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13. ABSTRACT ***(Maximum 200 Words)***

These studies examine the role estrogens play in the initiating events in cancer. We hypothesize that to develop cancer, one first must have DNA damage, which escapes normal repair and is set as a mutation in a critical gene. DNA damage can occur by direct damage to DNA by estrogen metabolites, as assayed in small oligonucleotides using MALDI-TOF mass spectrometry. Moreover, culturing cells in high, physiological levels of estradiol (E_2, 0.35 nM) or 4-OHE_2 (0.18 nM) results in detectable depurinating adducts in the estrogen receptor-positive human cell line T47D and in the estrogen receptor-negative cell line MDA MB-468. In addition, the repair of an oligo containing a stable adduct and an apurinic (AP) site or just an AP site, was assayed using cellular extracts from MCF-10A1 human breast cell line. The MCF-10A1 cell extracts repaired oligos containing both a stable adduct and an AP site, as well as an AP site alone. However, the relative amount of repair depended on the relative portions of the sites of damage.

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Kimberly Chapman

PI - Signature Date

Table of Contents:

Introduction:

These studies were designed to investigate the role estrogens play in DNA damage, and how this damage affects repair mechanisms. Endogenous estrogens can be activated by enzymes (i.e. cytochrome P450s and peroxidases) to activated forms, including catechols and quinones, which react with DNA resulting in DNA damage (1-3). How repair mechanisms approach this damage determines whether a mutation is set, passed on to subsequent daughter cells, or avoided (4). Failure to repair certain DNA damage sequence motifs or DNA damage combinations can lead to mutations in critical genes and subsequent development of cancer (4). Estrogen levels are greatly increased in female reproductive organs, and so, the risk of cancer development in these organs is theoretically greater than in non-estrogen-dependent organs, making this a useful study in terms of breast cancer.

Annual Summary Body:

Technical Objective #1: Unscheduled DNA synthesis in ACI rat mammary gland. This objective was completed and reported in the 1998 report.

Task 1: **completed**
Task 2: **completed**

Technical Objective #2: Determination of up-regulated repair enzymes in human cell culture.

*Task 3:***Completed** (reported 1999 report)
Task 4: Probe for expected enzymes and determine when they appear during the cell cycle. **Not Done**

It was decided to postpone task 4 (probe for expected enzymes to determine when they appear during the cell cycle) of this technical objective until useful DNA oligos containing DNA damage were developed. The development of these vectors is discussed in Technical Objective #4.

Technical Objective #3: Determination of up-regulated repair enzymes in human breast from women with and without breast cancer.

Task 5: **Partially completed**

Cellular extracts prepared from MCF-10A1 cells, which are derived from a human fibrocystic breast, were used in the *in vitro* repair assays discussed in technical objective #4. Consequently the proteins involved in normal repair of oligos containing different damage types were determined. We are not familiar with any reports in the literature implicating these proteins in human breast cancer. As for the up-regulated enzymes of estrogen metabolism, these have yet to be determined in normal controls versus breast cancer patients.

Technical Objective #4: Determination of the effects of different catechol estrogen-induced damaged on repair enzyme function.

Task 6: Design and obtain oligonucleotides (18 mers and 54 mers) **Completed and Expanded.**
Design and make GFP plasmid template. **Completed**
Establish MCF-10A1 (and HeLa S3) culture for repair assay and set up *in vitro* repair assay. **Completed**
Produces cellular extract from MCF10A1, HeLa S3 cells and MCF-10A1 cell extract immunodepleted of XPA protein or AP-1 protein. **Completed**
Task 7: Determine best conditions for repair assay and carry out assay with all designed oligonucleotides. **Completed**
Learn to microinjection MCF-10A1 cells with GFP and determine best conditions. **Completed and abandoned.**
Task 8: Carry out DNAse footprinting to determine location of repair subunits in relation to DNA damage. **Concluded**

As previously described in these reports, studies were undertaken to examine DNA damage by catechol estrogen quinones by using a matrix assisted laser desorption ionization-time of flight (MALDI-TOF) mass spectrometry technique. In this new method, the presence of a stable adduct or an AP site can be identified within a short defined oligonucleotide (oligo) without losing the sequence information of the oligo. Data from these studies confirmed that estradiol-2,3-quinone (E_2-2,3-Q) when it reacts with a purine within an 18 base long oligo forms a stable adduct. In comparison, estradiol-3,4-quinone (E_2-3,4-Q) forms AP sites at the single purine in these 18 mers. The AP sites are identified by strand breaks formed in the MALDI-TOF instrument. In both cases, the sequence of the reactive oligos remains intact, so that the sequence surrounding the adduct could be determined if it were unknown (although this method works more effectively with defined oligos).

