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Award Number: DAMD17-00-1-0248

TITLE: Pharmacogenetic Factors Contributing to Variation in Response to Tamoxifen and Raloxifene

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REPORT DATE: July 2002

TYPE OF REPORT: Annual

PREPARED FOR: U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

DISTRIBUTION STATEMENT: Approved for Public Release;
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1118 072

REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 074-0188

Public reporting burden for this collection of information is estimated to average 1 hour per response, including the time for reviewing instructions, searching existing data sources, gathering and maintaining the data needed, and completing and reviewing this collection of information. Send comments regarding this burden estimate or any other aspect of this collection of information, including suggestions for reducing this burden to Washington Headquarters Services, Directorate for Information Operations and Reports, 1215 Jefferson Davis Highway, Suite 1204, Arlington, VA 22202-4302, and to the Office of Management and Budget, Paperwork Reduction Project (0704-0188), Washington, DC 20503

1. AGENCY USE ONLY (Leave blank)	2. REPORT DATE	3. REPORT TYPE AND DATES COVERED
	July 2002	Annual (1 Jul 01 - 30 Jun 02)

4. TITLE AND SUBTITLE
Pharmacogenetic Factors Contributing to Variation in Response to Tamoxifen and Raloxifene

5. FUNDING NUMBERS
DAMD17-00-1-0248

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8. PERFORMING ORGANIZATION REPORT NUMBER

9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES)
U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

10. SPONSORING / MONITORING AGENCY REPORT NUMBER

11. SUPPLEMENTARY NOTES

20021118 072

12a. DISTRIBUTION / AVAILABILITY STATEMENT
Approved for Public Release; Distribution Unlimited

12b. DISTRIBUTION CODE

13. ABSTRACT The purpose of these studies is to elucidate the pharmacogenetic factors that contribute to variation in human response to tamoxifen (TAM) and raloxifene (RAL). We had previously identified and partially characterized common genetic polymorphisms in two human drug -metabolizing genes, SULT1A1 and UGT1A6. We hypothesized that these polymorphisms contributed to variation in TAM or RAL metabolism. These studies were divided into three aims with the purpose of 1) biochemically characterizing the contribution of these enzymes to the metabolism of TAM and RAL; 2) developing cell model systems to study allele-specific differences in cellular response to these molecules and; 3) perform a clinical pharmacogenetic study to evaluate the association of common genetic polymorphisms in drug metabolizing genes with variable clinical response to TAM. Thus far we have determined that SULT1A1 and UGT1A6 contribute to the inactivation of 4-hydroxytamoxifen (OHT), the active metabolite of TAM, and that a separate enzyme, UGT1A9 catalyzed the glucuronidation of RAL. We have determined genotype/phenotype correlation for UGT1A6 alleles in a bank of human liver tissue and have generated HEK 293 cell lines that stably express each of the four UGT1A6 allozymes. The UGT1A6 *2 allozyme, when expressed homozygously, is associated with high UGT1A6 activity. We established MCF-7 breast cancer cell lines stably expressing the wildtype and variant SULT1A1 alleles and have measured allele-specific differences in the response of these cells to estrogens and OHT. These studies suggest that pharmacogenetic factors might contribute to variable cellular response to antiestrogens.

14. SUBJECT TERMS	15. NUMBER OF PAGES
SULT1A1, UGT1A6, pharmacogenetics, tamoxifen, sulfation and glucuronidation	10
	16. PRICE CODE

17. SECURITY CLASSIFICATION OF REPORT	18. SECURITY CLASSIFICATION OF THIS PAGE	19. SECURITY CLASSIFICATION OF ABSTRACT	20. LIMITATION OF ABSTRACT
Unclassified	Unclassified	Unclassified	Unlimited

