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A DIPHENYLMETHANE DERIVATIVE SELECTIVE FOR THE ANTI-ESTROGEN BINDING SITE MAY HELP DEFINE ITS BIOLOGICAL ROLE

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SUMMARY: By employing as a probe the new compound, N,N-diethyl-2-[(4-phenylmethyl)-phenoxy]-ethanamine·HCl (N,N-DPPE), which preferentially binds the anti-estrogen binding site, it is demonstrated that this site appears to contribute to the growth inhibitory action of tamoxifen on MCF-7 human breast cancer cells, even at lower concentrations of this anti-estrogen (1×10^{-7} M to 1×10^{-6} M) at which the major effect is clearly mediated via estrogen receptor. The combination of N,N-DPPE and tamoxifen is additive and this effect is not abolished by 17β-estradiol. This suggests that the anti-estrogen binding site is not simply a passive reservoir for binding tamoxifen, but may itself mediate the cytotoxic effects of specific ligands.

The triphenylethylene derivative tamoxifen (TAM) is widely used in the clinical therapy of breast cancer (1). This anti-estrogenic compound competes both for the estrogen receptor (ER) (2) and the more recently described anti-estrogen binding site (AEBS) (3). Recent in vitro studies with human breast cancer cell lines suggest that at concentrations of TAM achievable in vivo (1×10^{-7} to 1×10^{-6} M) an estradiol (E_2)-reversible inhibition of cell growth occurs which correlates with ER-status but not AEBS (4). At in vitro concentrations of TAM $\geq 1 \times 10^{-6}$ M a rapid non-E_2 reversible cytotoxic effect is observed irrespective of ER status (5), suggesting to some that this latter effect may be non-specific and of little relevance to the in vivo situation (6), while others have speculated that a cytotoxic effect of TAM, possibly mediated by interaction of the alkylaminoethoxy side chain with AEBS may still be important (7,8).

In order to better assess a possible role for AEBS independent from ER in the mechanism of action of TAM, a ligand which binds preferentially to AEBS is necessary. We have recently described the synthesis of a novel com-

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pound, N,N-diethyl-2-[4(phenylmethyl)-phenoxy]-ethanamine·HCl (N,N-DPPE) which binds with high affinity to AEBS in rat liver microsomes while showing no affinity for ER in rat uterine cytosol at concentrations $\leq 1 \times 10^{-5}$ M and no ability at concentrations of 1×10^{-8} M to induce progesterone receptor (PgR) in MCF-7 breast cancer cells (9). This paper describes the growth inhibitory properties of N,N-DPPE and its interaction with TAM on the ER-positive, AEBS-positive MCF-7 human breast cancer cell line and suggests a possible role for AEBS in mediating cytoxicity.

MATERIALS AND METHODS

Chemicals: Tamoxifen citrate (trans-(p-dimethylamino-ethoxy-phenyl)-1,2-diphenyl but-1-ene) and 17β-estradiol were purchased from Sigma Chemical Co., St. Louis, MO. N,N-DPPE (N,N-diethyl-2-[(4 phenylmethyl)-phenoxy]ethanamine-HCl) was synthesized in our laboratory as described previously (9).

Cell Line and Receptor Studies: The MCF-7 human breast cancer cell line was maintained at 37°C in 5% CO_2 in 75cm^2 tissue culture flasks (Corning Glassware, Corning, N.Y.) containing Dulbecco's modified Eagle's medium (Grand Island Biological Co., Grand Island, N.Y.) supplemented with 10 µg/ml insulin, 3.5 mg/ml glucose and 10% fetal calf serum (GIBCO). ER and AEBS assays were carried out using a microplate adaptation of the standard dextran-coated charcoal assay as described previously (9,10). For MCF-7 cells, ER concentration was 105 ± 23 fmoles/mg cytosol protein while that for AEBS was 1120 ± 299 fmoles/mg protein in the microsomal fraction.