One of the most important efforts to legitimize the study of repair of catechol-estrogen-induced DNA damage was to demonstrate that this damage occurs in environments that are physiologically possible. Studies were undertaken to examine

whether detectable levels of depurinating and stable adducts were formed. Depurinating adducts are those free adducts produced following estrogen-quinone binding to a nitrogenous base, destabilizing the glycosyl bond, leaving an apurinic site (AP). Stable adducts remain within the DNA. Using the estrogen receptor positive cell line T47D and the estrogen receptor cell line MDA MB-468, the ability of estradiol (E_2) at the physiological levels of 100 pg/ mL (0.35 nM) or 1 pg/ mL (2.3 pM) to form stable adducts and depurinating adducts was determined. In addition, the ability of 4-OHE_2 to form adducts at the same levels was determined.

Cells were treated for 0, 2, 4, or 6 days with the appropriate E_2 levels, 4-OHE_2 levels, or DMSO (the negative control and estrogen carrier). Medium was collected, saved and cells were refed with medium containing their specific treatment. At each of the time points, the cells were harvested and DNA was made.

To determine the amount of depurinating adducts produced by the cells treated with the estrogens, the medium was used. Cells ordinarily release depurinated adducts, and so, the depurinating adducts can be isolated from their medium. The medium was dried, Soxhelet extracted, and concentrated. Then, it was purified using a preparation with a 660 Waters HPLC equipped with a photodiodearray (PDA) 990 Waters UV detector in a water/methanol gradient and fractions at the retention times corresponding to the depurinating adducts, 4-OHE_2-1-N7Gua and 4-OHE_2-1-N3Ade, were collected. These adducts were identified and quantitated using an HPLC equipped with a CoulChem electrochemical detector (ESA) by using an ESA MD-150 RP C18 column eluted by 50 mM ammonium hydrogen sulfate/ 0.4% acetic acid/ acetonitrile solution. 4-OHE_2-1-N7Gua levels were also confirmed by running HPLC with a reverse phase Luna(2) C18

column (250 x 4.6 mm, 5μm) equipped with a 12-channel CoulArray (ESA) electrochemical detector in a linear gradient beginning with mobile A phase (0.1 M ammonium acetate pH 4.4/ acetonitrile/ methanol, 80:15:5) and ending in 90% mobile B phase (0.1M ammonium acetate, pH 4.4/ acetonitrile/ methanol, 30:50:20) over 50 min with a flow of 1 mL per min (gradient designed by Dr. Rosa Todorovic, 5). The detector measured potentials on 12 channels set from 0 mV to 590 mV, and data were processed using CoulArray software confirming retention times and peak heights between the dominant peaks and two adjacent channels. The samples in which adducts were recovered were then sent to our collaborators at Washington University in St. Louis for confirmation by LC/MS/MS, a very sensitive mass spectrometry technique using liquid chromatography and two tandem mass spectral bombardments. The levels of depurinating adducts detected listed in Table 1.

Table 1: Levels of 4-OHE_2-1-N7G and 4-OHE_2-1-N3A depurinating adducts produced by cells in culture.(femtomolar/cell)

DMSO treated cells

Days	**T47D**		**MDA MB-468**	
	4-OHE_2-1-N7G	4-OHE_2-1-N3A	4-OHE_2-1-N7G	4-OHE_2-1-N3A
2	50.6	6.55	30.07	0.101
4	409	5.99	89.2	2.67
6	42.7	nd*	201	1.65

E_2 treated cells (100 pg/mL)

Days	**T47D**		**MDA MB-468**	
	4-OHE_2-1-N7G	4-OHE_2-1-N3A	4-OHE_2-1-N7G	4-OHE_2-1-N3A
2	1420	24.3	178	50.6

4	870	41.6	480	25.6
6	1960	179	1610	59.1

4-OHE_2 treated cells (100 pg/mL)

Days	**T47D**		**MDA MB-468**	
	4-OHE_2-1-N7G	4-OHE_2-1-N3A	4-OHE_2-1-N7G	4-OHE_2-1-N3A
2	1590	56.2	22.4	30.5
4	970	69.3	237	6.07
6	960	14.8	866	100

nd* not detected

The level of stable adducts produced by each of these estrogen treatments was below our level of detection (10^{-9} μmol adduct/ μmol DNA) in a ^{32}P-postlabeling with the P1 nuclease method described in Cavalieri, et al (2). ^{32}P-postlabeling is a two-dimensional chromatography technique originally described by Randerath and Reddy in which the stable adducts within a DNA strand are ^{32}P-labeled and separated, whereas the undamaged bases cannot be labeled (6).

Perhaps we are unable to detect stable adducts because the level of stable adducts in these cells must be particularly low to begin with following E_2 treatments in the pM range. Moreover, the cells have time to repair their DNA following treatment and prior to DNA isolation (at least 48 h).

Having established that (1) catechol estrogen metabolites can cause direct DNA damage, and that (2) physiologically relevant E_2 was capable of producing adducts in cell

culture, the next step was to look at the efficiency of repair in the presence of a stable adduct and an AP site in close proximity.

Estrogen-quinones are capable of producing both AP sites and stable adducts, so initial studies explored repair in the presence of both damage types. We suspect that the presence of a stable adduct will inhibit the repair of an AP site because of competition by two repair mechanisms, nucleotide excision repair (NER) and base excision repair (BER).