Table of Contents

INTRODUCTION

The goals of this proposal are to elucidate the pharmacogenetic factors that influence cellular response to tamoxifen (TAM) and raloxifene (RAL). Specifically, the work described represents a stepwise approach to the study of genetic polymorphisms in human sulfotransferase (SULT) and UDP-glucuronosyltransferase (UGT) genes. Those studies will progress from basic biochemical studies to the use of cell models and will culminate in a clinical pharmacogenetic study. In the first aim, we proposed to biochemically characterize the capacity of wildtype and variant SULT1A1, SULT1A2 and UGT1A6 proteins to conjugate 4-hydroxytamoxifen (OHT) and raloxifene RAL. The second aim focussed on the development of cell models to study allele-specific differences in cellular response to these antiestrogens. Finally, the third aim will determine the association of genetic polymorphisms in several metabolic pathways with human response to TAM in a clinical setting.

BODY

Specific Aim1. Biochemically characterize the capacity of recombinant wildtype and variant SULT1A1, SULT1A2 and UGT1A6 proteins to conjugate OHT and RAL in *in vitro* assays. We proposed four sub aims associated with this aim—each to be completed within the first year of funding. Those included:

1) Generation of recombinant wildtype and variant UGT1A6 allozymes
2) Biochemical characterization of the SULT1A1 and SULT1A2 allozymes with regard to their capacity to sulfate OHT and RAL.
3) Optimization of the glucuronidation assay.
4) Biochemical characterization of the UGT1A6 allozymes with regard to their capacity to glucuronidate OHT and RAL.

In our previous progress report we stated that we had completed subaim 2 but were having difficulty expressing UGT1A6 enzymes. We previously reported that in a bank of human liver tissues, the UGT1A6*2 allele, when expressed homozygously, was associated with a relatively high level of enzyme activity (see 2001 progress report). We have now successfully established HEK 293 cell lines that stably express each of the four UGT1A6 allozymes that we had previously identified. We are currently using those cell lines as a source of recombinant UGT1A6 proteins to test the hypothesis that the four UGT1A6 allozymes exhibit different glucuronidation kinetics. We have determined, in concordance with activities determined in human tissues, that cells expressing the UGT1A6*2 allele exhibit a high level of glucuronidating activity (Table 1). We have completed subaim 3 and have developed a thin layer chromatography assay for measuring kinetics of UGT1A6 proteins using OHT as the substrate. We are now evaluating the HEK293 cell lines for capacity to glucuronidate OHT. In our previous report we stated that RAL was not a substrate for either UGT1A6 or SULTs 1A1 or 1A2. Therefore that substrate will no longer be evaluated in this study (though we did determine that UGT1A9 glucuronidates RAL and that has become the basis for a separate DOD study -- DAMD 17-0101-0523).

Specific Aim 2. Determine the antiestrogen response to OHT and RAL of cells in which wildtype and variant SULT1A1 and UGT1A6 allozymes have been expressed. These studies were designed to evaluate whether allele-specific differences in the response of cells to estrogens and antiestrogens could be measured. Sulfation of E2 and OHT results in inactive molecules (ie., they do not interact with the estrogen receptor). Therefore, we hypothesized that cells expressing the less active SULT1A1*2 allozyme would exhibit an enhanced proliferative response to E2 and OHT because those cells would have lower capacity to inactivate those molecules. There were originally six sub aims associated with this specific aim to be completed in months 9 through 30 of the funding period. In our previous progress report we justified modification of the subaims to the following (new aims are underlined while deleted aims are in italics) :

- Generation of expression constructs for SULTs and UGTs
- *Generation of transformed yeast cells*
- *ß-galactosidase assays*
- Generation of stably transfected human MCF-7 and HEK 293 cell lines
- Cell proliferation assays
- Quantitative RT-PCR of stably transfected MCF-7 cell lines
- Correlation of UGT1A6 Genotype with phenotype in a bank of human liver tissues

We previously reported that subaims 1,4 and 5 were completed for the SULTs. We have also compared estrogenic response of cells that natively express SULT1A1*1 or SULT11*2 in a homozygous fashion. Those data are consistent with results from the MDF-7 cell model system in that the T47 cells, which are homozygous for SULT1A1*1, exhibit abrogated proliferative response to E2 compared to MCF-10F cells which are homozygous for SULT1A1*2 (Figure 1A). This is presumably because the cells expressing *1 have a larger capacity to metabolically inactivate E2. To test that hypothesis further, we treated T47 cells with mefanamic acid, a SULT1A1 inhibitor, and showed that in the presence of the inhibitor the proliferative response of the T47D cells reaches that of the MCF-10F cells (Figure 1B).