Growth Inhibition Assays: For 7 day experiments approximately 2×10^4 MCF-7 cells were seeded into replicate 9.62cm^2 wells (Linbro, Flow Laboratories, McLean, VA.) containing Dulbecco's modified Eagle's medium supplemented with insulin, glucose and 3.5% dextran-coated charcoal (DCC)-stripped fetal calf serum. After 24 hours, increasing concentrations of either TAM or N,N-DPPE were added to each set of replicate wells, both in the presence and absence of 1×10^{-8} M 17β-estradiol (E_2). After 7 days cells were removed by treatment with Isoton II buffer. Cell numbers were determined by Coulter counter. For 24 hour cytotoxicity assays approximately 5×10^5 cells were seeded as above; 2% DCC-stripped fetal calf serum was employed. After removal by Isoton II buffer, to 10 µl of cell suspension was added an equal volume of trypan blue dye and surviving cells were estimated by hemocytometer. To assess the effects of N,N-DPPE on growth inhibition of MCF-7 cells in the presence of TAM, increasing ratios of N,N-DPPE (1x,2x,4x,8x), based on a concentration of 5×10^{-7} M TAM, were added in the presence and absence of 17β-estradiol (E_2) for 7 days. Growth inhibition was assessed as described above. All results were analyzed using Student's 2-tailed t-test.

RESULTS AND DISCUSSION

The effect of increasing concentrations of TAM and N,N-DPPE on the growth of MCF-7 cells over 7 days is shown in Fig. 1. For TAM (Fig. 1A) 3 types of inhibition are seen: 1) a significant inhibition of cell growth occurring in the absence of E_2 between 1×10^{-7} M and 7.5×10^{-7} M (50% inhibition at 5.6×10^{-7} M) which is almost completely reversible by the addition of 1×10^{-8} M E_2,

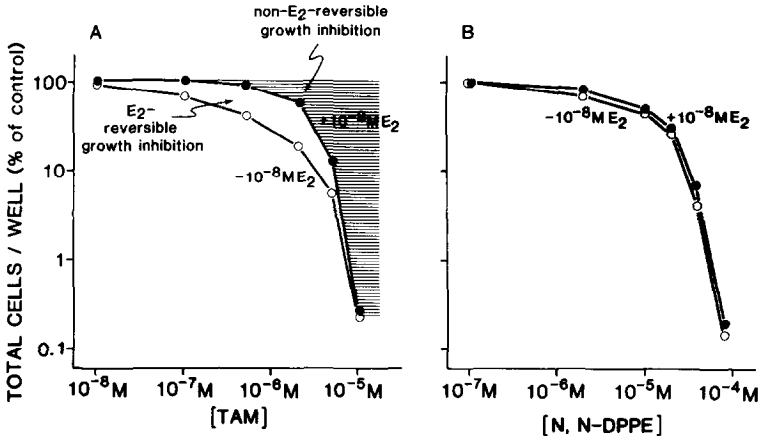

Figure 1:(A) Growth inhibition of ER-positive, AEBS-positive MCF-7 cells after 7 days of exposure to increasing concentrations of tamoxifen (TAM), in the presence (●) and absence (O) of 1 x 10^{-8} M 17β-estradiol (E_2). Approximately 2 x 10^4 MCF-7 cells were seeded into replicate 9.62cm^2 wells containing Dulbecco's modified Eagle's medium supplemented with insulin (10μg/ml), glucose (3.5mg/ml) and dextran-coated charcoal (DCC)-stripped fetal calf serum (3.5%) to which, after 24 hours, was added increasing concentrations of TAM. After 7 days of growth the cells were removed from the wells by treatment with Isoton II buffer. Total cell number at each concentration was determined by Coulter counter and expressed as a % of cells incubated for 7 days in medium containing 0.1% dimethylformamide (DMF) as vehicle control.

(B): Growth inhibition of MCF-7 cells after 7 days of exposure to increasing concentrations of N,N-DPPE in the presence (●) and absence (O) of E_2.