Initial studies, reported in a previous annual report, used an 18 mer with a single AP site to establish that *in vitro* repair assays were possible in our hands using a whole cell extract isolation method based on Biade, et al. (7). From this point, we designed a larger template oligo that could bind the enzymes required for both NER and BER, and that could incorporate a stable adduct and an AP site at 16 nt or 7 nt apart. NER is recognized to repair stable bulky adduct. To do so, it excises 28-34 nt and then ligates a newly synthesized strand to replace the damaged one. On the other hand, BER is dependent on glycosylases with or without lyase to repair AP sites or abnormal nitrogen bases and excises 1 to 8 nt. Important to this discussion, short patch BER uses AP-1 to remove AP sites created by spontaneous depurination, depurinated chemical adducts, or glycosylases (excising 1-2 nt). Long patch BER repairs damage that is susceptible to repair by its glycosylases that can excise 3-8 nt, because they contain their own lyase activity.

One of the templates built to probe the ability of the MCF-10A1 cell to repair different types of DNA damage was created from a plasmid coding for green fluorescent protein. Using molecular biology techniques, a dU was cloned into the sequence of the

plasmid that codes for the green fluorescent protein. This plasmid with an AP site built by UDG reaction, alone, or other plasmids with stable adducts and AP sites, were going to be microinjected into MCF-10A1 cells to study repair *in vivo* of different damage configurations. Theoretically, the depurination of the dU by UDG should result in loss of the fluorescing protein translated from the plasmid. Unfortunately, optimization of microinjection of MCF-10A1 cells, of which there are no reports in the literature, was not accomplished in six months of attempts. It was decided to abandon this approach.

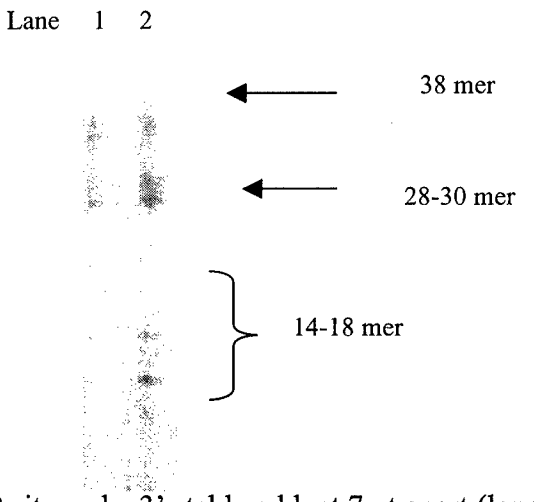
Then, a 50 bp long oligo was designed to contain an AP site and a stable adduct 7 nt or 16 nt apart (Table 2). Repair of these oligos were studied using the *in vitro* repair assay described for repair of the 18 mers.

In general, oligos with an AP site and a stable adduct 17 nt apart are less efficiently repaired than oligos with only an AP site, if the AP site is 5' of the stable adduct. If the AP site is 3' of the stable adduct and separated by 16 nt, the oligos are repaired as well as those with only an AP site. On the other hand, oligos with an AP site and a stable adduct 7 nt apart are repaired to similar levels. Perhaps this is due to co-repair, which describes the ability of long patch BER and NER to excise and repair both damage sites and repair them simultaneously.

Figure 1 compares the repair of a 50 mer with a 5'AP site and a 3' stable adduct 17 nt apart (lane 1) with a similar oligo containing only an AP site (lane 2). None of the bands in lane 1 contain quantitatively the same amount of product as in lane 2 according to the Quantitative Analysis Program (Molecular Dynamics, CA). This is an indication that the presence of the stable adduct decreases the amount of repair of the product. This differs from a similar oligo with damage only 7 nt apart (Figure 2). In the case of this

oligo, the damage may not be repaired due to competition between repair mechanisms for the damage.

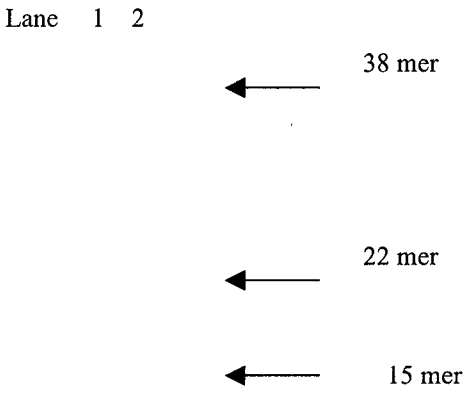
Figure 1: Repair of an oligo with a 5'AP site and 3'stable adduct 16 nt apart, compared to repair of an oligo with only an AP site (lane 2).



