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Inhibition of Angiogenesis by Antiestrogens[1]

A. Gagliardi and D. C. Collins[2]

Research Service, Veterans Affairs Medical Center [A. G., D. C. C.], and Departments of Physiology [A. G.] and Obstetrics and Gynecology [D. C. C.], University of Kentucky College of Medicine, Lexington, Kentucky 40536

ABSTRACT

In this study, we have determined the ability of the partial estrogen antagonists, clomiphene, tamoxifen, and nafoxidine, and the pure estrogen antagonists, ICI 164,384 and ICI 182,780, to inhibit angiogenesis in the chick egg chorioallantoic membrane. All of the partial estrogen antagonists and the pure estrogen antagonist, ICI 182,780, showed significant angiostatic activity in a dose-related manner. The addition of up to 5-fold of 17β-estradiol to the disks containing clomiphene, tamoxifen, or ICI 182,780 did not alter the angiostatic activity of these antiestrogens.

These novel findings show that the antiestrogens are effective inhibitors of angiogenesis. The finding that angiostatic activity is not altered in the presence of excess estrogens suggests that this activity is exerted via mechanisms other than their inhibition of estrogen action. This angiostatic activity may contribute to the therapeutic effect of antiestrogens in estrogen receptor-negative tumors.

INTRODUCTION

Lerner *et al.* (1) first described a group of nonsteroidal compounds that inhibited the trophic effects of naturally occurring estrogens. This work has resulted in the development of a large number of antiestrogens that competitively bind to the estrogen receptor and have been used as therapeutic agents for endocrine responsive tumors. Jordan and Murphy (2) questioned whether the antitumor effect of the antiestrogens is due solely to the blockade of the estrogen receptor. Antiestrogens have been shown to alter the activity of a number of growth factors that are important in the control of cellular proliferation (3, 4) and to inhibit ornithine decarboxylase activity (5), cholesterol synthesis (6), protein kinase C (7), and microsomal lipid peroxidation (8).

Recent results from several laboratories suggest that tamoxifen is an effective therapeutic agent, even in estrogen receptor-negative breast cancer (9, 10). Knabbe *et al.* (10) reported that TGFβ was enhanced by tamoxifen treatment in estrogen receptor-negative breast cancer and suggested a role for TGFβ in the inhibition of tumorigenesis in these cells.

Tumor growth and metastasis are dependent on vigorous angiogenesis (11). For this reason, inhibitors of angiogenesis have become an important potential approach for cancer therapy. TGFβ peptides have been shown to inhibit neovascularization by reducing endothelial cell mitosis (12). Nonsteroidal antiestrogens are also potent inhibitors of protein kinase C, which also plays an important role in angiogenesis (13).

In this study, we have demonstrated that three nonsteroidal antiestrogens, clomiphene, nafoxidine, and tamoxifen, and two steroidal antiestrogens, ICI 164,384 and ICI 182,780, inhibited angiogenesis in a dose-related manner. In addition, the interaction of the antiestrogens with heparin and 17β-estradiol is described.

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[2] To whom requests for reprints should be addressed, at A-301 Kentucky Clinic, University of Kentucky Medical Center, Lexington, KY 40536-0284.

[3] The abbreviations used are: TGFβ, transforming growth factor β; CAM, chorioallantoic membrane; DMSO, dimethyl sulfoxide; ICI 164,384, *N-n*-butyl-*N*-methyl-11-[3,17β-dehydroxyestra-1,3,5(10)-triene-7α-yl]undenanamide; ICI 182,780, 7α-[9-(4,4,5,5,5-pentafluoropentasulfinyl)nonyl]estra-1,3,5(10)-triene-3,17β-diol.

MATERIALS AND METHODS

Heparin sodium USP (PM-20487; activity, 171 units/mg) was a gift from Dr. Judah Folkman, Boston, MA. The antiestrogens and 17β-estradiol were obtained from Sigma Chemical Co., St. Louis, MO, or Steraloids, Inc., Wilton, NH. The two steroidal antiestrogens, ICI 164,384 and ICI 182,780 were a gift of Dr. A. E. Wakeling, ICI Pharmaceuticals, Cheshire, England.

