Systemic treatment approaches in breast cancer

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Cover design and lay-out:
F.J. Smits (Dept. Medical Oncology, Rotterdam Cancer Institute/Dr. Daniel den Hoed Kliniek).

Printed by:
ICG Printing b.v., Dordrecht, The Netherlands

ISBN: 90-9010433-X

The author is grateful for the financial support provided by: Rhône-Poulenc Rorer, Bristol-Myers Squibb, SmithKline Beecham Farma, Amgen B.V.

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Medicamenteuze behandelingsmodaliteiten
voor borstkanker

PROEFSCHRIFT

ter verkrijging van de graad van doctor aan
de Erasmus Universiteit Rotterdam
op gezag van de rector magnificus
Prof. dr P.W.C. Akkermans M.A.
en volgens besluit van het college van promoties.

De openbare verdediging zal plaatsvinden
op woensdag 4 juni 1997, om 15.45 uur

door

Marijke Bontenbal
geboren te Woubrugge

PROMOTIECOMMISSIE

Promotor	:	Prof. dr G. Stoter
Overige leden	:	Prof. dr F.H. de Jong Prof. dr J.W. Oosterhuis Prof. dr A.T. van Oosterom
Co-promotor	:	Dr J.G.M. Klijn

Ter nagedachtenis aan mijn ouders,
voor Anieta

CONTENTS

CHAPTER 1

INTRODUCTION

1.0 INTRODUCTION

Breast cancer is the most common malignant tumor among women, with an estimated 135,000 new cases and 58,000 recorded deaths per year in the European Community in 1990 [1]. With respect to the Netherlands, the most recent data of The Netherlands Cancer Registry show an incidence of nearly 10.000 new cases of primary breast cancer and about 3500 breast cancer deaths per year [2]. In women breast cancer comprises one-third (33,2%) of all types of cancer. Ultimately about one out of 10 women will get breast cancer during her life and one out of every 22 women will sooner or later die as a consequence of metastatic disease [2].

Among the solid tumors breast cancer is one of the few types of cancers sensitive to different systemic treatment regimens both with respect to endocrine- and chemotherapy. Both treatment modalities are nowadays standard practice in the treatment of breast cancer, either to achieve cure, or prolongation of (relapse-free) survival in the adjuvant setting, or to palliate metastatic disease. The efficacy of the different forms of systemic treatment is dependent on patients- and tumor characteristics [3].

1.1 Endocrine treatment of breast cancer

More than 100 years have past since Beatson empirically performed the first oophorectomy in a patient with metastatic breast cancer, and documented the regression of skin metastases [4]. It is only since a few decades however that we are beginning to understand the biology that explains why Beatson was successful. For many years standard endocrine manipulation for the treatment of breast cancer has been directed toward inhibiting, ablating, or otherwise interfering with estrogen activity (Table I) [5,6].

The efficacy of such manipulation depends on the phase of the disease and tumor-load. While cure is possible in a subset of patients with primary disease, in contrast, in unselected patients with metastatic disease only prolongation of survival can be reached. The median duration of response of first-line endocrine therapy is between one and two years [7-10]. The question was raised whether combinations of hormones or sequential hormonal therapy with agents with different mechanisms of action could improve treatment outcome. Several trials have been initiated addressing this issue. Most of the randomized trials performed were based on rudimental understanding of the mechanisms of action of endocrine therapy and have not led to a real successful combination of endocrine therapies.

Table I 100 years in the development of endocrine therapy[6]

1896	oophorectomy
1922	ovarian irradiation
1939	androgens
1944	synthetic estrogens
1951	progestins
1952	pituitary irradiation
1953	adrenalectomy
1953	hypophysectomy
1971	anti-estrogens
1973	aromatase inhibitors
1982	LHRH agonists
1987	antiprogestins
1993	"pure" anti-estrogens

In spite of higher initial response rates with some of the hormonal combinations, survival was not influenced and toxicity was often increased [11-19].
It is only since the last decades that we learned that the development and function of the mammary gland depends on the coordinated action of estrogen, prolactin, progesterone, adrenal corticosteroids, insulin, growth hormone, and thyroid hormone [20]. Besides estrogen, many steroid and peptide hormones, but also growth factors and other trophic substances, have been recognized that are involved in the growth regulation of the normal breast and of breast cancer (Table II).
Together with this enhanced knowledge of endocrine/paracrine/growth factor involvement in breast cancer growth, the number of available endocrine agents has been drastically increased in the past twenty years (Table III) [9,20-24]. This offers now many new points of action in the endocrine therapy of breast cancer, making combinations of (new) endocrine agents more attractive.

Table II Hormones and other factors involved in the growth regulation of breast cancer (directly and indirectly)[21]

1. Steroid hormones	:	estrogens, progesterone, androgens, glucocorticosteroids
2. Peptide hormones	:	prolactin, growth hormone, insulin, somatostatin, calcitonin (LH, FSH, ACTH).
3. Other trophic factors	:	iodothyronines (T4, T3), vit. D, retinoids, polyamines, melatonine.
4. Growth factors	:	insulin-like growth factors (IGF-1, IGF-2), epidermal growth factor (EGF), transforming growth factors (TGF-α, and β), platelet-derived growth factor (PDGF), fibroblast growth factors (FGF), mammary derived growth factor 1 (MDGF-1).
5. Secretory proteins		

Table III New endocrine agents and new treatment modalities[22]

1) Pure antiestrogens
2) LHRH analogues
3) Aromatase inhibitors
4) Antiprogestins
5) Somatostatin analogues with or without prolactin inhibitors
6) Potent metallopeptide analogues
7) Radiolabelled hormones (SS-A-Yttrium)
8) Specific growth factor pathway interfering therapy
9) - Inhibition of angiogenesis
- Inhibition of metastatic capacity
10) Sensibilisation for chemotherapy by recruitment of tumor cells in S-phase

1.2 Chemotherapy of breast cancer

Cytotoxic chemotherapeutic agents form the other main group of systemic treatment of breast cancer [25-29]. Since the initial use of cytotoxic drugs in humans in 1942, virtually every new member of this class of drugs developed has been tested

for breast cancer. In the adjuvant setting chemotherapy is as effective as hormonal therapy in pre-menopausal patients, but less effective in post-menopausal patients. Used as single agent in disseminated breast cancer anthracyclines, cyclophosphamide, methotrexate and fluorouracil induce objective responses in about 20-40% of the patients. These agents are the cornerstones of breast cancer chemotherapy nowadays, together with promising new agents as paclitaxel and docetaxel (Table IV) [30-34].

Table IV Activity of single agent chemotherapy in metastatic breast cancer[30-34]

	Response rate (%)
Fluorouracil	27
Methotrexate	28
Cyclophosphamide	33
Doxorubicin	32
Epirubicin	34
Mitoxantrone	20
Paclitaxel	17-62
Docetaxel	44-73

Also combination chemotherapy has been extensively tested in breast cancer. When treating patients with metastatic disease, response rates in first-line therapy of about 40-50% can be achieved with the combination of CMF (cyclophosphamide, methotrexate and fluorouracil), whereas CAF or CEF, anthracycline (doxorubicin or epirubicin) containing schemes are claimed to be the most effective ones with response rates of 50-60% (35-37). However, with these schemes only 10-20% of the patients achieve a complete remission usually of short duration, and side-effects of chemotherapy are often substantial.

1.3 Selected new treatment modalities

Although combination chemotherapy and new endocrine agents have improved the therapeutic benefit for breast cancer patients in the past decades, a plateau in response

rates, duration of response and survival has been reached, and no further improvements can be expected with the standard treatment schemes. Extension of the pharmacopeia and enhanced knowledge about the mechanisms of response and resistance of tumor cells to different kinds of therapy have led to investigations based on more basic knowledge. Interest arose in testing:

a) endocrine stimulation of breast cancer followed by chemotherapy, because of the preclinically acquired knowledge that hormonal agents can recruit tumor cells into S-phase and because proliferative cells are more sensitive for chemotherapy,

b) frequently administered low-dose or chronic low-dose chemotherapy, to increase exposure time of tumor cells to cell cycle phase specific drugs,

c) diminishing side-effects of chemotherapy by administering a low-dose per time period, without loss of efficacy,

d) new analogues of chemotherapeutic drugs,

e) combinations of growth-inhibiting endocrine agents, using the extended knowledge of hormonal growth regulation of breast cancer.

It was the scope of this thesis to investigate some of these new strategies in breast cancer treatment.

1.3.1 Combinations of growth-stimulating hormones and chemotherapy

Lippman et al. were among the first to describe kinetic changes in breast cancer cells caused by estrogens and antiestrogens [38]. Tamoxifen decreased the incorporation of thymidine and resulted in a growth inhibition as compared to untreated controls. When the "growth-blockade" by tamoxifen was removed, DNA synthesis in these cells increased rapidly. It was suggested that tamoxifen had led to synchronization of the tumor cells, and estrogen rescue was suggested as the mechanism to explain the rapid increase in DNA synthesis after tamoxifen removal. Later on other investigators found that anti- hormones and pharmacologic concentrations of steroid- or peptide hormones could block hormone dependent breast cancer cells in the G_0G_1-phase of the cell cycle, thereby reducing the percentage of cells in the S- and G_2M-phases of the cell cycle [39-41]. On the other hand, physiologic concentrations of hormones and several growth factors were found to induce the semi-synchronous recruitment of a population of G_0/G_1-phase cells into the cell cycle (S/G_2M-phase) and shorten the overall cell cycle time [38,42]. It was hypothesized that recruitment of a population of G_0/G_1-phase breast cancer cells into the active phases of the cell cycle followed by cell cycle specific drugs might lead to an augmented

cytotoxic effect of such manipulation compared to unstimulated controls. In 1978 Weichselbaum et al. were the first to describe an augmented cytotoxic effect of 1-β-D arabinofuranosylcytosine, an S-phase specific drug, in hormone responsive MCF-7 human breast cancer cells in vitro pretreated with 10^{-9} M 17β estradiol [43]. Since then this concept of hormonal recruitment has been addressed by several investigators.

Pretreatment with estradiol or a combination of estradiol, insulin, EGF and dexamethasone enhanced the effect of several cytotoxic drugs in MCF-7 cells, and in primary breast tumor cells cultured in agar [44-46]. The same results were found for estrogen pretreatment followed by cyclophosphamide in the hormone responsive MXT mouse mammary tumor, in vitro and in vivo [47-49] and for testosterone stimulation preceding chemotherapy in the androgen responsive Shionogi mammary carcinoma tumor in castrated mice [50]. Results from these preclinical studies indicated that growth promotion could indeed result in an augmented cytotoxic effect of subsequent administered chemotherapy under certain experimental conditions.

Several clinical trials have been performed using combinations of growth stimulating hormones and chemotherapy in metastatic and locally advanced breast cancer [9,51-62]. The growth promoting hormones were used to create a higher proliferation index in the tumor, in order to make the cells more vulnerable to subsequent cytotoxic therapy. Some trials reported a high complete remission rate (up to 49%), or survival advantage [51-54], others did not find any benefit from hormonal recruitment followed by chemotherapy [55-63]. Furthermore, clinical investigations have shown that estrogen stimulation can enhance the labelling index in primary tumors [55], and a significant positive relationship was found between clinical regression after neo-adjuvant chemotherapy and pretreatment proliferative activity in primary breast cancer [64]. However, in the various trials different treatment schemes have been used preventing clear conclusions about the value of growth promotion followed by chemotherapy. At present, we do not know which combinations and time intervals of the hormones and chemotherapy are most optimal. We investigated *in vitro* (chapter 2-5) and *in vivo* (chapter 6) the effects of hormonal manipulation followed by administration of chemotherapy.

1.3.2 Frequent or chronic low-dose chemotherapy

Weekly low-dose doxorubicin plus mitoxantrone

Metastatic breast cancer is still an incurable disease. Therefore, for this stage of the disease the development of treatments which are effective and (relatively) well

tolerated is important for palliation. Recently acquired knowledge of the action of chemotherapeutic drugs on the cellular level, and of the influence of multidrug resistance and kinetic resistance on the efficacy of the drugs has led to the development of new schemes with the standard drugs.
Doxorubicin is a major drug in cancer therapy, and nowadays one of the cornerstones of breast cancer therapy. Although the precise mechanism of antineoplastic action of doxorubicin is not fully understood, the drug can intercalate DNA and inhibit replication and transcription of DNA and inhibit DNA repair, inhibit topoisomerase II activity and affect regulation of gene expression and integrity and activity of cellular membranes [65-71]. Furthermore the drug can be reduced to semiquinone free radicals by NADPH cytochrome p450 reductase [72-73]. For many years doxorubicin has been the most effective drug as a single agent in the standard three-weekly schedules in the treatment of metastatic breast cancer with a 40% response rate in the first line and about 20%-30% response in second-line therapy. Toxicity of the drug with the higher dosed schemes however, is impressive in terms of induction of cytopenia, complete alopecia in all patients and frequent induction of nausea and emesis. The most important long-term side-effect is the cardiotoxicity. This cardiotoxicity is associated with lipid peroxydation of the membrane of myocardial cells by free radicals, and impairment of mitochondrial function such as disturbances in the intracellular calcium transport, depressed adenosine diphosphate-stimulated respiration and alterations of membrane structure and function [74-77]. Therefore, application of a weekly low-dose schedule of doxorubicin might be attractive in order to decrease the occurrence and severity of side-effects without loss of efficacy [78-80].

Mitoxantrone is an anthraquinone derivate of doxorubicin. The drug displays at least 3 modes of action: stabilization of the topoisomerase-DNA cleavable complex, aggregation and compaction of DNA via electrostatic cross-linking, and oxidative activation with free radical generation [81]. The drug is active in breast cancer treatment, but in direct comparison with doxorubicin, it induces slightly lower response rates [81-83]. However, mitoxantrone is less toxic as manifested in the frequency of alopecia, nausea/vomiting and mucositis [83,84]. Mitoxantrone is also less cardiotoxic for the drugs produces few if any free radicals via the NADPH cytochrome P-450 reductase pathway nor via membrane lipid peroxidation [81]. In weekly low-dose therapy the drug has shown responses in a small study in elderly patients [85]. Preclinical data suggested that mitoxantrone caused a concentration-dependent inhibition of doxorubicin stimulated lipid peroxydation in liver microsomes

of rabbits [86]. Therefore, in the study described in chapter 7 we investigated the effects of weekly low-dose mitoxantrone plus doxorubicin as second-line chemotherapy in metastatic breast cancer.

Chronic low-dose etoposide

Etoposide has been used in the treatment of various kinds of tumors, but has shown unsatisfactory activity in breast cancer when used in a short-term i.v. or oral (mostly day 1-5) schedule [87]. Etoposide is a podophyllotoxin derivate, and the drug exerts its action by inhibiting the topoisomerase II enzyme involved in the process of unwinding and cleaving DNA during replication. Topoisomerase II expression increases in the S-phase and peaks in the late G_2M-phase of the cell cycle, making etoposide a phase specific drug. Moreover, the binding of etoposide to the cleavable complex is rapidly reversible [88]. This makes the drug attractive for use in a prolonged administration scheme for slowly proliferating tumors like breast cancer. Furthermore the bioavailability of etoposide is high for low doses [89-91].
Etoposide has shown activity as a protracted low-dose oral scheme in several solid tumors, with acceptable toxicity [92-95]. Chapter 8 describes the results of a study with long-term low-dose oral administration of etoposide as second-line chemotherapy in patients with metastatic breast cancer.

1.3.3 New chemotherapeutic analogues

Carboplatin plus etoposide

Cisplatin and cisplatin-based combination chemotherapy has shown significant effectiveness in breast cancer when used as first-line therapy [96]. However, because of its toxicity and relative inconvenience of administration, cisplatin has not become standard therapy in breast cancer. The advent of cisplatin analogues with less nephrotoxicity and the feasibility of administration in the outpatient setting has offered new possibilities for this class of drugs.
Like cisplatin, carboplatin forms DNA-DNA interstrand- as well as DNA-protein cross links [97]. Also nuclear protein phosphorylation has been described in rat tissues, and the drug may form intrastrand DNA cross links [97].
Monotherapy with carboplatin or combinations of carboplatin with etoposide, or fluorouracil, have been used mainly as second-line chemotherapy in metastatic breast cancer, and have shown low response rates of less than 20 % objective responses [98-100]. Little is known about the efficacy and toxicity of a combination of carboplatin

and etoposide as first-line chemotherapy in breast cancer. In chapter 9 we describe the results of a phase II study with the combination of carboplatin and etoposide as first-line therapy in metastatic breast cancer.

Epirubicin

Epirubicin is the 4'epimer of doxorubicin and differs only from the mother compound by the 4'-hydroxyl group in the equatorial rather than the axial orientation. The mechanism of antitumor activity of epirubicin is comparable to that of doxorubicin. However, due to this steric modification there are several biochemical differences compared to doxorubicin: a) the DNA-epirubicin complex is less stable, b) influx of epirubicin into tumor cells is faster but retention is lower, c) the ability of the epirubicin-iron complex to peroxidize lipids is lower, and the epirubicin coordination complex with metal ions is different from that of doxorubicin [101]. It can be expected that these alterations in biochemistry may result in a different toxicity profile of epirubicin compared to doxorubicin. In the first clinical trials it was suggested that use of epirubicin might result in an antitumor efficacy comparable to that of doxorubicin, but would induce less myelo- and cardio-toxicity [102,103]. There are however only a few studies comparing directly both drugs in an equimolar or equimyelotoxic dose [104]. The results of these (mostly smaller) studies are conflicting. No significant differences in response rates were found between the two drugs when administered in an equimolar dose, nor in an expected equimyelotoxic dose. In the latter trials the dose of epirubicin ranged from 1.4 to 1.5 times the dose of doxorubicin. When equimolar doses of epirubicin and doxorubicin were used in combination treatment with cyclophoshamide and fluorouracil no statistical differences in response rates or survival were observed between the two drugs, but the epirubicin combination resulted in less myelosuppression and acute gastrointestinal toxicity, and less cardiac effects when similar cumulative doses were given. The EORTC Breast Cancer Cooperative Group performed the largest study directly comparing both anthracyclines on an expected equimyelotoxic base. Results of this study are shown in chapter 10.

1.3.4 Combinations of growth-inhibiting hormonal therapy

Successfully combining two or more hormonal agents remains theoretically and practically an important challenge [105]. Standard endocrine therapy usually employs

antisteroidal agents to inhibit estrogen stimulation of tumor growth [5]. However, other steroid and peptide hormones are also important in breast development and function [Table III] [23,106]. Recent preclinical research has shown that in addition to estradiol insulin-like growth factors (IGF-1 and IGF-2) are potent stimulators of breast cancer growth in vitro [107,108]. In vivo the pituitary-derived growth hormone regulates the secretion of IGF-1 by the liver and other tissues.

A new approach in breast cancer treatment is manipulation of the hypothalamic-pituitary function to inhibit prolactin and growth hormone secretion. Single agent treatment of metastatic breast cancer with dopamine agonists (inhibitors of prolactin secretion) or with somatostatin (growth hormone release inhibiting hormone) analogues, however, has shown only moderate benefit [22,109-113]. An explanation for this lack of success of single agent dopamine agonists can be the fact that, in humans, growth hormone also acts as a potent ligand for the lactogenic receptor [114-119]. Furthermore, estrogens can counteract the growth inhibitory effects of dopamine agonists or somatostatin (analogues) [120,121]. Therefore, and in view of the observations that somatostatin analogues can a)decrease growth hormone and IGF-1 secretion, b)inhibit human tumor cell growth in vitro and in animal models directly when somatostatin receptors are present, and because 40-75 % of the primary breast cancers are somatostatin receptor positive [122-126], clinical treatment with a combination of a somatostatin analogue and an antiprolactin might be more worthwhile. Results of this combined treatment thusfar published show a low response rate in heavily pretreated patients [21,127-129]. However, the most attractive combination for the treatment of breast cancer might be the combination of an antisteroidal agent with a somatostatin analogue and an antiprolactin. In chapter 11 of this thesis the clinical implications and endocrine effects of such combination treatment in post-menopausal metastatic breast cancer are investigated.

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CHAPTER 2

OESTRADIOL ENHANCES DOXORUBICIN UPTAKE AND CYTOTOXICITY IN HUMAN BREAST CANCER CELLS (MCF-7)

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European Journal of Cancer and Clinical Oncology 1988; **24**: 1409-1414

ABSTRACT

The cytotoxic effect of doxorubicin on human breast cancer cells (MCF-7) appeared to be correlated with drug concentration, exposure time and cellular uptake of doxorubicin. The effects of short-term stimulation of the growth of MCF-7 cells with 30 pM oestradiol was investigated with respect to the uptake of doxorubicin and cell kill. Culture of MCF-7 cells in steroid hormone-deprived medium resulted in an approx. 90% arrest of the cells in the G0G1-phase of the cell cycle. Growth stimulation with 30 pM oestradiol caused a 3-5-fold increase in the number of cells in S-G2M phase at between 18 and 24 h after administration of oestradiol to the medium. Incubation of oestradiol-stimulated cells with 0.37 μM doxorubicin during both 1 and 6 h resulted in an augmented inhibition of cell growth compared to unstimulated controls. An enhanced cellular uptake of doxorubicin after administration of oestradiol was observed only after an incubation period of 6 h and not of 1 h. These observations suggest that both an increased sensitivity to doxorubicin and an augmented cellular uptake of the drug may underlie the cytotoxic effects of doxorubicin after pretreatment with oestradiol.

INTRODUCTION

In the past 10-15 years combination chemotherapy as well as treatment with new (anti-)steroidal agents have improved the therapeutic benefit for breast cancer patients [1,2]. However, for several years a plateau phase has been reached with respect to response rate, duration of response and survival. At its best hormonal treatment or chemotherapy can reach a maximal response rate of 50-75% in (subgroups of) patients with metastatic breast cancer, with a mean duration of response of about 12 or 8 months respectively. Combination therapy with cytostatics and growth inhibitory hormones may cause a modest improvement in treatment results compared to single treatment modalities, especially in postmenopausal patients with steroid receptor positive tumours [3-6]. However, response rates generally do not surpass 75%, and the results of different studies are conflicting. Treatment with growth inhibitory hormones can even decrease the efficacy of chemotherapy in subgroups of patients [4]. Growth inhibitory hormones can interfere with the action of cytostatic drugs indirectly by influencing drug metabolism and immune function, and directly by influencing cellular uptake of cytostatic drugs or cell kinetics [7]. One of the reasons for treatment failure could be the apparently high proportion of tumour cells in the resting phase of

the cell cycle, in which phase cells are generally less sensitive to chemotherapeutic agents [7,8].

A new approach in the treatment of breast cancer patients involves short-term stimulation of tumour cell growth to improve the therapeutic ratio of cytotoxic agents [7,9]. Oestradiol and some other hormones and growth factors can induce recruitment of quiescent cells into the proliferative phase of the cell cycle, causing cells to be more vulnerable to subsequent administration of cytotoxic agents [10,11]. The results of different experimental studies have shown that pretreatment of tumour cells with physiological and low pharmacological dosages of oestrogens can enhance the cytotoxic effect of chemotherapeutic agents *in vitro* [7,9-11].

The first clinical studies involving such hormonal manipulation showed an improvement in the percentage of complete remissions and/or survival [12-15], while in only a few small studies no benefit of oestrogen recruitment with subsequent chemotherapy has been reported [16,17]. However, the points most open to discussion are the following: (a) the importance of cell synchronization using tamoxifen prior to stimulation of tumour cells; (b) the choice of the growth stimulatory agents to be used in addition to oestrogens (if necessary); (c) the optimal dosages of the growth stimuli; and (d) the optimal duration of the stimulation period. Furthermore, at present very little is known on the effects of short-term hormonal stimulation of tumour cells upon the degree of cellular uptake of cytostatic agents.

Therefore, using human breast cancer (MCF-7) cells as an experimental model we have investigated the effects of pretreatment of these cells with oestradiol on their uptake of doxorubicin and the subsequent tumour cell kill. This study shows the results of experiments in which the effects of various time periods of oestradiol stimulation and doxorubicin incubation were investigated.

MATERIALS AND METHODS

Cell culture

MCF-7 human breast cancer cells were originally obtained from EG&G Mason Research Institute, Worcester, U.S.A. in its 219th passage. Cells were routinely cultured in RPMI 1640 medium containing 5mg/l phenol red and supplemented with penicillin (100 units/ml), streptomycin (100 μg/ml), insulin (10 μg/ml) and 10% heat-inactivated foetal calf serum (i.e. complete growth medium). Cultures were maintained at 37°C in a humidified atmosphere of 5% CO_2 in air and cells were passaged weekly.

For experiments, cells of exponentially growing cells in culture were harvested after incubation for 5 min at 37°C with 2 ml of 0.25% trypsin in Dulbecco's phosphate buffered saline, lacking Ca^{2+} and Mg^{2+} (DPBS). Subsequently, cells were seeded in T25 flasks (ca 10^5 cells/flask) in complete growth medium or in RPMI 1640 medium containing phenol red and supplemented with 10% steroid-depleted male human serum, penicillin (100 units/ml) and streptomycin (100 μg/ml) (i.e. experimental medium). Previous experiments in our laboratory had shown only an approx. 10% stimulation of growth by oestradiol, when cells were cultured in RPMI-1640 medium containing phenol red, supplemented with steroid-depleted foetal calf serum [18]. Replacing the foetal calf serum by 10% steroid-depleted male human serum, as applied by Devleeschouwer et al., revealed a 4-6-fold stimulation of growth with already very low concentrations of oestradiol, 10-100 pM [19, 20]. Doxorubicin as doxorubicin hydrochloride/lactosum (in H_2O) and oestradiol (in ethanol) were added as indicated in the legends to the figures. Steroid depletion of male human serum was performed by two 45 min incubation at 50°C with 0.5% charcoal-0.05% dextran T-70 (w/v), with an intermediate 2 h incubation at 37°C with sulphatase (2 U/ml).

Estimation of cell survival following exposure to doxorubicin

Monolayer cultures were incubated for different time periods (1-72 h) in the absence and presence of doxorubicin at concentrations as indicated in the legends to the figures. Immediately after the incubation period the monolayer cells were washed with 0.15 M NaCl and supplied with drug-free complete growth medium. After daily medium refreshment, cells were harvested by trypsinization and cell number determined with a haemocytometer.

Hormonal manipulation and flow cytometer analysis

Medium containing 30 pM oestradiol or the equivalent volume of the solvent ethanol (final ethanol concentration 0.025%) was added to MCF-7 cell cultures after 6 days of incubation in steroid-deprived experimental medium. At different time points after oestradiol addition cells were harvested by trypsinization and pelleted by centrifugation for 5 min at 100 g. Cells were subsequently resuspended in 150 μl PBS containing 0.01 mg/ml propidium iodide (PI), 1 mg/ml ribonuclease A and 1% Triton X-100. Incubation with PI was performed for 10 min at room temperature. After washing of the cells with 0.15 M NaCl, the PI fluorescence of the nuclei was determined with a Fluorescence Activated Cell Sorter (FACS 440, Becton and Dickinson).

Incubation with doxorubicin and determination of cellular doxorubicin content

Monolayer cells were incubated with different concentrations of doxorubicin, ranging from 0.037 to 37 μM, as mentioned in the legends to the figures. At the indicated time points, cells of triplicate incubations were harvested by trypsinization and washed with 0.15 M NaCl. Cells from three T25-flasks were used for the extraction of cellular anthracycline as previously described by Kokenberg et al. [21]. The other T25-flasks of replicate cultures were used for the estimation of cell survival. Doxorubicin contents of cellular extracts were measured by high performance liquid chromatography (HPLC) according to a method adapted from Israel et al. [22]. The detection limit after extraction from biological fluid was 10 ng doxorubicin/ml. The metabolite doxorubicinol was detected.

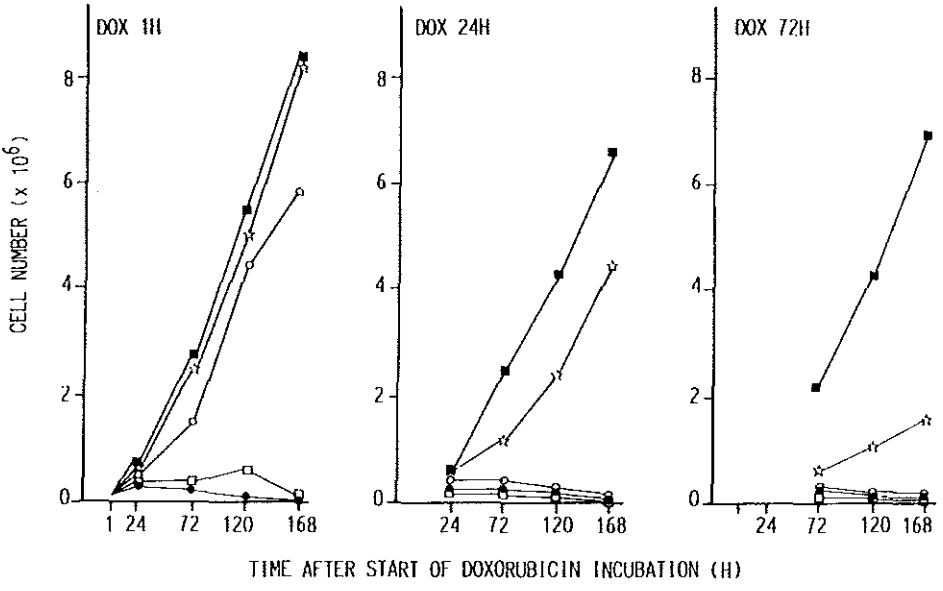

Figure 1

Survival of MCF-7 cells after incubation with doxorubicin. MCF-7 cells grown for 2 days in complete growth medium were incubated at 0 h in the absence (■) or presence of 0.037 (*), 0.37 (o), 3.7 (□), 37(●) μM doxorubicin. The incubation times with doxorubicin were 1 (left panel), 24 (middle panel) and 72 h (right panel). At the indicated time points the number of cells in the flasks of triplicate cultures was determined.

Statistical analysis

The significance of differences between the results obtained with the different incubation conditions was calculated using Wilcoxon's test.

RESULTS

The effects of drug concentration and exposure time on cellular content of doxorubicin and cell growth

Exposure of MCF-7 human breast cancer cells *in vitro* to different concentrations of doxorubicin results in a dose-dependent antiproliferative effect of the drug (Fig. 1). Moreover, the antiproliterative effect in the sense of inhibition of cell growth or even decrease in cell number appeared not only to be correlated with the drug concentration of doxorubicin but also with the duration of exposure. In this respect, the cellular uptake of doxorubicin appeared positively correlated with both drug concentration and duration of exposure (Table 1).

		CELLULAR DOX (p mol/10^6 cells ± SD)	
		DOX (1h)	DOX (24h)
EXTRACELLULAR DOX (μM)	0.037	85 (± 32)	48 (± 9)
	0.37	330 (± 103)	565 (± 56)
	3.7	3486 (± 2333)	7694 (± 1747)
	37	7378 (± 2116)	41066 (± 6108)

Table 1

The effects of the drug concentration in medium and drug exposure time on cellular content of doxorubicin (DOX). Monolayer cells were incubated with doxorubicin (0.037, 0.37, 3.7 and 37 μM) for 1 and 24 h. The cellular uptake of the drug was measured by HPLC.

At the lowest extracellular concentration of doxorubicin (0.037 μM) there was no significant difference between cellular uptake after 1 and 24 h of incubation. The

absence of an expected increased uptake after 24 h might be caused by the fact that such low concentrations were at the lower limits of detection.
With respect to growth inhibition, long-term incubation with the lower dosages of doxorubicin was as efficient as short-term exposures with 10 times higher dosages (Fig. 1).

Effects of oestradiol on cell cycle

Incubation of resting MCF-7 cell cultures with a low concentration of oestradiol (30 pM) resulted in an increase in the number of actively proliferating cells, as measured by PI-fluorescence with flow cytometry (Fig. 2).

