Arch. Pharm. Res. Vol.20, No.5, pp. 465-470, 1997

Expression of Human Liver 3, 4-Catechol Estrogens UDP-Glucuronosyltransferase cDNA in COS 1 Cells

Mee Ryung Ahn[1], Ida S. Owens[2] and Yhun Yhong Sheen[1]

[1]College of Pharmacy, Ewha Womans University, Seoul 120-750, Korea and [2]Section of Drug Metabolism, Heritable Disease Branch, National Institute of Child and Human Development, National Institute of Health, USA

(Received June 23, 1997)

The human cDNA clone UDPGTh2, encoding a liver UDP-glucuronosyltransferase (UDPGT), was isolated from a λgt 11 cDNA library by hybridization to mouse transferase cDNA clone, UDPGTm1. The two clones had 74% nucleotide sequence identities in the coding region . UDPGTh2 encoded a 529 amino acid protein with an amino terminus membrane-insertion signal peptide and a carboxyl terminus membrane-spanning region. In order to establish substrate specificity, the clone was inserted into the pSVL vector (pUDPGTh2) and expressed in COS 1 cells. Sixty potential substrates were tested using cells transfected with pUDPGTh2. The order of relative substrate activity was as follows: 4-hydroxyestrone > estriol >2-hydroxyestriol > 4-hydroxyestradiol > 6α-hydroxyestradiol > 5α-androstane-3α, 11β, 17β-triol= 5β-androstane-3α, 11β, 17β-triol. There were only trace amounts of gulcuronidation of 2-hydroxyestradiol and 2-hydroxyestrone, and in contrast to other cloned transferase, no gulcuronidation of either the primary estrogens and androgens (estrone, 17β-estradiol/testosterone, androsterone) or any of the exogenous substrates tested was detected. A lineweaver-Burk plot of the effect of 4-hydroxyestrone concentration on the velocity of glucuronidation showed an apparent Km of 13 μM. The unique specificity of this transferase might play an important role in regulating the level and activity of these potent and active estrogen metabolites.

Key words : UDP-glucuronosyltransferase cDNA, UDPGTh2, 4-Hydroxyestrone, Estriol

INTRODUCTION

The broad physiolgical role of estrogens in sexual development, the menstrual cycle and reproduction in human has been well characterized (Horwitz and McGuire, 1978b). Estrogenic effects were altered but not abolished by oxidative metabolism, whereas these activities were blocked by glucuronidation and sulfation (Horwitz and McGuire, 1978a). In the liver, primarily, brain, pituitary, and other peripheral tissues at lesser extent (Katzellenbogen, *et al.*, 1985), oxygenation of the steroids by specific cytochrome P 450-dependent monooxygenases converted the compounds to 2,3-catechol esterogens (Katzellenbogen *et al.*, 1984), 3,4-catechol estrogens (Katzellenbogen *et al.*, 1979), or 16α-hydroxylated forms (King *et al.*, 1984). Although catechol estrogens were rapidly cleared when injected *in vivo* (Miller and Katzellenbogen, 1983), these derivatives, formed in estrogen-responsive tissue, bound the estrogen receptor with high affinity (Roke and Katzellenbogen, 1982) and were, thus, thought to be involved in the neuroendocrine regulation of estrogen action. The 2,3-catechol isomers were antiestrogen (Sheen and Katzellenbogen, 1987), while the 4-hydroxyestrogens were quite potent etrogens with central effects on gonadotropin release (Sheen *et al.*, 1984), sexual behavior (Sheen *et al.*, 1985), and puberty (Sudo *et al.*, 1983) in rat. The 16α-hydroxy derivatives, estriol, was thought to exert antiestrogenic effects during pregnancy (Sutherland and Jordan, 1981). Because of these differences in biological activity, researchers considered that the catechol estrogens represent two classes of compounds with, most likely different pathways of regulation and clearance. UDP-glucuronosyltransferase (UDPGT) was a family of integral membrane enzymes that catalyze the glucuronidation of many potentially toxic xenobiotics and endogenous compounds. An undetermined number of isoforms catalyze the transfer of the glucuronic acid moiety from UDP-glucuronic acid to any of a large number of lipophilic acceptor molecules to generate water-soluble glucuronides which can be readily excreted from cells. Due to the difficulties associated with the purification of this labile and membrane-bound

