Timo Ylikomi
Department of Cell Biology
University of Tampere
Tampere, Finland

ISBN 978-951-29-3497-3 (PRINT)
ISBN 978-951-29-3498-0 (PDF)
ISSN 0355-9483
Painosalama Oy – Turku, Finland 2008

To my family

ABSTRACT

Anu Kallio
THE EFFECTS OF SELECTIVE ESTROGEN RECEPTOR MODULATORS ON THE DEATH OF BREAST CANCER CELLS AND OSTEOBLASTIC CELLS
Institute of Biomedicine, Department of Anatomy and Turku Graduate School of Biomedical Sciences, University of Turku, Turku, Finland
Annales Universitatis Turkuensis
Painosalama Oy, Turku, Finland 2008

Selective estrogen receptor modulators (SERMs) are structurally different compounds that interact with intracellular estrogen receptors (ERs) in target organs as estrogen receptor agonists and antagonists. Tamoxifen (Tam) is a SERM compound widely used in chemotherapy of ER-positive breast cancer. It inhibits proliferation and facilitates apoptosis of breast cancer cells by ER-dependent modulation of gene expression. Recent reports have shown that Tam has also rapid nongenomic effects. In this thesis we studied the mechanisms by which Tam exerts rapid effects on breast cancer cell viability. We demonstrate that Tam at pharmacological concentrations causes rapid mitochondrial death program in breast cancer cells. In addition, we evaluated the upstream signaling events leading to the mitochondrial disruption by Tam. Our results suggest that Tam rapidly induces sustained activation of extracellularly signal-regulated kinase (ERK1/2) in ER-positive breast cancer cell lines, which effects can be opposed by 17β-estradiol (E_2). Tam-induced rapid death appears to be primarily ER-independent, but it can possibly be modulated by ERs. However, epidermal growth factor receptor (EGFR)-associated mechanisms were shown to be involved in Tam - induced cell death.

We also compared the ability of E_2 and different SERMs to promote survival of osteoblast-derived cells against etoposide-induced apoptosis. In order to compare the roles of the two ER isotypes, we created an U2OS human osteosarcoma cell line stably expressing either ERalpha (ERα) or ERbeta (ERβ). We present data that E_2 and a novel SERM compound ospemifene are able to protect osteoblastic cells from apoptosis. The protective effect could be mediated via both ERα and ERβ although the responses of the cell lines expressing either of the two receptors differed from each other. Moreover, we show that the osteoblast-protective effect is associated with changes in the levels of osteoblast-produced cytokine expression. Information about the anti- and proapoptotic actions of Tam and other SERMs in different target tissues could possibly be exploited in development of new tissue specific SERM compounds.

Keywords: Apoptosis, tamoxifen, SERM, breast cancer, estrogen, estrogen receptor, mitochondria, ERK, osteoblast, ospemifene

TIIVISTELMÄ

Anu Kallio
SELEKTIIVISTEN ESTROGEENIRESEPTORIN MUUNTELIJOIDEN (SERM) VAIKUTUS RINTASYÖPÄSOLUJEN JA LUUN SOLUJEN KUOLEMAAN
Biolääketieteen laitos, anatomia ja Turun Biolääketieteen tutkijakoulu, Turun yliopisto, Turku, Suomi
Annales Universitatis Turkuensis
Painosalama Oy, Turku 2008

Selektiiviset estrogeenireseptorin muuntelijat (SERMit) ovat ryhmä kemialliselta rakenteeltaan erilaisia yhdisteitä jotka sitoutuvat solunsisäisiin estrogeenireseptoreihin toimien joko estrogeenin kaltaisina yhdisteinä tai estrogeenin vastavaikuttajina. Tamoksifeeni on SERM –yhdiste, jota on jo pitkään käytetty estrogeenireseptoreita (ER) ilmentävän rintasyövän lääkehoidossa. Tamoksifeeni sekä estää rintasyöpäsolujen jakaantumista että toisaalta aikaansaa niiden apoptoosin eli ohjelmoidun solukuoleman muuntelemalla ER-välitteisesti kohdesolun geenien ilmentymistä. Viimeaikaiset tutkimustulokset ovat kuitenkin osoittaneet tamoksifeenilla olevan myös nopeampia, nongenomisia vaikutusmekanismeja. Tässä väitöskirjatyössä tutkimme niitä nopeita vaikutusmekanismeja joiden avulla tamoksifeeni vaikuttaa rintasyöpäsolujen elinkykyyn. Osoitamme että tamoksifeeni farmakologisina pitoisuuksina aikaansaa nopean mitokondriaalisen solukuolemaan johtavan signallointireitin aktivoitumisen rintasyöpäsoluissa. Tämän lisäksi tutkimme myös tamoksifeenin aiheuttamaan mitokondriovaurioon johtavia tekijöitä. Tutkimustuloksemme osoittavat että ER-positiivisissa rintasyöpäsoluissa tamoksifeeni indusoi pitkäkestoisen ERK-kinaasiaktivaation, joka voidaan estää 17-β-estradiolilla. Tamoksifeenin aikaansaama nopea solukuolema on pääosin ER:sta riippumaton tapahtuma, mutta siihen voidaan vaikuttaa myös ER-välitteisin mekanismein. Sen sijaan epidermaalisen kasvutekijäreseptorin (EGFR) voitiin osoittaa osallistuvan tamoksifeenin nopeiden vaikutusten välittämiseen.

Tämän lisäksi vertailimme myös estradiolin ja eri SERM-yhdisteiden kykyä suojata apoptoosilta käyttämällä osteoblastiperäisiä soluja. Pystyäksemme vertailemaan ER-isotyyppien roolia eri yhdisteiden suojavaikutuksissa, transfektoimme U2OS osteosarkoomasolulinjan ilmentämään pysyvästi joko ERalfaa (ERα) tai ERbetaa (ERβ). Tulostemme mukaan sekä estradioli että uusi SERM-yhdiste ospemifeeni suojaavat osteoblastin kaltaisia soluja etoposidi-indusoidulta apoptoosilta. Sekä ERalfa että ERbeta pystyivät välittämään suojavaikutusta, joskin vaikutukset erosivat toisistaan. Lisäksi havaitsimme edellä mainitun suojavaikutuksen olevan yhteydessä muutoksiin solujen sytokiiniekspressiossa. Tietoa SERM-yhdisteiden anti-ja proapoptoottisten vaikutusmekanismeista eri kohdekudoksissa voidaan mahdollisesti hyödyntää kehiteltäessä uusia kudosspesifisiä SERM-yhdisteitä.

Avainsanat: Apoptoosi, tamoksifeeni, SERM, rintasyöpä, estrogeeni, mitokondrio, ERK, estrogeenireseptori, osteoblasti, ospemifeeni

TABLE OF CONTENTS

ABBREVIATIONS

AP-1	activating protein-1
BA	bongkrekic acid
Bcl	B-cell leukemia
bp	base pair
BSA	bovine serum albumin
cDNA	complementary deoxyribonucleic acid
DFH-DA	dichlorodihydrofluorescein diacetate
DMEM	Dulbecco´s modified Eagle´s medium
DMSO	dimethylsulfoxide
DPI	diphenylene ionodium
DPN	diarylpropionitrile
E2	17β-estradiol
EGFR	epidermal growth factor receptor
ELISA	enzyme-linked immunosorbent assay
ERα	estrogen receptor alpha
ERβ	estrogen receptor beta
ERK	extracellularly signal-regulated kinase
ERT	estrogen replacement therapy
Eto	etoposide
GFP	green fluorescent protein
GPCR	G-protein coupled receptor
iFBS	inactivated fetal bovine serum
IL	interleukin
kDA	kilodalton
MAPK	mitogen-activated protein kinase
nRFP	nuclear red fluorescent protein
OMM	outer mitochondrial membrane
OPG	osteoprotegerin
Osp	ospemifene
PARP	poly (ADP-ribose) polymerase-1
PBS	phosphate buffered saline
PCD	programmed cell death
PKC	protein kinase C
PPT	propyl-pyrazole-triol
PTP	permeability transition pore
qRT-PCR	quantitative reverse transcription polymerase chain reaction
Ral	raloxifene

RANK	receptor activator of NF-κB
RANKL	receptor activator of NF-κB ligand
RNA	ribonucleic acid
ROS	reactive oxygen species
SDS-PAGE	sodium dodecyl sulfate polyacrylamide gel electrophoresis
SERM	selective estrogen receptor modulator
Tam	tamoxifen
TMRM	tetramethylrhodamine methyl ester
TNF	tumor necrosis factor
Tor	toremifene
Z-VAD-FMK	z-Val-Ala-Asp-fluoromethylketone
$\Delta\psi$ m	mitochondrial membrane potential

LIST OF ORIGINAL PUBLICATIONS

This thesis is based on the following original articles, which are referred to in the text by the Roman numerals (I-III).

I Anu Kallio, Aiping Zheng, Johanna Dahllund, Kaisa M Heiskanen and Pirkko Härkönen (2005): Role of Mitochondria in Tamoxifen-induced Rapid Death of MCF-7 Breast Cancer Cells. Apoptosis, 10:1395-1410.

II Aiping Zheng, Anu Kallio and Pirkko Härkönen (2007): Tamoxifen-Induced Rapid Death of MCF-7 Breast Cancer Cells Is Mediated via Extracellularly Signal-Regulated Kinase Signaling and Can Be Abrogated by Estrogen. Endocrinology, 148: 2764-2777.

III Anu Kallio, Tao Guo, Elisa Lamminen, Jani Seppänen, Lauri Kangas, Kalervo Väänänen and Pirkko Härkönen: Estrogen and selective estrogen receptor modulator (SERM) protection against cell death in estrogen receptor alpha and beta expressing U2OS cells. *Submitted.*

INTRODUCTION

Breast cancer is the most common malignancy among western women and the second overall cause of death in developed countries. Bone is the most common site of breast cancer colonization and metastases usually bring about bone loss with accompanying pain and loss of function. An association between female hormones and breast cancer is evident and estrogens have been shown to regulate multiple activities in breast cancer cells, including cell proliferation and escape from apoptotic cell death (Dong *et al.,* 2001; Leung and Wang 1999; Zhang *et al*., 2001; Fernando and Wimalasena 2004; Patel *et al.,* 2007). The estrogen receptor (ER) is present in nearly two-thirds of breast tumors and the ER status of breast tumors serves as an important indicator of likelihood to benefit from endocrine therapy. In addition to genomic effects of estrogen, it has more recently been demonstrated that estrogen is capable of enacting also rapid, membrane initiated signaling events in a variety of cell types (Migliaccio *et al.,* 1996; Song and Santen 1996; LeMellay *et al.,* 1997; Boyan *et al.,* 2003; Adamski and Benveniste 2005; Edwards 2005). However, the nature of plasma membrane ER is still a matter of debate.

Selective estrogen receptor modulators (SERMs) are structurally different compounds that interact with intracellular estrogen receptors in target organs as estrogen receptor agonists and antagonists. Tamoxifen (Tam) is the first clinically relevant SERM which has been used extensively for the treatment of breast cancer over three decades. Tamoxifen acts primarily through ERs by modulation of gene expression, which leads to inhibition of proliferation and increase of apoptosis (Perry *et al.,* 1995; Otto *et al.,* 1996). Besides ER-mediated genomic effects, pharmacological concentrations of Tam have more recently been shown to have ER-independent nongenomic effects in various cell types (Ferlini *et al.,* 1999; Kim *et al.,* 1999; Zhang *et al.,* 2000). Due to the side effects related to Tam-therapy, second generation SERMs have been developed. The ultimate goal of drug discovery is to find a SERM that prevents breast cancer and conserves the skeleton without increasing the risk of endometrial cancer and venous thromboembolism, and without inducing hot flushes.

This study was undertaken to study the mechanisms and pathways of Tam -induced death of breast cancer cells. Furthermore, we wanted to study the potential roles of different ER subtypes in anti-and proapoptotic actions of estrogen and other SERM molecules using ER-transfected U2OS osteosarcoma cell lines as models.

REVIEW OF THE LITERATURE

1. BREAST CANCER

There are approximately 1000000 new cases of breast cancer in the world each year (Patel *et al.,* 2007) and the incidence of breast cancer is still rising. Breast cancer is slowly becoming more prevalent in countries which previously had low rates of cancer as well as becoming a leading cause of cancer death in some countries. Indeed, breast cancer has the highest incidence of all types of cancer in women. Age and family history are the strongest risk factors, but sex hormones also play an important role, as demonstrated by epidemiological studies reporting a consistent association by reproductive personal history and breast cancer risk. In addition, the acceptability of preventive strategies by healthy women is closely related to their lifetime risk of developing breast cancer.

The main cause of morbidity and mortality in most cancers is metastasis. Breast cancer most commonly metastasizes to lymph nodes, bone, lung and liver. Especially ER-positive breast tumors often metastasize to bone, whereas ER-negative breast cancers more aggressively colonize visceral organs (Nguyen and Massague 2007). Bone metastasis results in pain, pathologic fractures, bone marrow suppression, hypercalcaemia, nerve-compression, cachexia and death (Coleman 2006). Up to 70-80% of patients with advanced breast cancer have bone metastasis (Woodhouse *et al.,* 1997; Coleman 2006). Breast cancer patients with bone metastasis are treated with antiestrogens and adjuvant therapies with aromatase inhibitors and cytotoxic agents. These treatments are used for palliation of pain from bone metastasis and for reduction of skeletal events. In addition, surgery, radiotherapy and antineoplastic therapy are used to control pain. However, the current treatments do not significantly prolong the lifespan of patients (Choueiri *et al*., 2006; Cicek and Oursler 2006). Thus, no effective treatments have been developed that specifically target the metastases in bone.

2. ESTROGENS

Estrogens are a group of steroid compounds functioning as the primary female sex hormones. The three major naturally occurring estrogens in women are estradiol, estriol, and estrone. Estrogens are produced primarily by ovaries but a smaller additional quantity of estrogens is produced by the adrenal glands and peripheral tissues such as fat, liver, and kidneys by converting androgens to estrogens. Estrogen hormones are also formed in the placenta during pregnancy. Estrogen signaling is essential for mammary gland development (Saji *et al.,* 2000) and for the development and maintenance of other female sex characteristics.

2.1 Estrogen and mammary gland

Estrogens, in particular 17β-estradiol (E_2), are well-characterized mitogens in mammary gland. In addition to normal developmental functions, estrogens are implicated in breast cancer initiation and promotion. The link between ovarian factor

and growth of breast cancer was established more than a century ago (Beatson 1896). Removal of the ovaries from premenopausal women with metastatic breast cancer was found in selected cases to cause temporary disease regression (Boyd 1900). However, until recently, it was generally accepted that estrogens do not directly cause breast cancer. Nevertheless, recent laboratory studies suggest that the presence of estrogen predisposes mammary epithelial cells to malignant transformation (Russo *et al.,* 2006; Cavalieri *et al*.; 2006). Because the strong signal that estrogen has on the mediators of cell cycle, cells that have oncogenic mutations may continue to divide in the presence of estrogen (Patel *et al.,* 2007). Eventually, these cells may develop into a tumor. A mechanistic link between estrogen action and breast cancer was established by the discovery of the estrogen receptor (ER). The estrogen receptor expression became widely used as a predictive marker of hormonal responsiveness and clinical aggressiveness. Breast cancer expression of ER positively correlates with prognosis and is associated with a more favorable response to antihormone treatment.

In addition to proliferative actions, estrogens have also been demonstrated to have an antiapoptotic influence in both ER-positive and -negative breast cancer cells (Huang *et al.,* 1997; Bynoe *et al.,* 2000; Haynes *et al.,* 2000; Perillo *et al.,* 2000; Ahamed *et al.,* 2001; Choi *et al.,* 2001; Zhang *et al.,* 2001). E_2 has been shown to protect breast cancer cells against apoptosis induced by for example TNF-α, H_2O_2, serum withdrawal, UV-radiation and chemotherapeutic drugs (Fernando and Wimalasena 2002; Razandi *et al.,* 2003; Zhang *et al.,* 2004; Pedram *et al.,* 2006). However, the molecular mechanisms of antiapoptotic estrogen action are not fully defined. It has been shown that E_2 induces transcription of genes that have antiapoptotic actions in many cell types (Dong *et al.,* 1999; Leung *et al.,* 1999). In addition to nuclear events, estrogen has been demonstrated to be capable of bringing about rapid membrane-initiated signaling events in a variety of breast cancer cell types (Edwards 2005). These include release of calcium, secretion of prolactin, generation of inositol triphosphate and nitric oxide, phosphorylation of Bad and activation of MAPK and PI3K/Akt (Pietras *et al.,* 1975; Pietras *et al.,* 1979; Pappas *et al.,* 1995; Le Mellay *et al.,* 1997; Razandi *et al.,* 1999; Migliaccio *et al.,* 1996; Vasudevan *et al.,* 2001; Fernando and Wimalasena 2004).

2.2 Estrogen and bone

Estrogen has an important role in the development and growth of bones and later in the maintenance of the bone mass. It is believed that the major action of estrogen on the skeleton in vivo is through the inhibition of bone resorption (Riggs *et al.,* 2002). The maintenance of bone mass is controlled by the relative activities of bone forming osteoblasts and bone resorbing osteoclasts. Classical estrogen receptors are present in bone marrow stromal cells, osteoblasts, osteoclasts and their progenitors indicating that the effects of estrogen on bone are mediated, at least in part, directly. Although some of the anti-resorptive effects of estrogen are via direct actions on osteoclasts and hematopoietic cells, estrogen has also been shown to have indirect effects by regulating osteoblasts and bone marrow stromal cells (Zallone 2006). Osteoclast formation is dependent on equilibrium between several cytokines acting on autocrine and paracrine manner and produced by osteoblasts, which indicates that bone resorption is coupled

with osteoblastic bone formation. These cellular and molecular bases for the effects of estrogen on bone are now being increasingly better understood.

2.2.1 Effects of estrogen on bone resorption

Estrogen has clear effects on osteoclast development, activity and apoptosis. The key, essential molecule for osteoclast development is receptor activator of NF-κB ligand (RANKL), which is expressed on the surface of bone marrow stromal/osteoblast precursor cells, T-cells and B-cells. RANKL binds to its cognate receptor, RANK, on osteoclast lineage cells, and is neutralized by the soluble decoy receptor, osteoprotegerin (OPG), which is also produced by the osteoblastic lineage cells. Combined *in vitro* and *in vivo* studies have now demonstrated that estrogen suppresses RANKL production by osteoblastic cells and also increases OPG production (Lindberg *et al*., 2001; Saika *et al*., 2001; Bord *et al*., 2003; Syed and Khosla 2005). It has been hypothesized by several authors that the protective action of estrogen can be due to a change of the ratio among RANKL, cytokine inducing osteoclastogenesis, and OPG. This statement, however, has not been clearly proven, due to the discrepancy between the data in the literature.

In addition to the effects of estrogen on RANKL and OPG expression, estrogen also regulates the production of additional cytokines in osteoblasts, thus modulating osteoclastic activity in a paracrine fashion. Estrogen suppresses production of pro-resorptive cytokines such as interleukin-6 (IL-6) and tumor necrosis factor-α (TNF-α) by the cells of osteoblastic lineage (Manolagas and Jilka 1992; Compston 2001). The effect of E_2 on IL-6 expression has been reported to be mediated via both ERα and ERβ (Brady *et al.,* 2002).

Estrogen also has direct effects on osteoclast lineage cells. Thus, estrogen induces apoptosis of these cells and suppresses RANKL-induced osteoclast differentiation (Kousteni *et al.,* 2002, Zallone 2006). Moreover, estrogens have also been shown to inhibit the activity of mature osteoclasts through direct, receptor-mediated mechanisms (Hughes *et al.,* 1996; Chen *et al.,* 2001).

2.2.2 Effects of estrogen on bone formation

Although the precise mechanisms that control the relative numbers of osteoblasts and osteoclats are not known at present, apoptosis in the regulation of bone metabolism has recently been demonstrated by several investigators (Urayama *et al*., 2000, Kameda *et al*., 1995, Kawakami *et al*., 1997, Nakashima *et al*., 1998, Kawakami *et al*., 1998, Weinstein *et al*., 1998). Especially apoptosis of osteoblasts is getting more attention since it has been considered to be an important determinant of bone formation and therefore of skeletal integrity (Manolagas 2000; Weinstein and Manolagas 2000), such that disorders that promote the process are associated with increased bone fragility (Weinstein *et al*., 1998), and treatments that inhibit it are associated with anti-fracture efficacy (Jilka *et al*., 1999). Indeed, at the cellular level, there is now considerable evidence that estrogen prolongs the lifespan of the osteoblasts by inhibiting osteoblast apoptosis (Syed and Khosla 2005). This, in turn, increases the functional capacity of each osteoblast. Conversely, estrogen deficiency is associated with accelerated osteoblast apoptosis.

Estrogen effects on osteoblast apoptosis appear to be mediated by activation of the Src/Shc/ERK signaling pathway and downregulation of JNK leading to alterations in the activity of key transcription factors (Kousteni *et al.,* 2001; Kousteni *et al.,* 2003).

The effects of estrogen on osteoblast proliferation and differentiation markers have been variable, depending on the model system used. These discrepancies, however, could be due to the fact that most of the studies were conducted before ERβ was discovered, since the ER isoform concentrations can greatly influence the estrogen-dependent response. A number of more recent studies using osteoblastic cell systems expressing ERα have found a consistent inhibitory effect of estrogen on proliferation, whereas no effect of estrogen was seen using cells expressing ERβ. It seems that estrogen directly regulates osteoblast proliferation and differentiation, although the net effect of estrogen on osteoblasts likely depends on factors such as species differences, cell system heterogeneity, differentiation stage, ER isoform expression and receptor concentration.

3. ESTROGEN RECEPTORS

3.1 ER structure

The estrogen receptors (ERs) are members of a large family of steroid receptors that act as nuclear transcriptional regulators (Olefsky 2001). ERα was the first estrogen receptor cloned and it was isolated from MCF-7 human breast cancer cells in the late 1980s (Walter *et al.,* 1985; Greene *et al.,* 1986; Green *et al.,* 1986). Ten years later, ERβ was cloned from rat prostate (Kuiper *et al.,* 1996). Mouse (Tremblay *et al.,* 1997) and human (Mosselman *et al.,* 1996; Enmark *et al.,* 1997; Ogawa *et al.,* 1998) forms of ERβ have also been cloned. The human ERα gene is located on chromosome 6 and the ERβ gene is on chromosome 14, demonstrating that they are encoded by separate genes and are distinct (Enmark *et al.,* 1997). Both ERs comprise eight exons and have six functional domains. At the amino-terminus the A/B domain contains the ligand-independent transactivation function 1 (AF-1). The highly conserved DNA binding domain (DBD) is located in the central C region, which is adjacent to region D, a flexible hinge which contains a nuclear localization signal. The ligand-dependent AF-2 is located at the multifunctional E/F domain at the carboxy-terminus. The most highly conserved areas are at DNA- and ligand-binding domains (96% and 60%, respectively).

3.2 ER ligands

A wide variety of structurally distinct compounds bind to the ER with differing affinities. Certain ligands act as ER agonists, and these include the natural ligand E_2, as well as the synthetic estrogen diethylstilbestrol (DES). Certain phytoestrogens, which are environmental compounds such as genistein, can also be estrogenic. Other compounds, such as ICI182780, are receptor antagonists. A final group of mixed agonists and antagonists are comprised of the SERMs. According to article by Kuiper *et al.* (Kuiper *et al.,* 1997), the relative binding affinities (RBAs) of ERα for various ligands are: DES (468)>4-OHT (178)>E_2 (100)>ICI 164,384(85)>genistein (5). In contrast, ERβ has been shown to display the following RBAs: 4-OHT (339)>DES (295)>ICI 164,384(166)>E_2 (100)>genistein (36). In this work RBA value for E_2 was

arbitrarily set at 100. In addition to ligands that bind both ERα and ERβ, also synthetic isotype specific ligands have been developed.

3.3 ER distribution to organs

Different ER ligands interact with ER subtypes in various parts of the human body. The abundance and distribution of the receptors will, in part, determine whether a ligand will have a particular effect. ERα and ERβ are known to be localized in the breast, brain, cardiovascular system, urogenital tract and bone (Enmark *et al.,* 1997; Kuiper *et al.,* 1997; Gustafsson 1999; Taylor and Al-Azzawi 2000). ERα is the main ER subtype in the liver, whereas ERβ is the main ER in the colon. ERα and ERβ may also localize to distinct cellular subtypes within each tissue. For example, within the ovary, ERα is largely present in the thecal and interstitial cells, whereas ERβ is predominantly in the granulosa cells (Hiroi *et al.,* 1999; Sar and Welch 1999). In the prostate, ERβ localizes to the epithelium, whereas ERα localizes to the stroma (Weihua *et al.,* 2001).

