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SHORT COMMUNICATIONS

Affinity of ligands other than triarylethylenes for liver microsomal antiestrogen binding sites*

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Tamoxifen (TAM) (Fig. 1) is a triarylethylene antiestrogen of use in the palliative treatment of breast cancer, and in biochemical studies of the mechanism of action of estrogens [1, 2]. While the molecular mechanisms by which TAM exerts its pharmacologic effects are not fully understood, interference with estrogen receptor function seems to be of the greatest significance [2]. Recently, TAM and other antiestrogens were reported to interact with binding sites distinct from estrogen receptors [3]. While these antiestrogen binding sites (AEBS) were found in estrogen receptor-containing tissue (ovary, uterus), highest concentrations were found in the liver, particularly in the microsomal fraction [4]. Thus, it has been suggested that these sites influence the effects of TAM and related antiestrogens indirectly, by affecting pharmacokinetic parameters.

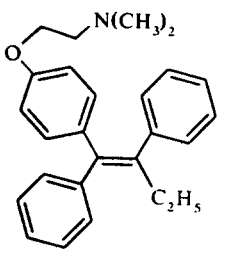

Fig. 1. Structure of tamoxifen.

The basic aminoether side chain in TAM and other triarylethylene antiestrogens seems to be an absolute requirement for AEBS affinity [4, 5]. Also the triarylethylene aromatic moiety appears to be necessary for optimal binding [5], although replacement of this with a diphenylmethyl moiety gave a compound with affinity for AEBS approaching that of TAM [6]. This latter finding suggested to us that compounds bearing aminoether side chains and aromatic moieties besides diphenylmethyl, such as the 1,2-diphenyl, phenothiazine, or dibenzazepine systems, might have affinity for the AEBS. Many drugs and related chemicals contain combinations of these substituents. Thus, the objective of this study was to determine the affinity, relative to that of TAM, of representative compounds bearing basic aminoether side chains and aromatic moieties different from that of TAM.

Materials and methods

Materials. The β-(dimethylamino)ethyl derivatives of *p*-bromophenol, *p*-hydroxybenzophenone, and *p*-hydroxy-α-ethyldeoxybenzoin were prepared as described previously [7–9]. Diphenhydramine hydrochloride, procaine hydrochloride, and metyrapone were purchased from the Sigma Chemical Co., St. Louis, MO. Other chemicals were obtained as gifts, from the manufacturers indicated in parentheses, as follows: brompheniramine maleate (Arnar-Stone Laboratories, McGaw Park, IL); chlorpromazine hydrochloride, SKF 525-A: β′-(diethylamino)-ethyl-α-α-diphenylvalerate hydrochloride, and isopropamide iodide (Smith, Kline & French Laboratories, Philadelphia, PA); imipramine hydrochloride (Ciba-Geigy, Inc., Summit, NJ); and tamoxifen citrate (Stuart Pharmaceuticals, Wilmington, DE).

[^{3}H]TAM (70 Ci/mmole, 0.19 Ci/mg), labeled at one of the N-methyl groups, was purchased from the Amersham Corp., Arlington Heights, IL. Radiochemical purity was checked by thin-layer chromatography using plastic backed precoated sheets (silica gel 60 F_{254}, EM Reagents No. 5775), and chloroform–methanol–28% aqueous ammonia (90:10:0.5, by vol.) as developing solvent. After storage at 8° for 6 months, 95% of the radioactivity present migrated with unlabeled tamoxifen, but after 9 months only 88% of the radioactivity did so. Preparative thin-layer chromatography of an aliquot of the stock solution containing 0.126 μg (24 μCi) was carried out under conditions described above. The zone with R_f equal to that of TAM was cut out and eluted with 3.4 ml of ethanol. The [^{3}H]-TAM in this solution was found to be 98% radiochemically pure.

TEA buffer was composed of Tris base (10 mM), disodium EDTA (1.5 mM) and sodium azide (3 mM). Solution pH was adjusted to 7.2 (25°) by addition of 10% aqueous HCl.

Dextran-coated charcoal suspension was prepared by stirring a suspension of 100 mg of Dextran C and 1 g of Norit A in 100 ml of deionized water for 12 hr at 8°. The mixture was centrifuged, and the precipitate was resuspended in 100 ml of cold TEA buffer. The mixture was stored at 8°.

