A-Ring Substituted Estrogens as Inhibitors of the MXT Transplantable Mammary Ductal Carcinoma

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ABSTRACT

A-ring substituted estrogens have been examined as growth inhibitors of the hormone dependent MXT murine mammary tumor. Certain of these estrogen analogues inhibited the growth of newly implanted as well as established MXT tumors when administered either by s.c. or i.p. injections or by intubation. These compounds were nontoxic over a broad range of active levels. Amino and nitro groups, introduced at position-4 of estrone 3-methyl ether were particularly carcinostatic, a property not shared by 4-bromoestrone 3-methyl ether. In addition tumor inhibition was greatly diminished by placing the nitro group at the other ortho position (i.e., carbon-2). Evidence indicates that the A-ring substituted estrogens may function as growth inhibitors via the estrogen receptor mechanism in the case of 4-nitro- and 4-aminoestrone. The 3-methyl ethers of these compounds also blocked tumor growth, possibly through in vivo dealkylation leading to the free phenolic A-ring substituted estrogens. On the other hand, A-ring substituted 3-deoxyestrogens (particularly 4-nitro- and 4-aminoestratrien-17β-ol), which do not bind to receptor, were also excellent inhibitors of hormone dependent MXT breast tumors and therefore must express their activity by mechanisms other than that mediated by receptor. The A-ring substituted estrogens are unlike tamoxifen and diethylstilbestrol which (a) display toxicity at optimum inhibitory doses and (b) are inactive or marginally active in rodent breast cancer models.

INTRODUCTION

Although there has been considerable interest in the triphenylethylenes as growth inhibitors of the hormone dependent mammary tumors through their interaction with the estrogen receptor, there has been little concern with estrogen analogues as antitumor compounds. Those derivatives previously studied have been ring D substituted estrogens [(e.g., 17α-thioestradiol (1), estradiol-16α- and estrolactone (2)]. Treatment with these compounds proved to be very unsatisfactory, bringing about only limited regressions of initial tumor size after 56 days of daily injections (40 mg/kg, rat) accompanied in one case (17α-thioestradiol) with toxicity. For the most part the early data gathered on steroid inhibitors of human mammary tumors involved the administration of androgen and progesterone derivatives (2). Both of these hormones proved to be unsatisfactory therapeutic agents with minimal activity and/ or undesirable side effects.

Pharmacological doses of 17β-estradiol or diethylstilbestrol have long been used as inhibitors of hormone dependent mammary tumor growth (3). Although these agents provided an effective antitumor regimen, the estrogenic activity of these compounds was deleterious to normal target tissues. Moreover, these treatments elevated the serum prolactin, a hormone which, incidentally, is stimulatory to rodent mammary tumors (3).

Discovery of the receptor mechanism for the activity of estrogen analogues has led to the design of antiestrogens, which took advantage of the receptor protein binding phenomenon. Tamoxifen (and nafostimate) became useful since they compete (at pharmacological levels) for the binding site of 17β-estradiol. Once bound, the receptor, complexed with these antiestrogens, is incapable of normal chromatin interactions, and the unstimulated neoplasm regresses. Although efficacious, the antiestrogens exhibit some undesirable side effects (5, 6) and they are, apparently, inactive in certain human tumors that are otherwise responsive to hormonal treatment (7). Thus, other agents, nontoxic and effective against hormone dependent tumors, are needed for breast cancer therapy in order to (a) expand the spectrum of neoplasms that respond to hormonal therapy and (b) provide further insight into the characteristics of hormone dependent cancers.

We have recently reported (8) that 4-nitroestrone 3-methyl ether is an effective growth inhibitor of certain DMBA1-induced rat mammary tumors. When administered at 24 mg/kg this nontoxic estrogen analogue was as active in the DMBA tumor system as tamoxifen (0.8 mg/kg), being surpassed in its carcinostatic activity only by ovarietomy or pharmacological doses of estradiol-17β 3-benzoate. This A-ring substituted estrogen proved not only to bring about regression of hormone dependent tumors in the rat but tumor formation was prevented when administered for 10 days prior to DMBA-intubation or when injected for 20 days just following treatment with DMBA (8). DMBA-induced mammary tumors, which reappeared in ovarietomized rats, were also prevented by 4-nitroestrone 3-methyl ether, suggesting that this analogue was effective against tumors that grew in animals free of ovarian steroids.

