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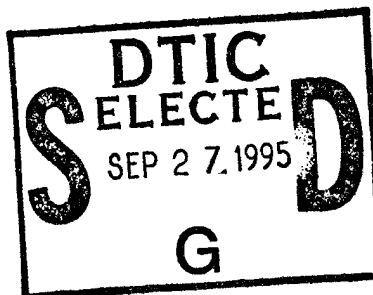
GRANT NO: DAMD17-94-J-4125

TITLE: An Organophosphorus Pesticide, Chlorpyrifos, Increases the Levels of the Multidrug-resistant Gene Product in Breast Cancer Cells

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REPORT DATE: 28 Jul 95



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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DTIC QUALITY INSPECTED 8

REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 0704-0188

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 the 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 28 Jul 95	3. REPORT TYPE AND DATES COVERED Annual 1 Sep 94 - 31 Aug 95
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4. TITLE AND SUBTITLE An Organophosphorus Pesticide, Chlorpyrifos, Increases the Levels of the Multidrug-resistant Gene Product in Breast Cancer Cells	5. FUNDING NUMBERS DAMD17-94-J-4125
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6. AUTHOR(S) Christine Lanning Robert Fine, M.D.	
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7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES) Duke University Medical Center Durham, North Carolina 27710	8. PERFORMING ORGANIZATION REPORT NUMBER
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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
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11. SUPPLEMENTARY NOTES

12a. DISTRIBUTION/AVAILABILITY STATEMENT Approved for public release; distribution unlimited	12b. DISTRIBUTION CODE
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13. ABSTRACT (Maximum 200 words)	<table border="1" style="width: 100%; border-collapse: collapse;"> <tr> <td colspan="2" style="padding: 2px;">Accession For</td> </tr> <tr> <td style="padding: 2px;">NTIS CRA&I</td> <td style="padding: 2px; text-align: center;"><input checked="" type="checkbox"/></td> </tr> <tr> <td style="padding: 2px;">DTIC TAB</td> <td style="padding: 2px; text-align: center;"><input type="checkbox"/></td> </tr> <tr> <td style="padding: 2px;">Unannounced</td> <td style="padding: 2px; text-align: center;"><input type="checkbox"/></td> </tr> <tr> <td colspan="2" style="padding: 2px;">Justification</td> </tr> <tr> <td colspan="2" style="padding: 2px;">By _____</td> </tr> <tr> <td colspan="2" style="padding: 2px;">Distribution / _____</td> </tr> <tr> <td colspan="2" style="padding: 2px; text-align: center;">Availability Codes</td> </tr> <tr> <td style="padding: 2px; text-align: center;">Dist</td> <td style="padding: 2px; text-align: center;">Avail and/or Special</td> </tr> <tr> <td style="padding: 2px; text-align: center; vertical-align: middle;">A-1</td> <td style="padding: 2px;"></td> </tr> </table>	Accession For		NTIS CRA&I	<input checked="" type="checkbox"/>	DTIC TAB	<input type="checkbox"/>	Unannounced	<input type="checkbox"/>	Justification		By _____		Distribution / _____		Availability Codes		Dist	Avail and/or Special	A-1	
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14. SUBJECT TERMS Organophosphorus Pesticide, Chlorpyrifos, Breast Cancer	15. NUMBER OF PAGES 9
	16. PRICE CODE

17. SECURITY CLASSIFICATION OF REPORT Unclassified	18. SECURITY CLASSIFICATION OF THIS PAGE Unclassified	19. SECURITY CLASSIFICATION OF ABSTRACT Unclassified	20. LIMITATION OF ABSTRACT Unlimited
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Annual Report
Christine Lynn Lanning

Summary of Proposed Goals for the First Year:

- 1). Establish a Protein Response
- 2). Demonstrate that Chlorpyrifos Induces MDR

Proposed Hypothesis:

We originally proposed that chlorpyrifos, the widely used organophosphorus pesticide, induces the expression of the multidrug resistance (mdr) gene product in a manner similar to that of chemotherapeutic agents and may contribute to a MDR phenotype seen in untreated and relapsed breast cancer patients.

