

# Interactions between radiation and endocrine therapy in breast cancer

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## Abstract

Adjuvant radiotherapy and adjuvant endocrine therapy are commonly given to patients with invasive breast cancer or with ductal carcinoma *in situ* (DCIS). Although both therapies have been well established through a number of randomized studies, little is known about a possible interaction of both treatment modalities if they are given simultaneously. A number of *in vitro* studies have indicated that tamoxifen treatment might reduce the intrinsic radiosensitivity of MCF-7 breast cancer cells. Conversely, estradiol treatment increases the intrinsic radiosensitivity of MCF-7 cells. In one available animal study, an antagonistic effect of tamoxifen and ionizing radiation (XRT) could not be observed. Retrospective analyses of randomized clinical studies have not indicated an antagonistic effect of tamoxifen on the effectiveness of XRT, since local control has been consistently higher when XRT was combined with tamoxifen, compared with treatment with XRT alone, regardless of whether tamoxifen was started simultaneously with radiotherapy or after completion of radiotherapy. Currently there are no clinical data available that would suggest an adverse effect of adjuvant tamoxifen treatment started prior to or simultaneously with radiotherapy in breast cancer or DCIS. However, since an antagonistic effect of tamoxifen and simultaneous chemotherapy has been reported recently, the issue of simultaneous versus sequential radiation and tamoxifen treatment in breast cancer should be addressed in further studies.

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## Introduction

Radiotherapy and endocrine therapy are both of major importance in the adjuvant treatment of breast cancer. Surprisingly, very few investigations are available on the possible interaction of endocrine therapy and ionizing radiation (XRT), if both are given simultaneously. This interaction could be additive, synergistic or antagonistic. Indeed, several studies have been published with conflicting results. Some *in vitro* studies have indicated that incubation of breast cancer cell lines with tamoxifen might induce an intrinsic radioresistance. Others have found an additive mode of interaction. Recent clinical studies in ductal carcinoma *in situ* (DCIS) have shown a positive effect of the combined treatment with tamoxifen and XRT on local recurrent-free survival. Due to these conflicting results, clinicians are often uncertain as to how to combine both treatment modalities.

This review will summarize the *in vitro* studies on the interaction of endocrine therapy and XRT in tumor cells and in non-malignant tissues. Possible mechanisms of interaction will be discussed. Secondly, the investigations in animal

experiments that are available will be presented. Finally, evidence from clinical studies will be compared with experimental results in order to develop a hypothesis for further studies and for the clinical application of both treatment modalities in breast cancer.

The interaction of endocrine therapy and chemotherapy will be covered briefly as far as clinical data are available. The possible *in vitro* interactions of cytostatic drugs with estrogen or tamoxifen in breast cancer would surpass the scope of this review.

## Abbreviations and nomenclature

Different names for genes and their products have been used in the literature. For standardization, nomenclature committees are maintaining different databases (e.g. Human Gene Nomenclature at [www.gene.ucl.ac.uk](http://www.gene.ucl.ac.uk) or Gene Cards at [www.bioinfo.weizmann.ac.il](http://www.bioinfo.weizmann.ac.il)). In this review, we use the most common synonyms. Table 1 shows these together with their standardized names.

**Table 1** Common synonyms and their standardized names.

Synonym	Standardized name
ATM	Ataxia telangiectasia mutated
ATR	Ataxia telangiectasia and Rad3-related
BRCA	BRCA1 (breast cancer susceptibility protein)
Cdc25A	CDC25A
Cdc25C	CDC25C
Cdk4	CDK4 (cyclin-dependent kinase)
Chk2	CHEK2 (checkpoint kinase 2)
CyclinA	CCNA
CyclinD	CCND
CyclinE	CCNE
DNA-PK	DNA-dependent protein kinase
Mdm2	MDM2 (mouse double minute 2)
NBs	NBS1 (Nijmegen breakage syndrome)
PIKK	KIP2 (phosphoinositide 3-kinase related kinases)
p21	CDKN1A
p27	CDKN1B
p53	TP53 (protein)
Rb	RB1 (retinoblastoma protein)

## Materials and methods

A medline search has been performed using the following combinations of key words: radiation and hormones; chemotherapy and hormones; radiation and endocrine treatment and breast cancer. Review articles on the subcellular action of XRT, tamoxifen and estrogen have been used.

## Results

### The concept of intrinsic radiation sensitivity

The cellular response after exposure to XRT is characteristic for each cell line. The portion of cells surviving a certain radiation dose is a measure of its intrinsic radiation sensitivity. This term is a description of the action of radiation on a cellular level, which corresponds to *in vitro* investigations. The curability of a tumor by XRT is determined by further important factors such as tumor oxygenation and repopulation of tumor cells after each fraction of XRT. Since the interaction of estrogens and anti-estrogens with XRT has mostly been studied *in vitro*, knowledge about the cellular response to XRT is most important for the purpose of this review.

At least four possible consequences of radiation interaction with cells can affect the long-term reproductive viability of the cell or its progeny: necrosis, apoptosis, accelerated senescence, and terminal differentiation. If none of these occurs, or if the radiation-induced damage of the cells can be repaired, the cell may survive without undergoing alterations in the divisional process.

A cell that is damaged by XRT and loses its reproductive integrity may divide once or more often before all the progeny are rendered reproductively sterile. Possible

consequences to the cell may be a rapid death by apoptosis, death during the next attempt at cell division, unusual forms as a result of aberrant attempts at division, or it may stay as it is, unable to divide, but physiologically functional for a long period. Such functional but sterile cells do not appear to be different from fertile cells. Some of these may be terminally differentiated cells. Irradiated cells may also divide, giving rise to one or more generations of daughter cells before some or all of the progeny become sterile. If some reproductively viable progeny emerge in those colonies, the colonies may regrow. All of these changes must be kept in mind when the endpoints of *in vitro* assays for XRT-induced cellular damage are evaluated.

In order to assess the radiosensitivity of tumor cells *in vitro*, colony-forming assays are performed after irradiation of the cells with different test doses. Survival is determined by the ability of the surviving cells to form macroscopic colonies within a given time (usually 7–14 days). The percentages of surviving cells are plotted against the doses given. The semilogarithmic plot of cellular survival (S) as a function of dose (D) is called a ‘survival curve’, which is specific for each tumor cell line, and which is best described by a linear quadratic model with the following formula  $S = e^{-(\alpha D + \beta D^2)}$ . The  $\alpha$  and  $\beta$  terms in this equation and their ratios are used to describe survival curve characteristics and to classify the cellular response to radiation (for review see Hellman 2001).