We have also examined SULT expression in breast cancer cell lines and tissues (Figure 2). SULT1A1 and SULT1E1 were evaluated because SULT1E1 represents a competing metabolic pathway for estrogen metabolism, thus variation in its expression might influence estrogen response of cells. Our data indicate that while SULT1E1 is expressed consistently in normal breast tissue -- it appears to be much less expressed in breast tumors and transformed cell lines. SULT1A1, on the other hand, is consistently expressed in tumors and cell lines. These data suggest that variation in SULT1A1 activity will likely impact estrogenic and antiestrogenic response of cells. Corroborating this prediction, data presented in our last progress report showed profound differences in response of MCF-7 cells to E2 and OHT depending on whether the cells expressed the SULT1A1*1 allele (high activity) or the SULT1A1*2 allele (low activity). Furthermore, we used the MCF-7 cell lines and pulse chase analyses to evaluate the half-life of

SULT1A1*1 and SULT1A1*2 proteins. Those studies determined that the SULT1A1*1 protein is six fold more stable than the SULT1A1*2 protein. This is in close agreement with the ten-fold difference in the steady state level of the two proteins that we determined in the MCF-7 cell lines. We also measured kinetic parameters associated with recombinant SULT1A1 allozymes. There was no difference in the affinity of the two allozymes toward OHT, E2 or p-nitrophenol. However, the SULT1A1*2 allozyme consistently exhibited lower Vmax (substrate turnover) toward all substrates studied. Collectively our data suggest that a common genetic polymorphism in the human SULT1A1 gene (SULT1A1*2 allele) may be associated with greater estrogenic response of breast epithelial cells but may also be associated with greater antiestrogenic response of those cells to OHT.

We have now generated HEK 293 cells stably expressing each of the four UGT1A6 allozymes. Those cells will be evaluated for antiestrogenic response to OHT after transient transfection with estrogen receptor alpha or, separately, estrogen receptor beta. These studies will help determine whether common alleles of the human UGT1A6 gene might be associated with variable cellular response to OHT.

Specific Aim 3. Determine the association of SULT1A1, SULT1A2, UGT1A6, CYP3A4 and CYP2D6 genotypes with the clinical response of women who are being prescribed TAM. The purpose of this aim is to determine the pharmacogenetic factors that impact clinical response to TAM. Because TAM is subject to several competing metabolic pathways—each of them polymorphic—we expanded the pharmacogenetic scope in this aim to encompass the oxidative metabolic pathways that activate TAM to OHT (CYP3A4 and CYP2D6).

In the last progress report we stated that due to complications with approval of our clinical protocol by the Army, accrual of patients was delayed. Accrual began in October of 2001. However, this timing coincided with release of clinical data suggesting that letrozole, an aromatase inhibitor, was more effective than tamoxifen in the treatment of certain breast cancers. These data have resulted in letrozole becoming the first line choice of antiestrogen therapy in women with certain breast cancers. Our current protocol stipulates in the inclusion criteria no concomitant use of other hormonal or antiestrogenic agents. Therefore, we have only accrued two women into our study. We are currently negotiating changes to our protocol to include women who are also taking aromatase inhibitors (such as letrozole). Naturally, such changes will be submitted to the Army for approval. We anticipate requesting a "no cost" extension of this grant to complete these studies once proper inclusion criteria are established.