2) a greater degree of inhibition in the absence of E_2 between 7.5 x 10^{-7} M and 3 x 10^{-6} M which is only partially reversible by the addition of E_2, and 3) a rapidly increasing cytotoxic effect above 3 x 10^{-6} M which becomes increasingly irreversible by E_2 over a very narrow range between 7 x 10^{-6} M and 1 x 10^{-5} M.

In contrast, for N,N-DPPE (Fig. 1B), as might be predicted by its lack of interaction with ER (9), the inhibition observed is not reversible by E_2 either at low or high concentrations; the curve obtained resembles that for TAM in the presence of E_2 (Fig. 1A) except that higher concentrations of N,N-DPPE are required to cause 50% growth inhibition (1 x 10^{-5} M vs. 3 x 10^{-6} M for TAM) and the rapid cytotoxic effect occurs over a narrow range between 2.5 x 10^{-5} M and 7.5 x 10^{-5} M.

To further investigate the cytotoxicity occurring at high concentrations of TAM and N,N-DPPE, time course studies were carried out (Fig. 2). As judged by trypan blue dye exclusion, cell kill occurs rapidly with TAM; 50% cytotoxicity is observed between 1 and 6 hours over a narrow range between 7.5 x 10^{-6} M and

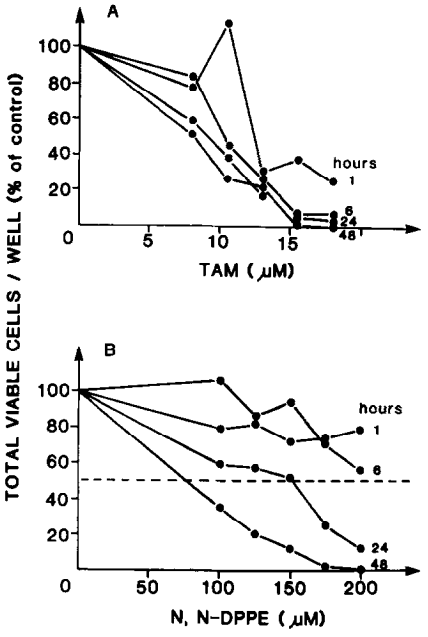

Figure 2:(A) Time course study of exposure of MCF-7 cells to high concentrations of TAM (7.5 x 10^{-6} M to 1.75 x 10^{-5} M) for 1,6,24 and 48 hours. Approximately 5 x 10^{5} MCF-7 cells were seeded into replicate wells as for Fig. 1; 2% DCC-stripped fetal calf serum was employed. After each time period cells were removed from the wells by treatment with Isoton II buffer. Cytotoxicity was assessed by trypan blue dye exclusion; viable cells were counted by hemocytometer and expressed as % of control cells exposed to 0.1% DMF as vehicle control.
(B): Time course study of exposure of MCF-7 cells to high concentrations of N,N-DPPE (10 x 10^{-5} M to 20 x 10^{-5} M) for 1,6,24 and 48 hours.

1.5 x 10^{-5} M; little added effect is seen after this time. In contrast, for N,N-DPPE, almost no effect is seen in the first 6 hours at a 10-fold higher concentration; however, significant cell kill is observed at 24 and 48 hours, with 50% cytotoxicity occurring at 15 x 10^{-5} M N,N-DPPE at 24 hours. The finding that an approximately 10-fold higher concentration of N,N-DPPE as compared to TAM is required to effect cytotoxicity may correlate with its correspondingly lower affinity for AEBS in rat liver microsomes (9).

