Cancer Chemother Pharmacol (1986) 18: 21–23

Cancer Chemotherapy and Pharmacology

The antiproliferative properties of tamoxifen and phenothiazines may be mediated by a unique histamine receptor ($?H_3$) distinct from the calmodulin-binding site*

Lorne J. Brandes[1,2,3], R. Patricia Bogdanovic[3], Max D. Cawker[3], and Ratna Bose[2]

[1] Departments of Medicine and [2]Pharmacology and The Manitoba Institute of Cell Biology[3] University of Manitoba, 100 Olivia Street, Winnipeg, Manitoba, R3E 0V9, Canada

Summary. *N,N*-diethyl-2-[(4-phenylmethyl)-phenoxy]-ethanamine HCl (DPPE), a novel histamine antagonist ($?H_3$), which selectively binds with high affinity to the antiestrogen-binding site (AEBS/$?H_3$), inhibits the activity of calmodulin-dependent myosin light chain kinase (MLCK) only at concentrations >1 m*M*, as opposed to tamoxifen (TAM), which has an $IC_{50}=4$ μ*M* in the same assay. This suggests that the antiestrogen-binding site is distinct from the site on calmodulin which binds TAM and phenothiazines. However, at an in vitro concentration of 1×10^{-6} *M*, the antiproliferative effects of DPPE and several phenothiazines, which also compete for binding to AEBS/$?H_3$, are about equal; this suggests that affinity for AEBS/$?H_3$ rather than that for the calmodulin-binding site may correlate with clinically relevant antigrowth effects of these compounds.

Introduction

Recent studies indicate that phenothiazine derivatives inhibit the growth of malignant cell lines in vitro [6, 17]; it has been suggested that their antiproliferative effects relate to the calmodulin-binding properties of these compounds [7]. In addition, the triphenylethylene-derivative antiestrogen tamoxifen (TAM) [11] has been demonstrated to bind to calmodulin with a somewhat greater affinity than trifluoperazine [9], suggesting to some [10] an additional mechanism for its antiproliferative and antitumor effects.

TAM also binds with higher affinity to a saturable microsomal binding site designated the antiestrogen binding site (AEBS) [16]. We have suggested that this site may be a unique growth-promoting histamine receptor ($?H_3$) [2, 4, 8] as a result of binding studies with what appears to be a selective antagonist for AEBS, *N,N*-diethyl-2-[(4-phenylmethyl)-phenoxy]-ethanamine HCl (DPPE) [1, 3]. DPPE, like TAM and phenothiazines, also demonstrates antiproliferative effects at micromolar concentrations in vitro [1]; at concentrations $>10^{-5}$ *M*, rapid cytotoxic effects are seen [1]. In vivo, DPPE demonstrates antiuterotrophic and antiestrogenic effects in oophorectomized immature rats [2], despite the fact that it does not bind to estrogen receptor (ER) [3]. On the basis of these data, we have suggested, as did Szego previously [13], that the action of estrogens may be mediated through histamine; the antiproliferative and antiestrogenic effects of TAM could be via inhibition of histamine binding to the AEBS/$?H_3$ moiety of a 'linked' ER + AEBS/$?H_3$ receptor + AEBS/$?H_3$ receptor, with TAM binding both sites simultaneously [2]. This may be supported by the findings of others, who have recently suggested that up to 20% of ER may be associated with microsomal membranes [12, 15].

In this study, conducted with DPPE as a selective probe, we present evidence that the microsomal AEBS/$?H_3$ binding site is distinct from the calmodulin site to which TAM and the phenothiazines bind. Furthermore, we show that like TAM, phenothiazine derivatives compete with [^{3}H]DPPE for binding to AEBS/$?H_3$ in rat liver microsomes, and that their affinity for this site, rather than that for the calmodulin binding site, may correlate best with in vitro antiproliferative effects at lower concentrations.

Materials and methods

DPPE hydrochloride was synthesized as previously described [3]. [^{3}H]DPPE (35.6 Ci/m*M*) was custom-synthesized by New England Nuclear (Boston, Mass) [2]. Tamoxifen citrate (Sigma Chemical Company; St Louis, Mo), prochlorperazine (Stemetil; Poulenc, Montreal, Quebec), chlorpromazine (Largactil; Poulenc), trifluoperazine HCl (Stelazine; Smith, Kline and French, Mississanga, Ontario), and promethazine HCl (Phenergan; Poulenc) were employed as cold competitors for [^{3}H]DPPE binding. Pure lyophilized bovine brain calmodulin was obtained from Bio-Rad (Mississanga, Ontario).