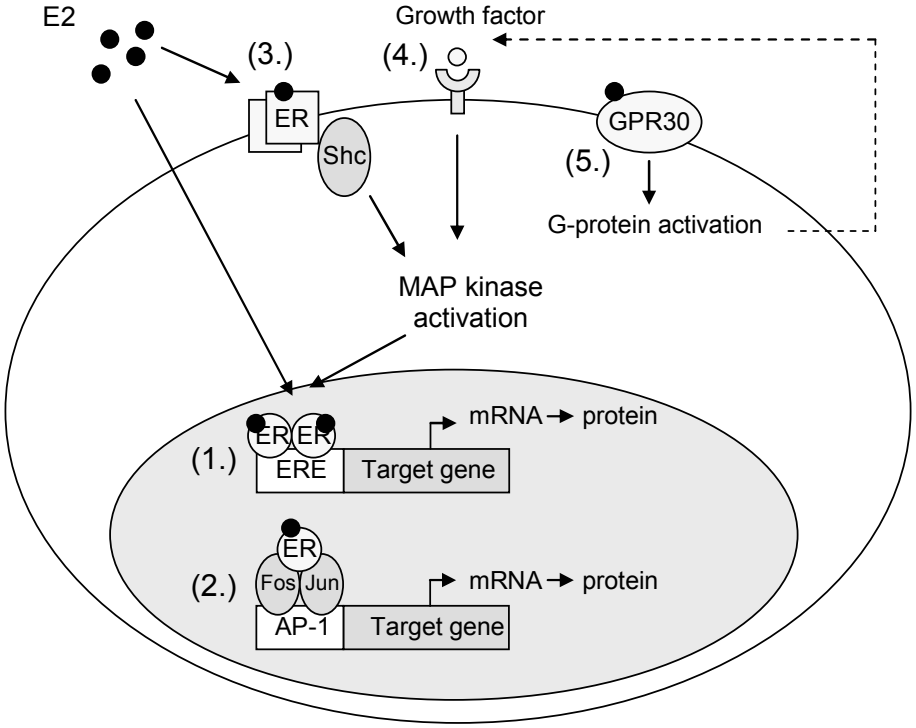

Figure 1. Simplified overview of ER-regulated gene transcription. Estrogens can initiate gene transcription via multiple pathways and receptors: (1.) and (2.) Classical pathway. (3.) Plasma membrane ER. (4.) Growth factor related pathways. (5.) G-protein activation.

3.4 ERα and ERβ activation

Originally it was considered that ER signalling was relatively straightforward: ligand bound to a single nuclear receptor, which resulted in a transcriptional change. It is now recognized that there are several mechanisms of action: ligand-dependent and independent, genomic and non-genomic.

3.4.1 Classical ligand-dependent activation of ER

In the absence of ligand, ER is sequestered in a multiprotein inhibitory complex within target cell nuclei. Ligand binding induces phosphorylation of ER, dissociation from chaperones such as heat shock protein 90, changes in protein conformation and dimerization (Hall *et al.,* 2001). Dimers (either α/α, β/β homodimers or α/β heterodimers) then interact with estrogen-response elements (EREs) in target genes to promote transcription (Huang *et al.,* 2004). This may be direct or indirect through the action of co-activators (e.g. SRC1, AIB1) (McKenna *et al.,* 1998), which enhance transcription or co-repressors (Dobrzycka *et al.*, 2003) that down-regulate expression. Co-activators such as AIB1 are important in breast cancer, where up to 65% of cases can show expression (Bouras *et al.,* 2001). Ligand-bound ER can modulate gene expression also in other ways. ER can bind to transcriptional complexes on other regulatory DNA sequences, such as activator protein 1 (AP-1), which is mediated by c-fos, and c-jun transcription factors (Webb *et al.,* 1995), or through GC-box bound specificity protein 1 (SP-1) (Kushner *et al*., 2000; Saville *et al*., 2000; Safe *et al*., 2001). Several genes important for tumour cell proliferation and survival may be regulated by estrogen in this way (Kushner *et al*., 2000).

3.4.2 Ligand-independent activation of ER

It is now recognized that ER can be activated by growth factors (epidermal growth factor, insulin growth factor-1, transforming growth factor α) and signalling molecules (cyclic AMP) (Lee *et al.,* 2001). Multiple kinases involved in growth factor, stress and cytokine signalling can phosphorylate ERα at the AF-1 domain or co-activators and co-repressors, so affecting dimerization, DNA binding, ligand binding and transcriptional activation (LeGoff *et al.,* 2004; Chen *et al.,* 2002). Activation of ERα function by growth factor signalling could be important in the development of resistance to tamoxifen by breast cancers (Schiff and Osborne 2005).

3.4.3. Membrane-initiated non-genomic steroid signaling

The concept of a putative membrane ER was proposed over 25 years ago, following observations of rapid responses of cells to estrogen (Pietras and Szego 1977). Originally termed 'nongenomic ER activity', this is also called 'membrane-initiated steroid signaling (MISS) (Nemere *et al.,* 2003). A series of E_2-induced MISS events has been described in benign cells and malignant cells of various origins. These effects occur from seconds to minutes after administration of E_2 and involve rapid activation of many signaling molecules (Cheskis 2004; Levin 2005; Shupnik 2004), such as 1) IGF1 and EGF receptors, 2) HRAS1 (also known as p21ras) and RAF1, 3) MAPK and PI3K/Akt, 4) protein kinase C, 5) calcium channel, 6) nitric oxide and prolactin secretion, and 7) Maxi-K channels. These rapid effects are also called "extranuclear", or "membrane-mediated effects", because they take place outside of the nucleus and are initiated predominantly in or near the plasma membrane.

Several possible candidates have been suggested to mediate binding of E_2 in the plasma membrane. These include 1) full-length ERα (Razandi *et al.,* 1996); 2) a truncated form of ERα with a molecular weight of 46 kDa (Li *et al.,* 2003); 3) a unique estrogen membrane protein called ER-X, whose ligand affinity differs from that of the classic

ERα but is recognized by antibodies directed against ERα ligand binding domain (Toran-Allerand *et al.,* 2002); 4) sex steroid binding protein acting in concert with a membrane protein megalin (Catalano *et al.,* 1997; Hammes *et al.,* 2005); 5) G protein-coupled receptor 30 (GPR30) (Thomas *et al.,* 2005); 6) growth factor receptors and 7) an unknown protein present in non-ERα-, non-ERβ-expressing CHO and COS-7 cells (Netrapalli *et al.,* 2005). Although these various E_2 binding proteins exist in specific systems, accumulating evidence supports the classic full-length ERα in or near the membrane functioning as the membrane estrogen receptor, which has been demonstrated in several types of cells, including breast epithelial cells, osteoblasts, endothelial cells, and vascular smooth muscle cells. Classical ERα has been shown to be expressed near the region of the cell membrane in MCF-7 cells (Song *et al.,* 2002). In addition, translocation of full-length ERα has been demonstrated into or near the plasma membrane in response to E_2 (Song and Santen 2006). Additional evidence supporting the classic ERα in the membrane was obtained from studies in which this receptor was knocked down by a selective small interfering RNA (siRNA) (Song *et al.,* 2004). In this study, E_2 activated MAPK in a matter of minutes, but abrogation of ERα with a selective siRNA abolished this effect in human breast cancer cells. Yet another study demonstrated that transfection of ER-negative breast cancer cells with ERα resulted in 5% of ERα located in the plasma membrane and the remainder predominantly in the nucleus (Razandi *et al.,* 1996). However, opinion is divided regarding whether or not membrane initiated steroid signaling occurs in breast cancer, as the most convincing studies supporting its existence have come from other systems, such as pituitary (Song *et al.,* 2004) and cardiovascular (Zhang *et al.,* 2002). Nevertheless, it has been postulated that this type of ER activity may be important in breast cancers that overexpress tyrosine kinase receptors and may be a mechanism for tamoxifen resistance (Toran-Allerand *et al.,* 2002).

Recent studies have implicated GPR30, a G protein-coupled seven transmembrane receptor target that resides in the plasma membrane, as a more plausible candidate for mediating membrane initiated steroid signaling in breast cancer (Thomas *et al.,* 2005), and in clinical breast cancer it was demonstrated that GPR30 facilitated both ERα- and EGFR-dependent activity (Filardo *et al.,* 2006). However, GPR30 and ERα showed different associations with HER-2/neu, tumor size and distant metastasis, suggesting that these mechanisms of estrogen activation are autonomous in breast cancer (Filardo *et al.,* 2006). *In vitro* studies of breast cancer cell lines have produced contrasting results, as estrogen was unable to activate multiple downstream signaling pathways in cells that lacked classical (nuclear) ERα, even in the presence of GPR30 (Pedram *et al.,* 2006).

Another hypothesis is that estrogen exerts extranuclear actions by interacting directly with growth factor receptors (Razandi *et al.,* 2004). Crosstalk from membrane-localized ERs to nuclear ERs has recently been proposed to be mediated through growth factor receptor tyrosine kinases such as epidermal growth factor receptor (EGFR) (Filardo *et al.,* 2002). Growth factor receptors, in turn, bring about signal transduction to kinases such as mitogen activated protein kinase (MAPK).

4. SELECTIVE ESTROGEN RECEPTOR MODULATORS

SERMs are a group a therapeutic agents available for the prevention and treatment of diseases such as osteoporosis and breast cancer (Jordan 2004). A selective estrogen receptor modulator is defined as a compound that binds with high affinity to estrogen receptors. A characteristic that distinguishes these substances from pure receptor agonists and antagonists is that their action is different in various tissues, thereby granting the possibility to selectively inhibit or stimulate estrogen-like action in various tissues. Clinically available SERMs fall into two chemical classes: triphenylethylenes (e.g., tamoxifen and its derivatives) and bentzothiophnenes (e.g. raloxifene). Currently, four SERMs are licensed for clinical use: tamoxifen, toremifene, clomifene and raloxifene. New-generation SERMs, such as bazedoxifene, arzoxifene, lasofoxifene and ospemifene, are also currently being evaluated. The aim is to find a SERM that conserves the skeleton and prevents breast cancer without increasing the risk of endometrial cancer and venous thromboembolism, and without inducing hot flushes.

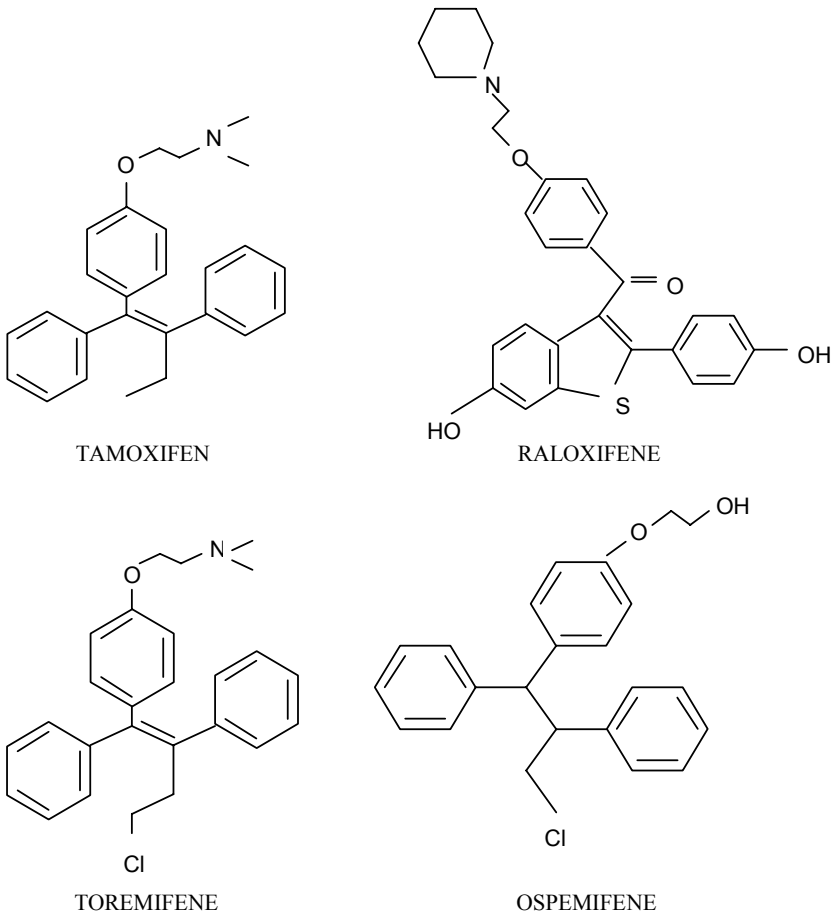

Figure 2. Chemical structures of four SERMs tamoxifen, toremifene, raloxifene and ospemifene

4.1 Tamoxifen: a first generation SERM

4.1.1. Clinical use

Tamoxifen (Tam), the trans-isomer of a substituted triphenylethylene, (Z)-1-[4-[(dimethylamino)ethoxy]phenyl]-1,1-diphenyl-1-butene, was synthesized and developed in the 1960s (Harper and Walpole 1966). It was initially discovered in a search for antifertility agents but later was recognized to have antitumoural potential. First reports of its use for the treatment of advanced breast cancer appeared in the early 1970s (Cole *et al.,* 1971). Currently, Tam is the endocrine treatment of choice for women diagnosed with all stages of hormone-responsive breast cancer (both estrogen-receptor positive and/or progesterone receptor positive). Furthermore, Tam also decreases the incidence of contralateral breast cancer in women who have had a first cancer (Jordan and Morrow 1999). Tam is also administrated for prophylactic chemoprevention in women identified as high risk for breast cancer and appears to reduce disease incidence by 50% in pre-or postmenopausal cohort (Fisher *et al.,* 1998; Cuzick *et al.,* 2003). Adjuvant Tam treatment for five years reduces the risk of breast cancer recurrence by 47% and mortality by 26% among patients diagnosed with hormone-responsive disease.

Toremifene, another SERM, was developed in Finland (Kangas 1990) and is used in the treatment of postmenopausal breast cancer (Valavaara 1990). Tor has been shown to have similar efficacy and adverse effects as Tam.

4.1.2 Beneficial effects of Tam use

Because of its pro-estrogenic action at many target tissue sites, Tam use is associated with many beneficial effects (Singh *et al.,* 2007). It appears to maintain and/or, to some degree, restore bone mineral density in postmenopausal women. In women being treated for breast cancer, Tam was also found to have cholesterol-lowering effects.

4.1.3 Deleterious effects of Tam use

There are several limitations to the long-term use of Tam in healthy, postmenopausal women. It has been demonstrated that adjuvant Tam treatment specifically for breast cancer or for prophylactic chemoprevention is associated with a 2-7 fold elevation in risk of subsequently developing endometrial carcinoma (Fisher *et al.,* 1998; Curtis *et al.*, 1996; Fisher *et al.,* 1994; Rutqvist *et al.,* 1995; Van Leeuwen *et al.,* 1994). Nevertheless, it is important to stress that the increase in endometrial cancer noted with Tam only occurs in postmenopausal women (EBCTCG 1998; Fisher *et al*., 1998). Thus, the future use of Tam for chemoprevention may be restricted to high-risk women who will develop breast cancer during their premenopausal years since the risk-benefit ratio has shown to be favorable in premenopausal women. Tam also significantly increases the risk of venous thromboembolism. It also causes vasomotor symptoms (i.e. hot flashes) which can be severe enough to lead to discontinuation of therapy in a minority of breast cancer patients.

4.1.4 Molecular mechanisms of Tam in breast cancer cells

Tam exerts its effects primarily by binding to ER and altering the conformation of the complex. This leads to modulation of gene expression, which in turn leads to inhibition of proliferation (Rochefort *et al.,* 1983) and increase of apoptosis (Wärri *et al.,* 1993; Watts *et al.,* 1994; Kandouz *et al.,* 1999; Thiantanawat *et al.,* 2003; Salami *et al.,* 2003; Somai *et al.,* 2003) of breast cancer cells. An example of the effects of Tam in induction of cell death is the transcriptional regulation of expression of members of the Bcl-2 protein family involved in apoptosis (Diel *et al.,* 1999). Besides ER-mediated genomic effects, pharmacological concentrations of Tam have been shown to have ER-independent nongenomic effects in various cell types (Couldwell *et al.,* 1994; Ferlini *et al.,* 1999; Kim *et al.,* 1999; Zhang *et al.,* 2000; Lehenkari *et al.,* 2003). The various molecular mechanisms of the rapid proapoptotic actions of Tam are not fully understood, but Tam has been shown for example to have an ionophoric effect on cell membranes, which is associated with rapid changes in membrane permeability and intracellular pH. This, in turn, leads to decreased viability and death of the cells (Lehenkari *et al.,* 2003). Also changes in membrane fluidity and alterations in intracellular calcium fluxes (Custodio *et al.,* 1998; Zhang *et al.,* 2000) have been reported. Tam has also been shown to rapidly inhibit estrogen-dependent PKC in MCF-7 cells, HCC38 cells and chondrocytes (Schwartz *et al.,* 2002; Boyan *et al.,* 2003).

Many cancer chemotherapeutic agents have been shown to exert their anticancer properties by inducing apoptosis through mechanisms that involve mitochondria. Indeed, there are reports demonstrating that mitochondria are also central in the Tam-induced increased rate of apoptosis after long-term treatment (Tuquet *et al.,* 2000; Dietze *et al.,* 2001). High concentrations of Tam have been shown to increase mitochondrial respiration and decrease mitochondrial transmembrane potential and oxidative phosphorylation (Tuquet *et al.,* 2000; Cardoso *et al.,* 2001; Cardoso *et al.,* 2003). More recently Tam at submicromolar concentrations has been shown to induce oxidative stress and mitochondrial apoptosis of breast cancer cells via stimulating mitochondrial nitric oxide synthase (Nazarewicz *et al.,* 2007) and induce release of cytochrome *c* from mitochondria. Tam treatment has also previously been demonstrated to induce ROS generation in Jurkat cells and ovarian cancer cells (Ferlini *et al.,* 1999). A recent report by Moreira *et al*. demonstrates induction of ROS generation by Tam in isolated liver mitochondria (Moreira *et al.,* 2006).

4.2 Raloxifene: a second generation SERM

4.2.1 Current clinical use and results of clinical trials

Currently Raloxifene (Ral) is the only SERM approved worldwide for the prevention and treatment of postmenopausal osteoporosis and vertebral fractures. It was initially developed as therapy for Tam-refractory breast cancer (Bryant and Dere 1998), but it has been found to have a superior target tissue profile and is by far the most widely studied of second generation SERMs. Clinical trials indicate that Ral has ER agonistic effects on bone and serum lipids in healthy, postmenopausal women. Recently

published results of the Study of Tam and Ral (STAR) show that Ral works as well as Tam in reducing breast cancer risk for postmenopausal women at increased risk of the disease (Vogel *et al.,* 2007) and that it is associated with a lower incidence of endometrial cancer, endometrial hyperplasia and total thromboembolic events than Tam. The controversial aspect of the trial appears to be the failure of Ral to control completely the development of noninvasive breast cancer after 2 years of treatment. Furthermore, Ral has not been tested in premenopausal women. Therefore, Tam remains the only proven intervention in premenopausal women. However, for the moment, Ral is proving to be an important advance in chemoprevention because it is a multifunctional medicine that can target women at low risk for breast cancer with osteopenia and healthy women with a high risk of breast cancer.

4.2.2. Molecular mechanisms of Ral action in bone

The molecular mechanism of bone protection by Ral is not currently well known. Ral acts on bone in a similar manner to estrogens, but its osteoblastic actions remain to be fully clarified. Studies in vitro show that Ral modulates the bone homeostasis inhibiting osteoclastogenesis and the bone resorption with dose-dependent activity (Messalli *et al*., 2007). The mechanism has been suggested to imply both direct and indirect activities on bone cells. However, it has been recently suggested that human osteoclast formation and bone resorption is inhibited by Ral mainly indirectly through osteoblastic cells and this is associated to increased production of OPG (Michael *et al.,* 2007). Ral-induced upregulation of OPG expression by osteoblastic cells has been demonstrated also in earlier studies (Viereck *et al.,* 2003). Moreover, recently Ral treatment in postmenopausal women showed a significant increase in OPG levels after 6 months of therapy, providing for the first time also *in vivo* data (Messalli *et al.,* 2007). In addition to the OPG/RANK/RANKL system, the antiresorptive activity of Ral has been suggested to involve also other cytokines, in particular IL-6 (Taranta *et al.,* 2002; Cheung *et al.,* 2003, Michael *et al.,* 2007). Indeed, Ral has been shown to inhibit the production of IL-6 from cultured human osteoblasts.

4.3 Ospemifene: a novel SERM compound

Ospemifene (Osp, previously named FC1271a) is a new selective estrogen receptor modulator (SERM) that is being developed for the treatment of urogenital atrophy and osteoporosis. It is a metabolite of toremifene (Kangas *et al.,* 1990; Sipilä *et al.,* 1990) which specifically binds to both ERs with an affinity very similar to that of toremifene and Tam. In the in vitro analysis, Osp has been found to exert estrogen-like anabolic effects in bone marrow cultures (Qu *et al.,* 1999). This compound presented tissue-specific agonistic and antagonistic effects also *in vivo* (Härkönen *et al.,* 1996). In addition, Osp was found to exert estrogen-like effects in bone marrow cultures by enhancing osteoblastic bone differentiation with a mechanism that differs from that of Ral (Qu *et al.,* 1999). The effects of Osp on osteoblastic differentiation could be inhibited by the pure antiestrogen ICI182780, suggesting an ER-mediated mechanism (Qu *et al.,* 1999). It has also been recently demonstrated that human osteoclast formation and bone resorption is inhibited by Osp mainly indirectly through

osteoblastic cells and this is associated to increased production of OPG (Michael *et al.,* 2007).

Clinically (Voipio *et al.,* 2002; Rutanen *et al.,* 2003), Osp is well tolerated and shows a dose-dependent reduction in bone turnover in postmenopausal women, suggesting that it may be useful for the treatment and prevention of osteoporosis (Komi *et al.,* 2004, Komi *et al.,* 2005). In addition, the bone-restoring activity of Osp has been reported to be similar to that of Ral (Komi *et al.,* 2006). Based on *in vivo* studies, it has also been recently suggested that Osp exhibits efficacy in breast cancer chemoprevention comparable to that of Tam (Namba *et al.,* 2005; Wurz *et al.,* 2005). Furthermore, Osp has neutral or beneficial effects on various vascular surrogate markers (Ylikorkala *et al.,* 2003). It does not cause clinically significant changes in endometrium, and it has a clear estrogenic effect in vaginal epithelium (Voipio *et al.,* 2002, Rutanen *et al.,* 2003, Komi *et al.,* 2005).

4.4 Molecular basis for the distinct biocharacteristics of SERMs

It is currently believed that SERMs exert at least their genomic effects mainly via the ERs. The mechanisms by which a single compound can demonstrate estrogen agonistic effects in some target tissues but antagonistic effects in others have not been fully understood. However, several of the possible alternatives have been suggested. First, it has been hypothesized that individual SERMs may induce specific and unique changes in receptor conformations, which accounts for their particular pharmacological properties in target tissues (McDonnell *et al.,* 1995; Brzozowski *et al.,* 1997; Pike *et al.,* 1999). Secondly, the ratio of ERα to ERβ and the differential affinity of SERMs for each of these receptors could also explain some of aspects of SERM selectivity (Kuiper *et al.,* 1997; Kuiper *et al.,* 1998; Gustafsson 1999; Sun *et al.,* 1999). Thirdly, the relative concentration of coactivators or corepressors and their selective interaction with ERα is another possibility to explain the target site specificity for SERM action (Voegel *et al.,* 1996; Hall *et al.,* 2001; Schiff *et al.,* 2003).

Fourthly, the actions of SERMs may depend on target gene promoter, since in addition to classical ERE, SERMs can interact with genes possessing the AP-1 element (Paech *et al.,* 1997) or Sp1 binding sites. Finally, SERMs are also likely to be able to enhance the signaling potential of ER through intracellular signaling pathways that are induced by extracellular factors (e.g. growth factors) (Smith 1998). SERMs may facilitate ERs interactions with cell surface receptors or induce ER-independent signaling pathways. Also nongenomic ER-independent signaling events have been described at least for Tam (Ferlini *et al*., 1999; Kim *et al.,* 1999; Zhang *et al.,* 2000)

5. CELL DEATH PATHWAYS

Balance between cell division and cell death is of utmost importance for the development and maintenance of multicellular organisms. Disorders of either process have pathologic consequences and can lead to disturbed embryogenesis, neurodegenerative diseases, or the development of cancer. Therefore, cell death is an

essential strategy for the control of the dynamic balance in living systems, and two fundamentally different forms of cell death, apoptosis and necrosis, have been defined.

5.1 Necrosis and apoptosis

Necrosis is an accidental passive process resulting in an early disruption of the cell membrane and in the progressive breakdown of ordered cell structures in response to violent environmental perturbations such as severe hypoxia/ischaemia, extremes of temperature and mechanical trauma. This type of cell death is associated with organelle swelling.

In contrast to necrosis, apoptosis or programmed cell death (PCD) involves the activation of energy-requiring intracellular machinery, which is tightly regulated and conserved throughout evolution (Yuan 1996). Apoptosis affects single cells asynchronously, typically in the absence of inflammatory changes (Wyllie *et al.,* 1980). It is involved in morphogenesis of embryonic tissues as well as in homeostasis of adult organs and tissues. Apoptosis for example eliminates cells exposing the organism to danger. Programmed cell death is a process with typical morphological characteristics including plasma membrane blebbing, cell shrinkage, chromatin condensation and fragmentation (Kerr *et al.,* 1972). A family of cystein-dependent aspartate-directed proteases, called caspases, is responsible for the proteolytic cleavage of cellular proteins leading to the characteristic apoptotic features, e.g. cleavage of caspase-activated DNAse resulting in internucleosomal DNA fragmentation. Currently, two pathways for activating caspases have been studied in detail. One (extrinsic pathway) starts with ligation of a death ligand to its transmembrane death receptor, followed by recruitment and activation of caspases in the death-inducing signalling complex (Ashkenazi and Dixit 1998). The second pathway (intrinsic pathway) involves the participation of mitochondria, which release caspase-activating proteins into the cytosol, thereby forming the apoptosome where caspases will bind and become activated (Green and Reed 1998). In addition, several other apoptotic pathways have been suggested: for example endoplasmic reticulum stress-induced apoptosis and caspase-independent apoptosis (Vermeulen *et al.,* 2005).

5.2 Death receptor dependent apoptotic pathway

Plasma membrane receptors triggering external apoptosis signalling belong to the tumour necrosis factor (TNF)-receptor superfamily. This family includes Fas (Apo-1 or CD95), TNF-receptor-1 (TNF-R1), death receptor-3 [DR3 or TNF-receptor-related apoptosis-mediating protein (TRAMP) or Apo-3], TNF-related apoptosis inducing ligand receptor-1 (TRAIL-R1 or DR4), TRAIL-R2 (DR5 or Apo-2) and DR6. Upon ligand binding, death receptors cluster and and form death-inducing signaling complexes (DISCs) consisting of adaptor proteins and several procaspase-8 molecules. Two linear subunits of procaspase-8 compact to each other followed by autocleavage of procaspase-8 to caspase-8 which in turn will activate downstream effector caspases (Thornberry and Lazebnik 1998). In addition to death receptors, decoy receptors (DcR1, DcR2, DcR3, osteoprotegerin) have been identified. These receptors compete

with the death receptors for ligand binding, but they do not transduce apoptotic signals (Sheikh and Fornance 2000).

