# Identification of Biomarker Candidate, Catechol Estrogen-DNA Adduct, in Human Breast Tissue

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# Overview

## Background

- Long term exposure to estrogens increases the risk of breast cancer in women;
- Estrogen metabolites can react with DNA, causing removal of bases from DNA via a process called depurination;
- The abasic sites of DNA, if not repaired, can lead to the mutation of DNA and thus initiate cancer;
- Amounts of the depurinating adducts, 4-OH-E1(2)-Ade and 4-OH-E1(2)-Gua , reflect the extent of DNA damage and may serve as biomarkers.

## Purpose

- To detect the DNA adducts, 4-OH-E1(2)-Ade and 4-OH-E1(2)-Gua, that may serve as biomarkers for early diagnosis of breast caner.

## Methods

- Extract DNA adduct from breast tissue samples by Soxlet extraction;
- Reduce matrix of extracts by Solid-Phase Extraction (SPE);
- Fractionate extracts by semi-preparative HPLC;
- Analyze fractions of HPLC by nano LC-MS/MS.

## Results

- A nano LC-MS/MS method was developed to detect the DNA adducts in human breast tissue;
- The DNA adduct: 4-OH-E1-Ade was detected in normal breast tissue from two femal donors;
- The amount of **4-OH-E1-Ade is 12 fmole/100 mg and 33 fmole/50 mg in normal breast tissue**;
- The detection limit is 1 fmole/100 mg tissue and 100 amole in 5 μL for control;
- A novel, "one-pot" synthesis of the biomarker candidates for use as reference compounds was also developed.

# Introduction

Compelling evidence has revealed that estrogens contribute to the development of breast cancer. The endogenous estrogens, estrone (E1) and estrodiol (E2), can be metabolized to catechol estrogens and then quinone estrogens, which can further react with DNA, leading to the modification of DNA. The consequent error-prone DNA repair can thus generate oncogenic mutations that initiate breast caner. The quinone estrogens can either form stable adducts with DNA or predominantly remove bases from DNA by a depurination process.

The depurinating products, catechol estrogen-DNA adducts, may be utilized to measure the extent of DNA damage and in this way serve as biomarkers for early diagnosis of breast cancer. Here we report the detection of these DNA adducts in human breast tissue. To support the analytical work, we developed a novel, "one-pot" synthesis of the biomarkers for use as reference compounds, and we also described the synthetic method.

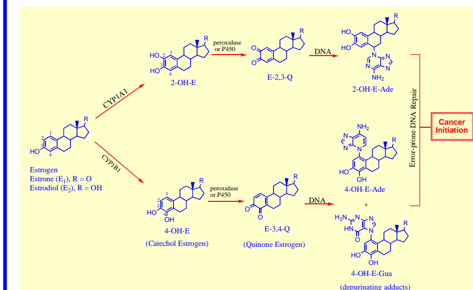



**Figure 1.** Initiation of breast cancer by endogenous estrogens. The process catalyzed by cytochrome P450 1B1 (CYP1B1) may be the major carcinogenic path leading to the formation of depurinating adducts, the potential biomarkers.

# Methods

## Treatment of Human Breast Tissue

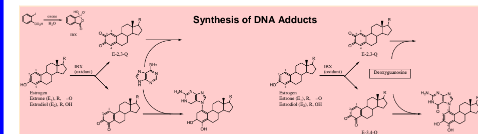
- Normal breast tissue
  1. Breast tissue was obtained from reduction mammaplasty surgery in a healthy female donors (two cases);
  2. Ground breast tissue was Soxhlet extracted with MeOH/CHCl3 for 24 h;
  3. Extract was reconstituted to 6 mL with 10% MeOH and applied to a solid-phase extraction (SPE) cartridge (Focus, Varian Inc.) and eluted with 1.25 mL of 60% MeOH, 30% ACN, 10% H2O and 0.1% TFA;
  4. Eluent was fractionated by a RP C18 5 μm 100 Å 10 × 150 mm column (P. J. Cobert, St. Louis, MO). Fractions were collected at 0.2 min intervals from 9 – 12 min whereas control experiments showed that the 4-OH-E1-Ade eluted at 10.8 min;
  5. Fractions of HPLC were reconstituted in 5 μL of 97% H2O 3% ACN 0.1% FA and injected into nano LC-MS/MS to identify 4-OH-E1-Ade adduct.
- Standard addition: spike 4-OH-E1-Ade into normal breast tissue
  1. Synthetic 4-OH-E1-Ade reference was spiked at 50 fmole/100 mg tissue;
  2. Steps 2-5 were repeated.

## Nano LC-MS/MS

- LC: Waters CapLC (Waters Corp.), before-column splitting;
- Column: uncoated 360/75 μm OD/ID Fused-silica capillary column with 15 μm PicoFritTM tip (New Objective Inc.);
- Packing material: C18 (Lunar 3 μm 100 Å, Phenomenex, Torrance, CA);
- Mobile phase: solvent A (97% H2O, 3% ACN, 0.1% FA) and B (3% H2O, 97% ACN, 0.1% FA);
- Gradient: 97% A for 8 min and then a 37 min linear gradient to 100% B;
- MS: LCQ Deca quadrupole ion trap mass spectrometer (Thermo Finnigan);
- Parent ions with m/z = 420.3 ±1.2 amu were selected in the total ion chromatography (TIC) to monitor the elution of protonated 4-OH-E1-Ade adduct.