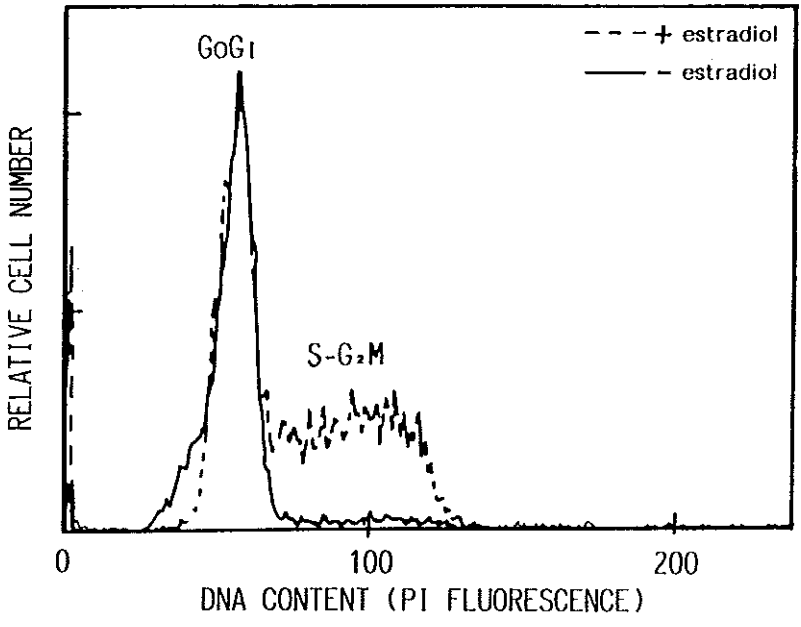

Figure 2
Effect of oestradiol on cell cycle distribution. MCF-7 cells were cultured for 6 days in steroid hormone-deprived experimental medium. Cell cycle distribution, measured by PI-fluorescence, is plotted for control cultures and cultures stimulated with oestradiol for 24 h. The percentage of cells in the S-G2M-phase was calculated from the areas of the peaks as depicted in this figure.

The maximal increase in the number of cells in S-G2M phase was found to occur 24 h after the oestradiol pulse (Fig. 3). This fraction increased in this time period from

approx. 20 to 50% (Fig. 3), while in the control group only 10% of the cells in the S-G2M phase of the cell cycle were observed at 24 h.

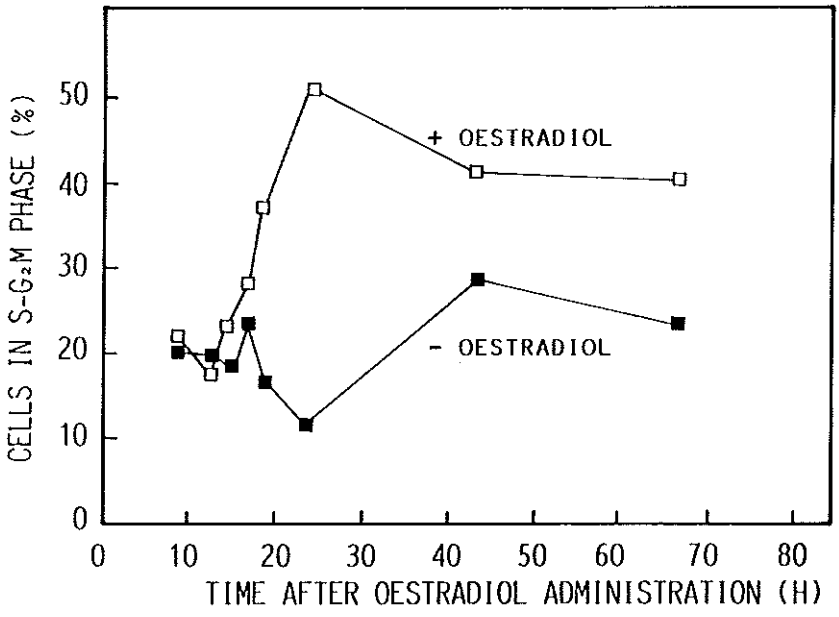

Figure 3

Kinetics of the oestradiol effect on cell cycle distribution. MCF-7 cells were cultured for 6 days in steroid hormone-deprived experimental medium. At the indicated time points the percentage of cells in S-G2M-phase, determined by flow cytometry, is plotted for the oestradiol (30pM) stimulated and unstimulated cultures.

Effect of oestradiol on cellular doxorubicin uptake and tumour cell growth

Pretreatment of MCF-7 tumour cells with 30 pM oestradiol caused a significantly ($P<0.05$) higher cellular uptake of doxorubicin (259 $\pm$ 11 pmol/10^6 cells; mean $\pm$ S.D., n=3), when incubated with 0.37 μM doxorubicin during 6 h (Fig.4) and compared to unstimulated cells (177 $\pm$ 16 pmol/10^6 cells; mean $\pm$ S.D., n=3). Such an increased cellular uptake of doxorubicin was not found after an incubation period of 1 h (94 $\pm$ 2 vs, 94 $\pm$ 8 pmol/10^6 cells; mean $\pm$ S.D., n=2). However, with respect to cell growth, pretreatment with oestradiol appeared to enhance the cytotoxic efficacy of doxorubicin (0.37μM) both during 1- and 6- h incubations (Fig. 5). It has to be noted that a net decrease in cell number caused by doxorubicin was observed not prior than 72 h after an incubation period of 6 hours with the drug (Fig. 5).

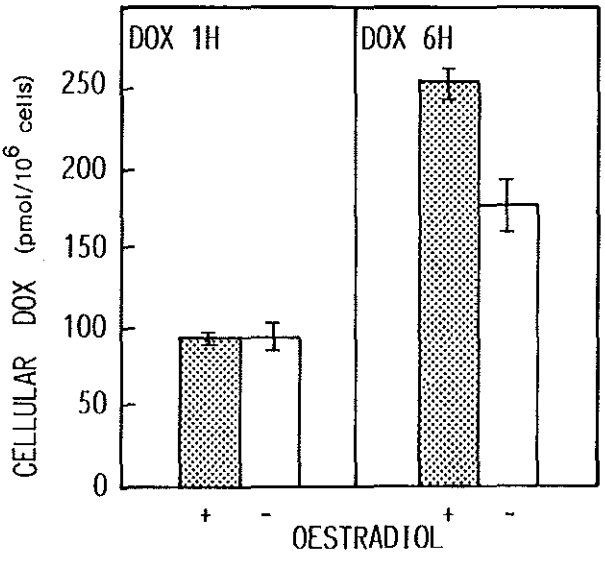

Figure 4
Effect of oestradiol pretreatment on the cellular uptake of doxorubicin. Cellular doxorubicin content was measured by HPLC after 1 and 6 h incubation with doxorubicin (0.37 μM) in oestradiol (30 pM) stimulated (+) and unstimulated (-) control cultures. The 6 h incubation with doxorubicin started 19 h after the administration of oestradiol. The 1 h incubation started 24 h after oestradiol stimulation.

DISCUSSION

Chemotherapy of metastatic breast cancer has currently reached a plateau in therapeutic results with response rates in the range of 50-75% [1,2]. Moreover, in early breast cancer adjuvant therapy mostly fails to accomplish cure [23]. It seems therefore, that no further improvement can be expected from the currently used multi-drug therapies alone [23,24]. Even combined hormono-chemotherapy, which at its best results in an additive effect in subgroups of patients, causes an objective but only temporary response in approx. 75% of the patients [3-6]. An explanation for the lack of cure and the temporary nature of the response in patients with metastatic disease may be found in the observation that, spontaneously or due to growth-inhibitory endocrine therapy, a significant proportion of the tumour cells are in the resting phase of the cell cycle. As a result, tumour cells display cytokinetic drug resistance even to

high dosages of chemotherapeutic agents. Most of these agents, including doxorubicin,

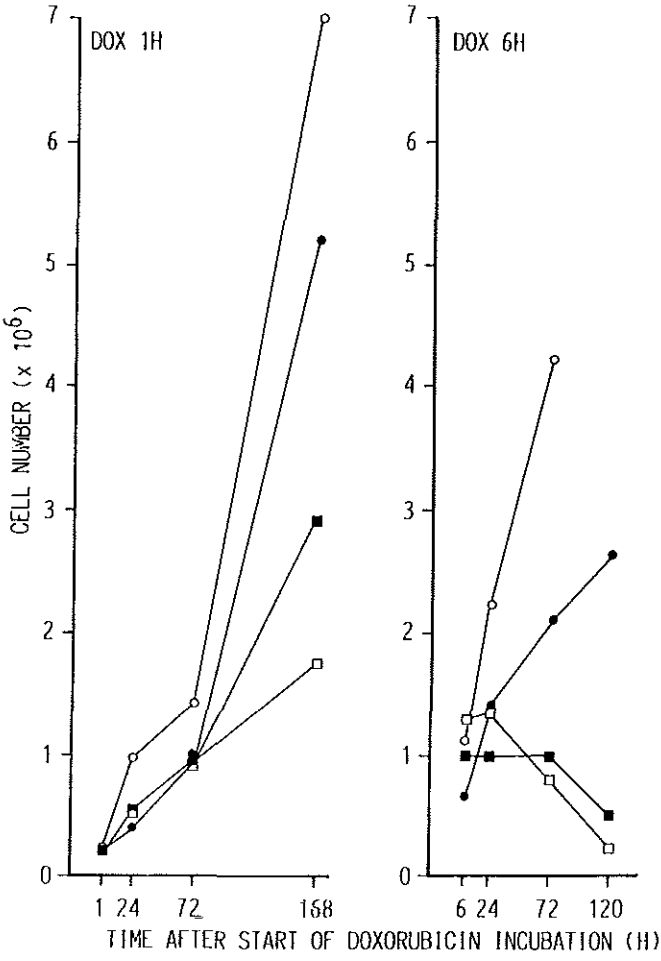

Figure 5

Effect of oestradiol pretreatment on the cytotoxicity of doxorubicin. After oestradiol (30 pM) stimulation and incubation with 0.37 μM doxorubicin during 1 and 6 h as indicated in Fig 4, replicate cultures were washed and drug-free complete growth medium was added to the flasks. At the indicated time points the number of cells in the flasks was determined and growth curves plotted. In the left panel it is shown that after 168 h, growth inhibition by a 1-h incubation with 0.37 μM doxorubicin is significantly higher when the cultures are pretreated with oestradiol ($1.7 \pm 0.14 \times 10^6$ cells vs $2.93 \pm 0.13 \times 10^6$ cells, mean $\pm$ S.D., n =3) ($P<0.05$). In the right panel the decrease in cell number 120 h after a 6 h incubation with doxorubicin is significantly higher in the cultures pretreated with oestradiol ($0.20 \pm 0.02 \times 10^6$ cells vs $0.49 \pm 0.04 \times 10^6$ cells; mean $\pm$ S.D., n=3) ($P<0.05$). [Control plus oestradiol (o), control without oestradiol (●), doxorubicin plus oestradiol stimulation (□), doxorubicin without oestradiol stimulation (■).

are primarily effective on proliferating cells. In agreement with these findings are the results of a study of Sulkes et al [25], which show that slowly growing primary breast cancers with a low labelling index (LI) are not or less sensitive to chemotherapy as compared to tumours with a high LI. Recently, it has been shown that growth-stimulatory hormones (such as oestrogens) can increase the cell sensitivity to cytotoxic chemotherapy by increasing the number of actively proliferating cells in these tumours [10,11]. Several clinical studies, in which the effects of oestrogen 'rescue' or recruitment of tumour cells in the treatment schedule were investigated, show high complete response rates and sometimes an increase in overall survival [12,15]. There are also a few reported studies in which no significant benefit of this treatment modality was found [16,17]. Nevertheless, the results of these studies are difficult to compare because of a great variation in treatment schedules. These differences involve the dose and the choice of chemotherapeutic agents and oestrogens, the use of tamoxifen, the way and duration of treatment, and time scheduling of the drug used. At present the optimal treatment protocol is unknown.

The results of our study suggest that for optimal cell kill the time interval between administration of oestrogens and doxorubicin should be around 18-24 h, as shown by an increase in the number of proliferating cells from about 10-20% to 50-60% in several experiments. Pretreatment of human breast cancer cells with oestradiol from 19-24 h before administration of doxorubicin results in increased cytotoxic efficacy of the drug. This is the first report showing that pretreatment with a physiological dose of oestradiol can increase the cellular uptake of doxorubicin. Inhibition of cell growth appeared to be correlated with the cellular content of doxorubicin. Moreover, pretreatment with oestradiol increased cytotoxicity also after short-term (1 h) incubation with doxorubicin without enhanced cellular uptake of the drug. These observations suggest that both an increased sensitivity to doxorubicin and an augmented cellular uptake of the drug may underlie the present cytotoxic effects of doxorubicin.

Apart from the time of administration, the dose of oestradiol probably is also important in view of the finding that pharmacological dosages (10^{-6}, 10^{-7}M) caused reduced cellular uptake and antimetabolic effects of methotrexate and nevertheless resulted in reduced cell growth. Thus, short-term pretreatment with a pharmacological dose of oestradiol may indeed enhance drug sensitivity of tumour cells, but it may possibly prevent additional beneficial effects by increased cellular uptake of cytotoxic drugs as observed after pretreatment of cells with physiological dosages of oestradiol.

However, the oestradiol-stimulated drug uptake may only affect the uptake of specific cytotoxic drugs, such as doxorubicin. Other drugs may even show a decreased uptake resulting form effects of oestradiol on drug transport via the cell membrane [26].

In conclusion, our study shows that the optimal interval between the start of pretreatment with oestradiol and administration of doxorubicin is 18-24 h. During this period a 3-5-fold increase in the number of proliferating MCF-7 cells was observed. Cells showed a higher uptake of doxorubicin and an increased sensitivity to doxorubicin, which resulted in increased cell kill. Combined treatment with specific hormones and growth factors can improve the cytotoxic ratio of doxorubicin [11]. Therefore, further studies should focus on combinations of hormones and growth factors with respect to recruitment of resting cells prior to chemotherapy.

Acknowledgements

The authors would like to thank Mrs E. Mooi-Kokenberg and Mr K. van der Stuyt for their assistance, Mrs A. Sugiarsi and Mrs M.J. Slotboom for their secretarial help, and J. Marselje for preparing the prints. This work was supported by grant RRTI 87-11 from The Dutch Cancer Society 'Koningin Wilhelmina Fonds'.

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CHAPTER 3

EFFECT OF HORMONAL MANIPULATION AND DOXORUBICIN ADMINISTRATION ON CELL CYCLE KINETICS OF HUMAN BREAST CANCER CELLS.

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British Journal of Cancer 1989; 60: 688-692

SUMMARY

Dual-parameter flow cytometry, following bromodeoxyuridine (BrdUrd) incorporation and propidium iodide (PI) uptake into DNA, was used to study the effect of oestradiol and/or insulin on cell cycle kinetics of human breast cancer cells *in vitro*. After a lag-period of 6-12 h, an optimum in the percentage of S-phase cells was reached between 18 and 24 h after hormone administration. A 1 h pulse of oestradiol was as effective as the continuous presence of oestradiol in pushing the cells from quiescent growing cultures into the cell cycle. A 1 h pulse of insulin was less effective than continuous administration. The addition of doxorubicin resulted in an accumulation of the cells in the late S/G_2M-phases. It is concluded that dual-parameter flow cytometry allows accurate assessment of the effects of hormones and chemotherapy on the cell cycle. Therefore this method is very suitable for studying the interaction of hormones and chemotherapy on cell growth.

INTRODUCTION

Slowly proliferating tumours like breast cancer are in general less sensitive to the lethal effects of cytotoxic drugs than rapidly proliferating malignancies. One of the explanations for this relative insensitivity can be kinetic resistance [1]. Growth stimulation of slowly growing breast tumour cells *in vitro*, followed by cell cycle active chemotherapy, results in an augmented cytotoxic effect of the chemotherapeutic drug [2-5]. This recruitment concept has been clinically applied with diverse results [6-10]. However, little is known with respect to optimal conditions for selection and scheduling of growth stimuli and cytostatics. Studies using cell cultures may provide valuable information for designing future treatment protocols in line with the recruitment principle. These studies require an accurate method to determine the number of cells in the distinct phases of the cell cycle, the duration of the cell cycle and the effects of growth stimuli and chemotherapeutic agents thereon. A commonly used method to establish the DNA distribution in cells involves the uptake of propidium iodide (PI). A major disadvantage of this rapid and reproducible method is that advanced mathematical models are needed to estimate approximately the percentages of the cells in the separate phases of the cell cycle. Moreover, after partial synchronisation of cells by growth arrest and subsequent stimulation, the amount of cells appearing in the early S-phase will remain undetected when using models based on a Gaussian distribution of cells. These disadvantages are circumvented by a dual-

parameter flow cytometric method involving BrdUrd-incorporation and PI-uptake [11]. We have studied recruitment of growth-delayed MCF-7 human breast cancer cells into the cell cycle after oestradiol and/or insulin administration, and the cytokinetic effects of doxorubicin administration thereon.

MATERIALS AND METHODS

Cell culture

The MCF-7 cell line was obtained from E.G. & G. Mason Research Institute (Worcester, MA, USA) in its 219th passage. Cells were grown in a humidified atmosphere of 5% CO_2 in air at 37°C in complete growth medium (RPMI-1640 medium containing 5 μg ml^{-1} phenol red, supplemented with 10% heat-inactivated (30 min at 56°C) fetal calf serum (FCS), 100 U ml^{-1} penicillin, 100 μg ml^{-1} streptomycin, 50 μg ml^{-1} gentamycin and 10 μg ml^{-1} porcine insulin. For experiments, logarithmically growing cell cultures were trypsinised and seeded in T25-flasks at a density of 0.5 x 10^6 cells per flask, in experimental medium, i.e. RPMI-1640 medium, without phenol red and insulin, supplemented with antibiotics and 4.5% steroid hormone depleted FCS (obtained by treatment twice with 0.5% charcoal, 0.05% dextran T-70 (w/v) for 45 min at 50°C, and an intermediate 2 h incubation at 37°C with 2 U ml^{-1} of sulphatase). Cells were precultured for 2 days. Experimental medium without additions (control), or supplemented with 0.03, 0.5 or 1.0 nM oestradiol (Merck, Darmstadt, FRG), 1.7 μM porcine insulin (Organon BV, Oss, The Netherlands), or the combination of 1 nM oestradiol and 1.7 μM insulin, was added to the cell cultures. Medium was renewed every day unless indicated otherwise in the legends to the figures. In experiments studying the effects of doxorubicin (Adriablastina; Farmitalia, Milan, Italy), medium containing 0.2 μg ml^{-1} doxorubicin + 1 nM oestradiol was added for 6 h to the cultures, which had been pretreated for 15 h with medium containing 1 nM oestradiol, i.e. the stimulated cultures. In the control groups the same procedure was used but without oestradiol addition. After two washes after doxorubicin incubation, the cells were allowed to continue growth in complete growth medium. Medium was renewed every 48 h.

Cell harvest

Thirty minutes before harvesting, BrdUrd (Serva, Heidelberg, FRG) was added to the monolayer cultures (final concentration of 10 μM) and incubated at 37°C in 5%

CO_2 in air. Cells were washed twice with phosphate buffered saline (PBS)' and were harvested by a 5 min incubation at 37°C with 0.5 ml trypsin/EDTA) 0.05/0.02%; Biochrom, Berlin) in 2 ml PBS, and addition of 1 ml trypsin inhibitor (0.1 mg ml^{-1}; Sigma, St. Louis, MO, USA) in PBS. An aliquot of the cell suspension was collected for cell count using a haemacytometer, and the remainder of the cells were pelleted at 100 g for 5 min, resuspended in 100 μl PBS, fixed for 30 min at 0°C with 2 ml 70% ethanol (-20°C), and stored at -20°C before preparation for analysis by flow cytometry.

Flow cytometry

Labelling and staining procedure (anti-BrdUrd FITC/PI)

Fixed cells were pelleted for 5 min at 100 g, incubated with 2 ml of 4 M HCl for 20 min at 18°C, and after centrifugation the nuclei were incubated for 10 min at 0°C, in 0.5 ml 0.1 M phosphate buffer (pH 4.5), containing 0.1 mg ml^{-1} pepsin (Sigma, St. Louis, MO, USA). The nuclei were pelleted and washed with 2 ml 0.1 M borate buffer (pH 8.5). Following centrifugation the nuclei were incubated for 30 min at 0°C with a 1:20 dilution of Anti-BrdU-FITC-conjugate (Becton & Dickinson, Mountain View, USA) in a final volume of 100 μl PBS containing 0.25% Tween-20, and 5% bovine serum albumin (BSA), spun down after addition of 2 ml 0.5% Tween-20 in PBS, and incubated for 10 min. at 0°C in 2 ml PBS containing 0.25% Tween-20 and 10 μg ml^{-1} PI. Pelleted nuclei were resuspended in 0.5-1.0 ml PBS containing 0.5% Tween-20 and were analysed by flow cytometry.

Measurement of FITC- and PI-fluorescence

The FITC- and PI-fluorescence of individual nuclei were measured using a Becton and Dickinson (Sunnyvale, CA, USA) fluorescence-activated cell sorter (FACS 440). In the FACS 440 system the nuclei traversed the light beam of a Spectra-Physics 5-W Argon laser tuned at 488 nm, 0.4 W. Emitted light passed a 560 nm dichroic beam splitter. Excitation and emission wavelengths of FITC and PI were 494/517 and 540/625 nm, respectively. Green (FITC) fluorescence was measured through a 530/30-nm band-pass filter and red (PI) fluorescence through a KV 550 cut-off filter. Emitted light was registered at a photomultiplier. Signals were amplified linearly. The instrument was calibrated with 1.0 and 2.83 μm diameter fluorescent standard beads (Polysciences Inc., Warrington, PA, USA). Cell debris was excluded from analysis

by elevating the threshold of the red fluorescence. The flow rate was set at 500-1000 nuclei s^{-1}. For each sample at least 10^4 cells were analysed.

Data analysis was performed using a Hewlett Packard 68B system. PI-fluorescence was recorded as a histogram of fluorescence intensity. From this histogram the percentage of nuclei in the different phases of the cell cycle was estimated with graphical methods and a fitting method (SFIT) using mean fluorescence [12]. Cell cycle distribution after labeling with anti-BrdUrd FITC and PI was performed using the windowing technique. Windows were set around the regions of G_0G_1/S/G_2M-phase cells in the dot plots [12].

RESULTS

The cell cycle distribution of MCF-7 cells in culture was established by analysis of DNA distribution using PI-uptake and by dual-parameter flow cytometry. The histogram obtained after PI-uptake in nuclei of MCF-7 cells 12 h after a 1 h pulse with 30 pM oestradiol is shown in Figure 1a. The CV of the G_0G_1-peak was 4.5%. By dual-parameter flow cytometry it is shown that of the total amount of cells present in the S-phase (35%), a high proportion is actually in the early S-phase (Figure 1b), cells which were not detected when only PI-uptake was used. Analysis of the DNA histogram (Figure 1a) by graphical and a 'simple' fitted method to assess the percentage of S-phase cells resulted in an underestimation of the amount of cells in S-phase. Depending on the methods used [12], 16-29% of the cells were observed in S-phase. Even sophisticated mathematical programs will result in an underestimation of the amount of S-phase cells, because these cells are hidden under the G_0G_1-peak. Moreover, by analysis of DNA histograms obtained with PI-fluorescence only, no discrimination can be made between cells which are arrested in the S-phase and cells which are actively synthesising DNA. For reasons mentioned above, the PI-method is not appropriate to study accurately changes in cell cycle kinetics resulting from perturbation with cell cycle active cytotoxic agents. We have therefore applied the method of dual-parameter flow cytometry with PI-and Anti-BrdUrd FITC to study cell cycle kinetics of MCF-7 breast cancer cells and the effects of growth-stimulating hormones and doxorubicin thereon.

Growth of MCF-7 cells which were seeded and maintained in medium deprived of steroid hormones was remarkably decreased. The amount of cells in the S-phase of the

cell cycle declines from 30-40% at the time of seeding to approximately 10-15% at the start of the experiment, i.e. time point zero. Figure 2 shows by dot plots the wave

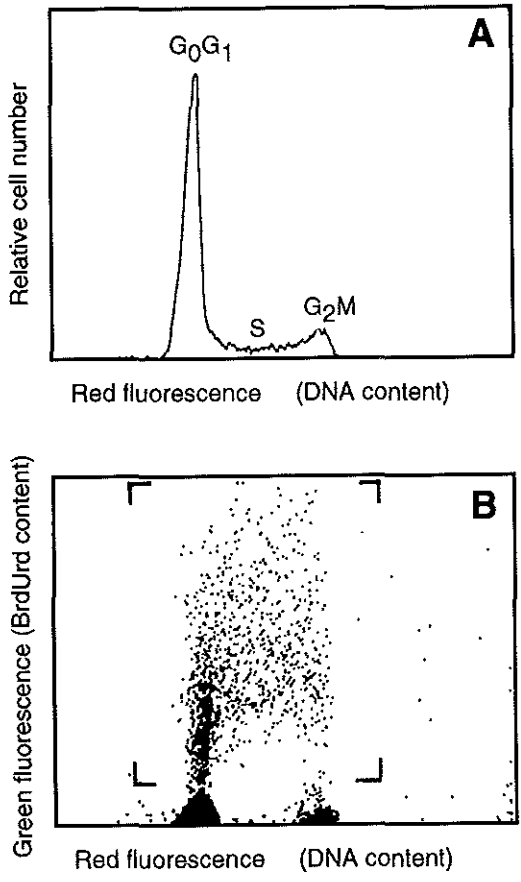

Figure 1

Cell cycle distribution of MCF-7 cells. Cells were harvested 12 h after a 1 h pulse with 30 pM oestradiol. **a**, Histogram of DNA, propidium iodide (PI) uptake, indicated by red fluorescence only. **b**, Dual-parameter flow cytometry with PI (*x* axis) and anti-BrdUrd FITC fluorescence (*y* axis). Cells in the marked area represent cells actively synthesising DNA, i.e. cells in S-phase. Black spot below the marked area on the left side represents G_0G_1-phase cells, and the black spot on the right side represents G_2M-phase cells. The G_0G_1-peak in histogram a corresponds with the left black spot in b plus the cells in early S-phase lying in-line above this black spot.

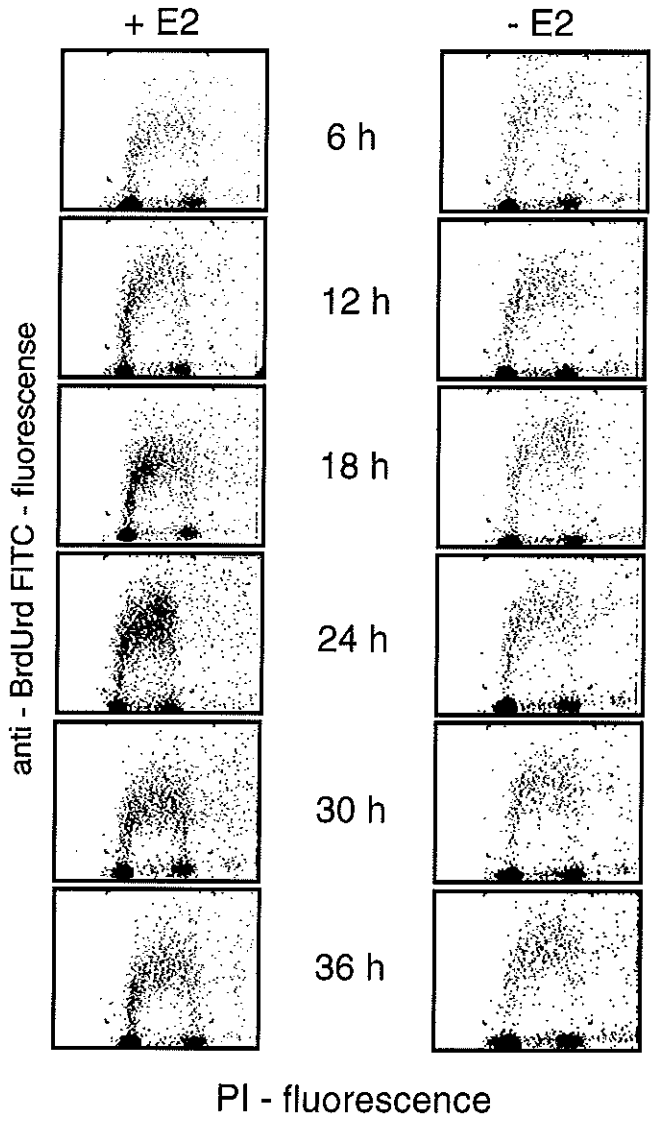

Figure 2

Cell cycle distribution of MCF-7 cells, measured by dual-parameter flow cytometry 6-36 h after start of stimulation with 1 nM oestradiol (E_2) compared to controls. The left column shows a clear increase in cells in S-phase, especially 18-24 h after oestradiol administration. For detailed information see: the method section and the legend to Figure 1.

of cells going into S-phase after oestradiol administration. Figure 3a shows the kinetics of accumulation of cells in the S-phase as a result of stimulation (for up to 26 h) with 1 nM oestradiol, 1.7 μM insulin and the combination of both hormones. After a lag period of about 6-12 h (as also concluded from additional experiments, data not shown), the percentage of cells in the S-phase augments rapidly with an optimum between 18 and 24 h after addition of hormones. Stimulation with insulin mimics the pattern obtained by oestradiol treatment, whereas the combination of both hormones shows a minor (9%) but significant (Wilcoxon, $2p < 0.05$) additional effect.

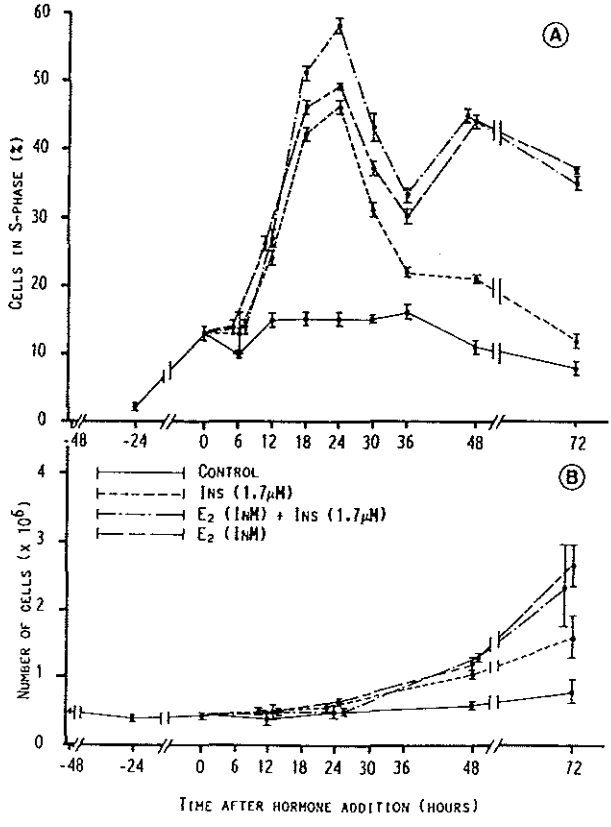

Figure 3
Effects of oestradiol 1nM (E_2), insulin 1.7 μM (Ins), or the combination (E_2 + Ins) on the increase of S-phase cells (a), and growth of MCF-7 cell cultures (b), compared to controls. Medium was renewed daily, and hormones were present from time 0 up to 26 h. Data for both a and b are plotted as means $\pm$ s.d. of triplicate incubations.