RESULTS

A. Chlorpyrifos & P-gp Expression in MCF-7 Breast Cancer Cells

Since treatment of rats with chlorpyrifos increased P-gp expression along the digestive tract (Lanning et al., 1995), we wanted to determine its potential to induce P-gp expression in breast cancer cells. Using flow cytometry with the C219 and UIC2 P-gp antibodies, P-gp expression in MCF-7 breast cancer cells exposed to chlorpyrifos for 3 days was examined. A significant reproducible increase in P-gp expression was not obtained with chlorpyrifos exposure. To ensure that the technique was working properly, another experiment with adriamycin, an agent known to induce P-gp expression, was conducted. A dose dependent increase in P-gp expression was observed (Figure 1). Since the technique was working properly, it was concluded that either chlorpyrifos did not induce P-gp expression or that the breast cancer cells could not metabolize chlorpyrifos to the oxon, the metabolite shown to interact with P-gp (Lanning et al., 1995).

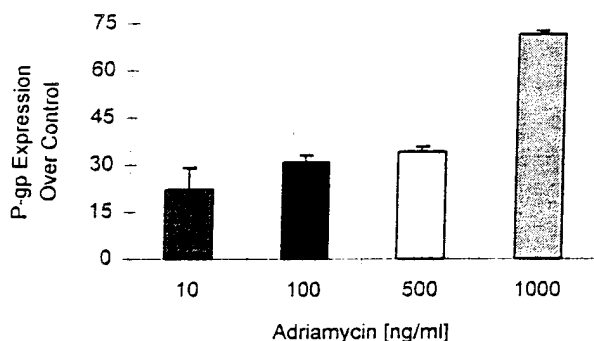


Figure 1. Adriamycin Induced P-gp Expression in MCF-7 Cells.

MCF-7 breast cancer cells were exposed 3 days to varying concentrations of adriamycin. P-gp expression was measured using flow cytometry with the UIC2 P-gp antibody. P-gp expression was reported as the increase over control, wild type MCF-7 cells without drug exposure.

B. DDT Interacts with Breast Cancer P-gp

Since we were unable to establish a reproducible increase in P-gp expression with chlorpyrifos treatment, we sought a new compound. An association between elevated organochlorine levels (DDT/DDE) and the development of breast cancer has been reported (Wolff et al., 1993). We decided to examine DDT and DDE to determine any association with P-gp in breast cancer cells.

Before dosing cells with DDT to determine its impact on P-gp expression, an ATPase assay was run to determine if DDT was a substrate for P-gp. The ATPase assay measures the organic phosphate liberated from the binding of agents to P-gp. Briefly, ADR10 breast cancer cells have been selected with the P-gp agent, adriamycin, and as a result over-express P-gp (Figure 2).

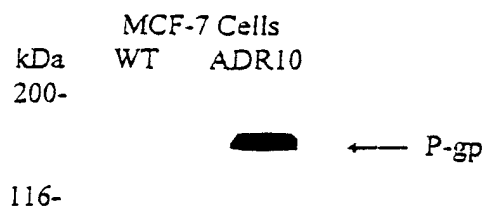


Figure 2. P-gp Expression in ADR10 Breast Cancer Cells.

P-gp expression was determined using the P-gp antibody, C219. Lane 1, wild type MCF-7 cells, lane 2, ADR10 selected MCF-7 breast cancer cells.

Microsomes prepared from the ADR10 breast cancer cells were incubated in the presence of verapamil, an agent known to bind to P-gp and stimulate P-gp ATPase activity, and in the presence of varying concentrations of DDT. Both the control, verapamil, and DDT stimulated ATPase activity (Figure 3). DDT at 1.0 μM stimulated maximal ATPase activity (258 nmol phosphate/min/mg protein). Interestingly, maximal stimulation with verapamil, a known agent for P-gp, (225 nmol/min/mg protein) occurred at 10 μM . These results suggest that P-gp had a greater affinity for DDT and suggest that DDT is a substrate.