5.3 Mitochondrial apoptotic pathway

Mitochondria play a central role in both cell life and death (Duchen 2004). They are essential for the production of ATP through oxidative phosphorylation and regulation intracellular Ca^{2+} homeostasis. In addition, mitochondria are the principal generators of intracellular reactive oxygen species (ROS). Furthermore, mitochondria also play a key role in controlling pathways that lead to apoptosis. Defects of mitochondrial function can result in excessive production of ROS, formation of the permeability transition pore (PTP) and the release of small proteins that trigger apoptosis (Moreira *et al.,* 2007). Therefore, changes in the structural and functional characteristics of mitochondria provide a number of primary targets for drug-induced toxicity and cell death (Wallace and Starkov 2000). In addition to mitochondia, organelles involved in programmed cell death include lysosomes and endoplastic reticulum. However, the majority of cytotoxic agents have been shown to trigger the mitochondrial pathway (Friesen *et al.,* 1999; Green 2000).

5.3.1. Bcl-2 family proteins

The mitochondrial apoptotic pathway is initiated and regulated by the key proteins of Bcl-2 family. Based on structure and their roles in apoptosis, the Bcl-2 family of proteins is divided into three groups: (1) the antiapoptotic proteins containing four BH homology domains (Bcl-2, Bcl-xL, Bcl-w, Mcl-1, A1/Bfl-1, NR-13, Boo/Diva/Bcl-2-L-10, Bcl-B and adenoviral E1B); (2) the proapoptotic proteins containing three BH homology domains (BH3; Bax, Bak, Bok/Mtd, Bcl-Xs); and (3) the single BH homology domain containing sensors of apoptosis that function via activation of proapoptotic proteins or inhibition of antiapoptotic proteins. These BH3-only members include Bid, Bad, Noxa, puma, Bmf, BimL,/Bod, Bik/Nbk, Blk, Hrk/DP5, Bnip3 and Bnip3 L (Gavrilova-Jordan and Price 2007). Most of these proteins contain a C-terminal hydrophobic α-helix, a potential transmembrane domain involved in their localization to the membranes of the mitochondria, the endoplasmic reticulum and the nucleus (Er *et al.,* 2006). The different family members can homo- or hetero-dimerize, and the relative ratios of anti- and pro-apoptotic proteins will determine the susceptibility of cells to apoptotic stimuli (Vermeulen *et al.,* 2005).

5.3.2 Mitochondrial outer membrane permeabilization

Mitochondrial outer membrane permeabilization (MOMP) is a cellular event that integrates upstream apoptotic signals such as activation of Bcl-2 family members into release of pro-apoptotic proteins from mitochondria to the cytosol and is often considered as point of no return in apoptotic programs. The mechanisms responsible for MOMP during apoptosis remain controversial. In general, two classes of mechanisms have been described and each may function under different circumstances; those in which the inner mitochondrial membrane participates, and those involving only the outer membrane (Green and Kroemer 2004). The first class of mechanism for

MOMP, which involves only the outer membrane, appears to be mediated by members of Bcl-2 family proteins acting directly on the outer mitochondrial membrane (OMM). Antiapoptotic Bcl-2 family members function to block MOMP whereas proapoptotic members promote it. In the second class of mechanism, a permeability transition pore (PTP) opens in the inner membrane, allowing water and molecules up to 1,5 kD to pass through. Most models of this pore postulate a role for adenine nucleotide transporter (ANT) and the voltage-dependent anion channel (VDAC) in the outer membrane. Opening of PTP leads to rapid loss of mitochondrial membrane potential (Martzo *et al.,* 1998; Mignotte and Vayssiere 1998; Lemasters *et al.,* 1999), matrix swelling and rupture of the OMM. Irrespective of its mechanisms, MOMP leads to release of proapoptotic substances such as cytochrome *c*, smac/DIABLO, Omi/HtrA2 and endonuclease G into the cytosol (Kim *et al.,* 2005; Skommer *et al.,* 2007).

5.3.3. Cytochrome *c* and AIF release

One of firstly found proteins to be released from the mitochondria to the cytosol upon apoptotic stimuli is cytochrome *c*, an essential component of the respiratory chain (Goldstein *et al.,* 2000). Once released into the cytosol, cytochrome *c* activates apoptotic protease-activating factor 1 (Apaf-1) which, together with procaspase-9, forms an active holoenzyme complex termed the apoptosome (Li *et al.,* 1997). Apoptosome-associated caspase-9 can then activate effector caspases. Effective induction of apoptosis requires that cytosolic inhibitor-of-apoptosis-proteins (IAPs) are removed from the complex by Smac/Diablo or Omi/htra2 (Ferri and Groemer 2001; Suzuki *et al*., 2001).

Apoptosis inducing factor (AIF) is a mitochondrial protein that plays a pivotal role in PCD (Susin *et al*., 1996). It is normally retained in the intermembrane mitochondrial space, where it performs an oxidoreductase function (Miramar *et al.,* 2001). Similar to the bifunctional role of cytochrome *c*, AIF becomes an active cell killer when it is released to the cytosol; it then translocates to the nucleus and triggers, possibly together with endonuclease G (Wang *et al.,* 2002), peripheral chromatin condensation and high molecular weight DNA loss (Loeffler *et al.,* 2001; Susin *et al.,* 1999; Yu *et al.,* 2002).

5.3.4. Caspases

Apoptotic signalling mainly converges in the activation of intracellular caspases, a family of cysteine-dependent aspartate-directed proteases which propagate death signalling by cleaving key cellular proteins (Nicholson and Thornberry 1997). Caspases are synthesized in normal cells as inactive proenzymes; they can rapidly be activated by autoproteolytic cleavage or cleavage by other caspases at specific aspartic acid (Asp) residues (Thornberry and Lazebnik 1998). Currently, 14 members of the caspase family have been identified, of which 7 mediate apoptosis. During apoptosis, caspases with a long pro-domain function as upstream signal transducers (”initiator” caspases) and proteolytically activate downstream caspases (”effector” caspases) which contain a short pro-domain (Thornberry *et al*., 1997). In the classic apoptotic pathway formation of apoptosome leads to ATP-dependent cleavage and activation of procaspase-9, the initiator caspase in mitochondrial apoptosis (Li *et al.,* 1997)

Activation of procaspase-9 results in activation of downstream executioner caspases, such as caspase-3, caspase-6 and caspase-7 (Leist and Jäättelä 2001; Strasser *et al.,* 2000).

Caspases specifically recognize and cleave a tetrapeptide sequence on their substrate with an absolute requirement for an aspartate residue. Effector caspases act on a variety of substrates resulting in proteolysis of cellular proteins and death by apoptosis. One well-known caspase substrate is poly-(ADP-ribose) polymerase (PARP), a nuclear protein implicated in DNA repair. PARP is one of the earliest proteins targeted for specific caspase cleavage (Kaufmann *et al.,* 1993; Duriez and Shah 1997; Kaufmann *et al.,* 1993; Los *et al.,* 1999). Caspase cleavage and inactivation of ICAD (inhibitor of caspase-activated DNAse) allow caspase-activated DNAse to translocate to the nucleus where it is responsible for internucleosomal DNA cleavage, generating oligonucleosomal DNA fragments (Sakahira *et al*., 1998; Liu *et al*., 1997). Caspase cleavage of lamins results in nuclear shrinkage; cleavage of cytoskeletal proteins like fodrin and actin leads to cytoskeletal reorganization and membrane blebbing (Orth *et al*., 1996; Mashima *et al*., 1995; Martin *et al*., 1995).

A molecular link between the death receptor and the mitochondrial apoptosis pathways can be found at the level of caspase-8 cleavage of cytosolic Bid, a member of the BH3 domain-only subgroup of Bcl-2 family (Li *et al.,* 1992). Cleaved Bid translocates from the cytosol to mitochondria and activates the mitochondrial-dependent apoptotic pathway (Desagher *et al.,* 1999) Caspase-3 can also cleave Bid, thereby inducing cytochrome *c* release and apoptosis (Luo *et al.,* 1998).

5.3.5. Reactive oxygen species

In addition to regulation of apoptosis, one of the best known functions of mitochondria is energy production. Concomitant with the mitochondrial production of adenosine triphosphaste (ATP) is the stepwise reduction of O_2 to H_2O, with several intermediate reactive oxygen species (ROS). Impairment of oxidative phosphorylation and excessive generation of ROS leads to damage of cell proteins, lipids and DNA (Garilova-Jordan and Price 2007). Indeed, ROS are important to apoptosis induced by radiation, chemotherapeutic agents, and many cell stressors (Benhar *et al*., 2002). Release of cytochrome *c* from mitochondria may lead to formation of ROS and cell death even if caspase function is inhibited.

5.4 Caspase-independent cell death

Some forms of cell death cannot be easily classified as apoptosis or necrosis, e.g. when cell death occurs in the presence of caspase inhibitor without DNA fragmentation, DNA condensation or caspase activation. Indeed, there is accumulating evidence indicating that cell death can occur in a programmed fashion but in complete absence of caspases. Alternative models of programmed cell death have therefore been presented.

Because it has become clear that inhibition of caspase activation does not necessarily protect against cell death stimuli but rather can reveal or even enhance underlying

caspase-independent death programs, several models have been proposed. One of them involves autophagy, which is characterized by sequestration of cytoplasm or organelles in autophagic vesicles and their subsequent degradation by the cell's own lysosomal system (Schweichel and Merker 1973, Kroemer and Jäättelä 2005). Paraptosis, on the other hand, involves cytoplasmic vacuolation and mitochondrial swelling in the absence of caspase activation or typical nuclear changes (Speradio *et al.,* 2000), whereas mitotic catastrophe occurs as a default pathway after mitotic failure and development of aneuploid cells (King and Cidlowski 1995). Slow cell death describes the delayed type of PCD that occurs if caspases are inhibited or absent. In contrast to these more specific definitions of PCD above, also a model has been proposed that classifies cell death into four subclasses, according to their nuclear morphology. Apoptosis is defined by stage II chromatin condensation into compact figures, which are often globular or crescent shaped. Slightly different is apoptosis-like PCD, which is characterized by less-compact chromatin condensation, so-called stage I chromatin condensation. In contrast, in necrosis-like PCD no chromatin condensation is observed, but at best, chromatin clustering to loose speckles, whereas necrosis is characterized by cytoplasmatic swelling and cell membrane rupture (Leist and Jäättelä 2001). In addition, attempts have been made to order caspase-independent cell death according to the cellular organelles involved (Ferri and Kroemer 2001). Organelles such as the mitochondria, lysosomes, or ER and plasma membrane death receptors can be involved in either of the subclasses but may play a more prominent role in certain types of PCD. The signals from the different cellular organelles are linked and may act both upstream and downstream of each other.

Although several models of caspase-independent cell death have been described, the various death routes may overlap and several characteristics may be displayed at the same time. Upon a lethal stimulus, a cell is likely to have access to different death programs that can be executed via caspases or independent of caspases. In addition, more than one death program may be activated at the same time (Unal-Cevik *et al.,* 2004). Furthermore, a cell may switch back and forth between different death pathways (Chi *et al.,* 1999). It has, therefore, been postulated that the dominant cell death phenotype is determined by the relative speed of the available death programs; although characteristics of several death pathways can be displayed, only the fastest and most effective death pathway is usually evident (Burch 2001). Taken together, the cellular death response triggered by cytotoxic agents depends on the type and dose of chemotherapeutic stress within the cellular context and may involve classic apoptosis, as well as various types of apoptotic or necrotic PCD.

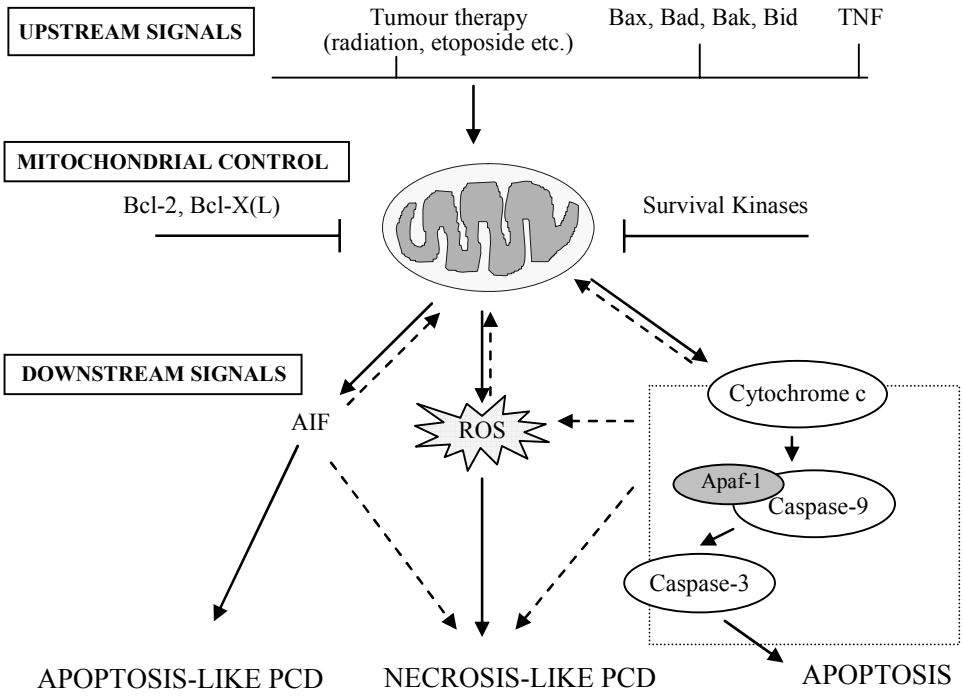

Figure 3. One proposed model for classification of mitochondrial cell death pathways

6. MAPK/ERK SIGNALLING

Mitogen-activated protein (MAP) kinases are serine/threonine-specific protein kinases that respond to extracellular stimuli (mitogens) and regulate various cellular activities, such as gene expression, mitosis, differentiation, and cell survival/apoptosis. Extracellular stimuli lead to activation of a MAP kinase via a signaling cascade ("MAPK cascade") composed of MAP kinase, MAP kinase kinase (MKK or MAP2K), and MAP kinase kinase kinase (MKKK or MAP3K). MAP3K that is activated by extracellular stimuli phosphorylates a MAP2K on its serine and threonine residues, and then this MAP2K activates a MAP kinase through phosphorylation on its serine and tyrosine residues. This MAP kinase signaling cascade has been evolutionarily well-conserved from yeast to mammals.

To date, four distinct groups of MAPKs have been characterized in mammals:

1. Extracellular signal-regulated kinases (ERKs). The ERKs (also known as classical MAP kinases) signaling pathway is preferentially activated in response to growth factors, such as TGF-β and EGF, and regulates cell proliferation and cell differentiation.

2. C-Jun N-terminal kinases (JNKs), also known as stress-activated protein kinases (SAPKs).

3. p38 isoforms. Both JNK and p38 signaling pathways are responsive to stress stimuli, such as cytokines, ultraviolet irradiation, heat shock, and osmotic shock, and are involved in cell differentiation and apoptosis

4. ERK5, which has been found recently, is activated both by growth factors and by stress stimuli, and it participates in cell proliferation.

MAPK pathway is considered to be one of the key pathways regulating breast cancer growth and apoptosis. For example, tumors of breast tissue frequently overexpress epidermal growth factor receptor (EGFR) and related family members. EGFR acts as a central point linking all upstream ligand signals on MAPK activation. Consistently, recent studies have found that breast cancers frequently contain an increased proportion of cells with activated form of MAP kinase. Of the four MAPK pathways described, the one involving ERK-1 and -2 is most relevant to breast cancer (Santen *et al.,* 2002). Estradiol dependent breast tumor cells have been suggested to utilize MAP kinase pathways in at least one of three ways. First, MAPK can phosphorylate the ER and enhance its transcriptional efficiency. Secondly, estradiol may stimulate growth factors which increase the level of MAPK and thirdly, estradiol may activate pathways which utilize a membrane associated ER to activate MAPK through nongenomic effects. Indeed, the rapid ERK phosphorylation by E_2 has been demonstrated in several reports (Filardo *et al*., 2002; Fernando and Wimalasena 2002; Song *et al*., 2002; Thomas *et al*., 2006).

Even though activation of ERK1/2 is generally considered to lead to cell survival (Marshall 1995; Zhang and Liu 2002) there is evidence that in several cell types ERKs may also transmit pro-apoptotic signals (Stanciu and DeFranco 2002; Tang *et al.,* 2002; Reccia *et al.,* 2004). Phosphorylation of ERK leads to translocation of activated ERK to nucleus. Nuclear translocation of MAPK is transient, although the duration time in the nucleus varies depending on the cell types and the stimuli used (Marshall 1995; Adachi *et al.,* 2000). Interestingly, a report by Chen *et al.,* suggests that the anti-apoptotic effect of E_2 in osteoblasts may be converted into a pro-apoptotic one by alteration of the temporal pattern the ERK activation (Chen *et al.,* 2005). Thus, the ability of ERK-1 and -2 to be involved in both proliferation and apoptosis may be explained by activation of a distinct set of transcription factors for genes which mediate these processes.

It is possible that the localization and duration of kinase signaling similarly contribute to different actions of Tam in breast cancer cells. Indeed, Tam has been shown to activate ERK in HeLa cells (Duh *et al.,* 1997) and human endometrial cancer cells (Acconcia and Kumar 2006). In addition, recent report from Visram and Greer suggests that both E_2 and Tam stimulate rapid and transient ERK activation in MCF-7 cells via distinct signaling mechanisms (Visram and Greer 2006).

AIMS OF THE STUDY

This work was undertaken to study the pathways and mechanisms of tamoxifen-induced death in breast cancer cells and the ability of estrogen and some selective estrogen receptor modulators (SERMs) to protect against cell death.

The specific aims of the present study were as follows:

1. To compare the ability of tamoxifen (Tam) and different SERM compounds to induce cell death in breast cancer cells and to study the molecular mechanisms of these actions.

2. To determine the upstream events leading to the previously observed mitochondrial disruption in breast cancer cells by Tam, and to study the effect of E_2 on Tam–induced rapid death of breast cancer cells.

3. To compare the ability of E_2 and different SERMs to promote survival of cells against apoptosis, to compare the roles of the two estrogen receptor isotypes in these actions and, finally, to study if the cells containing either ERalpha or ERbeta differentially respond to E_2 at the level of cytokine expression.

MATERIALS AND METHODS

1. REAGENTS (I-III)

RPMI-1640, McCoy´s 5A, DMEM/F-12, L-glutamine, insulin, diphenylene ionodium (DPI), bongkrekic acid (BA), E2, Tam, and trypan blue solution were purchased from Sigma (St. Louis, MO) and FBS was purchased from Life Technologies, Inc. (Paisley, Scotland, U.K.). Tor, Ral and Osp from Hormos Medical Oy (Turku, Finland), z-Val-Ala-Asp-fluoromethylketone (Z-VAD-FMK) from Enzyme System Products (Aurora, Ohio) and ICI 182780 from Tocris (Ellisville, MO). AG1478 and BIBX1382 were from CalBioChem (La Jolla, CA), PD98059, tetramethylrhodamine methyl ester (TMRM), MitoTrackerRed CMXRos fixable mitochondrial dye and dichlorodihydrofluorescein diacetate (DFH-DA) were from Molecular Probes (Eugene, Oregon, USA). All drugs were dissolved in DMSO (Sigma, St. Louis, MO). Vectashield mounting medium with DAPI was obtained from Vector Laboratories Inc. (Burlingame, CA) and FuGENE 6 Transfection Reagent from Roche Diagnostics Corporation, (Indianapolis). Western blotting nitrocellulose membranes were from Millipore (Billerica, MA) and culture dishes with glass bottoms from MatTek, (Ashland, MA). Antibody for cytochrome c was purchased from BD Pharmingen (San Diego, CA), for PARP from Jackson Immunochemicals (West Grove, PA), for caspase-9 from Santa Cruz Biotechnology (Santa Cruz, CA), for Bcl-2 and Bcl-XL from BD Pharmingen, for ERα, ERK1/2 and p-ERK1/2 from Cell Signaling (Beverly, MA) and for β-actin from Sigma. Enhanced chemiluminescence detection system was purchased from Amersham Pharmacia Biotech (Uppsala, Sweden) and colored size markers for western blotting from BioRad (Hercules, CA). Micro-BCA Protein Assay Kit was from Pierce (KMF, Germany), Trizol reagent from Invitrogen (Carlsbad, CA), AMV reverse transcriptase enzyme from Finnzymes (Espoo, Finland) and SYBR Green PCR Master Mix from Applied Biosystems (Foster City, CA). The ELISA kits for detection of OPG and IL-6 were purchased from Biomedica (Wien, Austria) and Sanquin (Amsterdam, The Netherlands), respectively.

2. CELL CULTURE (I-III)

2.1 Breast cancer cells lines (I,II)

The estrogen-sensitive MCF-7 and T47D human breast tumor cell lines were originally obtained from the laboratory of Dr. C. K. Osborne (University of Texas Health Science Center, San Antonio, Texas). The cells were maintained in RPMI 1640 culture medium supplemented (10%) with heat-inactivated fetal bovine serum (iFBS), 2 mM L-glutamine, insulin (4 μg/ml) and 1 nM 17β-estradiol (E2). For estrogen-deprivation and Tam pre-culture studies cells were grown in phenol red-free RPMI-1640 culture medium supplemented (5%) with dextran charcoal-treated fetal bovine serum (dcFBS) and 2 mM L-glutamine. The MDA-MB-231 cells were a gift from Dr. T. Guise (University of Texas Health Science Center, San Antonio, Texas). The cells were maintained in DMSO culture medium supplemented (10%) with iFBS and 2 mM L-glutamine.

2.2 Osteoblast-like cell lines (III)

Parental U2OS cells were maintained in McCoy´s 5A culture medium supplemented (10%) with heat-inactivated fetal bovine serum (iFBS), 2 mM L-glutamine, 50 U/ml penicillin and 50 µg/ml streptomycin. For ERα-, ERβ- and vector transfected U2OS cells medium was supplemented with G-418 (0,25 mg/ml). SaOS-2 cells were cultured in McCoy´s 5A culture medium supplemented (15%) with heat-inactivated fetal bovine serum (iFBS), 2 mM L-glutamine, 50 U/ml penicillin and 50 µg/ml streptomycin. For experiments cells were grown overnight in phenol-free DMEM/F-12 media containing 0,1% BSA supplemented with E_2, ER ligands or SERMs at the concentrations indicated in the results. Treatment times were one hour for TNFα and six hours for etoposide.

2.3 Production of U2OS/ERα and U2OS/ERβ cell lines (III)

The U2OS (human osteosarcoma) cells were stably transfected with human ERα or ERβ. The following constructs were transfected to U2OS cells together with pcDNA3.1 expression vector which contains a neomycin resistance gene:

1) pSG5-hERα:

Human estrogen receptor α coding sequence: 1,8 kb in pSG5-vector (=HEO), cloned at EcoR1 site. In HEO the ERα sequence contains artificial Gly400Val mutation (GGG to GTG), which destabilizes it in the absence of ligand. Thus, it gives a lower backgroung and clearer ligand-induced transactivation data than that with wild-type ERα (=HEGO) (Bruder *et al.,* 1997).

2) pSG5-hERβ

Human estrogen receptor β coding sequence: 1,6 kb in pSG5-vector, cloned at BAMH1 site encoding a full length ERβ (Fuqua *et al.,* 1999). ERβ cDNA was PCR-cloned from human ovary mRNA.

Transfection reagent used was Fugene™ 6 and clones were selected with the antibiotic G418 (0,5 mg/ml).

Expression and functionality of the ER constructs on U2OS cells was confirmed by RT-PCR, western blotting and immunofluorescence microscopy.

2.3.1. RT-PCR

For RT-PCR analysis the RNAs were isolated from MCF-7, parental U2OS and ERα or ERβ transfected U2OS cells by using the Trizol reagent (Invitrogen) and purified according to manufacturer´s instructions. cDNA was synthetized from 2 µg of total RNA in a 20 µl reaction containing random hexanucleotides and AMV reverse transcriptase enzyme (Finnzymes, Espoo, Finland). RT-PCR was performed using the Eppendorf Mastercycler. Amplification reactions for ERα were performed for 35 cycles. Successful cDNA synthesis was verified by PCR for GAPDH. The following primers were used:

ERα (forward)	5` ACA AGC GCC AGA GAG ATG AT --3`
ERα (reverse)	5`- AGG ATC TCT AGC CAG GCA CA -3`
ERβ (forward)	5`- TGA AAA GGA AGG TTA GTG GGA ACC -3`
ERβ (reverse)	5`- TGG TCA GGG ACA TCA TCA TGG -3`
GAPDH (forward)	5`-AGC CAC ATC GCT CAG ACA C-3`
GAPDH (reverse)	5`-GCC CAA TAC GAC CAA ATC C-3`

2.3.2. Western blotting

For analysis of expression of ERα and ERβ by Western blotting, aliquots of whole cell lysate protein (50 μg) were separated by SDS-PAGE and transferred to nitrocellulose membranes (Millipore, Billerica, MA). The membranes were blocked with 8% skimmed milk in TBS/0.05% Tween-20 and incubated with the primary antibodies for of ERα and ERβ (Santa Cruz Biotechnology). The proteins were visualized using an enhanced chemiluminescence detection system (Amersham Pharmacia Biotech, Uppsala, Sweden) with colored markers (BioRad, Hercules, CA) as size standards.