The ability of these compounds to inhibit angiogenesis was determined by using a modification of the chick CAM assay described by Crum *et al.* (14) and Gagliardi *et al.* (15). The chicken embryos, obtained from Sunrise Farms, Catskill, NY, were cracked and then placed in Petri dishes in a humidified CO_2 incubator (3% CO_2/air) at 37°C on day 3. Each compound or combination of compounds was dissolved in methylcellulose (0.45%) containing 2% DMSO. The solution (10 μl) was air dried on a Teflon-coated metal tray (2-mm diameter) and implanted on the outer one-third of a 6-day chorioallantoic membrane where capillaries were still growing. The zone around the methylcellulose disk was examined 48 h after implantation. An inhibition of angiogenesis was indicated by an avascular zone of ≥4-mm diameter around the methylcellulose disk. The results were expressed as the percentage of embryos which showed inhibition of angiogenesis. The blank methylcellulose disk and cortisol-21-phosphate (100 μg) plus heparin (80 μg) were used as negative and positive controls.

RESULTS

A major problem with some steroids and antiestrogens is their low solubility in aqueous media. DMSO or DMF was used to increase the solubility of the steroids and antiestrogens in this study. The effect of DMSO and DMF on the percentage of inhibition of angiogenesis is shown in Table 1. No significant inhibition of angiogenesis occurred when up to 4% DMSO or 2% DMF was added to the methylcellulose disks. On the basis of these results, the methylcellulose disks used in this study included 2% DMSO, a level which showed no significant inhibition of angiogenesis.

The effects of increasing amounts of the nonsteroidal antiestrogens, tamoxifen, clomiphene, and nafoxidine, are shown in Tables 2–4 and Fig. 1. The inhibition of angiogenesis increased in a dose-related manner for all three nonsteroidal antiestrogens. The results with clomiphene (Table 2; Fig. 1) showed significant inhibition when 25 μg were added to the disk (23% inhibition) and increased in a dose-related manner to 73% inhibition of angiogenesis when 150 μg of clomiphene were added. Tamoxifen (Table 3; Fig. 1) showed a similar pattern, with significant inhibition at 25 μg (10%) and increased to 74% inhibition of angiogenesis when 150 μg of tamoxifen were added. Nafoxidine (Table 4; Fig. 1) showed a similar pattern, but a slower increase from 11% inhibition at 25 μg to 56% inhibition of angiogenesis at 150 μg.

The two 7α-alkylamide analogues of 17β-estradiol (ICI 164,384 and ICI 182,780), which show pure antiestrogenic activity, also inhibited angiogenesis (Table 5; Fig. 1). The angiostatic effect increased in a dose-related manner from 21% at 25 μg to 68% at 100 μg of ICI 182,780. Because of the limited amount of ICI 164,384 available, only two levels, 50 and 100 μg/disk were studied. ICI 164,384 showed high levels of angiostatic activity with 57 and 80% inhibition at 50 and 100 μg/disk, respectively.

The effect of 17β-estradiol on the percentage of inhibition of angiogenesis by clomiphene is shown in Tables 1–5. Table 1 shows the effect of 17β-estradiol alone on angiogenesis. A small but significant

Table 1 *Percentage of inhibition of angiogenesis by DMSO, DMF, heparin, or 17β-estradiol*

The number of positive eggs/total (+eggs/total) and the percentage showing inhibition of angiogenesis (% of positive) are shown.

Compound	+eggs/total	% of positive
DMSO only (%)		
1	0/42	0
2	0/26	0
4	0/24	0
8	3/27	11
16	15/36	45
32	8/20	40
DMF only (%)		
1	0/23	0
2	0/25	0
4	6/37	16
8	7/22	32
16	6/24	25
32	9/23	39
Heparin only (μg/disk)		
50	0/65	0
100	0/27	0
17β-estradiol only (μg/disk)		
50	2/26	8
100	4/27	15
500	0/21	0
750	0/19	0
Cortisol-21-PO_4 (μg/disk)		
50	2/22	9
100	15/55	27
100 + 80 μg heparin	27/41	66

Table 2 *Percentage of inhibition of angiogenesis by the antiestrogen, clomiphene alone, and in the presence of 17β-estradiol or heparin*

The number of positive eggs/total eggs (+eggs/total) and the percentage showing inhibition of angiogenesis (% of positive) are shown.