When cells are irradiated, lethal damage can occur, or the damage may be modified and not lead irrevocably to cell death. Such amelioration of radiation damage is called repair. If post-irradiation conditions are modified to allow repair, cells that would have died can be salvaged. In general, post-irradiation conditions that suppress cell division are the ones most favorable to the repair of potentially lethal damage. The influence of estrogens or anti-estrogens on cell cycle progression is probably a major factor for the interaction of endocrine therapy with the cellular repair of radiation damage.

The modification of the intrinsic radiosensitivity of tumor cells by chemical or biological influences can be divided into the following four categories: independent, additive, synergistic or antagonistic interaction (see Table 2).

In the *in vivo* situation, the modulation of the

**Table 2** Terminology of interaction between drugs and XRT.

Terminology	Description
Independence	The agents act independently, their mechanisms of damage are independent
Additivity	The agents act on the same loci, and therefore their sublethal and lethal damages are additive.
Synergism	The two agents have a result that is more effective than pure additivity.
Antagonism	The cell killing is less than independent action.

radiosensitiveness of a tumor is much more complex. Here the intrinsic radiosensitivity is only one of several factors which influence the curability of a tumor by XRT. The microenvironment of the cells, such as oxygenation, has a major influence on radiosensitivity. Angiogenesis and repopulation of tumor cells during fractionated irradiation are further determinants, which might be influenced in order to modify the curability of a tumor by XRT (see below).

## Radiation and apoptosis

Apoptosis is an important response to XRT in many cells. The proportion of cells undergoing apoptosis, rather than interrupting the cell cycle to repair radiation damage, may be a very important determinant for radiation curability of a tumor. Radiation-induced apoptosis follows several distinct pathways dependent on the time-course and the cell cycle position. Premitotic apoptosis is a rapid apoptotic cell death associated with the fast activation of caspase-3. In contrast, post-mitotic apoptosis is a delayed cell death which occurs after cell division and does not require the activation of caspase-3 (Shinomiya 2001). Certain normal cells, such as lymphocytes and germ cells, show apoptosis in response to very small doses of radiation. The reciprocal nature of radiation repair and apoptosis may explain the correlation between potentially lethal damage repair and radiocurability. Cells with a great capacity for potentially lethal damage repair have little apoptotic response to radiation. It may be the latter that is the determining characteristic (Rupnow *et al.* 1998). The loss of the apoptotic response seems to be correlated with tumor progression.

## Radiation, repair and the cell cycle

The cell cycle starts with a resting phase after previous cell division (G1, gap), followed by DNA replication to copy the DNA for the next division (S, synthesis). After a second resting phase (G2), the segregation of the DNA strands begins (M, mitosis). During the cell cycle, the integrity of the genome is regularly monitored by a complex network of different proteins. They can be divided roughly into a sensor, a transducer and an effector component (Abraham 2001, Kastan 2001). If genotoxic stress (e.g. double strand breaks (DSB) after XRT) is sensed, this is translated into a biochemical signal. Kinases and other regulatory proteins transduce the signal, until effector proteins are reached. Three different responses to damaged DNA are possible: apoptosis (programmed cell death), cell cycle arrest to gain time for sufficient DNA repair, and the formation of chromosomal aberrations as a consequence of misrepair (Abraham 2001). The latter might lead to cell death at the next cellular division. Cell cycle progression can be stopped at distinct phases: at the G1 checkpoint during transition from G1 to S phase, at the S checkpoint (throughout S phase) and at the

G2 checkpoint during transition from G2 to M (Kastan 2001). Failure of these controls and checkpoints leads to genomic instability, resulting in a predisposition to cancer and hypersensitivity to XRT.

One Gy (which is half of the daily dose given during conventional fractionation) is thought to cause about one DSB per chromosome. DSBs represent the damage which is most difficult to repair. Cells are able to detect DNA damage caused by XRT (for review see Lowndes & Murguia 2000).

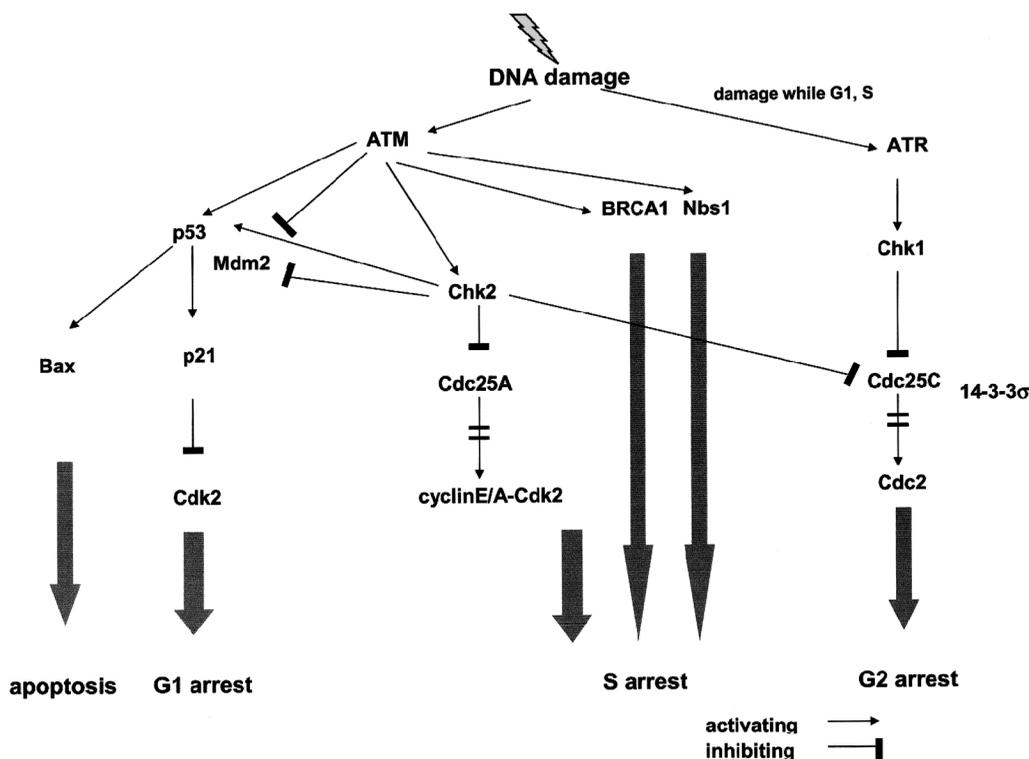
After the detection of abnormal DNA, biochemical signals are transduced by cascades of protein kinases to activate effector mechanisms. The apical key reaction is a catalytic activation of the ATM protein kinase (Kastan *et al.* 2001, Lavin & Shiloh 1997) (Fig. 1). ATM belongs to the family of PIKK (including DNA-PK and ATR among others). The mechanism whereby ATM is activated following XRT is still controversial (for review see Abraham 2001). There is evidence that activation is a matter of direct or indirect binding to DNA.