2.3.3. Immunofluorescence microscopy

Immunofluorescence microscopy was used for localization of transfected ERs in U2OS/ERα and U2OS/ERβ cells. The cells were incubated for 2 hours with ERα or ERβ specific monoclonal antibodies (Santa Cruz Biotechnology) after which the media were replaced with media containing Alexa Fluor 488-conjugated secondary antibody (Molecular Probes) and the cells were further incubated 45 minutes. The cells were then incubated with DAPI nuclear dye for 10 minutes. Microscopic imaging was carried out on a Zeiss Wide-Field microscope (Zeiss, Jena, Germany).

2.3.4. Reporter gene assays

Luciferase reporter gene assays were used to characterize the functionality of transfected ERs. The U2OS/ERα and U2OS/ERβ cells were transfected with only a luciferase reporter plasmid under the control of two ER response elements or a human ERα or ERβ plasmid and the same luciferase reporter plasmid by using Fugene transfection reagent (Roche, Madison, WI). Twenty-four hours after transfection the cells were treated for 24 hours with 10 nM E_2 and the luciferace activity was measured using a dual-luciferase reporter assay according to manufacturer´s instructions (Promega, Madison, WI). For quantitation, in the absence of hormone, the luciferase activity was set to 1.

3. CELL DEATH DETERMINATION (I-III)

3.1 Trypan blue analysis (I, II)

Cells were cultured overnight in 3.5-cm diameter tissue culture plates at a density of 1-1.2 × 10^5 cells/plate. Culture media were replaced with phenol-red-free media containing either 5-40 μM BA, 10 nM E2, 50 μM Z-VAD-FMK, 10 μM DPI, 1 μM ICI 182780 and/or antiestrogens at the concentrations indicated in the results.

Alternatively, cells were incubated for 48 hours in phenol-red-free media containing dcFBS (5%) and 2 mM L-glutamine and then treated with 20 μM PD98059, 1 μM ICI182780, 10 μM AG1478, 10 μM BIBX1382, 10 nM E2 and/or various concentrations of Tam. Pre-treatment times were either a) 3 hours for Z-VAD-FMK (50 μM), b) one hour for DPI (10 μM) and BA (5-40 μM) or c) 30 min or 4 hours for ICI 182780 (1 μM) d) 1 hour for PD98059 (20 μM), AG1478 (10 μM) and BIBX1382 (10 μM).

Control plates had equivalent volumes of DMSO solvent. After treatment for 10–60 min, the cells were washed with PBS and cell death analysis was performed by way of trypan blue exclusion. Four to six separate areas of approximately 400 cells were calculated for each plate. The percentage of cells taking up blue dye determined relative cell viability.

3.2 Nuclear staining (III)

Apoptotic cells were quantified by visualization of changes in nuclear morphology by DAPI staining. Cells (1×10^5) were grown in 3.5-cm diameter Petri dishes with coverslips for overnight. Culture media were replaced with DMEM/F12 phenol-red-free media containing 0,1% BSA and various concentrations of either E2, PPT, DPN or SERMs. Non-treated cells served as controls and these cultures had equivalent volumes of DMSO solvent. After 24 hours etoposide was added to half of the plates for 6 hours. After treatment, the cells were fixed with 3% paraformaldehyde and the coverslips were mounted on the slides with Vectashield mounting medium with DAPI. Microscopic imaging was carried out on a Zeiss Wide-Field microscope (Zeiss, Jena, Germany). Fifteen areas of 1300 × 1000 pixels in one sample were randomly selected from the image for quantification of apoptotic cells. Data of three independent experiments are presented as the percentage of dead cells (means ± SE) for each treatment.

4. MICROSCOPIC IMAGING (I-III)

4.1 Confocal Microscopic Imaging of Cytochrome c Release (I)

MCF-7 cells (1×10^5) were grown in 3.5-cm diameter Petri dishes with coverslips for two days. Culture media were replaced with phenol-red-free media containing 2 mM L-glutamine, 400 nM MitoTrackerRed CMXRos fixable mitochondrial dye and drugs at the concentrations indicated in the results. Non-treated cells served as controls and these cultures had equivalent volumes of DMSO solvent. After treatment, the cells were fixed with 3% paraformaldehyde + 0.5% Triton X-100 for 15 min at RT. The specimens were blocked with normal goat serum (NGS) in PBS for 30 minutes. The cells were incubated with primary antibody (mouse anti-cytochrome c) in PBS-0.01% Triton X-100 with 1.5% NGS at room temperature for 2 hours, then washed and incubated with secondary antibody (goat anti-mouse-Alexa 488), after which coverslips were mounted on the slides. Confocal imaging was carried out on a Zeiss LSM 510 confocal microscope equipped with an argon-ion laser and helium-neon lasers (Zeiss, Jena, Germany). For excitation of Alexa 488 the 488 nm line and for MitoTrackerRed CMXRos the 543 nm line was used and a ×63 oil objective lens

(numerical aperture 1.4) was employed. The beam path for Alexa 488 contained a 488 nm main dichroic mirror and a 500–530IR nm bandpass filter and the beam path for MitoTrackerRed CMXRos contained a 543 nm main dichroic mirror and a 560 nm long pass filter for detection of emitted fluorescence.

4.2 Confocal Microscopic Imaging of the Mitochondrial Membrane Potential (I)

MCF-7 cells (1×10^5) were grown in 3.5-cm diameter tissue culture dishes with glass bottoms for two days, after which they were incubated with 50 nM tetramethylrhodamine methyl ester (TMRM) in culture medium for 60 min at 37 °C and then rinsed with serum-free medium (phenol-red-free, NaHCO3-free RPMI-1640 supplemented with 2 mM L-glutamine and 25 mM HEPES buffer). After this, the cells were treated with serum-free medium supplemented with 50 nM TMRM and either 0.2% DMSO, 10 nM E2, or 7.0 μM Tam. Living cells were then placed on the microscope stage (with a heater to maintain the temperature at 37 °C) and cells of one area were monitored with a Leica DM IRB confocal microscope (Leica, Wezlar, Germany) and a PL Apo 40.0 × 1 objective. Confocal images were collected at the time points indicated using a 568 nm excitation light from an argon/krypton laser, a 560 nm beam splitter and a 500–550 nM long pass filter.

4.3 Determination of ROS Levels by Fluorescence Microscopy (I)

The method to detect intracellular ROS levels has been described previously (Zhang *et al*., 2002). In brief, dichlorodihydrofluorescein diacetate (DFH-DA) is an uncharged nonfluorescent cell-permeable compound. Once inside the cells, the diacetate bond is cleaved by nonspecific esterases to form the polar and nonfluorescent DFH. Upon oxidation by ROS, this compound gives rise to DF, which yields green fluorescence. MCF-7 cells (1 × 105) were seeded in 3.5-cm diameter tissue culture dishes with glass bottoms and grown for two days, after which the culture media were replaced with phenol-red-free media containing 5 μM DFH-DA, 2 mM L-glutamine and drugs at the concentrations indicated in the Results for 20 min at 37 °C. Non-treated cells served as controls and these cultures received equivalent volumes of the DMSO solvent. The cells were viewed under a fluorescence microscope (Olympus IX 70) using excitation and emission wavelengths of 488 nm and 530 nm. Images of 1300 × 1000 pixels were collected and identical parameters, such as contrast and brightness, were used for all samples. Five areas in one sample were randomly selected from the image and the fluorescence intensity was recorded. The experiments were repeated at least three times, with similar results.

4.4 Transient transfection and fluorescence and confocal microscopic imaging of the subcellular localization of ERK2 (II)

To investigate the subcellular localization of transiently expressed ERK2-GFP fusion protein, 5×10^4 MCF-7 cells were grown in 3.5-cm diameter Petri dishes with coverslips in MCF-7 maintenance culture medium (RPMI-1640 supplemented with iFBS [10%], 2 mM L-glutamine, 10 nM E2, and insulin at 4 μg/ml). After two days, plasmids carrying GFP-ERK2 (a kind gift from Dr. Rony Seger, Department of Biological Regulation, The Weizmann Institute of Sciences, Rehovot, Israel) and nRFP

(red fluorescent protein targeted to the nucleus, a kind gift from Dr. Stavros C. Manolagas, Division of Endocrinology and Metabolism, University of Arkansas for Medical Sciences, Little Rock, Arkansas, USA) were introduced into the MCF-7 cells using FuGENE 6 Transfection Reagent, following the manufacturer's instructions. The following day, the culture media were replaced with phenol-red-free media containing dcFBS (5%) and 2 mM L-glutamine. Transient expression of GFP-ERK2 protein was assayed 48–72 h after gene transfection by fluorescence microscopy. The cells were then treated with serum-free medium supplemented with drugs at the concentrations indicated in the results for 5, 10, 20 or 30 minutes. After treatment, the cells were fixed with 4% paraformaldehyde. Fluorescence imaging was carried out using a Zeiss Axiovert microscope with a 63× oil-immersion objective and an appropriate filter set. The percentage of cells showing nuclear accumulation of GFP-ERK2 was quantified by enumerating cells exhibiting increased GFP in the nucleus compared with the cytoplasm. Fluorescence of nRFP was used to visualize the nuclei. At least 20 fields (images of 1300 × 1000 pixels) selected by random sampling were examined for each experimental condition.

5. WESTERN BLOTTING EXPERIMENTS (I, II)

Cells (3×10^4 or 1×10^5) were plated in 10-cm Petri dishes and allowed to attach for 24 h before treatment after which the cells were treated for the various times with drugs indicated in the results. Non-treated or vehicle (DMSO) treated cells served as controls. Alternatively, the culture media were replaced for 48 hours with phenol-red-free media containing dcFBS (5%) and 2 mM L-glutamine before treatments. The cells were lysed in standard Laemmli sample buffer and lysates were sonicated for 10 sec and boiled for 5 min in a water bath at 100 °C with β-mercaptoethanol. Aliquots (30 µl) of whole cell lysate protein were separated by SDS-PAGE and transferred to nitrocellulose membranes. The membranes were blocked with 8% skim milk in TBS/0.05% Tween-20 and incubated with the primary antibodies for ERα, ERK1/2 and p-ERK1/2, PARP, caspase-9, Bcl-2 and Bcl-XL or β-actin, and appropriate secondary antibodies. The proteins were visualized using an enhanced chemiluminescence detection system with colored markers as size standards. Quantification of the bands was carried out with an MCID Image Analyzer (Imaging Research, Ontario, CA). The relative pERK values were obtained from normalization of pERK1/2 values were normalized against the total ERK1/2 values.

For analysis of cytochrome c, cells were lysed in buffer A (20 mM Hepes-KOH, pH 7.5, 10 mM KCl, 1.5 mM MgCl2, 1 mM EGTA, 1 mM dithiothreitol, 250 mM sucrose, 100 mM phenylmethylsulfonyl fluoride, pepstatin A [1 µg/ml], leupeptin [2 µg/ml] and aprotinin [2 µg/ml]). The cells were homogenized using a glass dounce homogenizer and a tight pestle (30 strokes). Cell homogenates were centrifuged at 15,000 × g for 15 min at 4 °C. The pellet obtained represented the mitochondria-enriched fraction. The supernatants were further centrifuged at 100,000 g for 1 hour at 4°C and the resulting supernatant represented cytosolic protein fraction. Protein content was determined by using a Pierce Micro-BCA Protein Assay Kit. Thirty micrograms of mitochondrial protein and fifty micrograms of cytosolic protein were used in SDS-PAGE. Proteins

were visualized by means of appropriate antibodies. A nonspecific band at 70 kD served as a control for loading of proteins.51 Quantification of the bands was carried out with an MCID Image Analyzer.

6. MOLECULAR BIOLOGY METHODS (III)

6.1 RNA extraction

Total cellular RNA was extracted from the U2OS and SaOS-2 cells using the Trizol reagent and purified according to manufacturer´s instructions. cDNA was synthetized from 2 µg of total RNA in a 20 µl reaction containing random hexanucleotides and AMV reverse transcriptase enzyme.

6.2 Semiquantitative RT-PCR Analysis

PCR was performed using the Eppendorf Mastercycler. The following primers were used:

TNFα (forward)	5`-TGC TTG TTC CTC AGC CTC TT-3`
TNFα (reverse)	5`-TGG GCT ACA GGC TTG TCA CT-3`
IL-6 (forward)	5`-CCT TCC AAA GAT GGC TGA AA-3`
IL-6 (reverse)	5`-AGC TCT GGC TTG TTC CTC AC-3`
GAPDH (forward)	5`-AGC CAC ATC GCT CAG ACA C-3`
GAPDH (reverse)	5`-GCC CAA TAC GAC CAA ATC C-3`

After an initial denaturation at 94ºC for 3 minutes 25 cycles of PCR amplification were performed, each consisting of a denaturing step of 94 ºC for 30 seconds, annealing at 60 ºC for 30 seconds and extension at 72 ºC for 30 seconds, followed by a final step at 72 ºC for 10 minutes. The number of 25 cycles was selected because in preliminary experiments we verified that with this number of cycles the reaction was still in a linear range for all three genes (data not shown). The amplified fragments were detected by 1,5% agarose gel electrophoresis and ethidium bromide staining. Quantification of the bands was carried out with an MCID Image Analyzer.

6.3 Quantitative real-time PCR

Real-time quantitative PCR analysis was done using a 5700 Sequence Detector (PE Applied Biosystems, Foster City, CA, USA). Amplifications reactions were set up in 25 µl reaction volumes containing amplification primers and SYBR Green PCR Master Mix. The primer concentrations and cDNA sample volumes were optimized for each primer set. Primer sequences for IL-6, OPG and β-actin were:

IL-6 (forward) 5`-TACCCCCAGGAGAAGATTCC-3`
IL-6 (reverse) 5`-AAAGAGGCACTGGCAGAAAA-3`
OPG (forward) 5`-TGCAGTACGTCAAGCAGGAG -3`
OPG (reverse) 5`-TGTATTTCGCTCTGGGGTTC -3
β-actin (forward) 5`-CGTGGGCCGCCCTAGGCACCA -3`
β-actin (reverse) 5`- TTGGCCTTAGGGTTCAGGGGG-3`

Amplifications were performed in 96-well reaction plates. Calibration curves were derived for each gene following serial dilutions of a stock cDNA which were then used as standards. Calibration curves were run in parallel in triplicates for each analysis. Each sample was analysed three times during each experiment. The experiments were carried out at three times on each cell line. Amplification data were analysed using the Sequence Detector System Software (PE Applied Biosystems). The results were normalized to β-actin and expressed as percentage of controls.

6.4 Enzyme-Linked ImmunoSorbent Assay (ELISA)

6.4.1 OPG assay

Cell culture media were collected, and OPG was determined with ELISA according to the manufacturer's instructions. Standards, samples, positive control, assay buffer, and detection antibody were pipetted into a 96-well plate precoated with monoclonal anti-OPG antibody and mixed. After incubation for 24 h at +4°C, the plate was washed five times with washing buffer, streptavidin peroxidase conjugate was added to the wells, and the cells were incubated for 1 h at room temperature. The plate was washed five times, and tetramethylbenzidine was added as a substrate to develop the color reaction. After incubation for 20 minutes at room temperature, stop solution was added, and OPG was measured with a Victor model 2 instrument (EG & G Wallac) at 450-nm absorbance.

6.4.2. IL-6 assay

Similarly to OPG assay, cell culture media were collected, and IL-6 was determined with ELISA according to the manufacturer's instructions. Standards, samples, positive control, assay buffer, and detection antibody were pipetted into a 96-well plate precoated with monoclonal anti-IL-6 antibody and mixed. After incubation for 24 h at room temperature, the plate was washed five times with washing buffer; standars and samples were added to the wells and incubated 1 h at room temperature. Wells were again washed as previously after which biotinylated antibody was added and plate was incubated for 1 hour at room temperature. After third wash streptavidin-HRP conjugate was added to the wells, and the plate was incubated for 30 min at room temperature. The plate was washed, and tetramethylbenzidine was added as a substrate to develop the color reaction. After incubation for 30 minutes at dark in the room temperature, stop solution was added, and IL-6 was measured with a Victor model 2 instrument (EG & G Wallac) at 450-nm absorbance.

7. STATISTICAL ANALYSIS (I-III)

Data were expressed as mean ± SE. Statistical significance was determined using either student’s t-test (I), one-way analysis of variance (ANOVA) (III), non-parametric Mann-Whitney and Kruskal-Wallis comparison tests or ANOVA followed by multiple comparison tests or non-parametric (II). The critical value for significance was $P<0,05$ (*).

RESULTS AND DISCUSSION

1. RAPID EFFECTS OF TAMOXIFEN ON BREAST CANCER CELLS (I AND II)

1.1 Tam and Tor induce rapid death in breast cancer cells

When this study was started, there was increasing interest in the rapid nongenomic effects of steroid hormones. During the past ten years estrogens have been demonstrated to have various rapid effects on breast cancer cells and the first two publications of this thesis work are based on the hypothesis that SERM compounds also exert antiestrogenic effects in breast cancer cells via nongenomic mechanisms. Revealing the multiple action mechanisms of SERMs could possibly be exploited to development of new tissue specific SERM compounds as well as improve therapeutic responses to the treatment of patients with these drugs.

We first studied the ability of different SERM compounds to induce rapid death of breast cancer cells. As a primary model system we used MCF-7 cell line which is the first hormone-responsive cell line described and is considered to be a suitable *in vitro* model for studying the mechanisms of apoptosis (Simstein *et al.,* 2003). Trypan blue analyses revealed that Tam induced death of MCF-7 cells already within one hour of treatment since nearly 90% of the cells were dead after 60-min treatment with Tam (I, Fig. 1a). We also found that Tam-induced death can be facilitated by pre-culturing the cells with low concentrations of Tam or in the absence of E_2. In addition to MCF-7 cells, the death-inducing effect of Tam was observed on another ER-positive cell line T47D. Furthermore, ERα-negative and estrogen unresponsive MDA-MB-231 cell line also responded to Tam. Corresponding effects were obtained with Tor, which is another antiestrogen used in breast cancer treatment. However, since chemical structure and the clinical profile of Tam and Tor resemble each other, we chose to study only the rapid effects of Tam in detail. The two other antiestrogens studied, Ral and Osp, were not able to induce death of MCF-7 cells under these conditions. This is interesting, since the mechanisms of the genomic effects of Tam, Tor and Ral on breast cancer cells closely resemble each other (Pasqualini 2004).

Pharmacological concentrations of Tam, which are higher than those needed to induce transcriptional effects, were required to induce acute death of breast cancer cells. Depending on pre-treatment there was fluctuation on the response of the MCF-7 cells, but generally a 5-7 μM concentration of Tam appears to represent threshold that is needed to trigger the rapid death pathway. This cannot be considered as a toxic dose; however, since clinically relevant steady-state plasma concentrations of Tam together with its biologically active metabolites measured in patient sera can be as high as close to 5 μM (Peyrade *et al.,* 1996). Moreover, owing to accumulation of lipophilic Tam in cellular membranes and lipid-rich tissues such as the mammary gland, intra-tumoral concentrations can be 5–11 times higher than that (Kisanga *et al.,* 2004), which suggests that the rapid death-inducing effects of Tam may also have biological significance *in vivo*.

1.2 Tam-induced mitochondrial disruption

Mitochondria are known to play an integral role in apoptosis induced by various cancer chemotherapeutic drugs. There are also reports demonstrating that high concentrations of Tam increase the mitochondrial respiration and proton permeability and decrease mitochondrial transmembrane potential and oxidative phosphorylation in isolated rat liver mitochondria (Tuquet *et al.,* 2000; Cardoso *et al.,* 2001). In order to reveal the mechanism by which Tam rapidly induces death of MCF-7 cells, we first tested the hypothesis that it affects the mitochondria.

1.2.1. The effect of Tam on mitochondrial permeability transition pore

Since disruption of mitochondrial function is considered to be one of the primary events in apoptotic cell death, we studied whether bongkrekic acid (BA), an inhibitor of mitochondrial permeability transition and mitochondrial depolarization, could protect the cells from Tam-induced death. We observed that BA extended MCF-7 cell survival time in the presence of Tam, since at 30 minutes time-point the difference between the numbers of dead cells in the samples treated with either Tam only or combination of Tam and BA was nearly 50% (I, Fig.2a).

1.2.2. The effect of Tam on release of cytochrome *c*

The translocation of cytochrome *c* from the intermembrane compartment of mitochondria to cytosol is known to be central for mitochondrial apoptotic death pathway. Thus, we aimed to visualize the subcellular localization of cytochrome *c* following treatments with either Tam or E_2 by using immunohistochemical staining and a secondary antibody conjugated to Alexa-488 dye. For visualization of mitochondria, we loaded the cells with MitoTrackerRed CMXRos dye. A 20 minutes treatment with Tam was found to induce release of cytochrome *c*, whereas comparable effect was not observed with E_2 (I, Fig. 3a). Corresponding results were also obtained by Western blotting in which the level of cytochrome *c* protein was detected from both mitochondrial and cytosolic protein extracts.

1.2.3. The effect of Tam on mitochondrial membrane potential

Decrease in the mitochondrial membrane potential ($\Delta\psi$ m) has been reported to accompany apoptosis in many cases and the release of cytochrome *c* from mitochondria to cytosol is considered to be a consequence of a change in $\Delta\psi$ m. To evaluate the effect of Tam treatment on the mitochondrial transmembrane potential, we loaded MCF-7 cells with tetramethylrhodamine methyl ester (TMRM), which is a red cationic fluorophore that accumulates electrophoretically into mitochondria in response to the negative mitochondrial membrane potential. In case of mitochondrial depolarization the intramitochondrial TMRM fluorescence disappears. In our experiment TMRM was added to the culture medium and living cells were monitored by confocal microscopy during Tam treatment and images were collected every 10 minutes. To quantify the changes in TMRM fluorescence over time, the total TMRM fluorescence of individual cells was measured at various time points (I, Fig. 4b). We first observed decrease of $\Delta\psi$m in Tam treated sample after 20 minutes of treatment

whereas in other samples $\Delta\psi m$ remained intact, which results indicate that Tam induces decrease of $\Delta\psi m$.

1.2.4. Downstream effects of Tam

In the established apoptotic model release of cytochrome *c* from mitochondria triggers activation of caspases which are divided into initiator caspases (caspases 8 and 9) and executioner caspases (such as caspases 3, 6 and 7). MCF-7 cells have lost caspase-3 as a result of a genomic deletion in the *casp-3* gene (Jänicke *et al.,* 1998; Zapata *et al.,* 1998). Other caspases or proteases are, however, probably operative in Tam-induced death of MCF-7 cells, since they have been shown to undergo caspase-dependent apoptosis following treatment with etoposide and doxorubicin (Jänicke *et al.,* 1998; Jänicke *et al.,* 2001). Thus, MCF-7 cells do not exhibit a total defect in the mitochondrial apoptotic pathway.

We investigated the effects of Tam treatment on death pathways downstream from cytochrome *c* by examining the possible activation of procaspase-9 by Tam and, due to lack of caspase-3 in MCF-7 cell line, the proteolytic cleavage of PARP which is an endogenous substrate of caspases. Western blot analyses showed that neither caspase-9 nor PARP were cleaved during 30-min treatment of MCF-7 cells with Tam (I, Fig. 5a, b). The results concerning lack of acute caspase-9 activation and PARP cleavage in Tam-treated cells were confirmed using a broad-spectrum caspase inhibitor, Z-VAD-FMK. Administration of Z-VAD-FMK together with Tam did not prevent Tam-induced death of MCF-7 cells, which indicates that Tam-induced cell death was caspase-independent. Such alternative death pathways have been described in the literature (Obrero *et al.,* 2002).

1.2.5. The effect of Tam on the production of ROS

The release of cytochrome *c* from mitochondria increases oxidative phosphorylation and generation of ROS, which may contribute to cell death even if caspases are not activated. Tam-treatment has previously been demonstrated to induce ROS generation for example in Jurkat cells and ovarian cancer cells (Ferlini *et al.,* 1999). To study the effect of Tam on production of ROS in MCF-7 cells, we used diphenylene ionodium (DPI). It is a flavoprotein inhibitor of NADPH oxidase, which is considered to be a major source of ROS. Trypan blue analysis revealed that DPI was able to delay Tam-induced death of MCF-7 cells significantly, thus indicating the involvement of ROS. Similar results were obtained with E_2-deprived MCF-7 cells and MDA-MB-231 cells. Furthermore, we used DPI to study the causal relationship between cytochrome *c* release and induction of ROS formation. As demonstrated by immunocytochemistry, DPI was not able to prevent Tam-induced release of cytochrome *c* from mitochondria indicating that ROS production is distal to cytochrome *c* release. It is also possible that production of ROS is a separate event from release of cytochrome *c*, but ROS can also feed back on mitochondria and further cause functional damage to trigger the release of cytochrome *c* (Mattsson 2000).

The effect of Tam on intracellular ROS levels was further confirmed by fluorescence microscopy. The cells were loaded with DFH-DA which, upon oxidation by ROS, yields green fluorescence. We observed that addition of Tam for 20 minutes produced a rapid and robust increase in ROS in the cells (I, Fig. 8a) which result further indicates mitochondrial disruption by Tam-treatment. Tam induced cytochrome *c* release and oxidative stress has recently been reported also by Nazarewicz *et al.* who demonstrated the induction of these effects by submicromolar concentration of Tam in isolated rat liver mitochondria and MCF-7 cells (Nazarewitz *et al.,* 2007).

1.2.6. Effect of Tam on production of antiapoptotic proteins

Because Bcl-2 family proteins are known to control the sensitivity of cancer cells to chemotherapeutic agents, the levels of antiapoptotic proteins Bcl-X_L and Bcl-2 were determined by Western blotting in E_2-deprived and Tam-pretreated cells. Both Bcl-2 and Bcl-X_L have previously been shown to inhibit release of cytochrome *c* from mitochondria to cytosol (Yang *et al.,* 1997) and Bcl-2 has also been shown to prevent both caspase-dependent and -independent programmed cell death induced by a variety of stimuli (Mathiasen and Jäättelä 2002). Even though both of these antiapoptotic proteins maintain mitochondrial integrity, some reports propose that Bcl-2 and Bcl-X_L could differentially protect cells from apoptosis (Lebedeva *et al.,* 2003; Yang *et al.,* 2003). However, we found that E_2 increased and Tam decreased the expression levels of both of these proteins, which could provide an explanation for the increased sensitivity of E_2-deprived or 1 μM Tam-treated MCF-7 cells to higher doses of Tam in our experiments. The pre-incubation-produced facilitation is likely to be a consequence of the transcriptional effects of Tam and E_2 on expression of pro- and /or antiapoptotic proteins.