In this report, the antihormonal effect of 4-nitroestrone 3-methyl ether was evaluated in the MXT transplantable hormone dependent mammary tumor. In addition this model was utilized to study the antitumor activity of various aspects of A-ring substitution in estrogens.

MATERIALS AND METHODS

Materials. [6, 7]3H-4-Nitroestrone 3-methyl ether (56.4 Ci/mmol) was custom synthesized by New England Nuclear (Boston, MA). The unlabeled compound and nitroestrone were synthesized in our laboratory according to published procedures (9). Tamoxifen (free base) was a gift from Dr. D. H. McCurdy, Stuart Pharmaceuticals (Wilmington, DE) and diethylstilbestrol was purchased from Sigma (St. Louis, MO). 4-Aminosterone 3-methyl ether, 4-bromoestrone 3-methyl ether, 2-nitroestrone 3-methyl ether, 4-nitroestratriene-17β-ol, and 4-aminosteratriene-17β-ol, 4-nitroestrone 3-(2-hydroxyethyl) ether, and 4-nitroestrone 3-(e-hydroxyethyl) ether were prepared according to published procedures (9-11). The following procedure for the preparation of the 4-nitroestrone 3-
O-n-butyl ether is considered typical for the corresponding 3-O-alkyl ethers listed in Table 1. A mixture of 4-nitroestrone (1.9 g; 6 mmol)-l-iodobutane (6.8 ml; 11.04 g; 60 mmol)-anhydrous potassium carbonate (8.26 g; 60 mmol) in dry N,N-dimethylformamide (15 ml) was stored under argon for 16 h. The reaction mixture was poured with stirring into 200 ml of water and the aqueous solution was extracted with methylene chloride (3 × 50 ml). The dried (Na2SO4) extract was evaporated to dryness under vacuum and the residue was triturated with ethanol. The off-white crystalline material (1.63 g) exhibited a single spot on thin layer chromatography (precoated Silica Gel F-254 in methylene chloride:methanol, 98:2, v/v). The product crystallized from ethanol in the form of colorless needles; weight 1.51 g, m.p. 192-194°C; \( \lambda_{max} \) (ethanol) 276.1 nm (ε 1535).

\[ \text{C}_9\text{H}_8\text{NO}_4 \]

Calculated: C 71.13, H 7.87, N 3.71
Found: C 70.98, H 7.75, N 3.50

[4-Nitroestrone 3-O-methyl ether: \( \lambda_{max} \) (ethanol) 275.4 nm (ε 1568)].

\[ \text{C}_{10}\text{H}_{12}\text{NO}_4 \]

Calculated: C 69.95, H 7.34, N 4.08
Found: C 69.94, H 7.37, N 4.18

\[ \text{C}_{18}\text{H}_{22}\text{NO}_4 \]

Calculated: C 70.56, H 7.61, N 3.92
Found: C 70.42, H 7.52, N 3.90

Animals. B6D2F1 hybrid mice were bred in these laboratories from C57BL/6J females and DBA/2J males. All were over 17 g and within a 5-g weight range at the start of the therapy trials. Mice were supplied Purina autoclavable rodent chow and tap water ad libitum. Animal rooms were kept at 23.3 ± 2.2°C with 40-70% relative humidity and 12-h light-dark cycles.

The MXT tumor (4.5-day tumor volume doubling time) was initially induced with urethan and contains estrogen receptors (12). This tumor is a mixture of hormone-dependent and hormone-independent cells as evidenced by the fact that it will usually grow in male mice after a long delay. The mice are treated by administering the estrogen analogues, dissolved or suspended in a solution of 3% ethanol, 3% polyoxyethylene glycol 400, 0.1 or 0.2 ml, or i.p. with 0.5 ml of this mixture daily for the indicated period. In certain experiments the compounds were intubated with similar solutions (0.1 or 0.2 ml) to determine the activity of the analogues when administered p.o.