ATM is involved in the initiation of all cell cycle checkpoints following XRT-induced damage. ATR, another PIKK family member, can also phosphorylate most of the substrates, but is especially active after UV- or a high level of XRT-induced DNA damage (Hirao *et al.* 2000). Corresponding to the different checkpoints, ATM targets different substrates and thus can affect different pathways.

The first step of the G1-arrest pathway is a rapid rise in the p53 protein level (Banin *et al.* 1998, Canman *et al.* 1998). Multiple regulatory links between ATM and p53 have been shown. First of all, ATM phosphorylates p53 directly (Unger *et al.* 1999). Secondly, ATM-dependent activated Chk2 phosphorylates p53 on another specific site (Hirao *et al.* 2000). This interferes with the binding with Mdm2, a protein that exports p53 out of the nucleus for ubiquitination and degradation. Thirdly, ATM targets Mdm2 directly and modifies its activity (Maya *et al.* 2001). Thereby p53 accumulates in the nucleus mainly by stabilization. In addition, histone acetyltransferase p300 increases p53 transcription by an additional acetylation of p53 (Gu & Roeder 1997).

p53 acts as a transcriptional factor for a wide range of genes. Among others, it induces transcription of G1-Cdks (p21<sup>CIP1/WAF1</sup>) and several apoptosis genes (e.g. Bax) (Deng *et al.* 1995, Hirao *et al.* 2000). An increase in p21 expression suppresses Cdk2 activity, in this way delaying progression from the G1 to the S phase.

The crucial phase of the cell cycle before mitosis is the DNA replication during the S phase. Errors during the replication process itself may lead to misincorporation errors or stalled replication forks (Abraham 2001). On the other hand, the most important repair mechanism during this phase of the cell cycle is homologous recombination, which allows precise repair (for review see Paques & Haber 1999). Several parallel pathways have been identified to delay the S phase.



**Figure 1** Irradiation-induced DNA damage leads to the activation of ATM. The crucial step preceding the G1 checkpoint is the phosphorylation of p53 by ATM or Chk2, interfering with Mdm2 binding. Mdm2 activity can also be modified directly by Chk2. p53 induces transcription of p21, which binds to and inhibits cyclinE–Cdk2 and, by redistribution, cyclinD1–Cdk4 complexes. p53 can also induce apoptotic pathways via Bax. Multiple pathways induce S phase arrest. The target of ATM-dependent activated Chk2 is Cdc25A. After inhibiting phosphorylation, it can no longer activate cyclinE–Cdk2. Direct links between ATM, BRCA1, Nbs1 and S arrest have been shown. If DNA is damaged in G2, G2 arrest is induced by ATM–Chk2–Cdc25C. If the irradiation occurs earlier in the cell cycle, ATR is activated and targets Chk1, which phosphorylates Cdc25C. Phosphorylated Cdc25C is exported by 14–3–3 $\sigma$  out of the nucleus, thus preventing activation of Cdc2, the key kinase of G2/M progression.

The first pathway is mediated, like a G1 arrest, by ATM and Chk2 (Falck *et al.* 2001). The latter phosphorylates Cdc25A on a specific site to prime it for degradation. The downstream target cyclinA–Cdk2 complex is no longer activated by Cdc25A. Its function is to load Cdc45 onto pre-initiation complexes at the start of DNA replication (Zou & Stillman 2000). In this way, DNA synthesis is delayed to gain sufficient time for repair (Falck *et al.* 2001).

The second pathway is transduced via p95/Nbs1, another substrate of ATM (Wu *et al.* 2000, Falck *et al.* 2002). This gene product is mutated in patients with Nijmegen breakage syndrome (Carney *et al.* 1998, Lim *et al.* 2000) Like ataxia telangiectasia, this syndrome is based on defects of cell cycle checkpoints, leading to chromosomal instability and increased radiation sensitivity (Wu *et al.* 2000). The gene product p95/Nbs1 acts downstream of ATM, and its phosphorylation is critical for the transient inhibition of replication during the S phase (Lim *et al.* 2000). Interestingly, p95/Nbs1 operates together with two other DNA maintenance proteins, hMre11 and hRad50. They are involved in the

recombinational repair of DSBs (Carney *et al.* 1998). So this protein may function as a link between DNA repair mechanisms and cell cycle control.

A third pathway has been identified via the direct and indirect (Chk2) ATM-dependent activation of BRCA1 (Xu *et al.* 2001). This protein seems to be important in the surveillance of DNA replication, participating in checkpoint and repair pathways like p95/Nbs1. Its failure is commonly referred to as radioresistant DNA synthesis (RDS).

The last checkpoint before entering mitosis and last option to repair abnormal DNA is the G2 checkpoint. The signaling pathway for this checkpoint depends on the cell cycle phase during which the cell is irradiated (Xu *et al.* 2002). If DNA is damaged during G2, ATM activates Chk2 (Matsuoka *et al.* 2000). This kinase phosphorylates Cdc25C on an inhibitory residue. 14–3–3 $\sigma$  protein binds to phosphorylated Cdc25C and mediates the export of the complex out of the nucleus (Chan *et al.* 1999). Ultimately, the Cdc25C-dependent activation of Cdc2 is reduced and, as Cdc2 activation and accumulation in the nucleus is crucial

for the progression from the G2 phase into mitosis, the cell cycle stops at G2 (Matsuoka *et al.* 1998, Piwnica-Worms 1999). Interestingly, 14–3–3 $\sigma$  transcription is markedly increased by p53. So attenuation of the G2 checkpoint is p53 dependent (Piwnica-Worms 1999).

If DNA is damaged during an earlier phase of the cell cycle (G1, S), the activation of this cell cycle checkpoint is ATM independent. Instead, ATR phosphorylates protein kinase Chk1, leading to G2/M arrest via inactivation of Cdc25C (Liu *et al.* 2000, Abraham 2001).

### **Influence of estrogens on cell cycle progression in estrogen receptor (ER)-positive breast cancer cells**

ERs are initially expressed in 60–80% of all breast cancers (Osborne 1998). Two subtypes of this receptor, ER $\alpha$  and ER $\beta$ , have been defined (Kuiper *et al.* 1997). They differ in the ligand binding- but not in the DNA-binding domain. The expression seems to differ between different types of tissues. Interestingly, some estrogenic substances, in particular phytoestrogens, show different binding affinities to the ER subtypes (Kuiper *et al.* 1998). In addition, ER $\beta$  can modulate the transcriptional activity of ER $\alpha$  especially at low doses of 17 $\beta$ -estradiol (E2) (Hall & McDonnell 1999). In this review, we will focus on ER $\alpha$  (referred to as ER) and the most important estrogenic metabolite, E2.