Correspondence to: Sheen, Yhun Y. Ph.D, College of Pharmacy, Ewha Womans University, #11-1, Daehyundong, Sudaemunku, Seoul 120-750, Korea

class of proteins (Burchell, 1981; Irshaid and Tephly, 1987), very little in the literature existed regarding purification of human UDPGTs. Complementary DNAs isolated from rat (Mackenzie, 1987; Iyanagi *et al.*, 1986), mouse (Kimura and Owens, 1987), and human (Jackson *et al.*, 1987; Harding *et al.*, 1988) have been reported which encode transferases that glucuronidate primary estrogens or exogenous compounds such as p-nitrophenol (Jackson *et al.*, 1987), 4-methylumbelliferone (Kimura and Owens, 1987). Based on glucuronidating activity following expression in COS 1 cells, we uncovered a human UDP-glucuronosyltransferase which specifically glucuronidated 3,4-catechol estrogens and some steroids. The form was encoded in a cDNA clone, UDPGTh2, which was isolated from a human liver λgt11 cDNA library using a mouse cDNA, UDPGTm1, as a probe (Dong *et al.*, 1997). UDPGTh2-encoded protein represented a unique transferase activity for active estrogen metabolites.

MATERIALS AND METHODS

Materials

UDP-glucuronic acid and all aglycones tested for substrate activity were obtained from Sigma chemical Co. (St. Louis, MO, USA). [^{14}C]UDP-glucuronic acid [^{35}S] methionine and [^{32}P]deoxycytidine triphosphate were purchased from Amersham (Arlington Heights, IL, USA). Restriction enzymes and other reagents used in molecular biology techniques were from Bethesda Research Laboratories (Gaithersbug, MD, USA), or Boehringer Mannheim (Mannheim, Germany). The pSVL vector and the oligo-labeling kit were products of Pharmacia Fine Chemicals (Uppsala, Sweden). COS-1 cells were supplied from the American Type Culture Collection (Rockville, MD, USA). The Bluescript plasmids and XL-1 Blue cells were products of Stratagene (La Jolla, CA, USA). The Erase-A-Base kit was from Promega (Madison, WI, USA), tunicamycin was from Boehringer Mannheim (Mannheim, Germany), and Lipofectin™ was from Bethesda Research Laboratories (Gaithersbug, MD, USA). Dulbecco's modified Eagle's medium, fetal calf serum, and other cell culture reagents were from GIBCO (Gaithersbug, MD, USA).

Expression of UDPGTh-2 in COS-1 cells

The cDNA from the pSKM13$^+$-subclone was digested with *Sma* I and *Eco* RV and then ligated into the *Sma* I-digested pSVL expression vector. After appropriate subclones were characterized for the correct or reversed orientation of the cDNA with respect to the promoter element in pSVL, plasmid preparations were purified by cesium chloride banding. The expression unit was designated as pUDPGTh-2. Mock-transfected cells were prepared with the cDNA inserted in the reverse direction with respect to the promoter element of pSVL. COS-1 cells (80% confluent) were plated at a density of 10^6/60×15 mm dish in Dulbecco's modified Eagle's medium with 10% fetal calf serum and grown overnight. The purified plasmid was then combined with the carrier, Lipofectin™, and added to the cell culture dishes according to the manufacturer's instructions. At the end of the 5-hour transfection period which was blocked by the addition of 10% fetal calf serum, the cells were allowed to incubate for from 48 to 96 hours as indicated. Cultures received no further treatment and were harvested and stored at -70°C until assayed.