Repair of the 50 mer with a 5' AP site and a 3' stable adduct 7 nt apart (lane 1) compared to the repair of a similar oligo with only an AP site (lane 2) by MCF-10A1 cellular extract is shown in Figure 2. In this example, the oligo with an AP site and a stable adduct is repaired more effectively than the one with only an AP site. In other words the repair of the AP site and stable adduct is equivalent to or better than that of the AP site alone. This is probably a consequence of co-repair. Perhaps the presence of the stable adduct improves repair because it recruits the repair enzymes better.

Moreover, all studies, including some not shown here, indicate an oligo with only an AP site incorporates very little label to visualize repair. This may be a consequence of primarily being repaired by short patch BER, thus incorporating a single labeled base. However, these studies also indicate that there is some long patch BER of AP sites (22

mer) and even some NER (28-34 mer). One would expect to see more fully ligated products of repair (or even non-ligated products) of AP site damage in the XPA-immunodepleted cellular extract because NER would not be competing with the BER system. This is not the case.



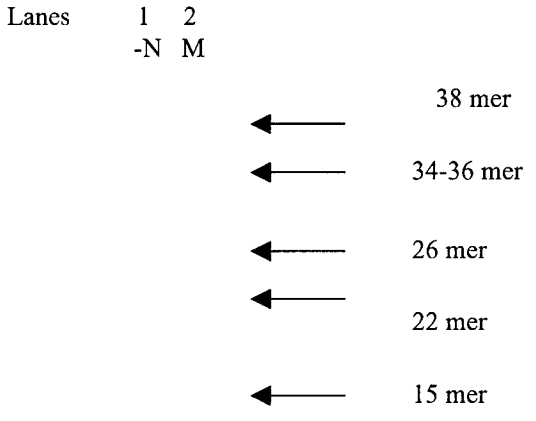
To determine the mechanisms that repair various damage conformations, immunodepletion of the MCF-10A1 cellular extract was carried out. By immunodepleting specific repair enzymes, a particular path of repair can be inhibited. As a result, the repair of a particular oligo with a particular damage must be undertaken by the remaining repair pathways. For example, by immunodepleting MCF-10A1 cellular extract of XPA, the cellular extract could not undergo NER. Providing evidence for this, the non-ligated products of NER produced by repair by complete MCF-10A1 cellular extract are no longer evident in lanes repaired by the XPA immunodepleted cellular extract. Likewise, AP-1 immunodepletion of MCF-10A1 cellular extract eliminated repair by short patch BER.

An example of the loss of a band in immunodepleted cellular extract, the repair of an oligo with a 5' AP site and a 3' stable adduct 7 nt apart by MCF-10A1 cellular extract (lane 2) only, MCF-10A1 CE immunodepleted of XPA (removing NER, lane 1) is illustrated in Figure 3.

The XPA immunodepleted cellular extract (lane 1) does not have the 34-36 mer, as is expected because it has lost NER, which can be seen in lane 2 (MCF-10A1 competent CE).

Although XPA is immunodepleted from the cellular extract used to repair this oligo, the AP site at position 14 is particularly well repaired by short patch BER, producing a 15 mer, which is evident in the MCF-10A1 CE repaired lane (lane 2) and the XPA-immunodepleted CE (lane 1). Interestingly, there is a 26 mer in both lanes, which can be a consequence of long patch BER of the stable adduct. The 22 mer in both lanes is suggestive of long patch BER of the AP site.

Figure3: Repair of an oligo with a 5'AP site and a 3'stable adduct 7 nt apart by XPA immunodepleted CE (-N, lane 1) or MCF-10A1 CE (M, lane 2)



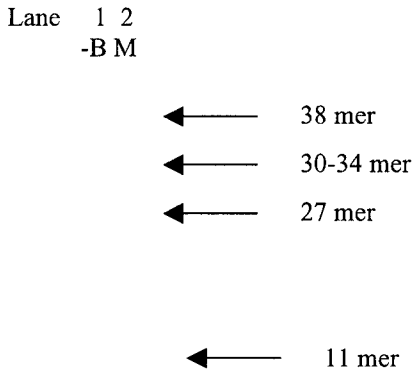
Having examined the results of XPA immunodepletion, Figure 4 examines the effects of AP-1 immunodepletion.

An oligo with a 5'stable adduct and a 3' AP site 16 nt apart repaired by MCF-10A1 CE (lane 2), and MCF-10A1 immunodepleted of AP-1 (lane 1) is illustrated in Figure 4.

Lane 2 of Figure 4, containing the MCF-10A1 repaired oligo has a 38 mer band suggestive of fully ligated repair. It also contains a 34-36 mer which is representative of the NER of the stable adduct. The 27 mer in lane 2 is evidence for short patch BER of the AP site at position 27. It is not evident in lane 1, which is immunodepleted of AP-1.