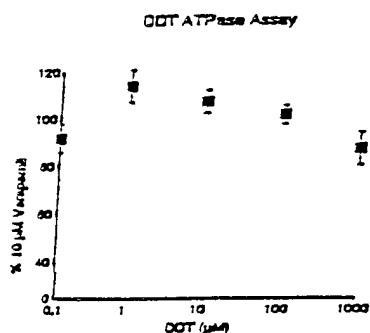


Figure 3. DDT Stimulation of P-gp ATPase Activity.

Membranes from ADR10 cells were incubated for 20 minutes in the presence and absence of varying concentrations of DDT. The differences between the ATPase activities measured in the presence and absence of DDT is plotted as percent of 10 μM verapamil stimulation. Experiments were repeated at least three times.

C. DDT and DDE Increase P-gp Expression in Breast Cancer Cells

Since DDT stimulated P-gp ATPase activity, DDT was examined to determine if it induced P-gp expression. MCF-7 breast cancer cells were treated with various concentrations of DDT and DDE for up to 17 days. P-gp expression was detected by immunoblot analysis using the P-gp antibody, C494. Increased P-gp expression was observed with both agents (Figure 4).

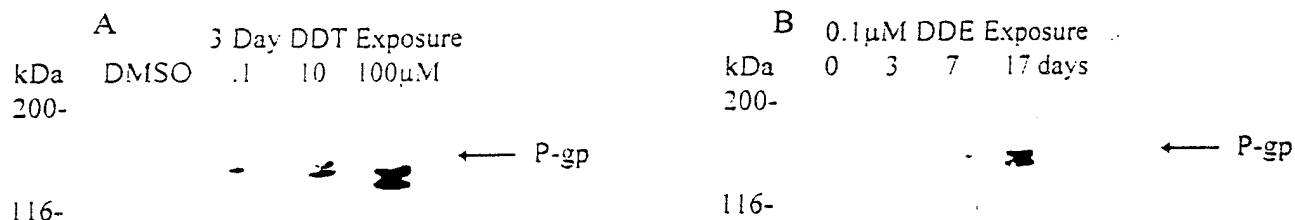


Figure 4. Immunoblot Analysis of DDT and DDE Treated Breast Cancer Cells.

P-gp expression was determined by immunoblot analysis of MCF-7 cells treated with various concentrations of DDT or DDE. DMSO was used as a negative control. Experiments were repeated at least three times.

D. DDT and DDE Induce a Functional P-gp

To demonstrate that the P-gp identified in the DDT and DDE treated MCF-7 cells was functional, a Rhodamine 123 assay was performed. Briefly, Rhodamine 123 is a P-gp transported mitochondrial dye; therefore, cells with functional P-gp (ADR10, DDT, and DDE treated cells) should appear "dull" whereas cells lacking P-gp (MCF-7 wild type cells) should stain brighter. After treating wild type, ADR10, 10 μM DDT, and 10 μM DDE cells with Rhodamine 123, the staining was analyzed through flow cytometry (Figure 5). Since DDT and DDE treated MCF-7 treated cells accumulated more dye than the ADR10 cells (cell over-expressing P-gp) and less dye than the wild type MCF-7 cells (cell expressing little to no P-gp), it appeared that the P-gp was functional.

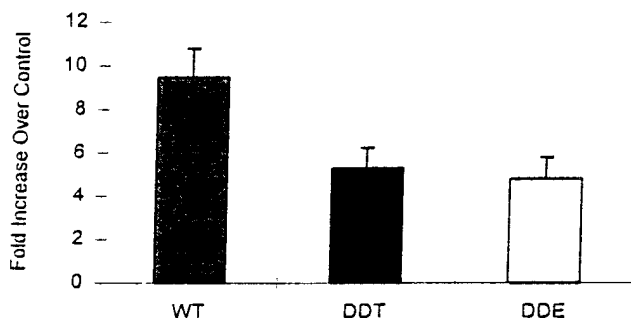


Figure 5. Rhodamine Accumulation in DDT and DDE Treated Breast Cancer Cells.

MCF-7 breast cancer cells were treated with 10 μM DDT or DDE for 6 days. Rhodamine accumulation was measured by flow cytometry. The results of accumulation were presented as % control (ADR10 accumulation).

SUMMARY

Since I was unable to obtain consistent increase in P-gp expression with chlorpyrifos treatment and was able to with DDT and DDE treatment, the original hypothesis was slightly modified.