E2 diffuses across cell membranes and binds to two independent activation domains with distinct binding characteristics (activation function (AF)-1 and AF-2) of the nuclear ER. A conformational change is induced by this process, which promotes its dimerization and enables DNA binding at estrogen-responsive elements (EREs) in promoter regions of a variety of genes (Hall & McDonnell 1999).

The most prominent effects of E2 are the recruitment of quiescent cells into G1, shortening the G1/S transition and an increase of about 50% in the S phase fraction of growth-arrested MCF-7 cells (Foster & Wimalasena 1996).

Cell cycle progress requires the sequential activation of different Cdk by the binding of specific cyclins (for review see Foster *et al.* 2001a). Entry into the G1 phase is mediated by Cdk4 and Cdk6, transition into and through the S phase by Cdk2 and the induction of mitosis by Cdk1. CyclinD binds to Cdk4/6, whereas cyclinA and cyclinE complex with Cdk2. Cdk-inhibiting proteins have also been identified. The INK4 family (including p16) inactivates Cdk4/6, whereas the CIP/KIP-family (e.g. p21) inhibits Cdk2 activity. Interestingly, p21 is redistributed by cyclinD–Cdk4 complexes, thus allowing cyclinA/E–Cdk2 complexes to mediate transition into the S phase (LaBaer *et al.* 1997).

At least two target genes of E2 which can initiate cell cycle progression at G1/S have been elucidated (Fig. 2).

In the first place, *myc* transcription is upregulated by the binding of ER to an atypical ERE in its promoter sequence.

Additionally, E2 can stabilize the MYC protein for several hours (Prall *et al.* 1998). MYC is an important regulator of cell proliferation and apoptosis. Among others, it induces the transcription of cyclinE and cyclinA (Foster *et al.* 2001b). These cyclins induce, together with Cdk2, the entry of cells into the S phase, although the contribution of each protein to this process remains controversial (Foster *et al.* 2001b). The retinoblastoma protein pRb is phosphorylated by the cyclinE/A–Cdk2 complex and releases transcription factor E2F, the crucial step preceding the S phase. Moreover, Cdc25A transcription is upregulated by MYC, a key activator of the cyclinE–Cdk2 complex. Vice versa, Cdc25A is activated by Cdk2 (Foster *et al.* 2001b).

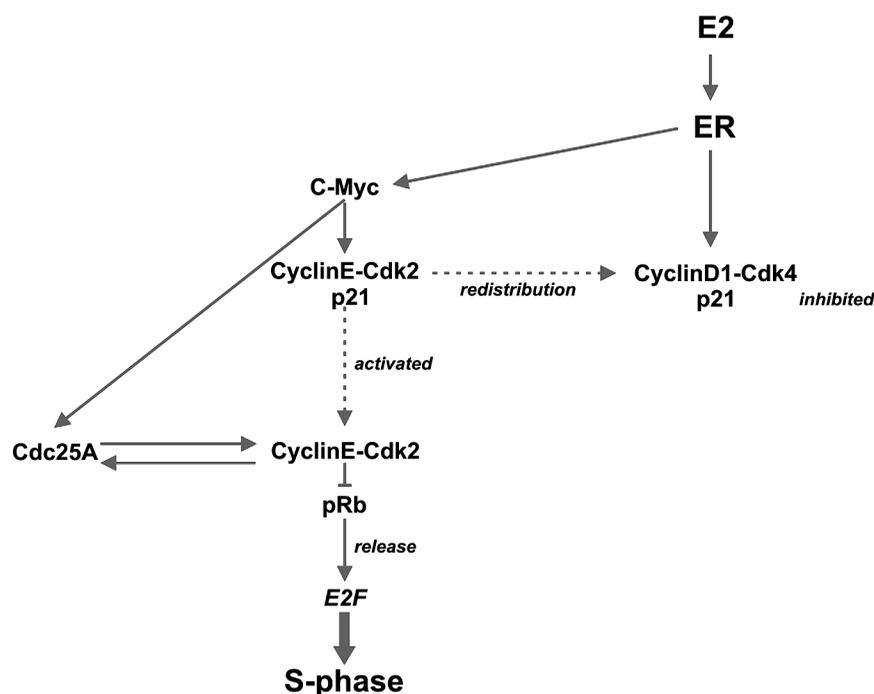
Secondly, E2 induces transcription of cyclinD via an imperfect ERE in the promoter (Foster & Wimalasena 1996). By forming a complex with Cdk4, p21 can be separated from the cyclinE–Cdk2 complex and redistributed towards cyclinD–Cdk4 (LaBaer *et al.* 1997, Planas-Silva & Weinberg 1997). This results in progressive pRb phosphorylation and S phase induction.

Independently of Cdk2/Cdk4 activation, E2 reduces Cdk-inhibiting proteins like p27 and p21 by the induction of proteasomal degradation (Foster *et al.* 2001a). Among other targets, it activates the Ras-Erk pathway by binding AF-1 and AF-2 to the p160 component of the coactivator complex recruited by Jun/Fos and so activating the coactivator (Kushner *et al.* 2000).

Taken altogether, E2 has regulatory influences throughout the cell cycle along many pathways. In contrast to classical growth factor concepts, it does not affect one single key mechanism, and its effects are not confined to one distinct phase of the cell cycle. In the near future, further studies will provide a deeper insight into the interaction of E2 and cellular pathways.

### **Possible sites of cell cycle interaction between XRT, E2 and anti-estrogens**

Repair of DNA damage following XRT is essential for cell survival, as chromosomal abnormalities will render the cells genomically unstable and eventually result in death of the daughter cells, referred to as clonogenic cell death. It is a hallmark of most cancer cells that the above-presented mechanisms for the adequate monitoring of DNA damage or the signal cascades for the regulation of repair or apoptosis following DNA damage have been impaired (Kastan *et al.* 2001, Xu *et al.* 2002). The induction of cell cycle progression by estrogens seems to play a role in hormone-sensitive carcinogenesis (Feigelson *et al.* 1996). On the other hand, during radiotherapy there might be a possibility to increase cytotoxicity. Even if one checkpoint is lost in carcinogenesis (especially the loss of G1 arrest, resulting from the mutation of p53), other checkpoints may still respond to the XRT and compensate for this loss (Piwnica-Worms 1999). This



**Figure 2** E2 binds to nuclear ER, which leads to increased transcription of a variety of genes, among them c-Myc and cyclinD1. By unknown mechanisms, E2 + ER induce the degradation of inhibiting kinase regulators (p21, p27). C-Myc is also directly stabilized by active ER and upregulates transcription of cyclinE and Cdc25A. CyclinD1 binds to Cdk4. This complex is inhibited by p21, thus redistributing p21 away from cyclinE–Cdk2. This complex is activated by Cdc25A and phosphorylates pRb. The conformational change releases transcription factor E2F.

potential may be markedly reduced by a stimulation of the cell cycle progression. A model of the interaction of estrogens with the checkpoints G1 and S leading to S phase entry and transition is proposed in Fig. 3. Note that this model is hypothetical, because the checkpoint pathways are based on results of studies on normal cells, not tumor cells. However, *in vitro* studies using MCF-7 cells support the model presented. Estrogen deprivation reduced radiosensitivity, while estrogen rescue abrogated the G1 checkpoint, led to a marked increase in the S and G2 fractions, and increased radiosensitivity (Wazer *et al.* 1989, Villalobos *et al.* 1996).