Preparation of rat liver microsomes was carried out as previously described [3]. Microsomal fractions were incubated for 5 min at 20 °C with 1.5 n*M* [^{3}H]DPPE and increasing duplicate concentrations of cold competitor as described previously [2]. Specifically bound label in the presence of competitor (Bc) was expressed as a ratio of that in the absence of competitor (Bo). %Bc/Bo [^{3}H]DPPE was plotted against the log molar *(M)* concentration of competitor.

For growth inhibition assays, 1×10^4 MCF-7 breast cancer cells were seeded into replicate 9.62-cm^2 wells (Linbro, Flow Laboratories, McLean, Va) containing Dulbecco's modified Eagle's medium supplemented with insulin,

* This work was supported by grants from the National Cancer Institute of Canada, the Medical Research Council of Canada, and by a generous gift from the estate of Alice Christle.

Offprint requests to: L. J. Brandes

glucose, and 5% decomplemented fetal calf serum. After 24 h, test compounds at a concentration of 1×10^{-6} *M* were added to replicate wells. After 7 days cells were removed by treatment with Isoton II buffer and counted by Coulter counter. For 24-h cytotoxicity assays, 5×10^5 cells were seeded as above in the presence of increasing concentrations of the test compounds; 2% fetal calf serum was employed. After removal with Isoton II buffer, to 10 µl cell suspension was added an equal volume of trypan blue dye, and surviving cells were estimated by hemocytometer.

Inhibition of activation of light chain phosphorylation by the calmodulin-dependent enzyme myosin light chain kinase (MLCK) was used as a measure of calmodulin binding. MLCK was measured by incorporation of ^{32}P from the substrate ATP-$\gamma^{32}P$. The assay mixture consisted of 15 µ*M* turkey gizzard smooth muscle myosin, 2–4 units MLCK, 1 µ*M* calmodulin, 2.5 m*M* $MgCl_2$, 10^{-5} *M* free Ca^{2+}, 40 m*M* imidazole buffer (pH 7.0), and 0.5 m*M* ATP with 1 µCi ATPγ32P. The reaction was started with ATP at 37 °C and terminated after 2 min with 6% trichloroacetic acid (TCA) containing 1.0% Na pyrophosphate. The pellet was washed twice with TCA, and the radioactivity counted. DPPE, TAM and hydroxyzine HCl (Atarax; Pfizer) were tested for their ability to inhibit light chain phosphorylation as a measure of calmodulin binding. It was previously determined that the inhibition of MLCK by DPPE and TAM was completely reversed by adding 1–2 µ*M* calmodulin; hydroxyzine was not tested in this way. All compounds were dissolved in 4% DMSO except for hydroxyzine, which was already in solution; controls contained an equal volume of 4% DMSO.

Results and Discussion

The ability of TAM, DPPE, and hydroxyzine to inhibit the activity of MLCK as a measure of calmodulin binding is shown in Fig. 1; the IC_{50} for TAM is 4 µM, that for hydroxyzine is 200 µ*M*, and that for DPPE is >1 m*M*. This provides strong evidence that calmodulin binding is distinct from binding to AEBS/?H_3, especially since all 3 compounds have approximately equal affinity for the latter site at a concentration of 1×10^{-6} *M* when [^{3}H] TAM is used as the label (4), while DPPE has a somewhat higher

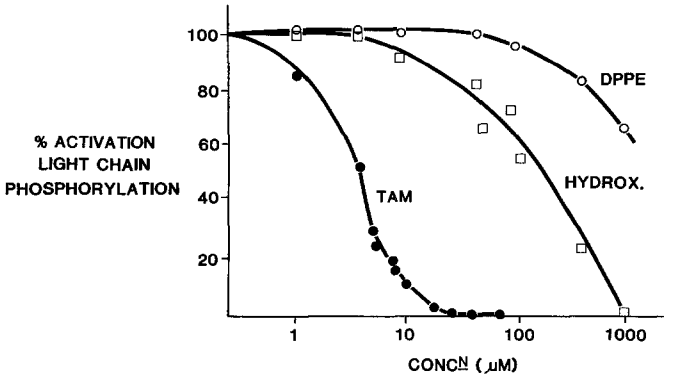

Fig. 1. Inhibition by tamoxifen *(TAM)*, DPPE, and hydroxyzine *(HYDROX)* of activation of light chain phosphorylation by the calmodulin-dependent enzyme myosin light chain kinase, as a measure of calmodulin binding. For TAM and DPPE, the inhibition observed was completely reversed by the addition of 1–2 µ*M* calmodulin (hydroxyzine was not tested for reversal)