Tissue preparation. Livers from groups of four 24-day-old female Sprague–Dawley rats were minced and homogenized in 5 vol. of ice-cold 1.15% KCl. The homogenate was centrifuged at 9000 *g* for 20 min. The resulting supernatant fraction was centrifuged at 105,000 *g* for 60 min. The combined microsomal pellets were homogenized in a volume of ice-cold 1.15% KCl equal to one-half that originally used. The homogenate was diluted with an equal volume of ice-cold TEA buffer. After mixing, 1-ml aliquots of the diluted homogenate were lyophilized. (The amount of [^{3}H]TAM-specific binding in reconstituted homogenates prepared in this way was constant for at least 6 weeks.) Lyophilized aliquots were reconstituted in 1.0 ml of ice water and 7.0 ml of ice-cold TEA buffer. Protein concentration was determined colorimetrically [10] and was adjusted to 0.5 mg/ml with ice-cold TEA.

Incubation mixtures. Incubations were carried out in triplicate at 4° in 12.5 × 75 mm polypropylene centrifuge tubes. In each tube was placed 0.2 ml of microsomal suspension (equivalent to 0.1 mg protein) and 0.01 ml of a solution of [^{3}H]TAM (1 pmole) in ethanol. Then 0.01 ml of a solution of unlabeled competitor in *N*,*N*-dimethylacetamide was added. After vortexing, incubation mixtures

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were stored for 4 hr. Then 0.4 ml of dextran-coated charcoal suspension was added to each tube, and after 15 min tubes were centrifuged at 500 *g* for 15 min. An aliquot (0.4 ml) of each supernatant fraction, in 8 ml of Scintiverse II (Fisher), was analyzed for bound [^{3}H]TAM by liquid scintillation spectrometry.

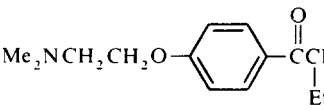
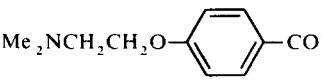
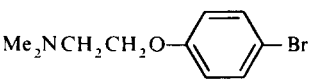
Results and discussion

Preliminary experiments showed specific [^{3}H]TAM binding to be directly proportional to protein concentration when the latter was between 0.05 and 0.12 mg per incubation (0.23 to 0.55 mg protein/ml). Higher protein concentrations resulted in reduced specific binding per mg of protein. Also, specific [^{3}H]TAM binding was unaffected by the presence of 1×10^{-5} M estradiol.

Most of the compounds listed in Table 1 were able to displace specifically bound [^{3}H]TAM from rat liver microsomal binding sites. Metyrapone, a compound containing two aromatic rings but no side chain, had apparent affinity comparable to that of several of the compounds containing both aromatic moieties and basic side chains (e.g. chlorpromazine). No apparent affinity was seen with isopropamide, in which the side chain nitrogen was quaternized, or in brompheniramine, in which the side chain and the aromatic moiety were not separated by an oxygen or a nitrogen atom.

Relative binding affinites (RBA) of the six compounds with the most pronounced effects in the one-point assays are listed in Table 2. TAM, which contains the 1,1,2-triarylethylene system, had the highest RBA. The next three compounds, which have 1,1-diphenylmethyl or 1,2-diphenyl moieties, had RBA values equal to 10–20% of that of TAM. The compound containing a single benzene ring, and the one containing a phenothiazine ring, each had RBA values equal to about 1% that of TAM.

Table 2. Affinity of compounds bearing *N,N*-dialkylaminoalkyl side chains for rat liver microsomal antiestrogen binding sites*

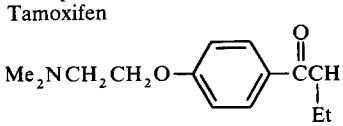
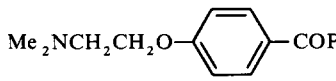
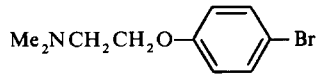
Compound	IC_{50}† (M)	RBA‡
TAM	5.8×10^{-8}	(100)
SKF 525-A	3.2×10^{-7}	18
	2.9×10^{-7}	20
	5.2×10^{-7}	11
	8.7×10^{-6}	0.7
Chlorpromazine	4.0×10^{-6}	1.5

* Six to eight concentrations (10^{-9} M to 10^{-4} M) of each unlabeled competitor were evaluated for ability to displace specifically bound [^{3}H]TAM at a set concentration of 4.5×10^{-9} M. Specific binding of [^{3}H]TAM at each competitor concentration was determined as described in Table 1.