In anticipation of accruing more study subjects, we have developed high throughput genotyping assays for CYP2D6 and CYP3A4. These assays, along with the previously established assays for SULT1A1 and UGT1A6, will be employed to establish drug metabolism genotypes relevant to tamoxifen in each study subject.

KEY RESEARCH ACCOMPLISHMENTS

- Established HEK 293 cell lines stably expressing UGT1A6*1, *2, *3 and *4
- Compared kinetic properties of recombinant UGT1A6 allozymes
- Determined that low activity of the SULT1A1*2 allele is attributed to a combination of lower enzymatic "turnover" of substrate (low Vmax) as well as significantly shorter half life of the SULT1A1*2 protein compared to SULT1A1*1.
- Established expression profile of SULTs in human breast tumors and transformed breast carcinoma cell lines
- Developed TLC assay for evaluating OHT glucuronidation kinetics
- Developed high throughput genotyping assays for CYP3A4 and CYP2D6

REPORTABLE OUTCOMES

Abstracts

Human Sulfotransferases and Cellular Response to Estrogens and Antiestrogens. Rebecca B. Raftogianis, Drug Metab Rev **33**:10, 2001.

Invited Symposia

Human Sulfotransferases and Cellular Response to Estrogens and Antiestrogens, 6th International ISSX Meeting, Munich Germany, October 2001

Pharmacogenetic Variation in a Human Phenol Sulfotransferase, SULT1A1, Seminar Speaker, Department of Pharmaceutical Sciences, School of Pharmacy, University of Colorado, Denver, CO, January, 2002

Functional Implications of Genetic Variation in the Human Cytosolic Sulfotransferases, Keynote Speaker, Research Triangle Park Drug Metabolism Discussion Group, Research Triangle Park, NC, February 2002

Manuscripts

Jeffrey J. Zalatoris and Rebecca Blanchard Raftogianis, Association of human UGT1A6 haplotype with level of enzyme activity in human liver tissue, submitted, *Pharmacogenetics*.

Susan E. Walther, William J. Geese and Rebecca Blanchard Raftogianis, Association of SULT1A1 pharmacogenetics with cellular response to estrogens and antiestrogens, *in preparation*

CONCLUSIONS

In summary, we have made significant progress in achieving the aims set forth in this grant. Specifically we have determined the SULT1A1 and UGT1A6 contribute to the conjugative metabolism of OHT, the active metabolite of TAM and that UGT1A9 is predominantly responsible for RAL glucuronidation. Furthermore, we have identified and determined the allele frequencies for four common UGT1A6 alleles in ethnically defined human populations and have determined genotype/phenotype relationships for those alleles in a bank of human liver tissues. Study of recombinant UGT1A6 allozymes suggest that the *2 allozyme, when expressed homozygously is associated with relatively high UGT1A6 activity. This is also true of human liver tissues expressing the UGT1A6*2 allele homozygously. We have developed cell model system to study SULT1A1 and UGT1A6 allele-specific cellular phenotypes and have implemented that system to identify allele-specific proliferative response of cells to estrogens and antiestrogens. Specifically, we have determined that cells expressing the SULT1A1*2 variant respond to those compounds significantly more than cells expressing the SULT1A1*1 allele. The mechanism for this phenotype appears to be attributable to both altered enzyme kinetics as well as altered intracellular protein stability. Approximately 30% of Caucasians and African Americans are homozygous for the SULT1A1*2 allele. These results are significant in that should these allele-specific responses also occur in tumors -- SULT1A1 genotype might be associated with clinical response to tamoxifen. That possibility will be explored in a clinical study as part of specific aim 3 of this grant.