Glucuronidation assay using [^{14}C] UDP-glucuronic acid

Appropriate control and transfected COS-1 cells were assayed for glucuronidating activity according to a published procedure, but with modifications. Transferase contained in cellular homogenates (50 µg of protein) was activated with 0.50 mg of CHAPS per mg of protein. The activated transferase was added to a 0.075 ml reaction containing 10 or 100 µM aglycone, 50 µM [^{14}C] UDP-glucuronic acid (0.125 µCi), 50 mM Tris-HCl, pH 7.7, 4 mM $MgCl_2$, 1 mM ascorbic acid, 1 mM phenylmethylsurfonyl fluroide, and 1.0 µg/ml leupeptin. Reactions were incubated for 16 hours at 25°C or for 1 hour at 37°C. To establish that glucuronides were formed, one-half of a 0.15 ml reaction was treated with 5.5 units of β-glucuronidase, pH 7.0, for 3 hours at 37°C (data not shown). Untreated and β-glucuronidase-treated reaction mixtures were analyzed by thin layer chromatography (TLC). Each aglycone was converted to the β-glucuronide by human microsomes for comparison (data not shown). The TLC plates were analyzed and quntitated by scanning on an Ambis Radioanalytical Imaging system Mark II (Ambis, San Diego, CA, USA). The minimum detectable amount of glucuronide product formed, under the assay conditions employed, was 1.7 pmol (signal/background ratio is 2, with a background of approximately 50 cpm). For the determination of the K_m value of the transferase using 4-hydoxyestrone, 500 µM [^{14}C] UDP-glucuronic acid (1.25 µCi) was added to each reaction and incubated for 1 hour at 37°C. It was predetermined that the generation of product was linear for at least 90 minutes. Plates were exposed to X-ray film for 21 days to obtain prints of the results.

RESULTS AND DISCUSSION

Screen for UDP-glucuronosyltransferase Activity

To establish substrate specificity, the expressed pUDPGTh2 was examined with some 60 compounds according to the details outlined under Materials and

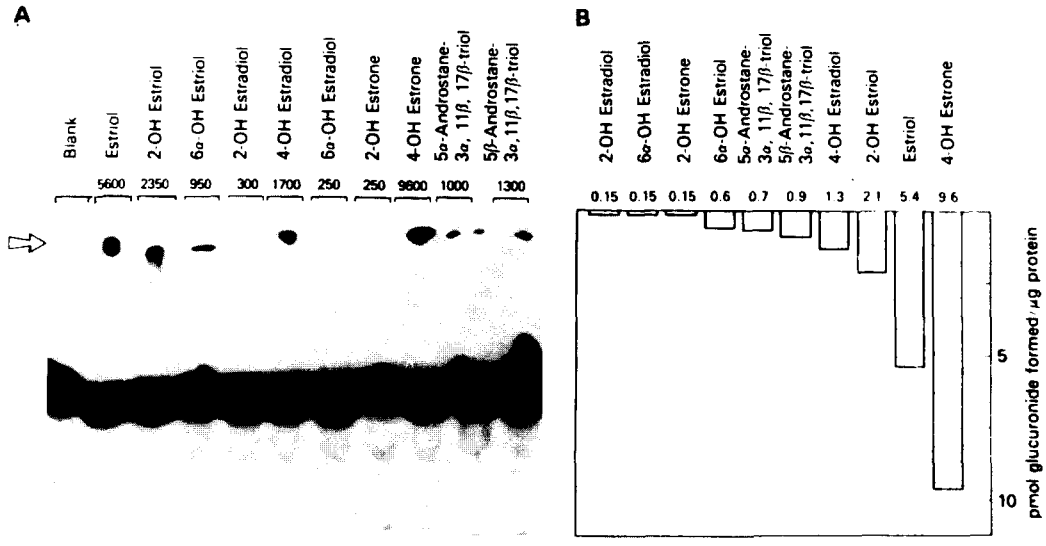

Fig. 1. Substrate specificity of the UDPGTh2-encoded UDP-glucuronosyltransferase expressed in COS 1 cells. A, pUDPGTh2-transfected cells which were incubated for 48 hours were assayed for the production of glucuronide (region of arrow) using 100 μM aglycone and 50 μM [^{14}C]UDP-glucuronic acid as described under "Materials and Methods". Reactions were incubated for 16 hours at 24°C and then analyzed by TLC chromatography, scanning the plate as described under "Materials and Methods" and exposed to x-ray film (20 days) for print. The aglycones and the radioactivity (in cpm) incorporated into glucuronides were recorded at the top of the chromatogram. B, substrate activities of aglycones expressed in picomoles of glucuronide formed/μg of protein. Counts shown in part A (arrow) were converted to picomoles of glucuronide formed based on counting efficiencies of the AMB1S system which ranged from 40% for 1,000 or less cpm to 27% for 10,000 cpm.