† The concentration required for 50% displacement of specifically bound [^{3}H]TAM.

‡ Relative binding affinity (RBA) = (IC_{50} of TAM/IC_{50} of the test compound) × 100.

Table 1. Inhibition of binding of [^{3}H]TAM to antiestrogen binding sites from immature female rat liver microsomes by compounds bearing *N,N*-dialkylaminoalkyl side chains*

Compound	Aromatic moiety	Conc. (μM)	Inhibition of specific binding (%)
Brompheniramine	Phenyl-2-pyridylmethyl	1.0	0
Diphenhydramine	Diphenylmethyl	10.0	56
SKF 525-A	Diphenylmethyl	1.0	92
Isopropamide†	Diphenylmethyl	10.0	0
Procaine	Phenyl	10.0	2
Imipramine	Dibenzazepine	10.0	60
Chlorpromazine	Phenothiazine	1.0	60
Tamoxifen	1,1,2-Triaryl	10.0	(100)
	1,2-Diphenyl	10.0	100
	Diphenylmethyl	10.0	100
	Phenyl	10.0	76
Metyrapone‡	1,2-Dipyridyl	10.0	59

* The [^{3}H]TAM concentration was 4.5×10^{-9} M in all incubations. Total binding was determined in incubation mixtures to which vehicle (*N,N*-dimethylacetamide) only was added. Nonspecific binding was determined in mixtures containing 1×10^{-5} M unlabeled TAM. Specific binding was calculated by subtracting non specific dpm bound from total dpm bound. Typically, specific binding in the absence of unlabeled competitor was *ca.* 20,000 dpm, which is equivalent to 134 fmoles per incubation (0.1 mg protein).

† This compound contains an *N,N,N*-trialkylammonioalkyl side chain.

‡ This compound does not contain a basic side chain.

The data in Table 1 indicate that compounds with a variety of aromatic moieties are able to compete with [^{3}H]-TAM for binding to rat liver microsomal AEBS. While the data in Table 2 reaffirm AEBS to bind triarylethylenes such as TAM with highest affinity, our results indicate that these sites will bind a relatively diverse collection of structural types, including SKF 525-A and metyrapone.

Since the last compounds are classical inhibitors of drug metabolism [11, 12], it may be that AEBS are a component of the liver microsomal drug-metabolizing enzyme system, which also is known to have relatively low ligand (substrate) specificity [13]. Cytochrome P-450, a component of this system, has been suggested to interact with SKF 525-A [14], metyrapone [12], and TAM [15, 16] in part on the basis of difference spectra generated by addition of each of these drugs to liver microsomal suspensions. Our results indicated an AEBS level of 1.34 pmoles/mg microsomal protein (Table 1), whereas rat liver contains about 1000 pmoles of cytochrome P-450/mg microsomal protein [17]. Thus, if one assumes that the AEBS are associated with cytochrome P-450, the amount involved must be a small percentage of the total amount present. Studies with purified components of the drug-metabolizing enzyme system, including isoenzymatic forms of cytochrome P-450, will be necessary to determine whether any of these constitute the AEBS.

The apparent distribution volume, and thus the extent and duration of action of triarylethylene antiestrogens, has been suggested to be influenced by the affinity of these compounds for AEBS [4, 18]. Our results implicate AEBS in modulation not only of the pharmacokinetics of TAM and related antiestrogens, but also of those of a wide variety of drugs bearing aminoether side chains and aromatic systems like those of compounds in this study.

