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TAMOXIFEN – 5-FLUOROURACIL SYNERGY IN HUMAN BREAST

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(Date)

TAMOXIFEN - 5-FLUOROURACIL SYNERGY IN HUMAN BREAST CANCER CELL LINES: CORRELATING IN VITRO SYNERGY WITH THE CURRENT ESTROGEN RECEPTOR MODEL

A Thesis submitted to the Yale University School of Medicine in partial fulfillment of the requirements for the Degree of Doctor of Medicine

BY

BRENT RODERICK WILFRED MOELLEKEN

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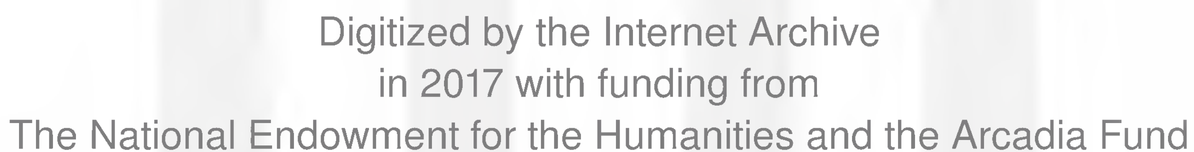
PREFACE

This thesis is dedicated to Dr. Chris Benz, who challenged and guided me with singular wisdom and patience, and to Dr. Ed Cadman, from whose enthusiastic support arose the means to conduct this research.

I thank both of these men deeply for the opportunity to represent our laboratory in Vienna, Austria at the Chemotherapy Congress, and attribute my desire to pursue a career in academics largely to the example they have set.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ABSTRACT

TAMOXIFEN - 5-FLUOROURACIL SYNERGY IN HUMAN BREAST CANCER CELL LINES: CORRELATING IN VITRO SYNERGY WITH THE CURRENT MODEL ESTROGEN RECEPTOR

Brent Roderick Wilfred Moelleken

1985

The potential mechanisms of in vitro synergy between the antiestrogen tamoxifen (TAM) and the pyrimidine analog 5-fluorouracil (5FU) in cultured breast cancer cells (MCF-7, T47-D) are explored from three aspects: the influence of TAM on 5-FU metabolite incorporation into RNA, the influence of 5-FU on the binding of (3H) estradiol to the estrogen receptor (ER), and the effects of 5-FU on ER binding to DNA-cellulose, an in vitro correlate of nuclear chromatin. While overall incorporation of 5-FU into RNA was decreased, TAM increased incorporation of metabolites of 5-FU into the 32-45S species RNA as determined by sucrose gradient centrifugation. At low, minimally toxic doses of 5-FU, (3H) estradiol binding to ER is increased. Since higher, more

cytotoxic doses of 5-FU actually decrease (3H)E2 binding to ER, this is an unlikely primary mechanism of synergy between TAM and 5-FU, in these cells. In other preliminary studies it appears that toxic doses of 5-FU increase binding of (3H) estradiol-ER to DNA-cellulose. From these results a mechanism can be postulated: 5-FU incorporation into RNA impairs processing of ribosomal and messenger RNA, inhibiting a negative feedback loop that specific low molecular weight RNA sequences exert on estrogen receptors. This, in turn, potentiates tamoxifen's growth-inhibiting effects on nuclear chromatin.

Alternate theories of TAM-5-FU synergy are also considered. The literature is reviewed with respect to applicable general aspects of breast cancer, MCF-7 cell culture and in vitro techniques for measuring ER activity and function, Scatchard analysis, the role of steroid receptors in human malignancy, known mechanisms of action of TAM and 5-FU, and lastly, the relationship of this information to the current model of estrogen receptor function is discussed.

CHAPTER I

BREAST CANCER IN GENERAL

Breast cancer continues to be a serious and widespread health problem for women in the United States. Death rates for breast cancer are tied at 18% with lung cancer for the highest percentage of deaths in women caused by cancer, followed by cancer of the colon and rectum at 15%.

Still, breast cancer has the highest cancer incidence in women, comprising 26% of all newly diagnosed cases of cancer. It is estimated that there were 115,000 newly diagnosed cases of invasive breast cancer in women in 1984 (144).

In spite of the many advances in chemo- and endocrine therapy, early detection and surgery, examination of the age-adjusted cancer death rates for females from 1930-1979 shows an unchanging rate for deaths from breast cancer at 27/100,000 females. Fortunately this does not mean that modern therapy has had no effect on the rate, which has risen slowly from a 5-year survival rate in 1960-63 of 63% for white and 46% for black women; in 1970-73, 68% for white women and 51% for black women; and in 1973-80, the most recent figures available, to 74% and 62% for white and black women, respectively. Two trends are noticeable -- increased survival at 5 years and decreased disparity between survival of white and black women with breast cancer.

On the surgical front, there is a recent trend away from radical operations when the primary tumors are small. A clinical trial of 701 women with breast cancers measuring

less than 2cm without palpable axillary nodes, in which women were randomized to one of two therapies - either radical mastectomy or a combination of quadrantectomy, axillary dissection and radiotherapy. Both groups received chemotherapy with the CMF regimen (cyclophosphamide, methotrexate, and fluorouracil). There were fewer recurrences in the quadrantectomy group; statistically, however, there was no difference, indicating that in women with tumors of less than 2cm, without palpable axillary nodes, mastectomy appeared to involve unnecessary mutilation (153).

Still, only about one half of all breast cancers are definitively cured; most of the curable patients have tumors confined to the breast and do not have extension of their disease to the axillary nodes.

From this information, the concept of breast cancer as a systemic disease has emerged; if the axillary nodes are positive, this can be operationally seen as a manifestation of a systemic disease warranting systemic treatment -- only in this fashion can microscopic metastases, not yet clinically evident, be cured.

If the present incidence rates at each age remain constant throughout the lifetimes of women now alive -- a reasonable expectation -- breast cancer may affect one of every eleven women in the United States (140). It is quite possible that the most decisive advances may be made against this disease on the cellular and ultra-structural levels.

This thesis will attempt to shed light upon one aspect of breast cancer therapy that shows considerable promise -- the combination of endocrine therapy with chemotherapy, to produce synergistic cytotoxicity against breast cancer cells, while sparing normal host tissues from added toxicity. This therapeutic innovation is being tested both in tissue culture and in preliminary clinical trials.

More specifically, in this thesis the mechanisms underlying TAM-5-FU synergy are explored. In spite of its laboratory and clinical promise, hormonal and cytotoxic therapy in general remains empirical and its mechanisms inadequately understood. To appreciate the therapeutic potential of chemo-endocrine therapy, a solid foundation in biochemical and receptor phenomena is necessary, as well as an exact knowledge of the independent mechanisms of action of the drugs under consideration. In addition, the literature is reviewed with the intention of providing the most up-to-date view of relevant background topics. In the near future it is quite conceivable that one will be able to design rationally formulated regimens of chemo- and endocrine therapy for each patient, tailored to the individual receptor, hormonal and tissue type, as well as to the patient's expectations of treatment.

EPIDEMIOLOGIC CONSIDERATIONS

The epidemiology of breast cancer holds considerable

information about the pathogenesis of breast cancer.

It is known that the frequency of breast cancer rises with increasing age in American women. Curiously, the reverse is true in Japanese women, but not in women of Japanese ancestry who have lived in the United States for two generations (143). The high dietary content of saturated fats in the American diet is sometimes implicated (supported by experimental evidence) (143); obesity has also been suggested (143). Interestingly, certain estrogens have been implicated which derive in large parts from peripheral conversion in adipose tissue -- as well as from the female sex organs.

Also, it is known that women who have menstrual patterns leading to lengthy, unopposed stimulation by estrogens (i.e. nulliparity, late age of first pregnancy, late menopause) have an increased risk. Other risk factors less readily subsumed under this theory are hypothyroidism and irregular menstrual cycles in many patients.

Certainly, genetic factors play a very important role in breast cancer: having a sister or mother with breast cancer -- particularly if it occurred premenopausally -- greatly increase a woman's chance of contracting it herself. In the absence of atypia on histologic examination, though, family history is not an important predictor (52).

The higher incidence of breast cancer in women with fibrocystic disease or previous breast cancer themselves is a well-described, but perhaps outdated concept (143), since

fibrocystic disease of the breast lacks specificity in predicting the development of breast cancer.

CHAPTER II

BREAST CANCER CELLS IN CULTURE

Most of the original work in this thesis, and a large portion of the background data, was obtained using human breast cancer cell lines, either from the MCF-7 or T-47-D cell lines.

Establishing a cell line is a matter of considerable difficulty: investigators tried for over 20 years to establish a human breast cancer cell line until they finally succeeded in 1958 by producing the BT-20 human breast cancer cell line (97).

The viability of malignant cells provided from freshly resected tumor samples or metastases is usually low. If the sample is obtained from a solid specimen, it may contain more supporting cells (especially fibroblasts) which tend to overgrow tumor cells in culture . Customarily, a lengthy "lag period" is noted after cells from a solid tumor have been implanted before a homogeneous culture grows. This lag period suggests that competition is occurring between the cells, and that the population arising ultimately is that most suited for the competetive environment of the culture flask. It is by no means necessarily the same cell type which predominated in the original tumor type; in fact, it may not even be representative of the original cells contained in the tumor. This seems to be a serious potential limitation of tissue culture experimentation (136). Once established, a cell line must be maintained on the proper culture medium and be demonstrated to be free of

bacterial and mycoplasma contamination. Several successful human breast cancer cell lines, among them the MCF-7 and T-47-D lines, are from pleural effusions; there is little contamination of malignant effusions by fibroblasts (57). From early studies, the concept of breast cancer as a population of cells with variable sensitivities to hormones emerges. Nenci's group in Italy demonstrated that many tumors contain a subset of cells that do not translocate bound hormone into the nucleus, although the hormones were taken up normally into the cytoplasm (119). This is a phenomenon which has important implications in assessing receptor status. Of 150 breast tumors examined, fewer than 10% of them were uniformly either receptor positive or receptor negative. This realization has direct clinical relevance and may explain the quick emergence of drug or endocrine resistant cell strains. It may be that the heterogeneity present in breast cancer specimens in vivo is not present in vitro.

Once a cell line has been established, it is necessary to document that this cell line indeed consists of breast cancer cells, which are epithelial in origin. In a recent comprehensive analysis of 47 reported human breast cancer cell lines at the NCI, only 22 were human, non He-La cells (a frequent contaminant) with epithelial morphology, i.e. possessed three structural markers under the electron microscope: desmosomes, tonofibrils and intracytoplasmic ductlike vacuoles. Among those 22 were the MCF-7 and T-47-D

cell lines, confirming their reputation as well-characterized cell lines (57). One important criterion of these cells is the presence of sex steroid hormone receptors, as well as its concomitant, hormone responsiveness. In the case of MCF-7 cells, this would mean that the cells possess estrogen receptors and be responsive to hormonal manipulation, i.e., cell growth rates should respond to estrogens and antiestrogens. McGuire's group has undertaken a steroid receptor analysis of several human breast cancer cell lines, and found that the estrogen receptors in the cell lines tested were predominantly within the nucleus; in solid tumors, receptors are characteristically located in the cytoplasm (110). By contrast, only 1 of 9 breast cancer cell lines contained cytoplasmic estrogen receptor. Moreover, when nuclear estrogen receptor was assayed, it was found that 5 of 9 cell lines had significant unfilled nuclear estrogen receptor. They postulated that this apparent translocation of the estrogen receptor into the nucleus might be attributable to "tissue culture conditions." It is important to note that the two MCF-7 strains tested, each obtained from different laboratories, were significantly different, in that one was cytoplasmic ER-postitive, the other cytoplasmic ER-negative and both were nuclear ER-positive.

This discrepancy may reflect an important limitation in receptor research using human breast cancer cell lines: although the two MCF-7 strains were presumably from an

identical source they subsequently developed different characteristics accounting for inter-laboratory variation. It is necessary to examine carefully what is meant by "estrogen receptor positive," since the methods used for isolating cytoplasmic and nuclear estrogen receptor differ.

Briefly, McGuire's group utilized a sucrose density gradient technique (See "Methods Section"). First, 100-fold excess unlabeled diethylstilbestrol (DES) was added to the sample and control tubes, then (3H) estradiol added to the sample, followed by a centrifugation step with dextran-coated charcoal to absorb free steroid. A sucrose density gradient centrifugation step further purified the protein, which was measured by the Lowry method (74). Nuclear receptors, on the other hand, were obtained by a protamine sulfate precipitation, a competetive binding assay similar to the one used for cytoplasmic receptors using a phosphate buffer extraction. It is notable that when nuclear estrogen receptor and not just cytoplasmic receptor was considered, the percentage of tissue culture cells that were ER-positive approximated those of solid tumors (5/9 vs. 50-60%, respectively). Moreover, all cell lines which had measurable nuclear estrogen receptor also had cytoplasmic estrogen receptor, while the converse did not always apply. It would seem, therefore, that the assay for nuclear estrogen receptor more accurately reflects the estrogen receptor status of the whole cell when human breast cancer cell lines are assayed. For solid tumors, this is not

necessarily the case.

Another disconcerting aspect about cell culture is that if the growth medium is not selected to mimic in vivo conditions, it is conceivable that certain populations of cells might be selected which will thrive in the new in vitro enviroment. This might explain why the androgen-sensitive breast cancer cell line in Yates' laboratory lost its sensitivity to androgens when cultured in a testosterone free medium (159). This possibility has been minimized in the MCF-7 and T-47-D breast cancer lines, which are routinely grown in an estradiol- containing medium (see "Methods Section").

A few words about basic cell kinetics are relevant when one is searching for synergy between drugs. The continuous cell cycle can be conveniently divided into four stages (Fig. 4): G1, S, G2 and M. During G1, or the "first gap phase," cells which have just divided and begin to grow synthesize RNA and protein, but not DNA. In the S phase they begin to produce both DNA and histones for chromatin, while continuing to grow. During S phase, the synthesis of protein and RNA continues until G2, the second growth phase, where DNA and histone synthesis stops, and baseline protein RNA synthesis continues. The cell continues to grow until M, or mitotic phase, when cell growth and all production of protein and RNA cease.

In culture, cells do not grow synchronously -- there are an unpredictable number of cells in any given phase,

though at any given time far fewer in the M Phase, since temporally this is the shortest phase. It is an interesting, and apparently a clinically very significant phenomenon, that cells in culture can be transiently synchronized to a particular growth phase by the use of cycle-specific agents, thereby maximizing the effects of drugs which act only during particular segments of the growth cycle. Necessarily, cytotoxic agents cannot be applied through the complete cycle of all cells at maximal doses, since associated toxicity to normal, non-target cells would be prohibitively high.

Another important aspect of cell growth kinetics is the growth pattern of cells in culture. Measured simply as number of cells/ml, there are three phases of growth: the latent phase, after cells have been seeded into a flask and before they grow in large numbers; the logarithmic phase, where numbers of cells increase exponentially until either limited space, low pH or inadequate nutrient media limit growth; and the final plateau phase (see Figure 5).

Although cells are not growth cycle synchronized, there are several patterns of metabolism which have relevance to the effectiveness of chemotherapeutic agents. Also, biochemical measurements which do not take these kinetics into account may be spurious. To demonstrate this Benz and Cadman measured several biochemical parameters in L-1210 cells, a murine leukemic cell line. They found that during the midportion of logarithmic growth in this cell line,

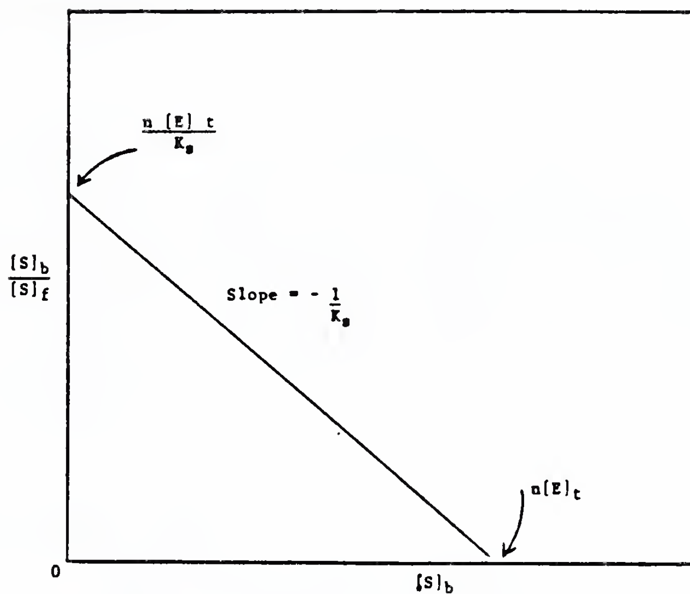
incorporation of glycine into purine bases, intracellular phosphoribosyl pyrophosphate (PRPP) pools and deoxyribonucleotides, the building blocks for nucleotide synthesis (Fig. 1), began to decrease significantly, as did cell volume. The amount of DNA per cell, however, did not change appreciably. As one might expect, the products of a rapidly growing cell, namely RNA, protein content and ribonucleotides all increased during mid-log phase; however they fell off sharply during late logarithmic-early plateau phase (18). The relevance of these phenomena to metabolism of various chemotherapeutic drugs is exemplified below. Benz et al demonstrated with the human colon carcinoma cell line, HCT-8, that cells synchronized with deoxythymidine (which causes an arrest at the G1-S phase boundary), were resistant to the 5-FU during the following G2/M phase (20).

Lastly, it is noteworthy that a cell type may also metabolize an active compound into several other species, which in turn have variable biological effects. The MCF-7 line, for example, produces both estrogens and androgens, such as dehydroepiandosterone and testosterone (128).

CHAPTER III

SCATCHARD ANALYSIS

Scatchard Plot of Equilibrium Substrate Binding Data Applied to Estrogen Receptor Analysis



[S]b= concentration of bound ligand (i.e. concentration of estrogen-receptor complexes)

[S]f= concentration of free ligand

[E]t= total concentration of enzyme (i.e. concentration of estradiol)

n= number of identical and independent ligand binding sites per molecule of enzyme

Therefore:

n[E]t= total concentration of ligand binding sites (i.e. total estrogen receptors)

Ks= ligand concentration at which the reaction rate is at half its maximal value (i.e. concentration of estradiol at which half the estrogen receptors are saturated; the lower the value, the greater the affinity of the receptor for its hormone)

Derived from the equation:

$$\frac{[S]b}{[S]f} = \frac{-1}{Ks}[S]b + \frac{n[E]t}{Ks}$$

Using this method of calculating affinity of substrate for receptor, as well as total number of binding sites, leads Benz (114) and Yang (158) to conclude that 5-FU treated MCF-7 cells have less estrogen receptor with identical affinity for estradiol when compared with control MCF-7 cells (Discussion Section).

CHAPTER IV

HORMONAL CONTROL OF CANCER

The treatment of breast cancer by hormonal manipulations is not new; as early as the end of the last century, advanced carcinoma of the breast was palliated by ovariectomy. Surgery, and later radiation were used to ablate these estrogen-producing structures, yet the rationale for this procedure was simply that the incidence of breast carcinoma was much higher in females than males (62).