Methods. The production of glucuronide was shown (Fig. 1, region of arrow) to occur preferentially with 4-hydroxyestrone > estriol > 2-hydroxyestriol > 4-hydroxyestradiol > 6α-hydroxyestriol > 5α-androstan 3α, 11β, 17β-triol=5α-androstane 3α, 11β, 17β-triol. 2-Methoxyestriol underwent glucuronidation at about the same rate as the 5β-androstan-3α, 11β, 17b-triol. There was only a trace of activity with 2-hydroxyestradiol, 2-hydroxyestrone, and 6α-hydroxyestradiol (Fig 1), and no detactable activity (<50 cpm) toward 17β-estradiol or estrone. The total radioactivity incorporated into each glucuronide was included in Fig. 1A and was converted to picomoles of product formed per g of protein in cell homogenate (Fig. 1B). Each glucuronide was shown to be sensitive to β-glucuronidase and mock-transfected cells did not contain transferase activity for any of the substrates metabolized by the UDPGTh2 encoded protein. There was a low constitutive activity for α-naphthol, as noted previously (Hardings *et al.*, 1988). This might account for the presence of trace amount of protein which immunocomplexed with anti-transferase IgG in mock-transfected cells. Endogenous and exogenous compounds which showed no detectable conversion to glucuronide were shown in Table I. We also determined the Km of this transferase for the preferred substrate, 4-hydroxyestrone. The effects of substrate corporation on the velocity of the conjugation reaction were plotted as their double reciprocal values as shown in Fig. 2. The x-intercept showed that the apparent Km was 12.5 μM. This report described the cloning and expression of a novel steroid UDP-glucuronosyltransferase, i.e., 3, 4-catechol estrogen/estriol UDP-glucuronosyltransferase. The predicted amino acid sequence of this transferase isoform, specified by a 1590 base pair open reading frame in the cDNA clone, UDPGTh2, showed strong identity with other cloned members of the other steroid transferase family: UDPGTm1 (66%), UDPGTr2 (69%), UDPGTr3 (66%), and UDPGTr4 (65%). It was, therefore, not surprising that the encoded UDPGT efficiently glucuronidates hydroxysteroids. Yet, this isoform clearly had a different acceptor substrate profile compared to the other cloned steroid transferases. The absence of defectable UDPGTh2 encoded activity toward 3-hydroxysteroids, 17-hydroxysteroids, or either 1-naphthol, estrone, and testosterone which were metabolized by activity encoded in UDPGTr4 (Mackinzie, 1986b), UDPGTr3/UDPGTr2 (Mackinzie, 1986a), and UDPGTm1 (Mackinzie, 1986b), respectively, indicated that this form was unique; it selectively glucuronidated two subsets of estrogen metabolites, 3, 4-catechol isomers and estriol. By using a highly sensitive radiometric assay to establish the aglycon specificity of the transferases, some 60 potential endogenous and exogenous substrates (Table I) were tested. There were three categories of chemical structures among metabolites of the primary estrogens and other steroids which supported glucuronidation by this enzyme. The highest activity was for the 3, 4-catecholic structure in 4-hydroxyestrone. A similar structure in 4-hydroxyestradiol ranks fourth in substrate activity. Steroids with dihydroxy substitutions at C-16 and C-17, but with a third

Table I. Compounds tested that showed no detectable conversion to glucuronide. Each aglycon was tested for substrate activity as described in the Legend to Fig. 1