1.3 ERK mediates rapid effects of Tam

Estrogen has been shown to indirectly affect the mitochondria by activation of MAPK (Gavrilova-Jordan and Price 2007). In turn, activation of MAPK may affect pro-and antiapoptotic proteins, thus changing mitochondrial susceptibility to apoptosis (Kirkland and Franklin 2003). It is speculated that also Tam, besides its uptake to the cells and nuclear translocation, generates a transmembrane signal transduction cascade by virtue of its high lipophilicity and partitioning in the cell membrane. According to the literature, Tam-induced activation of ERK has previously been demonstrated in HeLa cells (Duh *et al.,* 1997) and human endometrial cancer cells (Acconcia and Kumar 2006). Furthermore**,** since ROS have been shown to contribute to cell death, in part, through an effect on various cellular signaling pathways including MAPK pathway (Guyton *et al.,* 1996; Chen *et al*., 1995; Lander 1997; Bhat *et al.,* 1999) we tested the hypothesis that activation of ERK could be associated with Tam–induced rapid death of MCF-7 cells.

Activation of ERK has usually been considered to be involved in cell proliferation (Zhang and Liu 2002; Zhang *et al*., 2002; Peyssonaux *et al*., 2001), and the role of ERK in cell death has only recently been hypothesized. However, our Western blotting experiments demonstrate that Tam was able to activate ERK and that this activation

was sustained for at least 40 minutes in MCF-7 cells, whereas in vehicle-treated cells phosphorylation of ERK was detected only at 5 minutes of incubation (II, Fig. 2B). When compared to Tam, E_2 treatment resulted in only transient activation of ERK and baseline conditions returned at 20 minutes. This is basically in parallel with the results of previous studies reporting that E_2 rapidly and transiently activates ERK1 and ERK2 in MCF7 cells (Filardo *et al.,* 2002; Fernando and Wimalasena 2002; Song *et al.,* 2002; Thomas *et al.,* 2006). Prolonged ERK phosphorylation by Tam was also observed in estrogen receptor positive T47D cells. However, in estrogen receptor-negative MDA-MB-231 cells Tam did not induce statistically significant phosphorylation of ERK. These results suggest that ERK has a role in the acute death response of at least ER-positive breast cancer cells.

Since phosphorylation of ERK is known lead to translocation of activated ERK to nucleus, we also studied the effect of Tam treatment on subcellular localization of phosphorylated ERK. MCF-7 cells were transiently transfected with GFP-ERK2 and nucleus-targeted red fluorescent protein and exposed for different time periods to vehicle, E_2, Tam, and combination of Tam and E_2 before fixation and visualization by fluorescence microscopy. The localization of GFP-ERK2 was determined as the percentage of cells exhibiting accumulation of GFP-ERK2 in the nucleus. Our results from studies concerning subcellular localization of ERK after Tam treatment were basically in parallel with our phosphorylation data. We observed that ERK1/2 phosphorylation by Tam was associated with a prolonged nuclear localization of ERK1/2, which reached a peak level at 20 min, returning to baseline at 40 min (II, Fig. 3B). Conversely, E_2 was shown to exert a different kind of temporal pattern of ERK nuclear localization in comparison with Tam; E_2 caused a rapid translocation of ERK to nuclei, which reached a peak level at 5 min and returned to baseline at 10 min. Of note is that the proportion of nuclear GFP-ERK2 in control cells was relatively high, approximately 30%, which might have been a consequence of overexpression of ERK in these cells. This may partly explain the temporal differences between ERK phosphorylation and translocation.

In order to further confirm the role of ERK in Tam induced rapid effects, we employed PD98059, an inhibitor of MEK, at an ERK phosphorylation-inhibiting concentration. ERK phosphorylation was determined at several timepoints up to 40 minutes and PD98059 was found to oppose Tam-induced ERK phosphorylation completely. We also observed that inhibition of ERK with PD98059 resulted in a decreased amount of cell death brought about by Tam, which results further demonstrate that Tam acts via ERKs to induce apoptotic signaling in MCF-7 breast cancer cells.

Consistently with our results, a recent report by Visram and Greer also suggests that both E_2 and Tam are capable of activating ERK in MCF-7 cells (Visram and Greer 2006). The dual role of ERK1/2 in both cell death and cell proliferation may be explained by several recent findings which demonstrate that phosphorylated ERKs may produce different outcomes in the same cell depending on the duration of ERK accumulation in the nucleus and perhaps also on cell context (Murphy *et al.,* 2002; Chen *et al.,* 2005; Jeon *et al.,* 2006; Shaul *et al.,* 2006). For example, in their recent article Chen *et al.* demonstrate that the anti-apoptotic effect of E_2 may be converted

into a pro-apoptotic one by alteration of the temporal pattern of the ERK activation, perhaps by determining the activation of a distinct set of transcription factors. Thus, it is possible that the localization and duration of kinase signaling similarly contribute to different actions of Tam in breast cancer cells. Persistent nuclear retention of activated ERK1/2 has also been considered as a critical factor in eliciting pro-apoptotic effects also in neuronal cells subjected to oxidative stress (Stanciu and DeFranco 2002).

2. INVOLVEMENT OF RECEPTORS IN THE RAPID EFFECTS OF TAM (II)

In addition to signaling pathways, we were also interested in knowing which receptor structures mediate rapid effects of Tam on breast cancer cells. Rapid nongenomic ER-related signaling has been proposed to occur through distinct cellular localization of the classical ER or ER-like receptors, through classical heterotrimeric G proteins, and through many of the effectors traditionally associated with growth factors and GPCRs (Levin 2005). Some investigators postulate that nongenomic signaling of estrogen is mainly mediated by membrane-associated ERs (Kelly and Levin 2001), and this hypothesis has been further supported by the recent demonstration of membrane-located ERα in MCF-7 and other cell types (Razandi *et al.,* 2004; Marquez and Pietras 2001; Powell *et al.,* 2001; Pedram *et al.,* 2006). Furthermore, it has been suggested that, as a G-protein coupled receptor, the membrane localized pool of ER might signal to the mitochondria (Levin 2005). In addition to membrane and nuclear ERs, both ERα and ERβ have been identified within the mitochondrial matrix of rabbit uterine and ovarian cells (Monje and Boland 2001). Additional study with MCF-7 cells also localized ERα and ERβ to the mitochondria (Chen *et al.,* 2004). Other studies suggested that ERβ is primarily mitochondrial, whereas ERα is mainly nuclear (Cammarata *et al.,* 2004; Yang *et al.,* 2004). A recent report by Pedram *et al.* shows high affinity ERs in the mitochondria of MCF-7 cells and endothelial cells, compatible with classical ERα and Eβ (Pedram *et al.,* 2006). They also provide evidence of functions of mitochondrial ER potentially impacting breast cancer cell survival. Mechanisms of estrogen-mediated cellular actions have thus been shown to be very complex and it is possible that Tam is also capable of activating various receptors and signaling pathways that are convergent. Also mitochondrial estrogen receptors may contribute to rapid effects of Tam, either directly or indirectly.

2.1 Estrogen receptors

The results concerning the role of ERs in Tam-induced rapid effects in breast cancer cells were somewhat conflicting. Cell death analyses indicated that, in addition to ER-positive MCF-7 and T47D cells, Tam was able to induce death in ERα-negative MDA-MB-231 breast cancer cells, although the effect not equally effective to that in MCF-7 cells. This finding favors the idea that rapid actions of Tam are independent of ERs. Surprisingly, however, the pure antiestrogen ICI 182780 could partly oppose Tam-induced cell death when it was added together with Tam to MCF-7 cells. This finding suggests a role for estrogen receptors in the rapid actions of Tam. When studying the role of ERs in Tam-induced phosphorylation of ERK, we observed that Tam could

activate ERK in ER positive MCF-7 cells and T47D cells, but not significantly in ERα negative MDA-MB-231 cells, which results again indicate the involvement of ERs in the induction of rapid effects by Tam. However, addition ICI182780 together with Tam did not decrease level of ERK phosphorylation in MCF-7 cells when compared to treatment with Tam alone.

There may be several explanations for the discrepancies observed concerning the role of ERs in rapid effects of Tam. The fact that MDA-MB-231 cells are known to be ERα-negative, but that they do express ERβ (Vladusic *et al.,* 1998) raises the possibility that the rapid actions of Tam could actually be mediated through ERβ. However, since the effect of ICI 182780 was not observed in MDA-MB-231 cells, it is probable that rapid effects of Tam are primarily ER-independent, but can be facilitated by ER dependent mechanisms, as shown in MCF-7 cells. The superiority of Tam in MCF-7 cells compared to MDA-MB-231 cells may be explained by the signaling through the small pool of ERα that has recently been demonstrated to localize at the plasma membrane of MCF-7 cells (Razandi *et al.,* 2003). Interestingly, a recent report by Heberden *et al.* (Heberden *et al.,* 2006) demonstrated a raft-located estrogen receptor-like protein distinct from ERα which remained insensitive to the pure estrogen antagonist ICI 182780. Another study recently showed the existence of a protein named ER-X which could cross-react to an antibody directed against the binding site of ERα (Toran-Allerand *et al.,* 2002). Thus, it is possible that rapid effects of Tam in breast cancer cells are mediated by a yet unknown receptor structure. Although the reason for the lack of statistically significant ERK activation in MDA-MB-231 cells is not clear, activation of ERK seems to be important at least in the apoptotic response of ER-positive breast cancer cells against Tam. On the other hand, it is possible that Tam-induced rapid cell death is mediated only partly via the ERK pathway.

2.2 Epidermal growth factor receptor

There are reports suggesting that, in addition to acting via a membrane ER, estrogen can exert extranuclear actions by interacting directly with growth factor receptor complexes, which in turn leads to signal transduction to kinases such as ERK that phosphorylate and activate nuclear ERs (Levin 2005). Shou and coworkers demonstrated that when an EGFR family member ErbB2 was experimentally overexpressed, Tam treatment activated both ER and ErbB2 to signal downstream through ERK and PI3K (Shou *et al.,* 2004). However, this cross-talk has been associated with resistance to endocrine therapy in breast cancer (Nicholson *et al.,* 1999; Osborne *et al.,* 2003), rather than induction of cell death. Nonetheless, our results suggest that Tam may occupy growth factor signaling pathways also for induction of cell death.

We show that Tam–induced rapid death of breast cancer cells is, at least in part, mediated through EGFR since our Western blotting experiments demonstrate that inhibition of EGFR with EGFR inhibitors AG1478 and BIBX1382 totally abolished Tam–induced ERK-phosphorylation in both MCF-7 and T47D cells (II, Fig. 7A and Fig. 7B). Furthermore, we also tested the effect of BIBX1382 on Tam-induced ERK

phosphorylation on T47D cells and the results were comparable to those obtained with MCF-7 cells. Involvement of EGFR in the effects of Tam has been reported also by Visram and Greer who demonstrated that in MCF-7 cells E_2 and Tam were both equally dependent on EGFR for activation of ERK (Visram and Greer 2006). Moreover, results of trypan blue exclusion analyses showed that both EGFR inhibitors significantly opposed Tam-induced rapid death of MCF-7 cells which results further suggest involvement of EGFR in the Tam-induced rapid effects on breast cancer cells.

3. THE PROTECTIVE ACTIONS OF E_2 AGAINST TAM-INDUCED EFFECTS (I and II)

When studying the effect of E_2 on Tam–induced rapid death of breast cancer cells, we found that E_2 protected ER-positive MCF-7 and T47D cells from Tam-induced rapid death. However, it is notable that addition of E_2 along with Tam opposed Tam-induced ERK activation statistically significantly only in MCF-7 cells, whereas this effect was not as clear in T47D cells. The discrepancy observed between MCF-7 cells and T47D cells may be due to differences in ERα/ERβ ratio between these two cell lines (Pedrero *et al.,* 2002). The MCF-7 cells express a high ERα/ERβ ratio whereas T47D express a low ERα/ERβ ratio, which might be relevant if the rapid effects of E_2 are mainly mediated via the membrane-associated ERα as suggested by Pedram *et al.* (Pedram *et al.,* 2006). The survival-effects of E_2 were not observed in ER-negative MDA-MB-231 cells indicating, in contrast to ER-independence of Tam-induced effects, involvement of ERs in the protective actions of E_2. Furthermore, even though we observed that E_2 is able to oppose the effects of Tam, it is likely that E_2 and Tam utilize separate intracellular signaling mechanisms. This hypothesis is further supported by recent findings of Visram and Greer who observed that, even though both E_2 and Tam induced activation of ERK, the inhibition of Src or metalloproteinases caused distinct effects on ERK activation by E_2 and Tam (Visram and Greer 2006).

The antiapoptotic effects of estrogens in both ER-positive and -negative cells, including MCF-7 cells, has previously been reported also by others (Bynoe *et al.,* 2000; Choi *et al.,* 2001; Haynes *et al.,* 2001; Zhang *et al.,* 2001; Huang *et al.,* 1997; Perillo *et al.,* 2000; Ahamed *et al.,* 2001). Fernando and Wimalasena reported that E_2 reduces apoptosis induced by TNF-α, H_2O_2 and serum withdrawal, but not that induced by paclitaxel (Fernando and Wimalasena 2002). However, the latter observation was in contrast to data published by Razandi *et al.* (Razandi *et al.,* 2003). In addition, the anti-apoptotic action of E_2 against resveratrol has been described by Zhang *et al*. (Zhang *et al.,* 2004). Interestingly, a recent report of Pedram *et al*. (Pedram *et al.,* 2006) suggests that E_2 inhibits UV radiation –induced apoptosis in MCF-7 cells by directly up-regulating manganese superoxide dismutase activity in these cells. They report that E_2 inhibits UV radiation-induced cytochrome *c* release, the decrease of mitochondrial membrane potential and apoptotic cell death. However, also conflicting results have been published. The work by Moreira *et al.* with isolated rat liver mitochondria suggests that Tam delays opening of mitochondrial PTP, whereas E_2 has deleterious effects on mitochondria due to increased H_2O_2 production. However, the E_2 and Tam concentration used in this study were relatively high, which might affect the results.

4. DIFFERENT ESTROGEN RECEPTOR LIGANDS PROTECT OSTEOBLASTIC CELLS FROM APOPTOSIS (III)

Apoptosis has been demonstrated to partly regulate bone metabolism (Urayama *et al.,* 2000, Kameda *et al.,* 1995, Kawakami *et al.,* 1997, Nakashima *et al.,* 1998, Kawakami *et al.,* 1998, Weinstein *et al.,* 1998) and especially apoptosis of osteoblasts is getting more attention since it has been considered to be an important determinant of bone formation and therefore of skeletal integrity (Manolagas, 2000 and Weinstein and Manolagas, 2000), such that disorders that promote the process are associated with increased bone fragility (Weinstein *et al.,* 1998), and treatments that inhibit it are associated with anti-fracture efficacy (Jilka *et al.,* 1999).

Our studies with breast cancer cells indicated that E_2 was able to protect the cells against Tam-induced cell death. Consistently, others have also suggested that estrogen protects different types of cells from apoptosis induced by various substances (Bynoe *et al.,* 2000; Haynes *et al.,* 2000; Perillo *et al.,* 2000; Choi *et al.,* 2001; Zhang *et al.,* 2001; Gu *et al*., 2005). A potential role for estrogens in protection of bone mass through inhibition of osteoblast apoptosis has also been proposed. Indeed, E_2 has previously been shown to protect calvaria derived osteoblastic cells from etoposide-induced apoptosis (Zallone 2006, Kousteni *et al*., 2001) as well as to protect osteoblasts from ethanol-induced bone-loss (Chen *et al*., 2006).

In our third work we hypothesized that SERMs, in contrast to pro-apoptotic actions in mammary gland, exert estrogen-like bone-protective and antiapoptotic actions in osteoblastic cells. In addition, we wanted to evaluate the roles of different ER subtypes in the cell-protective effects of E_2 and other ER ligands. Furthermore, we studied the ability of these compounds to modulate etoposide-induced effects on the expression of IL-6 and OPG, which are two important bone regulatory cytokines. To examine the individual actions of ERα and ERβ we used U2OS human osteosarcoma cell line that lack of detectable endogenous ER expression. These cells were stably transfected with expression constructs for the human ERα and ERβ full lenght sequences. Transfected cells expressed the receptors at a RNA and protein level as demonstrated by RT-PCR and Western blotting. The transactivation capacity of transfected ERs was studed by ERE-luciferase reporter gene assays. In addition, we confirmed the functionality of transfected ERs by verifying with RT-PCR that ERα and ERβ were able to restore the effects of E_2 on TNFα –induced cytokine production. Corresponding cell lines have also been created and used by others for various purposes (Monroe *et al.,* 2003; Stossi, *et al.,* 2004 and Kian Tee *et al.,* 2004). As control, we also tested E_2 and SERM effects on apoptosis of another osteoblast-like cell line SaOS-2, which is known to express both ERα and ERβ (Sutherland *et al.,* 1996, Vidal *et al.,* 1999). For induction of apoptosis we used the chemotherapy drug etoposide at an osteoblast apoptosis inducing concentration (Urayama *et al.,* 2000).

4.1 Antiapoptotic effects of different ER ligands

In order to compare the ability of E_2 and synthetic ERα and ERβ agonists PPT and DPN to protect osteoblast-derived cells from apoptosis and to study the role of

different ER subtypes in these effects, we incubated the ERα or ERβ transfected U2OS cells with various ER ligands and induced apoptosis of the cells with etoposide. Apoptotic cells were quantified by visualization of changes in nuclear morphology. Our findings suggest that both ER isotypes play a role in mediating the protective effects of E_2 since estradiol was able to prevent etoposide-induced apoptosis in both ERα and ERβ -transfected U2OS cells. The protective effect of E_2 was not observed in parental or vector-transfected U2OS cells, which results support the conclusion that the antiapoptotic effect is mediated via ERs. In addition, in SaOS-2 cells both PPT and DPN were able to oppose etoposide-induced effect, thus further indicating the involvement of both ERα and ERβ.

Of the three SERMs examined in our experimental setup only Osp could oppose etoposide-induced apoptosis in ERα-transfected U2OS cells. For this reason, we chose to study only Osp instead of all three SERMs in the following experiments. Also in SaOS-2 cells Osp treatment significantly opposed the cell death induced by etoposide. Osp has previously been shown to exert estrogen-like effects in bone marrow cultures by enhancing osteoblastic differentiation with a mechanism that differs from that of Ral (Qu *et al*., 1999). However, the ability of Osp to protect osteoblasts from apoptosis has not been demonstrated before. In contrast, Ral has previously been reported to decrease sodium nitroprusside-induced apoptosis of osteoblasts (Olivier *et al*., 2004). However, in our experimental setup Ral did not protect the osteoblastic cells against etoposide-induced apoptosis.

4.2 Regulation of IL-6 and OPG by ER ligands

The inhibitory effects of estrogens on bone resorption have been suggested to involve regulation of RANKL-RANK-OPG system (Syed and Khosla 2005). In addition, estrogens have been shown to prevent bone loss also by regulating the production IL-6 and of several other cytokines that modulate osteoclastic bone resorption (Messalli *et al*., 2007, Compston *et al*., 2001). Furthermore, E_2 has previously been demonstrated to dose-dependently increase osteoblastic OPG mRNA and protein levels (Saika *et al*., 2001, Lindberg *et al*., 2001, Bord *et al*., 2003). This effect has also been shown in human osteoblasts transfected with estrogen receptors (Hofbauer *et al*., 1999).

To compare the response of ERα and ERβ transfected cells to E_2 and other ER ligands, we tested the ability of these compounds to modulate etoposide-induced effects on the expression of IL-6 and OPG by quantitative real-time PCR method. Our results suggest that in U2OS/ERβ cells Osp and DPN prevented the effects of etoposide on the expression of OPG, while in ERα-transfected cells none of the ligands tested had statistically significant etoposide opposing effect. This suggests involvement of ERβ in the regulation of the OPG mRNA expression. The role of ERβ was supported by the finding that in SaOS-2 cells only DPN had a significant etoposide-opposing effect. To further test the ability of ER ligands to induce OPG and IL-6 production, we used a sensitive sandwich ELISA method to measure protein concentrations in the cell media collected after different treatments. The results from these experiments also suggested a role for ERβ, rather than ERα, in regulation of OPG although on protein level the results were somewhat conflicting when compared to data obtained with PCR.

In addition to expression of OPG, also RANKL and the ratio of RANKL/OPG have been suggested to be important in modulation of bone homeostasis by estrogen. However, we could not to assess RANKL mRNA expression since the cell lines studied appear to produce very low levels of RANKL and were thus unable to give information about the estrogen-dependent RANKL/OPG ratio. Low RANKL expression by osteoblastic cell lines has been previously reported also by others (Hofbauer *et al.,* 1999, Cheung *et al.,* 2003).

The stimulatory effect of etoposide on IL-6 production has previously been demonstrated (Verdenqh *et al*., 2002, Wood *et al*., 2006, Tozava *et al*., 2002). However, there are also conflicting reports (Verdrenqh *et al*., 2003, De Vita *et al*., 1998). We found that etoposide strongly induced expression of IL-6 in osteoblastic cells and in case of ERα both E_2 and PPT opposed etoposide-induced expression of IL-6. In contrast, Osp did not have corresponding effect on IL-6 expression. E_2 and PPT also opposed the etoposide-induced IL-6 protein secretion. In case of U2OS/ERβ cells all ER ligands seemed to inhibit the etoposide-induced IL-6 expression but the effects were not statistically significant. These results suggest that E_2 regulates IL-6 expression via ERα, whereas Osp does not have an effect on IL-6. However, in SaOS-2 cells, all ligands did oppose the effects of etoposide on IL-6 expression suggesting ERβ contribution and cell-specific effect.

SUMMARY

In this thesis work we have studied the effects of selective estrogen receptor modulators on the death of breast cancer cells and osteoblastic cells. Cumulatively, our data suggest that Tam causes death of breast cancer by several mechanisms, one of which is rapid induction of cell death by mitochondrial mechanisms. Our results further show that susceptibility of breast cancer cells to Tam-induced death can be increased by a pre-treatment of the cells with antiestrogens such as Tam itself, or by E_2 withdrawal. These effects are most probably dependent on transcriptional regulation of the levels of antiapoptotic and/or pro-apoptotic proteins. Furthermore, our results suggest that Tam rapidly induces sustained activation of ERK1/2 in ER-positive breast cancer cell lines, which effects can be opposed by E_2. Tam-induced rapid death appears to be primarily ER-independent, but it can possibly be modulated by ERs. However, epidermal growth factor receptor (EGFR)-associated mechanisms were also shown to be involved in Tam -induced cell death.

Further work is needed to better understand possible relationships between nongenomic and genomic effects of Tam in breast cancer cells, but it is possible that these two mechanisms have different functions. Tam-initiated genomic signaling may represent a way through which the target cells are programmed for complex functions that require a long time to get in action and ultimately determine the fate of the cells. Nongenomic signaling mechanisms, on the other hand, may represent systems by which cells are rapidly activated to adjust to dynamic changes of the cell environment. Identification of the multiple mechanisms underlying Tam-induced cell death is important, because this information can be applied to improve therapeutic responses to the treatment of patients with this selective estrogen receptor modulator.

In addition to the Tam-induced effects on breast cancer cells, we present data that E_2 and a novel SERM compound ospemifene are able to protect osteoblastic cells from apoptosis. The protective effect could be mediated via both ERα and ERβ although the responses of the cell lines expressing either of the two receptors differed from each other. Moreover, we show that the osteoblast-protective effect is associated with changes in the levels of osteoblast-produced cytokine expression. Revealing the routes that lead to different biological effects of E_2 and selective estrogen receptor modulators used as therapeutics is of importance for understanding the mechanisms by which estrogenic/antiestrogenic compounds maintain cell survival in various tissues and homeostasis in bone. Our findings could have clinical relevance for example in the case of osteolytic breast cancer bone metastasis. Because of their direct role in bone degradation, osteoclasts are currently the major target of pharmaceutical interventions. Nevertheless, targeting the osteoclasts does not cure the disease or result in bone repair (Lipton *et al*., 2000) indicating that normal osteoblasts function is also impaired in osteolytic breast cancer metastasis (Mercer *et al*., 2004). Moreover, breast cancer cells have been demonstrated to directly induce apoptosis of osteoblasts (Mastro *et al*., 2004, Mercer *et al*., 2004). It has been previously suggested that apoptotic osteoblasts signal for decreased osteoclastic activity through OPG and our results imply that this

signaling may modulated by ER ligands. Thus, it is possible that the same group of drugs that are used to treat primary tumor might also have beneficial effects on tissue that is a potent site of metastasis. Information about the anti- and proapoptotic actions of Tam and other SERMs in different target tissues could possibly be exploited in development of new tissue specific SERM compounds. In this respect, it is of importance to understand that SERM compounds having chemically related structures might have clinically different effect profiles to patients.

The most important findings of this thesis are summarized in figures 4-6.