To test whether N,N-DPPE might influence the effect of TAM on growth of MCF-7 cells at a concentration of TAM normally reversible by E_2, increasing ratios of N,N-DPPE were added to a fixed concentration of TAM (5 x 10^{-7} M), both in the presence and absence of E_2 (Fig. 3). Significant inhibition after 7 days of exposure to TAM alone is seen; this effect is largely but not completely reversed by the addition of 1 x 10^{-8} M E_2 (55 $\pm$ 10% vs. 14 $\pm$ 8% inhibition). The

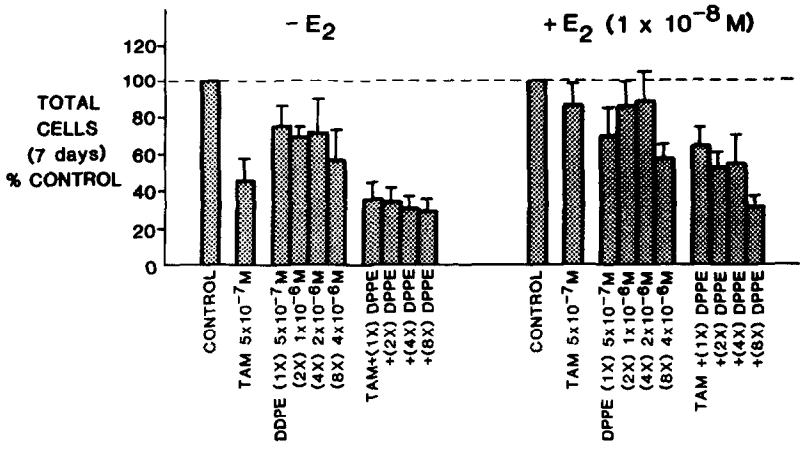

Figure 3: Growth inhibition of MCF-7 cells after 7 days of exposure to TAM alone (5×10^{-7} M), increasing ratios of N,N-DPPE alone (1x,2x,4x,8x TAM concentration) and TAM + N,N-DPPE, all in the presence and absence of E_2. Methodology was as for Figure 1. Cells grown in the absence or presence of 1×10^{-8} M E_2 alone in 0.1% DMF served as control. Bars, S.D. for results of 3 separate experiments.

MCF-7 cells treated with increasing concentrations of N,N-DPPE alone in the absence of E_2 also show significant growth inhibition, but, in all cases, less than for TAM; however, in the presence of E_2, N,N-DPPE demonstrates significantly ($p<.001$) more inhibition than TAM because, as opposed to TAM, there is no significant reversal of its effect by E_2 ($32 \pm 11\%$ vs. $25 \pm 12\%$ inhibition). The combination of N,N-DPPE plus TAM demonstrates significantly ($p<.05$) more inhibition than TAM alone in the absence of E_2; this effect is even more significant ($p<.001$) in the presence of E_2. This strongly argues against a passive effect of N,N-DPPE binding AEBS, allowing a redistribution with more TAM competing for ER (11); if this were the case one would expect to see a lesser effect in the presence of E_2 of TAM plus N,N-DPPE over TAM alone since all available ER should be occupied by E_2, thus preventing any additional interaction with TAM at this site; the abolition of ER-mediated TAM inhibition in the presence of E_2 may thus serve to "unmask" that due to AEBS and make more apparent any additive effects of N,N-DPPE.

The data suggests that TAM may act through AEBS as well as ER, even at lower concentrations achievable in vivo where ER-mediated inhibition predominates; conversely at higher concentrations the AEBS effect, as demonstrated by N,N-DPPE, may predominate. Whether TAM and N,N-DPPE act as drugs rather than specific ligands at higher concentrations causing cytotoxicity is not known, especially as

the nature of AEBS has not been determined. It is possible that cytoxicity is non-specific and occurs when AEBS binding of TAM or N,N-DPPE is exceeded at high concentrations allowing for free drug to act as a cytotoxin; on the other hand, the high concentrations required to effect cytotoxicity may simply reflect the fact that both might be weak ligands competing for AEBS with a putative natural ligand (12) capable of anti-growth effects at significantly lower (? physiological) concentrations. The ubiquitous distribution of AEBS (13) suggests that if this is so, it may play an important role in growth processes in general.

Finally, it is apparent that N,N-DPPE may, in addition to its use as a probe to investigate the biological relevance of AEBS, possess therapeutic potential.

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