Estrogens/derivatives	Type I	Progesterone/derivatives
Estrone	Harmol	21-OH Pregnenolone
17β-Estradiol	α-Maphtol	16α-OH Pregnenolone
16α-OH-17α-Estradiol	p-Nitrophenol	16α-OH Progesterone
2-Methoxyestradiol	4-aminophenol	17α-OH Progesterone
16α-OH Estrone	4-Methylumbelliferone	5β-Pregnane-3α,6α-diol-20-one
16β-OH Estrone	3-OH Benzo(α)pyrene	
Diethylstilbesterol		
Neuromodulators	**Type II**	**Androgen/derivatives**
Dopamine	Menthol	Testosterone
5-OH Tryptamine	Morphine	Dihydrotestosterone
6-OH Melatonin	Bilirubin	11β-OH Testosterone
Glucocorticoids/Mineralcorticoids	4-OH-Biphenyl	16α-OH Testosterone
Hydrocortisone	4-Acetaminophenol	Androsterone
Deoxycorticosterone	Phenolphthalein	5α-Androstane-17β-ol, 3-one
Aldosterone	Chloramphenicol	5α-Androstane-3β, 11β, 17β-triol
Vitamins	9-OH Benzo(α)pyrene	
Ergocalciferol	5-(p-Hydroxyphenyl)-5-phenylhydantoin	**Steroid precursors**
vitamin E	5-ethyl-5-(p-hydroxyphenyl) barbituric acid	17β-OH Cholesterol
vitamin K		19-OH Cholesterol
Cardiac glycosides	**Thio-containingsubstrates**	**Others**
Digitoxigenin-monodigitoxiside	Diethyldithiocarbamic Acid	2, 2, 2 trichloroethanol
Digitoxigenin-bisdigitoxiside	2-Mercaptobenzothioazole	
	4-Nitrothiophenol	

hydroxy group in the A-ring, were excellent substrates. Estriol and 2-hydroxyestriol rank second and third, respectively in terms of conversion to glucuronides. In constrast, two similar structures 2-methoxyestriol and 6 -hydroxyestriol were among the weakest substrates, suggesting that the 2-methoxy and 6-hydroxy substituents, respectively, interfere with substrate activity. The failure of 16α-hydroxytestosterone (C16, C17-dihydroxy) to generate glucuronides supported the notion that an additional hydroxy group was needed in the A ring for this type of substrate. Finally, the trihydroxysubstituted compounds, 5β-androstane 3α, 11β, 17β-triol, its isomer 5α-androstane 3α, 11β, 17β-triol, and 6α-hydroxyestradiol (3-, 6-, and 17-trihydroxy), were also weak substrates. The poor substrate activity of these compounds suggested that the positions of the three hydroxyl groups around the molecules were less optimal than those in estriol. It is noteworthy that the 2, 3-catechol structures in 2-hydroxyestrone and 2-hydroxyestradiol showed only a trace of glucuronidation (Fig. 1). Hence, the 3, 4-catecholic structure was a strict requirement. Estradiol (C-3, C-17-dihydroxy, aromatic A ring), which lacks either a catechol or a 16, 17-diol substitution, was not glucuronidated. The position of glucuronide linkage could not be established for either category of substrates because two or more hydroxyl groups were present on all active substrates.

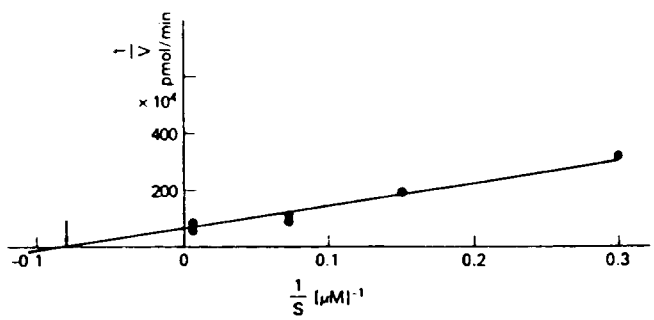

Fig. 2. Lineweaver-Burk plot of 4-hydroxyestrone concentration versus reaction velocity using UDPGTh2 transfected COS1 cells. pUDPGTh2-transfected cells which were incubated for 48 hours were assayed and analyzed as in Fig 2, except that 500 μM [^{14}C] UDP-glucuronic acid (1.25 μCi) was used with 3.3, 6.7, 13.3 and 133 μM 4-hydroxyestrone, and the reaction were incubated for 1 hour at 37°C.