The 34-36 mer remains evident in lane 1, which contains oligos repaired by AP-1 immunodepleted cellular extract, because long patch BER is not dependent on AP-1 to excise nucleotides. The 11 mer evident in the lanes, predominantly with the competent cellular extract could be the result of short patch BER of the stable adduct. It is much less evident in the AP-1 immunodepleted extract. This could support the fact that this extract is immunodepleted. It could also have implications for the presence of a polycyclic aromatic hydrocarbon (PAH) glycosylase. Some short patch BER is not dependent on AP-1 if the glycosylase contains lyase activity. A dibenzo[a,l]pyrene diol epoxide (DBPDE) or PAH glycosylase has yet to be described, although there is some supposition that there is one located in humans. Some PAH adducts can depurinate without the presence of a glycosylase. Moreover, the presence of this slight band may be due to the stable adduct not being repaired and acting as a polymerase block.

Figure 4: Repair of the oligo contaning a 5' stable adduct and a 3'AP site 16 nt apart by MCF-10A1 CE (M, lane 2), or AP-1 immunodepleted CE (-B, lane 1).



DNase I footprinting (Task 8) of repaired 18 and 50 mers was attempted. Although some footprinting was done, the study was not continued due to technical issues. Footprinting repair with whole cell extracts provided little useful data since so many different repair proteins bound to the oligo. The studies would have been more useful if single purified proteins were used. In addition, studies by Wasasuga et al. (8) well characterized the sites of binding of XPA/ RPA and XPG (which are the essential proteins for excision in NER).

Conclusions

These studies were able to demonstrate that (1) estrogen-quinones directly damage DNA, (2) physiological levels of E_2 can form depurinating adducts in human

breast cell culture, whether an estrogen receptor is present or not, and (3) repair is decreased in short oligonucleotides with AP sites 5' of stable adducts.

This work is significant in that it challenges the notion that breast cancer results from action of the estrogen receptor to increase proliferation. Data from these studies and others indicate that estrogen not only works as a proliferation inducer, but also can cause direct DNA damage. A general model for cancer development, proposed almost 40 years ago by Miller and Miller, states that DNA in a cell must be initially damaged by a damaging agent, an initiator (9). The cell containing this initiated DNA then must be promoted by a second stimulus to proliferation for tumors to develop. Estrogen by its very nature can act as a mutation inducer, by its ability to form depurinating adducts, and as a promoter, by its ability to induce breast cell proliferation. As a consequence, human breasts are particularly vulnerable to the development of cancer since they are constantly exposed to a weak carcinogen, E_2 that happens to also be a promoter that can induce cellular proliferation.

Future work should probe the ability of the presence of a stable adduct and an AP site in close proximity to induce mis-repair. These studies merely examine the ability of particular damage configurations to be repaired or not—i.e. failure to repair damage. Although important, failure to repair is not the only way a mutation can occur following DNA damage. Studies by Chakravarti, et al. implicate mis-repair as a significant player in the induction of papillomas by PAH (4, 11).

To do this, the presently used oligos need to be inserted into a plasmid. The plasmid can then be repaired by the cellular extracts and mis-repair probed by ligation-

mediated polymerase chain reactions (LM-PCR). In this way, mis-repair as well as failure to repair can be determined and quantified.

Bibliography :

1. Dwivedy, I., P. Devanesan, P. Cremonesi, E. Rogan, and E. Cavalieri (1992) *Chem. Res. Toxicol.* **5**: 828.

2. Cavalieri, E.C., D.E. Stack, Stack, P.D. Devanesan, R. Todorivic, I. Dwivedy, S. Higginbotham, S.L. Johansson, K.D. Patel, M.L. Gross, R.L. Cerny, and E.G. Rogan (1997) *PNAS* **94**: 10937

3. Stack, D., E.J. Byun, M.L. Gross, E.G. Rogan, and E.L. Cavalieri (1996) *Chem. Res. Toxicol.* **9**: 851.

4. Chakravarti, D., J.C. Pelling, E.L. Cavalieri, and E.G. Rogan (1995) *PNAS* **92**: 10422.

5. R. Todorovic, P. Devanesan, S. Higginbotham, J. Zhao, M. Gross, E. Rogan, E Cavalieri. (2000 *91st Annual Meeting of the American Association for Cancer Research.* **Abstract #686,** April 2000.

6. Reddy, V.M. and K. Randerath. (1986 *Carcinogenesis* **7**: 1543-51

7. Biade, S., R.W. Sobol, S.H. Wilson (1998) *J. Biol. Chem.* **273**: 898-902.

8. Wakasugi, M., and A. Sancar (1998 *PNAS* **95**: 6669-157

9. Miller, E.C. and Miller, J.A. (1981) *Cancer* **47**: 2327-45

10. Chakravarti, D., Mailander, P., Franzen, J., Higginbotham, S. Cavalieri, E.L., Rogan, E.G. (1998) **16**: 3203-3210

Appendix 1: Key Research Accomplishments:

• Demonstrated that E_2-2,3-Q directly forms stable adducts in short 18 base long oligonucleotides, detected by Matrix-Assisted Laser Ionization Desorption-Time of Flight (MALDI-TOF) mass spectrometry

• Demonstrated that E_2-3,4-Q directly forms AP sites in short 18 base long oligonucleotides, detected by MALDI-TOF mass spectrometry.