Compared with E2, anti-estrogens exhibit contrary effects on the cell cycle. In ER-positive breast cancer cells, tamoxifen leads to a G0/G1 arrest (Cariou *et al.* 2000). This is caused by the upregulation of p21 and p27, an increase in their binding to cyclinE–cdk2, and kinase inhibition. The pure estrogen antagonist ICI 182,780 could additionally induce a decreased cyclinD1 expression, thus inducing quiescence (G0) of MCF-7 cells (Carroll *et al.* 2000).

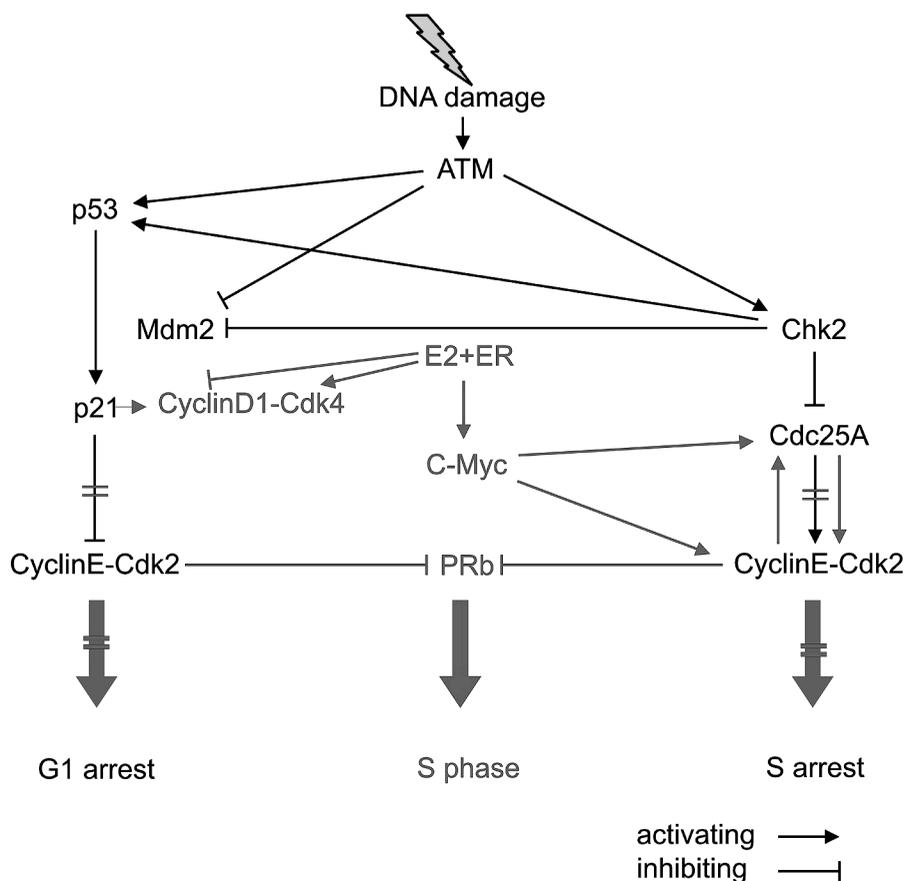
There are still other pathways to be considered. Some evidence suggests that XRT can result in the autophosphorylation of epidermal growth factor receptor (EGFR), independent of DNA damage (Schmidt-Ullrich *et al.* 1996). This triggers a response cascade ultimately leading to increased cell proliferation (for review see Schmidt-Ullrich *et al.*

2000). Consequently, adding epidermal growth factor to MCF-7 cell cultures increases radioresistance (Wollman *et al.* 1994). The induction of EGFR by XRT seems to depend on the basal level of EGFR expression (Milas *et al.* 2000). It is important to note that breast carcinomas show a high level of EGFR expression, especially in combination with ER $\alpha$  expression (Sun *et al.* 2001). Furthermore, there is evidence that estrogens can positively regulate the expression of EGFR in some model systems (Wollenhaupt *et al.* 2001). Thus, estrogens may exert a radioprotective effect via additional EGFR-pathway stimulation.

Further research is necessary to elucidate the deregulation of these pathways and to evaluate the changes in the radiosensitivity of ER-positive cancer cells.

### ***In vitro* experiments on the interaction of estrogens, anti-estrogens and XRT**

The first *in vitro* investigation on the interaction of tamoxifen and irradiation on breast cancer cells was published by Wazer *et al.* (1989). They found an antagonistic interaction between XRT and tamoxifen in the receptor-positive MCF-7 cell line. Incubation of MCF-7 cells with 1  $\mu$ M and 5  $\mu$ M tamoxifen 2 days prior to irradiation decreased the radiosensitivity of the cells. In contrast, incubation of the MCF-7 cells



**Figure 3** Solid arrows indicate irradiation-induced DNA damage pathways, arrows show estrogen-induced processes. The inhibition of cyclinE–Cdk2 by p21, transduced by ATM and p53, is disrupted by redistribution of p21 towards cyclinD1–Cdk4. The inhibition of Cdc25A by Chk2 is reduced by C-Myc-upregulated transcription and the reciprocal activation of Cdc25A and cyclinE. Thus G1/S arrests are stopped and the cell enters the S phase.

with E2 increased the radiosensitivity of the cells. Tamoxifen treatment altered the survival of the irradiated MCF-7 cells by widening the shoulder portion of the survival curve, pointing towards an increased repair of radiation-induced DNA damage. The same effect could be obtained by altered culture conditions. When MCF-7 cells were grown to confluence prior to XRT, and delayed plating was performed after irradiation, a similar decrease of intrinsic radiosensitivity, as after tamoxifen treatment, could be observed. Under these growth conditions of delayed plating, tamoxifen did not further decrease radiosensitivity. These observations indicated that the interaction of tamoxifen and XRT may be mediated by an influence on the repair of potential lethal DNA damage. Interestingly, the influence of tamoxifen on the intrinsic radiation sensitivity of MCF-7 cells could be abolished by co-incubation with 5  $\mu$ M or 100  $\mu$ M E2, whereas the tamoxifen-induced growth arrest on MCF-7 cells could not be reversed. Therefore these observations might

not primarily be explained by an alteration of the proliferation rate of the cell populations.