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REFEERENCES

1. R. L. Sutherland and L. C. Murphy, *Molec. cell. Endocr.* **25**, 5 (1982).
2. R. L. Sutherland and V. C. Jordan (Eds.), *Nonsteroidal Antiestrogens. Molecular Pharmacology and Antitumor Activity.* Academic Press, Sydney (1981).
3. R. L. Sutherland, L. C. Murphy, M. S. Foo, M. D. Green, A. M. Whybourne and Z. S. Krozowski, *Nature, Lond.* **288**, 273 (1980).
4. K. Sudo, F. J. Monsma, Jr. and B. S. Katzenellenbogen, *Endocrinology* **112**, 425 (1983).
5. C. K. W. Watts, L. C. Murphy and R. L. Sutherland, *J. biol. Chem.* **259**, 4223 (1984).
6. L. J. Brandes and M. W. Hermonat, *Biochem. biophys. Res. Commun.* **123**, 724 (1984).
7. P. C. Ruenitz, J. R. Bagley and C. M. Mokler, *J. med. Chem.* **25**, 1056 (1982).
8. R. E. Allen, F. P. Palopoli, E. L. Schumann and M. G. Van Campen, Jr., U.S. Pat. 2,914,561-4 (1959); *Chem. Abstr.* **54**, 5581e (1960).
9. M. J. K. Harper, D. N. Richardson and A. L. Walpole, Br. Patent 1,064,629 (1967); *Chem. Abstr.* **67**, 90515g (1967).
10. O. H. Lowry, N. J. Rosebrough, A. L. Farr and R. J. Randall, *J. biol. Chem.* **193**, 265 (1951).
11. J. R. Cooper, J. Axelrod and B. B. Brodie, *J. Pharmac. exp. Ther.* **112**, 55 (1954).
12. H. G. Jonen, B. Hüthwohl, R. Kahl and G. F. Kahl, *Biochem. Pharmac.* **23**, 1319 (1974).
13. B. Testa and P. Jenner, *Drug Metabolism: Chemical and Biochemical Aspects* pp. 3–135. Marcel Dekker, New York (1976).
14. J. B. Schenkman, H. Remmer and R. W. Estabrook, *Molec. Pharmac.* **3**, 113 (1967).
15. P. C. Ruenitz and M. M. Toledo, *Biochem. Pharmac.* **29**, 1583 (1980).
16. N. M. Meltzer, P. Stang and L. A. Sternson, *Biochem. Pharmac.* **33**, 115 (1984).
17. E. J. Flynn, M. Lynch and V. G. Zannoni, *Biochem. Pharmac.* **21** 2577 (1972).
18. V. C. Jordan and R. A. Bowser-Finn, *Endocrinology* **110**, 1281 (1982).

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Differential inhibition by T-2 toxin of total protein, DNA and isoprenoid synthesis in the culture macrophage cell line J774

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T-2 toxin (4β,15diacetoxy-8α-(3-methylbutyryloxy)-12,13-epoxytrichothec-9-ene-3α-ol.) is part of a major family of mycotoxins known as trichothecenes, which have world-wide distribution and which are implicated in a diverse variety of disease processes in animals and man [1–4]. The clinical picture in both man and animals is determined principally by arrest of normally rapidly proliferating tissues followed by their necrosis, as in bone-marrow, skin and gastrointestinal tract [5–6]. Of major interest is the effect of T-2 toxin on immune function, as leukopenia and agranulocytosis are life threatening complications of toxicity states.

In vitro studies have shown a number of the trichothecene toxins to be potent inhibitors of protein synthesis. These compounds, including T-2 toxin, would bind to ribosome thereby inhibiting peptidyl transferase and blocking initiation of synthesized polypeptides [7]. Although the mechanism of protein synthesis inhibition has been studied *in vitro* there have been very few detailed studies on whole cells aimed at accurately elucidating the effects of T-2 toxin on integrated cell function [8–9].

This study was undertaken to more precisely define the differential stability of J774 macrophage cell enzymes important in normal growth behaviour to the toxicity of T-2, by following the inhibition of protein, DNA and isoprenoid synthesis.

Materials and methods

The cells used in this study were derived from the J774 macrophage cell line originally isolated by Ralph and Nakoinz [11] and cloned in the laboratory of Dr. B. Bloom, Albert Einstein College of Medicine, NY. Cells were cultivated in Eagle's Minimal Essential Medium (MEM) supplemented with glutamine, glucose, 20% horse serum (inac-