However, this small additional effect regarding the percentage of cells in S-phase after 24 h did not result in an increase of cell number after 72 h. The maximal increase in the percentage of cells in the S-phase occurred 24 h after start of stimulation. A decline in the percentage of S-phase cells was observed after 24 h. In subsequent experiments we observed that this decline occurred irrespective of a medium change 2 h after reaching maximal stimulation. In cultures treated with oestradiol for 1 h (data not shown) or 26 h (Figure 3a), followed by incubation in the absence of oestradiol, a second wave of S-phase cells, starting after 36 h from time point zero, was observed. This second wave was not observed after preincubation with insulin only. Figure 3b shows the growth curves. Twenty-four hours after hormone addition the amount of cells per flask appeared identical in both the stimulated and the control groups. This implies that the increase in the percentage of cells in S-phase during this time period is due to recruitment of cells of these quiescent growing cultures into the cell cycle, and not to an increase in cell number due to a subpopulation of rapidly proliferating cells. The pattern (as shown in figure 3a for 1 nM oestradiol) and extent of stimulation were identical for lower dosages of oestradiol (0.03 and 0.5 nM used) (data not shown). In addition, a short 1 h pulse of 1 nM oestradiol resulted in a similar stimulatory effect after the pulse compared to the continuous presence of oestradiol (at 21 h, 60 *vs* 60%, and at 30 h, 38 *vs* 36% cells in S-phase). In contrast a 1 h pulse of insulin was not as effective as the continuous administration (Table I).

In separate experiments the effects of doxorubicin were studied. The presence of doxorubicin during the last 6 h of a 21 h incubation with or without 1 nM oestradiol did not affect the amount of S-phase cells at 21 h (Table II). However, after the subsequent addition of complete growth medium at 21 h, the S-phase cells, in the doxorubicin treated cultures completely accumulated in the late S- and G_2M-phases, measured 2 (Figure 4) and 5 days (Table II) later. After 5 days 59% of the oestradiol stimulated cells were accrued in the late S/G_2M-phases and 34% of the cells in the unstimulated controls. The accumulation of doxorubicin treated cells in the late S/G_2M-phases of the cell cycle has also been described for lymphoblasts [13].

DISCUSSION

Kinetic resistance can be one of the explanations why slowly growing tumours like breast cancer fail to respond to cytotoxic therapy [1]. Preclinical research has shown that growth of breast tumours can be accelerated by several hormones and growth

factors. Theoretically this growth stimulation can be used to recruit quiescent cells into the cell cycle, rendering them more vulnerable to the lethal effects of concomitant cytotoxic drugs. *In vitro* studies indicated that the combination of growth stimulation and cytotoxic therapy can lead to an enhanced cell kill in breast cancer [2-5].

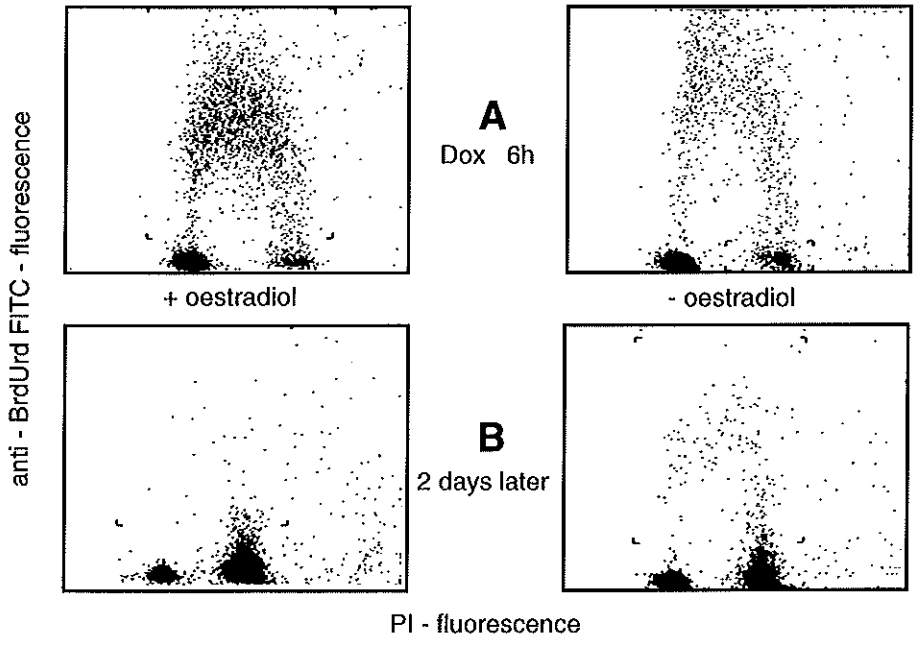

Figure 4
Effects of a 6 h incubation with doxorubicin on MCF-7 cells in S-phase (**a**) administered for the latter 6 h of a 21 h stimulation period with oestradiol (left) compared to control (right), measured immediately after doxorubicin incubation. **b** shows accumulation of cells (black spot on the right in the figures) in the late S/G_2M-phases 2 days after this 6 h incubation with doxorubicin.

Several clinical studies already make use of this concept of recruitment. Most of the studies report a higher complete remission rate and/or a longer survival [6-9]. Little is known, however, about the optimal duration, scheduling and dosages of this

hormono-chemotherapy, and about the effect of this combined modality on cell cycle kinetics.

In order to establish the magnitude of cytokinetic resistance in the treatment of breast cancer and to investigate optimal conditions to overcome this phenomenon, accurate measurement of changes in cell cycle kinetics due to therapy must be available. DNA histograms obtained with PI-fluorescence are widely used for the study of cell cycle kinetics. In this study we have shown that using this method the amount of (semi-) synchronised cells which appear in the early S-phase of the cell cycle after growth stimulation is underestimated when graphical or simple fitting methods are used to establish the amount of S-phase cells.

Table I Effect of time to exposure to oestradiol or insulin on percentage of cells in S-phase

	Cells in s-phase (%)			
	Continous stimulation		1 h phase	
Additions	at 21 h	at 30 h	at 21 h	at 30 h
Control	14 ± 1	16 ± 1	14 ± 1	15 ± 2
Oestradiol (1 nM)	60 ± 1	36 ± 1	60 ± 4	38 ± 2
Insulin (1.7 μM)	42 ± 7	29 ± 1	21 ± 1	18 ± 1

MCF-7 cells were stimulated with hormones for 1 h or continously for 21 or 30 h, and were harvested at 21 or 30 h after start of hormone addition. Percentage of cells in S-phase was measured by dual-parameter flow cytometry. Data are the means ± s.d. of duplicate incubations.

When there is a non-Gaussian distribution of cells in the S-phase, only very sophisticated mathematical methods can predict with some accuracy the amount of S-phase cells from the histogram.

Moreover, DNA histograms do not discriminate between cells arrested in S-phase and cells actively synthesising DNA. Dual-parameter flow cytometry can overcome these problems by a sharp discrimination between the cells in the separate phases of the cell cycle. With the method of BrdUrd incorporation followed by anti-BrdUrd FITC incubation, cells exhibiting green fluorescence are cells in S-phase actively synthesising DNA. This method allows us: (i) to define the time period required for cells to appear in the early S-phase after growth-stimulation; (ii) to assess small differences in the maximal percentages of cells in the S-phase after different treatment

modalities; (iii) to establish kinetic changes after cytotoxic treatment; and (iv) to investigate changes in the duration of the different phases of the cell cycle after hormonal-chemotherapeutic perturbation. In this study we have shown that a 6 h incubation period with doxorubicin (0.2 μg ml^{-1}) does not affect the percentage of cells in S-phase at the end of the doxorubicin incubation period. Recruitment of quiescent growing MCF-7 cells into the cell cycle was not blocked in these first 6 h. However, after 2 and 5 days all cells in S-phase have accumulated into the late S/G_2M-phases in both oestrogen-stimulated and control groups.

Table II Effect of doxorubicin incubation on the cell cycle distribution of MCF-7 cells

	Cells actively synthesising DNA (%)	
Additions	At the end of dox incubation	5 days later
Control	25 ± 1	27 ± 1
Oestradiol (1 nM)	57 ± 1	15 ± 1
Control + doxorubicin	25 ± 2	6 ± 1 (34% late S/G_2M)
Oestradiol + doxorubicin	57 ± 1	3 ± 1 (59% late S/G_2M)

MCF-7 cells were incubated with and without oestradiol (1 nM) for 21 h, and with and without doxorubicin (0.2 μg ml^{-1}) for the last 6 h of this period. Cell cycle distribution was assessed at the end of doxorubicin incubation and 5 days later. Data are the means ± s.d. of duplicate incubations.

In addition hardly any cells were synthesising DNA at these timepoints, indicating the absence of cells going from G_0G_1- to S-phase. This suggests that doxorubicin in this concentration and for this incubation period blocks MCF-7 cells, not only in the late S/G_2M-phases, but also in the G_0G_1-phase of the cell cycle. In view of the fact that cells in the G_2M-phase are most sensitive to radiotherapy [14], treatment of cancer patients with doxorubicin followed by radiotherapy might be of clinical value. In conclusion, dual-parameter flow cytometry is a reliable method to investigate cytokinetic changes in perturbed cells. The method can be of help in designing the optimal timespan, dosages and combinations of growth factors and chemotherapeutic drugs, resulting in an optimal cytotoxic effect in the recruitment concept, with respect to the management of breast cancer.

Acknowledgement

We thank Mrs. J. van der Meij-van der Vlis for her secretarial help and the department of photography for preparing the prints. This study is supported through grant RTTI 87-11 by the Dutch Cancer Society (KWF).

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CHAPTER 4

MANIPULATION OF CELL CYCLE KINETICS: INFLUENCE ON THE CYTOTOXICITY OF DOXORUBICIN IN HUMAN BREAST CANCER CELLS

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Journal of Steroid Biochemistry and Molecular Biology 1990; **37**: 1097-1101

SUMMARY

In vitro exposure of estrogen-receptor negative (ER-) EVSA-T human breast cancer cells to insulin and/or estradiol had no effect on cell cycle distribution, in contrast to a 3 to 5-fold increase in the percentages of cells in the S-phase of the cell cycle in the ER+ MCF-7 cell line. Estrogen pretreatment of MCF-7 cells followed by incubation with doxorubicin resulted in an augmented inhibition of cell growth compared to unstimulated controls. This delay in growth was accompanied by a decrease in the percentages of cells actively synthesizing DNA, and by an augmented percentage of cells exhibiting a G_2M-amount of DNA at the end of a 6-9 days period of culture in complete growth medium.

INTRODUCTION

Anti-hormones decrease the growth fraction of hormone responsive breast tumors, leading to an accumulation of cells in the G_0G_1-phase of the cell cycle. This influence of anti-hormones on cell cycle kinetics could be one of the reasons of the disappointing results of combined endocrine- and chemotherapy, because most chemotherapeutic agents exert their effect mainly on cells in the S- and G_2M-phases of the cell cycle. Even adverse effects of the combination of tamoxifen with chemotherapy have been reported in subsets of patients [1].

In contrast to this growth inhibition, several hormones and growth factors are known to accelerate the growth of slowly proliferating breast tumors, leading to the recruitment of a population of quiescent or G_0G_1-phase cells into the S- and G_2M-phases of the cell cycle. Several clinical studies have already applied the principle of hormonal recruitment of breast cancer cells into the active phases of the cell cycle followed by chemotherapy [2-6]. No uniform conclusions can be made yet due to a) the use of different therapeutic schemes; b) the possibility of inadequate recruitment due to incomplete reversion of the anti-estrogenic effect of tamoxifen, the synchronizing agent in several trials; c) the absence of proof of concealed recruitment in most of the studies.

We investigated the following in the ER+ MCF-7 and the ER- EVSA-T breast cancer cell line:

1) The effects of growth-promoting hormones and of doxorubicin on the kinetics of the tumor cells as measured by dual-parameter flow cytometry (PI/anti-BrdUrd FITC).

2) The effects of estrogen pretreatment followed by incubation with doxorubicin on the growth of the cell cultures.

MATERIALS AND METHODS

Cell culture

Culture conditions for both cell lines were identical to those described previously [7]. MCF-7 cells were maintained in RPMI-1640 medium containing 5 μg/ml phenol red, supplemented with 10% heat -inactivated (30 min at 56°C) bovine calf serum (BCS), 100 U/ml penicillin, 100 μg/ml streptomycin, 50 μg/ml gentamycin and 10 μg/ml porcine insulin. EVSA-T cells were maintained in Ham/F12 DMEM medium containing 8.6 μg/ml phenol red, supplemented with 5% heat-inactivated BCS, antibiotics and insulin. For experiments, logarithmical growing cell cultures were trypsinized and seeded in T25-flasks at a density of 0.5 x 10^6 cells per flask in experimental medium, i.e. RPMI-1640 or Ham/F12 DMEM medium without phenol red and insulin, supplemented with antibiotics and 4.5% (MCF-7) or 2.5% (EVSA-T) steroid-hormone depleted fetal calf serum. Cells were precultured for 2 days. Experimental medium without additions (control) or supplemented with 1 nM estradiol (Merck, Darmstad FRG), 1.7 μM insulin or the combination of both was successively added to the cell cultures for upto 24 h. In hormone-chemotherapy experiments MCF-7 cell cultures pretreated with estradiol (1 nM) for 19 hours were incubated for 30 minutes or 23 hours with experimental medium containing respectively 3 and 0.06 μM doxorubicin (Adriablastina, Farmitalia, Milan, Italy). After the 23 hour incubation period with doxorubicin, cells were washed and cultured further in complete growth medium. After the 30 minutes doxorubicin incubation and washing, the cells were cultured for another 24 hour in experimental medium in the absence of doxorubicin, followed by culturing in complete growth medium. The cell number per flask was set at regular intervals, as indicated in the figures.

Assessment of cell cycle distribution by flowcytometry

Bromodeoxyuridine (BrdUrd, Serva, Heidelberg, FRG) at a final concentration of 10 nM, was added to the cultures 30 min before harvesting. After this incubation cells were washed twice and were harvested by a 5 min incubation at 37°C with 0.5 ml trypsin/EDTA (0.05:0.02%; Biochrom, Berlin, FRG) in 2 ml PBS, and the addition of 1 ml trypsin inhibitor (0.1 mg/ml; Sigma St Louis, MO, USA) in PBS. An

aliquot of the cell suspension was collected for assessment of the cell number, using a hemocytometer. The remainder of these cells were stored in 2 ml of 70% ethanol (-20°C) before preparation for analysis by flow cytometry. The labeling- and staining procedures as well as the fluorescence measurement by flow cytometry were performed as described previously [7].

RESULTS

We have previously shown that the addition of estradiol and/or insulin to the medium of slowly proliferating ER^+ MCF-7 cell cultures results, after a lag-period of 6-12 h, in a rapid increase of the percentage of cells in the S-phase of the cell cycle.

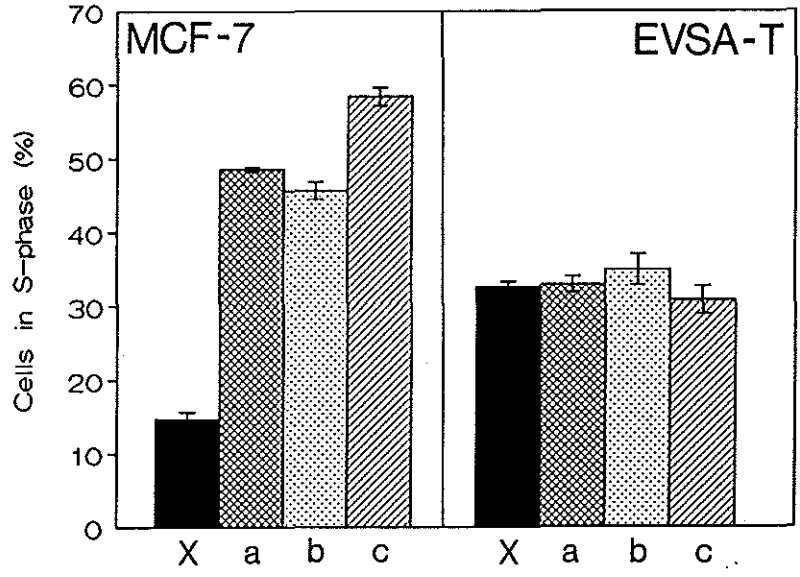

Figure 1
Effects of a 24 hour incubation with 1 nM estradiol (a), 1.7 μM insulin (b), or a combination of both hormones (c) on the increase in the percentage of S-phase cells in MCF-7 and EVSA-T cells, compared to controls (x=control: 0.01% ethanol). Data are the means $\pm$ SD of triplicate incubations.

The maximum of this increase is reached 18-24 h after the addition of the hormones [7]. In the initial experiments of the present study, the effects of estradiol (1 nM), insulin (1.7 uM) or a combination of both were examined in the ER^- EVSA-T cell

line. In Fig. 1, the effects of a 24 h incubation with estradiol and/or insulin on the percentages of cells in the S-phase of the cell cycle in EVSA-T cells are compared with the effects of the hormones in the MCF-7 cell line. Under steroid-hormone deprived culture conditions the EVSA-T cell line proliferates faster than the MCF-7 cell line, and no growth-stimulating effects of the hormones are observed in EVSA-T cells in contrast to the MCF-7 cells.

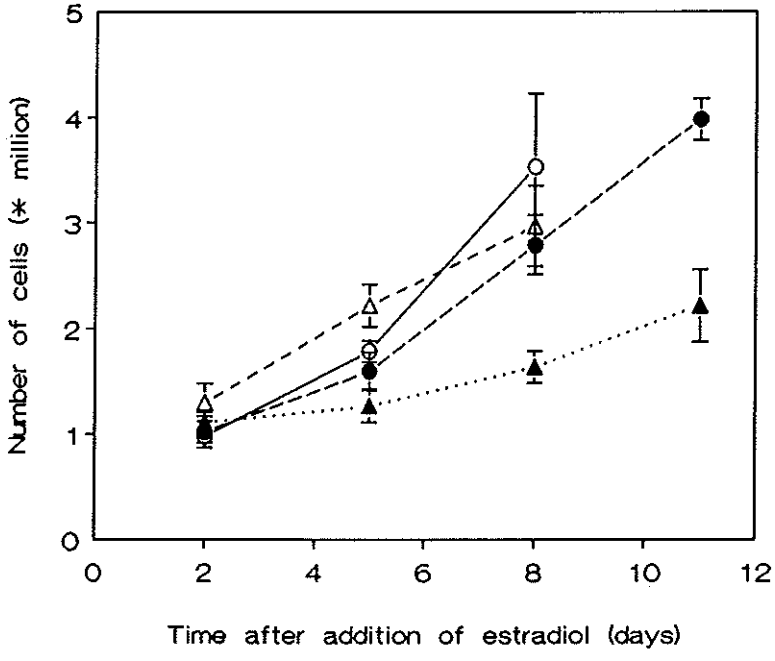

Figure 2
Effects of a 19-h pretreatment with estradiol (1 nM), followed by a 23-h incubation with doxorubicin (60 nM) (E_2/dox: ▲) on the growth of MCF-7 cells, as compared to unstimulated cultures (dox:●) and to the control cultures not incubated with doxorubicin (with E_2: Δ, control without E_2: 0). Data are the means ± SD of triplicate incubations.

The impact of estradiol pretreatment on the ultimate cytotoxicity of doxorubicin was investigated in subsequent experiments. MCF-7 cells were incubated with 1 nM estradiol 19 hours prior to incubation with doxorubicin. Doxorubicin was administered to the cultures at a low concentration (60 nM) for 23 h. or at a high concentration (3 μM) for 30 min. Figure 2 shows an example of growth of MCF-7 cells pretreated for

19 h. with or without 1 nM estradiol followed by a 23-h. incubation with 60 nM doxorubicin.
Estradiol pretreatment resulted in an enhanced cytotoxic effect of doxorubicin, as can be seen in the much slower increase in cell number in these cultures from day 5 onwards.

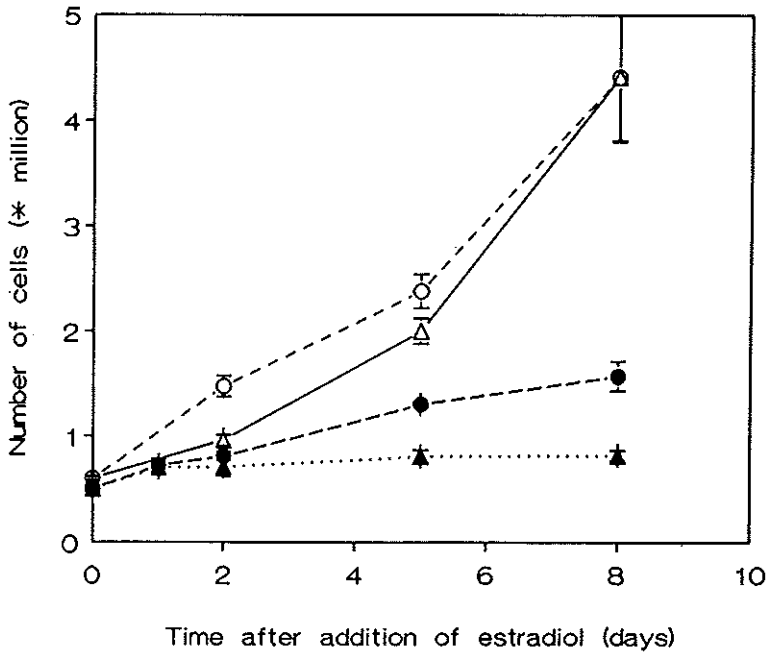

Figure 3
Effects of a 19 h-pretreatment with estradiol (1 nM), followed by a 30 min-incubation with doxorubicin (3 μM) (E_2/dox: ▲), on the growth of MCF-7 cells, as compared to unstimulated cultures (dox: ●) and to control cultures not incubated with doxorubicin (with E_2: Δ, control without E_2: 0). Data are the means $\pm$ SD of triplicate incubations.

The unstimulated cultures incubated with doxorubicin grew almost as rapidly in the presence of doxorubicin as the unstimulated control cultures. Fig. 3 shows the growth curves of MCF-7 cells stimulated with 1 nM estradiol for 19 h. followed by incubation with doxorubicin (3 μM) for 30 min. Growth in the unstimulated cultures is markedly retarded by doxorubicin compared with the cultures not incubated with doxorubicin, but there is nevertheless a small increase in cell number after 5 and 8 days of culture

inhibited in growth by doxorubicin. However, in the cultures pretreated with estradiol followed by doxorubicin even no increase in cell number is observed during this period.

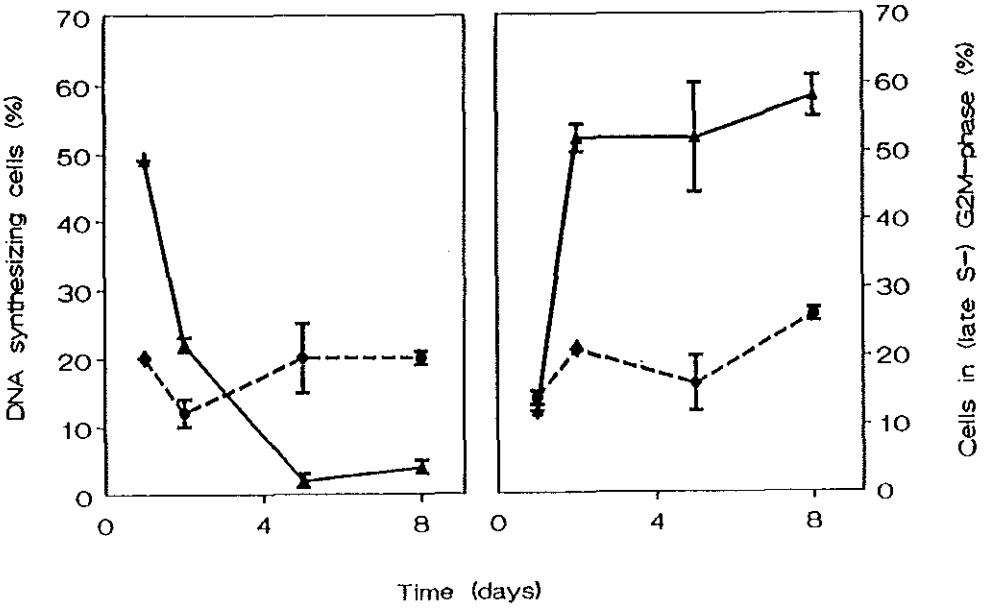

Figure 4

Effects of estradiol pretreatment followed by a 30 min-incubation with doxorubicin (3 μM) on the cell cycle distribution of MCF-7 cells (E_2/dox: ▲, dox: ●). Data are the means $\pm$ SD of duplicate incubations.

In addition to assessment of cell number as a result of hormono-chemotherapy, the cell cycle distribution was measured at the same time points as the cell counts were obtained. Fig. 4 shows the cell cycle distribution on days 1 to 8 from the experiment depicted in Fig. 3. A 30 min-incubation with doxorubicin has no immediate effect on the percentages of cells in the S-phase of the cell cycle both in the non-stimulated (18 vs 20% S-phase cells) and estradiol-stimulated (46 vs 49% S-phase cells) cultures. Cells present in the S-phase directly after estrogen pretreatment and doxorubicin incubation (= day 1 in Fig. 4) accumulated in the (late S-) G_2M-phase of the cell cycle at day 2, and remained there for the following days.

On the other hand when doxorubicin was administered in a low concentration for 23-h, after or without estradiol pretreatment (Fig. 2), accumulation of S-phase cells into the late S-G_2M-phase was less pronounced (maximal amount of cells in the late S-G_2M-phase: 30%, data not shown).

DISCUSSION

Endocrine therapy and chemotherapy act in different ways, and possibly exert their effects on different tumor cell populations in heterogenous breast cancer. Therefore, combinations of growth inhibitory anti-hormones with chemotherapy are applied. However, the results of clinical trials are so far not spectacular and even conflicting results were obtained [1].

On the other hand, evidence was found that estrogens could promote the growth of (slowly proliferating) breast cancers. Weichselbaum et al. [8] used this principle of hormonal recruitment in MCF-7 cells, and showed that growth stimulation of MCF-7 cells with 17β-estradiol resulted in a enhanced cytotoxic effect of successively administered 1-β-D-arabinofuranosylcytosine, an S-phase specific chemotherapeutic drug. Our results show that estradiol and/or insulin can recruit hormone-responsive MCF-7 breast cancer cells into the proliferative phase of DNA-synthesis of the cell cycle, whereas no growth stimulation of the hormones was found in the ER^- EVSA-T cell line. When these growth-stimulated MCF-7 cells were incubated with doxorubicin at the moment that the highest percentages of S-phase cells were found, a significantly improved cytotoxic effect of doxorubicin was observed. Assessment of the cell cycle distribution, as a result of hormone-chemotherapeutic perturbation with the high doxorubicin concentration, shows that the percentage of cells actively synthesizing DNA drops rapidly in the cultures pretreated with estradiol. The drop in the percentage of S-phase cells is accompanied by a long-term accumulation of cells into the (late) S-and G_2M-phases of the cell cycle, an effect which lasts at least until the end of the experiments. The lower concentration of doxorubicin, applied for a prolonged period, has a less pronounced effect on cell cycle distribution.

These results support findings reported by several investigators. In ER+ breast cancer cells a higher cytotoxic effect of chemotherapy after hormonal priming was observed [2,8-11]. Paridaens et al. [2] found a synergistic cytotoxic effect in the MXT mouse mammary tumor when cyclophosphamide was administered 24 h, and not 48 h, after estradiol pretreatment, whereas the synergistic effect was optimal when

estradiol was used in the dose that had led to the greatest mitogenic stimulation. This suggests that the improved cytotoxicity of cyclophosphamide on the tumor cells is due to recruitment of the cells into the active phases of the cell cycle, rather than to the simultaneous presence of both drugs in the cell. Epstein et al. [12-14] found that estrogen potentiates the cytotoxicity of VP-16 in the T-47D breast cancer cell line, not dependent upon cellular commitment to DNA synthesis, but by recruiting a clonogenic subpopulation characterized by increased topoisomerase II levels localized to an activated G1-phase cell subset. On the other hand Shaikh et al. [15,16] reported a synergistic cytotoxic effect in MCF-7 cells pretreated for 48 hours with medroxyprogesterone acetate (MPA) followed by incubation with vincristine, methotrexate or doxorubicin. The concentration of MPA used in these experiments had little effect on cell cycle distribution. Even exposure to doxorubicin before MPA led to an additive, but not synergistic cytotoxic effect. Osborne et al. [17] described an improved cytotoxic effect on MCF-7 cells from the combination of tamoxifen pretreatment followed by administration of doxorubicin or hydroxy-cyclophosphamide but a decreased cytotoxic effect was found when tamoxifen was followed by melphalan or fluorouracil. This might be caused by effects of tamoxifen on the intracellular transport of these cytotoxic drugs.

The results reported in most studies show that growth stimulation of slowly proliferative breast cancer with estradiol followed by chemotherapy seems worthwhile. Even specific combinations of anti-hormones and chemotherapy may be of interest. However, little is still known about the effects of combined- or sequential treatment of hormones and chemotherapy on tumor cells. Besides the kinetic implications of (anti-) hormones on breast cancer cells, combinations and sequences of (anti-) hormones and chemotherapy can lead to changes in: the fluidity of the cellular membrane, enzyme activation, drug-uptake and -metabolism, binding of drugs to molecular targets, or in the repair of drug-induced damage, all with possible consequences for the cytotoxicity. For clinical reasons it seems to be advisable not to combine both treatment modalities, outside controlled trials, until more of the mechanisms of action is revealed.

Acknowledgement

We thank the Department of Photography for preparing the prints.

This study is supported through grant RRTI 87-11 by the Dutch Cancer Society (KWF).

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CHAPTER 5

UPTAKE AND DISTRIBUTION OF DOXORUBICIN IN HORMONE-MANIPULATED HUMAN BREAST CANCER CELLS IN VITRO

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Submitted

SUMMARY

Kinetic resistance to cytotoxic drugs may account for the moderate responsiveness of breast cancer to chemotherapy. In the present study the *in vitro* effects of estradiol-mediated DNA stimulation on the cellular uptake of the DNA intercalating drug doxorubicin (DOX) were examined in MCF-7 human breast cancer cells. Using the fluorescent properties of the drug, the cellular uptake was investigated by high performance liquid chromatography (HPLC), and by flow cytometry. The uptake of DOX (0.01-2 μg/ml) by MCF-7 cells in suspension, incubated for 1 and 6 h, showed a strong correlation between the incubation concentration of DOX and the cellular uptake of the drug as measured by HPLC and flow cytometry. Simultaneous exposure of MCF-7 cells, in monolayer culture, to DOX (0.04-0.2 μg/ml) and estradiol (1 nM) for 1-24 h showed no significant difference in uptake of the drug compared to control cultures exposed to DOX in the absence of estradiol. Neither was there a significant difference in uptake of DOX when MCF-7 cells were pretreated with estradiol (1 nM) for 16-24 h followed by a 0.5, 1, 6 and 21/23 h incubation with DOX (0.01-2 μg/ml). Pretreatment with estradiol did not affect the retention of DOX as measured 24 h after a 0.5 h incubation with DOX (2 μg/ml). Furthermore, fluorescence microscopy revealed no difference in the cellular DOX distribution pattern of estradiol-stimulated MCF-7 cultures compared to unstimulated cultures. From this study we can conclude that, for the human MCF-7 breast cancer cells *in vitro*, the uptake, retention, and cellular distribution of DOX is not influenced by estrogenic manipulation.

INTRODUCTION

Doxorubicin (DOX) is a major drug in the treatment of breast cancer. Used as first-line single agent therapy, the drug induces a response in about 40% of the patients with disseminated breast cancer [1]. With multi-drug regimens containing DOX, response rates up to 60% can be achieved [1-3]. However, in spite of the promising results of multi-drug chemotherapy regimens in adjuvant therapy, where curation can be achieved in a small subset of patients [4], responses in disseminated breast cancer are short term and few patients remain in a durable complete remission.

