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TITLE: Studies on the Novel Anticancer Agents Metabolically Formed from 17-beta-Estradiol

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Studies on the Novel Anticancer Agents Metabolically Formed from 17-beta-Estradiol

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This is the final report for my Predoctoral Traineeship Award (No. DAMD17-02-1-0566). The studies described in the original grant proposal have been completed. Our major findings included the following: (1) We demonstrated, for the first time, that a novel class of nonpolar estradiol (E2) metabolites were formed by human liver microsomes and also by certain human cytochrome P450 (CYP) enzymes using NADPH as a cofactor. (2) Among a total of some 20 nonpolar E2 metabolite peaks detected, M15 and M16 were only selectively formed with a few of the human CYP isoforms (namely CYP3A4, CYP3A5, CYP1A1, CYP2C8, and CYP2C9). The formation of these two representative nonpolar estrogen metabolites by human CYP isoforms did not correlate with their overall catalytic activity for the oxidative metabolism of E2. (3) The structures of the metabolically-formed M15 and M16 were unequivocally identified to be the dimers of E2, linked together through a diaryl ether bond between a phenolic oxygen atom of one E2 molecule and the 2- or 4-position aromatic carbon of another E2. (4) M15 and M16 were chemically synthesized by using estradiol as the starting material.
Introduction

This is the final report for the Predoctoral Traineeship Award (No. DAMD17-02-1-0566). Recently, the PH.D. Dissertation Committee of our graduate program approved me for graduation with a PH.D. Degree in Basic Pharmaceutical Sciences. The studies described in the original grant proposal have been completed.

Body

The Specific Aims of the Original Proposal:

1. To determine the chemical structure of X2 (a major anticancer nonpolar E2 metabolite) by using various chemical and analytical methods.

2. To evaluate each of the nonpolar E2 metabolites formed by human liver microsomes for their inhibitory effects on the proliferation of ER(+) human breast cancer cell lines (MCF-7, T-47D, and ZR-75-1) and ER(-) human breast cancer cell lines (MDA-MB-231 and MDA-MB-435s).

3. To characterize the complete profiles of the nonpolar E2 metabolites formed by representative human liver and placental microsomes.

Significance: Studies described in this application will characterize the metabolic profiles of various nonpolar E2 metabolites that are formed by human tissues. More importantly, I will determine the biological activities of various nonpolar E2 metabolites for inhibiting the growth of human breast cancer cell lines. The structural information of the endogenous anti-breast cancer compound (X2) will be useful for the chemical synthesis of large amounts of this compound for further testing of its actions in animal models and eventually in humans for the treatment of breast cancers. I believe that success of my proposed studies will form the basis for future research efforts to further understand the mechanisms of their actions and to develop these novel estrogen metabolites as potential anti-breast cancer agents.

Major findings:

1. By using a versatile HPLC method (total elution time ~135 min) I developed, I detected the formation of some 20 nonpolar radioactive metabolite peaks (designated as M1 through M20), in addition to a large number of polar hydroxylated or keto metabolites, following incubations of $[^3]$H$\beta$-estradiol with human liver microsomes or cytochrome P450 3A4 in the presence of NADPH as a cofactor. The formation of most of the nonpolar estrogen metabolite peaks (except M9) was dependent on the presence of human liver microsomal proteins, and could be selectively inhibited by the presence of carbon monoxide. Among the four cofactors (NAD, NADH, NADP, NADPH) tested, NADPH was the optimum cofactor for the metabolic formation of polar and nonpolar estrogen metabolites in vitro, although NADH also had a weak ability to support the
reactions. These observations suggest that the formation of most of the nonpolar estrogen metabolite peaks requires the presence of liver microsomal enzymes and NADPH. Chromatographic analyses showed that these nonpolar estrogen metabolites were not the monomethyl ethers of catechol estrogens or the fatty acid esters of 17ß-estradiol. Analyses using liquid chromatography/mass spectrometry (LC/MS) and nuclear magnetic resonance (NMR) showed that M15 and M16, two representative major nonpolar estrogen metabolites, are diaryl ether dimers of 17ß-estradiol. These data suggest a new metabolic pathway for the NADPH-dependent, microsomal enzyme-mediated formation of estrogen diaryl ether dimers, along with other nonpolar estrogen metabolites.