• Demonstrated that depurinating adducts can be formed at detectable levels by physiological levels of E_2 or 4-OHE_2 (100 pg/mL) in human breast cell cultures.

• Undertook a series of *in vitro* repair assays and determined that an AP site 16 nt 5' of a stable adduct has decreased repair levels.

• Also determined that human repair mechanisms have significant overlap in their ability to repair different damage types

Appendix 2: Reportable Outcomes:

Manuscripts:

Chapman, **K.**, L. Zhang, M. Gross, E.C. Cavalieri, and E. Rogan. Identification of estrogen quinone-induced stable DNA adducts and apurinic sites by MALDI-TOF mass spectrometry. In preparation. (Abstract is Appendix 3c.)
Chapman, K., L. Zhang, M. Gross, E.C. Cavalieri, and E. Rogan. Production and analysis of 4-hydroxyestradiol-1-N7guanine and 4-hydroxyestradiol-1-N3adenine in breast caner cell lines treated with 0.35 nM or 0.18 nM estradiol or 4-hydroxyestradiol. In preparation. (Abstract is Appendix 3d.)
Chapman, K. and E.G. Rogan. Repair of oligonucleotides with a single site of damage by MCF-10A1 cellular extract. In preparation (Abstract is Appendix 3e.)
Chapman, K. and E.G. Rogan. Repair of short oligonucleotides containing both apurinic sites and stable adducts in close proximity. In preparation (Abstract is Appendix 3f).

Presentations and Abstracts at national meetings:

Chapman, K. and E. Rogan. Catechol estrogen-quinone-induced DNA damage. MD/PhD student meeting in Aspen, CO. July 1998 (Abstract is 3g).
Chapman, **K.**, L. Zhang, E. Cavalieri, M. Gross and E. Rogan. Identification of estrogen quinone-induced AP sites and stable adducts. Hormonal Carcinogenesis, Gordon Research Conference, Tilton, NH. August 1999 .
Chapman, K. and E.G. Rogan. Identification of depurinating and stable DNA adducts in human breast cancer cell lines following treatment with 2.3 nM estradiol or 4-OHE_2. 91st Annual American Association for Cancer Research Meeting, San Francisco, CA, April 2000, Proc. Amer. Assoc. Cancer Res. Vol 41: page 109. (Abstract is in Appendix 3a).

Chapman, K. and E.G. Rogan. DNA damage by estradiol metabolites and repair of oligonucleotides containing both stable and apurinic sites by MCF-10A1 and HeLa cellular extracts. Department of Defense, U.S. Army Medical Research and Materiel Command, Breast Cancer Research Program, Era of Hope Meeting, Atlanta, GA, June 2000. (Abstract is in Appendix 3b).
Li-Kang, Zhang, **Kimberly A. Chapman**, Michael L. Gross, Ercole L. Cavalieri, and Eleanor G. Rogan. Identification of the Apurinic sites in modified oligodeoxynucleotides by MALDI-TOF MS and ESI-MS. American Society for Mass Spectrometry, CA, June 2000.

Seminars:
K. Chapman. "Catechol Estrogen-DNA damage", Eppley Institute for Research in Cancer, Student/ Post-doc Seminar Series, March 1997.
K. Chapman. "Catechol Estrogen-DNA damage", Eppley Institute for Research in Cancer, Student/ Post-doc Seminar Series, October, 1998.
K. Chapman. "Catechol Estrogen-DNA damage", Department of Biochemistry and Molecular Biology seminar series, August 1999.
K. Chapman. "Repair of DNA damage in oligonucleotides", Eppley Institute for Research in Cancer Student/ Post-doc Seminar Series, February 2000.

Degrees: Completion of Ph.D. degree in August 2000

This work was included in the competitive renewal application for a program project grant: Application by Ercole L. Cavalieri, Dc.S. entitled Molecular Origin of Cancer: Catechol Estrogen-3,4-Quinones. Data from these studies were included in the application in

Project 3: Induction of DNA damage, misrepair, and tumor initiation, Dhruba Chakravarti
Project 5: Tandem Mass spectrometry for assessing DNA damage, Michael L. Gross

This work was also included in an NCI R01 application by Dhruba Chakravarti entitled 'Induction of DNA damage, misrepair and tumor initiation'.

Appenix 3a: Abstract for the 91st annual AACR meeting:

"Identification of depurinating and stable DNA adducts in human breast cancer cell lines following treatment with 2.3 nM estradiol or 4-OHE_2" **Chapman, K.** and E.G. Rogan. 91st Annual American Association for Cancer Research Meeting, San Francisco, CA, April 2000, Vol 41, pg 109.