The 16/C mammary adenocarcinoma is a rapidly growing (1.2-1.8 day tumor volume doubling time), highly metastatic tumor and is estrogen receptor positive although not hormone dependent (13).

Tumor Assessment. Methods of protocol design, tumor transplantation, drug treatment, end point determination, toxicity evaluation, data analysis, and the biological significance of drug treatment with transplantable tumors have been presented (13-17).

Briefly stated, the methods used are as follows: the animals to be used in an experiment are pooled, given s.c. implants of 30- to 60-μg tumor fragments by trocar (bilateral, flank) and again pooled before unselective distribution to the various treatment and control groups. Hormonal therapy is started 1-5 days after tumor implantation while the number of cells are relatively small (10^4-10^6 cells, early-stage disease). Tumors are measured with a caliper once or twice weekly depending on the growth rate of the tumor. Tumor weights are estimated from 2-dimensional measurements

\[ \text{Tumor wt (mg) = } (a \times b)^{3}/2 \]

where \( a \) and \( b \) are the tumor length and width (mm), respectively.

The following quantitative end points were used to assess antitumor activity: (a) tumor growth inhibition, treated/control. This is the most commonly used end point for the evaluation of antitumor activity.

\[ \% \text{T/C} = \frac{\text{Median tumor wt of the treated group (T)}}{\text{Median tumor wt of the untreated group (C)}} \]

The T/C value was determined when the control group tumors are in the 700- to 2000-μg weight range (median) for mouse tumors. Zeros are included in median tumor weight determinations. A T/C value less than 42% is considered significant antitumor activity; (b) tumor growth delay (T-C value), where \( T \) and \( C \) are the median time (days) required for the treatment group and the control group tumors, respectively, to reach a predetermined size (e.g., 500 mg). Tumor-free survivors are excluded from these calculations.

RESULTS

The MXT tumor does not grow readily in ovariectomized B6D2F1 mice (Fig. 1). However, within 2 weeks of implantation into intact mice this mammary tumor can be measured, reaching a mass greater than 2 g by 45 days. Injections (i.p.) of 4-
nitroestrone 3-methyl ether (800 mg/kg twice daily for 17 days) resulted in tumor growth delay greater than that experienced in mice free of gonadal hormones (Fig. 1). On the other hand, tamoxifen administered (i.p.) at a nontoxic level (25 mg/kg) had little effect on MXT tumor growth. After 81 days 4 of 9 surviving female mice (10 were initially treated) in the 4-nitroestrone 3-methyl ether group were tumor free. There were no mice without tumors in the other groups at this time.

The MXT tumor has displayed properties of both hormone dependence and independence (18). For example, this heterogenous tumor will grow in male B6D2F1 mice, albeit to a lesser extent. The ability of this mammary tumor to grow in male mice varies with the passage, often displaying increased independence of female gonadal hormones with a greater number of passages. In these studies, dilution experiments in which the minimum number of cells (10⁶) were implanted have shown that separate lines of MXT tumors can be obtained which grow 7, 32, 42, 50, 80, and 100% as well in male as in female B6D2F1 mice. Interestingly, 4-nitroestrone 3-methyl ether has been able to inhibit the growth of these tumors to values below that observed in the male mouse. In an experiment in which MXT transplants grew approximately one-half as rapidly in intact males relative to the growth rate in intact females (Fig. 2), it was seen that the growth of this tumor in castrated males and females was similar and approximated that in intact males. Ovarian hormones influenced the growth rate whereas testicular hormones were ineffective. Nevertheless treatment of intact male and female hosts with 4-nitroestrone 3-methyl ether (200 mg/kg, injected s.c. daily) inhibited growth to 7% the tumor size in untreated female B6D2F1 mice (Fig. 2, days 41–55).