Experiments in 1936 on a strain of mice with a propensity to develop breast carcinoma showed a much higher female incidence. Yet when male rats were administered estrogen, they demonstrated pre-cancerous cell types, and eventually metastatic breast adenocarcinoma. Similarly, in a strain of mice with a low natural incidence of breast carcinoma, administration of high doses of estrogen yielded tumors in nearly all the mice tested (95).

In prostatic cancer, hormonal therapy, particularly with DES, provides excellent palliation; estrogen therapy also provides an alternative for men not permitting orchiectomy. Therapy with the anti-androgen cyproterone acetate (Androcur) has been disappointing, despite its theoretical promise. In fact, hormonal therapy is the main treatment modality for advanced prostatic cancer. At least 80% of the cases will respond for varying periods of time to hormonal therapy (55). Gustafsson and his group in Sweden correlated clinical response rate in prostatic cancer patients with tumor androgen receptor status (68). Of 16

patients whose prostatic tumors were assayed for estrogen receptor, 9/11 who had measureable receptor levels responded well (82%), while those without measureable receptor levels responded poorly (1/4 = 25%). Notably, the two receptor-positive individuals who did not respond to hormonal therapy had the lowest detectable levels of steroid receptor. Gustafsson's group is quick to point out, however, that although the correlation between estrogen receptor positivity and clinical response was excellent, the results were not performed in an isolated system; it is therefore possible that the estrogen therapy administered acted via a negative feedback loop on the luteinizing hormone secretion of the pituitary gland resulting in decreased testicular secretion of testosterone, and therefore less androgenic stimulation to the prostate.

Endometrial cancer responds well to progestins in a third of all cases. This well-documented phenomenon is most closely correlated, as one might imagine, with the presence of progesterone, as well as estrogen receptor. Estrogen receptors may be necessary for synthesis of progesterone receptors.

Renal cancer also shows promise of responding to androgens or progestins in small clinical trials (148). Concolino's group demonstrated that 61% of human renal cell tumors tested had estrogen receptor, and 61% had progesterone receptor. After nephrectomy, 18 patients received progestational therapy; in 14 of them, objective

benefit was noted (47). Others have reported success using adrenal cortical hormones, testosterone and progesterone (48), in both laboratory animals and man.

The discovery that some tumors traditionally thought not to be endocrine responsive may actually contain hormone receptors and respond to endocrine therapy raises many new therapeutic possibilities. Specifically regarding pancreatic cancer, Greenway's group has found that pancreatic cancer cells contain high affinity binding sites for estrogen, presumably estrogen receptors. Moreover, they found similar receptors in fetal but not in normal pancreas, suggesting derepression of fetal genes might be occurring with pancreatic cancer(66). Benz et al have reported on the in vitro sensitivity of pancreatic carcinoma cell lines to TAM, suggesting the presence of estrogen receptor. More exciting is the possibility of combining hormonal with endocrine therapy to treat pancreatic carcinoma (26). (Figure 13). For example, the pancreatic cancer cell line, COLO-357, is sensitive to estradiol, tamoxifen and progesterone in vitro (21) (Figure 13). This cell line contains estrogen receptor; however, by Scatchard analysis its Kd is greater (hence its estrogen receptor has a lower affinity for estrogen) than the Kd for ER measured in the MCF-7 human breast cancer cell line (Figure 14).

Estrogen receptors have been identified in numerous other tissues, such as malignant melanoma, colon carcinoma, gallbladder carcinoma, liver carcinoma, squamous cell

carcinoma and even some sarcomas (147). Clinical studies have not yet been performed to evaluate the possibility of incorporating endrocrine therapy into the treatment programs involving those tumor types, although the possibilities are vast.

Because of their ability to reduce mitosis in lymphocytes and destroy lymph tissue, adrenal steroids have been, and currently are being used to treat malignant lymphoma and leukemia. Numerous studies have shown a correlation between glucocorticoid receptors and clinical response to glucocorticoid therapy in non-Hodgkins malignant lymphoma (27), acute lymphoblastic leukemia (ALL) and thymoma (78), and lysis of both normal and malignant lymphoid cells (73). Using cytoplasmic extracts from lymphoblasts, Lippman and his group used a competetive binding assay similar to the one described in this thesis, ("Methods Section") with [3H] dexamethasone, to determine by Scatchard analysis the number of specific glucocorticoid binding sites and a Kd of 2-8nM (104).

While the effects of estrogen upon breast cancer cell lines (stimulatory) and glucocorticoid upon lymphoblast cell lines (inhibitory) are opposite, they seem to exhibit a similar cause-and-effect relationship between hormone action upon a specific receptor, and the corresponding metabolic consequence (160). Although the tumor growth patterns are not as clearly delineated as in breast carcinoma, there seem to be parallels both in the remissions and patterns of

resistance (160).

Similarly, when corticosteroid therapy is employed concomitantly with an antimetabolite such as vincristine, more satisfactory clinical remissions are evident; in breast cancer, combined chemo- and endocrine therapy shows great promise in both in vitro and clinical trials (115, 3).

CHAPTER V

HORMONES IN BREAST CANCER

Hormone withdrawal therapy has found a secure place in the treatment of breast cancer. Ovariectomy, preferably surgical because of its faster induction of remission, had been advocated as the primary treatment for premenopausal women with inoperable advanced breast cancer. The clinical response rate varies between 10% and 35%, and lasts 10-25 months on the average (148).

Regarding the commonly employed androgens used to treat disseminated breast cancer, regimens frequently include pharmacologic doses (much larger than needed for physiological function) of fluoxymesterone, calusterone and droomostanolone proprionate (62, 148). Testosterone propionate and testosterone enanthate, though clinically as effective as the other androgens, have unacceptably virilizing effects. Progestins, androgens, medical or surgical hypophysectomy or adrenalectomy (eg. aminoglutethamide) are endocrine agents of last resort. Response rates vary greatly, from 20-25% for androgens and progestational agents, to 30% with adrenalectomy.

Paradoxically, estrogens themselves have found a place in treating breast cancer. The therapeutic regimen most frequently DES and ethinyl estradiol have been employed successfully. Traditionally, endocrine therapy is continued for 8 to 12 weeks, at which time progress is evaluated. If the tumor appears to have responded, therapy is continued until a recrudescence is evident. Interestingly, after this has occurred and endocrine therapy is then stopped, another

remission frequently ensues, suggesting that a population of tumor cells dependent upon the pharmalogical doses of estrogen has developed.

The response rate to DES therapy approaches 30-40% of all postmenopausal patients treated, with the length of remissions lasting from 6 months to 1 year.

Recently, tamoxifen has replaced DES as the initial treatment for patients who are more than 5 years past the menopause (148). The dosage generally employed is around 20-40 mg daily. A regression in tumor size of >50% has been noted in 30%-40% of patients treated for 12-24 months (148).

It is now clinically well-established that patients with breast tumors containing measureable levels of ER respond better, and with greater frequency, than patients without detectable estrogen receptor levels (81). The response rate for ER-positive tumors to endocrine therapy is 60% compared to 10% for ER-negative tumors. Roughly half of tumors assayed are found to be ER-positive (130).

There are very few studies which dispute the importance of ER status. Leake's group in Scotland reported finding estrogen receptors in only 1 of 72 tumors sampled. Their finding that none of the 25 patients with advanced breast cancer who were treated with tamoxifen responded clinically, suggested to them that breast cancer did not contain ER (98). In this particular study, no effort was made to quantitate ER by Scatchard analysis. Instead, Leake used a one-spot competetive binding assay introducing a possible

source of error which he acknowledged.

Although, patients with breast cancer generally respond best to endocrine therapies if their tumor specimen contains ER, this correlation is not perfect. A better correlation is obtained by assaying both estrogen and progesterone receptor (PGR). McGuire's group noted that response rates for patients with ER levels of <3, 3-100 and 101-1000 fmol/mg cytosol protein was 6%, 46% and 80%, respectively (111). This represents a better correlation than the often quoted figures of 10% and 60% response rates for ER-negative and ER-positive tumors. When ER status is noted as either positive or negative, and PGR status is also considered, 7 series of patients are compared, with the following results (111), indicating partial responses.

ER-negative PGR-negative	9/63 = 14%	ER-positive PGR-negative	20/71 = 28%
ER-negative PGR-positive	(3/6 = 50%)	ER-positive PGR-positive	67/91 = 74%

Ratios shown indicate the number of patients in category (numerator) over the total number of patients tested (denominator). Patients in the ER-negative, PGR-positive group are in parentheses to indicate the small sample size. It is noted that some ER-negative, PGR-negative samples may spuriously be PGR-negative; it is known

that progesterone receptor synthesis is estrogen dependent (75), and that in ER-negative tumors, the PGR may be unexpressed. Thus, it might be considered feasible to give women a small dose of estrogens prior to the assay for PGR (75).

Israel and Saez have noted another limitation: they have found that in women who had plasma progesterone levels higher than 100 ng/100ml, the determination for PGR was invariably negative; possibly, progesterone has inhibited the production of its own receptor in a negative feedback loop (79). Estrogen receptor status alone remains a more clinically important prognosticator than age, menopausal status or nodal status alone in women with operable breast cancer (90). McGuire found a correlation between menopausal status, ER status, and clinical response to chemotherapy: premenomenopausal women seem to develop more aggressive, ER-negative tumors which respond poorly to endocrine therapy (this would be predicted by the ER-negative status), and yet retain their responsiveness to chemotherapy. This can be explained because such tumors grow more quickly, have a higher growth fraction (112), and, accordingly, are more susceptible to the antimetabolites active against rapidly dividing cells).

Allegra and Lippman's group have made another observation -- that the presence of estrogen receptor correlates with a <u>poor</u> response to chemotherapy. In a retrospective study, 34/45 (76%) of women with ER-negative

adriamycin alone or in combination with other agents (2). Most regimens contained 5-fluorouracil. This observation is intuitively surprising, but is in good accord with McGuire's previous observation that tumors with estrogen receptors are slower growing than their ER-negative counterparts. It seems logical that a tumor without detectable estrogen receptor is phenotypically more divergent from the differentiated cell type that makes up normal breast epithelial tissue; these anaplastic tumor cells might therefore be more rapidly growing. The opinion that ER-positive tumors respond worse to chemotherapy than ER-negative tumors is by no means uniform; there are also several studies suggesting that ER-positive tumors respond better, (28, 89) or at least the same (28) as ER-negative tumors.

In a recent review of the literature, almost every series has shown that women with ER-positive tumors have better survival and 3-year relapse-free survival rates than ER-negative patients (28). Conversely, postmenopausal women have more ER-positive tumors, which respond better to hormonal therapy and worse to chemotherapy.

These observations are so well substantiated that many investigators have recommended categorically that every woman with breast cancer should have estrogen receptor assays performed (111). The data, in total, suggest that most premenopausal women with breast cancer have less differentiated, more anaplastic, ER-negative tumors, which

most premenopausal women with breast cancer have less differentiated, more anaplastic, ER-negative tumors, which are probably best treated with chemotherapeutic regimen. Postmenopausal women, on the other hand, tend to have less undifferentiated, slower growing tumors which respond best to hormonal therapy.

Correlating receptor status with tumor cytokinetics, Allegra and others have suggested it is theoretically possible to arrest cells in one uniform stage of the cell cycle by means of antiestrogens. It would then be possible to advance cells synchronously by means of estrogens, and use of an S-phase specific chemotherapeutic agent when the cells enter S-phase, resulting in maximal tumor cell cytotoxicity with the lowest possible dose of chemotherapeutic agent (2).

Kute and Wittliff have shown that there exists a correlation between clinical response and a certain molecular form of estrogen receptor. Estrogen receptor separates into 4S and 8S components when sedimented on a linear sucrose gradient. Women with tumors containing the 8S form were more likely to respond to hormonal therapy. Each separate form (i.e. 8S, 4S) has several specific estrogen binding components. Those components elute out at different ionic strengths on diethylaminoethyl (DEAE) cellulose columns. Wittliff's group has postulated that the 8S aggregate is necessary for normal activation of the estrogen receptor, and that its apparent components,

would predictably not be responsive to hormonal therapy (94).

Accordingly, when patients who were ER-positive but whose receptor did not demonstrate the 8S species were eliminated from consideration, the response rate to hormonal therapy rose to 75% (as compared to the frequently cited figure of 55-60%) (94).

It is conceivable that amidst all tumors there is a heterogeneous population of cells, some of which contain estrogen receptor and will respond to hormonal therapy, while still other cells will remain resistant to hormonal therapy because of deficient or absent estrogen receptor. Such tumor heterogeneity may await the selective forces that will favor the eventual preponderance of a resistant cell type.

The interrelations between the estrogen receptor and other hormones are complex and poorly understood. It is known that tumors containing positive ER and PGR respond to hormonal therapy, and, predictably, are stimulated by estradiol. These tumors are also stimulated by insulin. On the other hand, tumors containing only ER, or neither ER nor PGR, do not respond to estradiol plus insulin(79). PGR, then, seems to signify the presence of a properly functioning ER.

In certain breast cancer cell lines, prolactin either stimulates or maintains ER. Estrogens, on the other hand, interfere with prolactin binding (71). Insulin seems to

regulate ER (71). Indeed, insulin has been implicated in mammary tumorigenesis (70). Estrogens, on the other hand, seem to decrease binding of insulin (71, 141), probably by reducing the number of insulin receptors. An interesting correlate can possibly be seen in women taking estrogen-containing contraceptives as during early pregnancy, in whom glucose tolerance abnormalities have been noted. Is this a manifestation of downregulation of insulin receptors?

Other studies have demonstrated an interaction between cyclic adenosine 3':5' monophosphate (cAMP) and estrogen. Rat mammary carcinomas show increased cAMP binding and decreased estradiol binding if the hosts are treated either with DBcAMP (N6,02-dibutyryl cyclic adenosine 3' : 5'-monophosphate) or with ovariectomy. Furthermore, these changes were reversible either with exogenously administered estradiol or cessation of DBcAMP treatment (38). This study suggests that an antagonistic action between estradiol and cAMP exists in rat mammary carcinomas.

Recently, some exciting studies have come out demonstrating the presence of receptor proteins for cAMP. It seems that there exist at least three subtypes, with molecular weights of 39,000, 48,000 and 56,000 daltons. The 39,000 dalton segment is most probably a fragment of one of the larger subunits, each of which is probably a protein kinase (cAMP-dependent protein kinase I and II, respectively). The association of charge alteration (as

determined by 2-d gel electrophoresis) with hormone independency has been noted in mammary tumors in rats (39), and the question of an association between cAMP receptors and steroid dependency of human has been tumors raised.

CHAPTER VI

TAMOXIFEN (TAM)

(Figures 3, 6, 10)

The use of antiestrogens to treat hormone-dependent malignancies is intuitively appealing. Its actions may be specific for ER (131), the site of the action of estrogens, which have been shown to promote the growth of mammary carcinomas and breast cancer cell lines, especially in those with high levels of estrogen receptor. After oral administration, peak concentrations of tamoxifen occur in 4-7 hours. It undergoes extensive metabolism, in particular to a monohydroxylated (OH-TAM) derivative that has even more antiestrogenic properties than tamoxifen itself (this property is experimentally important; discussed later). Its metabolism is enterohepatic, and its metabolites are excreted in stool. Its final T1/2 is at least 7 days (62). Tamoxifen is a relatively poor binder to estrogen receptor; its Kd is from 30 to 300 times as high as the Kd for estradiol binding. However, OH-TAM (as assessed by its radioactive form (3H) OH-TAM), binds as well to estrogen receptor as estradiol (46). TAM provides an attractive alternative to surgical ovariectomy, which is invasive and does not completely eliminate the available source of estrogens. Antiestrogens, on the other hand, attack the effector site. Thus, it may be irrelevant that peripheral estrogens continue to be produced by adipose tissue and the adrenal medulla.

The systemic toxicity of antiestrogens in general, and tamoxifen in particular, is low (100). Serious toxicity is usually restricted to hot flashes, nausea, vomiting, and

various other minor side effects, all of which seldom necessitate stopping therapy: only 25% of patients treated have these adverse effects. Less frequent side effects include menstrual irregularities, vaginal bleeding and discharge, pruritis vulvae and dermatitis (62), probably representing withdrawal effects of estrogen from these tissues. The usual dosage of TAM is 20 to 40 mg daily. Responses may be paradoxically associated with inflammation and increase in the size of lesions, as well as bone pain at the site of metastases, and usually occur in 4-10 weeks (62).

Antiestrogens may also effect a reduction in binding of prolactin to mammary tumors, reducing the effects of another factor which may enhance tumor growth (84).

It was once believed that antiestrogens acted by binding to the estrogen receptor, effectively rendering it unavailable for estrogen. The full story is a considerably more complicated, however. Indeed, antiestrogens may prevent estrogens from expressing themselves, and most probably, they act through the estrogen receptor (131). Evidence suggests that antiestrogens have several cellular effects. They compete with estrogens for formation of a receptor-steroid complex; they alter binding to the nuclear binding sites; and may even disrupt the regeneration of the cytoplasmic receptor (82). Importantly, they must bind to ER and enter the nucleus in order to bind to chromatin and induce their effects. But the specifics of the process from

this point on are not clear.

Katzenellenbogen has found that the TAM-ER complex is translocated to the nucleus. Usually, this event occurs identically with estrogens and antiestrogens, but with estrogens, the level of cytoplasmic receptor is replenished relatively soon after exposure: in the case with antiestrogens, the antiestrogen-receptor complex is retained for prolonged periods in the nucleus and the level of cytoplasmic receptor is depleted for prolonged periods (41).

Moreover, experiments on estrogen-responsive uterine tissue showed that the uterus was refractory to the effects of estrogens during the period where cytoplasmic receptors were depleted. And estrogen responsiveness returned linearly with the return of cytoplasmic estrogen receptor levels (84). This relationship is dose-dependent, that is, the higher the dosages of antiestrogens the greater the antagonism of the actions of estrogens. It is unknown why an antiestrogen can exert its effects at levels so low that no depletion in cytoplasmic receptor can be detected at all.

By depleting cytosolic ER, an antiestrogen can render an estrogen-dependent tumor cell incapable of growing. Whether this reflects a decrease in the synthesis of estrogen receptor (45) or, more probably, a sequestration in the nucleus is not fully clear (84). There is a small body of evidence suggesting TAM has its own receptor, similar to, but separate from the estrogen receptor (150). Some antiestrogens, most of them investigational agents, have

been found to act through a mechanism different from the estrogen receptor (15).

The effects of antiestrogens on thymidine utilization have also been studied. Estrogen treatment of MCF-7 cells increased total cellular thymidine synthesis two to threefold, but tamoxifen treatment reduced total thymidine synthesis by 15 to 30% below control rates. The rate of extracellular salvage uptake of dThd increased initially, but decreased significantly after 24-48 hours. There is a marked shift in TAM-treated MCF-7 cells toward the salvage pathway, accounting for 60-70% of the total thymidine production (compared to 5% for estrogen-treated MCF-7 cells). It would seem, therefore, that tamoxifen disrupts the de novo pathway preferentially (105).

The effect of TAM treatment is an overall reduction in intracellular dThd pools (105), and a resultant inhibition of DNA synthesis. Overall tamoxifen treatment results in decreased DNA, RNA and protein synthesis, as one would expect (53). TAM has been noted to have an effect on 3Urd incorporation into RNA. Specifically, TAM increases the radioactive nucleotide's incorporation into the <4S segment of RNA (23).

