Table 2. Inhibition of norepinephrine secretion by TFP-treated cells stimulated with acetylcholine or 56 mM potassium medium*

	Norepinephrine released (ng/10^5 cells)
Zero-time control	33.8 ± 2.0
Zero-time + 2 μM TFP	33.9 ± 1.52
Incubated control	42.6 ± 2.2
Incubated control + 2 μM TFP	49.3 ± 1.4
Stimulated, 0.1 mM acetylcholine	89.0 ± 5.0†
Stimulated, 0.1 mM acetylcholine + 2 μM TFP	53.0 ± 1.2
Stimulated, 56 mM potassium	129.8 ± 4.2†
Stimulated, 56 mM potassium + 2 μM TFP	103.0 ± 3.4†

* Cells were suspended at 2 × 10^6/ml in medium in the presence or absence of 2 μM TFP for a 5-min incubation period at 37°. Aliquots were then pipetted into microfuge tubes containing an equal volume of medium (controls), medium containing 0.2 mM acetylcholine, or 112 mM potassium medium. For TFP-treated groups, the media also contained 2 μM TFP. Each value is the mean ± S.E. for six replicates.

† Significantly different from the incubated control at $P < 0.01$.

inactivation of calmodulin results in inactivation of the secretory system. Inhibition of chromaffin cell secretion by low micromolar concentrations of TFP parallels the results of Schubart *et al.* [13] for protein phosphorylation and insulin secretion by hamster insulinoma cells. They found half-maximal inhibition of these two calcium-dependent functions at less than 3 μM TFP and hypothesized that the drug influenced secretion at an early calcium-dependent step in the secretory pathway. Krausz *et al.* [4] have demonstrated inhibition of an early step of insulin secretion by isolated pancreatic islets but at much higher TFP concentrations (30–100 μM). Our results with chromaffin cell secretion also indicate a predominant role of TFP at an early stage of the secretory process, probably with events surrounding activation of the cholinergic receptor, while events subsequent to membrane depolarization appear to be less affected. It also appears that sufficient calcium is available for the exocytotic phase of secretion since potassium-induced secretion is inhibited by only 21% in the presence of TFP (2.2 mM extracellular calcium).

Our results, and those of others [14, 15] with high TFP concentrations, indicate detrimental effects of the drug on cell function that are probably unrelated to an effect on calmodulin. Thus, considerable care must be exercised in the definition of calmodulin-dependent functions based upon treatment of cells with TFP concentrations in the high micromolar range.

Acknowledgements—We wish to thank Peck Meat Packing, Inc., for the gift of tissue and Smith Kline & French Laboratories for the gift of trifluoperazine. We are especially grateful to Dr. Frank L. Siegel for helpful suggestions.

Marquette University School of Dentistry
Milwaukee, WI 53233, U.S.A.

JACK C. BROOKS*
SUZANNE TREML

* Correspondence concerning this manuscript should be addressed to: Dr. Jack C. Brooks, Department of Basic Sciences, Marquette University School of Dentistry, Milwaukee, WI 53233, U.S.A.

REFERENCES

1. W. Y. Cheung, *Science* **207**, 19 (1980).
2. R. J. DeLorenzo and S. D. Freedman, *Biochem. biophys. Res. Commun.* **80**, 183 (1978).
3. R. J. DeLorenzo, *Ann. N.Y. Acad. Sci.* **356**, 92 (1980).
4. Y. Krausz, C. B. Wollheim, E. Siegel and G. W. G. Sharp, *J. clin. Invest.* **66**, 603 (1980).
5. J. C. Brooks and F. L. Siegel, *J. biol. Chem.* **248**, 4189 (1973).
6. R. M. Levin and B. Weiss, *Molec. Pharmac.* **12**, 581 (1976).
7. R. M. Levin and B. Weiss, *Molec. Pharmac.* **13**, 690 (1977).
8. P. O. Seglen, *Expl Cell Res.* **74**, 450 (1972).
9. E. M. Fenwick, P. B. Fajdiga, N. B. S. Howe and B. G. Livett, *J. Cell Biol.* **76**, 12 (1978).
10. J. C. Brooks, *Endocrinology* **101**, 1369 (1977).
11. U. S. von Euler and U. Hamburg, *Acta physiol. scand.* **19**, 74 (1949).
12. L. R. Hegstrand and B. Eichelman, *J. Chromat.* **222**, 107 (1981).
13. U. K. Schubart, N. Fleischer and J. Erlichmann, *J. biol. Chem.* **222**, 11063 (1980).
14. M. Osborn and K. Weber, *Expl Cell Res.* **130**, 484 (1980).
15. S. D. Levine, W. A. Kachadorian, D. N. Levin and D. Schlondorff, *J. clin. Invest.* **67**, 662 (1981).