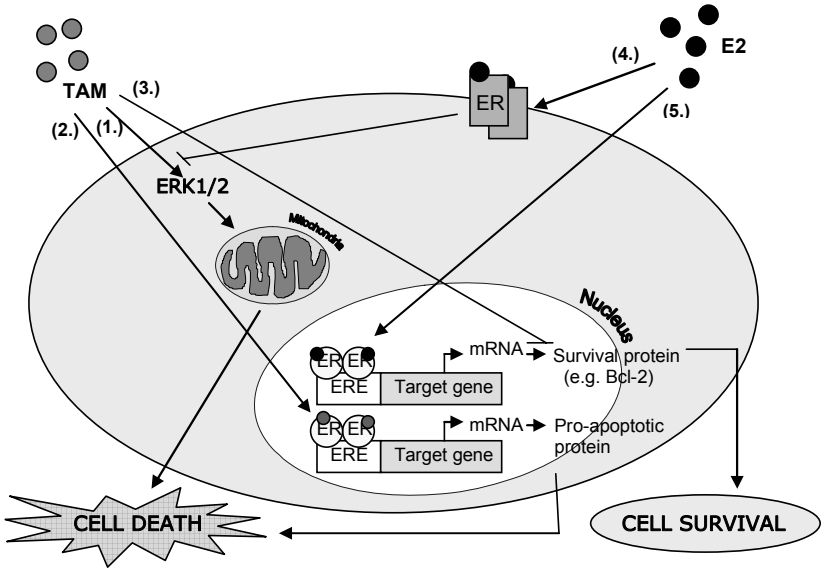

Figure 4. The protective effects of estrogen against Tam-induced death of breast cancer cells. Tam-induced activation of ERK1/2 leads to mitochondrial disruption and eventually death of the cells (1). Alternatively, Tam may induce transcription of proapoptotic genes via nuclear ERs (2) or block translation of antiapoptotic proteins (3). Tam-induced effects can be abrogated by E_2 which acts either via membrane ERs to block Tam-induced ERK1/2 activation (4) or via nuclear ERs to upregulate expression of antiapoptotic genes. The model is based on the results presented in this study.

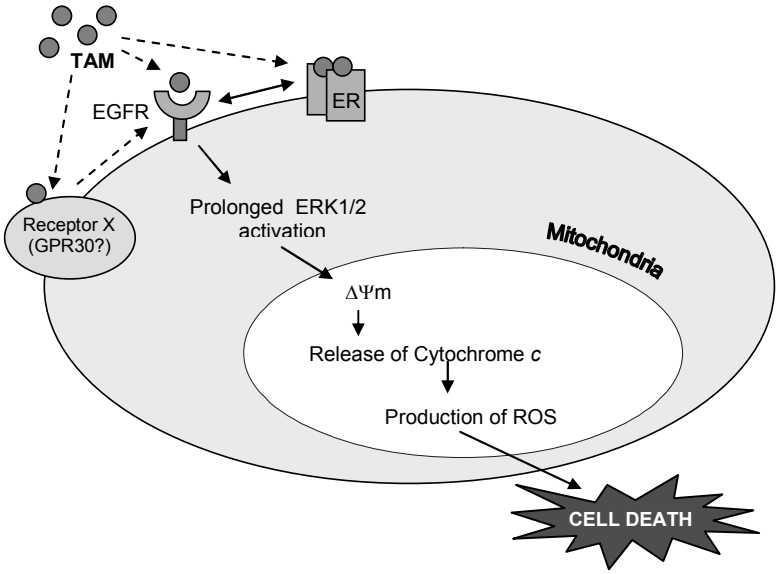

Figure 5. A model for **Tam-induced death pathway in breast cancer cells.** Tam may interact with several putative receptor structures (ER, EGFR, "receptor X") on cell membrane. This triggers prolonged ERK1/2 activation leading to decrease of mitochondrial membrane potential, release of cytochrome *c* from mitochondria to cytosol and excessive production of reactive oxygen species (ROS) eventually leading to death of breast cancer cells. The model summarizes the results presented in this study.

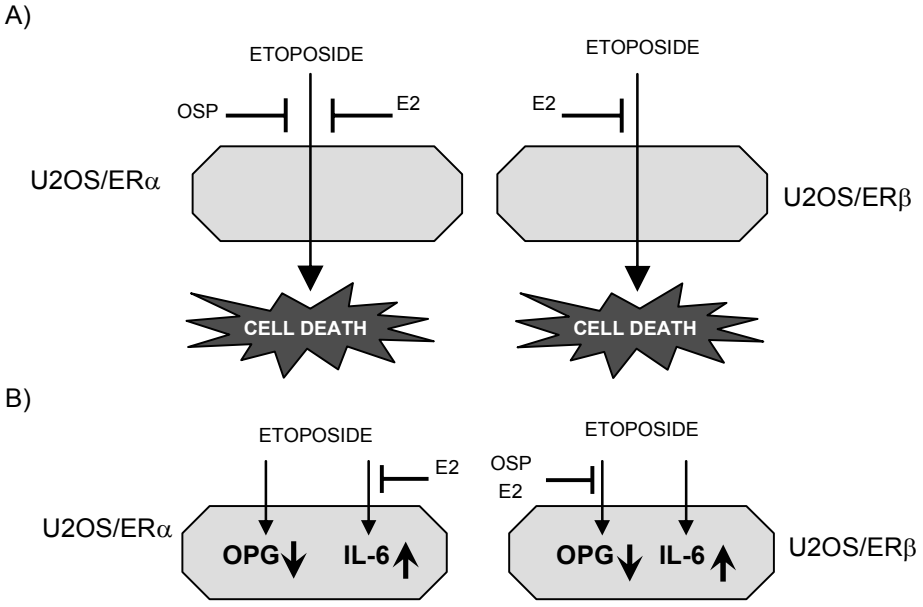

Fig.6. **E_2 and SERM protection against cell death in ERα and ERβ expressing osteoblastic cells.** Both ER subtypes, alpha and beta, mediate the protective effects of E_2 against etoposide-induced apoptosis on osteoblast-like U2OS cells. In addition, Osp extends survival of etoposide-treated U2OS/ERα cells (A). The U2OS cells containing either ERα or ERβ differentially respond to E_2 and Osp at the level of cytokine expression upon etoposide challenge (B).

CONCLUSIONS

On the basis of the results and discussion presented in this thesis, I draw the following conclusions:

1. Tamoxifen at pharmacological, clinically achievable concentrations is able to initiate a rapid mitochondrial death pathway in MCF-7 breast cancer cells. Tam-induced rapid death program has features of both apoptosis and necrosis, and it is facilitated by pre-culturing the cells with low concentrations of Tam or in the absence of E_2.

2. Tamoxifen, in addition to previously demonstrated genomic effects, is capable of acting via rapid membrane-initiated signaling and ERK pathways. It appears to have a primary role in the acute death response of at least ER-positive breast cancer cells to Tam.

3. E_2 is able to oppose the rapid effects of Tam in ER-positive breast cancer cells. However, due to lack of protective effect in ER-unresponsive cell line, it is likely that E_2 and Tam utilize separate signaling mechanisms.

4. The rapid effects of Tam are primarily ER-independent, but can be facilitated by ER-dependent mechanisms. On the other hand, EGFR-associated mechanisms appear to be involved in Tam-induced death.

5. The protective actions of E_2, as well as novel SERM compound ospemifene, against apoptosis are mediated via both ERalpha and ERbeta. In addition, the cells containing either ERalpha or ERbeta differentially respond to E_2 and ospemifene at the level of cytokine expression.

ACKNOWLEDGEMENTS

This study was carried out at the Department of Anatomy, Institute of Biomedicine and MediCity Research Laboratory, University of Turku.

I warmly thank my supervisor Professor Pirkko Härkönen for support and guidance during these years. There were several times when I felt like giving up but your encouragement kept me going on. I truly admire your innovative mind and extensive knowledge in cell biology!

I want to thank Professor Kalervo Väänänen for giving me the opportunity to work at the Department of Anatomy and for providing the excellent laboratory facilities for this study.

Professor Olli Lassila acknowledged for enabling me to work as a PhD student at the well-organized Turku Graduate School of Biomedical Sciences (TuBS). I am grateful for Professor John Eriksson and Docent Päivi Koskinen for their guidance during the early years of my thesis work.

Docent Paula Martikainen and Docent Juha Klefström are acknowledged for revising the thesis manuscript and for their valuable remarks.

I wish to thank my co-authors Kaisa Heiskanen, Johanna Dahllund, Tao Guo, Elisa Lamminen, Jani Seppänen and Lauri Kangas for their contribution.

Aiping Zheng is acknowledged for collaboration of many years, for excellent company during many scientific trips and for introducing me many new laboratory techniques.

I want to express my thanks to all the former and present members of Härkönen-lab: Maija Valta, Sanna Virtanen, Annikki Vaisto, Roope Huttunen, Tiina Pöyhönen, Natalija Eigeliene, Mirjami Mattila, Johanna Ruohola, Leena Strauss, Anni Wärri, Merja Tasanen, Johanna Ojala, Anu Lillbacka, Marika Ojala, Tero Hautamäki, Noora Mykkänen, Huili Zheng, Tommi Ahonen, Kalle Mikola, Pirkko Rauhamäki, Päivi Lakkakorpi, Michael Husheem, Niina Loponen, Jorma Määttä and Kati Raepalo.

My warmest thanks belong to Kati Tarkkonen and Teresa Elo who shared the lab and office room with me for many years. Your friendship and support has been extremely important to me, especially on “bad lab days.” I wish to thank Johanna Tuomela for friendship and numerous inspiring conversations. Our non-scientific trips have been truly refreshing! Eeva Valve is acknowledged for encouragement and always keeping up the good spirit.

I want to thank Soili Jussila for excellent maintenance of the lab and Taina Malinen for providing tools to everyday lab work. Iris Dunder and Soili Huhta are acknowledged for their excellent secretarial help. In addition, numerous people from Department of Anatomy, Medicity and BTK are acknowledged for providing practical help, tips and reagents.

I want to thank all my wonderful friends. Special thanks belong to Marika; your support during these years has been irreplaceable. I wish to thank the members of Saunalaudat ry; Reeta, Virpi, Ansku, Emma and Sanna for the many memorable moments we have shared. I am grateful to Riikka for being my "running therapist". Janne & Riika, Jouni, Jonnu & Ville, Aleksi & Mira are warmly acknowledged for the great times together.

I owe my deepest gratitude to my dear mom-in–law Kikka. There are numerous times when your help with children has "rescued" me and my cells. This thesis would not have been finished without your contribution.

I can not thank enough my parents Tarja and Martti. You have always provided me with love and support when ever I have needed it. I am so fortunate to have parents like you!

Our children, Mikael, Anni and Elias are especially thanked for their patience on all those evenings and weekends when mom had to leave home to feed her cells or sit beside the computer. You three definitely make my life worth living!

Last, but certainly not the least, I want to acknowledge my husband Kristian. I have absolutely no idea what I had done without your love and support. Thank you so very much Love!

This study was financially supported by the Turku Graduate School of Biomedical Sciences, the Sigrid Jusélius Foundation, the Academy of Finland, the Cancer Association of South-western Finland, the Finnish Cultural Foundation, the Turku University Foundation, the Foundation of Jalmari and Rauha Ahokas, the Orion-Farmos Research Foundation, the Finnish Breast Cancer Group and the Lilly Foundation.

REFERENCES

Acconcia F And Kumar R. Signaling Regulation Of Genomic And Nongenomic Functions Of Estrogen Receptors. *Cancer Lett* 2006; 238: 1-14

Adamski J and Benveniste E. 17β-Estradiol activation of the c-Jun N-Terminal kinase pathway leads to down-regulation of class II major histocompatibility complex expression. *Mol Endo* 2005; **19(1):** 113-124

Ahamed S, Foster JS, Bukovsky A and Wimalasena J. Signal transduction through the Ras/ERK pathway is essential for mycoestrogen zeralenone-induced cell-cycle progression in MCF-7 cells. *Mol Carcinog* 2001; **30:** 88-98

Arpino G, Green SJ, Allred DC, Lew D, Martino S and Osborne CK *et al.*, HER-2 amplification, HER-1 expression, and tamoxifen response in estrogen receptor-positive metastatic breast cancer: a Southwest Oncology Group Study. *Clin Cancer Res* 2004; **10**: 5670–5676

Askenazi A and Dixit VM. Death receptors: signaling and modulation. *Science* 1998; **281**: 1305-1308

Battersby S, Robertson BJ, Anderson TJ, King RJB and Mcpherson K. Influence of menstrual cycle, parity and oral contraceptive use on steroid hormone receptors in normal breast. *Br J Cancer* 1992; **65**: 601-607

Beatson GT. On the treatment of inoperable cases of carcinoma of the mamma: suggestions for a new method of treatment with illustrative cases. *Lancet* 1896; **2**: 104-107

Benhar M, Engelberg D and Levitzki A. ROS, stress-activated kinases and stress signaling in cancer. *EMBO Rep.* 2002; **3**: 420-425

Bhat N and Zhang P. Hydrogen peroxide activation of multiple mitogen-activated protein kinases in an oligodendrocyte cell line: role of extracellular signal-regulated kinase in hydrogen peroxide-induced cell death. *J Neurochem* 1999; **72(1):**112-9

Bord S, Ireland DC, Beavan SR and Compston JE. The effects of estrogen on osteoprotegerin, rankl, and estrogen receptor expression in human osteoblasts. *Bone* 2003; **32**: 136-141

Bouras T, Southey MC and Venter DJ. Overexpression of the steroid receptor coactivator aib1 in breast cancer correlates with the absence of estrogen and progesterone receptors and positivity for p53 and her2/neu. ***Cancer Res*** 2001; **61**: 903-907

Boyan BD, Sylvia VL, Frambach T, *et al*. Estrogen-dependent rapid activation of protein kinase C in estrogen-receptor positive MCF-7 breast cancer cells and in estrogen-receptor negative HCC38 cells is membrane-mediated and inhibited be tamoxifen. *Endocrinology* 2003; **144:** 1812-24

Boyd S. On oophorectomy in the cancer of the breast. *Br Med J* 1900; **2**: 1161- 1167

Bryant HU and Dere WH. Selective estrogen receptor modulators: an alternative to hormone replacement therapy. *Proc Soc Exp Biol Med* 1998; **217**: 45-52

Brzozowski AM, Pike AC, Dauter Z, Hubbard RE, Bonn T, Engström O, Ohman L, Greene GL, Gustafsson JA and Carlquist M. Molecular basis of antagonism in the oestrogen receptor. *Nature* 1997; **389**: 753-758

Bursch W. The autophagosomal-lysosomal compartment in programmed cell death. *Cell Death Differ* 2001;**8**:569–81

Bynoe M, Grimaldi C and Diamond B. Estrogen up-regulates Bcl-2 and blocks tolerance induction of naive B cells. *Proc Natl Acad Sci* 2000; **97:** 2703-2708

Bruder JM, Sobek L, Oettel M *et al*. Dehydro-epiandrosterone stimulates the ERE. *J Steroid Biochem Mol Biol* 1997; **62**: 461-466

Cammarata PR, Chu S, Moor A, Wang Z, Yang SH and Simpkins JW. Subcellular distribution of native estrogen receptor alpha and beta subtypes in cultured human lens epithelial cells. *Exp Eye Res* 2004; **78**: 861-871

Cannings E, Kirkegaard T, Tovey SM, Dunne B, Cooke TG and Bartlett JM. Bad expression predicts outcome in patients treated with Tamoxifen. *Breast Cancer Res Treat* 2007; **102**: 173–179

Cardoso CM, Custodio JB, Almeida LM, Moreno AJ. Mechanisms of the deleterious effects of tamoxifen on mitochondrial respiration rate and phosphorylation efficiency. *Toxicol Appl Pharmacol* 2001; 176: 145-52

Cardoso CM, Moreno AJ, Almeida LM and Custodio JB. Comparison of the changes in adenine nucleotides of rat liver mitochondria induced by tamoxifen and 4-hydroxytamoxifen. *Toxicol In Vitro* 2003; 17: 663-70

Caristi S Galera JL, Matarese F, Imai M, Caporali S, Cancemi M, Altucci L, Cicatiello L, Teti D, Bresciani F and Weisz A. Estrogens do not modify MAP kinase-dependent nuclear signaling during stimulation of early G(1) progression in human breast cancer cells. *Cancer Res* 2001; **61:** 6360-6366

Catalano MG, Comba A, Fazzari A, Benedusi-Pagliano E, Sberveglieri M, Revelli A, Massobrio M, Frairia R AND Fortunati N. Sex steroid binding protein receptor (sbp-r) is related to a reduced proliferation rate in human breast cancer. *Breast Cancer Res Treat* 1997; **42**:227-234

Cavalieri E, Chakravati D, Guttenplan J, Hart E, Ingle J, Jankowiak R, Muti P, Rogan E, Russo J, Santen R and Sutter T. catechol estrogen quinines as initiators of breast and other human caners: implications for biomarkers of susceptibility and cancer prevention. *Biochim Biophys Acta* 2006; **1766**: 63-78

Chen D, Pace PE, Coombes and Ali S. Phosphorylation of human estrogen receptor alpha by protein kinase a regulates dimerization. ***Mol Cell Biol*** 2002; **20:** 5041-5047

Chen JQ, Delannoy M, Cooke C and Yager JD. Mitochondrial localization of ERα and ERβ in human MCF-7 cells. *Am J Physiol Endocrinol Metab* 2004; **286**: E1011-E1022

Chen JR, Kousteni S, Bellido T, Han L, DiGregorio GB, Jilka RL and Manolagas SC. Gender-independent induction of murine osteoclast apoptosis in vitro by either estrogens or non-aromatizable androgens. *J Bone Miner Res* 2001; **16**: S159

Chen JR, Plotkin L, Aguirre J, Man L, Jilka RL, Kousteni S, Bellido T and Manolagas SC. Transient versus sustained phosphorylation and nuclear accumulation of ERKs underlie anti- versus pro-apoptotic effects of estrogens. *J Biol Chem* 2005; **280:** 4632-4638

Chen JR, Haley RL, Hidestrand M, Shankar K, Liu X, Lumpkin CK, Simpson P, Badger T and Ronis M. Estradiol protects against ethanol-induced bone loss by inhibiting up-regulation of receptor activator of nuclear factor-κB ligand in osteoblasts. *J Pharm Exp Ther* 2006; **319**: 1182-1190

Chen S, Masri S, Wang X, Phung S, Yuan YC and Wu X, What do we know about the mechanisms of aromatase inhibitor resistance?, *J. Steroid Biochem Mol Biol* 2006; **102**: 232–240

Chen Q, Olashaw N and Wu J. Participation of reactive oxygen species in the lysophosphatidic acid-stimulated mitogen-activated protein kinase kinase activation pathway. *J Biol Chem* 1995; **270(48):** 28499-502

Cheskis BJ. Regulation of cell signalling cascades by steroid hormones. *J Cell Biochem* 2004; **93**:20-27

Cheung J, Mak YT, Papaioannou S, Evans BAJ, Fogelman I and Hampson G. Interleukin-6 (IL-6), IL-1, receptor activator of nuclear factor kappaB ligand (RANKL) and osteoprotegerin production by human osteoblastic cells: comparison of the effects of 17-beta oestradiol and raloxifene. *J Endo* 2003; **177**: 423-433

Chi S, Kitanaka C, Noguchi K, *et al*. Oncogenic Ras triggers cell suicide through the activation of a caspase-independent cell death program in human cancer cells. *Oncogene* 1999;**18**:2281–90

Choi K, Kang S, Tai C, Auersperg N and Leung P. Estradiol up-regulates antiapoptotic Bcl-2 messenger ribonucleic acid and protein in tumorigenic ovarian surface epithelium cells. *Endochrinology* 2001; **142:** 2351-2360

Choueiri MB, Tu LY, Yu-Lee SH and Lin SH. The central role of osteoblasts in the metastasis of prostate cancer. *Cancer Metastasis Rev* 2006; **25**: 601-609

Cicek M and Oursler MJ. Breast cancer bone metastasis and current small therapeutics. *Cancer Meatastasis Rev* 2006; **25**: 635-644

Clarke R, Leonessa F, Welch JN and Skaar TC. Cellular and molecular pharmacology of antiestrogen action and resistance. *Pharmacol Rev* 2001; **53**: 25–71

Cole MP, Jones CT and Todd ID. A new anti-osestrogenic agent in late breast cancer. An early clinical appraisal of ici46474. ***Br J Cancer*** 1971; **25:** 270-275

Coleman RE. Clinical features of metastatic bone disease and risk of skeletal morbidity. *Clin Cancer Res* 2006; **12**: 6243-6249

Compston JE. Sex steroids and bone. *Physiol Rev* 2001; **81**: 419-447

Cosman F and Lindsay R. Selective estrogen receptor modulators: clinical spectrum. *Endocr Rev* 1999; **20**: 418-434

Couldwell WT, Hinton DR, He S. Protein kinase C inhibitors induce apoptosis in human malignant glioma cell lines. *FEBS Lett* 1994; **345:** 43-46

Curtis RE, Boice Jr JD, Shriner DA, Hankey BF and Fraumeni Jr JF. Second cancers after the adjuvant Tamoxifen therapy for breast cancer. *J Natl Cancer Inst* 1996; **88**: 832-834

Custodio JB, Moreno AJ, Wallace KB. Tamoxifen inhibits induction of the mitochondrial permeability transition by Ca2+ and inorganic phosphate. *Toxicol Appl Pharmacol* 1998;152: 10-17

Cuzick J, Powles T, Veronesi U *et al.* Overview of the main outcomes in breast cancer prevention trials. *Lancet* 2003; **36**: 296-300

Desagher S, Osen-Sand A, Nichols A, Eskes R, Montessuit S, Laupher S, Maundrell K, Antonsson B and Martinou J. Bid-induced conformational change of Bax is reponsible for mitochondrial cytochrome c release during apoptosis. *J Cell Biol* 1999; **144**: 891–901

Dietze EC, Caldwell LE, Grupin SL, Mancini M, Seewaldt VL. Tamoxifen but not 4-hydroxytamoxifen initiates apoptosis in p53(-) normal human mammary epithelial cells by inducing mitochondrial depolarization. *J Biol Chem* 2001; 276: 5384-94

Dobrzycka KM, Townson SM, Jiang S and Oesterreich S. Estrogen receptor corepressors: a role in human breast cancer? *Endocr Rel Cancer* 2003; **10**: 517-536

Dong L, Wang W, Wang F, Stoner M, Reed JC, Harigari M, Samudro I, Kladde MP, Vyhlidal C and Safe S. Mechanisms of transcriptional activation of bcl-2 gene expression by 17beta-estradiol in breast cancer cells. *J Biol Chem* 1999; **274:** 32099-32107

Dowsett M, Johnston S, Martin LA, Salter J, Hills M and Detre S *et al.*, Growth factor signalling and response to endocrine therapy: The Royal Marsden Experience. *Endocr Relat Cancer* 2005; **12**: S113–S117

Duchen MR. Mitochondria in health and disease; perspectives on a new mitochondrial biology, *Mol Aspects Med* 2004; **25**: 365–451

Duh J, Yu R, Jiao J, Matwyshyn GA, Li W, Tan TH and Kong AN. Activation of signal transduction kinases by tamoxifen. *Pharmaceutical Res* 1997; **14:** 186-189

Dupont J, Karas M and LeRoith D. The potentiation of estrogen on insulin-like growth factor I action in MCF-7 human breast cancer cells includes cell cycle components. *J Biol Chem* 2000; **275:** 35893-35901

Duriez PJ, Shah GM (1997) Cleavage of poly(ADP-ribose) polymerase: a sensitive parameter to study cell death. Biochem Cell Biol 75:337–349

Early Breast Cancers Trialist´s collaborative group. Tamoxifen for an early breast cancer: an overview of the randomized trials. *Lancet* 1998; **351:** 1451-1467

Enmark E, Pelto-Huikko M, Grandien K *et al.*, human estrogen receptor beta-gene structure, chromosomal localization, and expression pattern. *J Clin Endocrinol Metab* 1997; **82**:4258–4265

Ferlini C, Scambia G, Marone M, Distefano M, Gaggini C, Ferrandina G, Fattorossi A, Isola G, Benedetti, Pancini P, Mancuso S. Tamoxifen induces oxidative stress and apoptosis in oestrogen receptor-negative human cancer cell lines. *Br J Cancer* 1999;**79**: 257-63

Fernando R and Wimalasena J. Anti-apoptotic effects of 17-b estradiol are mediated via PI3K/Akt and ERK/p90RSK and Bad phosphorylation. *Proceedings of AACR 43* 2002. Abstract.