Tamoxifen cytotoxicity has its correlate in the cell cycle. It produces a decrease in S-phase cells and an accumulation of G1 phase cells. When tamoxifen is withdrawn, the cells can be synchronized (19). This will be useful later in showing TAM- 5-FU synergy is not based on

synchronization of cells alone.

Recently the question has arisen whether TAM is also acting through a completely different, calmodulin-related mechanism. An article, which appeared in the 1984 Proc. Natl. Acad. Sci. (113), showed that a phosphorylation reaction of tyrosine residues is necessary for hormone binding to estradiol receptor, a step catalyzed by a protein kinase. Ca++ and calmodulin can stimulate estradiol binding in the presence of this protein kinase. It is also known that TAM may have an inhibitory effect upon calmodulin (96), which may in turn slow the Ca++-augmented protein kinase reaction necessary for receptor activation.

The structure of the antiestrogen-receptor complex is remarkably like the estrogen-receptor complex. On sucrose gradient centrifugation, the complexes are indistinguishable (82). Antiestrogens appear to have a very long in vitro half life -- 18-24 hours, compared with 1/2 hour in the case of estradiol (82).

As is the case with other drug antagonists, antiestrogens are by no means solely antagonistic to estrogens. However, their agonistic properties are, by definition, limited. The long-term retention in the nucleus of estrogen receptor can have corresponding long-term stimulatory effects, especially on uterine tissue (155, 43) (Figure 6). In spite of being bound longer to the nucleus, however, TAM-ER does not induce typical estrogen mediated effects (92).

Thus, it is known that TAM binds to ER, and that the complex is translocated to the nucleus. Benz, Cadman and coworkers have found that the effects of TAM upon the 47-DN human breast cancer cell line are completely reversible in a dose-dependent fashion with 1uM estradiol administration. Its effects become irreversible if estradiol is not administered within 48 hours (19). It is unlikely that TAM's effects are cell-membrane mediated. Benz has found that the human osteogenic sarcoma cell lines MG-63 and G-292, which have no measureable ER, were not inhibited by TAM at doses of less than 10 uM. However, above this concentration, cell growth was inhibited by 20-30%. But this growth inhibition could not be reversed with estradiol administration, nor was it synergistic when 5-FU was administered along with the TAM (13).

These data suggest TAM's effects are not cell membrane mediated at concentrations of less than 10uM, but are at concentrations above this. At least initially, presumably when TAM binds to ER, estradiol is able to reverse the TAM-induced cytotoxicity, but not thereafter.

CHAPTER VII

FLUOROURACIL (5-FU)

(Figures 1, 4, 10)

5-FU has seen wide clinical use. It is of at least palliative benefit with several types of cancer, particularly with neoplasms of the breast and gastrointestinal tract, pancreas, ovaries, cervix, bladder and head and neck, as well as in dermatological preparations.

Unfortunately, clinical toxicity is formidable, and limits the dosage of drug possible. The value of combining it with another drug with less systemic toxicity, particularly if the combination were synergistically toxic against cancer cells, is obvious.

Indeed, 5-FU has numerous clinical toxicities. Anorexia, nausea (78-90%) and vomiting (50-65%) are common, and frequently precede diarrhea (34-85%) and stomatitis (63-75%). When these clinical symptoms supervene, the correct dosage has been attained. It is clear from those symptoms that the primary actions of 5-FU are upon rapidly dividing tissues, especially in the gastrointestinal tract, oral mucosa, but also in the bone marrow. Leukopenia, thrombocytopenia, anemia, loss of hair and atrophy of the skin are also common (62, 32). The typical dosage is 12mg/kg per day for four days, followed by 6mg/kg every other day up to a theoretical maximal daily dose of 800 mg.

Classically, it is thought that 5-FU acts as an inhibitor of the enzyme thymidilate synthetase, the enzyme which converts dUMP to dTMP. Inhibition of DNA synthesis is

mediated primarily by one of 5-FU's metabolites, FdUMP (134).

It now seems that there are many important mechanisms accounting for 5-FU's cytotoxicity. Briefly, its toxicity can be categorized as membrane-directed, DNA-directed, and RNA directed, depending on the actions of its many metabolites (Fig 1).

Its DNA-mediated toxicity is mediated by 5-fluorodeoxyuridylate (FdUMP). Experimentally, the compound florodeoxyuridine (FdUrd, FUDR) is metabolized primarily into FdUMP by a phosphorylation reaction. It is useful experimentally to assess the DNA-directed component (i.e. by inhibiting thymidylate synthetase and subsequent DNA formation). Surprisingly, it was discovered that the main effects of 5-FU are not DNA-mediated but RNA-mediated (25). Cory's group concurred, finding no significant inhibition of thymidylate synthetase (50).

The RNA-mediated effects of 5-FU are primarily mediated through fluorouridine triphosphate (FUTP). The 5-FU-related compound used experimentally to obtain maximum RNA-directed cytotoxicity is 5-fluorouridine (FUrd). It is phosphorylated to FUMP, another metabolite of 5-fluorouracil which is preferentially incorporated into RNA. This is a very important aspect of the cytotoxicity of 5-FU, and will be commented upon later.

Another relatively less important source of cytotoxicity of 5-FU is 5-fluorouridine diphosphate:

glucose, which has its primary effects upon the cell membrane (87).

The cytotoxicity of 5-FU has its correlates in the cell cycle. One might expect its DNA-directed effects (through FdUMP) to show S-phase (i.e. when DNA synthesis takes place) specificity (20). In the HCT-8 colorectal adenocarcinoma cell line synchronized with dThd, the amount of FdUMP generated, 5-FU incorporated into cells, and 5-FU metabolites incorporated into RNA did not demonstrate a cell phase specifically correlating with either G1, S, G2 or M phase. There was, however, slight G2/M resistance.

The RNA-directed effects of 5-FU metabolites warrant particular attention. The evidence that incorporation of 5-FU metabolites into RNA correlate with less of clonogenic survival is overwhelming in the MCF-7 line. Kufe and Major demonstrated that this relationship is highly significant ($p < 0.0001$), dependent upon both time and concentration. The widely accepted mechanism of action previously proposed by Santi and McHenry (134), namely that 5-FU bound irreversibly to thymidylate synthetase, decreasing DNA synthesis, is challenged with the finding that when thymidine is administered to MCF-7 cells in culture and the thymidylate synthetase step in pyrimidine biosynthesis bypassed, cytotoxicity is not reversed. This suggests that the FdUMP-mediated cytotoxicity of 5-FU is not a critical mechanism.

These results were obtained utilizing cesium sulfate

gradients, which permit separation of RNA and DNA into separate bands, allowing one to compare the relative contribution of radiolabeled nucleotide to each fraction.

The effects of 5-fluorouracil upon cellular RNA have been measured. Every species of RNA incorporates 5-FU metabolites to some extent. But it is its incorporation into RNA that precedes abnormal protein synthesis, probably because the secondary structure of RNA is altered and base modification is impaired (96). In particular, uridine derivatives in RNA molecules are reduced, reflecting a significant substitution of uridine by 5-fluorouridine (152). This in turn has a direct effect on the enzyme uracil 5-methyltransterase, an enzyme whose activity is significantly elevated in malignant tumors (146, 150). The incorporation of 5-fluorouracil appears to occur into mRNA. Polyadenylation step of mRNA is impaired, and production of mature mRNA is decreased (64).

Incorporation of 5-FU into polysomal RNA has been measured. The synthesis of poly (A) RNA -- polyadenylic acid containing RNA -- was unaffected by a moderate dose of 5-fluorouracil (35). Data from sucrose gradient analysis and polyacrilamide gel electrophoresis show that the 32S - 45S species of RNA (pre-ribosomal RNA) are reduced (50). Other investigators have shown that the formation of 18S ribosomal RNA is blocked -- albeit by a combination inosine and 5-FU in a cell line otherwise unresponsive to the effects of 5-FU -- resulting in altered maturation of

precursor rRNA (50). Overall, rRNA synthesis has been found to be decreased by about 60% (35) in experiments on liver tissue of rats.

Unlike other chemotherapeutic agents (i.e. azacytidine). 5-fluorouracil is not believed to affect methylation of the 45S pre-rRNA or hnRNA (heterogenous nuclear RNA). Methylation reactions of LnRNA and tRNA were shown to be markedly reduced (154). Nuclear methylation reactions are critically important to cellular function: they regulate the initiation of transcription, allow for ribosomal stability and acylation of amino acids -- by means of the 4-8S RNA structure. The overall effect of the incorporation of 5-fluorouracil into RNA is an accumulation of defective LnRNA, and probably deficient protein synthesis. One species of LnRNA in particular, the 4-8S RNA, has been implicated in tamoxifen-5-fluorouracil synergy.

It will be recalled that antiestrogens, in this case tamoxifen, resulted in reduced DNA, RNA and protein synthesis (53). The implications of this common denominator are especially exciting, and will be discussed along with the synergy of TAM and 5-FU.

CHAPTER VIII

ESTROGEN-RECEPTOR BINDING TO DNA-CELLULOSE

The binding of the estrogen receptor to tritiated estradiol complex (ER- (3H)E2) to the nucleus is affected by numerous factors, most prominent among them are various species of RNA, suggesting an autoregulatory circuit operating at the receptor level. Furthermore, the complex has selectivity in its binding, suggesting an ability of the estrogen receptor complex to recognize specific sites on nuclear template. But even before any binding can occur, the ER-E2 complex must be activated (121).

Several investigators have shown that RNA inhibits estrogen-receptor complex binding to DNA-cellulose. Sato's group demonstrated that dialysis of cytosol containing [3H] E2-ER complexes resulted in a 3 to 6-fold increase in nuclear binding of the complexes to nuclei in their cell-free system obtained from ovariectomized rat uterus. They postulated low molecular weight inhibitors which were normally present in the cytosol inhibited the binding of ER-E2 complex to the nucleus (137). It has been shown in other systems that certain poly- and oligonucleotides promote the release of androgen-receptor and rat uterine estrogen- receptor complexes from DNA-cellulose (101). Poly (U,G) nucleotides were particularly effective. Again, the relationship was shown not to be solely charge-dependent. These data are consistent with an estrogen receptor model having a relatively base specific "autoregulatory" binding site for RNA, perhaps important in establishing

post-transcriptional, negative feedback control loop. An excellent study in a rat mammary tumor model demonstrated that a high molecular weight inhibitor of estrogen-receptor complex binding to DNA-cellulose existed which was destroyed by RNase but not DNase. Moreover, when different polynucleotides were were tested for their inhibitory effect on binding, Feldman et al discovered that poly(G) and poly(U) were by far the most inhibitory species tested (61), in close agreement with Liao's results (101). Moreover, 4S RNA increased the inhibition while rRNA did not (61). More specifically, Poly(U) and Poly(G) were most able to release receptor complexes when the U:G ratio was 1:5. A minimum of 15-20 nucleotides was necessary for activity (101), from which one can infer that sequence specificity exists.

Chong and Lippman performed similar experiments in the MCF-7 cell line and demonstrated that under low-salt (but not under high salt) conditions, RNA was associated with estrogen-receptor complexes. Moreover, purified RNA from MCF-7 cells inhibited estrogen-receptor complex binding to DNA-cellulose (40). Estrogen-receptor has shown a preference for A-T containing regions of double-stranded DNA. Yet the apparent correlation of increased electro-negativity of binding sites with binding does not hold, since tRNA does not compete for nuclear binding sites (88). Another investigator, Dickerman at SUNY at Albany, has proposed a model based largely on these observations, as well as on his finding that DNA-cellulose has a preference

for dG and dT sites, suggesting nuclear template may have specific sites recognized by dG and dT sites on the estrogen-receptor complex, which are possibly quite important in receptor recognition of the appropriate binding sites necessary to initiate transcription. It seems that binding of receptor-steroid complex to DNA is a linear function, nonsaturable because of the great excess of nuclear binding sites. The affinity of the complex for DNA is relatively low (157). Importantly, the greater the binding to DNA, the greater the magnitude of the response (i.e. a "trigger" as "cascade" phenomenon does not apply). It is also quite possible that the effects of steroid-receptor complexes are mediated by just a few high affinity nuclear binding sites, as some recent data suggest (51), and that their presence is masked by the overwhelming preponderance of low-affinity, nonspecific binding (157).

This thesis presents results relating these phenomena to the synergy observed in vitro between 5-FU, which has been shown to inhibit certain aspects of RNA processing, and TAM, which binds to estrogen receptor and has its effects in the nucleus.

CHAPTER IX

ESTROGEN RECEPTOR MODEL

A considerable amount is known about the estrogen receptor. Upon sedimentation on linear sucrose gradients, it can be resolved into 8S and 4S species. It appears that only the 8S species has typical estradiol binding activity, although each species can be broken up by diethylaminoethyl cellulose (DEAE) chromatography into separate, smaller binding components (94).

The estrogen receptor has been recognized as pivotal in stimulation of cellular growth and metabolism. A number of excellent reviews of this topic exist (i.e. 12, 44) The estrogen receptor will be discussed here only as it relates to TAM-5-FU synergy.

In Scatchard analysis of estradiol binding to estrogen receptor, Clark et al found that the classical estrogen receptor has a Kd of 0.8nM for the high-affinity "type I" site, and a lower affinity (Kd ≐ 30nM) "type II" site. There is evidence that the 8S region contains type II, and the 4S region type I binding sites (44). Zava and McGuire found specifically in the MCF-7 line that the cytosolic estrogen receptor (ERc) and the nuclear estrogen receptor (ERn) have high-affinity binding sites with different sedimentation characteristics. For ERc, Kd = 0.8nM, sedimentation coefficient 4S-5S on sucrose gradient analysis (161). Thus, it is the nuclear estrogen receptor that has the characteristics of the "classic" estrogen receptor. The modern view of the different subunits of the estrogen

receptor is that the cytoplasmic estrogen receptor sediments at ~8S on sucrose gredients (or if deaggregated with trypsin, at ~4S and ~3.6S), and has a molecular weight of 70,000 daltons; the nuclear estrogen receptor sediments at ~5S (124, 142). They have equal densities, and roughly equal DNA-binding abilities and molecular weights (6). Unbound receptor of either type is susceptible to photooxidation (59).

Type I binding sites are classically high-affinity, easily saturable binding sites, while type II sites are of lower affinity, but have a higher capacity for estradiol. It is very possible that they represent extracellular binding proteins.

Dickerman's model contained two binding domains: a steroid binding domain with an estradiol binding site; and a polynucleotide binding domain with a deoxyguanylate binding site (dC = dT>dA), and a histone binding site (Fig 8). Dickerman notes that the deoxyguanylate binding site corresponded to the deoxynucleotide with the greatest binding affinity, but that oligo (dT) and oligo (dC) follow oligo (dG) by a close margin in their respective binding affinities (51).

Importantly, his model does not specifically consider the role of RNA discussed earlier, namely one of negative feedback inhibition, a role which has become increasingly probable. Benz et al have proposed such a model (Fig 9), which considers all the data presently available (114). It

provides for estradiol diffusing through the cell membrane, binding to cytosolic ER, and activation of the ER-E2 complex (dependent upon temperature, ionic strength, unknown factors). After diffusing through the nuclear membrane, the activated ER-E2 complex binds to nuclear chromatin, with specific binding sites containing poly (G,T) sites (cf. 51).

RNA transcription can then take place. Products of RNA breakdown have specific sequences with an affinity for poly (U,G) (cf.101), probably based on ionic or electrostatic rather than covalent forces (131, 127).

This model will ultimately prove very useful in incorporating the data presented in this thesis into a unified theory accounting for tamoxifen-fluorouracil synergy.

CHAPTER X

MATERIALS AND METHODS

CELL LINES

The breast cancer cell line MCF-7 is a well-characterized, continuously growing monolayer cell line with a doubling time of 35 hr. It was grown in RPMI 1640 media (obtained from Gibco Labortories, Grand Island Biological Co., N.Y.). Unless otherwise indicated, it was supplemented with insulin (0.2 IU/ml), estradiol (1nM) and fetal calf serum (10%; Gibco). Stock cultures were grown in 75 cm.2 and 150 cm2 sterile plastic culture flasks (Costar Data Packaging, Cambridge, MA) with 25 ml and 50 ml, respectively, of supplemented media. They were incubated in 5% CO2 incubators at 37 degrees C. Single cell suspensions were prepared from cultures using a trypsin (0.05%) - EDTA (0.02%) solution. Cell counts were performed on a model ZBI Coulter Counter (Coulter Electronics, Inc., Highleah, FL). When indicated, cells were grown with a 1:1 mixture of fetal and neonatal calf serum, dialyzed fetal calf serum (Gibco), as media not supplemented with estradiol. The estradiol content of undialyzed commercial serum was measured by radioimmunoassay to be < 100 pM. Therefore, cultures not supplemented with estradiol contain < 1% of the estradiol of stock cultures. Where indicated, cells were grown in serum stripped of endogenous estradiol by dextran coated charcoal adsorption. All drugs were purchased from Sigma (St. Louis, MO) except tamoxifen citrate (Stuart Pharmaceuticals, Wilmington, DE). Flasks were seeded with 4 million cells

48-72 hours before adding drugs in order to obtain cells in logarithmic phase. Drugs were rinsed off the monolayer colonies with phosphate-buffered saline (PBS) and fresh media was added.

RNA FRACTIONATION AND ANALYSIS

Sucrose gradient centrifugation was performed on RNA extracted from whole cells using the procedure of Lizardi (107). This technique is capable of resolving 4S, 8S, 28S and 45S RNA. RNA from labeled cells was layered onto 5-47% linear sucrose gradients and run for 18 hours at 26,000 rpm in an SW27 rotor at 4 degrees. The 0.6 ml fractions were collected and run through a UV monitor directly into scintillation vials, into which 1.4 ml of water and 10 ml of scintillation fluid (Aquasol, New England Nuclear, Boston, Mass.) was placed before counting on a Packard Tricarb scintillation counter.

RECEPTOR BINDING TO DNA-CELLULOSE

Purified estrogen receptor of cells treated with 5-fluorouracil, tamoxifen or control cells, as noted (isolated as described below), was assayed for its effects binding to DNA cellulose by the modifications of the methods of Hollander et al (88, 61) and Liao et al (101). Combined cytosol and nuclear extracted (3H)ER was prepared in connection with receptor studies described below. DNA-cellulose (400 g/ml) was prepared by the drying procedure of Alberts and Herrick (1) using calf thymus DNA (P-L Biochemicals) and Cellex grade cellulose Bio-Rad

Laboratories, Richmond, CA). Cellulose treated without DNA was used as a control.

Where indicated, the Liao DNA cellulose column assay was used (101). DNA-cellulose (0.5 mg), prepared as indicated above, was packed into a glass column (this was 0.5 mg DNA/column) and washed with Medium ET (20 mM Tris-HCl, pH 7.5, 1.5mM EDTA) with 0.1 M KCl was added to the column (this was ~ 10,000 cpm) and incubated at 20 degrees C for 30 minutes. Columns were washed seven times with 0.5 ml Medium ET with 0.1 M KCl in order to remove free steroid or steroid-receptor complex which did not bind to DNA-cellulose. The steroid-receptor complex that remained attached to DNA-cellulose was eluted with 0.5 ml Medium ET with 0.6 M KCl and counted in 2 ml Liquiscint (toluene-based) scintillation cocktail.