REFERENCES

None

APPENDIX

Determined in microsomal preparations from HEK 293 cells stably expressing each allele

Table 1. Kinetic Data for UGT1A6 Allozymes

rUGT1A6 Allozyme	Km (uM)		Vmax (nmol/min/mg)		Vmax/Km	Variation from *1
*1	1203	+/- 365	35.4	+/- 4.11	0.0294	1
*2	609	+/- 152	34.1	+/- 2.69	0.0561	1.9x
*3						
*4	626	+/- 233	18.1	+/- 2.45	0.0289	1x

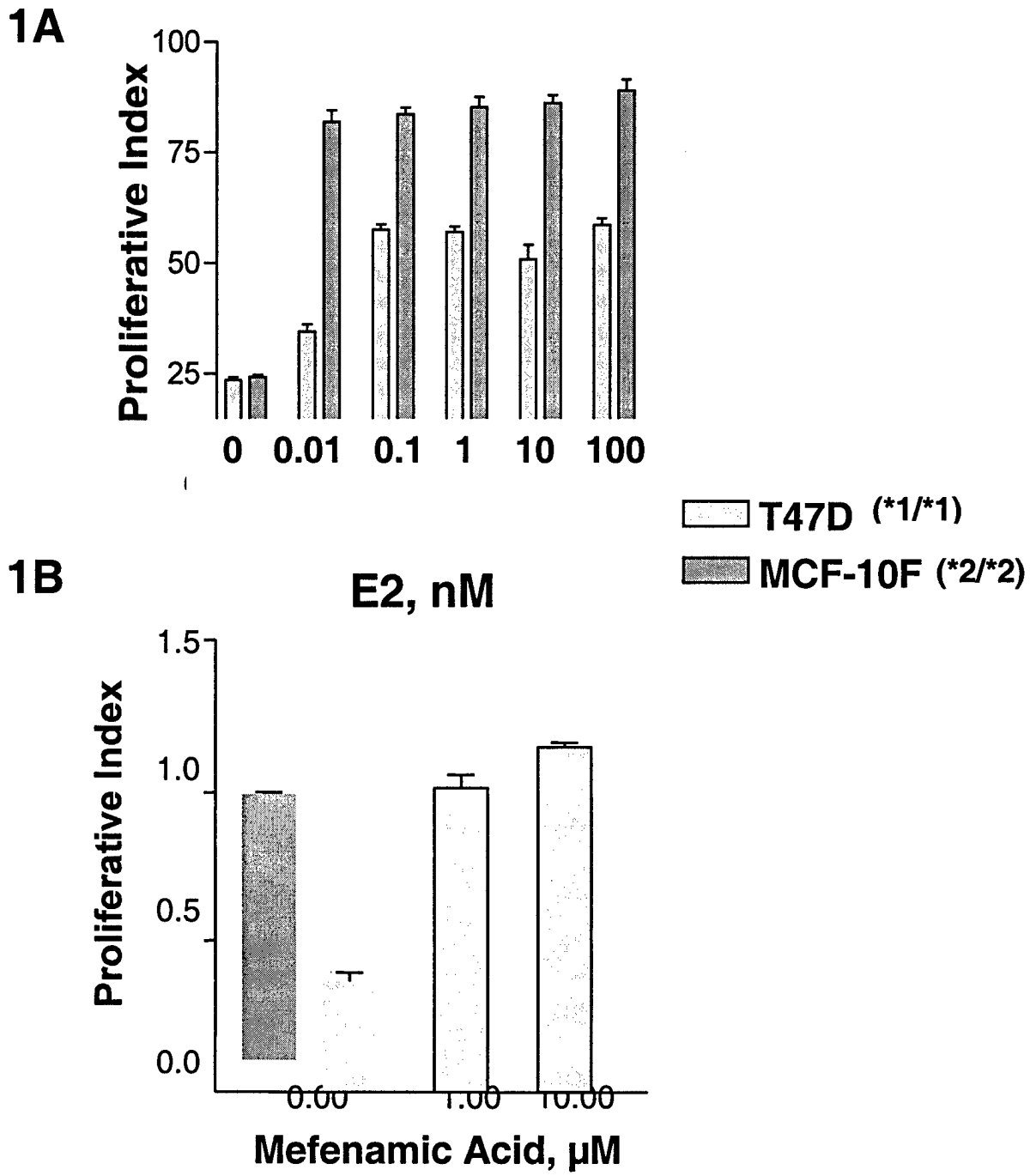

Figure 1. Proliferative response of native breast cell lines to E2. A) T47D cells are homozygous for SULT1A1*1 and exhibit an abrogated response to E2 compared to MCF-10F cells (homozygous for SULT1A1*2). B) In the presence of10 nM E2 and mefanamic acid, an inhibitor of SULT1A1, T47D cells exhibit estrogenic response similar to MCF-10F. This suggests that cellular capacity to sulfate estrogens impacts the proliferative response of cells to estrogens.