Biochemical Pharmacology, Vol. 32, No. 2, pp. 373–375, 1983.
Printed in Great Britain.

0006-2952/83/020373-03 $03.00/0

Identification of a new metabolite of tamoxifen in patient serum during breast cancer therapy

(*Received* 6 *May* 1982; *accepted* 23 *July* 1982)

Tamoxifen, ICI 46,474, *trans*-1-(*p*-β-dimethylaminoethoxyphenyl)-1,2-diphenylbut-1-ene, is a non-steroidal antiestrogen used as a palliative agent in the treatment of advanced breast cancer [1]. This antagonist competes with

estradiol for high affinity binding sites found in some tumors [2]. Upon uptake by a target cell, tamoxifen or a metabolite binds to the cytosolic estrogen receptor and is thought to be translocated to the nucleus whereupon the cell is rendered refractory to estrogen-stimulated mitosis [3]. In humans, 70% of the drug is metabolized within 7 hr of administration [4], and more recent studies have shown that the primary route of metabolism is by hepatic demethylation to *N*-desmethyltamoxifen [5]. After chronic administration, the metabolite *N*-desmethyltamoxifen (Fig. 1) attains serum levels similar to those of tamoxifen [6]. Daniel and others [7, 8] have identified the metabolite 4-hydroxytamoxifen (Fig. 1) as a minor serum component with a circulating level less than 10% of that attained by the parent drug. Studies *in vitro* have shown that the binding affinity of this metabolite is 100-fold greater than that of tamoxifen [9, 10]. Fabian *et al.* [8] have suggested that the low serum levels of 4-hydroxytamoxifen may be sufficient, in view of its activity, to contribute significantly to the net effect of this drug. The purpose of the present report is to present evidence for the identification of a novel tamoxifen metabolite found in breast cancer patients during therapy. This compound has been designated Metabolite Y or *trans*-1-(*p*-*β*-hydroxyethoxyphenyl)-1,2-diphenylbut-1-ene (Fig. 1).

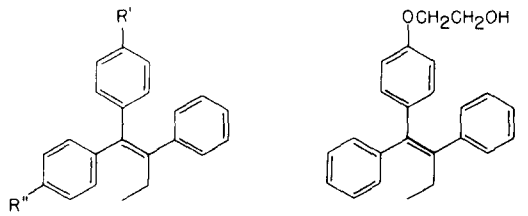
Metabolite Y has been identified in breast cancer patients receiving 10 mg b.i.d. (three patients) and 150 mg b.i.d. (two patients) tamoxifen orally. The apparent concentration of this compound changes with dose elevation and reduction. The relative amounts of Metabolite Y produced at the high dose are disproportionately higher than amounts produced at the lower dose. This compound has antiestrogenic activity *in vivo* and *in vitro* (results being prepared for publication). Preliminary studies indicate that Metabolite Y has approximately 50% the potency of tamoxifen as an antiestrogen in rat uterine weight tests.