Fernando R and Wimalasena J. Estradiol abrogates apoptosis in breast cancer cells through inactivation of BAD: Ras-dependent nongenomic pathways requiring signalling through ERK and Akt. *Mol Biol Cell* 2004; **15:** 3266-3284

Ferri KF and Kroemer G. Organelle-specific initiation of cell death pathways. *Nat Cell Biol* 2001;**3**:E255–63

Filardo E, Quinn J, Frackelton AR Jr and Bland K. Estrogen action via the g-protein-coupled receptor, gpr30: stimulation of adenylyl cyclase and camp-mediated attenuation of the epidermal growth factor receptor-to-MAPK signaling axis. *Mol Endo* 2002; **16**: 70-84

Filardo E, Quinn J, Frackelton AR Jr and Bland K. Estrogen action via the G-protein-coupled receptor, GPR30: stimulation of adenylyl cyclase and cAMP-mediated attenuation of the epidermal growth factor receptor-to-MAPK signaling axis. *Mol Endo* 2002; **16:** 70-84

Filardo EJ, Graeber CT, Quinn JA, Resnick MB, Giri D, Delellis RA, *et al.* Distribution of GPR30, a seven membrane-spanning estrogen receptor, in primary breast cancer and its association with clinicopathologic determinants of tumour progression. *Clin Cancer Res* 2006; **12**: 6359-6366

Fisher B, Constatntino JP, Redmond CK, Wickerman DL and Cronin WM. Endometrial cancer in Tamoxifen treated breast cancer patients: findings from the National Surgical Adjuvant Breast and Bowel Project (NSABP) B-14. *J Natl Cancer Inst* 1994; **86**: 527-537

Friesen C, Fulda S and Debatin KM. Cytotoxic drugs and the CD95 pathway. *Leukemia* 1999;**13**:1854–8

Gee JM, Robertson JF, Gutteridge E, Ellis IO, Pinder SE and Rubini M *et al.*, Epidermal growth factor receptor/HER2/insulin-like growth factor receptor signalling and oestrogen receptor activity in clinical breast cancer. *Endocr Relat Cancer* 2005; **12**: S99–S111

Fuqua SA, Schiff R and Parra I *et al*. Expression of wild-type ERβ and variant isoforms in human breast cancer. Cancer Res 1999; **59**: 5425-5428

Gavrilova-Jordan L and Price T. Actions of steroids in mitochondria. *Semin Reprod Med* 2007; **25**:154-164

Gelmann EP. Tamoxifen for the treatment of malignancies other than breast and endometrial carcinoma. *Semin Oncol* 1997; **24:** S65-S70

Green DR and Kroemer G. The pathophysiology of mitochondrial cell death. *Science* 2004; 305: 626-629

Green DR and Reed JC. Mitochondria and apoptosis. *Science* 2005; **281**: 1309-1312

Green DR. Apoptotic pathways: paper wraps stone blunts scissors. *Cell* 2000;**102**:1–4

Green S, Walter P, Kumar V *et al.* Human oestrogen receptor cdna: sequence. *Nature* 1986; **320**: 134–139

Greene GL, Gilna P, Waterfield M, Baker A, Hort Y and Shine J. Sequence and expression of human estrogen receptor complementary dna. *Science* 1986; **231**: 1150–1154

Gu G, Hentunen TA, Nars M, Härkönen PL and Väänänen HK. Estrogen protects primary osteocytes against glucocorticoid-induced apoptosis. *Apoptosis* 2005; **10**:583-595

Gustafsson JA. Estrogen receptor beta—a new dimension in estrogen mechanism of action. *J Endocrinol* 1999; **163**: 379–383

Gutierrez MC, Detre S, Johnston S, Mohsin SK, Shou J and Allred DC *et al.*, Molecular changes in tamoxifen-resistant breast cancer: relationship between estrogen receptor, HER-2, and p38 mitogen-activated protein kinase. *J Clin Oncol* 2005; **23**: 2469–2476

Hall JM, Couse JF and Korach KS. The multifaceted mechanism of estradiol and estrogen receptor signaling. *J Biol Chem* 2001; **276**: 36869-36872

Hammes A, Andreassen TK, Spoelgen R, Raila J, Hubner N, Schultz H, Metzger J, Schweigert FJ, Luppa PB, Nykjaer A and Willnow TE. Role of endocytosis in cellular uptake of sex steroids. *Cell* 2005; **122**:751-762

Harper MJ and Walpole AL. Contrasting endocrine activities of cis and trans isomers in a series of substituted triphenyletylenes. *Nature* 1966; **212**:87

Haynes M, Sinha D, Russell K, Collinge M, Fulton D, Morales-Ruiz M, Sessa WC and Bender JR. Membrane estrogen receptor engagement activates endothelial nitric oxide synthase via the PI3K-kinase-Akt pathway in human endothelial cells. *Circ Res* 2000; **87:** 677-682

Heberden C, Reine F, Grosse B, Henry C, Zagar Y, Chaumaz G and Lieberherr M. Detection of a raft-located estrogen receptor-like protein distinct from ERα. *Int J Biochem Cell Biol* 2006; **38**: 376-391

Heerdt AS and Borgen PI. Current status of tamoxifen use: an update for the surgical oncologist. *J Surg Oncol* 1999; **72:** 42-49

Helguero LA, Faulds MH, Gustafsson JA and Haldosen LA. Estrogen receptors alpha (er alpha) and beta (er beta) differentially regulate proliferation and apoptosis of the normal murine mammary epithelial cell line hc11. *Oncogene* 2005; **24**: 6605–6616

Helzlsouer KJ and Couzi R. Hormones and breast cancer. *Cancer* 1995; **76**: 2059-2063

Hiroi H, Inoue S, Watanabe T *et al.* Differential immunolocalization of estrogen receptor alpha and beta in rat ovary and uterus. *J Mol Endocrinol* 1999; **22**: 37–44

Hofbauer LC, Khosla S and Dunstan CR. Estrogen stimulates gene expression and protein production of osteoprotegerin in human osteoblastic cells. *Endocrinology* 1999; **140**: 4367-4370

Holm C, Rayala S, Jirstrom K, Stal O, Kumar R and Landberg G. Association between Pak1 expression and subcellular localization and tamoxifen resistance in breast cancer patients. *J Natl Cancer Inst* 2006; **98**: 671–680

Honig SF. Treatment of metastatic disease: hormonal therapy and chemotherapy. In: J.R. Harris, M.E. Lippman, M. Morrow and S. Helman, Editors, *Diseases of the Breast*, Lippincott-Raven, Philadelphia 1996: 669–734

Huang J, Li X, Yi P, Hilf R, Bambara RA and Muyan M. Targeting estrogen responsive elements (eres): design of potent transactivators for *ere*-containing genes. *Mol Cell Endocrinol* 2004; **218**: 65-78

Huang Y. Estrogen increases intracellular p26Bcl-2 to p21Bax ratios and inhibits taxol-induced apoptosis of human breast cancer MCF-7 cells. *Breast Cancer Res Treat* 1997; **42:** 73-81

Hughes DE, Dai A, Tiffee JC, Li HH, Mundy GR and Boyce BF. Estrogen promotes apoptosis of murine osteoclasts mediated by TGF-β. *Nat Med* 1996; **2**: 1132-1136

Hui A-M, Zhang W, Chen W, *et al*. Agents with selective estrogen receptor (ER) modulator activity induce apoptosis *in vitro* and *in vivo* in ER-negative glioma cells. *Cancer Res* 2004; **64**: 9115-9123

Härkönen PL, QuQ, Dahllund J, Laine J, Hemminki K, Kangas L and Väänänen HK. FC1271a is a a novel tissue-spesific antiestrogen with bone-sparing effect. 10th international congress of endocriniology USA 1996; 256

Jensen EV, Cheng G, Palmieri C, Sajv S, Makela S, Van Noorden S, *et al.* Estrogen receptors and proliferation markers in primary and recurrent breast cancer. *Proc Natl Acad Sci USA* 2001; **98**: 15197-15202

Jeon ES, Lee MJ, Sung SM and Kim JH. Sphingosylphosphorylcholine induces apoptosis of endothelial cells through reactive oxygen species-mediated activation of ERK. *J Cell Biochem;* 2006; **100**:1536-47.

Jilka RL, Weinstein RS, Bellido T, Parfitt AM and Manolagas SC. Osteoblast programmed cell death (apoptosis): modulation by growth factors and cytokines. *J Bone Miner Res* 1998;**13**:793-802

Jordan VC and Morrow M. Tamoxifen, raloxifene, and the prevention of breast cancer. *Endocr Rev* 1999; **20**: 253-278

Jordan VC. Selective estrogen receptor modulatin: concept and concequences in cancer. *Cancer Cell* 2004; **5:** 207-213

Jänicke RU, Engels IH, Dunkern T, *et al*. Ionizing radiation but not anticancer drugs causes cell cycle arrest and failure to activate the mitochondrial death pathway in MCF-7 breast carcinoma cells. *Oncogene* 2001; **20:** 5043-5053

Jänicke RU, Sprengart ML, Wati MR and Porter AG. Caspase-3 is required for DNA-fragmentation and morphological changes associated with apoptosis. *J Biol Chem* 1998; **273:** 9357-9360

Kameda T, Ishikawa H and Tsutsumi T. Detedtion and characterization of apoptosis in osteoclasts in vitro. *Biochem Biophys Res Commun* 1995; **207**: 753-760

Kandouz M, Lombert A, Perrot JY, *et al*. Proapoptotic effects of antiestrogens, progestins and androgen in breast cancer cells. *J Steroid Biochem Mol Biol* 1999; **69:** 463-71

Kangas L. Biochemical and pharmacological effects of toremifene metabolites. *Cancer Chemother Pharmacol* 1990; **27**: 8-12

Kaufmann SH, Desnoyers S, Ottaviano Y, Davidson NE and Poirier GG. Specific proteolytic cleavage of poly(adenosinediphosphate ribose) polymerase: an early marker of chemotherapy-induced apoptosis. *Cancer Res* 1993; **53:** 3976-3985

Kawakami A, Eguchi K, Matsuoka N, Tsuboi M, Koji T and Urayama S. Fas and fas ligand interaction is necessary for human osteoblast apoptosis. *J Bone Miner Res* 1997; **12**: 1637-1646

Kelly M and Levin ER. Rapid actions of plasma membrane estrogen receptors. *Trends Endocrinol Metab* 2001; **12;** 152-156

Kerr JF, Wyllie AH, Currie AR. Apoptosis: a basic biological phenomenon with wide-ranging implications in tissue kinetics. *Br J Cancer* 1972; **26**: 239–257

Kian Tee M, Rogatsky I, Tzagarakis-Foster C, Cvoro A, An J, Christy R, Ymamamoto K and Leitman D. Estradiol and selective estrogen receptor modulators differentially regulate target genes with estrogen receptors α and β. *Mol Biol Cell* 2004; **15**: 1262-1272

Kim J-A, Kang YS, Jung M-W, Lee SH and Lee YS. Involvement of ca influx in the mechanism of tamoxifen-induced apoptosis in hepg2 human hepatoblastoma cells. *Cancer Lett* 1999; **147**: 115-23

King KL and Cidlowski JA. Cell cycle and apoptosis: common pathways to life and death. *J Cell Biochem* 1995; **58**:175–80

Kirkegaard T, Witton CJ, McGlynn LM, Tovey SM, Dunne B and Lyon A *et al.*, AKT activation predicts outcome in breast cancer patients treated with Tamoxifen. *J Pathol* 2005; **207**: 139–146

Kirkland RA and Franklin JL. BAX, reactive oxygen and cytochrome c release in neuronal apoptosis. *Antioxid Redox Signal* 2003; 5: 589-596

Kisanga ER, Gjerde J, Guerreri-Gonzaga A, *et al*. Tamoxifen and metabolite concentrations in serum and breast cancer tissue during three dose regimens in a randomized preoperative trial. *Clin Cancer Res* 2004; **10**: 2336-2343

Komi J, Heikkinen J, Rutanen EM, Halonen K, lammintausta R and Ylikorkala O. Effecst of ospemifene, a novel SERM, on biochemical markers of bone turnover in healthy postmenopausal women. *Gynecol Endocrinol* 2004; **18**: 152-158

Komi J, Lankinen K, DeGregorio M, Heikkinen J, Saarikoski S, Tuppurainen M, Halonen K, Lammintausta R, Väänänen K, Ylikorkala O and Erkkola R. Effects of ospemifene and raloxifene on biochemical markers of bone turnover in postmenopausal women. *J Bone Miner Metab* 2006; **24**: 314-318

Komi J, Lankinen KS, Härkönen P, DeGregorio MW, Voipio S, Kivinen S, Tuimala R, Vihtamäki T, Vihko K, Ylikorkala O and Erkkola R. Effects of ospemifene and Raloxifene on hormonal status, lipids, genital tract, and tolerability in postmenopausal women. *Menopause* 2005; **12**: 202-209

Kousteni S, Bellido T, Plotkin LI, O´Brien CA, Bodenner DL, Han L, Han K, Digregorio GB, Katzenellenbogen BS, Katzenellenbogen PK, Roberson PK, Weinstein RS, Jilka RL AND Manolagas SC. Nongenotropic, sex-nonspesific signalling through the estrogen or androgen receptors: dissociation from transcriptional activity. *Cell* 2001; **104:** 719-730

Kousteni S, Chen J-R, Bellido T, Han L, *et al*.. Reversal of bone loss in mice by nongenotropic signaling of sex steroids. *Science* 2002; **298**: 843

Kousteni S, Han L, Chen JR, Almeida L, Plotkin T, Bellido S and Manolagas SC. Kinase-mediated regulation of common transcription factors accounts for the bone-protective effects of sex steroids. *J Clin Invest* 2003; **111**: 1651-1664

Kroemer G and Jäättelä M. Lysosomes and autophagy in cell death control. *Nat Rev Cancer* 2005; **11**: 886-897

Kuiper GG, Carlsson B, Grandien K, Enmark E, Haggblad J, Nilsson S and Gustafsson JA. Comparison of the ligand binding specificity and transcript tissue distribution of estrogen receptors alpha and beta *Endocrinology* 1997; **138**: 863-870

Kuiper GG, Enmark E, Pelto-Huikko M, Nilsson S and Gustafsson JA. Cloning of a novel receptor expressed in rat prostate and ovary. *Proc Natl Acad Sci USA* 1996; **93**: 5925–5930

Kuiper GG, Lemmen JG, Carlsson B, Corton JC, Safe SH, van der Saag PT, van der Burg B and Gustafsson JA. Interaction of estrogenic chemicals and phytoestrogens with estrogen receptor beta. *Endocrinology* 1998; **139**: 4252-4263

Kushner PJ, Agard DA, Greene GL, Scanlan TS, Shiau AK, Uht RM, *et al.* Estrogen receptor pathways to ap-1. *Steroid Biochem Mol Biol* 2000; **74**: 311-317

Lander H. An essential role for free radicals and derived species in signal transduction. *FASEB J* 1997;**11:**118-24

Lawson JS, Field AS, Champion S, Tran D, Ishikura H and Trichopoulos D. Low oestrogen receptor [illegible] expression in normal breast tissue underlies low breast cancer incidence in japan. *Lancet* 1999; **354**: 1787-1788

Lebedeva I, Sarkar D, Su Z, *et al*. Bcl-2 and Bcl-X_L differentially protect human prostate cancer cells from induction of apoptosis by melanoma differentiation associated gene-7, *mda*-7/IL-24. *Oncogene* 2003; **22**: 8758-8773

Le Goff P, Montano MM, Schodin DJ and Katzenellenbogen BS. Phosphorylation of the human estrogen receptor. Identification of hormone-regulated sites and examination of their influence on transcriptional activity. *J Biol Chem* 1994; **269**: 4458-4466

Le Mellay V, Grosse B and Lieberherr M. Phospholipase C beta and membrane action of calcitriol and estradiol. *J Biol Chem* 1997; **272:** 11902-11907

Lee AV, Cui X and Oesterreich S. Cross-talk among estrogen receptor, epidermal growth factor and insulin-like growth factor signalling in breast cancer. *Clin Cancer Res* 2001; **7**: 4429-4435

Lehenkari P, Parikka V, Rautiala TJ, *et al*. The effects of tamoxifen and toremifene on bone cells involve changes in plasma membrane ion conductance. *J Bone Miner Res* 2003; **18:** 473-8

Leist M and Jäättela M. Four deaths and a funeral: from caspases to alternative mechanisms. *Nat Rev Mol Cell Biol* 2001;**2**:589–98

Lemasters JJ, Qian T, Bradham CA, *et al*. Mitochondrial dysfunction in the pathogenesis of necrotic and apoptotic cell death. *J Bioenerg Biomembr* 1999; **31**: 305-319

Leung LK and Wang TT. Paradoxical regulation of Bcl-2 family proteins by 17beta-oestradiol in human breast cancer cells MCF-7. *Br J Cancer* 1999; **81:** 387-392

Levin ER. Integration of the extra-nuclear and nuclear actions of estrogen. *Mol Endocrinol* 2005; **19**:1951-1959

Lewis JS and Jordan VC. Selective estrogen receptor modulators (SERMs): mechanisms of anticarcinogenesis and drug resistance. *Mutat. Res* 2005; **591**: 247–263

Li H, Zhu H, Xu CJ and Yuan J. Cleavage of BID by caspase 8 mediates the mitochondrial damage in the Fas pathway of apoptosis. *Cell* 1998; **94** :491–501

Li L, Haynes mp AND Bender Jr. Plasma membrane localization and function of the estrogen receptor alpha variant (er46) in human endothelial cells. *Proc Natl Acad Sci U SA* 2003; **100**:4807-4812

Li P, Nijhawan D, Budihardjo L, *et al*. Cytochrome c and dATP -dependent formation of Apaf-17 - caspase-9 complex initiates an apoptotic protease cascade. *Cell* 1997; **91:** 479-489

Lindberg MK, Erlandsson M, Alatalo SL, Windahl S, Andersson G, Halleen JM, Carlsten H, Gustafsson JA and Ohlsson C. Estrogen receptor alpha, but not estrogen receptor beta, is involved in the regulation of the OPG/RANKL (osteoprotegerin/receptor activator of NF-kappa B ligand) ratio and serum interleukin-6 in male mice. J Endocrinol 2001; **171**: 425-433

Linke SP, Bremer TM, Herold CD, Sauter G and Diamond C. A multimarker model to predict outcome in tamoxifen-treated breast cancer patients. *Clin Cancer Res*2006; **12**: 1175–1183

Lipfert L, Fisher JE, Wei N, Scafonas A, Su Q, Yudkovitz J, Chen F, Warrier S, Birtzin ET, Kim S, Chen HY, Tan Q, Schmidt A, Dininno F, Roherer SP, Hammond MH, Rodan GA, Freedman L and Reszka AA. Antagonist-induced, AF-2 independent estrogen receptor α phosphorylation. *Mol Endo* 2006; **20(3)**: 516-33

Lipton A, Theriault RL, Hortobagyi GN, Simeone J, Knight RD, Mellars K, Reitsma DJ, Heffernan M And Seaman J. Pamidronate prevents skeletal complications and is effective palliative treatment in women with breast carcinoma and osteolytic bone metastases: long term follow-up of two randomized, placebo-controlled trials. *Cancer* 2000; **88**: 1082-1090

Liu X, Zou H, Slaughter C, Wang X (1997) DFF, a heterodimeric protein that functions downstream of caspase-3 to trigger DNA fragmentation during apoptosis. Cell 89:175–184

Lobenhofer EK, Huper G, Iglehart J and Marks J. Inhibition of mitogen-activated protein kinase and phosphatidylinositol 3-kinase activity in MCF-7 cells prevents estrogen-induced mitogenesis. *Cell Growth Differ* 2000; **11:** 99-110

Lobo RA. Benefits and risks of estrogen replacemet therapy. *Am J Obstet Gynecol* 1995; **173**: 982-989

Los M, Wesselborg S and Schulze-Osthoff K. The role of caspases in development, immunity and apoptotic signal transduction: lessons from knockout mice. *Immunity* 1999*;* **10:** 629-639

Luo X, Budihardjo I, Zou H, Slaughter C and Wang X. Bid, a Bcl2 interacting protein, mediates cytochrome c release from mitochondria in response to activation of cell surface death receptors. *Cell* 1998; **94**:481–490

Magnusson C, Holmberg L, Norden T, Lindgren A and Persson I. Prognostic characteristics in breast cancers after hormone replacement therapy. *Breast Cancer Res Treat* 1996; **38**: 325-334

Manolagas SC and Jilka RL. Cytokines, hematopoiesis, osteoclastogenesis and estrogens. *Calsif Tissue Int* 1992; **50**: 199-202

Manolagas SC. Birth and death of bone cells: basic regulatory mechanisms and implications for the pathogenesis and treatment of osteoporosis. *Endocr Rev* 2000; **21**:115–137

Manolagas SC, Kousteni S and Jilka RL. Sex steroids and bone. *Recent Prog Horm Res* 2002; **57**:385–409

Marshall CJ. Specificity of receptor tyrosine kinase signaling: transient versus sustained extracellular signal-regulated kinase activation. *Cell* 1995; **80:** 179-185

Martin SJ, O' Brien GA, Nishioka WK, McGahon AJ, Mahboubi A, Saido TC, Green DR (1995) Proteolysis of fodrin (non-erythroid spectrin) during apoptosis. J Biol Chem 270:6425–6428
Marquez D and Pietras R. Membrane-associated binding sites for estrogen contribute to growth regulation of human breast cancer cells. *Oncogene* 2001; **20:** 5420-5430

Marsaud V, Gougelet A, Maillard S and Renoir J-M. Various phosphorylation pathways, depending on agonist and antagonist binding to endogenous estrogen receptor alpha (ERalpha), differentially affect ERalpha extractability, proteasome-mediated stability, and transcriptional activity in human breast cancer cells. *Mol Endo* 2003; **17:** 2013-27

Marzo I., Brenner C., Zamzami M., *et al*. Bax and adenine nucleotide translocator cooperate in the mitochondrial control of apoptosis. *Science* 1998; **281**: 2027-2031

Mashima T, Naito M, Fujita N, Noguchi K, Tsuruo T (1995) Identification of actin as a substrate of ICE and an ICE-like protease and involvement of an ICE-like protease but not ICE in VP-16–induced U937 apoptosis. Biochem Biophys Res Commun 217:1185–1192

Mastro A, Gay C, Welch D, Donahue H, Jewell J, Mercer R, Digirolamo D, Chislock E and Guttridge K. Breast cancer cells induce osteoblast apoptosis: a possible contributor to bone degradation. *J Cell Biochem* 2004; **91**: 265-276

Mathiasen I and Jäättelä M. Triggering caspase-independent cell death to combat cancer. *Trends in Mol Med* 2002; **8**: 212-220

Mattson MP. Apoptosis in neurodegenerative disorders. *Nat Rev Mol Cell Biol* 2000; **1:** 120-129

McDonnell DP. The molecular pharmacology of SERMs. *Trends Endocrinol Metab* 1999; **76**: 301-311

Mckenna NJ, Nawaz Z, Tsai SY, Tsai H-J and O'malley BW. Distinct steady state nuclear receptor co-regulator complexes exist *in vivo*. *Proc Natl Acad Sci USA* 1998; **95**: 11697-11702

Mercer R, Miyasaka C and Mastro A. Metastatic breast cancer cells suppress osteoblasts adhesion and differentiation. *Clin Exp Metastasis* 2004; **21**: 427-435

Messalli E, Mainini G, Scaffa C, Cafiero A, Salzillo PL, Ragucci A and Cobellis L. Raloxifene therapy interacts with serum osteoprotegerin in postmenopausal women. *Maturitas* 2007; **56:** 38-44

Michael H, Härkönen PL, kangas L, Väänänen HK and Hentunen TA. Differential effects of selective oestrogen receptor modulators (SERMs) on human osteoclasts in vitro. *Br J Pharm.* 2007: 1-12

Migliaccio A, Di Domenico M, Castoria G, deFalco A, Bontempo P, Nola E and Auricchio F. Tyrosine kinase/p21 ras/ Map-kinase pathway activation by estradiol-receptor complex in MCF-7 cells. *EMBO J* 1996; **15:** 1292-1300

Mignotte B and Vayssiere JL. Mitochondria and apoptosis. *Eur J Biochem* 1998; **252**: 1–15

Monje P and Boland R. Subcellular distribution of native estrogen receptor alpha and beta isoforms in rabbit uterus and ovary. *J Cell Biochem* 2001; **82**: 467-479

Monroe D, Getz B, Johnsen S, Riggs B, Khosla S and Spelsberg T. Estrogen receptor isoform-spesific regulation of endogenous gene expression in human osteoblastic cell lines expressing either ERα or ERβ. *J Cell Biochem* 2003; **90:** 315-326

Moreira PI, Custodio J, Moreno A, Oliveira CR and Santos MS. Tamoxifen and estradiol interact with the flavin mononucleotide site of complex I leading to mitochondrial failure. *J Biol Chem* 2006; **14**:10143-10152

Moreira P, Custódio J, Nunes E, Moreno A, Seiça R, Oliveira C and Santos M. Estradiol affects liver mitochondrial function in ovariectomized and tamoxifen-treated ovariectomized female rats. *Toxicol Appl Pharmacol* 2007; **221**: 102-110

Mosselman S, Polman J and Dijkema R. ER BETA: identification and characterization of a novel human estrogen receptor. *Febs Lett* 1996; **392**: 49–53

Murphy LO, smith S, Chen RH, Fingar DC and Blenis J. Molecular interpretation of ERK signal duration by immediate early gene products. *Nat Cell Biol* 2002; **4(8):** 556-64

Nakashima T, Sasaki H, Tuboi M, Kawakami A, Fyjiyama K and Kiriyama T. Inhibitory effect of glugocorticoid for osteoblast apoptosis induced by activated peripheral blood mononuclear cells. *Endocrinology* 1998; **139**: 2032-2040

Namba R, Young LJ, Maglione JE *et al*. Selective estrogen receptor modulators inhibit growth and progression of premalignant lesions in a mouse model of ductal carcinoma in situ. *Breast Cancer Res* 2005; **7**:R881-R889

Nazarewicz RR, Zenebe WJ, Parihar A, Larson SK, Alidema E, Choi J and Ghafourifar P. Tamoxifen induces oxidative stress and mitochondrial apoptosis via stimulating mitochondrial nitric oxide synthase. *Cancer Res* 2007; **67**:1282-1290

Nemere I, Pietras RJ AND Blackmore PF. Membrane receptors for steroid hormones: signal transduction and physiological significance. *J Cell Biochem* 2003; **88**: 438-455

Nethrapalli IS, Tinnikov AA, Krishnan V, Lei CD and Toran-Allerand CD. Estrogen activates mitogen-activated protein kinase in native, nontransfected cho-k1, cos-7, and rat2 fibroblast cell lines. *Endocrinology* 2005; **146**:56-63

Newcomb PA, Longnecker MP, Storer BE, Mittendorf R, Baron J, Clapp RW, Bogdan G and Willett WC. Long-term hormone replacement therapy and risk of breat cancer in postmenopausal women. *Am J Epidemiol* 1995; **142**: 788-795

Nguyen DX and Massague J. Genetic Determinants Of Cancer Metastasis. *Nat Rev Genet* 2007; **8**: 341-52

Nicholson DW, Thornberry NA. Caspases: killer proteases. *Trends Biochem Sci* 1997; **22** :299–306

Nicholson R, McLelland R, Robertson J and Gee J. Involvement of steroid hormone and growth factor crosstalk in endocrine response in breast cancer. *Endocr Relat Cancer* 1999; **6:** 373-387

Obrero M, Yu DV and Shapiro DJ. Estrogen receptor-dependent and estrogen recptor -independent pathways for tamoxifen and 4-hydroxytamoxifen-induced programmed cell death. *J Biol Chem* 2002; **277:** 45695-45703