Abstract

Many chemicals can cause DNA damage, which occasionally leads to cancer. Perhaps not surprisingly, some of these compounds are endogenously formed, including the

catechol estrogens (CE). Estrone (E_1) and E_2 can be oxidized to their 2-CE and 4-CE by cytochrome P450s. Further oxidation by cytochrome P450s or peroxidase produces the E_1/E_2-2,3-quinones and/or E_1/E_2-3,4-quinones, which form stable or depurinating adducts, respectively, when they react with DNA. The human breast cancer cell lines MDA-MB468 and T47D were treated with high, physiological levels (0.2 nM) of E_2 or 4-OHE_2 for 2-6 days. The depurinating adducts 4-OHE_2-N3Adenine and 4-OHE_2-N7Guanine were isolated by reverse phase HPLC with UV detection, and then identified by subsequent HPLC with an ESA Coulchem electrochemical detector. The identities of the adducts produced were then confirmed using tandem mass spectrometry. The levels of stable adducts were quantified by using the ^{32}P- postlabeling method. Depurinating adducts were formed in MDA-MB468 (estrogen receptor negative) and T47D (estrogen receptor posistive) cells following treatment with 0.2 nM E_2 or 4-OHE_2. The levels of depurinating adducts were in the pM range and were more than 100 fold higher than those of the stable adducts. These results suggest that at physiological levels of E_2 or 4-OHE_2, human breast cells form depurinating DNA adducts of E_2.

Appendx 3b : Abstract for the Era of Hope Meeting:

"DNA damage by estradiol metabolites and repair of oligonucleotides containing both stable and apurinic sites by MCF-10A1 and HeLa cellular extracts **Chapman, K.** and E.G. Rogan. Department of Defense, U.S. Army Medical Research and Materiel Command, Breast Cancer Research Program, Era of Hope Meeting, Atlanta, GA, June 2000.

Abstract: Breast cancer is a widespread health risk for thousands, if not millions, of women each year. These studies examine the role estrogen plays in the initiating events in cancer. To develop cancer, one first must have DNA damage, which escapes normal repair and is set, as a mutation in a critical gene. One way DNA damage can occur is by direct damage to DNA by estrogen metabolites. Estradiol can be activated by endogenous enzymes, such as cytochrome P450s, to its catechol forms (2-OH or 4-OH) and further oxidized to quinone forms (E_2-2,3-Q or E_2-3,4-Q), which can cause DNA damage. Culturing of cells with high, physiological levels of E_2 (0.35 nM) or 4-OHE_2 (0.35 nM) resulting in detectable depurinating and stable DNA adducts in the estrogen receptor-positive cell line T47D an in the estrogen receptor-negative cell line MDA MB-468. DNA damage occurs by many mechanisms, but failure to repair must occur for this damage to progress to mutation and, then potentially, to cancer. Additional studies described here examine the ability of cellular extracts from MCF-10A1 human cell lines to repair oligonucleotides (oligos) containing two different types of damage, stable DNA adducts and apurinic sites. Cytosine-thymine-rich 36 base long oligos were prepared containing a single adenine and a single deoxyuridine. In studies looking at the effect of stable adducts on various repair mechanisms, the oligo were treated with *anti*-dibenzo[a,l]pyrene diol epoxide (DB[*a,l*]PDE), which forms stable adducts 99% of the time. Then, the 36 mers were complemented to a 68 mer containing stem loops on each end and Xba I and HinD III restriction sites. Apurinic sites were created by treatment of the oligos with uracil DNA glycosylase (UDG) to remove the uracil base. The MCF-10A1 cell extracts repaired both stable and apurinic sites.

Appendix 3c: Abstract from MALDI-TOF paper :

Analysis of oligonucleotides containing estrogen quinone-induced stable adducts or apurinic sites by MALDI-TOF mass spectrometry. **Kimberly A. Chapman**, Li-Kang Zhang, Michael L. Gross, Ercole L. Cavalieri, and Eleanor G. Rogan. **in preparation**.

Abstract: Stable adducts and apurinic sites have been directly identified in DNA for the first time by using matrix assisted laser desorption ionization/time of flight (MALDI-TOF) mass spectrometry. These results demonstrate that the estrogen metabolite, estradiol-2,3-quinone, reacts with DNA to produce stable adducts , whereas estradiol-3,4-quinone affords depurinating adducts that lead to apurinic sites. The data presented in this article support the hypothesis that metabolites of endogenous estrogens can react with DNA to cause damage that may lead to mutations.

Appendix 3d: Adducts paper:

Production and analysis of 4-hydroxyestradiol-1-N7guanine and 4-hydroxyestradiol-1-N3adenine in breast cancer cell lines treated with 0.35 nM or 0.18 nM estradiol or 4-hydroxyestradiol. **Kimberly A. Chapman**, Ercole L. Cavalieri, Li-Kang Zhang, Michael Gross and Eleanor G. Rogan. **in preparation**

Abstract: Depurinating adducts have been identified and quantified from T47D (estrogen receptor positive) and MDA MB-468 (estrogen receptor negative) human breast epithelial cells following treatment with physiologically relevant levels of estradiol (E_2) and 4-OHE_2. In general, the level of depurinating adducts in each cell line is similar (in the fM/cell range), suggesting that the estrogen receptor plays no significant role in depurinating adduct formation. Stable adducts were not found to be present by ^{32}P-postlabeling at the time points assayed.