Sera from two breast cancer patients receiving 150 mg b.i.d. tamoxifen were extracted (5 vol. hexane–butanol, 98:2) and subjected to analysis by TLC [Kieselgel 60 plates (Merck), toluene–triethylamine–methanol, 90:10:0.2]. In this system, tamoxifen and its metabolites may be converted to fluorescent phenanthrene analogs by brief exposure to intense ultraviolet light (λ_{max} = 254 nm) [11]. This unique feature allows for great specificity and detection to 1 ng tamoxifen. After *in situ* conversion to fluorescent derivatives and scanning with a fluorescence densitometer (U.V.-V.I.S. II, Farrand Optical Co.), an unidentified compound was observed in extracts from these patients (above). This peak exhibited fluorescence only after u.v. activation. The R_f value for this compound (0.25) was distinct from R_f values for tamoxifen (0.41), *N*-desmethyltamoxifen (0.13), and 4-hydroxytamoxifen (0.04). The R_f values for synthetic Metabolite E, *trans*-1-(*p*-hydroxyphenyl)-1,2-diphenylbut-1-ene, and synthetic Metabolite Y were identical (R_f = 0.25) Using high performance liquid chromatography (HPLC) [Perkin–Elmer M 601 Liquid Chromatograph, Alltech 600-Si 10 *μ*m Silica column, P.E. L.C.-55 U.V. Detector, hexane–isopropanol (99:1), 3 ml/min, 900 psi], only synthetic Metabolite E (ret. time = 4.9 min) and synthetic Metabolite Y (ret. time = 10.5 min) would elute. Tamoxifen, *N*-desmethyltamoxifen and 4-hydroxytamoxifen were retained in this system due to the intrinsic polarity of their side chains. Serum extracts from patients on tamoxifen therapy (10 mg b.i.d. or 150 mg b.i.d.) showed a fluorescent peak with a retention time of 10.5 min. No Metabolite E was observed. These compounds were detected by ultraviolet absorption (A = 220 nm) and by fluorescence (Fluorichrome, Varian Instruments, Ex = 220 nm, Em = 370 nm). Fluorescence was generated by post-column u.v. activation (λ_{max} = 254 nm). The description of this novel detection system and its inherent advantages over existing methodology has been reported recently [12]. The serum component eluting with Metabolite Y standard exhibited fluorescence only after u.v. activation. Analysis of the column eluate by u.v. absorption (A = 220 nm) showed this peak (ret. time = 10.5 min) to be a single component with no interference by non-fluorescent compounds.

Serum taken from a patient receiving 150 mg b.i.d. tamoxifen (4 ml) was extracted, concentrated, and purified by HPLC as described above. Detection was by u.v. absorption to avoid conversion to the phenanthrene. This fraction was collected, concentrated under N_2 at 55°, and dissolved in 0.1 ml acetyl chloride (Mallinckrodt AR). This reaction was allowed to proceed for 16 hr at room temperature. The reaction was terminated by evaporation under N_2 at ambient temperature. The acetylated product was dissolved in a minimal volume of methanol (Burdick & Jackson, Spectrograde) and subjected to further purification by gas chromatography (Hewlett Packard, model 5700A Gas Chromatograph). Synthetic Metabolite Y, extracted from normal human serum, HPLC purified, and derivatized to the acetate had a retention time of 12.2 min using 3% OV-17 on 80/100 Supelcoport (Alltech Scientific) at 250° with a gas flow rate of 30 ml/min. The major peak from the derivatized HPLC-purified extract from the patient had a retention time of 12.1 min. The gas chromatograph was coupled to a dodecapole electron impact mass spectrometer (Hewlett Packard model 5930A). The mass spectrum of synthetic Metabolite Y, extracted from normal human serum, and the unknown compound from the patient, both showed three fragments. The mass spectra of the peaks and their relative intensities are compared in Table 1. It seems that this compound fragments primarily by cleavage of its ether linkage.

Serum concentrations of Metabolite Y in breast cancer patients were measured by HPLC (*vide supra*), using synthetic Metabolite E as an internal recovery standard [12]. Recovery of this marker was 82% (100 ng spike), using extraction conditions detailed above. This was consistent and linear between 10 and 200 ng. One patient receiving 10 mg b.i.d. tamoxifen had a serum concentration of 6 ng/ml Metabolite Y after 278 days of continuous therapy. Another patient receiving 150 mg b.i.d. tamoxifen had a serum level of 721 ng/ml Metabolite Y after 291 days of therapy. The serum levels of tamoxifen for these patients were 180 and 960 ng/ml respectively. A series of normal and high dose tamoxifen-treated patients is at present being monitored during 1–2 years of therapy. All patients have detectable serum levels of Metabolite Y.