Where indicated, the Liao DNA-Cellulose Centrifugation Assay was used (101).

Into large microfuge tubes, 20-100 ul of DNA-cellulose (this makes 20 to 100 mg DNA) and the radioactive complex (usually 2,000-10,000 cpm) along with 0.5 ml Medium ET were placed. Where indicated, a test compound was added to the microfuge tube at this stage. The contents were mixed and incubated at 20 degrees C for 5 minutes, then spun for 5 minutes in a Beckman microfuge. The DNA-cellulose pellet was washed 5 times with 1.0 ml Medium ET with 0.1 M KCl. The entire contents of the microfuge tubes were placed into scintillation vials with 2.0 ml toluene-based scintillation

cocktail. Quench was found to be within 1% in both control and drug-treated groups.

ESTROGEN RECEPTOR ISOLATION AND EXTRACTION

MCF-7 cells were taken from storage at - 70 degrees C after having been harvested as described previously. (61). To the partially thawed pellet of cells was added 1.0-1.5 volumes of Buffer A (10mM Tris, pH 7.4, 1 mM EDTA, 1 mM dithiothreitol, prepared < 4 hours before use) at 0 degrees C. Keeping on an ice bath at 0-4 degrees C, the cell pellet was sonicated at 250 W for two 10-second bursts, the minimum sonication to obtain more than 90% cell lysis (verified with light microscopy using a trypan blue stain). Benz et al have determined that sonication performed in this manner does not have significant effect upon estradiol to receptor (13). The mixture was incubated for 60 minutes at 0-4 degrees C with intermittent (every 15 minutes) vortexing. The mixture was spun at 2800 RPM for 20 min in Beckman Centrifuge. The pellet was discarded and the supernatant was washed twice with 2.0 ml of PBS containing 1% Tween 80; this improved the ratio of specific to nonspecific binding.

The supernatant was incubated at 0-4 degrees C in 10 nM (3H) E2 +/- 100-fold excess DES for 5 hours to determine nonspecific binding. DES is used because, unlike unlabeled estradiol which could theoretically also be used, DES has a very low affinity for alpha-fetoprotein (12), a source of

spurious specific binding. A 42.8% volume of chilled (0-4 degrees C) saturated (NH4)2 SO4 was added and incubated at 0-4 degrees C for 30 minutes with intermittent mixing (each thirty minutes).

Where indicated, a protamine sulfate precipitation was performed after a modification of the method of Horwitz et al (71). The mixture was spun at 2800 RPM for 20 minutes and the supernatant discarded. The pellet was resuspended in 1.0 ml Buffer A + 0.1 M KCl and mixed thoroughly by vortexing, standard Sephadex LH-20 columns were prepared (126). These were used to separate macromolecular bound from free radioactivity. Two 100 ml samples were taken and assayed for protein content by the Bradford protein assay (30) (obtained from Bio-Rad Laboratories). Samples were vortexed and placed in a toluene-based scintillation cocktail for measurement of radioactivity.

CHAPTER XI

RESULTS

DEVELOPMENT OF ASSAY

The methods described in "Materials and Methods" were used to culture MCF-7 human breast cancer cells. It was important to have adequate amounts of estrogen receptor in order to perform very accurate specific binding estrogen receptor assays, and to use the same estrogen receptor for DNA-cellulose binding assays.

Cells were compared with respect to the amount of specific binding (and hence level of estrogen receptor) in control MCF-7 cells, MCF-7 cells raised exclusively in Gelding serum (which contained < 3 fmol/mg protein estrogen receptor -- undetectable levels), and MCF-7 cells raised in dextran coated charcoal-stripped 10% fetal calf serum (see "Materials and Methods").

	Gelding	DCC	Control
Specific E2 binding relative to control	10-19%	97-105%	100%

Two seperate experiments were carried out, with the results shown. Quantitatively, the amount of estrogen receptor was compared in MCF-7 cells raised in dextran coated charcoal-stripped serum with MCF-7 cells raised in gelding serum. In two seperate experiments, Dcc-stripped serum cells had 3.63-7.26 times as much specific binding (mean 5.4, SD 2.56) (expressed as fmol/mg protein bound). This range may be accounted for by the variability in estradiol content of Dcc-stripped serum.

Next, Dcc-stripped serum MCF-7 ER and ER from control MCF-7 cells were compared (isolated as indicated in "Material and Methods"). There was no difference is specific binding of the two groups in two separate trials.

We attempted to determine if estrogen receptor was heat labile. Experiments in control MCF-7 cells were carried out at 0 degrees C and at 17 degrees C. There was no detectable specific binding in cells in which the incubation step was performed at 17 degrees C, while experiments conducted at 0 degrees C showed expected levels of specific binding.

Two different assays for specific binding of (3H) estradiol were compared with respect to their levels of specific binding, the amount of protein (determined by Bradford assay) and the ability of the estrogen receptor to be applied to a DNA-cellulose column (a step necessitating resolubilization of the estrogen receptor fraction). The methods were the protamine sulfate precipitation assay of Chamness et al (14) and the ammonium sulfate precipitation reaction of Feldman et al (56).

Protamine sulfate and ammonium sulfate precipitation experiments produced identical levels of specific binding of (3H)E2 to ER, and similar amounts of protein, making them equally good assays for assessing fmol (3H)E2 bound per mg protein. However, the protamine sulfate precipitate showed poor resolubilization characteristics in heparin, making it difficult to apply samples to the very sensitive Liao DNA-cellulose column. As a result, the 40% ammonium sulfate

precipitation reaction was employed for DNA-cellulose studies.

The levels of specific binding were compared when the precipitation step of the estrogen binding assay was performed before and after the incubation step with (3H)E2 +/- excess DES. The yields were significantly higher when the precipitation step was conducted after the incubation step (data not shown).

The limits of resolution of the ammonium sulfate precipitation assay were explored. It was found that specific binding was detectable at 1/20 of standard sample sizes of labeled estrogen receptor fraction described in "Materials and Method."

3Urd INCORPORATION INTO RNA

Sucrose gradient centrifugation was performed on the MCF-7 RNA fraction (isolated according to "Materials and Methods") in control cells treated with 3Urd (Figure 17), 6 hours of 5uM 5-FU (Figure 18) and 5uM 5-FU after pretreatment with 72 hours TAM (Figure 19).

Standard RNA samples of 4S, 18S and 28S were applied to a sucrose gradient in order to determine standard RNA peaks. The fractions on the X-axis represent groups into which the indicated RNA species fall, and not fraction number.

There was an increase in incorporation of 3Urd over controls (3Urd was used in all gradients except the RNA

standards) into the 32-45S RNA segment in both the 5-FU treated and the TAM-pretreated, 5-FU treated MCF-7 cells. There was less (85% of controls) incorporation of 3Urd into the 18-28S RNA segment in the 5-FU treated cells, and markedly less (38% of controls) 3Urd incorporation into the 18-28s RNA segment in the case of the TAM-pretreated, 5-FU treated cells, compared with control MCF-7 cells.

Similarly, the incorporation of 3Urd into the <4S segment was less (74% of controls) in 5-FU treated cells, and much less (26% of controls) in TAM-pretreated, 5-FU treated cells. The area under the curves was calculated for each subsection.

The significance of these observations is discussed in "Discussion Section."

ER - (3H)E2 BINDING

In three separate trials, specific binding of estrogen receptor to (3H)E2 was examined. MCF-7 cells were treated with a 6 hour exposure to 5uM 5-fluorouracil. Specific binding was increased in each case, whether it was expressed as fmol (3H)E2 bound per million cells (mean 1 60% of controls, SD 42.1%) or fmol (3H)E2 bound per mg protein (195% of controls, SD 60.7%) by Bradford assay). The methods used are described in "Materials and Methods." See figure 21 for results.

RECEPTOR-DNA-CELLULOSE BINDING

Modifications of the Liao centrifugation and column assays (see "Materials and Methods") were used to assess the effect of 6 hours of 5uM 5-FU pretreatment in MCF-7 cells on estrogen receptor binding to DNA-cellulose. The results are presented in Table 16. The centrifugation and column assays were compared with respect to their ability to detect whether or not 1200 cpm of (3H) estradiol-receptor complex applied to the column bound to DNA-cellulose. It was found that detectable binding of receptor to DNA-cellulose resulted only in the centrifugation assay at this low level of radioactivity.

In two trials, 5uM 5-FU pretreatment for 6 hours increased binding of MCF-7 estrogen receptor to DNA-cellulose. Methods of calculating binding are described in the legend of Figure 16. The significance of these results is discussed under "Discussion Section."

CHAPTER XII

DISCUSSION

TAMOXIFEN - 5-FLUOROURACIL SYNERGY

A number of studies have shown significant clinical as in vitro benefits from combined chemo- and endocrine therapy involving tamoxifen.

Mouridsen's group found a significant benefit in combining CMF (cyclophosphamide, methotrexate, 5-fluorouracil) with tamoxifen, increasing the response rate (partial or complete remission) from 45% to 70% (115) in postmenopausal women with advanced breast cancer. A recent series in the New England Journal of Medicine summarized the results of the NSABP (National Surgical Adjuvant Breast and Bowel Project): when tamoxifen was added to a regimen of C-phenylalanine mustard combined with 5-fluorouracil, there was a suggestion of benefit in women over 50 years old with low levels of estrogen receptor (61).

One study in particular, by Allegra's group at the University of Louisville, demonstrates the necessity of attempting to establish a chemotherapeutic regimen based upon rational rather than empiric principles: Allegra's group found, in a Phase II clinical trial with a protocol of TAM, premarin, methotrexate and 5-FU, that the response rate in patients with advanced breast cancer was fully 72%, with a complete remission rate of 56%, all with minimal systemic toxicity. While his sample size was small (25 patients), these results are extremely encouraging; the usual complete remission rate is less than 10-15% (147).

Allegra attributes his apparent success to the broad

coverage of TAM and 5-FU of slow-growing, ER containing, as well as rapidly-growing, ER-negative tumors, respectively. Significantly, his patients did not have dominant visceral metastases, in contrast to patients of other investigators. Furthermore, he believes that his chemotherapeutic regimen, allows for the methotrexate pretreatment to synchronize cells, releasing them when thymidine incorporation, and hence DNA synthesis, is at its maximum, and therefore most vulnerable to chemotherapy (3).

This explanation is not completely satisfactory, since RNA synthesis, which is cell-cycle constitutive (except for the mitotic phase), accounts more for the toxicity of 5-FU than do the DNA-directed effects, as we have seen.

In spite of these very encouraging studies, a number of investigators have demonstrated equivocal (123, 36) or even antagonistic (120) results in combined chemo-endocrine therapy. In vitro, a number of studies have shown that tamoxifen pretreated breast cancer cells are synergistically cytotoxic with 5-FU treatment (Figure 10). For the MCF-7, the expected percent clonal growth of cells exposed to 5uM 5-FU for 6 hours, 12 hours before harvesting is 91%. For the same cells exposed to 10uM tamoxifen alone continuously, the percent clonal growth is 21%. If both drugs acted independently of each other, and none of the effects were overlapping (i.e. if none of the 9% killed by 5-FU were the same as the 79% killed by TAM), the expected combined cytotoxicity would be (.91) (.21) = .19, or 19%. The actual

observed cytotoxicity is 6%, less than third of that predicted; the O/E ratio, or ratio of observed cytotoxity to expected cytotoxity, is .31. Similar experiments under identical drug conditions in the 47-DN human breast cancer cell line concur (21). Here, the results were 93% clonal growth with 5-FU treatments 58% clonal growth with TAM, 26% with TAM and 5-FU, for an O/E ratio of .48, indicating synergy.

Clearly, these drugs are not acting independently. It is very tempting, and potentially of extreme clinical usefulness, to explain this drug synergy on the basis of cellular and biochemical phenomena, much as Cadman's group explained, thus far unassailably, the synergy between methotrexate and 5-FU on the basis of an increase in intracellular 5-phosphoribosyl-1-pyrophosphate pools induced by the dihydrofolate reductase inhibition of methotrexate, and a resultant increase in conversion of 5-FU to its toxic metabolites (34).

It is conceivable that cells that are weakened by one form of therapy (and therefore not be evident in cells killed) would be killed if another damaging but non-lethal drug were applied. Young's group, for example has made the observation that lymphocytes resistant to the effects of glucocorticoids succomb to them if maximally weakened with other, non-lethal agents (160). While this theory is possible, the method of measuring clonal growth assesses maximally weakened, and therefore probably non-dividing

cells, as contributing to overall reduction in clonal cell growth (when extrapolated to a significant subpopulation of cells).

We have examined the effects of 5-FU on RNA processing and protein synthesis. It is likely these RNA-directed effects play a role in explaining TAM-5-FU synergy.

Benz et al have found that TAM pretreatment of MCF-7 cells resulted -- surprisingly, if one considers the synergy -- in an overall reduction in intracellular accumulation of 5-FU; moreover, total incorporation of its metabolites was decreased from 20-60% (23).

In spite of an overall net reduction of intracellular 5-fluorouracil accumulation, tamoxifen enhances its toxicity; in this sense, it is unique.

Nor does it seem that TAM-5-FU synergy is mediated by synchronization alone (19), since some synergy is observed at a wide variation of doses and exposure times. Moreover, tamoxifen synchronization of cells subsequently treated with 5-fluourouracil did not appreciably alter fluorouracil toxicity (19).

It seems likely that 5-FU toxicity is mediated primarily through incorporation into RNA. It has been shown that while overall intracellular accumulation of 5-fluorouracil into cells is decreased by 20-60%, and total RNA decreased by 10-20%, cells treated with tamoxifen alone contained 10-20% less cellular RNA and exhibited altered RNA turnover -- independent of treatment with 5-fluorouracil.

Sucrose gradient centrifugation of newly synthesized RNA reveals more specifically how TAM and 5-FU affect RNA processing. The specific incorporation of a 6 hour exposure to 5-FU was compared with a 2 hour exposure to FUrd and radiolabeled uridine as a control. In one particular segment in cells pretreated with 5-FU namely the 32-45S segment, incorporation increased nearly two-fold. More significantly still, when the 5-fluorouracil metabolite 5-fluorouridine (FUrd) was used (whose effects are predominantly due to FUTP incorporation into RNA) incorporation into the 32-45S is increased almost three-fold (23) (Figures 7, 11).

Accumulation into this high molecular weight segment of RNA is most directly correlated with an inhibition of rRNA processing. There is a significant amount of data suggesting that low molecular weight species exert the maximal inhibitory binding between estrogen receptor and DNA-cellulose. Feldman et al (61) found that 16S and 23S species of RNA were most inhibitory; however, they did not specifically test the 32-45S segment, nor did they test 5-FU treated RNA (problems addressed in this thesis).

The results presented under the "3Urd Incorporation into RNA" Section of this thesis demonstrate increased incorporation of 3Urd into 32-45S RNA, a finding consistent with preliminary data from Benz and Cadman (23).

The results presented indicate that at low, growth-inhibiting doses of 5-fluorouracil, binding of (3H)

estradiol to estrogen receptor is markedly increased over controls. This would imply that the binding of TAM, acting through the estrogen receptor, would also be increased, resulting in more TAM-ER complex to bind to nuclear chromatin.

This finding has found support from several other techniques (Figure 20), among them the cytosol protein extract assay of Horwitz (74), the Shafie and Brooks whole cell assay (141) and flow cytometric data using Raber's methods (127), all of which revealed increased binding of 5-FU (at a minimally toxic concentration)-treated MCF-7 ER to (3H) estradiol.

However, Benz and coworkers have also found that (3H) estradiol binding to ER actually decreases when more toxic doses of 5-FU are used (14), consistent with the preliminary finding of other investigators (i.e. Yang and coworkers) (158), that a dose-dependent reduction in binding of (3H) estradiol to ER is found in the MCF-7 cell line with increasing doses of cytotoxic drugs (i.e. 5-FU) (Figure 15). Yang's group utilized the whole cell assay of Shafie and Brooks. These apparently conflicting results may be explained by different effects on intracellular metabolism and ER function between toxic and non-toxic doses of 5-FU. It is also possible that MCF-7 strains differ in the nature of their estrogen receptors (cf. Horwitz findings of two completely different MCF-7 strains) (71). All things considered, it is very likely that increased ER binding of

(3H) estradiol is not a major mechanism in TAM-5-FU synergy.

Interestingly, the Scatchard analysis of the binding data (Figure 15) reveals that after treatment with toxic concentrations of 5-FU, receptor number is reduced, while affinity of (3H) estradiol for receptor is unchanged. This indicates that cytotoxic drugs may cause a reduction in the amount, but not in the quality, of ER, consistent with the finding of decreased protein synthesis in 5-FU treated MCF-7 cells (53).

It is known that TAM, along with other antiestrogens, must bind to nuclear chromatin before exerting its cytotoxic effects (7). According to the estrogen receptor model by Benz et al, RNA -- particularly of the sequence poly (U,G) -- feeds back on the estrogen receptor, preventing its binding (and therefore its initiation of transcription) to nuclear chromatin. Any factor promoting TAM binding to nuclear chromatin would effectively increase the "intranuclear dose" of TAM, resulting in reduced RNA, DNA, protein synthesis.

By inhibiting RNA processing, 5-FU may be performing this function, and if the RNA recognition site on the estrogen receptor is specific, as several studies have suggested (i.e. 61, 101, 40), the 5-FU-induced change could conceivably be a small structural one, with a large resultant decrease in RNA binding to receptor. This is very likely, especially considering the wide variability of binding of different nucleotides and polynucleotides to

DNA-cellulose exhibited in these studies (esp. 108). One can conclude that this step augmenting TAM's effects on chromatin is qualitatively more important than the dose-dependent reduction in (3H)E2 specific binding after administration of cytotoxic drugs reported by Yang's group (158,), and independently by Benz et al (114).

In both of the latter cases, no qualitative difference in the ER was noted after treatment with 5-FU: in both cases Scatchard analysis showed no change in the affinity of the receptor for its ligand -- (3H)E2 -- but a reduction by 25-64% of ER levels at varying concentrations (160), and 50% reduction in ER at a single concentration.

It is therefore more important how much TAM-ER binds to template, and less important how much free cytosolic ER is present.

This finding is substantiated by preliminary findings that not all tumors which contain ER respond to TAM: in a malignant melanoma cell line, for example, ER has been found which exhibits normal Scatchard behavior for specific binding, i.e. ER is present; however, TAM has no effect upon the cell line. Another finding which elucidates the matter is that no transfer of receptor-ligand to the nucleus occurs, similar to the situation in liver cells, where glucocorticoid receptors are present (as in lymphocytes) but hepatocytes do not respond to glucocorticoid administration, while lymphocytes do. Again, there is no translocation of receptor-hormone complex to the nucleus.

Preliminary data presented here (Figure 16) suggest that pretreatment of MCF-7 breast cancer cells with 5-FU (5uM for 6 hours) results in increased binding of (3H)ER to DNA cellulose, an in vitro correlate of nuclear template.