The mechanism of action of DOX has been extensively studied in the past years, and it is the common view that the anti-tumor activity and most of the toxicities are the result of free radical formation and/or DNA intercalation, the latter resulting in inhibition of the activity of the topoisomerase II enzyme [5-7]. One of the major

problems of treatment with DOX is the intrinsic or acquired resistance of tumor cells to the drug. The P-glycoprotein-associated multidrug resistance, or MDR-1, is one of the mechanisms of resistance to cytotoxic agents that has been extensively studied in recent years [8-10]. Several modifiers of MDR-1 have emerged that have shown, *in vitro*, to be able to restore cytotoxicity in resistant cells. It appeared that this recovery of cytotoxicity is accompanied by decreasing drug-efflux and increasing intracellular drug concentration, or by inducing redistribution of the drug from the cytoplasm to the nucleus [11-13].

Because breast cancer is usually a slowly proliferating tumor with a low percentage of cells present in the S- and G_2M-phase, kinetic resistance is another possible mechanism of resistance of tumor cells to DOX. Although not strictly phase specific, DOX exerts its main action on proliferating cells. We and other investigators have shown, *in vitro*, that quiescent growing human breast cancer can be pushed into the cell cycle, with an optimum in the percentage of S-phase cells (up to 60%) between 18 and 24 h after hormone administration [14]. These hormone manipulated cells appeared to be more vulnerable for the cytotoxic effects of subsequently administered chemotherapeutic drugs [15-18]. Furthermore, also *in vivo* Remvikos *et al.* found evidence of a positive relationship between the proliferative activity (high S-phase fraction) of breast cancer before treatment and the clinical regression after neoadjuvant chemotherapy [19]. However, others could not confirm this observation [20].

In this study we investigated whether the observed increase in cytotoxicity after hormonal manipulation was accompanied by alterations in cellular uptake, retention and distribution pattern of DOX. We monitored, *in vitro*, in the estrogen-receptor positive MCF-7 cell line, the effects of estradiol on the cellular uptake, retention and distribution pattern of DOX using high performance liquid chromatography, flow cytometry and fluorescence microscopy.

MATERIALS AND METHODS

Cell culture

Culture conditions were identical to those described before [18]. MCF-7 cells were maintained at 37°C in 5% CO_2 in air in RPMI-1640 medium containing 21 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES) and 5 μg/ml phenol red, supplemented with 10% heat-inactivated (30 min at 56°) bovine calf serum (BCS), 10

mM $NaHCO_3$, 100 U/ml penicillin, 100 μg/ml streptomycin, 45 μg/ml gentamicin, 2 mM glutamine and 10 μg/ml porcine insulin (growth medium). To compare measurement of DOX fluorescence by HPLC and flowcytometry, logarithmically growing cell cultures were trypsinized and 1 x 10^6 cells were suspended in 4 ml growth medium containing 0.01-2 μg/ml DOX (Adriablastina, Farmitalia, Milan, Italy). A cell sample without DOX was used in parallel incubations in order to measure endogenous fluorescence of the cells. The cells were incubated in the dark, in a shaking water bath at 37^0C for 1 and 6 h.

For growth stimulation experiments cells were seeded in T25-flasks at a density of 0.5 x 10^6 cells per flask in 4 ml experimental medium, *i.e.* RPMI-1640 medium with HEPES but without phenol red and insulin, supplemented with antibiotics, glutamine, $NaHCO_3$, and 4.5% steroid-hormone-depleted fetal calf serum (DCC-FCS). DCC-FCS was obtained by treating FCS twice with 0.5% charcoal and 0.05% dextran T-70 (w/v) for 45 min at 50^0 C and an intermediate incubation with 2 U/ml sulfatase for 2 h at 37^0C. Medium was renewed after 24 h. Twenty-four hours later 4 ml experimental medium supplemented with estradiol (1 nM) (Merck, Darmstad, FRG), or the vehicle alone (control), was added to the cells for up to 24 h. DOX was added to the cultures simultaneously with estradiol and control, or 16 - 24 h after the addition of estradiol and control. The duration of incubation with DOX as well as the incubation concentrations are indicated in the text or legends to the figures.

Cell harvest

DOX incubations were terminated by washing monolayer cultures and cells in suspension twice with phosphate buffered saline (PBS). Immediately after incubation with DOX or 24 h later, in which case cells were refed for 24 h with drug-free hormone-depleted experimental culture medium, monolayer cultures were harvested by a 5 min incubation at room temperature with 0.5 ml trypsin/EDTA (0.05%/0.02%; Biochrom, Berlin, FRG) in 2 ml PBS, followed by addition of 1 ml trypsin inhibitor (0.1 mg/ml; Sigma St. Louis, MO, USA) in PBS. Cells incubated in suspension were centrifuged for 5 min at 100 g, whereafter DOX-containing medium was discarded. An aliquot of the cell suspension was collected for measurement of DOX fluorescence by flow cytometry and/or for determination of DOX distribution in the cell by fluorescence microscopy. The remainder of the cells was stored at -20°C until extraction of DOX and measurement of fluorescence by high performance liquid chromatography (HPLC) was performed.

The impact of washing and trypsinization on the DOX content of the monolayer cultures was investigated with estradiol-stimulated and control cultures. After a 4 h incubation with 0.2 μg/ml DOX, cells were washed twice with icecold PBS and scraped from the flasks, or washed and trypsinized at room temperature. No difference in uptake of DOX in the cells, as measured with flowcytometry, was observed between both cell harvest procedures (data not shown).

Measurement of DOX uptake by HPLC

The method used to measure DOX uptake by the tumor cells is a modification from the method described by Israel *et al.* [21]. Borate buffer (200 μl, 0.5 M, pH 9.8) and 3 ml chloroform/methanol (4:1) were added to 1 ml of cell suspension (cell numbers were approximately the same in estradiol-stimulated and control cultures) in a borosiliconized glass tube and shaken for 15 min. After centrifugation for 10 min at 1000 g, the lower organic layer was dried by vacuum centrifugation. Dried samples were reconstituted with 130-250 μl methanol and an aliquot of 100 μl was injected onto a reverse phase Bondapack Phenyl column (Waters Associates, Milford, USA), prepacked with 10 μm particles in a 250 x 4.6 mm stainless steel column, which was used for separation. The mobile phase consisted of (0.45 μm filtered) 0.1% ammonium-formate buffer, 70% acetonitrile (Baker Chemical Co., Jackson, USA) (70:30), pH 4.0. Ammonium-formate buffer was prepared by mixing 5 ml ammoniumhydroxide 20% (Baker Chemical Co., Jackson, USA) with 1000 ml H_2O (HPLC grade). The pH was set at 4.0 with formic acid 98% (Baker Chemical Co., Jackson, USA). The flow was set at 1.5 ml/min. Detection of DOX was accomplished at an excitation wavelength of 480 nm, using a Gilson Spectra-Glo fluorometer (Middleton, WI, USA). Retention times and areas of the peaks were recorded and integrated by a Shimadsu Model CR 3A integrator (United Technologies Packard, Delft, The Netherlands). Concentrations were quantified by using 4-deoxyrubicin as an internal standard. The detection limit after extraction from biological fluid was 10 ng DOX/ml. The metabolite doxorubicinol was detected.

Measurement of DOX uptake by flowcytometry

The DOX content of individual cells was measured with a Becton and Dickinson flow cytometer (Facscan), using a 15 mW argon-ion laser lightbeam tuned at 488 nm, which is close to the absorption maximum of DOX. At least 1 x 10^4 cells were analyzed from each sample. Data analysis was performed using the Facscan research

program of a Hewlett Packard 310 system. Fluorescent intensities were recorded with linear amplification. The value of the DOX uptake of a sample was obtained as the difference of the mean fluorescence intensity of the sample and the corresponding blanc sample.

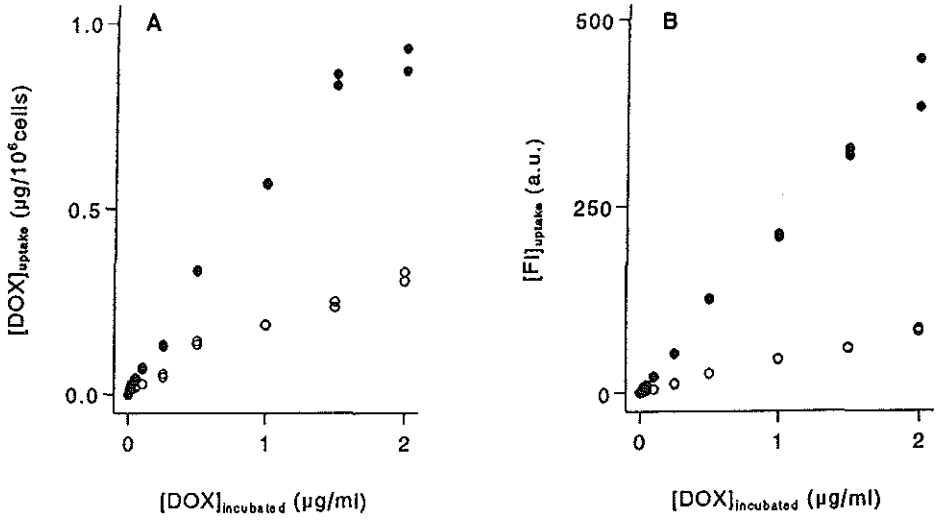

Figure 1
Correlation of DOX incubation concentrations and resulting cellular fluorescence as measured by HPLC (A) and flow-cytometry (B). MCF-7 cells (1 x 10^6/4 ml), in suspension were incubated for 1 (o) and 6 h (●) with 0.01-2 µg/ml DOX at 37^0 C. Data of duplicate incubations are shown.

Assessment of cellular distribution of DOX

For measurement of DOX distribution, cells pretreated with and without estradiol followed by incubation for 0.5-72 h with 0.2-1 µg/ml DOX were viewed with a Zeiss Axioskop (Carl Zeiss BV, Weesp, The Netherlands) fluorescence microscope equiped with a HBO 100 lamp (6 V, 20 W) and a 450 - 490 nm filter. Photographs were made after 2-5 min exposure time.

Statistics

In 21 experiments with varying doses and duration of DOX exposure, DOX uptake was measured with and without estradiol pretreatment. The number of replications for each condition varied between 2 and 8. The mean DOX uptake was calculated for each experiment over the replication with and without estradiol pretreatment. Analysis of variance (ANOVA), adjusted for experiment, was applied to test for the effect of estradiol pretreatment on DOX uptake.

RESULTS

Comparison of HPLC and flowcytometry for measuring DOX uptake

Measurement of DOX uptake by HPLC is time consuming, whereas measurement of fluorescence by flow cytometry is a much more simple method, but is said to be hampered by quenching. Therefore we compared both methods with MCF-7 cells exposed to a wide range of DOX concentrations. MCF-7 cells in suspension were incubated for 1 and 6 h with DOX concentrations ranging from 0.01-2 μg/ml. The cellular fluorescence was measured by HPLC and flow cytometry. Figure 1 shows the relation between the incubation concentration of DOX and the uptake of the drug by the tumor cells as measured by HPLC (Fig. 1A) and flowcytometry (Fig. 1B). For both methods a strong correlation exists between the incubation concentration of DOX and the cellular uptake of the drug.

Effect of simultaneous administration of estradiol and DOX on cellular DOX uptake

To investigate whether simultaneous incubation of estradiol and DOX influences the uptake of the drug, MCF-7 cells growing in a monolayer culture in hormone-deprived experimental medium for 48 h were exposed to 1 nM estradiol or the vehicle alone (control) and to 0.04-0.2 μg/ml DOX for a period of 1-24 h. In both hormone-treated and control cultures no significant differences in the uptake of DOX were observed as measured by flowcytometry (Fig. 2) and HPLC (data not shown).

Effect of estrogen pretreatment on the uptake of DOX

In previous studies we showed that estradiol stimulation resulted after 18-24 h in an about 3 to 5-fold increase in the percentage of cells in the S-phase of the cell cycle [14]. In MCF-7 cells growing as a monolayer culture we investigated whether this stimulation resulted in changes in the cellular uptake of DOX. MCF-7 cells cultured

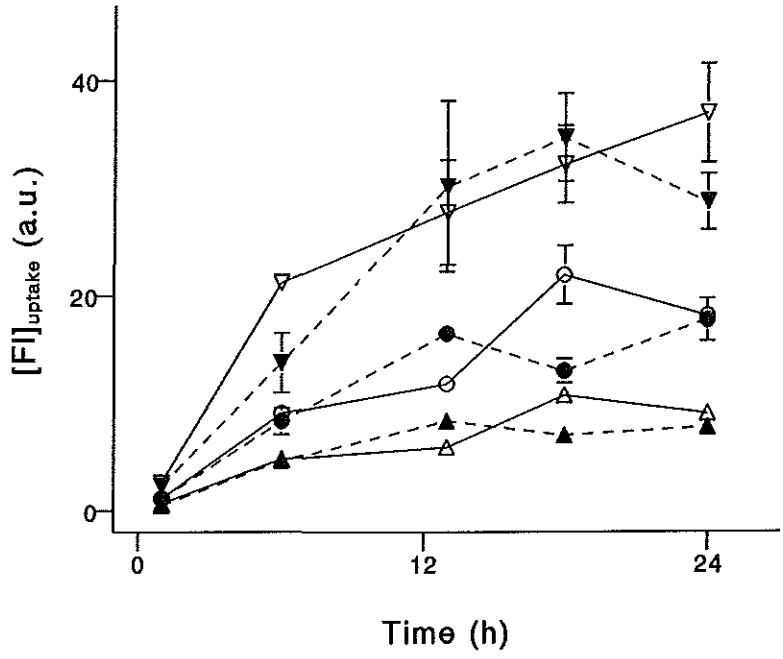

Figure 2

MCF-7 cells, in monolayer culture, grown for 2 days in hormone-deprived culture medium were incubated for 1-24 h with estradiol (1 nM, open symbols) and DOX (0.04 [Δ] - 0.1 [o] - 0.2 [∇] μg/ml), or no estradiol and DOX (solid symbols). Mean cellular fluorescence was measured with flowcytometry, at regular time intervals as indicated in the figure. Data are the means of duplicate incubations ± S.D.

in hormone-deprived experimental medium were incubated with 1 nM estradiol. After 16-24 h cultures were exposed for 0.5, 1, 6 or 21/23 h, to different concentrations of DOX (2 μg/ml for the 0.5 h, 0.1 and 0.2 μg/ml for the 1 h, 0.2 μg/ml for the 6 h, and 0.02 μg/ml for the 21/23 h incubations). In these 21 experiments the uptake of DOX in hormone-stimulated and control cells was measured by HPLC. Table 1 shows the mean uptake per experiment together with the number of replications and the standard deviations. A large variation in the DOX uptake was observed between experiments. For all incubation periods and incubation concentrations of DOX tested, there is no significant difference in the uptake of the drug as a result of estradiol pretreatment (Fig. 3). The same results were found when cells were only stimulated

with estradiol for 1 h (followed by a 19 h culture period in hormone-deprived medium) followed by incubation with DOX (data not shown).

Table 1: Uptake of DOX by MCF-7 cells as measured by HPLC

Exp.	DOX inc. conc. (μg/ml)	Duration of DOX exposure (h)	N	DOX uptake (ng/10^6 cells) Estradiol stimulated (mean)	(SD)	Not stimulated (mean)	(SD)
A	2	0.5	2	119	1	102	12
B	2	0.5	6	45	12	65	18
C	2	0.5	6	75	14	66	10
D	0.1	1	4	25	4	17	2
E	0.2	1	4	64	10	26	2
F	0.2	1	4	36	4	49	9
G	0.2	1	4	52	14	54	12
H	0.2	1	4	51	5	54	12
I	0.2	1	3	31	3	25	5
J	0.2	1	3	29	7	28	5
K	0.2	6	3	93	13	81	10
L	0.2	6	6	121	41	84	22
M	0.2	6	4	179	23	271	59
N	0.2	6	8	71	19	55	21
O	0.2	6	6	176	76	155	62
P	0.2	6	3	100	16	75	5
Q	0.2	6	2	105	9	102	25
R	0.2	6	6	289	54	236	63
S	0.02	21	4	34	8	41	5
T	0.02	23	4	23	4	33	10
U	0.02	23	2	30	6	63	32

N: number of incubations per experiment
SD: standard deviation

Effect of hormonal manipulation on cellular retention and distribution pattern of DOX

Estradiol-mediated growth stimulation can result in an enhanced cytotoxicity of chemotherapy as we and other investigators have shown before [15-18]. To investigate whether cellular retention of DOX rather than a higher uptake of the drug could

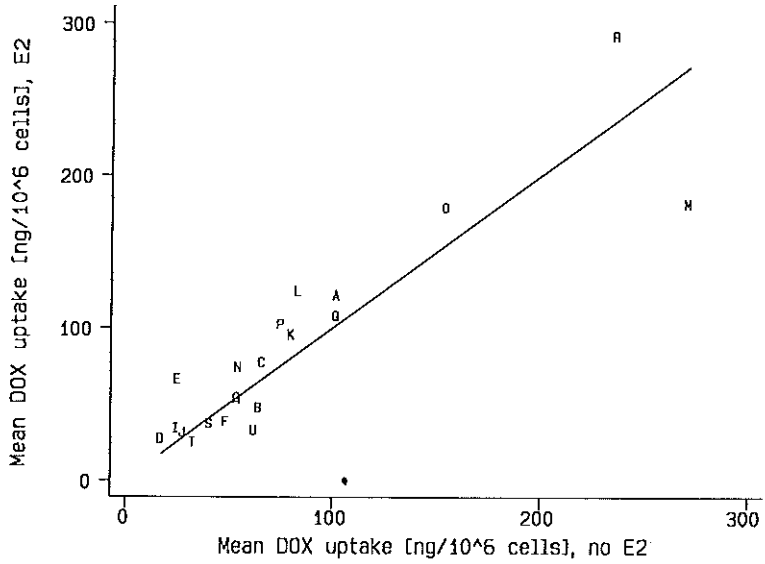

Figure 3

MCF-7 cells (0.5 x 10^6 cells) grown for 2 days in hormone-deprived culture medium, were stimulated with 1 nM estradiol for 16-24 h prior to incubation with DOX (2 μg/ml for 0.5 h; 0.1/0.2 μg/ml for 1 h, 0.2 μg/ml for 6 h; and 0.02 μg/ml for 21/23 h). The uptake of DOX in the estradiol-stimulated and control cultures as measured by HPLC are shown with the letters depicting the code of experiment as described in Table 1. Data shown from 21 independent experiments with n=2 to n=8 incubations per experiment.

account for the improvement of cytotoxicity as we observed under certain experimental conditions, cellular uptake of DOX was measured by HPLC 24 h after a 0.5 h incubation with DOX (2 μg/ml). Uptake of DOX as measured immediately after incubation was 74.6 $\pm$ 14.2 ng/10^6 cells and 66.3 $\pm$ 9.8 ng/10^6 cells in respectively estradiol-stimulated (n = 6) and control (n = 6) cultures. After 24 h, no differences in DOX content of the cells were found, estradiol pretreated cultures had retained 33.9 $\pm$ 10.7 ng DOX/10^6 cells (n = 4) vs 35.8 $\pm$ 7.3 ng DOX/10^6 cells (n = 4) in the control cultures.

DOX exerts its main effectivity as intercalating drug. Therefore we assessed the distribution pattern of the cellular fluorescence using a fluorescence microscope. MCF-7 cells growing in a monolayer culture and pretreated with and without 1 nM estradiol for 18 h were incubated for 0.5, 1, 3, 24, 48 and 72 h with 0.2-1 μg/ml DOX. Under all conditions tested, fluorescence was mainly localized in the nucleus

of the cells. An example of the fluorescence of the cells pretreated with estradiol followed by incubation with DOX (0.2 μg/ml) for 3 h is shown in Fig. 4.

DISCUSSION

Measurement of DOX fluorescence by HPLC is a reliable but time consuming method. Analysis of the DOX content of cells by flowcytometry is a very elegant and simple method, but is said to be influenced by quenching when the drug is intercalated in the DNA. Therefore we compared the DOX-associated fluorescence, as measured by flow cytometry, with the DOX content of breast cancer cells as measured by HPLC, using a wide range of DOX incubation concentrations. Like others [22] our data show a good correlation between the anthracycline incubation concentration and the mean cellular fluorescence in the tumor cells as measured by both methods, whereas measurement of DOX fluorescence by HPLC has the advantage of quantifying DOX uptake. Compared to germ-cell tumors and hematological malignancies, which can be cured by chemotherapy, breast cancer is primarily relatively sensitive to the lethal effects of chemotherapeutic drugs, but development of drug-resistance in the course of therapy is a major problem. From research in the field of multidrug resistance we know that reversal of multidrug resistance with calcium-channel blockers, steroids, calmudolin antagonists or cardiac agents, can be accompanied by an increased uptake of the cytotoxic drug as well as by a shift in intracellular drug-distribution from cytoplasmatic to nuclear localization [11-13].

Kinetic resistance may also account for the moderate responsiveness of breast cancer to the cytotoxic effects of chemotherapy. Several authors described an increasing uptake of DOX progressively with cell cycle traverse, and an enhanced cytotoxic effect of chemotherapy on cells in the S-G_2M phase of the cell cycle [23-25]. This observation could explain the better cytotoxic effect of chemotherapy in the rapidly proliferating tumors, containing a high percentage of cells in the S-phase of the cell cycle.

We have previously shown that stimulation of hormone responsive MCF-7 human breast cancer cells *in vitro* with estradiol, resulted in an increase in the percentage of S-phase cells within 24 hours [14]. When these stimulated cells were subsequently incubated with DOX an enhanced cytotoxic effect of the drug was observed [18]. Therefore we investigated in this study whether treatment with estradiol and DOX was accompanied by an altered uptake, differences in retention or by a shift in intracellular

drug distribution. In spite of a clear increase in the percentage of cells in S-phase due to a 16-24 h pretreatment with estradiol (the percentages of cells in S-phase augmented between 2- and 6-fold due to this hormonal manipulation; data not shown), no significant increase in uptake of DOX in estradiol pretreated MCF-7 cells was found in 21 separate experiments using a 0.5, 1, 6, and 21/23 h incubation with different concentrations of DOX.

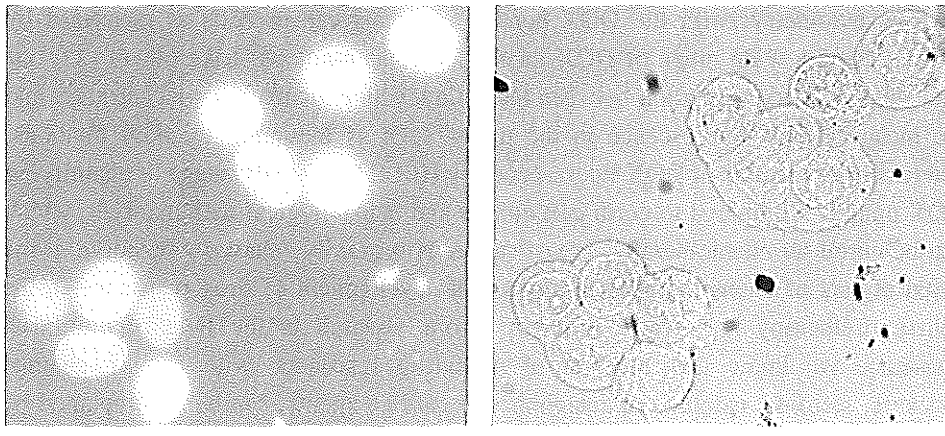

Figure 4

MCF-7 cells grown for 2 days in hormone-deprived culture medium, were stimulated with 1 nM estradiol for 18 h prior to a 3 h incubation with DOX (0.2 ug/ml). The figure shows at the right side the light microscopic picture of the cells and at the left side the cellular distribution of DOX. Photographs of fluorescent cells were made after 180 sec exposure time. Distribution of cellular fluorescence showed only nuclear fluorescence in both estradiol stimulated and control cultures.

To investigate whether drug efflux could be influenced by estradiol pretreatment, the DOX concentration of the tumor cells was measured after another 24 hours of culture in hormone- and drug-free experimental medium. Again no differences in intracellular drug concentration were found in MCF-7 cells.

Intracellular drug distribution was investigated using fluorescence microscopy. No difference in DOX distribution within the cells was observed due to hormonal manipulation. The fluorescence was predominantly localized in the nucleus of the cells.

Several other investigators have found an enhancement of the cytotoxic effect of chemotherapeutic drugs after growth stimulation of breast cancer cells in vitro [15-17]. However, it is clear from the work of Epstein *et al.* [26-28], Shaikh *et al.* [29,30], and Osborne *et al.* [31] that not only the stimulatory effect on DNA-synthesis by the hormones can be considered responsible for the enhancement of the cytotoxic effect of chemotherapy. Already minimal estradiol-mediated growth stimulation of T47D cells [26-28], or medroxyprogesterone acetate pretreatment of MCF-7 cells (without any effect on cell cycle distribution) [29,30] could enhance the cytotoxic effects of several chemotherapeutic drugs. Even tamoxifen pretreatment followed by doxorubicin or hydroxycyclophosphamide [31] resulted in an increased cell kill in MCF-7 cells, whereas tamoxifen followed by melphalan or fluorouracil resulted in the opposite effect. Therefore, mechanisms other than growth-stimulation may play a role to explain the augmented cytotoxic effect of some combinations of hormones and chemotherapy, for estrogen may even have an opposite effect. Estradiol is for instance also found to induce topoisomerase-II, the enzyme critical for replication, transcription and recombination of DNA [32], and in rat pituitary cells estradiol can induce the MDR resistance pump, leading to drug efflux [33], both mechanisms that can protect cancer cells from the lethal effect of chemotherapy. In contrast to our preliminary experiments using culture medium with male human serum, from the present study (using culture medium with DCC-FCS) we have to conclude that simultaneous administration or pre-treatment of MCF-7 breast cancer cells with estradiol and DOX has no influence on the uptake of DOX by the tumor cells, neither did we find an enhanced retention of DOX due to estradiol stimulation, nor was there a difference in the localization of DOX in the tumor cells due to the hormonal manipulation.

Acknowledgement

The authors would like to thank Mr. Reinier van der Linden, Department of Immunology, for his assistance. This study was financially supported by the Dutch Cancer Society (Nederlandse Kanker Bestrijding) by grant RRTI 87-11.

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CHAPTER 6

THE VALUE OF ESTROGENIC RECRUITMENT BEFORE CHEMOTHERAPY: FIRST RANDOMIZED TRIAL IN PRIMARY BREAST CANCER

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Modified version submitted

ABSTRACT

Purpose: In this randomized phase III study the efficacy of estrogenic recruitment followed by chemotherapy was compared with the efficacy of chemotherapy alone as adjuvant treatment in resectable breast cancer.

Patients and Methods: A total of 328 patients less than 66 years of age with stage II/IIIA breast cancer were randomly allocated to FAC or FAC + EE_2 (ethinyl-estradiol), irrespective of receptor status. FAC was given every 4 weeks for 4 cycles and consisted of 5-fluorouracil 500 mg/m^2, doxorubicin 50 mg/m^2, cyclophosphamide 500 mg/m^2, i.v., day 1. In the recruitment arm FAC was preceded by 0.5 mg EE_2 given orally, both 24 hours and just before the chemotherapy. There were no significant differences regarding patient and tumor characteristics between the treatment arms.

Results: From 318 evaluable patients, 124 showed a relapse and 70 patients have died after a median follow-up of 3.3 years. No significant differences between the two treatment groups were found with respect to relapse-free, local recurrence-free and overall survival both in univariate and in multivariate analysis adjusted for age, menopausal status, tumor size, number of positive nodes, grade and steroid receptor status.

Conclusion: Hormonal recruitment of breast cancer cells had no influence on the efficacy of subsequent chemotherapy with FAC compared to FAC alone in stage II/IIIA breast cancer patients, after a relatively short follow-up. In view of the fact that slowly proliferative tumors might be more sensitive to this hormonal manipulation, longer follow-up is necessary for definite conclusions.

INTRODUCTION

Both adjuvant endocrine treatment and chemotherapy of primary breast cancer result in a significant improvement of disease-free and overall survival, but the absolute benefit is limited.[1] New treatment modalities or new drug combinations are warranted to improve clinical treatment outcome. Combining anti-hormonal treatment with chemotherapy generally has led to no, or only very modest improvement in subgroups of patients.

Experimental studies of our and other groups have shown that estrogens or other steroids can induce the semi-synchronous recruitment of a population of quiescent

breast cancer cells into the cell cycle.[2-4] The hormone-induced proliferation can make these cells more vulnerable for the cytotoxic effect of subsequent chemotherapy.[3,5-9] Apart from the beneficial long-term effects of chemotherapy-induced castration it might be possible that in premenopausal women the relative good results of adjuvant chemotherapy can also partly be explained by accidental natural hormonal priming during some courses of chemotherapy, when the chemotherapy is administered after rising plasma estradiol levels during the menstrual cycle. Indirect clinical support for this hypothesis can be the observation of a negative interaction of the combination of the anti-estrogen tamoxifen with chemotherapy in premenopausal women.[10,11]

In a number of initial phase II studies in metastatic or locally advanced disease the application of priming of tumor cells by estrogens appeared to be promising as shown by relatively high objective (65%-75%) and complete (22%-47%) response rates.[12-14] Lippman et al.[13] found also a longer duration of response with improved survival in subgroups of patients. Based on these (pre)clinical studies we initiated this early randomized adjuvant study, comparing the effects of 4 courses of chemotherapy with FAC (5-fluorouracil, doxorubicin, and cyclophophamide) with the same chemotherapy preceded by estrogenic recruitment in stage II and IIIA breast cancer patients.

PATIENTS AND METHODS

Eligibility criteria

Women with primary resectable breast cancer, and at least one histologically verified positive axillary lymph node were eligible for this study if the following conditions were fulfilled: age < 66 years, pathological staging classification T_1-$T_{3A}N+M0$ according to the definitions of the American Joint Committee on staging, treated by modified mastectomy or surgery according to Halsted, and chemotherapy initiated within 15 days after the surgical procedure. Patients with breast conserving therapy were excluded. Oral contraceptives should have been stopped at least 2 weeks before the start of chemotherapy. Ineligibility criteria were: Karnofsky performance status < 80, white blood cell counts < 3.5 x 10^9/l or platelet counts < 100 x 10^9/l, abnormal liver function or disturbed renal function (bilirubin > 30 μmol/l, γGT > 30 IU/l, serum creatinine > 120 μmol/l), previous treatment for breast cancer, bilateral breast cancer, other neoplasms (except adequately treated basalioma or stage 0-I squamous carcinoma of the cervix uteri), current pregnancy or lactation, rapidly progressive fatal illness other than carcinoma, and cardiac diseases as congestive heart

failure or myocardial infarction less than 6 months before diagnosis of breast cancer. There were no exclusion criteria for receptor status or histological grades. All patients gave informed consent to the study. This study was approved by the Dutch Cancer Society.

Pre-study screening and follow-up studies

Investigation procedures performed before entry to this trial included history, physical examination, laboratory investigations including hemoglobin, white blood cell count, platelet count, liver function test (bilirubin, alkaline phosphatase, ASAT, ALAT, LDH, γGT) and renal function (urea, creatinine), chest X-ray, mammogram and bone-scan. Tumor specimens were assayed for estrogen receptor (ER) and progesterone receptor (PgR) levels by ligand binding assay or enzyme immunoassay as described before.[15] Following completion of the treatment the patients were checked every 6 months for 5 years and than yearly thereafter. At each follow-up visit history and physical examination were performed. Chest X-ray, mammography, a complete blood cell count and chemistry profile were performed on an annual basis.