Ogawa S, Inoue S, Watanabe T *et al.*, the complete primary structure of human estrogen receptor beta (her beta) and its heterodimerization with er alpha in vivo and in vitro. *Biochem Biophys Res Commun* 1998; **243**: 122–126

Olefsky JM. Nuclear receptor minireview series. *J Biol Chem* 2001; **276**: 36863-36864

Olivier S, Fillet M, Malaise M, Piette J, Bours V, Merville MP and Franchimont M. Sodium nitroprusside-induced osteoblast apoptosis is mediated by long chain ceramide and is decreased by raloxifene. *Biochem Pharmacol* 2005; **69**: 891-901

Orth K, Chinnaiyan AM, Garg M, Froelich CJ, Dixit VM (1996) The CED-3/ICE-like protease Mch2 is activated during apoptosis and cleaves the death substrate lamin A. J Biol Chem 271:16443–16446

Otto A, Paddenberg R, Schubert S and Mannhertz H. Cell-cycle arrest, micronucleus formation, and cell death in growth inhibition of MCF-7 breast cancer cells by tamoxifen and cisplatin. *J Cancer Res Clin Oncol* 1996; **22:** 603-612

Osborne CK, Bardou V, Hopp T, Chamness GC, Hilsembe SG, Fuqua SA, Wong J, Allred DC, Clark GM and Schiff R. Role of the estrogen receptor coactivator AIB1 (SRC-3) and HER-2/neu in Tamoxifen resistance in breast cancer. *J Natl Cancer Inst* 2003; **95:** 353-361

Paech K, Webb P, Kuiper GG, Nilsson S, gustafsson J, Kushner P and Scanlan TS. Differential ligand activation of estrogen receptors ERalpha and Erbeta at AP1 sites. *Science* 1997; **277**: 1508-1510

Pappas TC, Gametchu B and Watson CS. Membrane estrogen receptors identified by multiple antibody labeling and impeded-ligand binding. *FASEB J* 1995; **9:** 404-410

Pasqualini JR. The selective estrogen enzyme modulators in breast cancer: a review. *Biochim Biophys Acta* 2004; **1654**: 123-43

Patel R, Sharma C and Jordan VC. Optimizing the antihormonal treatment and prevention of breast cancer. *Breast Cancer* 2007; **14**: 113-122

Pedram A, Razandi M and Levin ER. Nature of functional estrogen receptors at the plasma membrane. *Mol Endocrinol* 2006; **20**: 1996-2009

Pedram A, Razandi M, Wallace DC and Levin ER. Functional estrogen receptors in the mitochondria of breast cancer cells. *Mol Biol Cell*. 2006; **17**: 2125-2137

Pedrero J, Del Rio B, Martinez-Campa C, Muramatsu M, Lazo P and Ramos S. Calmodulin is a selective modulator of estrogen receptors. *Mol Endo* 2002; **16(5)**: 947-960

Perillo B, Sasso A, Abbondanza C and Palumbo G. 17beta-estradiol inhibits apoptosis in MCF-7 cells, inducing bcl-2 expression via two estrogen-responsive elements present in the coding sequence. *Mol Cell Biol* 2000; **20:** 2890-2901

Perry R, Kang Y and Greaves B. Effects of tamoxifen on growth and apoptosis of estrogen-dependent and –independent human breast cancer cells. *Ann Surg Oncol* 1995; **2:** 238-245

Peyssonaux C and Eychene A. The Raf/MEK/ERK pathway: new concepts of activation. *Bio Cell* 2001; **93: 53-62**

Pietras RJ and Szego CM. Endometrial cell calcium and oestrogen action. *Nature* 1975; **253:** 357-359

Pietras RJ and Szego CM. Metabolic and proliferative responses to estrogen by hepatocytes selected for plasma membrane binding sites specific for estradiol-17beta. *J Cell Physiol* 1979; **98:** 145-159

Pietras RJ and Szego CM. Specific binding sites for oestrogen at the outer surfaces of isolated endometrial cells. *Nature* 1977; **265**: 69-72

Pike AC, Brzozovski AM, Hubbard RE, Bonn T, Thorsell AG, Engström O, Ljunggren J, Gustafsson JA and Carlquist M. Structure of the ligand-binding domain of oestrogen receptor beta in the presence of a partial agonist and a full antagonist. *Embo* J 1999; **18:** 4608-4618

Pines A. Lessons from RUTH. *Climacteric* 2006; **9**:323-324

Planas-Silva MD, Bruggeman RD, Grenko RT and Smith JS. Overexpression of c-Myc and Bcl-2 during progression and distant metastasis of hormone-treated breast cancer. *Exp Mol Pathol.* 2007; **82**: 85–90

Powell C, Soto A and Sonnenschein C. Identification and characterization of membrane estrogen receptor from MCF-7 estrogen-target cells. *J Steroid Biochem Mol Biol* 2001; **77:** 97-108

Qu Q, Härkönen PL and Väänänen HK. Comparative effects of estrogen and antiestrogens on differentation of osteoblasts in mouse bone marrow culture. *J Cell Biochem* 1999; 73: 500-507

Rahko E, Blanco G, Bloigu R, Soini Y, Talvensaari-Mattila A and Jukkola A. Adverse outcome and resistance to adjuvant antiestrogen therapy in node-positive postmenopausal breast cancer patients-the role of p53. *Breast* 2006; **15**: 69–75

Razandi M, Pedram A, Greene GL and Levin ER. Cell membrane and nuclear estrogen receptors (ERs) originate from single transcript: Studies of ERα and ERβ expressed in Chinese hamster ovary cells. *Mol Endo* 1999; **13:** 307-319

Razandi M, Pedram A, Park ST and Levin ER. Proximal events in signalling by plasma membrane estrogen receptors. *J Biol Chem* 2003; **278:** 2701-2712

Razandi M, Pedram A, Merchenthaler I, Greene G and Levin E. Plasma membrane estrogen receptors exist and function as dimers. *Mol Endo* 2004; **18**: 2854-2865

Reccia I, Rucci N, Funari A, Migliaccio S, Taranta A, Longo M, Kneissel M, Susa M, Fabbro D and Teti A. Reduction of c-Src activity by substituted 5,7-diphenyl-pyrrolo[2,3-d]-pyrimidines induces osteoclast apoptosis in vivo and in vitro. Involvement of ERK1/2 pathway. *Bone* 2004; **34:** 65-79

Riggins RB, Bouton AH, Liu MC and ClarkeR. Antiestrogens, aromatase inhibitors, and apoptosis in breast cancer. *Vitam Horm* 2005; **71**: 201–237

Riggs BL, Khosla S and Melton 3rd LJ. A unitary model for involutional osteoporosis: estrogen deficiency causes both type I and type II osteoporosis in postmenopausal women and contributes to bone loss in aging men. J Bone Miner Res 1998; **13**:763–773

Riggs BL, Khosla S and Melton LJ. Sex steroids and the construction and concervation of the adult skeleton. *Endocr Rev* 2002; **23**: 279-302

Rochefort H, Borgna JL and Evans E. Cellular and molecular mechanism of action of antiestrogens. *J Steroid Biochem* 1983; 19: 69-74

Russo J, Fernandez SV, Russo PA, Fernbaugh R, Sheriff FS, Lareef HM, garber J and Russo IH. 17-beta estradiol induces transformation and tumorigenesis in human braest epithelial cells. Faseb J *2006*; **20**: 1622-1634

Rutanen EM, Heikkinen J, halonen K, Komi J, Lammintausta R, and Ylikorkala O. Effects of ospemifene, a novel SERM, on hormones, genital tract, climateric symptoms, and quality of life in postmenopausal women: a double-blind, randomized trial. *Menopause* 2003; **10**: 433-439

Rutqvist LE, Johansson H, Signomklao T *et al.* Stockholm breast cancer study group. Adjuvant Tamoxifen therapy for early stage braest cancer and second primary malignancies. *J Natl cancer Inst* 1995; **87**: 645-651

Safe S. Transcriptional activation of genes by 17β-estradiol through estrogen receptor-sp1 interactions. *Vitam Horm* 2001; **62**: 231-252

Saika M, Inoue D, Kido S and Matsumoto T. 17β-estrdiol stimulates expression of osteoprotegerin by a mouse stromal cell line, ST-2, via estrogen receptor-α. *Endocrinology* 2001; **142**: 2205-2212

Sakahira H, Enari M, Nagata S (1998) Cleavage of CAD inhibitor in CAD activation and DNA degradation during apoptosis. Nature 391:96–99

Salami S and Karami-Tehrani F. Biochemical studies of apoptosis induced by tamoxifen in estrogen receptor positive and negative breast cancer cell lines. *Clin Biochem* 2003; **36**: 247-53

Santen RJ, Song RX, Mcpherson R, Kumar R, Adam L, Jeng MH and Yue W. The role of mitogen-activated protein (map) kinase in breast cancer. *J Steroid Biochem Mol Biol* 2002; **80**: 239-56

Santen RJ, Song RX, McPherson R, Kumar R, Adam L, Jeng MH and Yue W. The role of mitogen-activated protein (MAP) kinase in breast cancer. *J Steroid Biochem Mol Biol.* 2002; **80(2):** 239-56

Sar M and Welsch F. Differential expression of estrogen receptor-beta and estrogen receptor-alpha in the rat ovary. *Endocrinology* 1999; **140**: 963–971

Saville B, Wormke M, Wang F, Nguyen T, Enmark E, Kuiper G, *et al*. Ligand-, cell-, and estrogen receptor subtype (α/β)-dependent activation at gc-rich (sp1) promoter elements. *J Biol Chem* 2000; **275**: 5379-5387

Schiff R and Osborne CK. New insights into estrogen receptor-α function and its implications for endocrine therapy resistance in breast cancer. *Breast Cancer Res* 2005; **7**: 205-211

Schiff R, Massarweh S, Shou J and Osborne CK. Breast cancer endocrine resistance: how growth factor signaling and estrogen receptor coregulators modulate response. *Clin Cancer Res* 2003; **9**: 447S-454S

Schwartz Z, Sylvia VL, Guinee T, Dean DD and Boyan BD. Tamoxifen elicts its anti-estrogen effects in growth plate chondrocytes by inhibiting protein kinase C. *J Steroid Biochem Mol Biol* 2002; **80:** 401-10

Schweichel JU and Merker HJ. The morphology of various types of cell death in prenatal tissues. *Teratology* 1973; **7**: 253–66

Shaul YD and Seger R. The MEK/ERK cascade: From signaling specificity to diverse functions. *Biochem Biophys Acta;* 2006 (in press).

Sheikh MS, Fornace AJ Jr (2000) Death and decoy receptors and p53-mediated apoptosis. Leukemia 14:1509–1513

Shoker BS, Jarvis C, Clarke RB, Anderson E, Hewlett J, Davies MP, *et al.* Estrogen receptor-positive proliferating cells in the normal and precancerous breast. *Am J Pathol* 1999; **155**: 1811-1815

Shou J, Massarweh S, Osborne CK, Wakeling AE, Ali S, Weiss H and Schiff R. Mechanisms of Tamoxifen resistance: Increased estrogen receptor-HER2/neu cross-talk in ER/HER2-positive breast cancer. *J Natl Cancer Inst* 2004; **96:** 926-935

Shupnik MA. Crosstalk between steroid receptors and the c-src-receptor tyrosine kinase pathways: implications for cell proliferation. *Oncogene* 2004; **23**:7979-7989

Simstein R, Burow M, Parker A, Weldon C and Backman B. Apoptosis, chemoresistance, and breast cancer: Insights from the MCF-7 cell model system. *Exp Biol Med* 2003; **228**: 995-1003

Singh M, Stringfellow H, Paraskevaidis E, Martin-Hirch P and Martin F. Tamoxifen: Important conciderations of a multi-functionla compound with organ-spesific properties. *Cancer Treat Rev* 2007; **33**: 91-100

Sipilä H, Kangas L, Vuorilehto L, Kalapudas A, Eloranta M, Sodervall M, Toivola R and Anttila M. metabolism of toremifene in the rat. *J Steroid Biochem* 1990; **36**: 211–215

Smith CL. Cross-talk between peptide growth factor and estrogen receptor signaling pathways. *Biol Reprod* 1998; **58**: 627-632

Somai S, Chaouat M, Jacob D, *et al*. Antiestrogens are pro-apoptotic in normal human breast epithelial cells. *Int J Cancer* 2003; **105**: 607-12

Song R and Santen RJ. Membrane initiated estrogen signalling in breast cancer. *Biol Reprod* 2006; **75**: 9-16

Song R, McPherson R, Adam L, Bao Y, Shupnik M, Kumar R and Santen R. Linkage of rapid estrogen action to MAPK activation by ERα-Sch association and Shc pathway activation. *Mol Endo* 2002; **16(1):** 116-127

Song RX, Barnes CJ, Zhang Z, Bao Y, Kumar R and Santen RJ. The role of shc and insulin-like growth factor 1 receptor in mediating the translocation of estrogen receptor alpha to the plasma membrane. *Proc Natl Acad Sci USA* 2004; **101**:2076-2081

Song RX, Mcpherson RA, Adam L, Bao Y, Shupnik M, Kumar R and Santen RJ, linkage of rapid estrogen action to mapk activation by erα-shc association and shc pathway activation. *Mol Endocrinol* 2002; **16**:116-127

Sperandio S, de B I, Bredesen DE. An alternative, nonapoptotic form of programmed cell death. Proc Natl Acad Sci U S A 2000;**97**:14376–81

Speroff L. Postmenopausal hormone therapy and breast cancer. *Obstet Gynecol* 1996; **87**: 44S-54S

Stanciu M and DeFranco DB. Prolonged nuclear retention of activated extracellular signal-regulated protein kinase promotes cell death generated by oxidative toxicity or proteasome inhibition in a neuronal cell line. *J Biol Chem* 2002; **277:** 4010-4017

Steinberg KK, Thacker SB, Smith SJ, Stroup DF, Zack MM, Flanders WD and Berkelman RL. A meta-analysis of the effect of estrogen replacement therapy on the risk of breast cancer. Jama 1991; **265**: 1985-1990

Strasser A, O´Connor L and Dixit VM. Apoptosis signaling. *Annu Rev Biochem* 2000; **69:** 217-245

Sun J, Meyers MJ, Fink BE, Rajendran R, Katzenellenbogen JA and Katzenellenbogen BS. Novel ligands that function as selective estrogens or antiestrogens for estrogen receptor-alpha or estrogen receptor-beta. *Endocrinology* 1999; **140**: 800-804

Sutherland MK, Hui DU, Rao LG, Wylie JN and Murray TM. Immunohistochemical localization of the estrogen receptor in human osteoblastic SaOS-2 cells, association of receptor levels with alkaline phosphatase activity. *Bone* 1996; **18**: 361-369

Syed F and Khosla S. Mechanisms of sex steroid effects on bone. *Biochem Biophys Res Commun* 2005; **328**: 688-696

Tang D, Wu D, Hirao A, Lahti JM, Liu L, Mazza B, Kidd VJ, Mak TW and Ingram AJ. ERK activation mediates cell cycle arrest and apoptosis after DNA damage independently of p53. *J Biol Chem* 2002; **277:** 12710-12717

Taranta A, Brama M, teti A, De Luca V, Scandurra R, Spera G, Agnusdei D, termine J and Migliaccio S. The selective estrogen receptor modulatorm Raloxifene regulates osteoclast and osteoblasts activity in vitro. *Bone* 2002; **30**: 368-376

Taylor AH and Al-AzzawI F. Immunolocalisation of oestrogen receptor beta in human tissues. *J Mol Endocrinol* 2000; 24: 145–155

Thiantanawat A, Long BJ and Brodie AM. Signaling pathways of apoptosis activated by aromatase inhibitors and antiestrogens. *Cancer Res* 2003; **63**: 8037-50

Thomas P, Pang Y, Filardo EJ and Dong J. Identity of an estrogen membrane receptor coupled to a G protein in human breast cancer cells. *Endocrinology* 2005; **146**:624-632

Thomas W, Coen N, Faherty S, O Flatharta C and Harvey B. Estrogen induces phospholipase A_2 activation through ERK1/2 to mobilize intracellular calcium in MCF-7 cells. *Steroids* 2006; **71:** 256-265

Thornberry NA, Lazebnik Y (1998) Caspases: enemies within. Science 281:1312–1316

Thornberry NA, Rano TA, Peterson EP, Rasper DM, Timkey T, Garcia CM, Houtzager VM, Nordstrom PA, Roy S, Vaillancourt JP, Chapman KT, Nicholson DW (1997) A combinatorial approach defines specificities of members of the caspase family and granzyme B. Functional relationships established for key mediators of apoptosis. J Biol Chem 272:17907–17911

Toran-Allerand CD, Guan X, Maclusky NJ, Horvath TL, Diano S, Singh M, Connolly ES, Jr, Nethrapalli IS and Tinnikov AA. ER-x: a novel, plasma membrane-associated, putative estrogen receptor that is regulated during development and after ischemic brain injury. *J Neurosci* 2002; **22**:8391-8401

Tozava K, Okamoto T, Hayashi Y, Sasaki S, Kawai N and Kohri K. N-acetyl_L-cysteine enhances chemotherapeutic effect on prostate cancer cells. Urol Res 2002; **30**: 53-58

Tremblay GB, Tremblay A, Copeland NG *et al.*, cloning, chromosomal localization, and functional analysis of the murine estrogen receptor beta. *Mol Endocrinol* 1997; **11**: 353–365

Tuquet C, Dupont J, Mesneau A and Roussaux J. Effects of tamoxifen in electron transport chain of isolated rat liver mitochondria. *Cell Biol Toxicol* 2000; **16:** 207-219

Unal-Cevik I, Kilinc M, Can A, Gursoy-Ozdemir Y and Dalkara T. Apoptotic and necrotic death mechanisms are concomitantly activated in the same cell after cerebral ischemia. *Stroke* 2004;**35**:2189–94

Urayama S, Kawakami A, Nakashima T, Tsuboi M, Yamasaki S, Hida A, Ichinose Y, Nakamura H, Ejima E, Aoyagi T, Nakamura T, Migita K, Kawabe Y and Eguchi K. Effect of vitamin K2 on osteoblast apoptosis: vitamin K2 inhibits apoptotic cell death of human osteoblasts induced by Fas, proteasome inhibitor, etoposide, and staurosporine. *J Lab Clin Med* 2000; **136**: 181-193

Valavaara R. Phase II trials with toremifene in advanced breast cancer; a review. Breast *Cancer Res Treat* 1990; **16** suppl: S31-S35

Van Leeuwen FE, Benraadt J, Coebergh JW, *et al.* Risk of endometrial cancer after Tamoxifen treatment of breast cancer. *Lancet* 1994; **343**: 448-452

Vasudevan N, Kow LM and Pfaff DW. Early membrane estrogenic effects required for full expression of slower genomic actions in nerve cell line. *Proc Natl Acad Sci* 2001; **98:** 12267-12271

Verdenq Wood LJ, Nail LM, Perrin NA, Elsea CR, Fisher A and Drucker BJ. The cancer chemotherapy drug etoposide (VP-16) induces proinflammatory cytokine production and sickness behavior-like symptoms in a mouse model of cancer chemotherapy-related symptoms. *Biol Res Nurs* 2006; **8**: 157-169

Vermeulen K, Van Bockstaele DR and Berneman ZN. Apoptosis: mechanisms and relevance in cancer. *Ann Hematol* 2005; **84** :627-39

Vidal O, Kindblom LG and Ohlsson C. Expression and localization of estrogen receptor-beta in murine and human bone. *J Bone Miner Res* 1999; **14:** 923-929

Viereck V, Grundker C, Blascheke S, Niederkleine B, Siggelkow H, froch K-H, Raddatz D, Emons G and Hofbauer L. Raloxifene concurrently stimulates osteoprotegerin and inhibits interleukin-6 production by human trabecular osteoblasts. *J Clin Endocr Metab* 2003; **88**:4206-4213

Visram H and Greer PA. 17beta-estradiol and Tamoxifen stimulate rapid and transient ERK activation in MCF-7 cells via distinct signaling mechanisms. *Cancer Biol Ther* 2006; **5**: 1677-1682

Vladusic E, Hornby A, Guerra-Vladusic F and Lupu R. Expression of estrogen receptor beta messenger RNA variant in breast cancer. *Cancer Res* 1998; **58:** 210-214

Voegel JJ, Heine MJ, Zechel C, Chambon P and Gronemeyer H. TIF2, a 160 kDa transcriptional mediator for the ligand-dependent activation function AF-2 of nuclear receptors. *Embo J* 1996; **15**: 3667-3675

Vogel VG, Costantino JP, Wickerham L *et al.*Effects of tamoxifen vs raloxifene on the risk of developing invasive braest cancer and other disease outcomes. *JAMA* 2007; 295; 2727-2926

VoipioSK, Komi J, Kangas L, Halonen K, DeGregorio MW and Erkkola RU. Effects of ospemifene (FC-1271a) on uterine endometrium, vaginal maturation index, and hormonal status in healthy postmenopausal women. *Maturitas* 2002; **43**: 207-214

Walter P, Green S, Greene G *et al.* Cloning of the human estrogen receptor cdna. *Proc Natl Acad Sci USA* 1985; **82**: 7889–7893

Wallace KB and Starkov AA. Mitochondrial targets of drug toxicity. *Annu Rev Pharmacol Toxicol* 2000; **40**: 353–388

Watts CK, Sweeney KJ, Warlters A, Musgrove EA and Sutherland RL. Antiestrogen regulation of cell cycle progression and cyclin D1 gene expression in MCF-7 human breast cancer cells. *Breast Cancer Res Treat* 1994; 31: 95-105

Webb P, Lopez GN, Uht RM and Kushner PJ. Tamoxifen activation of the estrogen receptor/ap-1 pathway: potential origin for the cell-specific estrogen-like effects of antiestrogens. *Mol Endocrinol* 1995; **9**: 443-456

Weihua Z, Warner M and Gustafsson JA. Estrogen receptor beta in the prostate. *Mol. Cell Endocrinol.* 2002; **193**: 1–5

Weinstein RS SC and Manolagas. Apoptosis and osteoporosis *Am J Med* 2000; **108**: 153-164

Weinstein RS, Jilka RL, Parfitt AM and Manolagas SC. Inhibition of osteoblastogenesis and promotion of apoptosis of osteoblasts and osteocytes by glucocorticoids. Potential mechanisms of their deleterious effects on bone. *J Clin Invest* 1998; **102**: 274-282

Wingo PA, Layde PM, Lee NC, Rubin g and Ory HW. The risk of breast cancer in postmenopausal women who have used estrogen replacement therapy. *JAMA* 1987; **257**: 209-215

Woodhouse E, Chuaqui R and Liotta L.General mechanisms of metastasis. *Cancer* 1997; **80**: 1529-1537

Wurz GT, Read KC, Marchisano-Karpman C, Gregg JD, Beckett LA, Yu Q and Degregorio MW. Ospemifene inhibits the growth of dimethylbenzanthracene-induced mammary tumors in Sencar mice.*J Steroid Biochem Mol Biol* 2005; **97**:230-40

Wyllie AH, Kerr JF and Currie AR. Cell death: the significance of apoptosis. *Int Rev Cytol* 1980; **68**: 251–306

Wärri AM, Huovinen RL, Laine AM, Martikainen PM, Harkonen PL. Apoptosis in toremifene-induced growth inhibition of human breast cancer cells in vivo and in vitro. *J Natl Cancer Inst* 1993; **85**:1412-1418

Yang C, Lin H, Chen C, *et al*. Bcl-X_L mediates a survival mechanism independent of the phosphoinositide 3-kinase7Akt pathway in prostate cancer cells. *J Biol Chem* 2003; **28:** 25872-25878

Yang J, Xuesong L, Bhalla K, *et al*. Prevention of apoptosis by bcl-2: Release of cytochrome c from mitochondria blocked. *Science* 1997; **275:** 1129-1132

Yang SH, Liu R, Perez EJ *et al*. Mitochondrial localization of estrogen receptor β. *Proc Natl Acad Sci* USA 2004; **101**: 4130-4135

Ylikorkala O, Cacciatore B, Halonen K, Lassila R, lammintausta R, Rutanen EM, Heikkinen J and Komi J. Effects of ospemifene, a novel SERM, on vascular markers and function in healthy, postmenopausal women. *Menopause* 2003; **10**:440-447

Yuan J. Evolutionary conservation of a genetic pathway of programmed cell death. *J Cell Biochem* 1996; **60**: 4–11

Zallone A. Direct and indirect estrogen actions on osteoblasts and osteoclasts. *Ann N Y Acad Sci* 2006;**1068**:173-179

Zapata JM, Krajewska M, Krajewski S, *et al*. Expression of multiple apoptosis-regulatory genes in human breast cancer cell lines and primary tumors. *Breast Cancer Res Treat*1998; **47:** 129-140

Zhang S, Cao H, Davis F, Tang HY, Davis PJ and Lin HY. Oestrogen inhibits resveratrol-induced post-translational modification of p53 and apoptosis in breast cancer cells. *Br J Cancer* 2004; **5:91:** 178-85

Zhang W and Liu H. MAPK signal pathways in the regulation of cell proliferation in mammalian cells. *Cell Res* 2002; **12:** 9-18

Zhang W, Couldwell WT, Song H, Takano T, Lin JH and Nedergaard M. Tamoxifen-induced enhancement of calcium signaling in glioma and mcf-7 breast cancer cells. *Cancer Res* 2000; 60: 5395-5400

Zhang Y, Touneksti O, Akerman B, Goodyer C and LeBlanc A. 17-beta-estradiol induces an inhibitor of active caspases. *J Neurosci* 2001; **21:** RC176

Zhang Z, Maier B, Santen RJ and Song RX. Membrane association of estrogen receptor alpha mediates estrogen effect on mapk activation. *Biochem Biophys Res Commun* 2002; 294:926-933

ORIGINAL PUBLICATIONS