The estrogen receptor was isolated using a modification of the Feldman, Kallos, Hollander (NH4)2 SO4 precipitation (61). ER from control cells were compared with respect to their ability to bind to DNA-cellulose by modifications of two techniques described by Liao et al (101): the column method and the centrifugation method. In both cases, cellulose without DNA was used as a control. As a second control, it was necessary to substitute a 100-fold excess of DES in order to obtain specific binding. Table 15 indicates that the centrifugation method may be the more sensitive assay: at a lower overall amount of radioactivity (1200 cpm) 52 cpm of specific binding in cells treated with 5uM 5-FU were detected, compared to 0 cpm for control cells not treated with 5-FU. This compares with only 17 specific cpm in the 5uM 5-FU treated MCF-7 cells in the column assay.

The centrifugation assay of Liao appeared to be quite specific. When greater concentrations of control (i.e. not treated with 5-FU) MCF-7 ER were applied, at cpm levels of 3600 and 8000 cpm, no nonspecific binding was detected, while with the column assay 113 nonspecific counts were detected when 3600 cpm were applied to the column.

These data suggest that 5-FU pretreatment of MCF-7 cells results in greater binding of the resultant ER to

nuclear template. It is known that certain RNA species inhibit binding of ER to DNA-cellulose, and that 5-FU impairs RNA processing.

It is entirely consistent with the ER model of Benz et al that inhibiting RNA processing would increase binding of ER to DNA-cellulose, and therefore also of TAM to DNA-cellulose, since evidence suggests TAM acts through the estrogen receptor. Pretreatment with 5-FU may effectively diminish the negative feedback loop at the RNA-receptor level.

This is an attractive hypothesis for several reasons, and is supported by three lines of evidence presented in this thesis: 5-fluorouracil, and to a lesser extent probably also tamoxifen, impair RNA processing; 5-fluorouracil by increased incorporation into 32-45S segment of RNA (Figures 18, 19), tamoxifen by other, less well-defined mechanisms. The binding of activated receptor to DNA-cellulose appears also to be increased, a necessary progression from the first data. Tamoxifen binding to nuclear template, the site of its presumed cytotoxic mechanism, is therefore also increased. The increased binding of receptor to (3H) estradiol (and therefore, according to our model, also to tamoxifen), is relegated to secondary importance, since its presence or absence has no affect on the ultimate TAM-5-FU synergy observed in vitro.

Drawing the latter two conclusions would have been impossible without a very specific assay in which estrogen

receptor levels could first be quantitated, then applied to the second DNA-cellulose assay.

receptor levels could first be quantitated, then applied to the second DNA-cellulose assay.

CHAPTER XIII

CONCLUSION

The study of the TAM-5-FU question is far from finished, since there are numerous studies that would clarify matters further. It is tempting, for example, to conduct ER specific binding assays with radioactive TAM, or better yet radioactive OH-TAM, which has a lower Kd than its nonhydroxylated relative, and follow through with DNA-cellulose binding studies. This would dispel any doubt of TAM's actions being mediated through a separate receptor. Are the actions of other antiestrogens than TAM mediated through other receptors? What is their optimal scheduling; or how can we maximize total synergy by maximizing each contributing component?

Since drugs such as 5-FU and TAM have such clear in vitro and clinical merit in treating breast cancer, and are so readily at hand, it would be a mistake not to explore methods to maximize their utility.

Ultimately, definitive treatment may come from other, not yet well-defined approaches. It may be possible to inhibit estrogen biosynthesis via aromatase inhibitors (Brodie, 31), to prevent new cellular growth by inhibiting prostaglandin synthesis specifically (Bennett, 8), by actually structuring a molecule consisting of an estrogen combined chemically to an antimetabolite (Leclerq, 99), or by using pituitary or hypothalamic releasing hormones to inhibit steroidogensis (Corbin, 49), among many, many others.

Our goal must be individualized treatment of cancer based on its receptor types, histological characteristics, growth characteristics and the patient's expectations of treatment.

It is not unreasonable to think that we have arrived in the era when pharmacology can be based upon molecules individually built for each disease, much in the same fashion as the angiotensin converting enzyme inhibitor antihypertensives were designed to minimize side effects and maximize enzyme inhibiting capabilities. It was first necessary to characterize receptor sites and functional groups in exquisite, three dimensional detail.

If this seems whimsical in the case at hand, TAM-5-FU synergy, witness the large body of recent data, each study suggesting a single aspect of estrogen receptor structure and function; our understanding of the current model is a vast and welcome improvement over the models extant even 5 years ago, and exciting questions continue to suggest themselves, as we draw inexorably nearer to optimization of our current regimens.

CHAPTER XIV

FIGURES

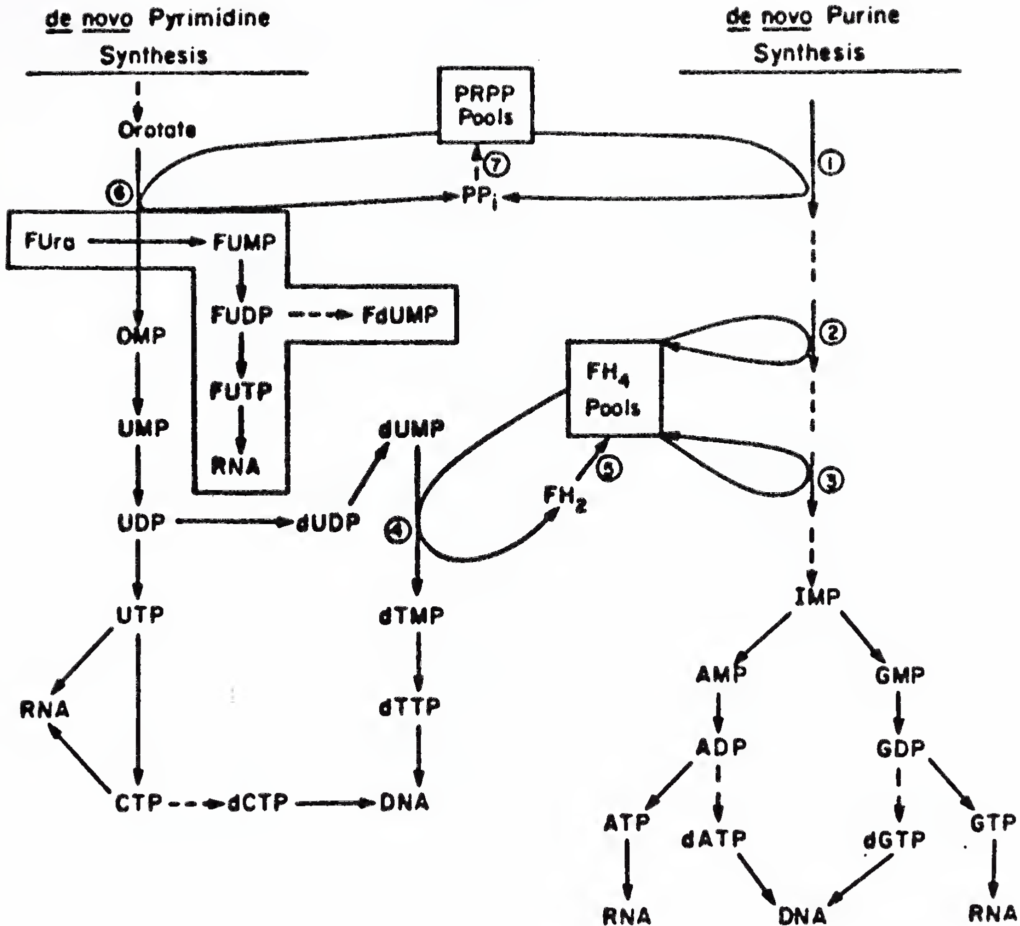


Figure 1. Proposed interaction of MTX and FUra. Broken arrows, multiple enzymatic steps. Enzymes (circled numbers): 1, amidophosphoribosyltransferase; 2, phosphoribosyl glycineamide formyltransferase; 3, phosphoribosyl aminoimidazole carboximide formyltransferase; 4, thymidylate synthetase; 5, dihydrofolate reductase; 6, orotate phosphoribosyltransferase; and 7, phosphoribosyl pyrophosphate synthetase. MTX inhibits Enzyme 5, and dTMP synthesis contimues until the tetrahydrofolate pools no longer support the methyl transfer to dUMP. Because of this reduction in tetrahydrofolate pools, purine synthesis is also inhibited. 5-fluorodeoxyuridine monophosphate directly inhibits Enzyme 4 in the presence of tetrahydrofolate. FUDP, 5-fluorouridine diphosphate: FdUMP, 5-fluorodeoxyuridine monophosphate; FUTP, 5-fluorouridine triphosphate; OMP orotidine monophosphate; FH_4, tetrahydrofolate; FH_2, didydrofolate. Reproduced with permission from Cadman et al (25).

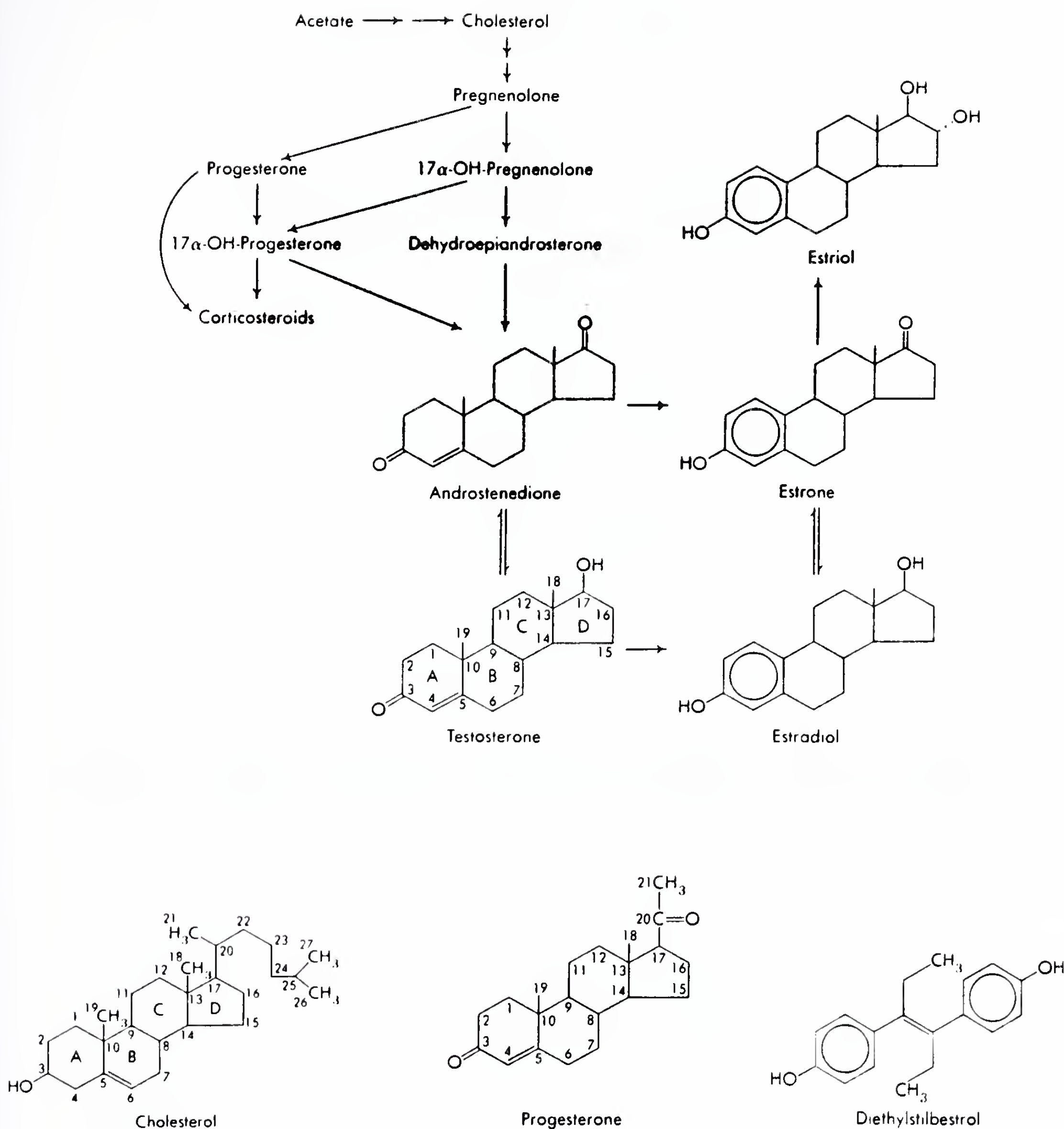
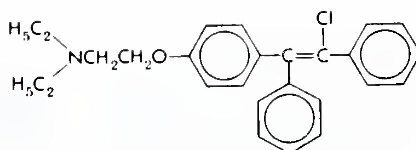
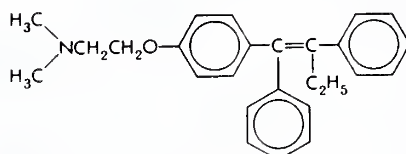


Figure 2. Synthesis of estradiol from cholesterol, showing related compounds, including diethystilbestrol (DES). Adapted from Goodman and Gilman (62).





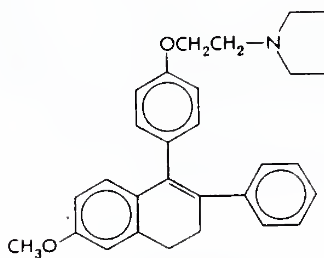
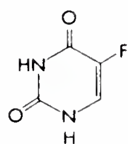


Figure 3. Three commonly used antiestrogens. From Goodman and Gilman (62).



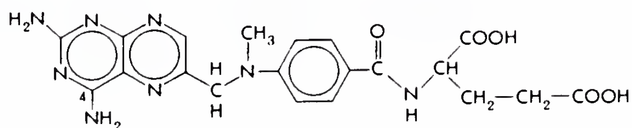


Figure 4. 5-Fluorouracil (5-FU) and Methotrexate (MTX). From Goodman and Gilman (62).

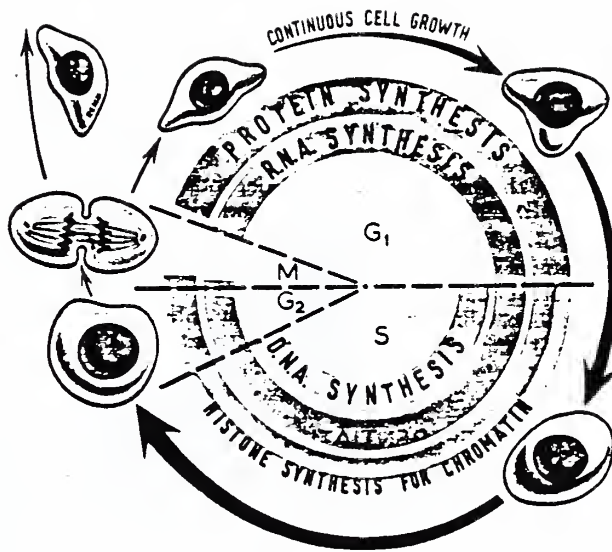


Figure 5. The Cell Cycle. From Gray's Anatomy (156). Note that protein synthesis and RNA synthesis are constitutive, DNA synthesis is not.

ANTIESTROGEN ACTION

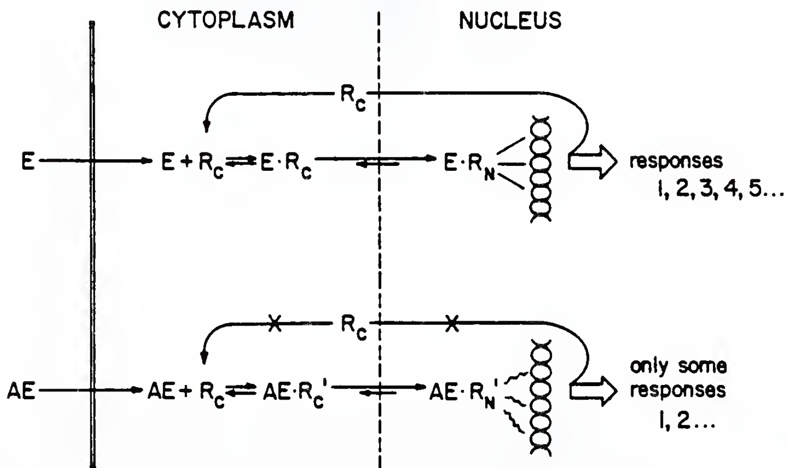


Figure 6. Model of Antiestrogen Action. E-estrogen AE-antiestrogen. From Katzenellenbogen et al (82).

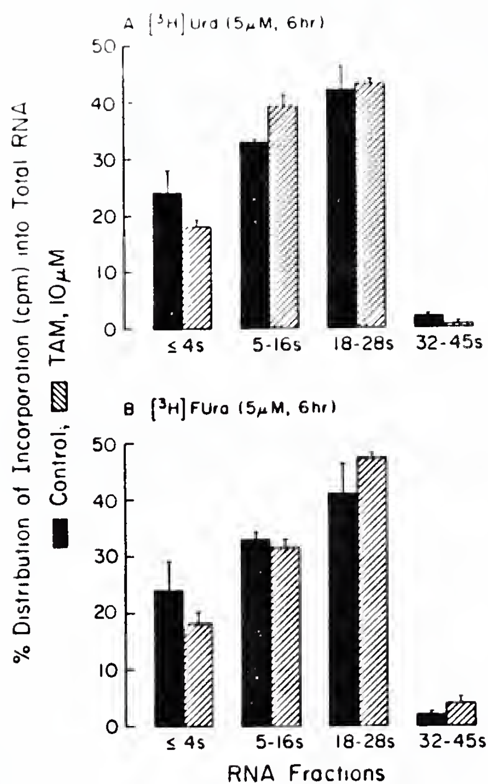


Figure 7. Percentage of distribution of (3H) uridine (Urd) and (3H) FUra incorporation into grouped fractions of 47-DN. Results from sucrose gradient centrifugation assay. From Cadman et al (19). Note greatly increased incorporation into 32-45S fraction upon (3H) FUra treatment.

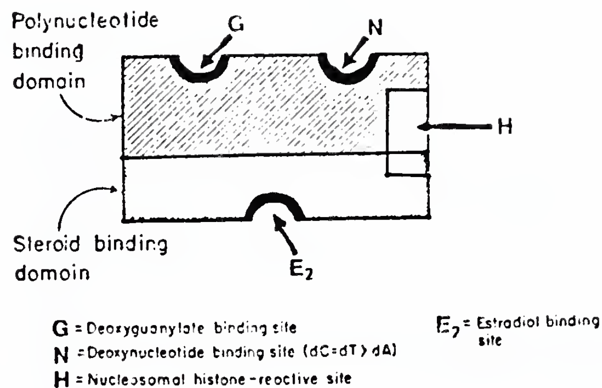


Figure 8. The Dickerman Estrogen Receptor Model. From Dickerman et al (51).