The biochemical significance of the strict specificity for a 3, 4-catechol estrogen UDP-glucuronosyltransferase could be related to the physiological requirement for differential regulation of the 2, 3- and 3, 4-catechol estrogens. The 2, 3-catechol estrogens were primarily inactive metabolites (Dutton, 1980) while the 3, 4-catechol estrogens had potent effects on gonadotropin release (Klassen and Doull, 1996). The very different metabolic profiles for the two types of catechol estrogens would be difficult to regulate with

one detoxification pathway.

In summary, we presented the cDNA of a human UDP-glucuronosyltransferase, UDPGTh2, which upon expression in COS1 cells, was found to encode a form highly selective for two important metabolites of estrogen, 3, 4-catechol derivatives and estriol.

ACKNOWLEDGEMENTS

The present study was supported by the Genetic Engineering Research Grant from the Education Ministry of Korea.

REFERENCES CITED

Bock, K. W., Lienblum, W. and Pfiel, H., Functional Heterogeneity of UDP-Glucuronosyltransferase activities in C57BL/6 and DBA/2 Mice. *Biochem. Pharmacol.*, 31, 1273-1277 (1982).

Church, G. and Gilbert, W. J., Genomic sequencing. *Proc. Natl. Acad. Sci. U.S.A.*, 81, 1991-1995 (1984).

Classen E. and Doull A., Biotransformation of xenobiotics: Cassaret & Doull's toxicology, MacMillan P.C., New York, pp. 113-198, 1996.

Dong, M. S., Owens, I. S. and Sheen, Y. Y., Cloning and expression of human liver UDP-glucuronosyltransferase cDNA, UDPGTh2. *Arch. Pharm. Res.*, 20, in press (1997).

Dutton, G. J., *Glucuronidation of drugs and other compounds.* CRC Inc., Florida USA pp. 69-78, 1980.

Fournel-Gigleux, S., Jackson, M. R., Wooster, R. and Burchell, B., Expression of a human liver cDNA encoding a UDP-glucuronosyltransferas catalysing the glucuronidation of hyodeoxycholic acid in cell culture. *FEBS Lett.*, 243, 119-122 (1989).

Harding, D., Fournel-Gigleux, S., Jackson, M. R. and Burchelle, B., Cloning and Substrate Specificity of a human phenol UDP-glucuronosyltransferase expressed in COS-7 cells. *Proc. Natl. Acad. Sci. U.S. A.*, 85, 8381-8385 (1988).

Horwitz, K. B. and McGuire, W. L., Estrogen control of progesterone receptor in human breast cancer. *J. Biol. Chem.*, 253, 2223-2228 (1978b).

Horwitz, K. B. and McGuire, W. L., Antiestrogen mechanisms of action and effects in breast cancer: Breast cancer advances in research and treatment. Plenum Press, New York vol 2, pp. 155-204, 1978a.

Jackson, M. R., McCarthy, L. R., Harding, D., Wilson, S., Coughtrie, M. W. H. and burchell, B., Cloning of a human liver microsomal UDP-glucurosyltransferase cDNA. *Biochem. J.*, 242, 581-588 (1987).

Katzenellenbogen B. S., Miller, M. A., Mullick, A. and Sheen, Y. Y., Antiestrogen action in breast cancer cells:Modulation of proliferation and protein synthesis and interaction with estrogen receptors and additional antiestrogen binding sites. *Cancer Res. Treat.*, 5, 231-243 (1985).

Katzenellenbogen B. S., Norman, M. J., Eckert, R. L., Peltz, S. W. and Mangle, W. F., Bioactive, estrogen receptor interations, and plasminogen activator-inducing activities of tamoxifen and hydroxytamoxifen in human breast cancer cells. *Cancer Res.*, 44, 112-119 (1984).