Chemotherapy and dose modifications

The patients were randomized to receive FAC chemotherapy alone or FAC preceded by hormonal recruitment. The FAC regimen consisted of 4 cycles of fluorouracil 500 mg/m^2, doxorubicin 50 mg/m^2 and cyclophosphamide 500 mg/m^2, administered intravenously (IV) once every 4 weeks, starting within 15 days after surgery. In the recruitment arm the same chemotherapy was administered, but each course was preceded by the oral administration of 0.5 mg ethinyl-estradiol 24 hours before, and at the time of the administration of the chemotherapy. A 4-weekly interval between the courses of chemotherapy was used in order to prevent postponement of chemotherapy after estrogen stimulation in a substantial number of patients due to cytopenia. The dose of ethinyl-estradiol (0.5 mg) was chosen because similar dosages of estrogens[16] caused plasma total- and unconjugated estradiol levels as found in the luteal phase of the menstrual cycle (200-700 pg/ml) which appeared to increase proliferation indices in endometrium, breast,[17] and breast cancer.[18] Adjuvant chemotherapy was started within two weeks after surgery because some initial trials showed that early chemotherapy before radiotherapy caused better relapse-free survival than delayed chemotherapy.[19,20] During the first course of chemotherapy the total dose of cyclophosphamide was divided over 5 days because some experimental studies

showed an increase of the labeling index in local[21] and distant[22] tumor foci after extirpation of the primary tumor. In addition Nissen-Meyer et al.[23] observed a 14% improvement of relapse-free survival by one short-course of cyclophosphamide administered immediately post surgery. At the time of scheduled retreatment, therapy was postponed for 1 or 2 weeks if WBC was $< 3 \times 10^9$/l and or platelets $< 100 \times 10^9$/l. When at 2 weeks of delay no full hematological recovery was reached, doses had to be modified according the following scheme: 50 % reduction of the dosage of all 3 agents when WBC was 2.0-3.0 x 10^9/l or platelets 75-100 x 10^9/l, and the patient went off study when WBC was $< 2 \times 10^9$/l or platelets $< 75 \times 10^9$/l.

Radiotherapy

Radiotherapy to the thoracic wall (or incidentally also to the regional lymphnodes) was administered in case of a narrow tumor resection area (< 1 cm from the muscular thoracic wall) and started after the 4 courses of chemotherapy. The total dose of radiotherapy administered was 46 Gy.

Statistics

Primary endpoints for this study were relapse-free and overall survival. The trial was designed to detect an increase of 50% in the median relapse-free survival with a power of 90% at 5% significance level. Therefore 432 patients has to be entered in the study. The expected duration of accrual was 4 years. The accrual rate of the trial was lower than expected and the trial was closed after 6.5 years when 328 patients had been entered. Acturial survival probabilities and curves were calculated with Kaplan-Meier method. In relapse free survival analysis patients counted as failure at relapse or at death; all other patients were censored at last contact. In the analysis of distant-metastasis free period, patients were censored at last contact or death, if no previous distant metastasis had been observed. The same applies to the analysis of local recurrence free period. Differences in survival or relapse free survival between groups and associations between continous covariates and failures rates were analysed and tested with Cox proportional hazards model. The test for difference in (relapse free) survival between the two treatment arms was done both without and with adjustment for prognostic factors.

Table 1 Patient characteristics

	FAC+ recruitment	FAC	Total
Patients entered	166	162	328
Patients evaluable	161	157	318
Mean age (yr)	48.4	48.5	48.5
Menopausal status			
pre	100	93	193
post	56	60	116
unknown	5	4	9
Tumorsize			
pT_1	26	24	50
pT_2	122	110	232
pT_3	13	23	36
Positive nodes			
1-3	80	85	165
4-10	68	50	118
> 10	13	20	33
unknown	0	2	2
Grade			
well differentiated	1	3	4
moderately differentiated	23	26	49
poorly differentiated	79	74	153
unknown	58	54	112
Surgery not radical	6	6	12
ER (fmol)			
≤ 10	37	33	70
≥ 10	112	113	225
unknown	12	11	23
PgR (fmol)			
≤ 10	53	39	92
> 10	74	88	162
unknown	34	30	64

RESULTS

Patient and tumor characteristics

In this multicenter study 16 institutions participated, each contributing between 2-80 patients. A total of 328 patients were randomized between October 1985 and May 1992, 162 to FAC alone and 166 to FAC preceded by estrogenic recruitment. Nine patients were found to be ineligible (5 on FAC and 4 on FAC recruitment). Reasons for ineligibility were: performed lumpectomy 3 patients, pT4 tumor 3 patients, distant metastases 2 patients, and ongoing oral anticonceptive therapy 1 patient. One patient was not evaluable because CMF chemotherapy was administered. The two treatment groups are well balanced with respect to the patient- and tumor characteristics as indicated in Table 1. For the whole group of patients the median follow-up is 3.3 years. The mean number of lymphnodes examined was 13 (range 2-31), and the mean number of positive nodes was 5 (range 1-28).

Treatment characteristics

Treatment was started at average 12 $\pm$ 2.7 days (mean $\pm$ SD) after surgery. For all 3 chemotherapeutic drugs the mean percentage of total dosages administered varied between 97.8% and 99.1%, without any difference between the two treatment arms. Treatment was well tolerated. The side effects were as expected for this well known regimen. There were no toxicity related hospital admissions and no treatment related deaths.

A total of 73 patients (38 in the FAC-recruitment arm and 35 in the FAC arm) were treated with radiotherapy. In 71 patients radiotherapy was applied to the thoracic wall and in 15 patients also the regional lymphnodes. Of the 71 patients who were treated with radiotherapy to the thoracic wall, 5 (7%) experienced a chestwall relapse, and of the 245 patients not treated with radiotherapy 23 (9.4%) had a chestwall recurrence.

Relapse-free and overall survival

With respect to relapse-free ($p=0.79$), local recurrence-free ($p=0.97$), distant metastasis-free ($p=0.60$) and overall survival ($p=0.69$) we found no significant differences between the two treatment groups. Neither were there any differences in the type of disease recurrences (Table 2). The respective survival curves are shown in Figure 1. The treatment group without estrogenic recruitment showed a slightly lower failure rate (relative hazard rate (RHR) between 0.91 and 0.99) but these differences were not significant ($p > 0.60$). For the whole patient population the

survival percentages for these 4 parameters after 1, 2, and 5 years are indicated in Table 3.

Table 2 Type of recurrences

	FAC+ recruitment	FAC	Total
Locoregional recurrences (n = 55)			
Scar	7	8	15
Chest wall s.c.	6	7	13
Skin diffuse	5	3	8
Supraclaviculair	11	10	21
Axillary	7	4	11
Distant metastases (n = 117)			
Bone	38	37	75
Liver	12	16	28
Lung	11	7	18
Lymph nodes	9	6	15
Brain	4	6	10
Skin	2	2	4
Second breast	3	0	3
Other	13	8	21

Table 3 Acturial survival rate

	1 yr	2 yr	5 yr
Local recurrence-free	96%	86%	74%
Distant metastases-free	90%	76%	50%
Relapse-free survival	88%	70%	46%
Overall survival	97%	89%	65%

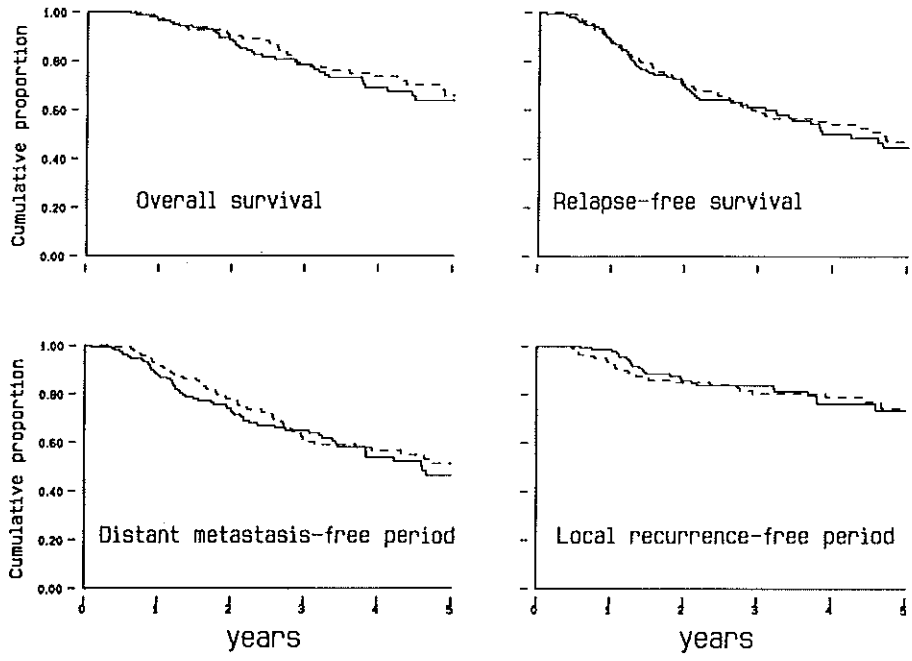

Figure 1
Overall; relapse-free; distant metastasis-free and local recurrence-free survival by arm (—— FAC+ estrogenic recruitment, ----- FAC)

The role of clinical prognostic factors

As expected, patients with large tumors ($p = 0.001$), high numbers of positive nodes ($p < 0.0001$) and negative steroid receptor status ($p = 0.42$) showed a worse relapse-free survival (Figure 2). With respect to age, in this study we found no association between menopausal status and relapse-free survival, but patients younger than 40 years tended to have a higher relapse rate. Also in a multivariate analysis including these clinical prognostic factors, we found no significant difference regarding relapse rate between the two treatment arms ($p = 0.57$).

In this study we investigated the clinical impact of the assessment of the percentage of positive nodes versus the number of positive nodes, using 3 subgroups for each parameter. For the number of positive nodes the subdivision was in 1-3 ($n=165$), 4-10 ($n=118$) and > 10 ($n=33$), while for the percentage of positive nodes the subdivision

was in < 40% (n=203), 40-80% (n=61) and > 80% (n=39). Both factors were statistically highly significantly related with relapse rate, but the percentage of positive nodes turned out to be a better predictor ($p = 2 \times 10^{-9}$ vs $p = 1 \times 10^{-6}$.

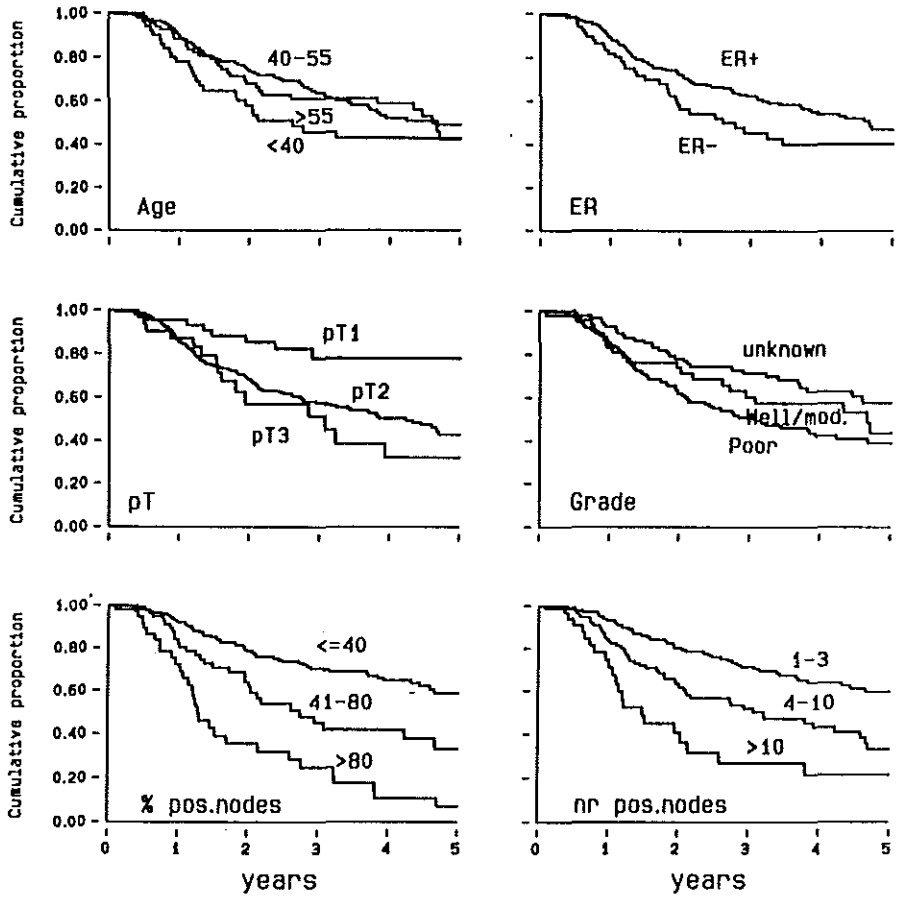

Figure 2
Relapse-free survival according to age, ER status, tumor size, differentiation grade, % of positive nodes, and number of positive nodes.

The 5 year relapse-free survival varied in the subgroups according to number of positive nodes between 22% and 60%, and int the subgroups according to percentage of postive nodes between 7% and 59%. Note that the subdivision according to

percentage of positive nodes gives a better separation with more patients in the good and very poor prognosis groups, than the subdivision by number of positive nodes (Figure 2).

DISCUSSION

The concept of hormonal stimulation before antitumor therapy was already applied many decades ago. Around 1940 radiotherapists used estrogen treatment in the waiting time prior to radiotherapy assuming that estrogens could make breast cancer more vulnerable for radiotherapy by growth stimulation in contrast to estrogen withdrawal by castration which can result in objective tumor remission. Later on similar studies with androgen-priming before radiotherapy were performed in metastatic prostate cancer.[24-26] However, these studies were more empiric and not based on preclinical testing of the principle in different models.

More recently, several laboratory studies showed that short-term pretreatment of tumor cells with estrogens can increase the antitumor efficacy of cytostatic drugs.[3,5-9] A few investigators demonstrated that also in patients with (locally advanced) breast cancer estrogenic pretreatment during one or several days increased the proliferation rate of the tumors.[27-29] However, this procedure of estrogenic recruitment of tumor cells prior to chemotherapy appeared to have disappointing antitumor effects in patients with advanced disease in comparison with the results of experimental preclinical studies.[30]

Initially, some of the phase II studies applying the principle of estrogenic recruitment therapy in locally advanced (Table 6) and metastatic disease (Table 7) showed relative high (complete) response rates.[12,14,29,31,32,34-36]

Subsequently a number of randomized studies using different treatment regimens were performed (Table 6 and 7).[13,33,37-42] Most studies found no difference in response rate between the treatment arms. Seymour et al. observed even a detrimental effect of estrogenic recruitment on the efficacy of chemotherapy, but in this study all chemotherapy was administered on day 1 and diethylstilbestrol (DES) from day 1 to day 6, which might have resulted in tumor flare after the chemotherapy.[41]

In contrast, Ingle et al. using the intravenous CMF regimen found a higher response rate after pretreatment with DES (39 vs 25%; $p = 0.06$).[42] Overall, in these randomized studies no significant effects were found with respect to progression-free and overall survival.

Table 6 Clinical trials of hormonal recruitment strategies in locally advanced breast cancer

Author (yr)	Pts. (N)	Hormonal therapy	Chemotherapy	CR (%)	OR (%)	TTP (months)	Survival (months)
Swain '87	70	Tam/Premarin	* FACM	49	93	34	39
Conte '87	39	DES	* FAC	15	72	NA	NA
Fabian '94	28	Estradiol	* FAC Vinblastine	89	NA	35	57
Baldini '93	108	DES	* FAC	6	60	22	47
		---	* id.	13	72	20	50

Abbreviations: CR: Complete response, OR: overall response, TTP: median time to progression, Survival: median overall survival, Tam: tamoxifen, DES: diethylstilbestrol, F: fluorouracil, A: doxorubicin, C: cyclophosphamide, NA: not available, id: identical chemotherapy.

Based on all these studies, it can be concluded that estrogenic recruitment before chemotherapy does not improve the antitumor effects of chemotherapy; only in various subgroups of patients some benefit of the hormonal manipulation was found The potential reasons for these disappointing results in locally advanced and metastatic disease can be suboptimal treatment regimens (with respect to type and dose of estrogen, tamoxifen therapy, type and timing of chemotherapy and duration of treatment), prior existence of chemotherapy drug resistance, and especially tumor heterogeneity. The latter two reasons are expected to be less important in early breast cancer. However, also in our first adjuvant study applying the principle of estrogenic recruitment therapy, we found no additional benefit of such hormonal manipulation. The absence of a benefit can be explained by several reasons. Apart from those mentioned above with respect to advanced disease, it might be possible that after a median follow-up of 3.3 years only those tumors with an initial high proliferation rate have already recurred. These at the start of treatment already rapidly proliferating tumor cells are potentially less sensitive for further stimulation by estrogens in contrast to slowly proliferating or dormant tumor cells. It might be anticipated that pretreatment with estrogens is more effective in tumors with a low S-phase fraction, which is a favourable prognostic factor associated with longer relapse-free survival.

Table 7 Clinical trials of hormonal recruitment strategies in metastatic breast cancer

Author (yr)	Pts. (N)	Hormonal therapy	Chemo-therapy	CR (%)	OR (%)	TTP (months)	Survival (months)
Allegra '83	32	Tam/Premarin	* FM	47	69	NA	NA
Eisenhauer '84	35	Tam/Premarin	* FM	NA	10	NA	NA
Paridaens '87	57	AG/Estradiol	* FAC	35	75	NA	NA
Benz '87	18	Tam/Estradiol	* FM	28	39	NA	NA
Fabian '94	22	Estradiol	* FAC Vinbl.	NA	50	4	17
Hug '94	63	Premarin	* FAC	13	76	17	29+
Lippman '84	108	Tam/Premarin	* FACM	22	65	16	19
		---	* id.	18	65	12	17
Lipton '87	35	AG/Estradiol	* FAC	14	62	NS	NS
		AG	* id.	7	64		
Paridaens '93	154	AG/Estradiol	* FAC	14	64	14	24
		AG	* id.	21	64	20	29
Conte '87	116	DES	* FEC	24	54	9	18
		---	* id.	16	57	9	14
Conte '96	258	DES	* FEC	15	50	11	20
		---	* id.	12	51	9	17
Seymour '93	39	DES[0]	* CNV	11	26	7	13
		---	* id.	25	55	10	15
Ingle '94	163	DES	* CMF	6	39	4	12
		---	* id.	4	25	4	13

Abbreviations: CR: complete response, OR: overall response, TTP: median time to progression, survival: median overall survival, Tam: tamoxifen, DES: diethylstilbestrol, AG: aminoglutethimide, F: fluorouracil, M: methotrexate, A: doxorubicin, C: cyclophosphamide, Vinbl.: vinblastine, E: epidoxorubicin, V: vincristine, N: mitoxantrone, NA: not available, NS: not significant, id: identical chemotherapy, [0]: postmenopausal patients on DES also received AG.

Therefore, it might be possible that after a longer follow-up the survival curves in our study will diverge.

In conclusion, in our adjuvant study thusfar we did not find any benefit of pretreatment with ethinyl-estradiol on the efficacy of FAC chemotherapy. However, we need longer follow-up before making definite conclusions.

Acknowledgement

We would like to thank KJ Roozendaal, Th.M. Dekkers, P.J.J. Leeuwerik, AJ van der Hoorn, WH Ezendonk, and C.M.A. Geers for their contribution, The Dutch Cancer Society for support and Mrs. F. Smits for typing of the manuscript.

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CHAPTER 7

WEEKLY LOW DOSE MITOXANTRONE PLUS DOXORUBICIN AS SECOND-LINE CHEMOTHERAPY FOR ADVANCED BREAST CANCER

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Breast Cancer Research and Treatment 1992; **21**: 133-138

SUMMARY

Weekly low dose mitoxantrone (3 mg/m^2) plus doxorubicin (8 mg/m^2) was administered as second-line chemotherapy to 33 patients with advanced breast cancer. Four out of 28 evaluable patients (14%) obtained a partial response with a median duration of 34 weeks (range 18-67$^+$ weeks), while 8 patients (29%) showed stable disease with a median duration of 28 weeks (range 11$^+$-60 weeks). Gastrointestinal toxicity and alopecia were mild. Grade II and III leukopenia occurred in 63% of the courses without serious infectious disease. Four patients experienced an asymptomatic drop of 16-20% in the left ventricular ejection fraction (LVEF) after relatively low cumulative doses of each drug, and one patient with a history of pericarditis carcinomatosa and mediastinal irradiation developed a heart failure. In conclusion, this second-line combination treatment had moderate activity in breast cancer and caused only few subjective side effects, especially with respect to gastrointestinal symptoms.

INTRODUCTION

Combination chemotherapy appears to be more effective than single agent therapy in inducing responses in disseminated breast cancer. First-line treatment with cyclophosphamide, methotrexate and fluorouracil (CMF) induces a response in about 40-50% of the patients with a median duration of response of less than a year [1]. Second-line chemotherapy in CMF-refractory patients often consists of single agent treatment with (4'-epi-)doxorubicin or mitoxantrone in a 3-weekly high dose schedule. However, only about 20-30% of these patients achieve a remission, frequently of short duration, while toxicity is often considerable especially by treatment with anthracyclines [2-4].

When (4'-epi-)doxorubicin or mitoxantrone are administered in low dose schedules every week or twice a month, the drugs can still be active, with remission rates of about 30% (0-59%) in patients with advanced breast cancer [5-22]. Gastrointestinal toxicity and alopecia are significantly less with these low-dose schedules.

Cardiotoxicity is a major problem of long-term (4'-epi-)doxorubicin treatment, while mitoxantrone occasionally produces such toxicity. Doxorubicin cardiotoxicity is probably induced by the intracellular formation of free radicals and stimulation of membrane lipid peroxidation in the heart muscle cells [23]. In experimental studies with hearts of rats, evidence was found that mitoxantrone did not form free radicals, and had a strong inhibitory effect on the lipid peroxidation [24,25]. In addition

mitoxantrone was found to cause a concentration-dependent inhibition of doxorubicin stimulated lipid peroxidation in liver microsomes of rabbits [26]. These data suggest that mitoxantrone might have an inhibitory effect on the occurrence of doxorubicin-induced cardiotoxicity. Furthermore, low-dose schedules of doxorubicin seem to produce less cardiotoxicity [5,6,27,28]. In view of these data it appeared attractive to combine doxorubicin and mitoxantrone at weekly dosages. Therefore, we initiated a phase II study with the combination of low doses of doxorubicin and mitoxantrone in a weekly schedule as second-line chemotherapy for patients with advanced breast cancer.

PATIENTS AND METHODS

Eligibility criteria of the protocol included: patients with measurable or evaluable lesions, age less than 80 years, World Health Organization (WHO) performance score 2 or less, life expectancy of more than 2 months, serum bilirubin less than 40 μmol/l, WBC above 3.0 x 10^9/l, platelets above 100 x 10^9/l, no prior therapy with anthracyclines or mitoxantrone. Patients with a history of recent cardiac disease, or with metastases in the central nervous system were excluded. Metastatic disease of all patients had to be resistant to previous endocrine therapy and to first-line chemotherapy with CMF (cyclophosphamide, methotrexate and fluorouracil). The protocol (DDHK 88-18) was approved by 2 different committees, both a protocol review and a medical ethics committee. All patients gave oral informed consent before entering the study.

On-study evaluation consisted of medical history, physical examination, tumor measurements, complete blood count (Hb, WBC, platelets), automated blood chemistry, left ventricular ejection fraction (LVEF, using radionuclide multigated analysis with intervals of initially 12, subsequently 8 or 4 weeks), bonescan, bone and chest X-rays and liver CT-scan (in case of liver metastases).

Treatment consisted of mitoxantrone 3 mg/m^2 plus doxorubicin 8 mg/m^2 as weekly sequential intravenous injections via a running infusion with physiologic saline during a few minutes. Responses were defined according to WHO criteria. Duration of partial response was measured from initiation of therapy until time of tumor progression. Drug toxicity was also evaluated according to WHO criteria.

Table 1 Patient characteristics		
Number of patients entered:	33	
Number of evaluable patients:	28	
Menopausal status:		
pre	1	
post	26	
peri or unknown	6	
Age:		
median (range)	57	(40-74)
WHO Performance Status:		
median (range)	1	(0-2)
Metastatic sites per patient:		
median (range)	3	(1-4)

RESULTS

Patients characteristics are indicated in Table 1. Thirty-three patients entered the study. Twenty-eight patients were evaluable for response and toxicity. Five patients were not evaluable because of early withdrawal or lack of response evaluation (within the first 8 weeks). Reasons to stop the treatment were: patient refusal (2), radiation therapy for pain (1), discovery of brain metastasis 1 week after start of treatment (1), and hyperbilirubinemia 1 week after start of treatment (1). These 5 inevaluable patients died between 0.5 and 8 months after start of treatment.

Table 2 Type of responses and time to progression (WHO criteria)

	Number of patients (%)	Mean duration in weeks (range)
CR	0	-
PR	4 (14)	34 (18-67^{+})
SD	8 (29)	28 (11^{+}-60)
PD	16 (57)	within 2-14 weeks

Responses are shown in Table 2. No complete responses were observed. A partial response (PR) was achieved in 4 out of 28 patients (14%) with a median duration of 34 weeks (range 18-67^{+} weeks), while 8 patients (29%) showed stable disease (SD) with a median duration of 28 weeks (range 11^{+}-60 weeks). One patient had early progressive disease after 2 weeks of treatment. Fifteen other patients showed tumor progression within 5-14 weeks after start of treatment.
Progression-free survival and overall survival curves of the evaluable patients are shown in Fig. 1.

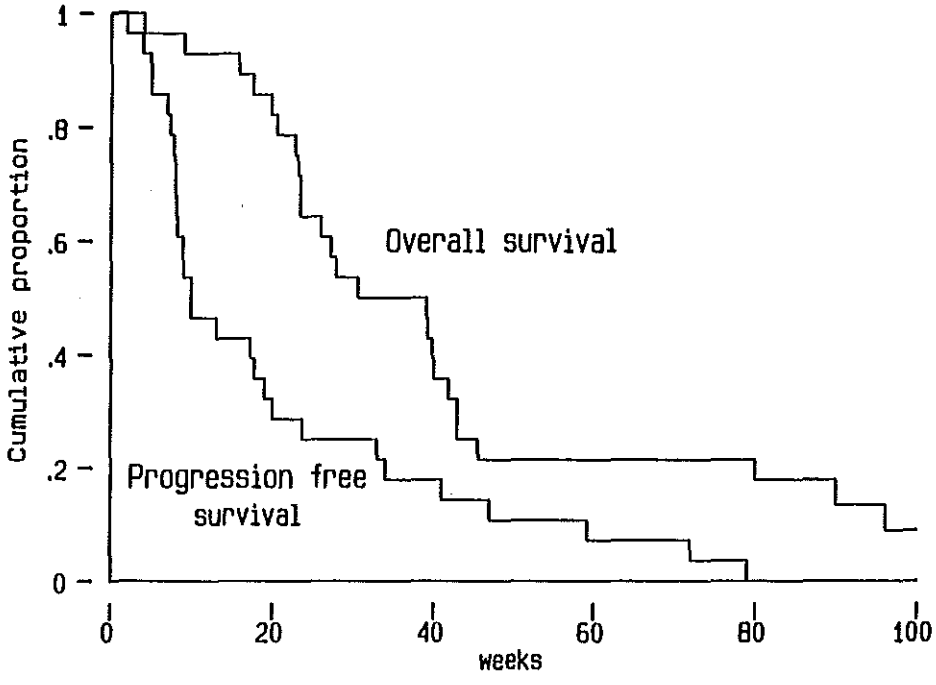

Figure 1
Progression-free and overall survival of all evaluable patients measured in weeks from start of treatment.

Toxicity is presented in Table 3. Leukopenia grade II occurred in 48% and grade III in 15% of all cycles. Gastrointestinal toxicity was very mild. Serious hair loss

grade II and III occurred in only a minority of the patients and was probably still related to the previous CMF treatment.

In 10 of the 12 patients who achieved a PR or SD, LVEF was repeated at least once. Three patients received a "doxorubicin equivalent" dose (cumulative doxorubicin dose + cumulative mitoxantrone dose x 5) of more than 550 mg/m^2, i.e. 720, 795, and 850 mg/m^2. LVEF of these patients dropped from 65 to 53%, 76 to 61%, and 80 to 73% respectively. LVEF of two other patients receiving a cumulative "doxorubicin equivalent" dose of 195 and 292 mg/m^2 dropped from 89 to 75% and from 76 to 64% respectively. In four other patients treated with "doxorubicin equivalent" cumulative doses of 209 to 500 mg/m^2 LVEF remained stable to the base-line value. Thus, in non of these 9 patients did LVEF decrease to below the critical limit of 50% and none of them showed any clinical sign of cardiotoxicity. One patient, with previous mediastinal irradiation and pericarditis carcinomatosa, developed a cardiac failure in the presence of a drop in the absolute level of the LVEF from 78% to 29%, after being treated with a "doxorubicin equivalent" cumulative dose of 478 mg/m^2. She was successfully treated with digoxin and diuretics.

Table 3 Percentage of side-effects (WHO grading)

Grade	0	1	2	3
Leukopenia*	15	22	48	15
Thrombocytopenia*	80	9	9	2
Mucositis*	96	4	-	-
Diarrhea*	94	6	-	-
Nausea/vomiting*	84	11	5	-
Alopecia**	82%		18%	

* Percentage of courses with side effect events as recorded in all (100%) weekly chemotherapy courses. **Alopecia is expressed as the percentage of patients experiencing various grades (0/1 and 2/3) of hair loss.

DISCUSSION

Several studies have shown that chemotherapy with weekly low doses of (4'-epi)doxorubicin or mitoxantrone can be as effective as the 3-weekly high-dose

schedules in inducing remissions in patients with advanced breast cancer, whereas toxicity of the low doses is considerable less [5-22,27,28]. Treatment results of 18 studies using "weekly" low dose (4'-epi-)doxorubicin or mitoxantrone in patients with advanced breast cancer are shown in Tables 4 and 5. Table 4 summarizes the treatment results of 7 studies concerning a total of 311 patients of whom less than 50% had been treated before with chemotherapy for advanced disease. Overall, 93 of the 311 patients (30%) treated with weekly low-dose anthracycline or mitoxantrone responded objectively. The mean percentage of response of the 7 separate series of patients was 32% (range 10-48%). Table 5 shows the treatment results of 11 studies concerning 361 patients of whom more than 50% had been pretreated with various types of chemotherapy. During this second-line chemotherapy 110 out of 361 patients (30%) responded. The mean percentage of response of the individual series was 29% (range 0-59%). Response durations in the studies vary widely. Based on response data available in 12 of these 18 studies, the median duration of response is about 6-7 months. In our study we combined mitoxantrone and doxorubicin in weekly low-dose schedules in order to achieve a low toxicity profile with preserved activity.

Table 4 Low-dose doxorubicin (Dox), 4'-epi-doxorubicin (Epi-dox), or mitoxantrone (Novantrone) mainly used as first-line chemotherapy in advanced breast cancer

Drug	Dosage	Treatment interval (weeks)	N of eval. pts	Responses (CR/PR)		Prior chemother. (% pts)	Ref.
				(n)	(%)		
Dox	15 or 20 mg	1	50	7	14	44	(5)
Dox	20 mg	1	62	19	31	0	(6)
vs							
VAC		3	66	24	35		
Dox	20 mg	1	81	29	36	0	(7)
vs							
Epi-dox	50 mg	2	68	15	22		
Epi-dox	12 mg/m^2	1	42	18	43	12	(8)
Epi-dox	20 or 40 mg	1	25	12	48	<24	(9)
Epi-dox	20 mg	1	41	4	10	17	(10)
Novantrone	3.3-6 mg/m^2	1	10	4	40	0	(11)

Number of eval. patients:311.

Number of patients achieving CR/PR:93/311 (30%).

Mean % CR/PR of all 7 individual series: 32% (range 10-48%).