Figure 2. SULT1A1 is More Highly Expressed in Breast Tumor Tissue and Tumor Cell Lines than SULT1E1

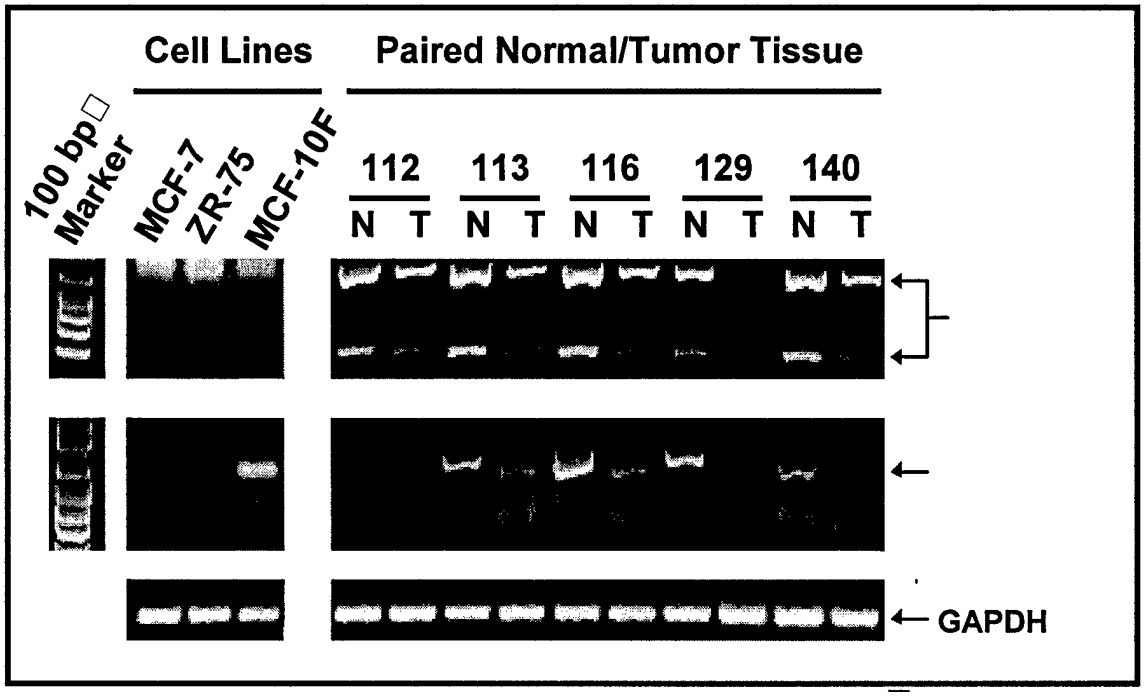

Figure 2. BamHI digestion of RT-PCR amplified phenol SULT transcripts indicated that SULT1A1 is expressed in normal breast tissue as well as breast tumors and, to a lessor extend in breast cell lines. MCF-7, ZR-75 and MCF-10F are cell lines, "N" indicates normal and "T" indicates tumor tissue. SULT1E1 was expressed in the cell line derived from normal epithelial cells (MCF-10F) but not in the tumor derived cell lines (MCF-7 and ZR-75) and is expressed at low levels if at all in breast tumors.