The presence of Metabolite Y in breast cancer patients receiving tamoxifen therapy suggests further, and as yet unidentified, routes of metabolism for this drug. The estimates given do not preclude the possibility of a much larger pool existing as conjugates. Biological studies indicate that Metabolite Y has antiestrogenic activity, but the overall



(1) TAMOXIFEN	R'=$OCH_2CH_2N(CH_3)_2$	R"=H
(2) N-DESMETHYLTAMOXIFEN	R'=$OCH_2CH_2NHCH_3$	R"=H
(3) 4-HYDROXYTAMOXIFEN	R'=$OCH_2CH_2N(CH_3)_2$	R"=OH
(4) METABOLITE E	R'=OH	R"=H

Fig. 1. Structures of the compounds described in the text.

Table 1. Mass spectral fragments (m/z) of an acetate derivatized unknown compound from a patient receiving 150 mg tamoxifen b.i.d. compared with authentic Metabolite Y derivatized as the monoacetate*

FRAGMENT	ION MASS AND RELATIVE ABUNDANCE: UNKNOWN COMPOUND	AUTHENTIC METABOLITE Y
A. MOLECULAR ION OF METABOLITE Y MONOACETATE	386(63.6%)	386(63.8%)
B. TRANS-1-(p-HYDROXY-PHENYL)-1,2-DIPHENYL-BUT-1-ENE	300(40.1%)	300(44.5%)
C. $^{+}CH_2CH_2O-\overset{\overset{O}{\|}}{C}-CH_3$	87(100%)	87(100%)

* The relative intensities are shown in parentheses.

pharmacology of this metabolite warrants further investigation before the significance of this finding can be fully understood.

Acknowledgements—The identification of Metabolite Y was supported by Core Fund P30-CA 14520. This work was facilitated by helpful comments and the dispatch of synthetic standard compounds by Drs. A. H. Todd and H. K. Adam of I.C.I., Ltd., Pharmaceutical Division, England. The highly competent technical expertise of Ms. Kendra Tutsch is gratefully acknowledged.

Department of Human Oncology
Wisconsin Clinical Cancer Center
University of Wisconsin Center for Health Sciences
Madison, WI 53792, U.S.A.

RICHARD R. BAIN
V. CRAIG JORDAN*

* Address requests for reprints to: Dr. V. C. Jordan, Department of Human Oncology, Wisconsin Clinical Cancer Centre, 600 Highland Ave., Madison, WI 53792, U.S.A.

REFERENCES

1. H. Mouridsen, T. Palshof, J. Patterson and L. Battersby, *Cancer Treat. Rev.* **5**, 131 (1978).
2. M. Lippman, G. Bolan and K. Huff, *Cancer Res.* **36**, 4595 (1976).
3. V. C. Jordan, C. Dix, L. Rowsby and G. Prestwich, *Molec. cell. Endocr.* **7**, 177 (1977).
4. J. Fromson, S. Pearson and S. Bramah, *Xenobiotica* **3**, 711 (1973).
5. H. K. Adam, E. J. Douglas and J. V. Kemp, *Biochem. Pharmac.* **28**, 145 (1979).
6. C. Fabian, L. Sternson and M. Barnett, *Cancer Treat. Rep.* **64**, 765 (1980).
7. C. P. Daniel, S. J. Gaskell, H. Bishop and R. I. Nicholson, *J. Endocr.* **83**, 401 (1979).
8. C. Fabian, L. Tilzer and L. Sternson, *Biopharm. Drug Dispos.* **2**, 381 (1981).
9. V. C. Jordan, M. Collins, L. Rowsby and G. Prestwich, *J. Endocr.* **75**, 305 (1977).
10. E. Coezy, J. Borgna and H. Rochefort, *Cancer Res.* **42**, 317 (1982).
11. F. Mallory, C. Wood and J. Gordon, *J. Am. chem. Soc.* **86**, 3094 (1964).
12. R. Brown, R. Bain and V. C. Jordan, *J. Chromat.* (in press).