Subjective side effects, gastrointestinal toxicity, and alopecia were mild with this combination therapy, but leukopenia regularly needed postponement of the chemotherapy. In spite of these clear toxic effects on bonemarrow function, the combination of weekly low-dose mitoxantrone and doxorubicin showed "moderate" antitumor activity (14% PR) as second-line chemotherapy in patients with metastatic breast cancer. However, in an additional 29% of the patients a SD was observed for 11^{+}-60 weeks with nearly the same median duration of progression-free survival as for partial responders (28 vs 34 weeks). This median duration of SD (about 6-7 months) in our patients is not different from that of the objective responders reported in the other studies (Table 4 and 5), i.e. 6-7 months. The overall response rate (CR/PR/SD) of 43% also is generally not different from that reported in other studies,

Table 5 Low-dose doxorubicin (Dox) or 4'-epi-doxorubicin (Epi-dox) mainly used as second-line chemotherapy in advanced breast cancer

Drug	Dosage	Treatment interval (weeks)	number of eval. pts.	Responses (CR/PR)		Prior chemother. (% pts)	Ref.
				(n)	(%)		
Dox	0.4 mg/kg*	1	29	11	38	100	(12)
Dox	0.5-1 mg/kg	1	31	11	35	heavily pretreated	(13)
Dox	20 mg/m^2	1,3,1,3	60	16	27	87	(14)
Dox	6-12 mg/m^2	1	34	20	59	> 62	(15)
Dox	5-11.5 mg/m^2	1	20	3	15	100	(16)
Dox	8-12 mg/m^2	1	17	2	12	70	(17)
Dox	10 mg/m^2	1	24	0	0	> 96	(18)
Dox	12 mg/m^2	1	30	8	27	97	(19)
Dox	20 mg	1	48	9	19	56	(20)
Epi-dox	20 mg	1	39	20	51	51	(21)
Dpi-dox	15 mg/m^2	1	29	10	34	52	(22)

Number of eval. patients: 361.

Number of patients achieving CR/PR: 110/361 (30%).

Mean % CR/PR of all individual series: 29% (range 0-59%).

* Therapy with initial loading course (days 1-3 and 8-10).

even in comparison with high-dose mitoxantrone 3-weekly in the first-line (40%) as reported by Harris et al. [29]. Striking in our study was the clear dose-limiting bonemarrow depression in spite of the low dosages of the drugs used. However it has to be noted that the relatively high incidence of leukopenia compared to 3-weekly schedules is influenced by the frequent weekly measurement of WBC in this weekly dose regimen.

In vitro studies with the combination of both drugs had suggested a possible protective effect of mitoxantrone towards doxorubicin-induced cardiotoxicity [26]. This possible protective effect of mitoxantrone towards doxorubicin-induced cardiotoxicity, however, was not observed in two clinical studies that combined the drugs in 3-weekly "high-dose" schedules [30,31]. From our study we cannot make definite conclusions in this respect. Although 4 patients showed a significant relative decrease (16-20% of the pretreatment value) of LVEF, non of them decreased below an absolute value of 50% with the exception of the patient with previous mediastinal irradiation and carcinomatous pericarditis.

It can be concluded that second-line combination treatment of weekly low-dose mitoxantrone plus doxorubicin is a well tolerated drug regimen for patients with CMF-resistant tumors. The antitumor efficacy is comparable to that of other second-line chemotherapeutic regimens in the absence of serious side effects, but postponement of drug administration was regularly needed because of the occurrence of leukopenia.

Acknowledgements

We wish to thank P.J. van Assendelft and J. Vuik for preparing the print, and DG Lederle Netherlands for support.

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CHAPTER 8

SECOND-LINE CHEMOTHERAPY WITH LONG-TERM LOW-DOSE ORAL ETOPOSIDE IN PATIENTS WITH ADVANCED BREAST CANCER

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Breast Cancer Research and Treatment 1995; **34**, 185-189

SUMMARY

In a phase II study, 27 patients with metastatic breast cancer were treated with oral etoposide as second-line chemotherapy at a dose of 50 mg/m^2/day for 21 days, which courses were repeated every 4 weeks. Twenty-one patients were evaluable for response, and twenty-five for toxicity. In two (10%) patients a partial response was observed with a duration of 60 and 122 weeks respectively, and seven patients (33%) showed stable disease. Gastrointestinal toxicity was usually mild, though relatively frequent. Anemia grade II and III was observed in 20% of all courses (< 10% of all measurements), and leukopenia grade III and IV was observed in 22% of all courses (< 10% of all measurements). There was one toxic death.

Reviewing the literature we calculated a response rate of intravenous etoposide treatment of 8% in 276 patients with metastatic breast cancer from 7 studies (response rates ranging between 0-14%), while (chronic) oral treatment caused a response rate of 19% in 145 patients from 8 different studies (response rates ranging between 0-35%).

INTRODUCTION

The prognosis of patients with disseminated breast cancer refractory to or relapsing from first-line chemotherapy is poor. All currently applied cytotoxic drugs yield low response rates (10-30%) with a median duration of response of about 6 months or less and frequently considerable toxicity (1). Therefore, new approaches are warranted. Because breast cancers contain usually a relatively low percentage of proliferating cells within the cell cycle, prolonged exposure to a cytotoxic drug is theoretically attractive.

Etoposide (VP16-213), a semisynthetic podophyllotoxin derivate with a wide antitumor activity, is a cell cycle phase-specific drug acting in the late S- and early G_2-phase of the cell cycle with schedule dependency (2). Activity of the drug is probably more related to duration of exposure to tumor cells rather than to the area under the curve (AUC) (3-5). Etoposide has been used in clinical trials for approximately 20 years without clear efficacy in breast cancer, but recently there is renewed interest in this drug because of the application of new treatment schedules (6,7). There is clinical evidence that chronic daily administration of oral etoposide can induce responses in patients with different chemotherapy refractory solid tumors (8,9).

Out of the four breast cancer patients in a phase I trial using prolonged low-dose oral etoposide (8), one had an objective response and one patient with non-measurable

disease had a subjective response. Based on these data we initiated a phase II study with daily oral administration of etoposide as second-line chemotherapy in patients with metastatic breast cancer.

PATIENTS AND METHODS

Eligibility criteria of this study included patients with measurable or evaluable lesions, age less than 80 years, World Health Organization (WHO) performance score (PS) 2 or less, life expectancy of more than 2 months, serum bilirubin less than 20 μmol/l, WBC above 3.0 x 10^9/l, platelets above 100 x 10^9/l, no prior therapy with etoposide. Patients with a history of recent cardiac disease, or patients with metastases in the central nervous system, were excluded. Metastatic disease of all patients was considered resistant to previous endocrine therapy and to first-line chemotherapy. All patients gave oral informed consent before entering the study.

On-study evaluation consisted of medical history, physical examination, tumor measurements, complete blood count (Hb, WBC, platelets), automated blood chemistry, bonescan, bone and chest X-rays and CT-scan or ultrasound of the liver in case of liver metastases. On follow-up complete blood count was performed weekly and response evaluation was performed after every second course.

Treatment consisted of etoposide 50 mg/m^2/day, orally for three consecutive weeks, in a twenty-eight day cycle. Responses and toxicity were defined according to WHO criteria. Duration of complete and partial response was measured from initiation of therapy till time of tumor progression.

RESULTS

Twenty-seven patients entered the study. Patients characteristics are indicated in Table 1. Two of the 27 patients were ineligible (PS: 3 and bilirubin 43 μmol/l). All 25 eligible patients were evaluable for toxicity. The total number of courses of etoposide administered was 90 (mean: 3.6; median 2, range 1-25). Gastrointestinal toxicity was usually mild. Twenty-one patients had no or only mild complaints of nausea (WHO grade 0-I), while four patients (16%) experienced nausea and vomiting grade II-III for which reason one of them was hospitalized. Alopecia was often related to previous chemotherapy, while in three patients the first hair loss (grade II-III) was undoubtedly etoposide-induced. Leukopenia grade III and IV occurred in 15 patients

Table 1 Patient characteristics

Number of patients entered	27	
Number of eligible patients	25	
Number of patients evaluable		
for response	21	
for toxicity	25	
Age		
median (range)	54	(37-76)
WHO performance status		
median (range)	1	(0-2)
Number of organ systems involved		
median (range)	3	(1-5)
Time from first sign of metastatic disease to start of etoposide (months)		
median (range)	19	(4-81)
No of prior hormonal therapies		
median (range)	1	(0-4)
Prior chemotherapy		
adjuvant	7	
chemotherapy for metastatic disease	27	
Cyclophosphamide/Methotrexate/Fluorouracil	18	
Cyclophosphamide/Doxorubicin/Fluorouracil	8	
Cyclophosphamide/Epirubicin/Fluorouracil	1	
Site of metastatic lesion		
liver	10	
lung	7	
pleura	6	
bone	17	
lymph node	5	
skin	11	
breast	6	

(60%), in 22% of all courses and in < 10% of all measurements; anemia grade II and III was observed in 9 patients (36%), in 20% of all courses and in < 10% of all measurements. Mild to moderate thrombocytopenia was infrequent (grade I-II: in 3% of all measurements). One patient died during the leukopenic period.

Four of the 25 eligible patients were not evaluable for response because of early withdrawal (within the first 3 weeks). Reasons to stop the treatment in these 4 patients were: severe nausea and vomiting (1), analysis of polyuria (1), anemia (1), and sudden

death in a patient with an axillary thrombosis two weeks after start of therapy. No complete responses were observed in 21 patients evaluable for response. Two patients (10%)(95% confidence interval 1-30%) achieved a partial response with a duration of 60 and 122 weeks, respectively. The first responding patient was treated before with hormonal therapy for 3 months without success, followed by 12 courses of FEC chemotherapy with stabilisation of the disease. During etoposide treatment the lytic bone metastases showed fair sclerosis. The second patient had been treated for three years with two lines of endocrine therapy, followed by 23 courses of CMF chemotherapy with a partial response. During etoposide therapy the pulmonary lesions showed a partial response and the bone lesions remained stable.

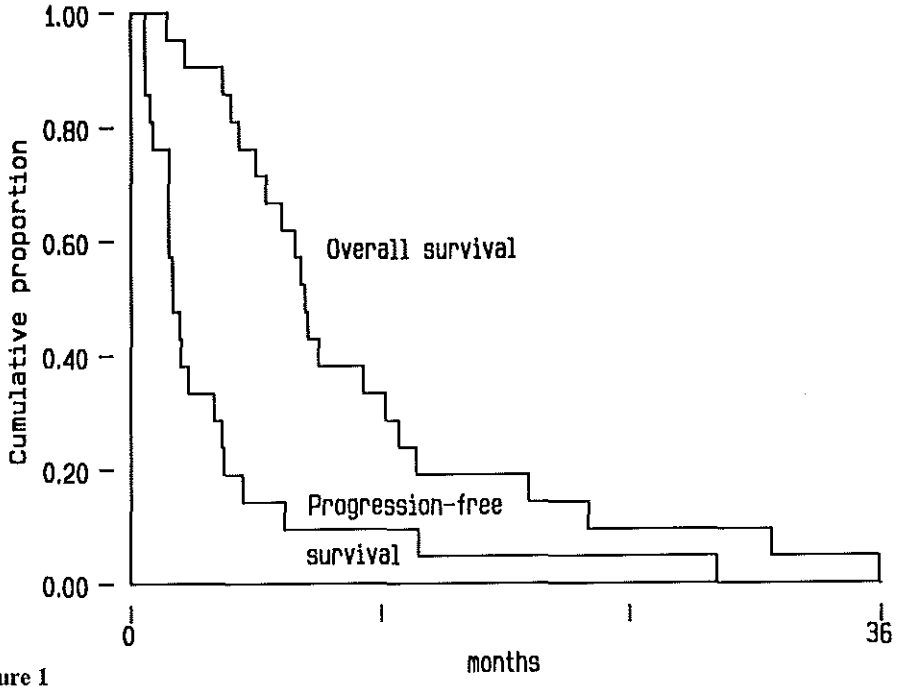

Figure 1
Median survival time

Seven patients (33%) had stable disease with a median duration of 19 weeks (range 9^{+}-32 weeks). Progressive disease from the start of treatment was observed in twelve patients. For the 21 evaluable patients the median time to progression was 2 months (mean : 4.3 months) and the median survival time was 8.3 months (mean : 11.3 months) (Fig. 1).

Table 2 Etoposide as ≥ second-line single agent therapy in metastatic breast cancer

Treatment schedule	No. of eval. pts	Response (CR+PR) n	%	Ref.
Intravenous administration				
100-250 mg/m^2 q every week	14	0	0	10
45 mg/m^2 days 1-5 q 3 wk	60	3	5	11
75 mg/m^2 days 1-5 q 3 wk	59	5	8	
50-70 mg/m^2 days 1-5 q 3 wk	35	5	14	12
50-70 mg/m^2 CI days 1-5 q 3 wk	31	4	13	
60-135 mg/m^2 twice weekly	24	1	4	13
125 mg/m^2 days 1,3,5 q 3-4 wk	19	0	0	14
100-125 mg/m^2 days 1,3,5 q 4-5 wk	19	2	11	15
300-450 mg/m^2 days 1-3 q 4 wk	15	1	7	16
Total	276	21	8	
Treatment schedule	No. of eval. pts	Response (CR+PR) n	%	Ref.
Oral administration				
75-125 mg/m^2 days 1-5 q 3 wk	14	0	0	10
200 mg/day days 1-5 q 2-3 wk or	20	0	0	17
300-400 mg/day days 1-5 q 2 wk				
50 mg/m^2/day; days 1-21 q 4 wk	4	1	25	8
50 mg/m^2/day; days 1-21 q 4 wk	18	4	22	18
50 mg/m^2/day; days 1-21 q 4 wk	43	15	35	19
50 mg/day; days 1-14 q 4 wk	10	1	10	20
50 mg/m^2/day; days 1-21 q 4 wk	25	5	25	21
50 mg/m^2/day; days 1-21 q 4 wk	21	2	10	This study
Total	145	28	19	

CI = continuous infusion

DISCUSSION

Treatment results of second-line chemotherapy in metastatic breast cancer are disappointing and remissions are usually of short duration (1). Therefore, testing of new treatment modalities remains of utmost importance.

In the past twenty years etoposide has been extensively used in the treatment of patients with a variety of solid tumors. Most experience with etoposide in breast cancer is obtained with intravenous (i.v.) treatment schedules. Table 2 summarizes the treatment results with etoposide in this disease. Seven studies (concerning 276 patients) applied i.v. etoposide as a single agent in previously treated patients. Response percentages varied from 0-14%, with an overall response rate of only 8% (10-16). Thus these short-term intravenous schemes of second-line chemotherapy with etoposide have shown only moderate activity in breast cancer, while toxicity was generally considered acceptable with myelosuppression emerging as the most frequent side effect.

Treatment results of eight studies (including our study) using oral etoposide as second-line chemotherapy in metastatic breast cancer are also shown in Table 2 (8,10,17-21). In the studies of Cavalli et al. (10) and Falkson et al. (17) using a high dose oral regimen for five days no responses were observed. However, because the cytotoxic effect of etoposide is more related to the duration of tumor cell exposure to the drug rather than to the AUC, prolonged exposure might theoretically result in an augmented anti-tumor effect (3-5). In a phase I trial (8) one out of 4 patients with breast cancer responded to a long-term low-dose etoposide regimen. The recommended dose for following phase II studies was therefore 50 mg/m^2/day for 21 days in a 28 day cycle. We performed a phase II study using this regimen but achieved only 10% remissions. Palombo et al. (18), Martin et al. (19) and Atienza et al. (21) performed similar studies in breast cancer patients pretreated with chemotherapy. They reported higher response rates i.e. 22%, 35% and 25%, respectively (Table 2). On the other hand Calvert et al. (20) reported the same response rate of 10% in a subgroup of 10 patients treated with 50 mg etoposide per day after previous chemotherapy. In this heterogeneous study higher response rates were observed in a subgroup of chemotherapy naive patients (45% response) and at a higher dose (100 mg/day) regimen (35% response, regarding mainly patients not treated with chemotherapy before). When taking together all literature data, (chronic) oral etoposide treatment caused an objective response in 19% of 145 patients (Table 2) (8,10,17-21), mostly of short duration. Responses can occur in all types of

metastatic sites. The toxicity observed in our study is comparable with that of other studies with leukopenia as the most serious side effect. Also Calvert et al. (20) and Atienza et al. (21) reported the occurrence of toxic deaths, although this outpatient regimen appeared to be quite manageable.

In conclusion, second-line chemotherapy with etoposide has only moderate activity in patients with metastatic disease in the presence of significant but manageable toxicity. Newer agents such as taxol and taxotere might therefore be of greater interest (22,23), but maybe etoposide can be of greater value in combination with these or other active agents.

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CHAPTER 9

PHASE II STUDY OF CARBOPLATIN AND ETOPOSIDE AS FIRST-LINE REGIMEN IN PATIENTS WITH METASTATIC BREAST CANCER

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Annals of Oncology 1994; **5**, 858-860

ABSTRACT

Background: The data available on the role of carboplatin and etoposide in breast cancer, especially in patients with no or minimal prior therapy are limited.

Patients and methods: We performed a phase-II study with carboplatin and etoposide as first line treatment in 34 patients with metastatic breast cancer. The treatment regimen was carboplatin 300 mg/m^2 day 1, and etoposide 100 mg/m^2 days 1, 3 and 5, every four weeks.

Results: Of 33 evaluable patients, two achieved complete responses (6%) lasting 4 and 5 months, 7 patients (21%) achieved partial responses with a median duration of 6+ (range 5 - 8) months, 15 patients had stable disease, and 9 progressed during treatment. The major toxicity was myelosuppression. WHO grades 3 or 4 leukocytopenia or thrombocytopenia were seen in 15 and 10 patients, respectively. One formally ineligible patient with an impaired renal function died 14 days after the start of treatment because of a septicaemia in the presence of a grade 4 leukocytopenia. Besides this patient no other patient presented with granulocytopenic fever.

Conclusion: In view of the observed response rate of 27% (95% confidence interval 11% - 43%) we think that carboplatin and etoposide given in this dose and schedule has probably no clear advantage over the more commonly used regimens.

INTRODUCTION

The data available on the role of carboplatin and etoposide in breast cancer, especially in patients with no or minimal prior therapy are limited. In previously chemotherapy treated patients a low activity has been reported for both carboplatin and etoposide (1-3), however when administered to previously untreated patients both agents have been shown to be active with a response rate of 15% for etoposide in 20 patients and a response rate of 14%, 35%, and 20% for carboplatin in 7, 34, and 20 patients, respectively (4-7). In a recently reported phase II trial with carboplatin given as a single agent using a pharmacokinetically guided dose schedule a response rate of 25% was reported in 40 patients. It is noteworthy that only one of the 13 previously treated patients responded compared to 9 of 27 patients who had not received previous chemotherapy (8). Also in a randomized trial the combination of etoposide and cisplatin given as first line treatment yielded a 63% response rate in 65 patients versus a 48% response rate on "CMF" in also 65 patients (9). In this study we investigated

the activity and toxicity of the combination of carboplatin and etoposide as first line treatment for metastatic disease.

MATERIALS AND METHODS

Between the end of 1989 and the beginning of 1993 patients with metastatic breast cancer were entered in the study. Eligibility requirements included measurable or evaluable disease, no prior chemotherapy for metastatic disease, WHO performance status ≤ 2, white blood cell count $\geq 3500/\mu L$, platelet count $\geq 100{,}000/\mu L$, serum creatinine ≤ 150 μmol/L, serum bilirubin ≤ 25 μmol/L, and informed consent. Adjuvant chemotherapy was allowed when given more than one year before entry in this study. Treatment consisted of carboplatin 300 mg/m^2 day 1, and etoposide 100 mg/m^2 days 1, 3, and 5, every four weeks. Complete blood counts were performed weekly. In case of a white blood cell count $<3000/\mu L$ or a platelet count $<100{,}000/\mu L$ on the day of retreatment, chemotherapy was postponed until recovery for a maximum of two weeks. Tumor response and toxicity were evaluated by standard WHO criteria (10). The initial planned statistical size of the study was 50 patients anticipating a true response rate of 40%. However after 34 patients the patients accrual was stopped because of the observed response rate.

RESULTS

Patients characteristics are listed in Table 1. Of the 34 entered patients, one patient was formally ineligible because of an elevated serum creatinine at the start of treatment. This patient was readmitted to the hospital 14 days after start of treatment because of a septicaemia with a clostridia species in the presence of a grade 4 thrombocytopenia and leukocytopenia. The patient died two days after admission.
Two patients (6%) achieved a complete response lasting 4 and 5 months. One of these patients had lymph node and liver metastases and the other had multiple skin metastases and a malignant pleural effusion. A partial response was observed in 7 patients (21%) with a median duration of 6+ months (range 5-8 months). Fifteen patients (45%) had stable disease with a median duration of 5 months (range 4-15 months), and 9 patients had progressive disease. After progression 28 patients were treated with second-line chemotherapy. The four patients who achieved an objective

Table 1 Patient charactistics

No. patients	34
Median age (years)	58
Range	29-73
Peformance (ECOG)	
0	15 (44%)
1	10 (29%)
2	9 (26%)
Adjuvant treatment	
Chemotherapy	11 (32%)
Hormonal treatment	4 (12%)
Previous hormonal treatment for metastatic disease	19 (56%)
Only one prior regiment	12
Two prior regimens	7
Menopausal status	
Premenopausal	8 (24%)
Postmenopausal	26 (76%)
Dominant metastic site	
Visceral	14 (41%)
Bone	9 (26%)
Soft tissue	11 (32%)
No. of metastatic site	
One	5 (15%)
Two	19 (56%)
≥ Three	10 (29%)
Disease-free interval	
None	4 (12%)
< 2 years	14 (41%)
≥ 2 years	16 (47%)
Interval from first metastases to study registration	
None	15 (44%)
< 2 years	11 (32%)
≥ 2 years	8 (24%)
Interval initial diagnosis and start chemotherapy	
Median (months)	31
Range	0-206

response to second-line chemotherapy all had responded to the previous treatment with carboplatin and etoposide. Median survival recorded from the start of chemotherapy for all patients was 15 months.

A total of 142 courses were administered with a median of 5 courses per patient. WHO grades 3 or 4 leukocytopenia or thrombocytopenia were observed in 15 (44%) and 10 (29%) patients, respectively. No cumulative hematological toxicity was observed. Besides the already mentioned ineligible patient no other patient presented with granulocytopenic fever during treatment. Nausea and vomiting was grade 1 in 16 (47%) patients, grade 2 in 8 (24%) patients and grade 3 in 10 (29%) patients. A grade 1 mucositis was observed in 2 (6%) patients and a grade 2 in 2 (6%) patients. Alopecia was common in those patients who received more than 2 cycles of chemotherapy.

DISCUSSION

The most commonly used chemotherapy regimens for patients with metastatic breast cancer are either "CMF" based or include an anthracycline. The observed differences in response rates between the various regimens are at least in part explained by differences in patient selection. Although the combination of carboplatin and etoposide has been given as second-line treatment and both agents have been included in high dose chemotherapy regimens, this combination has not properly been tested as a first-line treatment.

We investigated the activity of the combination of carboplatin and etoposide in patients with metastatic breast cancer who were either chemotherapy-naive or had only been treated with adjuvant chemotherapy. The predominant toxicity with this regimen consisted of thrombocytopenia and leukocytopenia. Although we observed one toxic death in a patient with renal impairment, the hematological toxicity was not cumulative and easily manageable in all other patients. The non-hematological toxicity with this regimen was mild and consisted mainly of nausea and vomiting and alopecia.

In 33 evaluable patients we observed with this regimen an objective response rate of 27% (95% confidence interval 11% - 43%) with a median response duration of 6 months. Although not tested in a randomized study we think that in view of the observed response rate and toxicity this regimen has probably no clear advantages above the more commonly used regimens. Higher response rates might be observed

with a dose intensification of both drugs. However such regimens necessitate the usage of intensive hematological support.

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CHAPTER 10

DOXORUBICIN VERSUS EPIRUBICIN, REPORT OF A SECOND-LINE RANDOMISED PHASE II/III STUDY IN ADVANCED BREAST CANCER

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submitted

SUMMARY

Purpose: The EORTC Breast Cancer Cooperative Group carried out a randomized trial to compare doxorubicin with epirubicin as second-line chemotherapy in patients with metastatic breast cancer.

Patients and methods: Two hundred and fifty-nine patients with at least one site of metastatic disease entered this trial, of whom 232 patients were eligible. Treatment consisted of doxorubicin 75 mg/m^2 or epirubicin 90 mg/m^2 i.v. every 3 weeks.

Results: The overall response rates for doxorubicin and epirubicin were 36% and 28% respectively ($p = 0.173$). The median time to progression was 23 weeks for doxorubicin and 19 weeks for epirubicin ($p = 0.063$) and the median duration of response was 40 weeks for doxorubicin and 32 weeks for epirubicin ($p = 0.059$). The median survival was 47 weeks for doxorubicin and 44 weeks for epirubicin ($p = 0.196$).

Leukocyte count on retreatment day ($p = 0.011$) and platelet nadir ($p = 0.031$) were significantly lower in the doxorubicin treated group. Also mucositis ($p < 0.001$), diarrhea ($p = 0.005$) and hemorrhage ($p = 0.048$) were significantly worse in the doxorubicin arm. Nine patients on doxorubicin and 2 patients on epirubicin experienced congestive heart failure (CHF).

Conclusion: At the dose levels used in this study, no statistical differences in response rate and survival were found between the two treatment arms. Treatment with doxorubicin tended to result in a slightly longer duration of response and time to progression but doxorubicin was more toxic than epirubicin.

INTRODUCTION

Doxorubicin is among the most effective chemotherapeutic drugs in the treatment of metastatic breast cancer. Used as a single agent doxorubicin induces response rates of approximately 40% as first-line chemotherapy and about 20% as second-line therapy after failure or relapse on combination chemotherapy [1,2].

Although the acute toxicities of doxorubicin are manageable, the major cumulative dose-limiting toxic effect of the drug is the development of congestive heart failure (CHF), which may be irreversible and lethal [3,4]. The risk of CHF induction with doxorubicin is limited at a cumulative dose of less than 550 mg/m^2, but increases rapidly thereafter, preventing the continuation of the use of the drug beyond this dose.

Since the early 1970's there has been a continuous search for anthracycline analogues with a more favourable therapeutic profile than doxorubicin. A number of anthracycline derivatives of lower cardiotoxic potential in animal models have been introduced into clinical trials, of which one is epirubicin, an analogue resulting from the epimerization of 4'-hydroxyl of doxorubicin [5]. The mechanism of action of epirubicin is similar to that of doxorubicin: binding to DNA and inhibiting synthesis and function of nucleic acid [6].

Experimental and phase I studies in breast cancer suggested that epirubicin had a more favourable therapeutic index than doxorubicin, i.e. similar antitumor activity but less toxicity [6-9]. In the early phase II studies in metastatic breast cancer epirubicin showed antitumor activity comparable to that of doxorubicin [10]. The toxicities encountered with epirubicin therapy were: leucopenia, nausea and vomiting and no severe life threatening cardiac toxicity up to a cumulative dose of about 1000 mg/m^2.

In view of these early data the EORTC Breast Cancer Cooperative Group decided to conduct a randomized phase II/III study comparing directly epirubicin with doxorubicin, both given as single agents in patients with metastatic breast cancer relapsing after previous chemotherapy without anthracyclines. The dose levels selected for epirubicin and doxorubicin were 90 mg/m^2 and 75 mg/m^2, respectively. Both drugs were administered as an i.v. bolus injection every 3 weeks. Based on data of previous investigations, epirubicin 90 mg/m^2 was anticipated to induce degrees of myelosuppression equivalent to doxorubicin 75 mg/m^2 [8,10]. Hematological growth factors were not used in this study.

PATIENTS AND METHODS

Eligibility criteria

Patients with metastatic breast cancer were eligible for this study, after giving informed consent according to the rules of the participating institution.
Measurable or evaluable progressive disease was required as well as a performance status (WHO) better than 3. Liver enlargement, pleural effusion, ascites, bone marrow involvement and osteoblastic lesions were not considered as evaluable disease. No more than one previous combination chemotherapy regimen without anthracyclines, as adjuvant therapy or for metastatic disease, could have been applied. Other exclusion criteria included renal (creatinine > 1,2 mg/dl) and/or hepatic (bilirubin > 1,5 mg/dl) dysfunction, congestive heart failure, significant arrhythmia, bilateral bundle branch

block or history of myocardial infarction as well as previous or concurrent malignancies (except adequately treated carcinoma in situ of the cervix and/or carcinoma of the skin).

Table I Accrual per institution

Institution	Responsible physicians	N	(%)
Finsen Center/Rigshospitalet, Copenhagen	Dr. H.T. Mouridsen Dr. M. Andersson	73	(28.2)
U.H. Gasthuisberg, Leuven	Dr. J. Wildiers	53	(20.5)
Azienda Ospedaliere, Parma	Prof. G. Cocconi	41	(15.8)
Rotterdam Cancer Institute	Dr. J.G.M. Klijn	32	(12.4)
Medical University, Gdansk	Prof. J. Jassem	27	(10.4)
Institut J. Bordet, Brussels	Prof. R. Paridaens	14	(5.4)
U.H. Leiden	Prof. A.T. van Oosterom	13	(5.0)
U.H. Dijkzigt, Rotterdam	Dr. T.A.W. Splinter	3	(1.0)
U.H. St. Radboud, Nijmegen	Dr. L.V.A. Beex	2	(0.8)
Spaarne Hospital, Haarlem	Dr. C.A.M. De Swart	1	(0.04)
Total		259	

Study design

Eligible patients were randomized between epirubicin and doxorubicin treatment by telephoning to the EORTC Data Center. Patients failing either epirubicin or doxorubicin, after 2, 3 or 4 courses, were to be crossed over to doxorubicin or epirubicin, respectively. Originally the study was started as a randomized phase II trial, but after an interim analysis had been performed, it was continued as a phase III trial.

Treatment protocol

The doses of epirubicin and of doxorubicin were 90 mg/m^2 and 75 mg/m^2 respectively. Both drugs were administered as an i.v. bolus injection and cycles were repeated every 3 weeks.

Table II

	DOX	EPI	Total
Patient material			
Entered	128	131	259
Ineligible	10	17	27
Total eligible	**118**	**114**	**232**
Non-evaluable	16	12	29
Evaluable	102	101	203
Reasons for ineligibility			
Prior or concomitant treatment	2	2	4
Too low WBC at entry	2	1	3
Poor performance status	2	5	7
Cardiovascular disease at entry	2	2	4
No measurable disease	2	2	4
No information	0	2	2
Brain metastases	0	2	2
Previous endometrial cancer	0	1	1
Total ineligible	**10**	**17**	**27**
Reasons for non evaluability			
Death before treatment started/poor condition preventing start of treatment	2	1	3
Early stop of treatment for toxicity	4	2	6
Refused treatment/lost to follow-up	5	1	6
Incomplete/missing data	1	6	7
Additional/incorrect treatment	4	3	7
Total eligible not fully evaluable	**16**	**13**	**29**

Dose modifications

Treatment was delayed by 1 week if WBC were $< 3 \times 10^9$/l or platelets $< 100 \times 10^9$/l at the scheduled time of the subsequent cycle. Further dose adjustments were made as follows: 50% of the dose if after 1 week delay the WBC count was between

2 and 2,9 x 10^9/l and/or platelets between 50-99 x 10^9/l, or postponement for another week if the WBC count was < 2 x 10^9/l or platelets < 50 x 10^9/l. If postponement was required for > 3 weeks the patient went off study. The dose was also reduced to 50% if bilirubin level ranged between 2 and 3 mg/dl, and to 0% with a bilirubin level above 3 mg/dl.