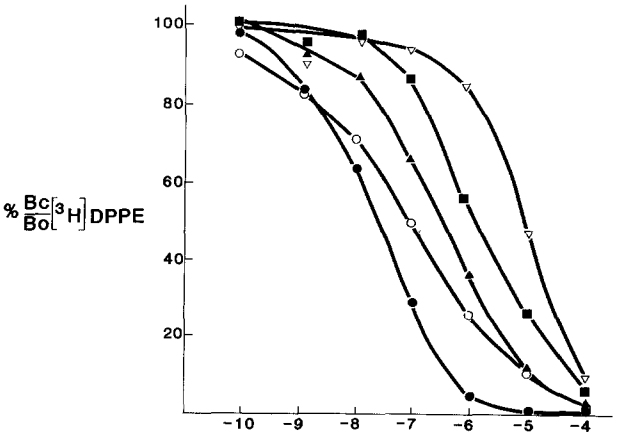

Fig. 2. Competition of cold DPPE (●), prochlorperazine (○), trifluoperazine (▲), chlorpromazine (■) and promethazine (▽) for [^{3}H]DPPE binding as a measure of affinity for AEBS/?H_3

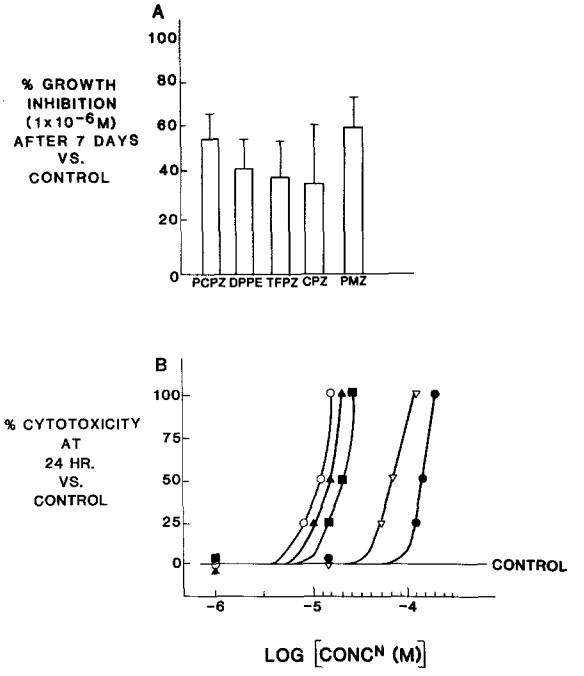

Fig. 3. **A** Growth inhibition vs control of MCF-7 human breast cancer cells after 7 days' exposure to a concentration of 1×10^{-6} *M* of the following compounds: prochlorperazine (PCPZ), DPPE, trifluoperazine *(TFPZ)*, chlorpromazine *(CPZ)*, and promethazine *(PMZ)*. *Bars*, SEM ($n=4$).
B 24-h cytotoxicity assay vs control for MCF-7 human breast cancer cells in the presence of increasing concentrations of prochlorperazine (○), trifluoperazine (▲), chlorpromazine (■), promethazine (▽) and DPPE (●)

affinity than TAM and hydroxyzine for the same site at a similar concentration with [^{3}H]DPPE as the label [2].

Figure 2 shows the ability of the various phenothiazines to compete with [^{3}H]DPPE for binding to the AEBS/?H_3 site in rat liver microsomes. It may be seen that the test compounds compete in the overall order DPPE > prochlorperazine > trifluoperazine > chlorpromazine > promethazine. However, at concentrations $<10^{-8}$ *M*, prochlorperazine has a slightly higher affinity than DPPE.

The antiproliferative effects of the same compounds at a concentration of 1×10^{-6} *M* are shown in Fig. 3 A. It may be seen that the growth inhibition of MCF-7 cells in the presence of DPPE and the phenothiazines vs control is significant and is similar to that observed previously for DPPE and TAM [1]. Although the degree of inhibition observed is not significantly different for any of the compounds, except for promethazine the trend reflects their order of affinity for AEBS/?H_3 (Fig. 2); the inhibition seen for promethazine appears to be at variance both with its much lower affinity for AEBS/?H_3 (Fig. 2) and that for calmodulin binding reported previously by others [18]. This suggests that there may be additional mechanisms by which this particular compound is acting.