HYPOTHESIS: DDT and DDE induce the expression of the multidrug resistance (mdr) gene product in a manner similar to that of chemotherapeutic agents and this may contribute to a MDR phenotype seen in untreated and treated breast cancer patients.

The first year goal of establishing a protein response was achieved with the immunoblot analysis demonstrating that both DDT and DDE increased P-gp expression. The second goal which was scheduled to be carried over into the first 3 months of the second year was to demonstrate the induction of MDR. In so far we have demonstrated that the P-gp identified in the DDT and DDE treated cells was functional. Next we plan to demonstrate that P-gp confers resistance, the last part of this aim.

In summary, this project is in agreement with the original time schedule and we do not foresee any major changes in the time line.

METHODS

1. Cell Culture

MCF-7 breast cancer cells were cultured in RPMI 1640 media supplemented with 5% heat inactivated fetal calf serum at 37°C in 5% CO₂.

2. Flow Cytometry

P-gp expression was determined using flow cytometry by the method of Mechetner and Roninson. After washing, the MCF-7 cells were incubated with rabbit serum (1:20) for 10 minutes. After removing the serum, the primary P-gp antibody, C219 or UIC2, was applied at 5 µg/ 4x10⁵ cells for 60 minutes at 4°C. After three washes with a 1% BSA/PBS solution, the cells were incubated for 60 minutes at 4°C with the secondary antibody, FITC conjugated rabbit antimouse IgG (1:32). The cells were washed three times and resuspended in 1 ml PBS. The fluorescence was measured by Dr. Michael Cook using a Coulter Epics 753 flow cytometer located in the Cancer Center Flow Cytometry Laboratory.

3. Membrane Preparation

Breast cancer cells were homogenized in a hypotonic lysis buffer (10 mM Tris, 10 mM NaCl, 2 mM EDTA, 2 mM PMSF, 10 mg/ml leupeptin, and 8 mg/ml aprotinin) and centrifuged at 400 x g for 10 minutes. The resulting supernatant was centrifuged at 100,000 x g for 60 minutes and the membrane pellet was resuspended in sample buffer and stored at -70°C.

4. Immunoblot Analysis

After determining the protein concentration using the BCA method, 100 µg membrane protein were resolved on a 7.5% polyacrylamide gel by the method of Laemmli. The gel was transferred for one hour to PVDF membrane and incubated for 1-2 hours in blocking solution containing 2.5% Carnation nonfat dry milk. The P-gp antibody, C219 or C494, was applied at 1 µg/ml for 1-2 hours. After washing, the membrane was incubated for 1 hour with biotinylated goat antimouse IgG diluted 1:600 in TBS containing 0.1% Tween 20. After washing, the membrane was incubated for one hour with streptavidin horseradish peroxidase diluted 1:6000 in PBS. After washing, the membrane was exposed to the ECL reagents and placed under film. Densitometry was used to quantitate the films.

5. P-gp ATPase Activity

The ATP hydrolysis assay was performed by the method of Sarkadi. Briefly, 10 µg ADR10 membrane proteins were incubated at 37°C in 0.1 ml of buffer [50 mM Tris-Mes (pH 6.8), 2 mM EGTA, 2 mM dithiothreitol, 50 mM KCL, and 5 mM sodium azide] with various concentrations of DDT. The ATPase reaction was initiated with the addition of 5 mM ATP and terminated with the addition of 0.1 ml 5% SDS solution. The SDS-containing samples were supplemented with 0.4 ml reagent A (2.5 M H₂SO₄, 1% ammonium molybdate, and 0.014% antimony potassium tartrate) and 1 ml distilled water.

Finally, 0.2 ml 1% ascorbic acid was added and the optical density was measured at 880 nm. ATPase activity was estimated by the difference obtained in P_i levels between 0 and 20 minute incubation periods.

6. Rhodamine 123 Accumulation Assay

Rhodamine accumulation was measured by flow cytometry following the method of Chaudhary and Roninson. Briefly, 2×10^5 cells were incubated with 100 ng/ml Rhodamine 123 for 2 hours at 37°C. After incubating, the cells were washed with PBS and analyzed by flow cytometry.

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