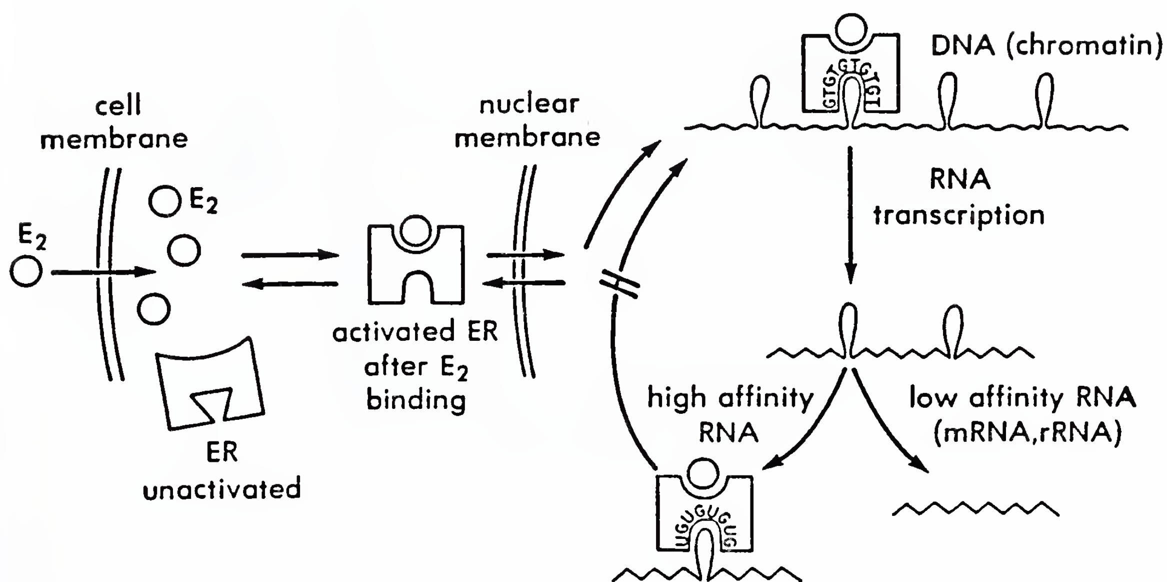


Figure 9. An Autoregulated Estrogen Receptor Model. From Miller and Benz (114).

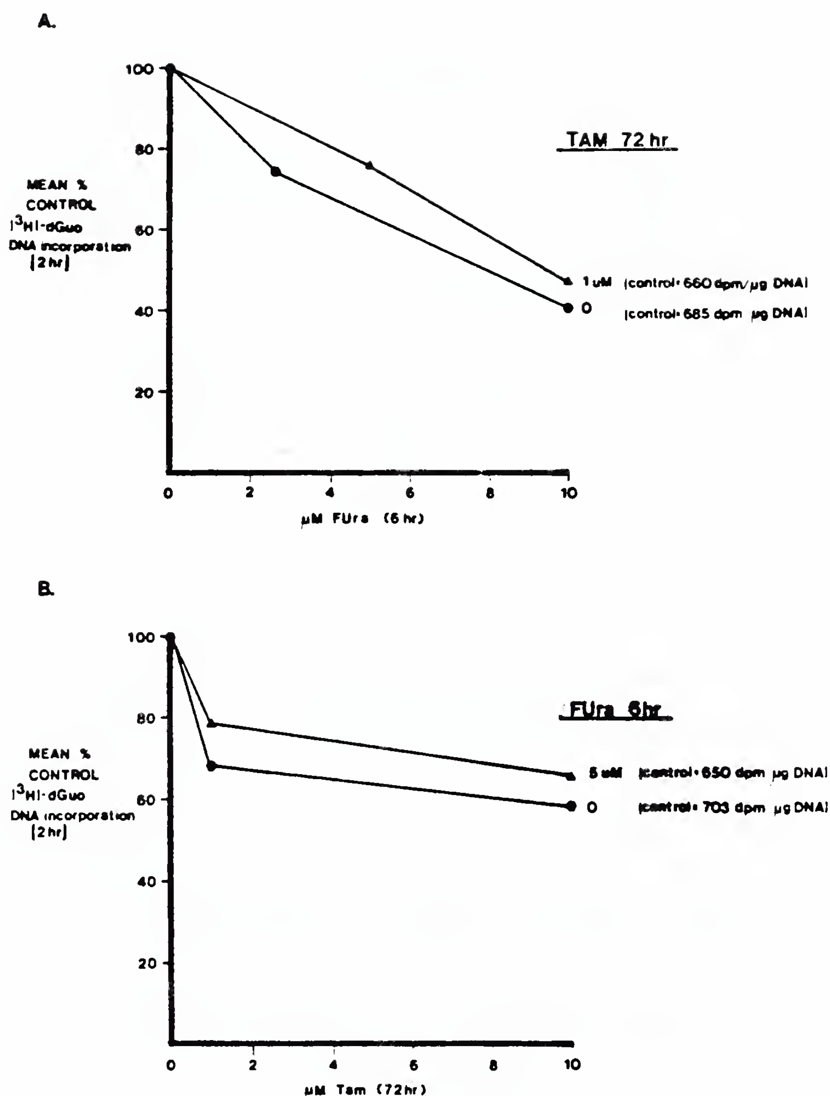


Figure 10. Unpublished data showing dose-dependent reduction incorporation of (3H) dGuo into DNA in T-47-D human mammary carcinoma cell line. From C. Benz (114).

Figure 10. Unpublished data showing dose-dependent reduction incorporation of (3H) dUo into DNA in T-47-D human mammary carcinoma cell line. From C. Benz (114).

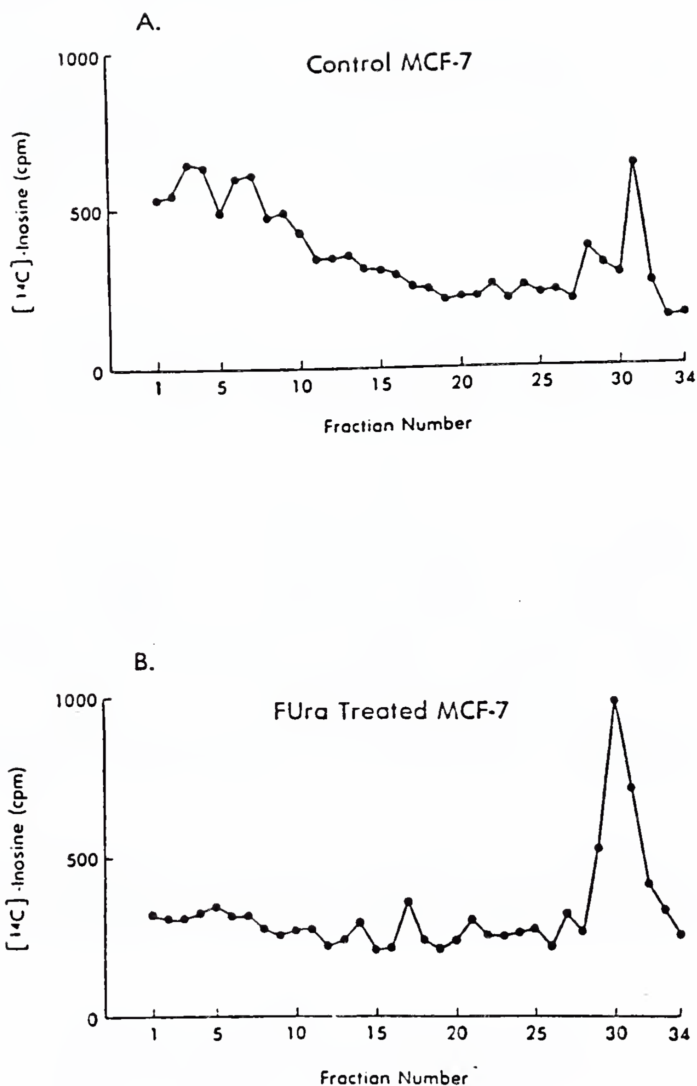


Figure 11. RNA fractionation on sucrose gradients showing increased incorporation of (3H) 5-FU into 32-45S segment of RNA in MCF-7 human breast cancer cell line.
S. Control
B. After 5uM 5-FU x 6 hr. From Benz et al (114).

Mean % Clonal Growth

Cell Line	FUra (5µM, 6hr)	TAM (10µM)*	TAM-FUra	O/E⁺
MCF-7	91%	21%	6%	.31
47-DN	93%	58%	26%	.48

*TAM administered continuously to MCF-7 and for 72 hr to 47-DN.

⁺Observed/Expected (O/E) clonal growth: < 1.0 ± .2 = synergism.
> 1.0 = antagonism

Figure 12. Tamoxifen (TAM) and 5-fluorouracil (5-FU) exhibit synergistic cytoxicity in two human breast cancer cell lines. Note that O/E ratios of less than 1.0 indicate synergy. From Benz, Moelleken, Benz, Wiznitzer (21).

Colo-357 Clonal Growth

	Control	TAM (10µM)	E_2 (5µM)	Pg (2.5µM)	Pg (5µM)
µM FUra 0	100%	86%	85%	44%	6%
15	91%	61%	25%	8%	1%
20	79%	43%	26%	7%	<1%
O/E*		.78%	.32	.2	.1
		.63	.39	.2	<.1

*Observed/expected (O/E) clonal growth: <1.0 ± .2 = synergism, >1.0 = antagonism

Figure 13. In COLO-357 human pancreatic carcinoma cell line TAM is synergistic with estradiol (E_2) or progesterone (Pg). From Benz, Moelleken, Benz, Wiznitzer (21).

Cell Line	ID_{25} TAM	K_d specific E_2 binding*
MCF-7	<1µM	0.1 nM
47-DN	2µM	1 nM
Colo-357	10µM	5 nM

*Dissociation constant by Scatchard analysis of whole cell ER assay using 0.2 - 3.0 nM [^{3}H]-E_2 ± 100 fold excess DES at 37°C x 1 hr.

Figure 14. Sensitivity to TAM (as indicated by concentration of tamoxifen necessary to inhibit clonal growth by 25%) parallels receptor affinity for estradiol. From Benz, Moelleken, Benz, Wiznitzer (21).

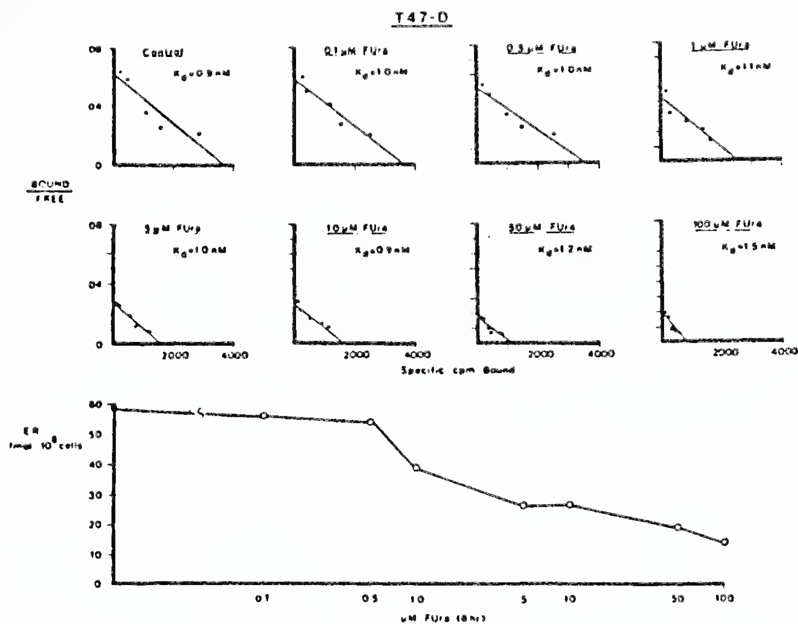


Figure 15. Fluorouracil decreases total specific binding to estrogen receptor. However this is not mediated by a change in receptor K_d (see 8 Scatchard plots above) but by a reduction in the number of estrogen receptors. Data from T47-D human breast cancer cells. From Miller and Benz (114).

Group	Specific cpm applied in assay	Centrifugation Method	Column Method
Control	1200	none detected	none detected
5uM 5-FU (6 hr.)	1200	52	none detected
Control	2000		none detected
5uM 5-FU (6 hr.)	2000		17
Control	3600	none detected	113
Control	8000	none detected	

Figure 16. Specific Binding of (3H)E_2 to DNA-Cellulose. Calculations were determined by (cpm of DNA-cellulose - cpm of control cellulose) - (cpm DNA-cellulose plus 100xs DES - cpm control cellulose with 100 xs DES).

"Specific cpm" denotes cpm of (3H) E_2 - receptor complexes applied to the column. "None detected" indicates that no binding of this complex to DNA-cellulose was observed.

Note that when 1200 "specific cpm" are applied to the column, binding to DNA-cellulose (52 cpm) is observed only in the centrifugation method, indicating that it is the more sensitive assay.

Notable also is that both at very low levels of radiation applied to the centrifugation method (1200 cpm) and at medium levels applied to the column method (2000 cpm), detectable binding resulted only in the 5uM 5-FU treated cells, leading to the preliminary conclusion: 5-FU increases binding of (3H)E_2 complex to DNA-cellulose.

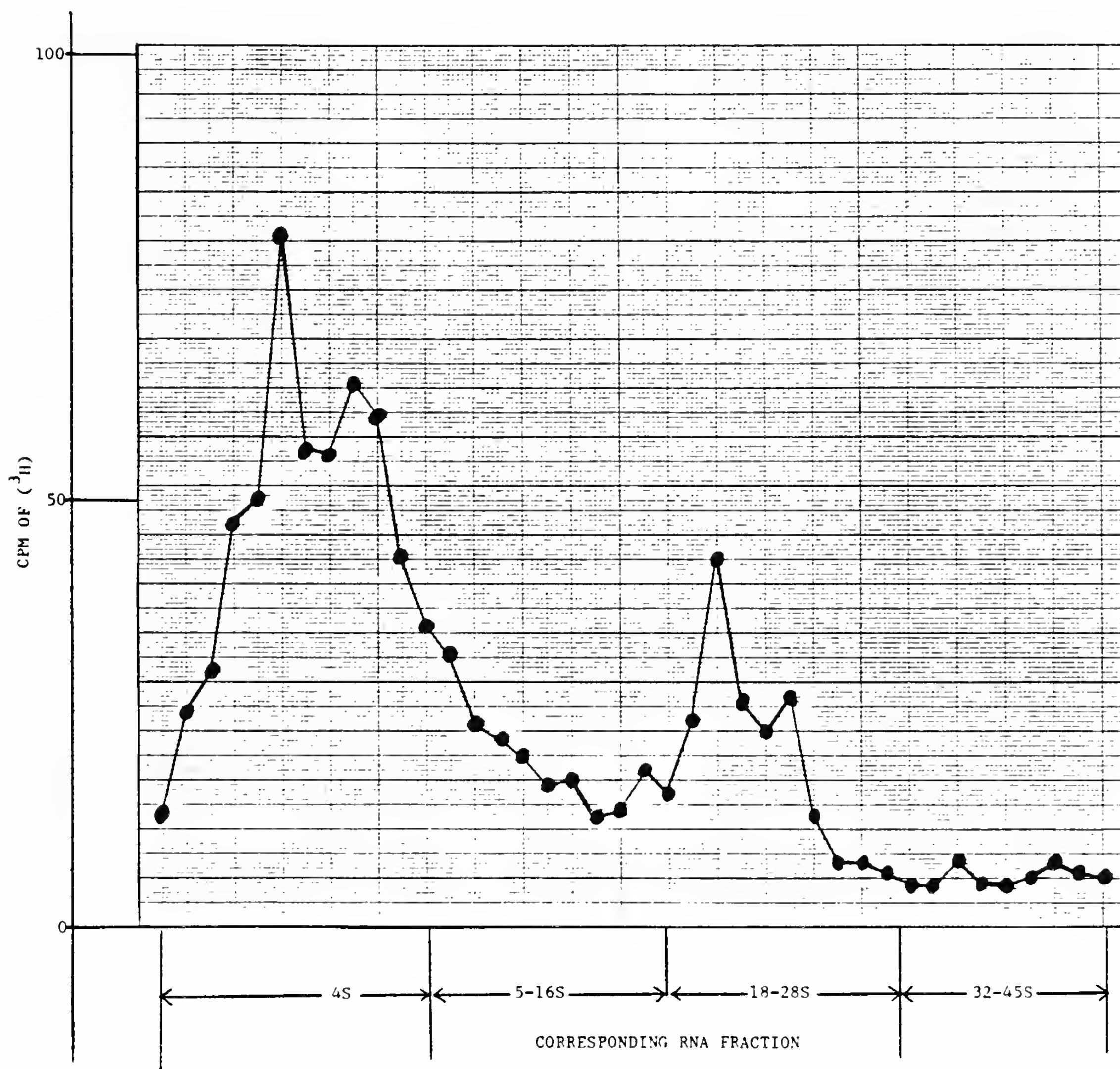


Figure 17. Sucrose gradient centrifugation of control MCF-7 cells. Note peaks at < 4S RNA and 18-28S RNA, and no significant incorporation of 3Urd into 32-45S segment.

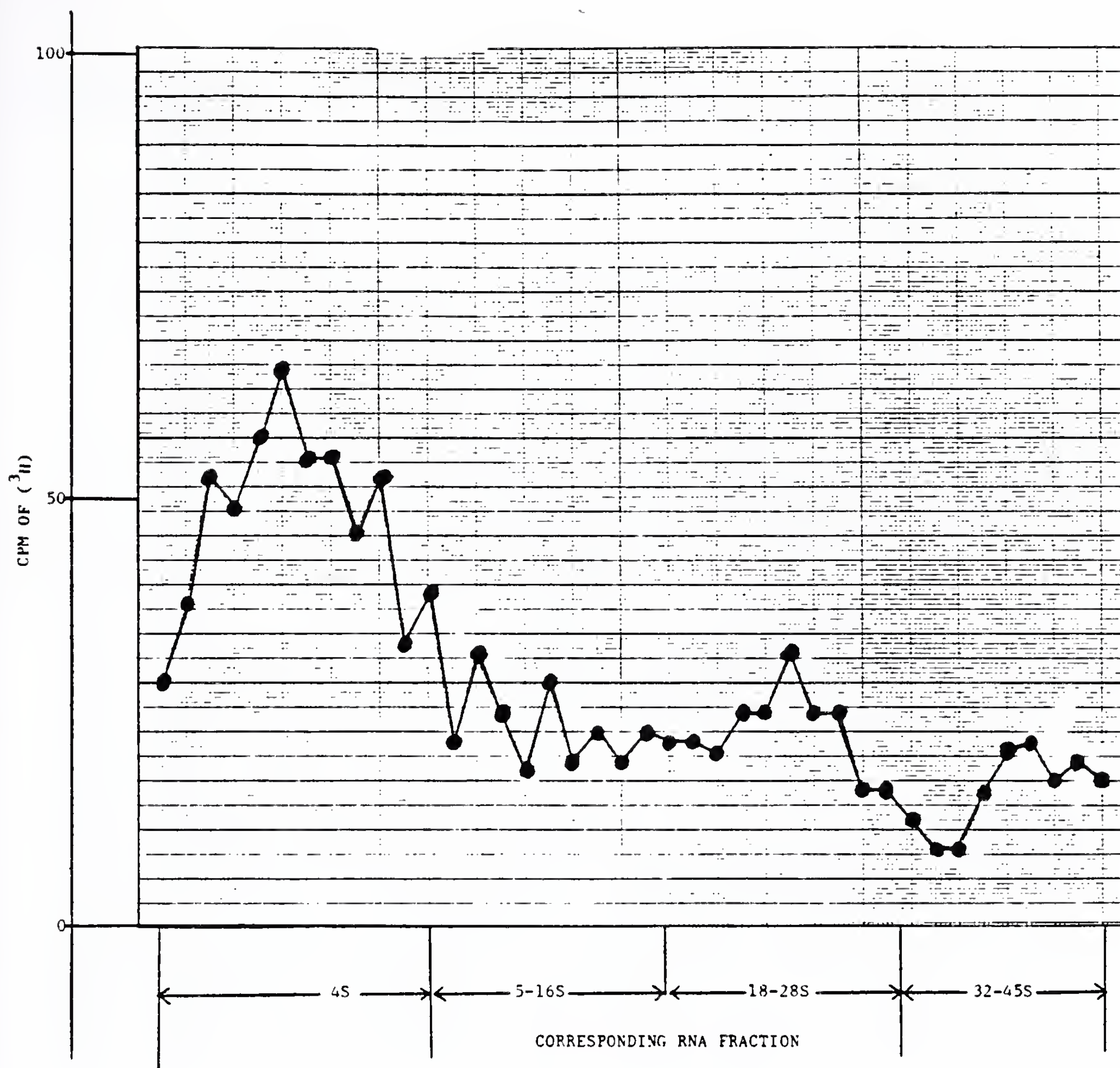


Figure 18. Sucrose gradient centrifugation of 5uM (3H) 5-Fu treated MCF-7 cells. Note decreased $\leq$ 4S, 18-28S Peaks, and increased 32-45S peak over control (cf. Figure 17).