The ability of certain A-ring substituted estrogens to slow the growth of any MXT tumor line below the growth in males (or castrates) was common to these investigations. Examination of a tumor line that grew equally in males and females showed that after 18 days of treatment with 4-nitroestrone 3-methyl ether (200 mg/kg) these tumors were one-half the size of the tumors in untreated mice of either sex.

The anti-breast cancer properties of 4-nitroestrone 3-methyl ether were observed over a wide range of administered levels. In an experiment in which the mice were given injections s.c. (twice daily) of varying levels of 4-nitroestrone 3-methyl ether, tumor growth inhibition was comparable over a dose range of 400–1600 mg/kg (T/C = 1.4–2.4%) and no toxicity was evident (Table 1, all mice gained weight). Again ovariectomized hosts displayed tumor growth inhibition similar to the treated groups.

Utilizing the regimen of once daily s.c. injections, it was determined that 33 mg/kg was the lowest active dose that inhibited the growth of MXT tumors in intact females (Table 2).

Eleven days of treatment of C3H/He mice bearing transplanted hormone independent 16/C mammary adenocarcinoma with twice daily injections (s.c.) of 4-nitroestrone 3-methyl ether (75-, 120-, and 220-mg/kg doses) yielded only slight tumor growth inhibition (T/C = 56% for the highest dose). Again no toxicity was evident.

A-ring substituted estrogens have shown activity against hormone dependent breast cancer (MXT) when administered p.o. (Table 3). In this group of experiments various ethers of 4-nitroestrone were examined for their ability to inhibit tumor growth. The propyl, isopropyl, and 3-hydroxypropyl as well as methyl ethers were active tumor inhibitors whereas the ethyl and n-butyl ethers would not be classified as growth inhibitors (to be classified as active an agent must inhibit growth more than 42%, by standards of the Drug Evaluation Branch of the National Cancer Institute). If, however, the β-carbon on the ethyl ether was oxidized to an alcohol the antitumor activity became apparent. In general, ethers with an even number of carbons were not carcinostatic whereas ethers with an odd number of carbons were active in either a straight or branched chain (Table 3, isopropyl ether).

Table 3 also contains data which show that tamoxifen at a moderately toxic p.o. dose (67 mg/kg; mean weight loss nadir = −2.0 g/mouse) was inhibitory to the MXT tumor. Likewise, a patently toxic dose of diethylstilbestrol (90 mg/kg, p.o.) only blocked tumor growth to 32% (not significant) of controls, whereas the administration of the nontoxic odd carbon chain ethers of 4-nitroestrone slowed the growth of hormone dependent mammary tumors from 8–19% of controls following p.o. administration (Table 3).

The character and position of the A-ring substituents also affected the growth inhibition of MXT tumors. Like 4-nitroestrone 3-methyl ether, the 4-amino derivative was highly inhibitory when injected s.c. (Table 4). The 4-bromoestrone 3-methyl ether, however, was inactive. Interestingly, if the nitro group was repositioned to the 2-carbon, the inhibitory activity was lost (Table 4). Since 4-nitroestrone which possesses a free phenolic group, was the most inhibitory to the growth of MXT tumors (33 mg/kg administered s.c. for 20 days brought about a T/C of 0% and a T − C of 78 days; Table 4) dealkylation of the 3-O-ethers must be considered as a prerequisite for the antitumor activity of A-ring substituted estrogens. The process
of dealkylation, however, did not play a role in the diminished activity of the 2-nitro derivative, for, in a separate experiment, 2-nitroestrone was inactive at the same administered level (33 mg/kg injected for 20 days s.c. yielded a T/C of 109% and a T – C of –1).

These experiments also demonstrated the relative unimportance of the 3-phenolic function to tumor inhibition (both 4-nitro and 4-aminoestratriene-17β-ol were highly inhibitory; Table 4).