Contrary to the findings at 1 μM concentrations, at higher concentrations (Fig. 3 B) the phenothiazines are cytotoxic at an IC_{50} ranging from 10 to 80 μM (in the order of potency; prochlorperazine > trifluoperazine > chlorpromazine > promethazine), while DPPE has a significantly higher IC_{50} of 200 μM. Thus, the order in which the phenothiazines effect high-concentration cytotoxicity appears to reflect their affinity for the calmodulin-binding site [18]. This suggests that calmodulin binding may contribute to high-dose cytotoxicity. However, the high-concentration cytotoxic effects of DPPE do not appear to be due to this mechanism, since the concentration at which 100% cytotoxicity occurs (300 μM; Fig. 3 B) is still far below the IC_{50} for calmodulin binding (> 1000 μM; Fig. 1). Since TAM, DPPE, and phenothiazines are all histamine antagonists [4, 8], and since at high concentrations histamine antagonists have been shown to release histamine [5], we suggest the additional possibility that at high concentrations the cytotoxic effects of these agents may result from enough intracellular histamine release from lysosomes [14] to cause severe perturbation of the cell membrane, resulting in rapid cell lysis. However they may occur, the high concentration effects of TAM, DPPE and the phenothiazines are unlikely to be of clinical relevance, whereas any clinically important antiproliferative effects may be in keeping with their affinity for AEBS/?H_3 at lower concentrations which appear to be achievable in vivo.

References

1. Brandes LJ (1984) A diphenylmethane derivative selective for the antiestrogen binding site may help define its biological role. Biochem Biophys Res Commun 124: 244
2. Brandes LJ, Bogdanovic RP (1986) New evidence that the antiestrogen binding site may be a novel growth-promoting histamine receptor (?H_3) which mediates the antiestrogenic and antiproliferative effects of tamoxifen. Biochem Biophys Res Commun 134: 601
3. Brandes LJ, Hermonat MW (1984) A diphenylmethane derivative specific for the antiestrogen binding site found in rat liver microsomes. Biochem Biophys Res Commun 123: 724
4. Brandes LJ, Macdonald LM, Bogdanovic RP (1985) Evidence that the antiestrogen binding site is a histamine or histamine-like receptor. Biochem Biophys Res Commun 126: 905
5. Goth A (1973) Histamine release by drugs and chemicals In: Schachter M (ed) Histamine and antihistamines, vol 1. Pergamon, Oxford, p 25
6. Hait WN, Lee GL (1985) Characteristics of the cytotoxic effects of the phenothiazine class of calmodulin antagonists. Biochem Pharmacol 34: 3973
7. Hait WN, Grais L, Benz C, Cadman EC (1985) Inhibition of growth of leukemic cells by inhibitors of calmodulin: phenothiazines and melittin. Cancer Chemother Pharmacol 14: 202
8. Kroeger EA, Brandes LJ (1985) Evidence that tamoxifen is a histamine antagonist. Biochem Biophys Res Commun 126: 750
9. Lam PHY (1984) Tamoxifen is a calmodulin antagonist in the activation of CAMP phosphodiesterase. Biochem Biophys Res Commun 118: 27
10. Lyman SD, Jordan VC (1985) Antiestrogen effect of trifluoperazine in mice. Biochem Pharmacol 24: 2221
11. Nicholson RI, Borthwick NM, Daniel CP, Syne JS, Davies P (1981) Biochemical basis of tamoxifen action in hormone-dependent breast cancer In: Sutherland RL, Jordan VC (eds) Non-steroidal antiestrogens, Academic, New York, p 281
12. Parikh I, Anderson WL, Neame P (1980) Identification of high-affinity estrogen binding sites in calf uterine microsomal membranes. J Biol Chem 255: 10266
13. Szego CM (1965) Role of histamine in the mediation of hormone action. Fed Proc 24: 1343
14. Szego CM (1974) The lysosome as a mediator of hormone action In: Greep RO (ed) Recent progress in hormone research, vol 30. Academic, New York, p 171
15. Watson GH, Muldoon TG (1985) Specific binding of estrogen and estrogen-receptor complex by microsomes from estrogen-responsive tissues of the rat. Endocrinology 117: 1341
16. Watts CKW, Sutherland RL (1984) High-affinity specific antiestrogen binding sites are concentrated in rough microsomal membranes of rat liver. Biochem Biophys Res Commun 120: 109
17. Wei JW, Hickie RA, Klassen DJ (1983) Inhibition of human breast cancer colony formation by anticalmodulin agents, trifluoperazine, W-7 and W-13. Cancer Chemother Pharmacol 11: 86
18. Weiss B, Prozileck W, Cimino M, Barnette MS, Wallace TL (1980) Pharmacological regulation of calmodulin. Ann NY Acad Sci 356: 319

Received January 27, 1986/Accepted April 11, 1986