Compound	+eggs/total	% of positive
Clomiphene only (μg/disk)		
25	8/35	23
50	24/55	44
100	13/22	59
150	24/33	73
Clomiphene + 17β-estradiol (μg/disk)		
50 + 50	20/55	37
50 + 250	7/23	30
150 + 50	22/30	75
150 + 150	17/23	74
150 + 300	13/20	65
Clomiphene + heparin (μg/disk)		
25 + 20	2/27	7
50 + 40	10/35	29
100 + 80	10/19	53
150 + 120	22/40	55

inhibition of angiogenesis occurred when 50 or 100 μg of 17β-estradiol alone were added as shown in Table 1. This small inhibition did not occur when increased amounts of 500 or 750 μg of 17β-estradiol were added. No significant reduction in the percentage of inhibition of angiogenesis was seen when 17β-estradiol (50–300 μg) was added to the disk with 25–150 μg of clomiphene. Similar results were obtained when up to 750 μg of 17β-estradiol were added to the disks containing tamoxifen (Table 3) and nafoxidine (Table 4). Levels of 17β-estradiol up to 5 times the antiestrogen level did not show a significant decrease in the percentage of inhibition of angiogenesis of these three nonsteroidal antiestrogens. In the case of the pure steroidal antiestrogen, ICI 182,780 (Table 5), there was a small decrease in the angiostatic activity when 5 times the amount of 17β-estradiol was added.

The effect of heparin on the angiostatic activity in the presence of cortisol-21-phosphate (Table 1) confirms our previous results that heparin in the presence of cortisol-21-phosphate greatly increased the angiostatic activity. On the other hand, the addition of heparin to disks containing the antiestrogens, clomiphene (Table 2) or nafoxidine (Table 4), significantly reduced their angiostatic activity.

DISCUSSION

The nonsteroidal antiestrogens, especially tamoxifen, have been extensively used in breast cancer therapy (16). The antiestrogens are thought to elicit their therapeutic effects by competing with endogenous estrogens for the estrogen receptor. However, more recent studies suggest that the antitumor effect of the antiestrogens is not due solely to the inhibition of estrogen receptor-mediated action (17, 18). Antiestrogens have been shown to affect the activity of many growth factors important in the control of cell proliferation (3, 4). They also alter a number of biochemical actions, such as the inhibition of ornithine decarboxylase (5), protein kinase C (7), and microsomal lipid peroxidation (8). In addition, tamoxifen has been shown to have positive therapeutic effects on women with estrogen receptor-negative breast cancer (2, 9, 10).

In this report, we have shown for the first time that the antiestrogens, clomiphene, nafoxidine, and tamoxifen, inhibited angiogenesis in the CAM assay. Tamoxifen and clomiphene have similar angiostatic activity with 73% inhibition of angiogenesis when 150 μg of either antiestrogen were added to the disks (see Fig. 1 and Tables 2–3).

Table 3 *Percentage of inhibition of angiogenesis by the antiestrogen tamoxifen, alone and in the presence of 17β-estradiol or heparin*

The number of positive eggs/total eggs (+eggs/total) and the percentage showing inhibition of angiogenesis (% of positive) are shown.

Compound	+eggs/total	% of positive
Tamoxifen only (μg/disk)		
25	2/20	10
50	8/21	38
100	25/40	61
150	16/23	74
Tamoxifen + 17β-estradiol (μg/disk)		
25 + 50	2/25	8
50 + 50	11/30	37
50 + 250	6/20	30
100 + 50	20/38	52
100 + 500	12/21	57
150 + 150	17/26	65
150 + 750	15/24	63
Tamoxifen + heparin (μg/disk)		
25 + 20	1/19	5
50 + 40	4/20	20
100 + 80	8/20	40
150 + 120	11/22	50

Table 4 *Percentage of inhibition of angiogenesis by the antiestrogen nafoxidine, alone and in the presence of 17β-estradiol or heparin*

The number of positive eggs/total eggs (+eggs/total) and the percentage showing inhibition of angiogenesis (% of positive) are shown.