High dose estrogens (10  $\mu$ M E2) inhibit cell proliferation in ER-positive cell lines by reasserting the cell cycle into the G0/G1 phase (Sutherland *et al.* 1983). However, such high estrogen concentrations did not alter the intrinsic radiosensitivity of the MCF-7 cells (Wazer *et al.* 1991), although a G1 arrest could be observed under such experimental conditions.

These *in vitro* experimental observations could be reproduced and also extended by the study of the receptor-negative cell line MDA-MB-231, in which tamoxifen did not alter the intrinsic radiosensitivity (Wazer *et al.* 1993). Flow cytometry of MCF-7 cells after 48 h of incubation with 1  $\mu$ M or 5  $\mu$ M tamoxifen showed an increased number of cells in the G0/G1 phase of the cell cycle and a decreased number of cells in the S phase, as observed for plateau phase cells. This supported the notion that tamoxifen increased the repair of potentially lethal damage following irradiation in hormone

receptor-positive cells. However, a direct influence on residual DNA damage could not be shown.

Wazer *et al.* assumed that the interaction of tamoxifen, estrogen and XRT in receptor-positive breast cancer cells would be mediated by the control of the G1 checkpoint of the cell cycle. Cells which underwent XRT-induced DNA damage would block the cell cycle at the transition from G1 to S phase in order to allow time for DNA repair. This block would be augmented by tamoxifen and antagonized by estrogen, since the latter would induce cell proliferation by antagonizing the G1 block.

These experimental observations have been confirmed by Villalobos *et al.* (1996) and by Böhning *et al.* (1996), using similar experimental conditions (Table 3). Böhning *et al.* (1996) showed that the duration of tamoxifen incubation prior to irradiation (24–96 h) influenced the experimental results: the longer the incubation period, the lower the radiation sensitivity of the cells. Villalobos *et al.* (1996) showed that estrogen withdrawal decreased radiosensitivity in ER-positive MCF-7 BUS cells that had intact p53. In ER-positive T47D B8 cells, which lack functional p53, the intrinsic radiosensitivity was not altered by estrogen withdrawal. However, in both cell lines estrogen withdrawal induced a G1 arrest of the cell cycle. The authors concluded that estrogens might influence the apoptotic pathway after radiation damage to ER-receptor positive cells. Beside the influence on G1 arrest and damage repair, this could be a second mechanism of interaction between endocrine treatment and XRT in breast cancer cells.

In contrast to these findings, Sarkaria *et al.* (1994) could not find a modulation of radiosensitivity by the treatment of MCF-7 cells with the active tamoxifen metabolite 4-hydroxy-tamoxifen (4OH-TAM). Newton *et al.* (1998) observed an

enhancement of radiation-induced apoptosis in MCF-7 cells which had been treated for 24 h with tamoxifen or the pure steroidal antiestrogen ZM 182780. The clonogenic survival of cells treated with ZM 182780 prior to irradiation was significantly reduced in comparison with irradiated untreated controls.

The conflicting results of the *in vitro* studies are summarized in Table 3. Most of the studies found a synergistic or additive interaction of estradiol and XRT, and an antagonistic interaction of tamoxifen and XRT. This interaction is mediated by influencing the G1 cell cycle checkpoint, thereby affecting the repair of potentially lethal DNA damage caused by XRT. A second target of interaction between estrogens, tamoxifen and XRT could be the regulation of apoptotic pathways. This possibility has not been studied in detail. Most of the *in vitro* studies have been performed on MCF-7 cells, which are known to be deficient in caspase 3 activity. Therefore these cells show an unusual form of apoptosis induction, mediated through the activation of caspase 7 (Mc Gee *et al.* 2002). MCF-7 might not be a proper model to study radiation or tamoxifen-induced apoptosis, and might therefore not be a representative *in vitro* model for breast cancer. In addition to the influence on cell cycle progression, apoptosis induction is the second major mechanism by which tamoxifen acts upon breast cancer cells. This has been shown *in vitro* and *in vivo* (Budtz 1999). Furthermore, the results of the randomized clinical studies that are available do not support an antagonistic interaction between XRT and tamoxifen (Fisher *et al.* 1996, 1999, 2001, Fowble *et al.* 1996, Dalberg *et al.* 1998). Therefore, the clinical relevance of the *in vitro* studies summarized in Table 3 is questionable, and likely reflects the pitfalls of experimental conditions.

**Table 3** *In vitro* studies on the interaction of estrogens and ionizing irradiation in breast cancer cell lines.

Reference	Cell line	Estrogen receptor	Incubation time (h) prior to irradiation	Hormone	Interaction
Wazer <i>et al.</i> (1989)	MCF-7	+	2	17 $\beta$ -estradiol Tamoxifen	+ –
Wazer <i>et al.</i> (1993)	MDA-MB-231	–	2	Tamoxifen	No interaction
Böhning <i>et al.</i> (1996)	MCF-7	+	1–4	17 $\beta$ -estradiol Tamoxifen	+ –
Villalobos <i>et al.</i> (1995)	MCF-7 BUS	+	3*	Estradiol	+
Villalobos <i>et al.</i> (1996)	MCF-7 BUS	+	3*	Estradiol	+
	T47D B8	+		Estradiol	No interaction
	EVSA-T	–		Estradiol	No interaction
Paulsen <i>et al.</i> (1996)	MCF-7	+	2	Tamoxifen	–
	MDA-MB-231	–		Estradiol Tamoxifen	No interaction No interaction
				Estradiol	+
Sarkaria <i>et al.</i> (1994)	MCF-7	+	5*	4OH-TAM	No interaction
Newton <i>et al.</i> (1998)	MCF-7	+	1	Tamoxifen ZM 182780	+ +

Interaction: +, increased sensitivity to ionizing irradiation; –, decreased sensitivity to ionizing irradiation.

\*Phenol red-free medium and charcoal-stripped bovine serum.