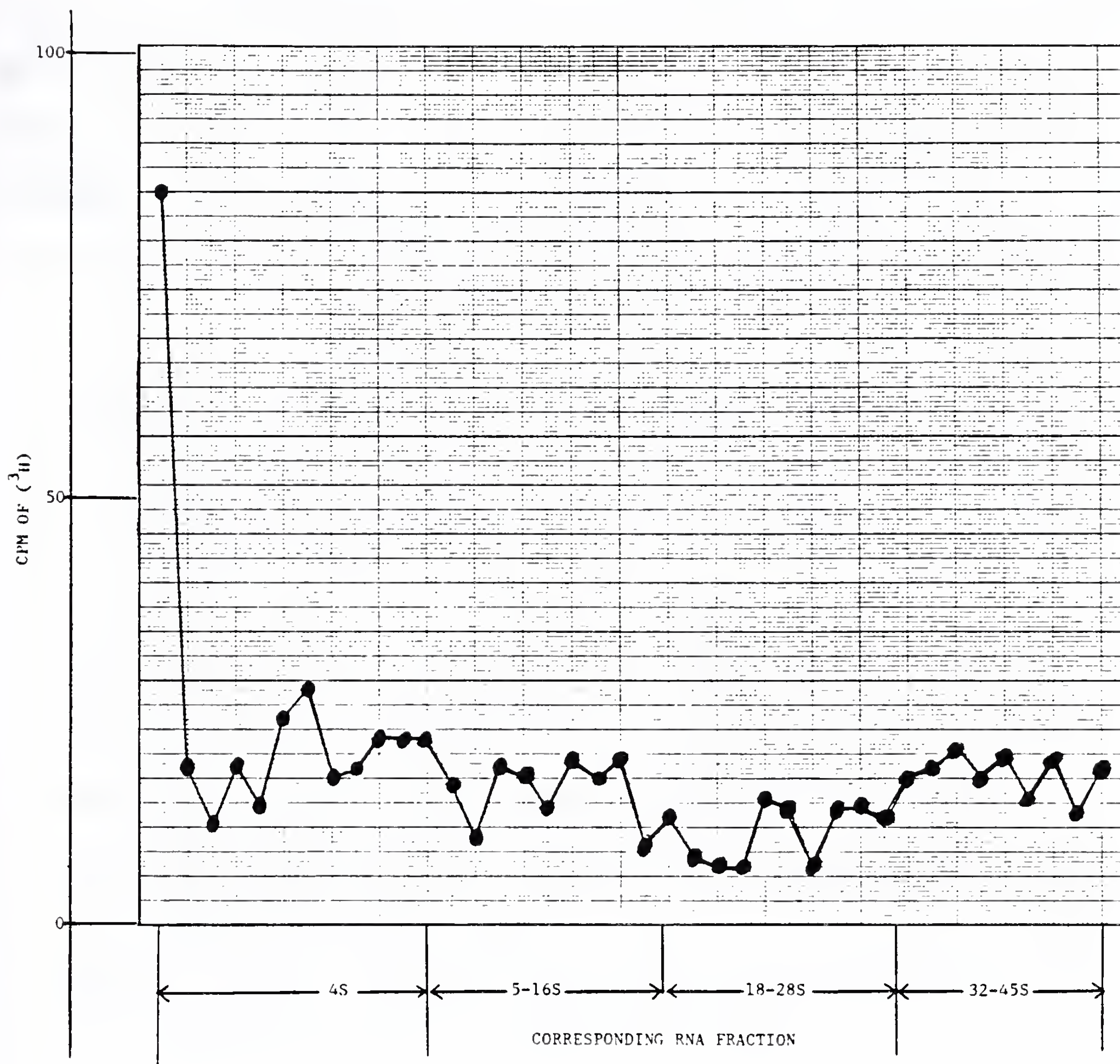


Figure 19. Sucrose gradient centrifugation of TAM-pretreated, (3H) 5-FU treated MCF-7 cells. Note very little $\leq$ 4S, 18-28S; and increased 32-45S peaks compared with control (cr. Figure 17).

Specific Estradiol Binding

	Control(fmol/mg protein) (method A)	5uM FUra x 6 hr (% control) (Method B)	(Method C)	(Method D)
MCF-7	44.0	174%	155%	-
47-DN	22.0	-	133%	-
Colo-357	19.5	-	-	156%

Figure 20. Several lines of evidence suggest spcific estradiol binding increases after treatment with 5uM 5-FU in MCF-7 cells. Methods employed were as shown below. Data for Method B are original. Otherwise, obtained from Benz and coworkers (23).

Method A: [^{3}H]-estradiol exchange on 100,000xg cytosol protein extract (Horwitz, et al) (74).

Method B: [^{3}H]-estradiol exchange on 30% NH_4SO_4 cut of 800xg whole cell lysate (Feldman, et al) (56).

Method C: [^{3}H]-estradiol exchange on intact cultured cells (Shafie and Brooks) (141).

Method D: Flow cytometric intensity of cellular bound fluorescein-conjugated estradiol (Raber et al) (127).

Group	Trial	fmol (^{3}H) E_2 bound per million cells	Ratio 5-FU to Control	fmol (^{3}H) E_2 bound per mg protein	Ratio 5-FU to Control
5-FU	1	0.194	1.67	2.09	2.65
Control		0.116		0.79	
5-FU	2	0.609	1.54	6.80	1.25
Control		0.396		5.44	
5-FU	3	0.0634	2.65	1.42	1.60
Control		0.0239		0.885	
		Mean	1.65		1.95
		SD	0.42		0.61

Figure 21. When MCF-7 cells are incubated in 5uM 5-FU for 6 hours, specific binding of (3H)E_2 to ER is increased compared to controls by 1.54 to 2.65 times (mean 1.65, SD 0.42) in the case of fmol (3H)E_2 bound per million cells, or from 1.25 to 2.65 times (mean 1.95, SD 0.61) in the case of fmol (3H)E_2 bound per mg protein.

Three separate experiments were conducted (Trials 1, 2 and 3). The results for each experiment are given under column 3, juxtaposing the value obtained for 5-FU with that for control cells. The ratio between the two is given in column 4. Columns 5 and 6 show an alternate method of quantitating estrogen receptor specific binding. Neither method is ideal, since neither simultaneously controls for cell size and varying amounts of total cellular proteins; thus both are given here.

Ideally, a dose-response relationship could have been established. However, these preliminary results do indicate a significant difference between control and 5-FU treated cells.

CHAPTER XV

REFERENCES

1. Alberts B, Herrick G. DNA-cellulose Chromatography. Methods Enzymol. 21D: 198, 1971.

2. Allegra JC, Lippman ME et al. An Association Between Steroid Hormone Receptors and Response to Cytotoxic Chemotherapy in Patients with Metastatic Breast Cancer. Cancer Res. 38: 4299-4304, Nov. 1978.

3. Allegra JC, Woodcock TM, Richman SP, Blaad KI, Witliff JL. A Phase II Trial of Tamoxifen, Premarin, Methotrexate and 5-Fluorouracil in Metastatic Breast Cancer. Breast Cancer Research and Treatment 2: 93-99, 1982.

4. Anderson KM et al. Comparison of Dextran-Coated Charcoal and Sucrose Density Gradient Analyses of Estrogen and Progesterone Receptors in Human Breast Cancer. Cancer Res. 40: 4127-4132, Nov. 1980.

5. Aranyi, Peter. Theroretical Considerations in Assessing Steroid Hormone Receptor Interactions: How Do Slow Kinetics Influence the Results of Equilibrium Measurements? In: Hormones and Cancer, ed S. Iacobelli, Raven: New York, 1980.

6. Baskevitch P, Rochefort H. Isopycnic Bonding in Metrizamide of the Uterine Cytosol and Nuclear Estradiol Receptors. Molecular and Cellular Endocrinology 22: 195-210, 1981.

7. Baudendistel L, Rush T. Antiestrogen Action: Differential Nuclear Retention and Extractability of the Estrogen Receptor. Steroids 28: 223-237, 1976.

8. Bennett, Alan. Prostaglandins and Their Synthesis Inhibiters in Cancer. In: Hormones and Cancer, ed S. Iacobelli et al Raven: New York, 1980.

9. Benz C. Unpublished data.

10. Benz C. Unpublished data.

11. Benz C. Personal Communication. Unpublished data, Jan. 18, 1985.

12. Benz C. Personal Communication. Unpublished data, Oct. 2, 1984.

13. Benz, Chris. Unpublished data, 1982.

14. Benz C, Cadman E. Unpublished data. Personal communication Jan. 20, 1985.

15. Benz C, Cadman E. Personal communication.

16. Benz C, Cadman E. Personal communication, 1982.

17. Benz C, Cadman E. Modulation of 5-Fluorouracil Toxicity via Estrogen Receptos. In: Biochemical and Biological Markers of Neoplastic Transformation. Ed Prakash Chandra, Plenum, 1983.

18. Benz C, Cadman E. Biochemical Alterations during Unperturbed Suspension Growth of L-1210 Cells. Cancer Res. 41: 157-163, Jan. 1981.

19. Benz C, Cadman E, Gwin J, Wu T, Amara J, Eisenfeld A, Dannies P. Tamoxifen and S-fluorouracil in Breast Cancer: Cytotoxic Synergism in Vitro. Cancer Res. 43: 5298-5303, Nov. 1983.

20. Benz C, Choti M, Newcomer L, Cadman E. Thymidine Enhancement of Methotrexate and 5-Fluorouracil Toxicity in Cultured Human Colon Carcinoma. Cancer Chemother. Pharmacol. 12: 104-110, 1984.

21. Benz C, Moelleken B, Benz CL, Wiznitzer I. Steroid Synergy in Human Breast and Pancreatic Carcinomas. Proc. 13th Congress of Chemotherapy, Vienna. 1983.

22. Benz C, Moelleken B, Cadman E: RNA and Estrogen Receptor Effects Associated with Tamoxifen- Fluorouracil Synergy in Breast Cancer. AACR 24, 1983.

23. Benz C, Santos G. Cadman E. Tamoxifen and 5-Fluorouracil in Breast Cancer: Modulation of Celluluar RNA. Cancer Res. 43: 4304-5308, Nov. 1983.

24. Benz C, Santos G. RNA Associated with Specific Binding in 5-Fluorouracil Treated Breast Cancer Cells. Breast Cancer Research and Treatment 4: 340, 1984.

25. Benz C, Tillis T, Tattleman E, Cadman E. Optimal Scheduling of Methotrexate and 5-Fluorouracil in Human Breast Cancer. Cancer Res. 42: 2081-2086, May 1982.

26. Benz C, Wiznitzer I, Benz C. Steroid Binding and Cytotoxicity in Cultured Human Pancreatic Carcinomas. In: Progress in Cancer Research and Therapy 31, ed. F. Bresciani et al, Raven: New York, 1984.

27. Bloomfield Clara D et al. The Therapeutic Utility of Glucocorticoid Receptor Studies in Non-Hodgkins Malignant Lymphoma. In: Hormones and Cancer, ed. S. Iacobelli et al, Raven: New York, 1980.

28. Bonnadonna Gianni, et al. Relationship Between Estrogen Receptor Status and Relapse Free Survival After Adjuvant CMF Chemotherapy. In: Hormones and Cancer, ed S. Iacobelli et al. Raven: New York, 1980.

29. Bonnadonna G, Rossi A, Valagussa P, Banfi A, Veronesi V. The CMF Program for Operable Breast Cancer with Positive Axillary Nodes. Cancer 39: 2904-2915, 1977.

30. Bradford MM. Protein Assay by Dye Binding. Anal. Biochem 72: 248, 1976.

31. Brodie AMH. Inhibition of Estrogen Biosynthesis: An Approach to Treatment of Estrogen-Dependent Cancer. In Hormones and Cancer, ed. S. Iacobelli et al, Raven: New York, 1980.

32. Cadman Ed. Toxicity of Chemotherapeutic Agents. From: Cancer, A Comprehensive Treatise, Vol 5, Ed. Frederick Becker. Plenum, 1977.

33. Cadman E, Benz C, Heimer R, O'Shaughnessy T. The Effect of de novo Purine Synthesis Inhibitors on 5-Fluorouracil Metabolism and Cytotoxicity. Biochem. Pharmacol. 30: 2469-2472, 1981.

34. Cadman E, Heimer R, Benz C. The Influence of Methotrexate Pretreatment on 5-Fluorouracil Metabolism in L-1210 cells. J. Biol Chem. 256: 1695-1704, 1981.

35. Carrico Christin, Glazer RI. Effect of 5-Fluorouracil on The Synthesis and Translation of Polyadenylic Acid-Containing RNA from Regenerating Rat Liver. Cancer Res. 39: 3694-3701, Sept. 1979.

36. Carter SK. The Interpretation of Trials: Combined Hormonal Therapy and Chemotherapy in Disseminated Breast Cancer. Breast Cancer Research and Treatment: 43-51, 1981.

37. Chamness GC, Huff K, McGuire WL. Protamine-Precipitated Estrogen Receptor: A Solid Phase Ligand Exchange Assay. Steroids: 628-635, May 1975.

38. Cho-Chung YS. Antagonistic Action between Cyclic Adenosine 3': 5' Monophosphate and Estrogen in Rat Mammary Tumor Growth Control. Cancer Res. 38: 4071-4075, Nov 1978.

39. Cho-Chung YS et al. Cyclic Adenosine 3': 5'- Monophosphate Receptor Protein in Hormone-dependent and Independent Rat Mammary Tumors. Cancer Res. 41: 1840-1846, May 1981.

40. Chong MT, Lippman ME. Effects of RNA and Ribonuclease on the Binding of Estrogen and Glucocorticoid Receptors from MCF-7 Cells to DNA-Cellulose. J. Biol. Chem 257: 2996-3002, 1982.

41. Clark JH, Anderson JN, Peck EJ. Steroids 22: 707-718. 1980.

42. Clark JH, Markavaich B, Upchurch S et al. Heterogeneity of Estrogen Responses. In: Recent Progress in Hormone Research 36, 1980.

43. Clark JH, McCormack SA et al. Effect of Clomiphene and other Triphenylethylene Derivatives on the Reproductive Tract in the Rat and Baboon. In: Hormones and Cancer, ed. S. Iacobelli et al, Raven: New York, 1980.

44. Clark JH, Peck EJ. Female Sex Steroids: Receptors and Function. Springer Verlag: Berlin, 1979.

45. Clark JH, Peck EJ, Anderson JN. Nature 251: 446-448, 1974.

46. Coezy E, Borgna J, Rochefort H. Tamoxifen and Metabolites in MCF-7 cells: Correlation between Binding to Estrogen Receptor and Inhibition of Cell Growth. Cancer Res. 42: 317, 1982.

47. Concolino Guiseppe et al. Human Renal Cell Carcinoma as a Hormone-dependent Tumor. Cancer Res. 38: 4340-4344, Nov. 1970.

48. Concolino G, Marocchi A et al. Endocrine Treatment and Steroid Receptors in Urological Malignancies. In: Hormone Cancer, ed. S. Iacobelli et al, Raven: New York, 1980.

49. Corbin Alan, Rosanoff Eugene. LHRH and LHRH-Like Synthetic Agonists: Paroadoxical Antifertility Effects and Their Therapeutic Relevance to Steroid-Dependent Tumors. In: Hormones and Cancer, ed. S. Iacobelli et al, Raven: New York, 1980.

50. Cory JG, Breland JC, Carter GL. Effect of 5-Fluorouracil on RNA Metabolism on Novikoff Hepatoma Cells. Cancer Res. 39: 4905, 1979.

51. Dickerman HW, Kuman SA. The Polynucleoticle Binding Sites of Estradiol Receptor Complexes. In: Proceedings of the Symposium on Hormones and Cancer, Showsbury, Mass. Plenum Press: New York, 1982.

52. Dupont WD, Page DL. Risk Factors for Breast Cancer in Women with Proliferative Breast Disease. N. Engl.

J. Med. 312: 146-51, 1985.

53. Edwards D, Adams D, McGuire W. Estrogen Regulation of Growth and Specific Protein Synthesis in Human Breast Cancer Cells in Tissue Culture. Adv. Exp. Med. Biol. 138: 133, 1982.

54. Ehrich CE et al. In: Endometrial Cancer, ed. MG Brush, RFB King, RW Taylor, Bailliere Tindall, London, 1978.

55. Ekman P, Dahlberg E, Gustafsson Jan-Ake et al. Present and Future Value of Steroid Receptor Assays in Human Prostatic Carcinoma. In: Hormones and Cancer, Ed. Stefano Iacobelli et al. Raven: New York, 1980.

56. El Etreby MF, Neumann F. Influence of Sex Steroids and Steroid Antagonists on Hormone-Dependent Tumors in Experimental Animals. In: Hormones and Cancer, ed S. Iacobelli et al, Raven: New York, 1980.

57. Engel LW, Young NA. Human Breast Carcinoma Cells in Continuous Culture: A Review. Cancer Res. 38: 4327-4339, 1978.

58. Fabian C, Sternson L, Bonnett M. Clinical Pharmacology of Tamoxifen in Patients with Breast Cancer: Comparison of Traditional and Loading Dose Schedules. Cancer Treat. Rep. 64: 765-773, 1980.

59. Feldman M, Kallos J, Hollander VP, Effect of Photoxidation on the Estrogen Receptor from Rat Mammary Tumor. J. Biol. Chem. 255: 8776-8779, 1980.

60. Feldman M, Kallos J, Hollander V. RNA Inhibits Estrogen Receptor Binding to DNA: J. Biol. Chem. 256: 1145, 1981.

61. Fisher B, Redmond C et al. Treatment of Primary Breast Cancer with Chemotherapy and Tamoxifen. New Engl. J. Med 305 1-5, 1981.