Treatment duration

Patients with remission or stable disease after two courses continued treatment until the disease progressed. On disease progression patients on epirubicin or on doxorubicin having received less than 5 courses were to be crossed over to doxorubicin or epirubicin, respectively. Treatment was to be stopped at a cumulative dose of 550 mg/m^2 of doxorubicin and initially also for epirubicin. After 100 patients had been entered, the maximum epirubicin cumulative dose was increased to 900 mg/m^2. Therapy was also discontinued in case of congestive heart failure, severe persistent side effects or hematological toxicities requiring treatment delay for more than 3 weeks, or due to patient refusal.

Pretreatment and follow-up studies

Baseline investigations included history and physical examination, performance status, tumor measurements, complete blood count, chemistries, chest X-ray, a bone scan or skeletal survey, ECG and preferentially measurement of isotopic left ventricular ejection fraction (LVEF). All baseline investigations were repeated after 2 courses and thereafter every 3-6 weeks. Chest x-ray, bone scan and/or bone surveys were repeated every 12 weeks. WBC and platelet nadirs were measured weekly during the first two treatment cycles.

Evaluation of response and toxicity

Patients were evaluable for response if they had received at least two courses of chemotherapy and if tumor measurements had been repeated at 6 weeks. Assessment of response was done according to the UICC criteria [11]. Toxicity was assessed according to the WHO criteria [12]. Duration of complete or partial response was measured from the date of randomization until the date of progressive disease. All cases were subjected to extramural review performed by both the study coordinator (A.T. van Oosterom) and an external reviewer with respect to eligibility and

evaluability, treatment effectiveness, toxicity, the correct reporting of the data described in the files and their representation on the forms.

Table III Patient characteristics at entry (all eligible patients)

	DOX (N = 118)		EPI (N = 114)	
Age (median, rage)	56 yr	(31-75) yr	56 yr	(34-73) yr
Performance status WHO	N	%	N	%
0	38	(32)	35	(31)
1	49	(42)	53	(46)
2	28	(24)	25	(22)
Unknown	3	(2)	1	(1)
Prior endocrine therapy				
None	31	(26)	33	(29)
Ablative	22	(19)	19	(17)
Additive	43	(36)	48	(42)
Both	13	(11)	10	(9)
Unknown	9	(8)	4	(4)
Prior chemotherapy	115	(98)	110	(97)
Dominant site				
Soft tissue	37	(31)	27	(24)
Bone	32	(27)	31	(27)
Visceral	49	(42)	56	(49)
Menopausal status				
Pre-menopause	0	(0)	4	(4)
Natural menopause	71	(60)	80	(70)
Artificial menopause	38	(32)	25	(22)
Unknown	9	(8)	5	(4)

Statistics

The response to treatment and degree of toxicity were compared using the chi-square test for proportions and the chi-square test for linear trend. Leukocyte and platelet values were compared using the Wilcoxon rank sum test. Progression and survival curves were computed based on the Kaplan-Meier product limit estimate and

compared using the logrank test. Adjustment for imbalances in prognostic factors was done by means of retrospective stratification.

RESULTS

Patient characteristics

Within this study (EORTC 10811) 259 patients were randomized by 10 institutions between June 1982 and May 1986, 128 to doxorubicin and 131 to epirubicin (Table I). The present analysis is based on a median observation time of 2.6 years. Twenty-seven patients were ineligible, and twenty-nine additional patients were not evaluable (Table II). The characteristics of the 232 eligible patients are given in Table III. The two treatment groups were well balanced with respect to age, performance status, and prior hormono- or chemotherapy.

Table IV Number of treatment courses without cross-over (all eligible patients)

	DOX (N = 118)		EPI (N = 114)	
	N	(%)	N	(%)
0	2	(2)	1	(1)
1	11	(9)	5	(4)
2	13	(11)	18	(16)
3	10	(9)	13	(11)
4	14	(12)	13	(11)
5	11	(9)	8	(7)
6	6	(5)	22	(19)
7	28	(24)	21	(18)
8	8	(7)	6	(5)
9	9	(8)	1	(1)
10	2	(2)	1	(1)
11	1	(1)	2	(2)
12	1	(1)	2	(2)
13	1	(1)	0	(0)
14	1	(1)	0	(0)
15	0	(0)	1	(1)
median number of cycles	5		5	

Table V Treatment duration and dosages (all eligible patients who started the treatment)

	DOX (N = 116)		EPI (N = 113)	
Duration of treatment (days)				
median (range)	127	(1-314)	113	(1-323)
N with information	116		113	
Dosage				
Total dose (mg)				
median (range)	568	(80-1476)	800	(140-2250)
N with information	101		97	
Total dose (mg/m^2)				
median (range)	383	(47-911)	447	(88-1452)
N with information	92		93	
Total dose (mg/m^2/week)				
median (range)	23	(10-38)	27	(12-49)
N with information	90		92	
Total dose as % of the planned dose				
median (range)	90	(41-154)	91	(40-162)
N with information	90		92	
Relative dose intensity	N	%	N	%
< 70%	14	(16)	13	(14)
70-89%	30	(33)	31	(34)
90-109%	32	(36)	35	(38)
≥ 110%	14	(16)	13	(14)
N with information	90		92	
Reductions				
No	52	(58)	64	(70)
At least once	38	(42)	28	(30)
Delay				
No	42	(47)	51	(55)
At least once	48	(53)	41	(45)

For the dominant site, a small imbalance was observed: visceral lesions were more frequent in the epirubicin arm and there were more soft tissue lesions in the doxorubicin arm.

Treatment duration and dosages

Three eligible patients never started their treatment, one patient in the doxorubicin arm had an episode of infection soon after randomization, and another one refused to start the treatment. One patient in the epirubicin arm had died suddenly before the treatment was started.

Table VI Response rate (all eligible patients)

	DOX (N = 118)		EPI (N = 114)	
	N	(%)	N	(%)
Complete response	5	(4)	2	(2)
Partial response	38	(32)	30	(26)
No change	37	(31)	45	(40)
Progression/ED/Not eval.	38	(32)	37	(33)
Progression	18	(15)	23	(20)
Early death due to malignant disease	1	(1)	0	(0)
Early death due to toxicity	3	(3)	0	(0)
Not evaluable	16	(14)	14	(13)
Two-sided P-value for trend		**P = 0.324**		
% of responders				
CR or PR	43	(36)	32	(28)
no objective response	75	(64)	82	(72)
Two-sided P-value for comparison of the % of response		**P = 0.173**		

Table IV shows the number of courses administered. The median number of treatment courses was 5 in both arms; 1 to 14 cycles in the doxorubicin arm and 1 to 15 cycles in the epirubicin arm.

In Table V treatment duration and total dosages administered are depicted. The median duration of treatment was 127 days in the doxorubicin arm (range 1 to 314 days) and 113 days in the epirubicin arm (range 1 to 323 days). The median dose of drug received per square meter was 383 mg in the doxorubicin arm (range 47 to 911 mg) and 447 mg in the epirubicin arm (range 88 to 1452 mg). The median dose intensity was 90% in the doxorubicin arm (range 41 to 154%) and 91% in the epirubicin arm (range 40 to 162%). Fifty-three percent of the patients in the doxorubicin arm and 45% of the patients in the epirubicin arm had at least one cycle delayed. Dose reductions occurred in 42% and 30% of the patients in the doxorubicin and epirubicin arms, respectively. Twenty-one patients on doxorubicin and 16 on epirubicin stopped prematurely the treatment because of toxicity or treatment refusal.

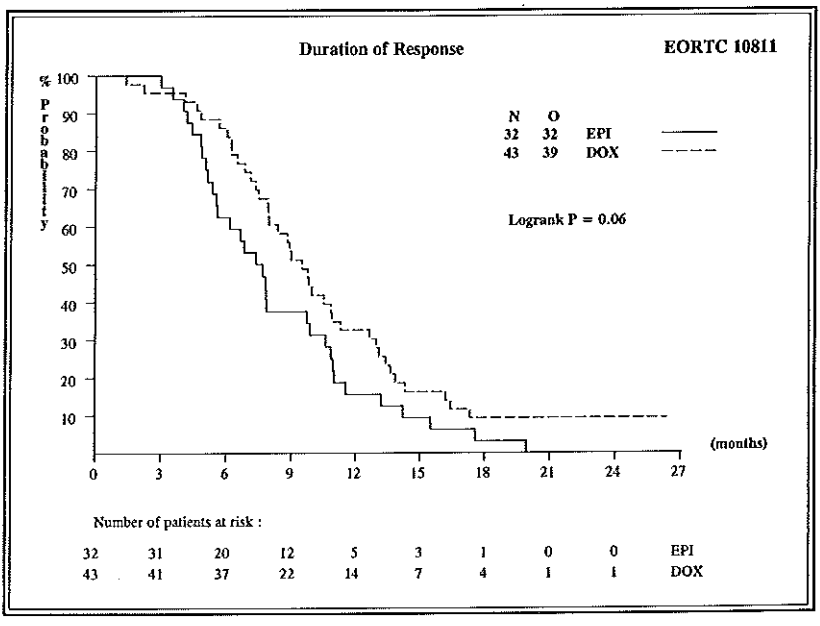

Figure 1
Duration of response

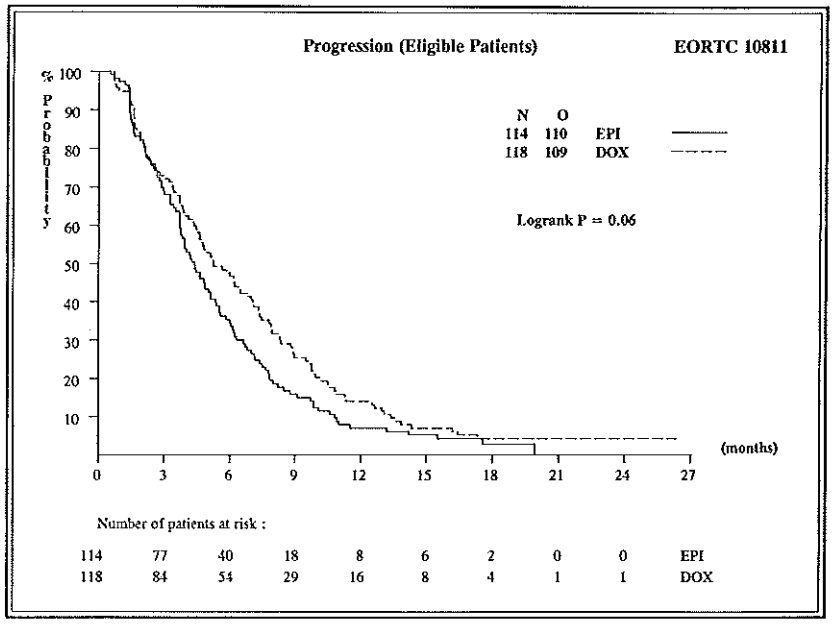

Figure 2
Time to progression

Treatment efficacy

Including the non evaluable patients, the response rate (CR + PR) was 36% for the doxorubicin arm (95% CI: 28-45%) and 28% for the epirubicin arm (95% CI: 20-36%, Table VI). This difference of 8% in the response rate (95% CI: -3% - +20%) is not significant using the chi-square test (p = 0.173). Likewise it is not significant using a test for linear trend (p = 0.324 for CR vs PR vs NC vs other). The response according to the dominant site of disease is presented in Table VII. When stratified by the dominant site of the disease, the conclusions are the same. The duration of response among the complete (CR) and partial (PR) responders is presented in figure 1. The median duration of response as measured from the date of randomization was 40 weeks for doxorubicin and 32 weeks for epirubicin. Using the logrank test, this difference is just not significant (p = 0.059).

Table VII Response by dominant site

	CR		PR		NC		PD/ED/NE		N
	N	(%)	N	(%)	N	(%)	N	(%)	
Soft tissue									
DOX	3	(8)	12	(32)	11	(30)	11	(30)	37
EPI	1	(4)	10	(37)	9	(33)	7	(26)	27
Bone									
DOX	1	(3)	12	(38)	12	(38)	7	(22)	32
EPI	1	(3)	9	(29)	13	(42)	8	(26)	31
Visceral									
DOX	1	(2)	14	(29)	14	(29)	20	(41)	49
EPI	0	(0)	11	(20)	23	(41)	22	(39)	56
P-value for a trend in response stratified by dominant site					**P = 0.436**				
P-value for the comparison of the % of response (CR + PR) stratified by dominant site					**P = 0.236**				

Time to progression for all eligible patients is presented in figure 2. The median time to progression was 23 weeks for doxorubicin and 19 weeks for epirubicin. Using the logrank test with or without adjustment for dominant site, this difference is not statistically significant (unstratified p = 0.063, stratified p = 0.085). Figure 3 shows the duration of overall survival for all eligible patients. The median survival for patients treated with doxorubicin was 47 weeks and for patients treated with epirubicin 44 weeks (unstratified p = 0.196, stratified p = 0.385). An analysis of time to progression and duration of survival in all randomized patients yielded similar results. A total of 5 patients initially treated with epirubicin were crossed over to doxorubicin treatment because of progressive disease. None of these patients responded. Among 9 patients not responding to first-line doxorubicin, 1 PR (11%) was observed with the use of second-line epirubicin treatment.

Toxicity

The hematological toxicity is presented in table VIII. It is expressed either as nadir or as retreatment day (first day of next cycle) values computed as the worst reported value over all cycles. The difference in the lowest leukocyte count on retreatment day

Table VIII Hematological toxicity (all eligible patients who started the treatment)

	DOX (N = 116)		EPI (N = 113)		P-value[1]
Leukocytes (10^9/l)					
Nadir over the whole treatment period					
median (range)	2.5	(0.2-3.9)	2.8	(0.2-4.0)	
N with information	60		56		P = 0.150
Worst retreatment value over the whole treatment					
median (range)	3.6	(2.0-10.3)	3.9	(2.3-12.7)	
N with information	103		107		P = 0.011
Nadir over the first two cycles					
median (range)	2.5	(0.2-3.9)	2.7	(0.2-4.0)	
N with information	42		38		P = 0.429
Worst retreatment value over the first two cycles					
median (range)	4.0	(2.2-10.5)	4.3	(2.5-17.5)	
N with information	103		107		P = 0.030
Platelets (10^9/l)					
Nadir over the whole treatment period					
median (range)	88	(8-206)	143	(27-335)	
N with information	24		22		P = 0.031
Worst retreatment value over the whole treatment					
median (range)	244	(50-592)	266	(105-552)	
N with information	102		107		P = 0.077
Nadir over the first two cycles					
median (range)	77	(8-301)	127	(27-335)	
N with information	19		17		P = 0.163
Worst retreatment value over the first two cycles					
median (range)	290	(50-656)	279	(107-692)	
N with information	102		107		P = 0.778

[1]Wilcoxon Rank Sum test

Table IX Non hematological toxicity (worst grade reported during the treatment period for all eligible patients who started the treatment)

	Grade 0	1	2	3	4	Total	P-value
Oral							
DOX	57	26	24	7	0	114	
EPI	89	13	10	1	0	113	P<0.001
Diarrhea							
DOX	88	11	12	2	1	114	P=0.005
EPI	103	4	6	0	0	113	
Haemorrhage							
DOX	104	5	4	1	0	114	
EPI	111	0	2	0	0	113	P=0.048
Nausea/ Vomiting							
DOX	9	20	46	31	8	114	
EPI	6	28	49	26	4	113	P=0.304
Fever							
DOX	90	11	12	0	2	115	
EPI	93	12	8	0	0	113	P=0.215
Alopecia							
DOX	10	4	15	81	2	112	
EPI	15	2	13	80	1	111	P=0.483
Infection							
DOX	89	14	9	1	2	115	
EPI	93	9	6	3	1	112	P=0.546

was significant between the doxorubicin and epirubicin arm ($p = 0.011$) with a lower value in the former arm. The platelet nadir (based on 46 patients) was significantly lower in the doxorubicin arm ($p = 0.031$). Three patients died in the doxorubicin arm with infectious complications during leukopenia, whereas no toxic deaths were observed in the epirubicin arm. The non-hematological toxicities are presented in Table IX, displaying the maximal toxicity assessed per patient excluding information after crossing over.

Mucositis and diarrhea were significantly lower in the epirubicin treated patients than in the doxorubicin treated patients ($p < 0.001$ and $p = 0.005$, respectively). There were also less hemorrhages with epirubicin than with doxorubicin ($p = 0.048$).

Concerning cardiotoxicity, 9 patients in the doxorubicin arm and 2 patients in the epirubicin arm experienced clinical CHF. However, sporadic measurements of LVEF precluded detailed assessment of cardiotoxicity.

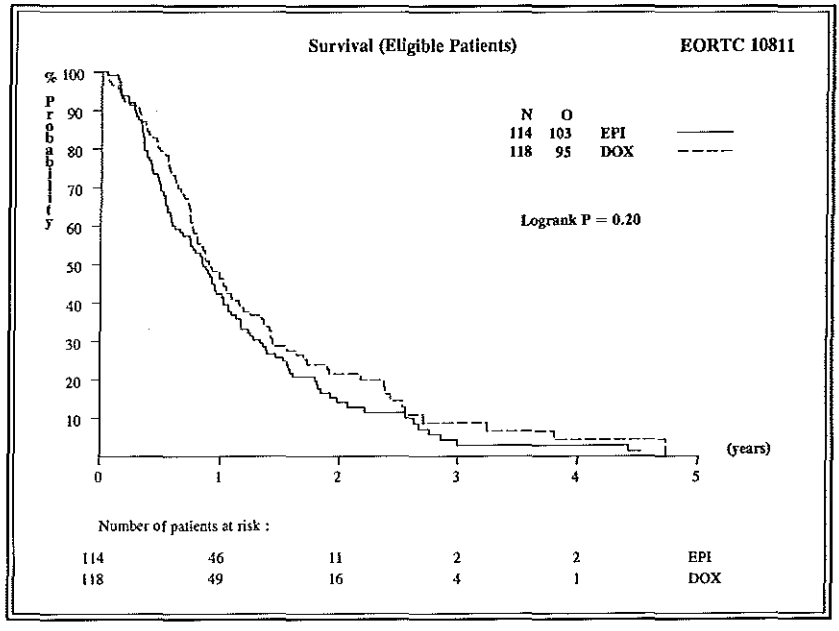

Figure 3
Duration of overall survival

DISCUSSION

There is virtually no cure of disseminated breast cancer. Therefore, treatment of metastatic disease is palliative, and aims at symptom relief and prolongation of life. In this situation side-effects of therapy should be minimal.

The objective of the present study (started in the eighties) was to compare the activity and toxicity of doxorubicin with its presumed equally effective but less toxic derivate epirubicin as second-line chemotherapy in patients with metastatic breast cancer. To the best of our knowledge this is the largest randomized trial directly comparing the two anthracyclines in advanced breast cancer. Eight smaller randomized studies in metastatic breast cancer have compared the efficacy of monotherapy with

both drugs given on an equimolar or equimyelotoxic base (Table X). In the four studies comparing epirubicin with doxorubicin on an equimolar base no differences in response percentages between the two treatment arms were observed [14,16,18,20]. However, the numbers of patients included in those studies were small, precluding any firm conclusions. Toxicity, however, was generally more pronounced in the doxorubicin treated patients.

Table X Doxorubicin vs Epirubicin as single agent therapy in metastatic breast cancer

Author	Year	No. of patients	Dose mg/m^2 DOX	EPI	Response rates % DOX	EPI	Ref
Brambilla	1986	42	75	75	52	62	14
Perevodchikova	1987	30	90	90	33	33	16
Lawton	1990	56	70	70	36	32	18
Gasparini	1991	43	20[1]	20[1]	38	36	20
Jain	1985	52	60	85	25	25	13
Taguchi	1986	63	40	60	35	56	15
Hortobagyi	1989	48	60	90	29	26	17
Perez	1991	138	60	90	47	49	19
Bontenbal	1996	233	75	90	36	28	this study

[1]: Weekly administrations

The four other studies compared the two drugs on an expected equimyelotoxic base with doses of epirubicin ranging from 1.4 to 1.5 times the dose of doxorubicin [13,15,17,19]. Similarly none of these studies showed significant differences in response rates, duration of response or survival between the two treatment arms. In the largest of these four studies, bone marrow toxicity of doxorubicin 60 mg/m^2 and epirubicin 90 mg/m^2 were almost superimposable, and the same results were found for gastro-intestinal toxicity [19].

The efficacy of equimolar doses of epirubicin and doxorubicin as part of a drug combination with fluorouracil and cyclophosphamide (FEC vs FAC) was investigated in two large randomized trials [21,22]. Response percentages, duration of response and time to progression did not differ in the two treatment arms. However, the

epirubicin combination showed a lesser degree of myelosuppression, nausea and vomiting.

In our study no significant differences in response rates were observed between the two drugs, with 36% versus 28% objective responses (CR + PR) for doxorubicin and epirubicin respectively. There was at best a trend in favor of doxorubicin for the median duration of response (40 vs 30 weeks), and for the median time to progression (23 vs 19 weeks), but the differences were small and duration of survival was the same in both groups. An explanation for the small differences we observed in time to progression and duration of response can possibly be found in the initial design of the study, where treatment had to be stopped after a cumulative dose of 550 mg/m^2 was reached for both drugs. Indeed, 14 patients in the epirubicin arm stopped treatment after a cumulative dose of 500-600 mg/m^2. Reduction of the treatment *period* could have influenced the time to progression and duration of response as was found in the studies of Ejlertsen et al. and Coates et al. [23,24]. On the other hand *dose* reductions occurred more frequently in patients treated with doxorubicin (42 vs 30%).

The increased toxicity seen with doxorubicin gives rise to the question if we used an insufficient dose of epirubicin. Bone-marrow toxicity is historically used to relate the dose of one drug to another [25]. At the time this study started clinical experience suggested that doxorubicin 75 mg/m^2 and epirubicin 90 mg/m^2 would induce comparable degrees of myelotoxicity [8,10]. In 1990 Mouridsen reviewed ten years of clinical experience with epirubicin, and calculated the equitoxic dose ratio for the hematologic toxicity of doxorubicin and epirubicin to be 1:1.2. [26]. Drug dosages chosen in this study fulfilled this criterium. Furthermore, Bastholt et al. compared the efficacy and toxicity of 4 different dose levels of epirubicin in patients with metastatic breast cancer [27]. An increase of the dose from 90 mg/m^2 to 135 mg/m^2 resulted in increased toxicity but had no impact on the efficacy of the drug.

In conclusion: a trend to but just no significant difference in efficacy is achieved with doxorubicin 75 mg/m^2 and epirubicin 90 mg/m^2 in the treatment of advanced breast cancer. On the other hand, the treatment with epirubicin is associated with significantly less side-effects.

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CHAPTER 11

FEASIBILITY, ENDOCRINE AND ANTITUMOR EFFECTS OF A TRIPLE ENDOCRINE THERAPY WITH TAMOXIFEN, A SOMATOSTATIN ANALOGUE AND AN ANTIPROLACTIN IN POSTMENOPAUSAL METASTATIC BREAST CANCER: A RANDOMIZED STUDY WITH LONG-TERM FOLLOW-UP

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submitted

SUMMARY

Suppression of the secretion of prolactin, growth hormone and insulin like growth factor 1 might be important in the growth regulation and treatment of breast cancer. Because estrogens may counteract the antitumor effects of such treatment, the combination of an anti-estrogen (tamoxifen), a somatostatin analogue (octreotide) and a potent anti-prolactin (CV 205-502) might be attractive. In this respect we performed a first exploratory long-term study on the feasibility of combined treatment and possible clear differences in endocrine and antitumor effects during such combined treatment versus standard treatment with tamoxifen alone. Twenty-two postmenopausal patients with metastatic breast cancer (ER and/or PR positive or unknown) were randomized to receive either 40 mg tamoxifen per day or the combination of 40 mg tamoxifen plus 75 μg CV 205-502 orally plus 3x 0.2 mg octreotide s.c. as first-line endocrine therapy. An objective response was found in 36% of the patients treated with tamoxifen alone and in 55% of the patients treated with combination therapy. Median time to progression was 33 weeks for patients treated with tamoxifen and 84 weeks for patients treated with combination therapy, buth the numbers are to small for hard conclusions. There was no difference in overall postrelapse survival between the two treatment arms. With respect to the endocrine parameters there was a significant decrease of plasma IGF-1 levels in both treatment arms, while during combined treatment plasma growth hormone tended to decrease and plasma prolactin levels were strongly suppressed; in some patients also insulin and TGF-α decreased during the triple therapy. Although there was no significant difference in mean decrease of plasma IGF-1 levels between the two treatment arms, combined treatment resulted in a more uniform suppression of IGF-1. Therefore, the addition of a somatostatin analogue and an anti-prolactin may potentially enhance the efficacy of anti-estrogens in the treatment of breast cancer due to favorable endocrine and possible direct antitumor effects. Large phase II trials using depot formulations (in order to increase the feasibility) of somatostatin analogues are warranted to demonstrate the potential extra beneficial antitumor effects of such combination therapy.

INTRODUCTION

Different steroid hormones, peptide hormones, growth factors and other trophic substances are involved in the growth regulation of human breast cancer (1-2). Estrogens, especially estradiol, are the most potent growth stimulatory hormones of

breast cancer. Therefore, endocrine treatment of metastatic breast cancer usually employs antisteroidal agents such as tamoxifen resulting in response rates of 30-40% (3).

Together with estradiol, insulin like growth factors (IGF1 and IGF2), are the most potent mitogens for breast cancer cells (1,4,5). The growth effects of both are mediated predominantly via IGF1 receptors, which have been demonstrated in 67-93% of primary human breast cancers (6-9) at higher density than in normal or benign breast tissue (10). In vivo, pituitary-derived growth hormone (GH) regulates endocrinologically the secretion of IGF-1 (11,12), but possibly has also regulatory effects on local IGF-1 secretion within (tumor) tissues (12-14). In addition, in breast cancer local production of GH with potentially a paracrine function has been described (15). In vitro, physiological concentrations of the lactotrophic hormones GH and prolactin (PRL) can stimulate the growth of breast cancer cells (16-19). In primary human breast cancers receptors for these lactotrophic hormones have been demonstrated in 13-72% of series of tumors investigated depending on the techniques used (19). Furthermore, increased plasma levels of both GH (20) and PRL (20,21) as well as of IGF-1 (22) have been found in patients with breast cancer. Therefore suppression of GH, PRL and IGF-1 secretion might be important in the treatment of breast cancer. Suppression of GH and IGF-1 secretion can be induced by somatostatin and its analogues (11,14,23-26). Interestingly, also receptors for somatostatin (SSTR) have been demonstrated in 36-67% of primary human breast cancers (2,9,27,28) and in even 75% by in-vivo receptor scintigraphy,(29) indicating that somatostatin analogues can affect directly tumor growth. Indeed, previously we (30) and others (11,31) showed direct growth inhibitory effects of somatostatin analogues on human breast cancer cell-lines.

Based on the data mentioned above, it can be concluded that somatostatin analogues and antiprolactins can have beneficial direct and indirect effects on the treatment of breast cancer. However, thusfar single treatment with these agents showed only minor activity in postmenopausal patients with metastatic breast cancer (2,26,32-38). Because unopposed estrogen action can overrule the growth inhibitory effects of somatostatin analogues (30) and/or antiprolactins, combination treatment with an antiestrogen, a somatostatin analogue and an antiprolactin might be of value and can potentially increase the efficacy of single treatment with tamoxifen alone. In view of the fact that also tamoxifen affects growth factor secretion (1,39-45) such combination treatment might be extra attractive. However, clinical results of such

combined treatment modality have not yet been reported. In the present paper, we report on the feasibility and the endocrine and long-term antitumor effects of combined treatment with tamoxifen, the somatostatin analogue octreotide and a new potent dopamine agonist (the antiprolactin CV 205-502) in comparison with those of single treatment with tamoxifen, in addition to an elaborate overview and discussion of literature data.

PATIENTS AND METHODS

The study was performed after approval by a local Human Investigations Committee (trial DDHK 88-30). Between August 1989 and May 1991, 22 postmenopausal patients with previously untreated metastatic breast cancer were randomized after prior informed consent to be treated within this trial. The patient characteristics are summarized in Table 1. Two patients were not evaluable: one stopped treatment with octreotide within 2 days not tolerating daily injections and another patient stopped single treatment with tamoxifen within two months because of detection of an endometrial carcinoma. Later on, one patient appeared to be perimenopausal according to a rise in estradiol levels after start of treatment with tamoxifen. Therefore, this patient was not included in the analysis for estradiol levels. Presently, the mean follow-up of all 20 evaluable patients is 3 years (range 3 months - 6 years). Within this follow-up period all but 2 patients showed progressive disease and 14 died.

The patients were randomized to be treated with either tamoxifen 40 mg per day or with the combination treatment consisting of 40 mg tamoxifen, 75 μg of the dopamine agonist CV 205-502 (®Norprolac) and the somatostatin analogue octreotide (®Sandostatin) 200 mg tid subcutaneously every day. Dose modification was not allowed. The duration of treatment varied from 6 weeks to more than 6 years. Patients were evaluated for toxicity and response every 6-12 weeks. Measurements of tumor response were performed according to the UICC criteria.

Plasma samples for measurement of basal hormone and growth factor concentrations (Table 2) were taken before and regularly between 4-24 weeks after start of treatment (fig. 1). Plasma peptide hormones and growth factors were measured by radioimmunoassays and radioreceptor assay (TGF-α) as described before (46). Plasma estradiol levels were measured by radioimmunoassay.

Table 1 Patient Characteristics

	Tamoxifen	Combination therapy	Total
Number of patients entered:	12	10	22
Number of patients evaluable:	11	9	20
Menopausal status:			
post	10	9	19
peri	1	0	1
Age: Mean (range)	59 (49-71)	62 (49-73)	60 (49-73)
WHO Performance Status:			
0	6	5	11
1	3	2	5
2	2	2	4
Disease sites:			
soft tissue	3	3	6
lymph nodes	1	1	2
bone	9	6	15
liver	4	1	5
lung	2	3	5
Number of disease sites:			
1	5	5	10
2	5	3	8
3	0	1	1
4	1	0	1
Receptor status (in tumor or metastases):			
ER and/or PR positive	10	6	16
ER and PR unknown	1	3	4

Statistical methods

The expected accrual rate per year was 60 patients. Because of a much lower actual recruitment, especially due to refusal of daily injections in the combined treatment arm, the trial was closed after inclusion of 22 patients in 2 years. Because of the relatively low number of patients in this study the analysis of the data has been primarily descriptive, directed at the calculation of response rate, progression-free

survival with actuarial methods and a description of the endocrine effects of the treatments by calculation of the change in plasma concentration levels from baseline. Because of the limited power of this study to detect differences between treatment arms, all p-values reported in this paper should be regarded as exploratory. The logrank test was used for the comparison of progression free survival. The Mann-Whitney non-parametric two sample test was used to compare the change in plasma levels in both treatment groups.