Compound	+eggs/total	% of positive
Nafoxidine only (μg/disk)		
25	2/20	10
50	8/71	11
100	9/24	38
150	14/25	56
Nafoxidine + 17β-estradiol (μg/disk)		
50 + 150	4/28	14
100 + 250	7/20	35
Nafoxidine + heparin (μg/disk)		
25 + 20	2/22	9
50 + 40	4/26	15
100 + 80	15/42	36
150 + 120	10/20	50

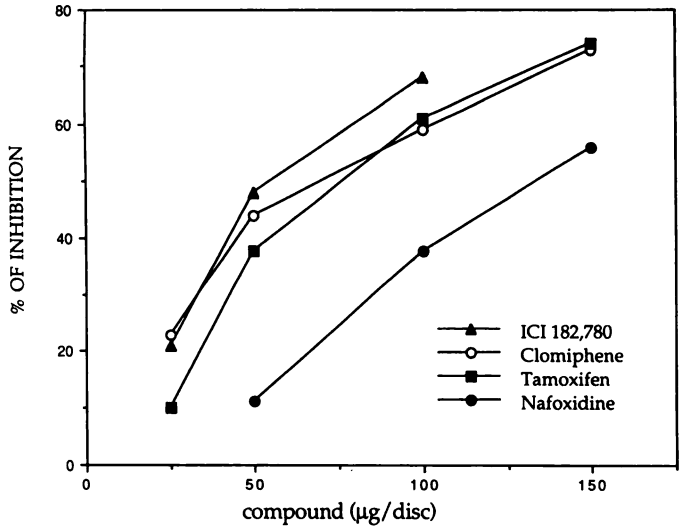

Fig. 1. Percentage of inhibition of increasing amounts of the antiestrogens, clomiphene, tamoxifen, nafoxidine, and ICI 182,780.

Table 5 *Percentage of inhibition of angiogenesis by two 7α-alkylamide analogues of estradiol (ICI 164,384 and ICI 182,780) which show pure estrogen antagonist activity, alone and in the presence of 17β-estradiol*

The number of positive eggs/total eggs (+eggs/total) and the percentage showing inhibition of angiogenesis (% of positive) are shown.

Compound	+eggs/total	% of positive
ICI 164,384 only (μg/disk)		
50	12/21	57
100	24/30	80
ICI 182,780 only (μg/disk)		
25	6/28	21
50	20/42	48
100	25/37	68
ICI 182,780 + 17β-estradiol (μg/disk)		
25 + 125	5/20	25
50 + 250	7/21	33
100 + 500	15/26	58

Nafoxidine had less angiostatic activity (56% inhibition with 150 μg of nafoxidine). Two steroidal antiestrogens, ICI 164,384 and ICI 182,780, which have pure antagonist activity (19), were tested for their ability to inhibit angiogenesis in the CAM assay. These 7α-alkylamide analogues of 17β-estradiol bind estrogen receptors with high affinity without activating any of the normal transcriptional estrogen responses. These pure antiestrogens are devoid of stimulatory activity and completely block the trophic activity of estrogens and partial antiestrogens (19). They displayed more angiostatic activity than the nonsteroidal partial antagonists. ICI 164,384 and ICI 182,780 showed 80 and 68% inhibition of angiogenesis at 100 μg/disk, respectively (see Table 5 and Fig. 1). The angiostatic activity increased in a dose-related manner for ICI 182,780. However, because of the small amount of ICI 164,384 available, we were only able to test it at two levels (50 and 100 μg). The data at these two points suggest that ICI 164,384 may be the most active angiostatic antiestrogen of the five tested in this report.

17β-Estradiol was added to the disk alone and with the two partial estrogen antagonists, clomiphene (Table 2), tamoxifen (Table 3), and the pure estrogen antagonist, ICI 182,780 (Table 5). The addition of up to 5-fold more 17β-estradiol did not significantly alter the angiostatic activity of either the partial or the pure estrogen antagonists. These results suggest that the inhibition of angiogenesis shown by the antiestrogens may not be through their competition for the estrogen receptor.

The novel findings reported here show that the antiestrogens are effective inhibitors of angiogenesis. The fact that angiostatic activity is not altered by the presence of estrogens provides further evidence that the antiestrogens exert an antitumor activity via mechanisms other than direct inhibition of estrogen action. Further studies are necessary to characterize this angiostatic activity by the antiestrogens.

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