Although transplantable tumors serve as excellent models with which to test carcinostatic agents by their administration soon after tumor implantation, the more potent tumor inhibitors are capable of halting growth after the neoplasm has established a growth rate. When the daily s.c. injections of [6,7-3H]-4-nitroestrone 3-methyl ether (200 mg/kg) were initiated 17 days following implantation of the MXT tumor, growth of the neoplasm was slowed considerably (Fig. 3), but regressions did not occur. During administration of this labeled antitumor agent, the plasma concentration of radioactivity was seen to increase within 5 days to a level corresponding to 12 μg/ml of injected compound, after which the plasma concentration remained relatively constant (Fig. 3). In other experiments where the injections were continued for 30 days, MXT tumor growth ceased entirely but again regressions did not occur. When the administration of antitumor compound was discontinued, tumor growth resumed, but at a rate slower than controls.

Examination of the more active A-ring substituted estrogens for their activity against established MXT tumors showed these agents to be growth inhibitory whereas tamoxifen was ineffective in this system (Table 5). The active carcinostatic agents in this experiment were 4-nitroestrone, 4-nitroestratriene-17β-ol, 4-aminoestratriene-17β-ol, and 4-aminoestrone 3-methyl ether. These A-ring substituted estrogens were not toxic (no weight loss) and increased the survival of the mice. In another experiment (data not shown) 4-nitroestrone 3-methyl ether inhibited the growth of established MXT tumors (treatment began 15...
DISCUSSION

A new class of compounds has been identified which is capable of inhibiting the growth of hormone dependent mammary tumors. The A-ring substituted estrogens inhibit the growth of newly implanted or established MXT tumors, when administered either by s.c. or i.p. injections or by intubation. These nontoxic agents exhibited specificity with regard to both the function and the position of the substituent added to the A-ring. Amino and nitro groups, introduced at position 4 of estrone 3-methyl ether were particularly carcinostatic, a property not shared by 4-bromoestrone 3-methyl ether. In addition, tumor inhibition was greatly diminished by placing the nitro group instead at the other ortho position (i.e., carbon 2). This was the case whether the nitroestrones were administered as 3-methyl ethers or as 3-phenolic nitroestrogens.

It has previously been observed in experiments with the DMBA-induced rat mammary tumor that approximately 15% of administered 4-nitroestrone 3-methyl ether was dealkylated in experiments where this compound significantly blocked tumor growth (8). Yet, the putative metabolic product, 4-nitroestriol, did not display inhibitory activity in the DMBA model (8). However, the studies reported herein clearly show 4-nitroestriol to be inhibitory. It, therefore, seems reasonable to conclude that this product of the in vivo dealkylation of 4-nitroestrone (and its reduced form, 4-nitroestradiol-17β) to be capable of binding to the estrogen receptor, bringing about tight chromatin association ("translocation") which was followed by the induction of increased levels of progesterone receptor in cultures of human breast cancer cells (MCF-7). Interestingly, the 2-nitro derivative (8) may be an active component in mammary tumor inhibition. This being the case, it is likely that 4-nitroestrone influenced tumor growth by binding to the estrogen receptor, and subsequently the complex may interact with chromatin of the neoplastic cell. Data from other studies in this laboratory (20, 21) have shown 4-nitroestrone (and its reduced form, 4-nitroestradiol-17β) to be capable of binding to the estrogen receptor, bringing about tight chromatin association ("translocation") which was followed by the induction of increased levels of progesterone receptor in cultures of human breast cancer cells (MCF-7). Interestingly, the 2-nitro derivative of 17β-estradiol did not induce progesterone receptor in this in vitro system. The information available would suggest that certain A-ring substitutions on the estrogen molecule (e.g., 4-nitro) may bring about a negative influence on estrogen receptor mediated responses such as growth while having no effect on the estrogenic induction of progesterone receptor.