Table 2 Mean plasma levels of hormones and growth factors before and during treatment

Hormone/ Growth factor	Treatment	No. of eval. patients	Pretreatment value (mean ± SD)	Absolute change from pretreatment value (mean ± SD)
E2 (pmol/l)	- TAM - Combined	N = 7 N = 6	87 ± 75 83 ± 80	-21 ± 63 -17 ± 74 **p=0.48***
nGH (μg/l)	- TAM - Combined	N = 10 N = 7	0.8 ± 1.2 2.6 ± 3.1	+1.23 ± 1.78 -1.3 ± 3.32 **p=0.10**
PRL (μg/l)	- TAM - Combined	N = 10 N = 7	5.2 ± 1.9 8.0 ± 5.2	-0.77 ± 2.62 -5.5 ± 4.85 **p=0.006**
Insulin (mU/l)	- TAM - Combined	N = 10 N = 7	25.7 ± 15.3 57.5 ± 41.4	+15.6 ± 39.8 -32 ± 37.6 **p=0.02**
TGF-α (ng/ml)	- TAM - Combined	N = 10 N = 8	0.30 ± 0.14 0.39 ± 0.13	+0.02 ± 0.09 -0.08 ± 0.12 **p=0.11**
IGF-1 (ng/ml)	- TAM - Combined	N = 10 N = 8	149 ± 64 137 ± 39	-62 ± 47 -69 ± 28 **p=0.63**

* p-values indicate differences in decrease between the two treatment groups
- TAM = tamoxifen
- Combined = combination treatment with tamoxifen, octreotide and CV 205-502

RESULTS

a.Endocrine effects of treatment

Figure 1 shows the absolute change from baseline in plasma hormone and growth factor concentrations for all patients with evaluable measurements. Since no trend was apparent in the values during treatment from one month after the start of treatment, for each patient all these values measured between 4 and 24 weeks are summarized by the mean. Table 2 shows the mean pretreatment values and the absolute mean change of each of the endocrine parameters from pretreatment values. Pretreatment basal estradiol levels were comparable in both treatment groups and the values during treatment did not show a systematic change. Basal GH showed a small decrease in 4 of 7 investigated patients during combined treatment and in none of 10 patients during single treatment with tamoxifen (fig. 1), but in view of differences in pretreatment values and a large variation during treatment there was only a trend for a difference ($p=0.10$) between the two treatment arms (Table 2). Most interesting was the significant decrease ($p < 0.0002$) of plasma IGF-1 levels during treatment (fig. 1), i.e. overall a mean decrease of 49% during combined treatment and 38% during single treatment with tamoxifen. This decrease showed no significant difference between the two treatment groups, either absolutely ($p=0.63$, Table 2) and percentually ($p=0.21$). However, IGF-1 suppression was more uniform during combined treatment in contrast to a strong variation in response during tamoxifen treatment (fig. 1). With respect to plasma prolactin levels the combined treatment caused a clearly significant suppression of prolactin secretion due to the antiprolactin CV 205-502, while tamoxifen had no significant effect (fig. 1, Table 2, $p=0.006$).

With respect to the other endocrine parameters, some patients showed a decrease of plasma insulin and TGF-α levels during combined treatment (fig. 1), but differences in overall results between the two treatment arms (Table 2) were only found for insulin ($p=0.02$) and not for TGF-α ($p=0.11$).

b.Antitumor effects

Five (55%) of 9 patients treated with the combination therapy showed an objective response compared to 4 (36%) out of 11 patients treated with tamoxifen alone (Table 3). Median time to progression was 84 weeks for the patients treated with combination therapy versus 32 weeks for patients treated with tamoxifen. Progression-free survival was slightly better for patients treated with the combination of drugs than those treated with tamoxifen alone (Fig. 2), but the numbers of patients in this feasibility study are

too small to draw definite conclusions. There was no difference between the two treatment arms with respect to overall postrelapse survival.

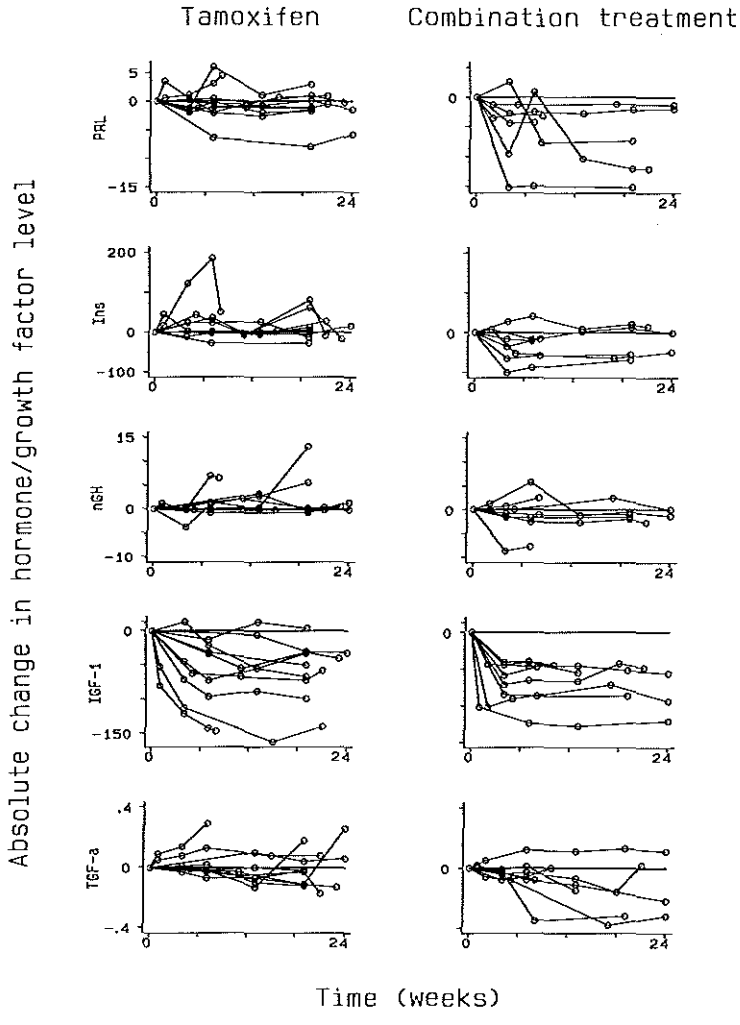

Figure 1
Effect of tamoxifen (left side of panel) and of combination treatment (right side of panel) on plasma hormone and growth factor concentrations. The zero-lines represent the basal pretreatment values, while the absolute individual changes are indicated as determined 4-24 weeks after start of treatment.

c.Toxicity

Treatment with the triple endocrine combination therapy appeared to be feasible, but a significant number of potentially eligible patients refused randomization because of the inclusion of three daily subcutaneous injections with Sandostatin within one of the treatment arms. However, subjective side-effects were minimal in both treatment arms. During combination therapy, shortly after start of treatment, slight nausea grade 1 (WHO) was observed in a minority of the patients, but no serious complaints were reported. One patient with diabetes mellitus had a persistent fall in plasma glucose levels during combined therapy needing reduction of daily insulin dosages (maybe as a consequence of suppression of glucagon secretion by octreotide). The most important side-effect was the development of asymptomatic gallbladder stones in one patient treated with combination therapy.

Table 3 Type of responses and time to progression (in weeks)

	CR	PR	SD	PD
Tam	1 (162)	3 (32,66,78)	3 (25,39,159)	4 (10-21)
Combination treatment	2 (171,209)	3 (84,86,115)	2 (22,36)	2 (7,11)

DISCUSSION

The relative role of PRL, GH and IGF-I in the development and treatment of human breast cancer is not clearly understood. All three peptides have been observed to be increased in plasma of a variable percentage of breast cancer patients (20-22). Growth stimulation of breast cancer cells by these peptides can be blocked by monoclonal antibodies (47-50). In addition, all three peptides (1,15,47,48,51,52) and their receptors (1,6-9,19) have been demonstrated in animal mammary tumors and/or human primary breast cancers suggesting a role in autocrine/paracrine cell growth regulation. However, nearly all endocrine therapies are focused on antagonism of estradiol, the primary mitogen for human breast cancer (3).

Some trials have tested the value of suppression of prolactin secretion by dopamine agonists (antiprolactins) (26,32, 34-38,53). Initial trials using single dopaminergic treatment with L-dopa or bromocriptine showed poor results (35-38). Two studies

investigated combination therapy of bromocriptine with antisteroidal treatment. Dogliotti et al. (54) found that bromocriptine in combination with high dose progestins reduced the percentage of patients with progressive disease, but Bonneterre et al. (55) observed no additional antitumor effect of bromocriptine to tamoxifen. This might be explained by the fact that progestins can increase plasma PRL levels (56), while in contrast tamoxifen has rather inhibitory effects on PRL secretion (57-59). Other authors (19,26,41) assumed that the lack of antitumor effects by single dopaminergic treatment may have been due to the presence of hGH, which is also a lactogen and can bind to lactotrophic receptors (19). However a few pilot studies using combined treatment with bromocriptine and a GH lowering drug such as a somatostatin analogue showed no impressive effects in heavily pretreated patients with metastatic breast cancer (2,26,32,34).

In view of the accumulating evidence regarding the importance of IGFs in the growth regulation of breast cancer (1,4), in the past decade there was an increasing interest in the GH/IGF axis especially due to the development of potent somatostatin analogues, which agents can suppress the function of the GH/IGF axis (11,14,23,24,26,46). This interest was further increased by the detection of SSTRs in breast cancer cell lines and tissues (30,31,60-62) and in about half of primary breast cancers (27-29). Indeed, we (30) and others (11,31) demonstrated direct growth inhibitory effects by various somatostatin analogues on different breast cancer cell lines. Inhibition of cell proliferation seems to be mediated especially by subtypes SSTR2 and SSTR5 (24,62). Also in some experimental animal models somatostatin analogues were able to cause inhibition of mammary tumor growth (11,14,23,24,63-66).

However, in 4 clinical studies (2,26,32-34), treatment of 38 (heavily) pretreated patients with octreotide caused only one objective response and 5 times stable disease (together 16%) (2). Also in previously untreated patients single first-line treatment with octreotide appeared to be less effective than common standard treatment modalities, which resulted in early stopping of this treatment arm in an ongoing randomized trial of the Mayo Clinics. These disappointing results of single somatostatin analogue treatment (or in combination with an antiprolactin) can be explained by our observation that estradiol abolished these growth inhibitory effects (30). Therefore, already at time of start of the present clinical study, in 1989, our study design testing these drugs in combination with an antiestrogen seems to be more appropriate. Later on, this approach was supported by the results of different

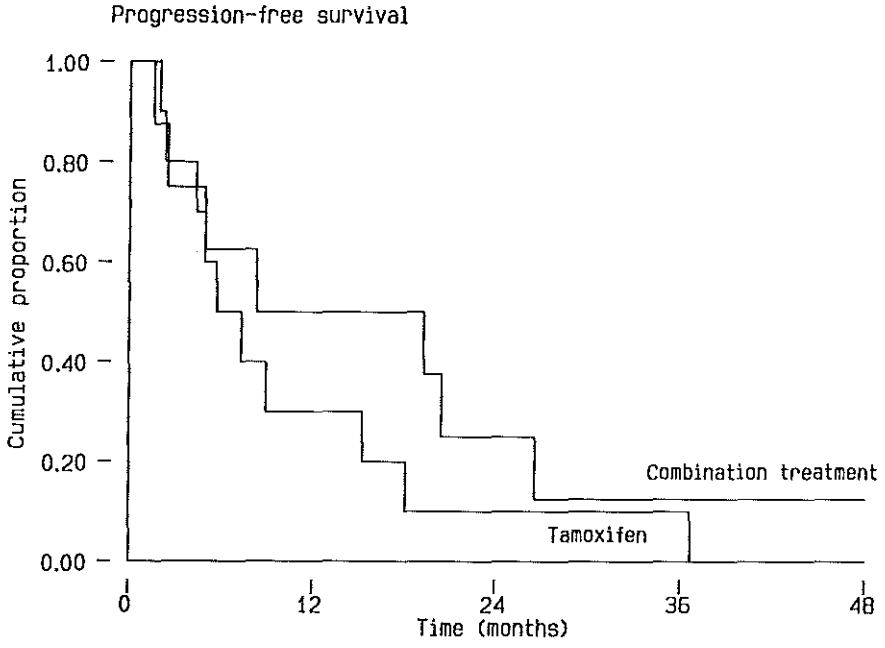

Figure 2
Actuarial progression-free survival curves for the two treatment groups.

preclinical studies indeed showing additive biological (67) and antitumor effects (66,68) of somatostatin analogues to endocrine therapy with tamoxifen or by surgical oophorectomy in hormone sensitive tumors in vivo. Meanwhile, tamoxifen appeared not only to act by blocking the growth stimulatory effects of estrogens but also to modify growth factor secretion (1,39-45,67,69-71) and to suppress the GH/IGF-I axis (41,59,72) and prolactin secretion (57-59). Tamoxifen and other antiestrogens can decrease especially plasma IGF-1-levels (39-45), but also can down-regulate IGF-I-R (73) and can suppress IGF-1-induced breast cancer cell proliferation (74). Furthermore, both octreotide (11) and anti-estrogens (43-45,74) affect IGF binding proteins. Thus additive endocrine and antitumor effects could be expected from combination therapy with tamoxifen plus a somatostatin analogue and an antiprolactin.

In our study tamoxifen caused rather an increase than a decrease of basal GH concentrations. In contrast, in several patients combination treatment tended to decrease basal GH levels. Previously, Pollak et al. (25) and Manni et al. (26) showed significant suppression of stimulated GH-levels (which are less affected by fluctuation than basal plasma GH levels during the day) by octreotide treatment. In contrast to the basal GH concentration, IGF-I levels are stable during the day. Strikingly, we found no additive suppressive effects of tamoxifen and octreotide on mean plasma IGF-1 concentrations, but combination treatment caused a more uniform suppression of IGF-1 (fig. 1). Both single tamoxifen and combination treatment caused a decrease of about 40-50%. This might partly be explained by the observation that tamoxifen already increases the release of endogenous hypothalamic somatostatin resulting in blunting of pituitary GH pulse amplitude (72). However, this does not exclude that clear additive endocrine effects might be found in studies using lower dosages of tamoxifen (20 instead of 40 mg/day) or higher dosages of somatostatin analogues than used in our trial. Recently, Kiang et al (42) reported an interesting observation indicating that the type of antitumor response is related with the extent of IGF-I suppression. In our study, we were not able to confirm this observation.

With respect to basal plasma insulin and TGF-α concentrations we found no impressive differences between the two treatment arms, although the combination therapy had a suppressive effect in some patients. Although somatostatin analogues might affect LH secretion in premenopausal patients (75), in our postmenopausal patients no significant effects on plasma E_2 levels were observed. This finding confirms the results of the study of Manni et al (26), who also found no effect of combined octreotide/bromocriptine treatment on plasma LH, FSH and E_2 levels. Finally, with respect to prolactin secretion (which is partly influenced by estrogens) the anti-estrogen tamoxifen tended to decrease plasma PRL levels, but not significantly. Interestingly, the new very potent antidopaminergic drug CV 205-502 used in the treatment of prolactinomas (76) caused a strong significant decrease of basal prolactin levels (with about 70%) also in our patients with normal PRL secretion. This suppression is more pronounced than previously reported for bromocriptine. However, in contrast to estradiol, it is presently unknown to which plasma levels PRL has to be suppressed in order to contribute to a (potential) extra antitumor effect.

With respect to the performance of our study, the triple endocrine therapy appeared to be feasible in the presence of only few non-serious side effects. However, a significant number of potentially eligible patients refused participation in the trial

because of the need of three daily injections in one of the treatment arms. This problem will be resolved by the application of depot preparations of somatostatin analogues, which are increasingly available. Furthermore continuous administration of drugs is generally more effective than daily injections as demonstrated for octreotide (66,77). In our pilot study the patients treated with the combination therapy showed less frequently progressive disease from start of treatment and a longer progression-free survival, but the numbers are undoubtedly too small for definite conclusions and our results have to be confirmed by other but much larger studies.
In conclusion: the results of different preclinical studies indicate that the addition of a somatostatin analogue (with or without combination with an antiprolactin) may enhance the antitumor efficacy of antiestrogens in the treatment of breast cancer (66-68). Our first randomized clinical study on triple therapy showed that in principle such an approach is clinically feasible and caused significant endocrine effects. A large multicenter randomized study in metastatic breast cancer, using a depot preparation of octreotide (instead of daily injections), is warranted to prove the presence of such potential extra beneficial antitumor effect.

Acknowledgements
We would like to thank the Dutch Cancer Society (grants RRTI 88-9 and IKR 90-18) and Sandoz for support, our colleagues J.Th.P. Janssen, P. van Liessum, J.J. Croles and C. van der Heul for their contribution, and Miss F. Smits and P. Bos for typing the manuscript.

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CHAPTER 12

SUMMARY

CONCLUSIONS AND PERSPECTIVE

Summary

Breast cancer is the most important cause of cancer death in women. Although adjuvant systemic therapy can cure a minority of the patients, metastatic disease is still lethal, and treatment in this stage of the disease is primarily palliative. Therefore, there is an urgent need for more effective therapy, and/or drugs with less side-effects. The first part of this thesis describes the results of preclinical studies testing the concept of recruitment of slowly proliferating (hormone-responsive) MCF-7 human breast cancer cells into the cell cycle by estrogens, in order to make the cells more vulnerable for the lethal effects of subsequently administered chemotherapy. The second part describes the results of several clinical trials a) applying the principle of recruitment of cells into the cell cycle followed by chemotherapy, b) administering low-dose chemotherapy frequently or chronically to a slowly proliferating tumor like breast cancer, and c) testing of new combinations or new analogues of chemotherapeutic drugs, and d) investigating the effects of new combinations of hormonal therapy.

Chapter 1 describes the background of the studies presented in this thesis.

Chapter 2 shows in MCF-7 human breast cancer cells *in vitro* that the cytotoxic effect of doxorubicin is correlated with the drug concentration, exposure time and cellular uptake of doxorubicin.

Chapter 3 shows that, in MCF-7 human breast cancer cells *in vitro,* dual-parameter flow cytometry (with bromodeoxyuridine (BrdUrd) incorporation and propidium iodide (PI) uptake into DNA) allows accurate measurements of the effects of hormones and chemotherapy on the cell cycle kinetics.

Chapter 4 describes in MCF-7 cells the effects of estradiol stimulation on the cytoxicity of subsequently administered doxorubicin. Pretreatment with estradiol shows an enhanced cytotoxic effect of the drug under the conditions tested.

Chapter 5 shows that hormonal manipulation has no influence on the uptake, retention or cellular distribution of doxorubicin. The drug content in the MCF-7 tumor cells was measured with high performance liquid chromatography and flowcytometry. The cellular distribution of doxorubicin was investigated using fluorescence microscopy.

Chapter 6 presents the results of the first randomized adjuvant trial in stage II/IIIA breast cancer comparing the effect of FAC chemotherapy alone, with estrogen pretreatment followed by FAC chemotherapy. After a median follow-up of 3.3 years there is no difference in (local-)relapse-free and overall survival between both treatment arms of the study.

Chapter 7 describes the results of a study investigating the efficacy of a combination of doxorubicin and mitoxantrone used as a weekly low-dose regimen in second-line therapy of metastatic breast cancer. This drug combination was well tolerated, but the response rate was moderate, with only 14% partial response.

Chapter 8 shows the results of daily oral low-dose therapy with etoposide as second-line treatment in metastatic breast cancer. Toxicity of this treatment scheme was significant, though manageable. Efficacy was disappointing, with only 10% of the patients achieving a partial response.

Chapter 9 describes the effects of the combination of carboplatin and etoposide as first-line chemotherapy in metastatic breast cancer. This combination regimen was well tolerated with myelosuppression as the most important side effect. Response rates showed no superior efficacy over the standard treatment schemes, with a response rate of 27%.

Chapter 10 presents the results of a study performed by the EORTC Breast Cancer Cooperative Group. The efficacy of doxorubicin as second-line therapy in metastatic breast cancer was compared with the efficacy of the analogue epirubicin. There was no significant difference in efficacy between both drugs, but there was a trend in favor of doxorubicin. However, the treatment with epirubicin was associated with significantly less side-effects.

Chapter 11 describes the endocrine- and antitumor effects of a new combination of hormonal therapy. The effects of monotherapy with the anti-estrogen tamoxifen were compared with the effects of combined treatment with tamoxifen, the somatostatin analogue octreotide, and the anti-prolactin CV 205-502. The study was closed early because a significant number of potential patients refused participation because of the

need of the three daily injections of octreotide in one of the treatment arms. Plasma IGF-1 levels decreased significantly in both treatment arms, but more uniformly during combined treatment, while during combination therapy plasma growth hormone tended to decrease and plasma prolactin levels were strongly suppressed.

Conclusions and perspective

The newly derived knowledge of kinetic changes in breast cancer cells by estrogens and anti-estrogens and the experience that tumors with a high proliferation index are most responsive to chemotherapy have led to the assumption that stimulation of DNA synthesis in slowly proliferating breast cancer cells followed by chemotherapy might result in enhanced cytotoxicity of subsequently administered chemotherapy. In the MCF-7 estrogen-responsive breast cancer cell line we observed a 2 to 6-fold increase in the percentages of S-phase cells after estrogenic-stimulation, and an enhanced cytotoxic effect of subsequently administered doxorubicin (chapter 2-5). Also *in vivo* estrogens have proven to be able to recruit tumor cells into the cell cycle, but the percentages of cells recruited into the active phases of the cell cycle are low and the efficacy of chemotherapy is not improved as it is shown in most studies, performed in metastatic or locally advanced breast cancer. Also in our adjuvant study, with a median follow-up of 3.3 years (chapter 6), we found no difference in efficacy of FAC chemotherapy alone, compared to FAC preceded by estrogenic recruitment. However, follow-up is still short, and differences may only become apparant after many years in a slowly proliferating tumor like breast cancer.

It is clear that estrogenic stimulation alone, followed by chemotherapy will not improve clinical outcome in advanced breast cancer. Other means of stimulation of DNA synthesis with hormones other than estrogens or with other growth-factors, or other sequences of hormones/growth-factors and chemotherapy may be necessary to improve treatment efficacy.

Treatment results of systemic therapies in advanced breast cancer have shown a plateau in response rates and survival in the past decades. Whilst awaiting more potent new drugs, new schemes and new combinations of chemotherapeutic drugs or hormones or new analogues of well known drugs have been explored in an attempt to improve clinical outcome and/or diminish side-effects. The new analogue, new drug-combinations and schemes tested in the studies described in this thesis (chapter 7-11) all showed efficacy in metastatic breast cancer, but demonstrated no clear superiority over the well known treatment schemes. At this moment we have to state that

metastatic breast cancer is still an incurable disease and palliation is the main goal in this stage of the disease.

New promising developments for the near future are the application of chemotherapy in high dosages, chemotherapy encapsulated in liposomes, and treatment with the new taxoid drugs. Novel approaches in endocrine therapy are the application of new anti-estrogens, anti-progestins, more potent aromatase inhibitors, long-acting analogues of somatostatin, analogues of vitamine A and D, growth factor antagonists, tyrosine protein kinase inhibitors, inhibitors of angiogenesis, protease inhibitors, and monoclonal antibodies.

CHAPTER 12

SAMENVATTING

CONCLUSIES EN PERSPECTIEF

Samenvatting

Borstkanker is de belangrijkste doodsoorzaak van kanker bij de vrouw. Adjuvante systemische behandeling met chemotherapie of hormonale therapie blijkt in staat in een klein deel van de patiënten micrometastasen te genezen. Het gemetastaseerde mammacarcinoom blijft echter een ongeneeslijke ziekte. Er is dus een grote behoefte aan effectiever behandelingsmogelijkheden en medicamenten met zo weinig mogelijk bijwerkingen.

In het eerste deel van dit proefschrift worden de resultaten beschreven van preklinisch onderzoek in de humane hormoon gevoelige mammacarcinoom cellijn MCF-7. Met behulp van oestrogenen worden tumorcellen vanuit de rustfase (GOG1-fase) van de celcyclus de S-fase (DNA synthese fase) ingedreven (recruitment). Onderzocht is of deze cellen in de S-fase van de celcyclus gevoeliger zijn voor de letale effecten van chemotherapie.

Het tweede deel van het proefschrift beschrijft de resultaten van klinische studies met betrekking tot a) toepassing van het principe van oestrogene recruitment gevolgd door chemotherapie, b) het effect van frequent of chronisch toedienen van lage doses chemotherapie, c) het effect van nieuwe combinaties of nieuwe analoga van chemotherapie en hormonale therapie.

Hoofdstuk 1: de achtergronden van de studies die in deze thesis worden beschreven.

Hoofdstuk 2: er is een positieve correlatie tussen het cytotoxisch effect van doxorubicine en de incubatie concentratie, tijd van blootstelling en mate van opname van doxorubicine in MCF-7 cellen.

Hoofdstuk 3: Dubbel-label flowcytometry met bromodeoxyuridine (BrdUrd) incorporatie en propidium iodide (PI) opname stelt ons in staat het effect van chemotherapie en hormonen op de celcyclus kinetiek nauwkeurig te meten.

Hoofdstuk 4: Oestrogene stimulatie van MCF-7 cellen gevolgd door doxorubicine chemotherapie resulteert in een toename van het cytotoxisch effect van het chemotherapeuticum, in vergelijking met het cytotoxisch effect van doxorubicine alleen.

Hoofdstuk 5: Hormonale manipulatie heeft geen invloed op de opname, de retentie en de verdeling binnen de cel van doxorubicine chemotherapie. High performance liquid chromatography, flowcytometry en fluorescentie microscopie werden gebruikt om de opname door en de verdeling van doxorubicine in de cel te bepalen.

Hoofdstuk 6: De eerste adjuvante studie bij patiënten met stadium II/IIIA borstkanker waarin hormonale recruitment gevolgd door FAC chemotherapie wordt vergeleken met FAC chemotherapie alleen, toont na een mediane follow-up van 3,3 jaar dezelfde overlevingsresultaten.

Hoofdstuk 7: De combinatie van wekelijks lage doses doxorubicine en mitoxantrone chemotherapie als 2e-lijn behandeling bij het gemetastaseerde mammacarcinoom wordt goed verdragen doch is matig effectief met 14% partiële remissies.

Hoofdstuk 8: Dagelijks lage dosis etoposide chemotherapie per os als 2e-lijn chemotherapie resulteert in slechts 10% partiële remissies. Enkele patiënten hebben forse maag-darm bezwaren tijdens deze behandeling.

Hoofdstuk 9: De combinatie van carboplatin en etoposide als 1ste-lijn chemotherapie wordt goed verdragen, doch resulteert in een respons percentage van slechts 27%.

Hoofdstuk 10: De EORTC Breast Cancer Cooperatieve Group vergelijkt in een gerandomiseerde studie de effectiviteit van doxorubicine en epirubicine als 2e-lijn chemotherapie. Er word geen significant verschil gevonden in effectiviteit tussen beide medicamenten hoewel er een trend in het voordeel van doxorubicine lijkt te zijn. Epirubicine heeft echter wel minder bijwerkingen dan doxorubicine.

Hoofdstuk 11: Het effect van tamoxifen als 1ste-lijn hormonale therapie wordt vergeleken met het effect van de combinatie van tamoxifen, octreotide (anti-groeihormoon) en CV 205-502 (anti-prolactine). Er is geen verschil in overleving tussen de twee studie armen. In beide groepen daalt de waarde van IGF-1 in het plasma significant, terwijl in de arm met combinatie therapie ook het antiprolactine gehalte in plasma duidelijk wordt gesuprimeerd.

Conclusies en perspectief

De nieuw verworven kennis omtrent kinetische veranderingen in borst kanker cellen veroorzaakt door oestrogenen en anti-oestrogenen en de ervaring dat tumoren met een hoge groeifractie het meest gevoelig zijn voor chemotherapie hebben geleid tot de veronderstelling dat stimulatie van de DNA synthese, in een vaak langzaam groeiende tumor als borstkanker, gevolgd door chemotherapie zou kunnen resulteren in een beter celdodend effect van het chemotherapeuticum. In de MCF-7 oestrogeen-gevoelige borstkanker cellijn vonden we na stimulatie met oestrogenen een 2 tot 6-voudige toename van het percentage cellen in de S-fase (DNA-synthese fase) van de celcyclus en een toename van het celdodend effect van aansluitend gegeven doxorubicine chemotherapie (hoofdstuk 2-5). Ook in de patiënt is bewezen dat oestrogenen in staat zijn tumorcellen te recruteren vanuit de rustfase in de actieve fasen van de celcyclus. Het percentage cellen dat gerecruteerd wordt blijft echter laag en de effectiviteit van aansluitend gegeven chemotherapie verbetert niet zoals blijkt uit de meeste studies die zijn verricht in patiënten met borstkanker in een vergevorderd stadium. Ook in onze adjuvante studie vonden we, na een mediane follow up van ruim 3 jaar (hoofdstuk 6), geen verschil in effectiviteit tussen FAC chemotherapie alleen en FAC chemotherapie voorafgegaan door oestrogene recrutering. De follow up van deze studie is echter nog maar kort en verschillen kunnen, bij een langzaam groeiende tumor als borstkanker, pas na vele jaren duidelijk worden. Het is wel duidelijk dat stimulatie met alleen oestrogenen gevolgd door chemotherapie niet zal resulteren in een betere klinisch resultaat voor patiënten met borstkanker in een vergevorderd stadium. Andere methoden van DNA stimulatie met andere hormonen en/of groeifactoren, of andere volgorden van de hormonen/groeifactoren en chemotherapie zijn waarschijnlijk noodzakelijk teneinde te komen tot een betere effectiviteit van de behandeling.

De resultaten van systemische behandeling met hormonen en/of chemotherapie van borstkanker in een vergevorderd stadium tonen de laatste decennia een plateau in het aantal responders, in de duur van de respons en in de duur van de overleving. Omdat effectievere middelen tegen de ziekte nog ontbreken zijn de laatste jaren nieuwe schema's, nieuwe combinaties en analoga van de bekende middelen onderzocht teneinde de klinische resultaten te verbeteren en/of de bijwerkingen te doen verminderen. De nieuwe combinaties van chemotherapie en hormonale therapie en het analogon uitgetest in de onderzoeken beschreven in dit proefschrift (hoofdstuk 7-11) waren allen effectief bij uitgezaaide borstkanker, doch leidden niet tot betere resultaten in vergelijking met de resultaten van de standaard behandelingen. Op dit moment

moeten we nog concluderen dat uitgezaaide borstkanker niet te genezen is, en dat palliatie in dit stadium van de ziekte het belangrijkste doel van de behandeling is.
Nieuwe veelbelovende ontwikkelingen voor de nabije toekomst zijn: de toepassing van chemotherapie in hoge doses, het toedienen van chemotherapie in liposomen en behandeling met een nieuwe groep chemotherapeutica, de taxanen. Nieuwe ontwikkelingen op het gebied van de hormonale behandeling zijn: de toepassing van nieuwe anti-oestrogenen, anti-progestagenen, effectievere aromatase remmers, langwerkende analoga van somatostatine, analogen van vitamine A en D, groeifactor antagonisten, tyrosine protein kinase remmers, angiogenese remmers, protease inhibitoren en monoclonale antilichamen.

DANKWOORD

Een proefschrift schrijft men niet alleen, dat is het werk van velen. Mijn dank gaat dan ook uit naar allen die direct of indirect hebben meegewerkt aan het tot stand komen van deze thesis.

Met name wil ik bedanken:
Gerrit Stoter, promotor, bij problemen altijd aanspreekbaar en blijvend stimulerend tot het voltooien van het proefschrift.
Jan Klijn, voor de begeleiding bij het onderzoek wat leidde tot deze thesis en voor het leren verwoorden van de resultaten.
Anieta Sieuwerts, lieve vriendin, jouw hulp en steun was op elke terrein van onschatbare waarde voor mij, het boek is dan ook mede aan jou opgedragen.
John Foekens, voor de hulp bij het verrichten van het laboratorium onderzoek, het interpreteren en verwoorden van de resultaten.
Florence Smits, voor het typen der manuscripten en editen van het proefschrift.
Willem Siliakus, voor de bijstand in moeilijke tijden en het zorgen voor ontspanning na inspanning.
Mijn collega-internisten (aanwezig in "de Daniel" of uitgevlogen naar andere oorden) voor de samenwerking als team en het steeds naar elkaar willen luisteren.
Alle verpleegkundigen van de afdelingen, het behandelcentrum en de doktersassistenten van de poliklinieken betrokken bij de behandeling van de patiënten, bedankt voor jullie bestede zorg.
Tevens wil ik de dames van de medische bibliotheek bedanken voor het voortdurend opzoeken van alle benodigde literatuur en de medewerkers van de audiovisuele dienst voor het maken van figuren en foto's.

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