2. I also characterized the NADPH-dependent formation of some 20 nonpolar estrogen metabolites by fifteen human CYP isoforms (CYP1A1, 1A2, 1B1, 2A6, 2B6, 2C8, 2C9, 2C18, 2C19, 2D6, 2E1, 3A4, 3A5, 3A7, and 4A11). I found that some of the nonpolar metabolites were formed at varying rates with each of the 20 human CYP isoforms tested, but M15 and M16 were selectively formed only with a few CYP isoforms. CYP3A4 and 3A5 had the highest catalytic activity for the formation of M15 and M16, but CYP1A1, 2C8 and 2C9 had weak but detectable catalytic activity for their formation. Kinetic analyses showed that the apparent $K_M$ values for CYP3A4 and CYP3A5-dependent formation of M15 and M16 ranged from 46–119 μM, and their apparent $V_{MAX}$ values ranged from 206–276 pmol/nmol of CYP/min. Using mass and NMR spectrometric analyses, we unequivocally identified the structures of M15 and M16 to be the dimers of 17ß-estradiol, which were connected together through a diaryl ether bond between a phenolic oxygen atom of one 17ß-estradiol molecule and the 2- or 4-position aromatic carbon of the other 17ß-estradiol.

3. Using estradiol as the starting material, I designed a four-step method for the chemical synthesis of these two estrogen dimmers with the Ullmann condensation reaction as a key step: STEP 1: Synthesis of 2- or 4-bromoestradiol from estradiol. STEP 2: Protection of the C-3 phenolic hydroxyl group of the 2- or 4-bromoestradiol. STEP 3: The Ullmann condensation reaction between the phenol-protected bromoestradiol and the estradiol potassium salt under our modified reaction conditions (with a 41% product yield). STEP 4: Removal of the C-3 benzyl group by catalytic hydrogenation. The chromatographic and various spectrometric properties of the two synthesized compounds were identical to those metabolically formed by human cytochrome P450 3A4.

**Key Research Accomplishments**

1. We have completed studies aimed at determining the chemical structures of M15 and M16 (originally called X1 and X2 in the grant proposal) by using various chemical and analytical methods.

2. We have chemically synthesized M15 and M16.
3. We have completed testing the inhibitory effects of M15 and M16 on the proliferation of ER(+) human breast cancer cell lines (MCF-7, T-47D, and ZR-75-1) and ER(-) human breast cancer cell lines (MDA-MB-231 and MDA-MB-435s).

4. We have systematically characterized the nonpolar E₂ metabolites formed by human liver microsomes and formed by 15 human cytochrome P450 isoforms.

Reportable Outcomes

Listed below are papers and abstracts that have come out of this award, with the P.I.'s name highlighted.


Conclusions

1. We demonstrated, for the first time, that a novel class of nonpolar E2 metabolites were formed by human liver microsomes and also by certain human CYP enzymes using NADPH as a cofactor.

2. Among a total of some 20 nonpolar E2 metabolite peaks detected, M15 and M16 were only selectively formed with a few of the human CYP isoforms (namely CYP3A4, CYP3A5, CYP1A1, CYP2C8, and CYP2C9). The formation of these two representative nonpolar estrogen metabolites by human CYP isoforms did not correlate with their overall catalytic activity for the oxidative metabolism of E2.
3. The structures of the metabolically-formed M15 and M16 were unequivocally identified to be the dimers of E2, linked together through a diaryl ether bond between a phenolic oxygen atom of one E2 molecule and the 2- or 4-position aromatic carbon of another E2.

4. M15 and M16 were chemically synthesized by using estradiol as the starting material.

References


Appendices

Not included.