In this connection, one must consider the inhibition of the MXT tumors implanted in male B6D2F1 mice (Fig. 2). As described in "Results," the MXT transplantable murine mammary tumor is a heterogeneous neoplasm with a portion of its cellular make up being hormone dependent. Depending on the passage, as much as 93% of transplanted cells require female hormones for optimal growth. It is apparent from the data in Fig. 2 that those tumor cells which grow in the male will also grow in castrates. These ovarian hormone independent cells will increase in the tumor's make up with passage number. Yet, certain A-ring substituted estrogens will inhibit the growth of these neoplastic mammary cells which progress in male and castrate B6D2F1 mice (Fig. 2). This observation is analogous to the experience with DMBA-induced mammary tumors in castrate B6D2F1 mice (Fig. 2).
rats, which regress upon ovariectomy but regrow in the castrate host as ovarian hormone independent tumors. The latter have been shown to regress after hormonal therapy (3). This often observed scenario is unrelated to the hormone independent estrogen receptor positive tumor (e.g., 16/C mammary adenocarcinoma) in which the receptor mechanism is inoperative.

The observation that 4-substituted nitro- and amino-estra-
trien-17β-ols also inhibit the growth of MX7 tumors increases the complexity of this phenomenon (Table 4). 4-Nitroestra-
trien-17β-ol (which lacks the 3-phenolic OH group) is not capable of competing with tritiated 17β-estradiol for receptor in cytosolic assays nor is this 3-deoxyestrogen derivative capable of induction of the progesterone receptor in MCF-7 cells (21). It would appear, then, that tumor inhibition is not wholly mediated via the receptor mechanism. Alternatively these com-
ounds may interact with receptor in a manner exclusive of 17β-estradiol competition which then negates the effect of the receptor complex on growth or progesterone receptor induction.

Estrogen sulfotransferase is a high affinity enzyme that is characteristic of many estrogen target tissues (22–24); however, its role has not been determined. All of the A-ring substituted estrogens, which inhibited growth of the MXT tumor have been shown to specifically inhibit estrogen sulfurylation (11, 25), with the 4-substituted species being most active. 4-Nitroestraftien-17β-ol has been shown to have the lowest Kᵢ for estrogen sulfotransferase (2.3 μM) (10). However, 4-nitroestrone 3-
 methyl ether is also an efficient enzyme inhibitor, and 4-nitroestrone would interfere with the endogenous activity of estrogen sulfotransferase since it is a preferred substrate (26). Sulfotrans-
ferase inhibition in hormone dependent breast tumors may possibly increase nuclear translocation of endogenous 17β-
estriadiol since the enzyme would be prohibited from forming estrogen sulfates, which, incidentally, do not bind to receptor (27). Whereas estrogen sulfotransferase is present in DMBA-
duced rat mammary tumors and its activity has been shown to vary with growth and regression (22), this enzyme does not appear to be functional in the MXT tumor. Furthermore this mechanism would appear to be less important since the A-ring substituted estrogens effectively blocked MXT tumor growth in male B6D2F₁ mice (Fig. 2).

Hendry et al. (28) have recently reported on the stereochemi-
ical complimentarity of certain regions of DNA with estrogens. These investigators have constructed models which demonstrated that a partially unwound area of DNA comprised of 5'-
dTdT-G-3'·5'-dCdA-3' allowed for estrogens to fit within the helix. This fit could tolerate 4-substitution but not 2-substitution. Such an explanation would have relevance only if the A-ring substituted estrogen was unable to function as an estrogen following insertion into DNA.

In summary, the evidence indicates that A-ring substituted estrogens may function as growth inhibitors via the estrogen receptor mechanism in the case of 4-nitro or 4-amoineostro-
ne, both of which bind to receptor and induce progesterone receptor (21). The 3-methyl ether of these compounds also blocked tumor growth, possibly through in vivo dealkylation (8) forming the above A-ring substituted estrogens. On the other hand, A-ring substituted 3-deoxyestrogens (particularly 4-nitro- and 4-
amoestra-trien-17β-ol), which do not compete with 17β-estradio-
il for receptor (21), were also excellent inhibitors of hormone dependent breast tumors and, therefore, must express their activity by mechanisms other than that usually attributed to receptor. All the above inhibitors exhibited properties distinct from tamoxifen which (a) displays toxicity at optimum inhibi-
tory doses, (b) is inactive or marginally active in the examined animal models for hormone dependent breast cancer, and (c) is cytotoxic to MCF-7 cells in culture (29).

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