## Synthesis of Biomarkers

- IBX oxidant was synthesized as shown in the reaction scheme for synthetic method;
- IBX (0.18 mmole) was added to a solution of estrone (0.18 mmole) in DMF (5 mL) in the dark;
- After 25 mins, a solution of Ade or dGuo in 10 mL of acetic acid and water (1:1, v/v) was added and allowed to react for ~ 4 h with stirring at room temperature;
- The final product consisted primarily of 3,4-catechol estrogen-DNA adduct;
- Four catechol estrogen-DNA adducts: 4-OH-E1-Ade, 4-OH-E1-Gua, 4-OH-E2-Ade and 4-OH-E2-Gua were synthesized by this method.




# Results

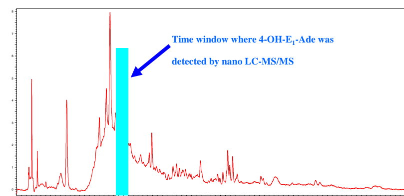



**Figure 2.** Typical liquid chromatograph of human breast tissue extract. Breast tissue was submitted to SPE/Focus for clean-up before fractionationed by HPLC. Fractions of HPLC were collected in 0.2 min interval then analyzed by nano LC-MS/MS. Negligible background of standard was achieved before analyzing the tissue extract.

## Tissue without standard addition

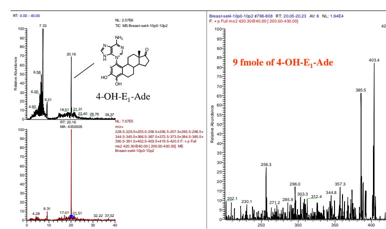



**Figure 3.** Fraction of HPLC (10.0-10.2 min) analyzed by nano LC-MS/MS. Biomarker candidate, 4-OH-$E_1$-Ade, was detected, and its amount in this HPLC fraction is 9 fmole determined by standard addition.

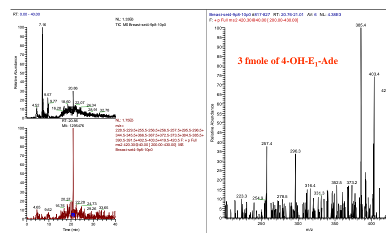



**Figure 4.** Fraction of HPLC (9.8-10.0 min) analyzed by nano LC-MS/MS. Biomarker candidate, 4-OH-$E_1$-Ade, was detected, and its amount in this HPLC fraction is 3 fmole determined by standard addition.

## Tissue with 50 fmole standard addition

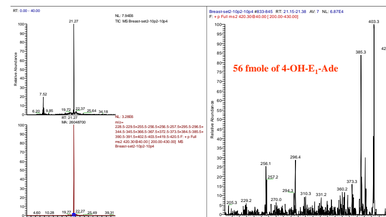



**Figure 5.** Addition of 50 fmole 4-OH-$E_1$-Ade standard into breast tissue extract. The amount of 4-OH-$E_1$-Ade in this HPLC fraction is 56 fmole determined by standard addition.

## Tissue with 50 fmole standard addition (cntd.)

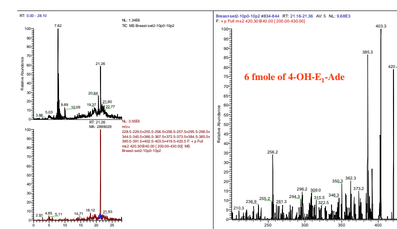



**Figure 6.** Addition of 50 fmole 4-OH-$E_1$-Ade standard into breast tissue extract. The amount of 4-OH-$E_1$-Ade in this HPLC fraction is 6 fmole determined by standard addition.

## Amount of 4-OH-$E_1$-Ade in normal breast tissue determined by standard addition

| Tissue (100 mg) | 4-OH-$E_1$-Ade | HPLC fractions 1 | 2 | 1 + 2 |
|---|---|---|---|---|
| Tissue w/o standard addition | Signal (counts) | $4.4 \times 10^6$ | $1.3 \times 10^6$ | $5.6 \times 10^6$ |
| | Amount (fmole) | 9 | 3 | 12 |
| Tissue + 50 fmole standard | Signal (counts) | $2.6 \times 10^7$ | $2.9 \times 10^6$ | $2.9 \times 10^7$ |
| | Amount (fmole) | 56 | 6 | 62 |

# Conclusions

- **A new nano LC-MS/MS method is available to detect biomarker candidate: 4-OH-E1-Ade in human breast tissue;**
- **The amount of 4-OH-E1-Ade is 12 fmole/100 mg and 33 fmole/50 mg in healthy breast tissue (two-case studies);**
- **The limit of detection is ~ 1 fmole/100 mg tissue (~ 5 pg/g).**

# Future Work

- **Statistically differentiate the level of catechol-estrogen DNA adducts in healthy tissue and in tumor;**
- **Search for biomarker candidates in serum and/or nipple aspirate;**

# Acknowledgement

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