62. Gilman AG, Goodman L, Gilman A. Goodman & Gilman. The Pharmacological Basis of Therapeutics, MacMillan: New York, 1980.

63. Glazer R, Legraverend M, The Effect of S-Fluorouridine 5-triphophate on RNA transcribed in Isolated Nuclei in vitro. Mol. Pharmacol. 17: 279, 1980.

64. Glazer R, Peale A. The Effect of 5-Fouorouracil on the the Synthesis of Nuclear RNA in 1210 cells in vitro. Pharmacol. 16: 270, 1979.

65. Greenberg ER, Barnes AB et al. Breast Cancer in Mothers Given Diethylstilbestrol in Pregnancy. N. Engl. J. Med 311, 1393-1398, 1984.

66. Greenway B, Williams R et al. Oestrogen receptor proteins in malignant and fetal pancreas. British Medical Journal 283, Sept. 1981.

67. Gullino Pietro M. The Regression Process in Hormone-Dependent Mammary Carcinomas In: Hormones and Cancer, ed. S. Iacobelli et al, Raven: New York, 1980.

68. Gustafsson, Jan-Ake et al. Correlation between Clinical Response to Hormone Therapy and Steroid Receptor Content in Prostatic Cancer. Cancer Res. 38: 4345-4348, Nov. 1978

69. Hamilton T. Control by Estrogen of Genetic Transcription and Translation. Science 161, 1968.

70. Henson JC, Legros N. Influence of Insulin and of Alloxan Diabetes on Growth of the Rat Mammary Carcinoma in vitro. European J. Cancer 6: 349-351, 1970.

71. Hilf R, Hissin PJ, Shafie SM. Regulatory Interrelationships for Insulin and Estrogen Action in Mammary Tumors. Cancer Res. 38, 4076-4085, Nov. 1978.

72. Homo F, Ouval D et al. Glucocorticoid Receptors in Normal and Neoplastic Human Lymphoid Cells. In: Hormones and Cancer, ed. S. Iacobelli et al, Raven: New York, 1980.

73. Horwitz K, Mcguire W. Nuclear Mechanisms of Estrogen Action Effects of Estradiol and Antiestrogens on Estrogen Receptors and Nuclear Receptor Processing. J. Biol. Chem. 253: 8185-8191, 1978.

74. Horwitz KB, Costlow ME, McGuire WL. MCF-7: A Human Breast Cancer Cell Line with Estrogen, Androgen, Progesterone and Glucocorticoid Receptor. Steroids 26: 785-795, 1975.

75. Horwitz KB, McGuire WL. Estrogen Control of Progesterone Receptor in Human Breast Cancer: Correlation with Nuclear Processing of Estrogen Receptor. J. Biol. Chem. 253; 2223-2228, 1978.

76. Horwitz KB, Zava DT, McGuire WL et al. Steroid Receptor Analysis of Nine Human Breast Cancer Cell Lines. Cancer Res. 38: 2434-2437, Aug. 1978.

77. Hutter Robert VP. Goodbye to "Fibrocystic Disease." N. Engl. J. Med 312; 179-181, 1985.

78. Iacobelli Stefano et al. Glucocorticoid Receptors and Steroid Sensitivity of Acute Lymphoblastic Leukemia and Thymoma. In: Hormones and Cancer, ed. S. Iacobelli et al. Raven: New York, 1980.

79. Israel N, Saez S. Relation Between Steroid Receptor Content and the Response to Hormone Addition in Isolated Human Breast Cancer Cells in Short-Term Culture. Cancer Res. 38: 4314-4317, Nov. 1978.

80. Jones VHT, Reed MJ. Steroid Hormones and Human Cancer. In: Hormones and Cancer, ed. S. Iacobelli. Raven: New York 1980.

81. Jonat W, Maas H. Some Comments on the Necessity of Receptor Determination in Human Breast Cancer, Cancer Research 38, 4305-4306, Nov. 1978.

82. Katzenellenbogen BS, Katzenellenbogen JA, Tsai Ten-lin et al. Antiestrogen Action in Estrogen Target Tissues: Receptor Interactions and Antiestrogen Metabolism. In: Hormones and Cancer, ed. S. Iacobelli et al. Raven: New York, 1980

83. Katzenellenbogen BS, Bhakoo HS, Ferguson ER, Lan NC, Tatee T, Tsai TS, Katzenellenbogen JA. In: Recent Progress in Hormone Research 35, ed RO Greep, Academic Press: New York: 259-300, 1979.

84. Katzenellenbogen BS, Ferguson ER. Endocrionlogy 97: 1-12, 1975.

85. Kaye AM, Reiss Nachum, Iacobbello S, Bartoccioni E, Marchetti B. The "Estrogen Induced Protein" in Normal and Neoplastic Cells. In: Hormones and Cancer, ed. S. Iacobelli et al. Raven: New York, 1980.

86. Keightley DD, Cressie NAC. The Woolf Plot is more Reliable than the Scatchard Plot in Analysing Data from Hormone Receptor Assays. J Steroid Biochem. 13: 1317-1323, 1980.

87. Kessel D. Cell Surface Alterations Associated with Exposure of Leukemia L-1210 cells to Fluorouracil. Cancer Res. 40: 322-324, 1980.

88. Khan S, Geldman M, Hollander V. The Biological Significance of Estradiol Receptor Binding to DNA. Cancer Res. 40: 1050, 1980.

89. Kiang BT, Frenning OH, Goldman AI, Ascentao VF, Kennedy

BJ. N. Engl. J. Med. 299: 1330-1334, 1978.

90. Knight WA, Livingston RB, Gregory EJ, McGuire WL. Estrogen Recetor: An Independent Prognostic Factor for Early Recurrence in Breast Cancer. Cancer Res. 37: 4669-4671, 1977.

91. Korenman SG, Dukes BA. Specific Estrogen Binding by the Cytoplasm of Human Breast Carcinoma. J Clin. Endocr. 30: 639-645, 1970.

92. Koseki Y, Zava DT, Chamness GC, McGuire WL. Oestrogen Receptor Translocation and Replenishment by the Antioestorgen Tamoxifen. Endocrinology 101: 1104-1109, 1977.

93. Kufe Donald W, Major Pierre P. 5-Fluorouracil Incorporation into Human Breast Carcinoma RNA Correlates with Cytotoxicity. J. Biol. Chem. 256, No 19: 9802-9805, Oct. 1981.

94. Kute TE, Heidemann P, Wittliff JL. Molecular Heterogeneity of Cytosolic Forms of Estrogen Receptors from Human Breast Tumors. Cancer Res. 38: 4307-4313. Nov. 1978.

95. Lacassagne, A. Hormonal Pathogenesis of Adenocarcinoma of the Breast. Am J Cancer 27: 217-228, 1936.

96. Lam, C. Biochem. Biophys. Research Commun. 118. 27-32, 1984.

97. Lasfargues, EY, and Ozzello L. Cultivation of Human Breast Carcinomas J. Natl. Cancer Inst., 21: 1131-1147, 1958.

98. Leake RE, Laing L, Calman KC, Macbeth FR. Estrogen Receptors and Antiestrogen Therapy in Selected Human Solid Tumors. Cancer Treatment Reports 64: 797-799, 1980.

99. Leclercq G, Devleeschouwer N et al. Estrogen-Linked Cytotoxic Agents of Potential Value for the Treatment of Breast Cancer. In: Hormones and Cancer, ed. S. Iacobelli et al, Raven: New York, 1980.

100. Legha SS, Slavik M, Canter SK. Hexamethylmelamine. Cancer 38: 27-35, 1976.

101. Liao S, Smythe S et al. Dependent Release of Androgen and other Steroid Receptor Complexes from DNA. J. Biol. Chem. 255: 5545, 1980.

102. Linder HR, Kohen F, Amsterdam A. An Approach to Site-

Directed Chemotherapy of Hormone-Sensitive Cancer. In: Hormones and Cancer, ed. S. Iacobelli et al, Raven: New York, 1980.

103. Lippman ME, Halterman RH, Leventhal BG, Perry, Thompson EB. Glucocorticoid-Binding Proteins in Acute Lymphoblastic Leukemic Blast Cells. J. Clin. Invest 52: 1715-1725, 1973.

104. Lippman ME, et al. Clinical Implications of Glucocorticoid Receptors in Human Leukemia. Cancer Res. 38: 4251-4256, Nov 1978.

105. Lippman ME, Aitken SC. Estrogen and Antiestrogen Effects on Thymidine Utilization by MCF-7 Human Breast Cancer Cells in Tissue Culture. In: Hormones and Cancer, ed. S. Iacobelli et al, Raven: New York, 1980.

106. Lippman ME (ed). John F. Fogarty Center Conference on Hormones and Cancer. Cancer Res. 38: 3981-4376, 1978.

107. Lizardi P, Engelberg A. Rapid Isolation of RNA using Proteinase K and Sodium Perchlarate. Anal. Biochem. 98: 116, 1979.

108. Maynard PV et al. Estrogen Receptor Assay in Primary Breast Cancer and Early Recurrence of the Disease. Cancer Res. 38: 4292-4295, Nov 1978.

109. McGuire WL. An Update on Estrogen and Progestrone Receptors in Prognosis for Primary and Advanced Breast Cancer. In: Hormones and cancer, ed. S. Iacobelli et al, Raven: New York, 1980.

110. McGuire WL, Carbone PP, Sears ME, Escher GC. Estrogen Receptors in Human Breast Cancer: An Overview. In: WL McGuire, PP Carbore and EP Vollmer (ed. S), Estrogen Receptor in Human Breast Cancer, New York: Raven Press: 1-7, 1975.

111. McGuire WL. Steroid Receptors in Human Breast Cancer. Cancer Res. 38: 4289-4291, Nov. 1978.

112. Meyer JS, Rao BR, Stevens SC, White WL. Low Incidence of Estrogen Receptors in Breast Cancers with Rapid Rates of Cellular Replication. Cancer 40: 2290-2298, 1977.

113. Migliaccio A, Rotondi A, Auricchio Ferdinando. Calmodulin-Stimulated Phosphorylation of 17B-estradiol Receptor on tyrosine. Proc. Natl. Acad. Sci. U.S.A. 81: 5921-5925, Oct. 1984.

114. Miller B, Benz C. Personal Communication. Unpublished

data, Oct 2, 1984.

115. Mouridsen HT, Palshof T, Engelsman E, Sylvester R. CMF versus CMF Plus Tamoxifen in Advanced Breast Cancer in Post-Menopausal women. An EORTC Trial. In: Breast Cancer-Experimental and Clinical Aspects, ed HT Morridsen, T Palshof. Pergamon Press: Oxford: 119-123, 1980.

116. Muller Enzio et al. Effects of Pyridoxal 5-Phosphate on Estrogen-Receptor Activation and Nuclear Binding. In: Hormones and Cancer, ed. S. Iacobelli, Raven: New York, 1980.

117. Nagy I, MacLeod Robert M. Effects of Estradiol and Anti-estrogens or Pituitary Hormone Production, Metabolism and pituitary Tumor Growth. In: Hormones and Cancer, ed. S. Iacobelli, Raven: New York, 1980.

118. Nandi S. Role of Hormones in Mammary Neoplasia. Cancer Res. 38: 4046-4049, Nov. 1978.

119. Nenci Italo et al. Hormone Receptor Cytochemistry in Human Breast Cancer. In: Hormones and Cancer, ed. S. Iacobelli, Raven: New York, 1980.

120. Osborne CK. Combined Chemo-Hormonal Therapy in Breast Cancer: a Hypothesis. Breast Cancer Research and Treatment 1: 121-123, 1981.

121. Park DC, Witliff JL. Assessment of "Activation" of Estrogen Receptors in Lactating Mammary Gland using DNA-Cellulose binding. Biochem. Biophys. Res. Commun 78: 151-258, 1977.

122. Pasqualini JR et al. Recent Data on Receptors and Biological Action of Estrogens and Antiestrogens in the Fetal Uterus of Guinea Pigs. In: Hormones and Cancer ed. S. Iacobelli et al, Raven: New York, 1980.

123. Patterson JS, Battersby LA. Tamoxifen: An Overview of Recent Studies in the Field of Onocology. Cancer Treatment Reports 64: 775-778, 1980.

124. Pavlik EJ, Nagell JR et al. Rapid Analysis of Estrogen and Progesterone Receptors Using Gel-Exclusion High-Performance Liquid Chromatography. Biochemistry 21; 139-145, 1982.

125. Pearson Olof H, et al. Role of Pituitary Hormones in the Growth of Human Breast Cancer. Cancer Res. 38: 4323-4326, Nov. 1978.

126. Pharmacia Fine Chemicals AB. Sephadex LH-20 Chromatography in Organic Solvents. Upplands Grafiska AB: Uppsala Sweden, 1978.

127. Raber MN et al. Flow Cytometric Analysis of Estrogen Receptor Content. Amer. Assoc. Clin. Res., Abstract #121, 1982.

128. Rademaker B, Vossenberg JBJ, Poortman J, Thijssen JHH. Metabolism of Estradiol-17B, 5-Androstene-3B, 17B-diol and Testosterone in Human Breast Cancer Cells in Long-Term Culture. J Steroid Biochem. 13: 787-91, 1980.

129. Ratajczak T, Hahnel R. Chromatographic and other Properties of the Estogen Receptors from Human Myometrium. J. Ster. Biochem. 7: 185-197, 1976.

130. Rich, MA Furmanski MAR, Brooks SC. Prognostic Value of Estrogen Receptor Determinations in Patients with Breast Cancer. Cancer Res. 38: 4296-4298, Nov. 1978.

131. Rochefort H et al. Hormonal Central of Breast Cancer in Cell Culture. In: Hormones and Cancer, ed Iacobelli et al, Raven: New York, 1980.

132. Rose DP, Davis TE. Ovarian Function in Patient Receiving Adjuvant Chemotherapy for Breast Lancet. Cancer 1: 1174-1176, 1977.

133. Rudland PS, Bennett DC, Warburton MJ. Growth and Differentiation of Cultured Rat Mammary Epithelial Cells. In: Hormones and Cancer, ed. S. Iacobelli et al. Raven: New York, 1980.

134. Santi DV, McHenry CS. 5-Fluoro-2'-deoxyuridylate: Covalent Complex with Thymidylate Synthetase. Proc. Natl. Acad. Sci. U.S.A. 69: 1855-1857, 1972.

135. Sartorelli S. Combination Chemotherapy with Actinomycin D and Ribonuclease: an Example of Complementary Inhibition. Nature (Lond.) 203: 877-878, 1964.

136. Sato Gordon H. Towards an Endocrine Physiology of Human Cancer. In: Hormones and Cancer ed. S. Iacobelli et al, Raven: New York, 1980.

137. Sato B, Nishizawa Y, Noma K, Matsumoto K, Yamamura Y. Estrogen-Independent Nuclear Binding of Receptor Protein of Post-Uterine Cytosol by Removal of Low Molecular Weight Inhibitor Endocrinology 104: 1474-1479, 1979.

138. Schrader WT, O'Malley BW. Structure of Chick Progesterone Receptors. Cancer Res. 38: 4199-4203, 1978.

139. Segel, Irwin. Enzyme Kinetics. Behavior and Analysis of Rapid Equilibrium and Steady-State Enzyme Systems. Wiley: New York: 217-223, 1976.

140. Seidman H, Silverberg E, Bodden A: Probablities of Eventually Developing and Dying of Cancer. CA 28: 35-46, 1978.

141. Shafie SM, Brooks SC. The Relationship of Insulin to Regulation of Breast Tumor Cells by 17B-Estradiol. In: 67th Meeting of the American Society of Biological Chemists: 1628, San Francisco, 1976.

142. Sica V, Bresciani F. Estrogen-Binding Proteins of Calf Uterus. Purification to Homogeneity of Receptor from Cytosol by Affinity Chromatography. Biochemistry 18, 2369-2378, 1979.

143. Siiteri PK, Nisker JA, Hammond GL. Hormonal Basis of Risk Factors for Breast and Endometrial Cancer. In: Hormones and Cancer, ed. S. Iacobelli et al, Raven: New York, 1980.

144. Silverberg Edwin. Cancer Statistics, 1984. CA-A Cancer Journal for Clinicians. American Cancer Society. CA 34: 7-23, 1984.

145. Soll D. Enzymatic Modification of Transfer RNA. Science 173: 293-299, 1971.

146. Speyer J, Collins J, Dedrick R et al. Phase I and Pharmacological studies of 5-Fluorouracil administered intraperitoneally. Cancer Res. 40: 567-572, 1980.

147. Stedman KE, Moore GE, Morgan RT. Estrogen Receptor Proteins in Diverse Human Tumors. Arch. Surg. 115: March 1980.

148. Stoll, Basil A. Endocrine Therapy in Cancer. The Practitioner 222: 211-217, Feb. 1979.

149. Stulberg MP, Sutton M, Isham KR. Undermethylated Transfer RNA Does Not Support Phase RNA-Directed in Vitro Protein Synthesis. Biochim. Biophys. Acta 435: 251-257, 1976.

150. Sutherland R, Murphy L. The Binding of Tamoxifen to Human Mammary Carcinoma Cytosol. Europ. J. Cancer 16: 1141, 1980.

151. Taylor RN, Smith RG. Pure Estrogen Receptor Complexes Stimulate RNA Transcription in Vitro. Biochim. and Biophys. Research Comm. 91: 136-142, 1979.

152. Tseng Wen-Cheng, Medina D, Randerath Kurt. Specific Inhibition of Transfer RNA Methylation and Modification in Tissues of Mice Treated with 5-Fluorouracil. Cancer Res. 38: 1250-1257, May 1978.

153. Veronesi, Umberto, et al. Comparing Radical Mastectomy with Quadrantectomy, Axillary Dissection and Radiotherapy in Patients with Small Cancers of the Breast. N. Engl. J. Med. 305: 6-11, 1981.

154. Waechter D, Baserga R. Effect of Methylation on Expression of Microinjected Genes. Proc. Natl. Acad. Sciences U.S.A., 1982.

155. Waters AP, Knowles JT. A Comparison of the Effects of Oestrogen and Tamoxifen on the Synthesis of Uterine RNA in Immature Rats. J. Ster. Biochem. 14: 625-630, 1981.

156. Williams PL, Warwick R. Gray's Anatomy. 36th British Edition. Saunders: Philpdelphia, 25: 1980.

157. Yamamoto K, Alberts B. Steroid Receptors: Elements for Modulation of Eucaryotic Transcription. In: Annual Review of Biochemistry 45: 721-741. Annual Reviews Inc: Palo Alto, 1976.

158. Yang KP, Samaan NA. Effect of Cytotoxic Drugs on Estrogen Receptor Level of Human Breast Cancer Cells (MCF-7). AACR Abstracts, Abstract No. 940, 1982.

159. Yates J, King RJB. Multiple Sensitivities of Mammary Tumor Cells in Culture. Cancer Res. 38: 4135-4137 Nov. 1978.

160. Young DA, Nicholson ML, Voris BP, Lyons RT. Mechanisms Involved in the Generation of the Metabolic and Lethal Actions of Glucocorticoid Hormones in Lymyphoid Cells. In: Hormones and Cancer, ed. S. Iacobelli et al, Raven Press: New York, 1980.

161. Zava D, McGuire W. Estrogen Receptor. J. Biol. Chem. 252: 3703-3708, 1977.

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