EVALUATION OF ALTERATIONS IN GENE EXPRESSION IN MCF-7 CELLS INDUCED BY THE AGRICULTURAL CHEMICALS ENABLE AND DIAZINON

A Thesis

by

TANMAYI PRADEEP MANKAME

Submitted to the Office of Graduate Studies of
Texas A&M University
in partial fulfillment of the requirements for the degree of

MASTER OF SCIENCE

May 2004

Major Subject: Genetics

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Approved to as style and content by:

David. L. Busbee (Chair of Committee)	Bhanu Chowdhary (Member)
Keith Murphy (Member)	Evelyn Tiffany-Castiglioni (Head of Department)
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ABSTRACT

Evaluation of Alterations in Gene Expression in MCF-7 Cells Induced by the Agricultural Chemicals Enable and Diazinon. (May 2004)

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Chair of Advisory Committee: Dr. David. L. Busbee

Steroid hormones, such as estrogen, are produced in one tissue and carried through the blood stream to target tissues in which they bind to highly specific nuclear receptors and trigger changes in gene expression and metabolism. Industrial chemicals, such as bisphenol A and many agricultural chemicals, including permethrin and fervalerate, are known to have estrogenic potential and therefore are estrogen mimics. Widely used agricultural chemicals, Enable (fungicide) and Diazinon (insecticide), were evaluated to examine their toxicity and estrogenicity. MCF-7 cells, an estrogen-dependent human breast cancer line, were utilized for this purpose. MCF-7 cells were treated with 0.033-3.3 ppb (ng/ml) of Enable and 0.3-67 ppm of Diazinon and gene expression was compared to that in untreated cells. Microarray analysis showed down-regulation of eight genes and up-regulation of thirty four genes in cells treated with 3.3 ppb of Enable, compared to untreated cells. Similarly, in cells treated with 67 ppm of Diazinon, there were three genes down-regulated and twenty seven genes up-regulated. For both chemicals, specific genes were selected for special consideration. RT-PCR confirmed results obtained from analysis of the microarray. These studies were designed to provide base-line data on gene expression-altering capacity of specific chemicals and will allow assessment of the deleterious effects caused by exposure to the aforementioned chemicals.

DEDICATION

To my parents,
for encouraging me to pursue my dreams and

To my teachers,
who helped me achieve them.

ACKNOWLEDGEMENTS

I would like to thank my committee chair Dr. David Busbee for giving me an opportunity to pursue this research. I am grateful to him not only for his academic guidance but also for his continuous support and encouragement during the course of my research.

I thank my committee members Dr. Bhanu Chowdhary and Dr. Keith Murphy for being a part of my committee and evaluating my research. I remain indebted to them for the invaluable advice they provided.

A special thanks to Regina Hokanson for constant guidance, which gave me better insights into my research. I thank Renuka Chowdhary for the help with microarray data analysis and for providing valuable insights during the course of my research.

I thank Dr. Laurie Davidson for guidance and troubleshooting with real time rt-pcr technology. I thank the administrative staff of the Departments of Veterinary Anatomy and Public Health and Genetics for their cooperation and help in administrative matters.

I thank my friends for their support, in times good and bad. Finally, I thank my family for their continuous support, encouragement and love through all these years.

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INTRODUCTION

Ligands are molecules that are bound by a protein. Certain ligands, such as the steroid hormones, are produced in one tissue and carried through the blood stream to target tissues, in which they bind to highly specific nuclear receptor proteins and trigger changes in gene expression and metabolism (1). Estrogen, a key steroid hormone, plays a vital role in the development and function of the mammary glands, and is active in neoplasia of this tissue. In both normal and cancer cells, estrogen receptor alpha (ERα), a nuclear receptor, is the primary target of estrogen. ERα is a transcription factor and its over-expression may lead to breast cancer.

Diethylstilbestrol (DES), a synthetic agonist of 17β-estradiol (E2) which was initially prescribed for pregnant women to lower the risk of spontaneous abortion, is now known to be associated with an increased risk of breast cancer and clear cell adenocarcinoma (CCA) of the vagina and cervix (2). Similarly, alkyl phenols, such as 4-nonyl phenol and 4-nonyl phenoxycarboxylic acid, appear to have intrinsic estrogenic activity because they compete for binding to the E2 receptor (3). Industrial chemicals, such as bisphenol A (bisA) and dibutyl phthalate (DBP), are known to exhibit estrogen-like activity and to enhance the proliferation of estrogen-responsive breast cancer cell lines (4). When the estrogenic potentials of several agricultural chemicals, such as permethrin and fervalerate, were examined they were found to be agonists of estrogen, competitively inhibiting the binding of estradiol to the estrogen receptor (ER) (5). All of these chemicals mimic estrogen.

In addition, a variety of chemicals that may alter, or in other ways dysregulate, essential gene expression in both developing embryos and adults may be correlated with altered morphological, neurological, or immunological development of the fetus. Chemically-altered gene expression in pre- and post-natal, developing, and adult humans may be correlated with specific physiological functions essential to good health.