Appendix 3e: Repair of single damage sites.

Repair of oligonucleotides with a single site of damage by MCF-10A1 cellular extract. **Kimberly A. Chapman** and Eleanor G. Rogan. **in preparation**

Abstract: *In vitro* repair assays were undertaken to determine the ability of cellular extracts from human breast epithelial MCF-10A1cells to repair oligonucleotides containing either a single deoxyuridine (dU), or an apurinic (AP) site. MCF-10A1 cellular extract is capable of repairing AP sites and dUs. Its repair capabilities are similar to the cellular extracts derived from HeLa S3 cells. Repair by either cellular extract results in fully repaired and fully ligated products of repair, but it also results in non-ligated fragments as well. Using immunodepleted extracts, the repair processes responsible for these fragments were identified. AP sites can be repaired by short patch base excision repair, long patch base excision repair and, rarely, nucleotide excision

repair. Repair of dU occurs by the same processes, but is predominantly dependent on long patch base excision repair.

Appendix 3f: Repair of two damage sites.

Repair of short oligonucleotides containing both apurinic sites and stable adducts in close proximity. **Kimberly A. Chapman** and Eleanor G. Rogan. **in preparation**

Abstract: Short oligonucleotides (oligos, 50 mers) containing an apurinic (AP) site and a stable adduct or a dU and a stable adduct were assayed for repair. Oligos containing an AP site and a stable adduct were repaired to a lesser extent than a single AP site, if the AP site was 5' to the stable adduct and 16 nucleotides (nt) away. If in this same configuration, an AP site 5' to a stable adduct was only spaced 7 nt away, its repair was equal to that of an AP site alone. This result may be a consequence of co-repair by which both of the sites of damages are repaired by one repair process at the same time. Oligos with the damage orientation reversed, having a stable adduct 5' to the AP site, are repaired almost equally to an AP site alone. Oligos that contain a dU and a stable adduct are often repaired better than similar oligos with only a dU.

Appendix 3g: M.D.-Ph.D. meeting poster abstract

Catechol Estrogen DNA Damage, **Kimberly Chapman** and Eleanor Rogan, MD-PhD student meeting, Aspen, CO July 1998

Abstract: Evidence from our laboratory and others indicates that estrogens act as complete endogenous carcinogens in initiating and promoting the development of cancer. Estradiol or estrone can be oxidized to its catechol form (CE) at the 2 or 4 position by cytochrome P450s and then oxidized by cytochrome P450s or peroxidases to quinone forms (CE-Q) (See figure 1).

These CE-Q damage DNA by directly binding to the DNA and forming stable adducts which remain in the DNA or depurinating adducts which are lost when the bond between the sugar and nucleobase is broken (See figure 2). *In vitro* and *in vivo* assays indicate that these CE-Q bind to the N7 of guanine or the N3 of adenine to form depurinating adducts that lead to apurinic sites in the DNA. CE-Q-initiated stable adducts form at the 2-amino of guanine and the 6-amino of adenine. CE-2,3-Q form only stable adducts, whereas CE-3,4-Q form more than 99% depurinating adducts.

DNA damage by CE-Q in ACI rat mammary glands treated in culture with CE-2,3-Q and CE-3,4-Q was demonstrated by [^{3}H]thymidine incorporation. Treated glands demonstrated higher levels of incorporation than untreated glands. Studies were then undertaken to develop molecular biological methods to visualize CE-Q-induced DNA damage. Using 18-base single-strand oligonucleotides, including one with a uracil-DNA glycosylase-induced apurinic site, we showed that electrophoresis on 20% polyacrylamide denaturing gels can be used to visualize apurinic site formation in these oligos. This method is being used to visualize CE-Q-induced apurinic site formation. Once the CE-Q-induced damage is visualized, double strand oligos containing apurinic sites will be treated with cellular extracts from

MCF-10A1 cells to see whether DNA repair can be initiated and visualized using this gel electrophoresis technique.

DEPARTMENT OF THE ARMY
US ARMY MEDICAL RESEARCH AND MATERIEL COMMAND
504 SCOTT STREET
FORT DETRICK, MARYLAND 21702-5012

REPLY TO
ATTENTION OF:

MCMR-RMI-S (70-1y) 23 Aug 01

MEMORANDUM FOR Administrator, Defense Technical Information Center (DTIC-OCA), 8725 John J. Kingman Road, Fort Belvoir, VA 22060-6218

SUBJECT: Request Change in Distribution Statement

1. The U.S. Army Medical Research and Materiel Command has reexamined the need for the limitation assigned to the technical reports listed at enclosure. Request the limited distribution statement for these reports be changed to "Approved for public release; distribution unlimited." These reports should be released to the National Technical Information Service.

2. Point of contact for this request is Ms. Judy Pawlus at DSN 343-7322 or by e-mail at judy.pawlus@det.amedd.army.mil.

FOR THE COMMANDER:

Encl

PHYLIS M. RINEHART
Deputy Chief of Staff for
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