In order to resolve this, more studies should be performed using different breast cancer cell lines, and other relevant endpoints representing possible targets of interaction should be analysed. It should be kept in mind that *in vitro* conditions such as the presence of phenol red or fetal bovine serum might mimic estrogenic activity on the cells. Some studies have been performed in the presence of one or both of these factors (Wazer *et al.* 1989, 1993, Böhning *et al.* 1996, Paulsen *et al.* 1996, Newton *et al.* 1998), others have used charcoal-stripped calf serum and phenol-free media (Sarkaria *et al.* 1994, Villalobos *et al.* 1995, 1996). The *in vitro* action of tamoxifen is dependent on the presence of estradiol in the medium. In estradiol-free medium tamoxifen failed to inhibit the growth of MCF-7 cells (Tanino *et al.* 1993).

A better understanding of the interactions of endocrine therapy and XRT will eventually allow an optimal use of the available drugs to augment the efficacy of XRT in hormone-sensitive cancer. Such possible targets for the interaction of endocrine and XRT treatment will be summarized in the next section.

### Animal studies on the interaction of tamoxifen and XRT in breast cancer

In an experimental model of female Sprague–Dawley rats, which carried chemically induced (1-methyl-1-nitrosourea) mammary tumors, therapeutic XRT or tamoxifen, each given as a single modality, reduced the size of the established tumors. The combination of tamoxifen and XRT did not show the antagonistic interaction which has been demonstrated in *in vitro* studies. Interestingly, tamoxifen decreased the rate of radiation-induced mammary tumors in this model (Kantorowitz *et al.* 1993).

Several animal studies have shown that treatment with tamoxifen does not only reduce the promotion of radiation-induced mammary tumors (Lemon *et al.* 1989), but also seems to reduce the initiation of radiation-induced mammary tumors (for review see Inano *et al.* 2002). This observation would confirm the hypothesis that the interaction of tamoxifen and XRT might take place at the cell cycle checkpoints. The augmentation of the radiation-induced cell cycle arrest by tamoxifen treatment would facilitate DNA repair and thus prevent radiation-induced genomic instability.

A possibly relevant interaction between tamoxifen and fractionated XRT might be the inhibition of repopulation of tumor cells between the fractions of XRT. Clinical and experimental studies showed that the time-intervals between the daily fractions of XRT will allow repopulation of tumor stem cells. Accelerated fractionation – with more fractions given per day and a reduction in overall treatment time – improved the local tumor control (Saunders *et al.* 1999). Agents which inhibit proliferation of tumor cells between the daily fractions will likely inhibit repopulation and thus

enhance the efficacy of XRT. This mechanism could only be observed by *in vivo* studies. Such studies have not been published so far.

### Clinical studies on the combined treatment of invasive breast cancer or DCIS with tamoxifen and XRT

Very few clinical studies are available in which the interaction of radiotherapy with tamoxifen, as a simultaneous and/or as a sequential application of the tamoxifen following radiotherapy, has been evaluated and compared with the effect of radiotherapy alone (Table 4, In the National Surgical Adjuvant Breast and Bowel Project B-14 (NSABP B-14) trial for node-negative ER-positive patients, tamoxifen was started after surgery and given during, as well as following, radiotherapy. Through 10 years of follow-up there was a significant increase in disease-free survival (69% versus 57%;  $P < 0.0001$ ; relative risk = 0.66; 95% confidence interval (CI) = 0.71–0.99) for those patients assigned to tamoxifen treatment compared with that of patients who received placebo. There was a 37% reduction in the cumulative incidence of tumor in the contralateral breast at 10 years follow-up, 3.8% for tamoxifen-treated patients versus 6.1% for those on placebo ( $P = 0.007$ ) (Fisher *et al.* 1996).

Dalberg *et al.* (1998) evaluated lymph node-negative postmenopausal patients with early breast cancer from the randomized Stockholm Adjuvant Tamoxifen Trial who had undergone breast-conserving therapy. Of 432 patients, 213 received 40 mg tamoxifen daily for 2 or 5 years. In all patients, radiotherapy was initiated following postmastectomy wound healing, usually 1 month after surgery. Tamoxifen was given starting 4–6 weeks after surgery. Although it was not stated specifically in this study, the majority of the patients received tamoxifen and irradiation simultaneously. The application of tamoxifen reduced the overall rate of ipsilateral breast recurrences (hazard ratio = 0.4, 95% CI = 0.2–0.9;  $P = 0.02$ ) and the risk of contralateral breast cancer (hazard ratio = 0.4; 95% CI = 0.1–1.1;  $P = 0.06$ ). This study strongly indicates that tamoxifen given simultaneously with XRT does not have an adverse effect on radiation sensitivity. In contrast, local control was improved by adjuvant application of tamoxifen with XRT compared with XRT alone.

In a retrospective analysis, Fowble *et al.* (1996) evaluated patients with stage I and stage II breast cancer who received tamoxifen and radiotherapy ( $n = 154$ ) versus patients who received radiotherapy alone ( $n = 337$ ). Unfortunately, the timing of tamoxifen and radiotherapy was unknown in 111 of the 154 patients who received the combined treatment. Twenty-three patients received tamoxifen during radiotherapy. Twenty patients received tamoxifen after the completion of radiotherapy. The study showed a decrease in distant metastasis for axillary node-positive patients who had received tamoxifen. There was no

**Table 4** Clinical studies on the interaction of tamoxifen and radiotherapy in breast cancer or DCIS.

Reference	Study design	Disease	Treatment	Disease-free survival (%)	P	Local recurrences (%)	P
Fisher <i>et al.</i> (1996)	Prospective randomized trial	EBC	Tamoxifen	69	0.0001	3.4	0.0001
			Placebo	57		10.3	
Dalberg (1998)	Retrospective analysis of a prospective randomized trial	EBC	Tamoxifen	80	0.03	7	0.02
			No endocrine therapy	70		19	
Fisher <i>et al.</i> (2002)	Prospective randomized trial	EBC	Tamoxifen			2.8	0.01
			Placebo			9.3	
Fisher <i>et al.</i> (1999)	Prospective randomized trial	DCIS	Tamoxifen	87.4 (95% CI 85.1–89.6)		8.2*	0.0009
			Placebo	83.3 (95% CI 80.8–85.8)		13.4*	

EBC, patients with node negative early breast cancer.

\*Cumulative incidence of invasive and non-invasive breast cancer at 5 years.

significant effect on local control or overall survival. Patients who received the combined treatment had a significant increase in the incidence of breast edema. Due to the unknown sequence of tamoxifen and radiotherapy for the majority of these patients, no conclusions can be drawn about the influence of tamoxifen on the intrinsic radiosensitivity of breast cancer.