Katzenellenbogen, B. S., Bhakoo, H. S., Fergson, E. R., Lan, N. C., Tatte, T., Tsai, T. L. and Katzenellenbogen, J. A., Estrogen and antiestrogen action in reproductive tissues and tumors. *Recent Pro. Hormon Res.*, 35, 259-300 (1979).

King, W. J. and Greene, G. J., Monoclonal antibodies localize estrogen receptor in nuclei of target cells. *Nature (Lodon)*, 307, 745-747 (1984).

Kimura, T. and Owens I. S., Mouse UDP glucuronosyltransferase cDNA and complete amino acid sequence and regulation. *Eur. J. Biochem.*, 168, 515-521 (1987).

Kirkpatrick, R. B., Falany, C. N. and Tephly, T. R., Glucuronidation of Bile Acids by Rat Liver 3-OH Androgen UDP-Glucuronyltransferase. *J. Biol. Chem.*, 259, 6176-6180 (1984).

Kornfeld., R. and Kornfeld. S., Assembly of asparagin-linked oligosaccharides. *Ann. Rev. Biochem.*, 50, 555-583 (1981).

Mackenzie, P. I., Hjelmeland, L. H. and Owens, I. S., Purification and Immunochemical Characterization of a Low Form of UDP Glucuronosyltransferase from Mouse Liver. *Arch. Biochem. Biophys.*, 231, 487-497 (1984).

Mackenzie P. I., Rat liver UDP-glucuronosyltransferase. *J. Biol. Chem.*, 262, 9744-9749 (1986a).

Mackenzie P. I., Rat liver UDP-glucuronosyltransferase. *J. Biol. Chem.*, 261, 14112-14117 (1986b).

Matern, H., Matern, S. and Gerok, W., Isolation and Characterization of rat Liver Microsomal UDP-Glucuronosyltransferase Activity toward Chenodeoxycholic Acid and Testosterone as a Single Form of Enzyme. *J. Biol. Chem.*, 257, 7422-7429 (1982).

Miller, M. A. and Katzenellenbogen, B. S., Characterization and quantitation of antiestrogen binding sites in estrogen receptor-positive and -negative human breast cancer cell lines. *Cancer Res.*, 43, 3094-3099 (1983).

Rorke, E. A. and katzenellenbogen B. S., Effects of estrogen and antiestrogens on estrogen receptor dynamics and the induction of progesterone receptor in MCF-7 human breast cancer cells. *Cancer Res.*, 42, 139-144 (1982).

Rose, T. K., Welsch, W. J., Sefton, B. M., Esch, F. S. and Ling N. C., Vesicular Stomatitis virus glycoprotein is anchored in the viral membrane by a hydrophobic domain near the COOH terminus. *Proc. Natl. Acad. Sci. U.S.A.*, 77, 3884-3888 (1980).

Sheen, Y. Y. and Katzenellenbogen B. S., Antiestro-

gen stimulation of the a 37,000 molecular weight secreted protein and estrogen stimulation of the 32, 000 molecular weight secreted protein in MCF-7 human breast cancer cells. *Endocrinol.*, 120, 1140-1151 (1987).

Sheen, Y. Y., Ruh, T. S., Mangel, W. F. and Katzenellenbogen B. S., An antiestrogenic potency and binding characteristics of the triphenylethylene H1285 in MCF-7 human breast cancer cells. *Cancer Res.*, 45, 4192-4199 (1984).

Sheen, Y. Y., Simpson, D. M. and Katzenellenbogen, B. S., An antiestrogen of the role of antiestrogen-binding sites in mediating the growth modulatory effects of antiestrogens:Studies using t-Butylphenoxy-ethyl diethylamine, a compound lacking affinity for the estrogen receptor., *Endocrinol.*, 117, 561-564 (1985).

Wishhart, G. J., Functional heterogenesity of UDP-glucuronosyltransferase as indicated by its differential development and inducibility by glucocorticoid. *Biochem. J.*, 174, 485-489 (1978).