The recent update of the NSABP B-21 study showed a clear benefit of sequential treatment with XRT and tamoxifen compared with XRT alone (Fisher *et al.* 2001, 2002). Radiotherapy was initiated within 2 weeks after surgery. Tamoxifen was begun within 35 days after surgery. An overlap of approximately 2 weeks in which both treatments were applied simultaneously can be assumed for the majority of the patients, although this was not stated clearly in the paper. Cumulative incidence of breast recurrences at 8 years after treatment was 9.3% for the patients treated with XRT and placebo compared with 2.8% for the patients treated with XRT and tamoxifen ( $P=0.01$ ). Survival was not affected.

Wazer *et al.* (1997) reported a non-randomized retrospective analysis of patients who received XRT alone or XRT followed by tamoxifen after breast-conserving surgery for early breast cancer. No difference was found in local control or cosmetic outcome.

Bentzen *et al.* (1996) found an association of tamoxifen treatment with marked lung fibrosis. Of the 84 patients who had participated in a randomized study, 38 patients received radiotherapy and tamoxifen, whereas 46 patients were treated with radiotherapy alone. The relative risk of the development of lung fibrosis in patients receiving tamoxifen was 2.0 (95% CI = 1.2–3.5;  $P=0.1$ ). This association was ascribed to the induction of transforming growth factor  $\beta$ , which is a known non-hormonal effect of tamoxifen.

A beneficial effect of tamoxifen in combination with XRT could be observed with respect to DCIS. In the NSABP B-24 study, 1804 patients received either tamoxifen or a pla-

cebo in combination with radiotherapy (50 Gy) for the breast-conserving treatment of DCIS (Fisher *et al.* 1999). Unfortunately there was no clear statement as to whether tamoxifen was applied concurrently with XRT or after radiotherapy. Since radiotherapy was started no later than 8 weeks after lumpectomy and tamoxifen was administered within 56 days following lumpectomy, and taking into account that the duration of radiotherapy up to 50 Gy is usually 5 weeks, a number of patients might have received tamoxifen and radiotherapy concurrently. Women in the tamoxifen-treated group had fewer breast cancer events at 5 years than did those on placebo (8.2% vs 13.4%;  $P=0.009$ ). The estimated ratio for all breast cancers was 0.63 (95% CI = 0.47–0.83). Interestingly, tamoxifen treatment reduced the risk of contralateral breast cancer (relative risk: 0.48, 95% CI = 0.26–0.87).

Whether the reduction of contralateral breast cancer risk was due to a decreased tumor promotion or a possible inhibition of radiation-induced tumor initiation, or both, is elusive. However, this observation confirmed the findings of the above-mentioned animal studies.

In summary, several large randomized clinical studies have shown that the combination of XRT with tamoxifen is more beneficial in the treatment of early breast cancer and DCIS than XRT alone. Although there was no clear distinction in most of the available studies as to whether tamoxifen was given concurrently with XRT or whether this treatment was started after XRT, a large number of the reported patients probably received concurrent treatment. It would be interesting to re-evaluate some of the randomized studies with regard to the mode of combined radiation and tamoxifen treatment, or to initiate a phase III study which would ask this question. Such a study has not been performed as yet.

Interestingly, two clinical studies (Albain *et al.* 2002, Pico *et al.* 2002) have been reported recently on the interaction of tamoxifen with adjuvant chemotherapy in breast cancer. In the randomized study SWOG-8814 trial (Albain *et*

al. 2002) postmenopausal women with risk factors received either adjuvant tamoxifen alone, adjuvant chemotherapy with adjuvant tamoxifen starting concurrently with chemotherapy or adjuvant chemotherapy with sequential application of tamoxifen. Chemotherapy consisted of oral cyclophosphamide, adriamycin and 5 Fluorouracil. The disease-free survival after 8 years was superior in the groups of patients who received chemotherapy and tamoxifen compared with tamoxifen alone. Patients in whom tamoxifen treatment was started after chemotherapy had a better disease-free survival (67%) than patients in whom tamoxifen treatment was started concurrently with chemotherapy (62%,  $P = 0.04$ ). Although overall survival was not significantly different yet there was a trend to better survival after sequential therapy.

A randomized study from Spain (GEICAM 9401) showed a similar trend (Pico *et al.* 2002). Postmenopausal node-positive patients with breast cancer received adjuvant epirubicin/cyclophosphamide chemotherapy. In one group, adjuvant tamoxifen was started concurrently with chemotherapy, in the other group adjuvant tamoxifen was started after chemotherapy. At 5 years after therapy, the disease-free survival of the patients who received sequential treatment showed a favorable trend compared with the group with concomitant treatment. However, the difference between both treatment groups was not statistically significant.

## Conclusions

The antagonistic interaction of tamoxifen and XRT which was observed in several *in vitro* studies has not been confirmed either in clinical or in animal studies. Possibly the experimental endpoints of the *in vitro* systems have not been relevant for the *in vivo* situation, since important determinants of radiation-induced tumor control, such as repopulation, cannot be assessed *in vitro*. Furthermore, *in vitro* studies might be influenced by culture conditions and the genetic properties of the cell lines used (in particular the MCF-7 cell line). The question whether tamoxifen will interfere with radiation-induced apoptosis has not been evaluated properly so far.

The reduction of radiation-induced mammary tumors in animal models is in keeping with the observation of reduced contralateral breast cancer in patients undergoing combined treatment with tamoxifen and XRT compared with patients receiving XRT alone. Whether tamoxifen treatment augments the repair of radiation-induced DNA damage by supporting cell cycle checkpoints in normal tissues or suppresses the promotion of breast cancer or both is speculative. In tumor cells, the mechanisms of recognition and repair of DNA damage might be different from those of normal cells since mutations of genes that monitor DNA integrity, repair and apoptotic pathways are an attribute of malignant transformation. Therefore, the mechanisms of interaction between hormones and anti-hormones with radiation-induced DNA

damage might be more complex in tumor cells compared with normal tissues.

Clinical studies suggest that the combined application of tamoxifen and XRT improves local control in DCIS as well as in invasive breast cancer. Available clinical studies do not indicate that the simultaneous application of tamoxifen and XRT is disadvantageous, as was suggested by *in vitro* studies. The tolerance of lung tissue to XRT might be slightly reduced if tamoxifen is given simultaneously with XRT. The duration of breast edema might be augmented. Cosmetic results have not been impaired by a combined treatment with tamoxifen.

Whenever indicated, both treatment modalities should be started early after surgery. They can be applied simultaneously. So far there is not sufficient evidence to withhold tamoxifen treatment during adjuvant irradiation of breast cancer. No randomized study exists which has tested the hypothesis that there is a difference in local control when tamoxifen treatment is started prior to rather than following adjuvant XRT. Such a study would be worth undertaking, since recent evidence from two clinical studies points towards an antagonistic interaction of tamoxifen with chemotherapy.

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