This thesis follows the style and format of Endocrinology.

Among the widely used agricultural chemicals Enable (α-[2-(4-chlorophenyl)ethyl]-α-phenyl-1*H*-1,2,4-triazole-1-propanenitrile), marketed by Rohm Haas, and Diazinon (*O,O*-diethyl*O*-2-isopropyl-6-methylpyrimidin-4-pyrimidinyl phos-phorothioate), distributed by Voluntary Purchasing Groups, Inc., are very interesting due to their toxicity in human cells (3,4,36,67). MCF-7 cells, an estrogen dependent human breast cancer line (Fig. 1), was used to examine the toxicity and estrogenicity of these chemicals.

With sequencing of the human genome as well as the advent of microarray technology, it is now possible to evaluate which genes have altered expression after chemical exposure, which may allow extrapolation to physiological changes at the organism level.

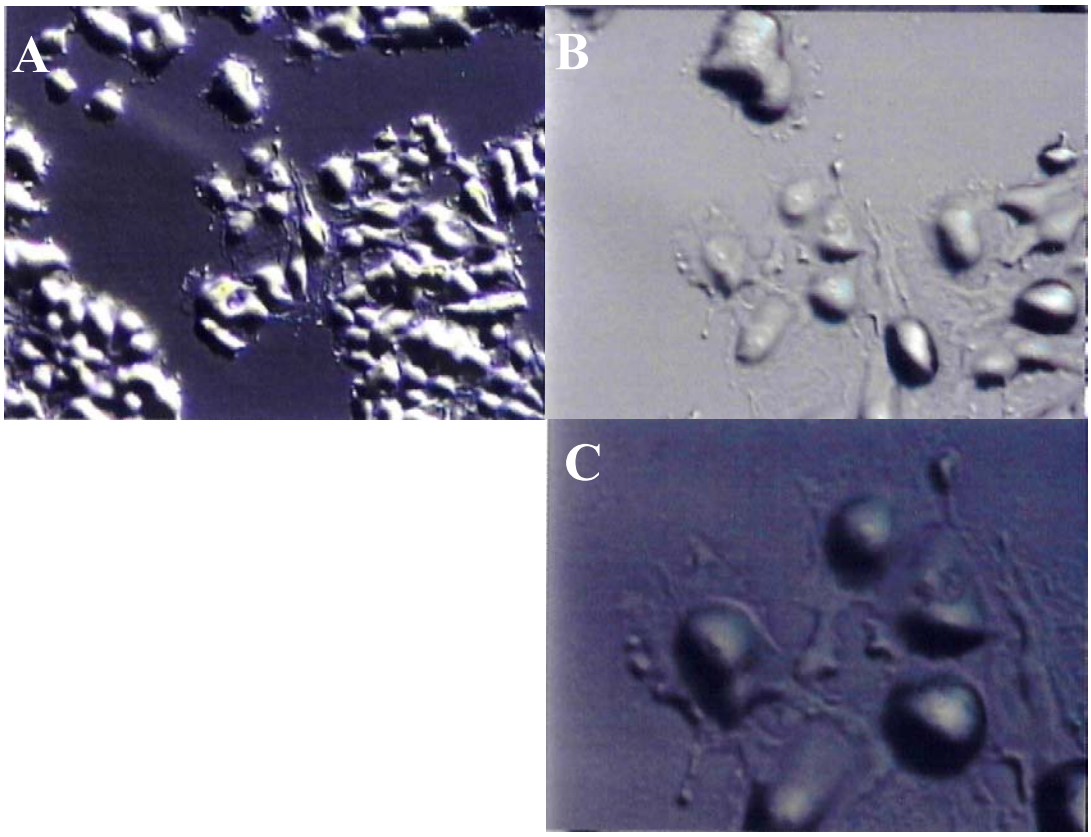

FIG.1. MCF-7 cells at different magnifications. A, B and C at 10, 20 and 40X respectively

FUNGICIDES

The action of most fungicides takes place outside the host and is called “protection”. A fungicide that acts outside the host is called a “protectant fungicide”. Most older fungicides sprayed on leaves and fruit are of this type. "Therapy" is chemical action inside the host. Fungicides may act to produce a toxic reaction in the fungus in several different ways. (l) Some may inhibit (slow down or stop) cell wall formation. Propiconazole and triadimefon act this way on cereal rusts. (2) The permeability of the cell wall may be affected, causing a leaking of nutrient materials from the cell. (3) Some fungicides may combine with essential metals to make them unavailable for normal cell functions, including the activities of essential enzymes. (4) Other fungicides may inhibit respiration, or nuclear division, or may break dormancy of spores (6).

Fungicides can be classified as those with a single mode of biochemical action (site specific) or those that affect a host of different processes (multi-site). The main groups of fungicides in use at present are listed below in order of their development (6). Dithiocarbamates have a broad spectrum of activity against downy mildews, leaf spots and rusts. They are non-systemic protectants that are usually used in formulated mixtures with other types of fungicides, to help minimize the risk of resistance problems occurring (6). Methyl benzimidazole carbamates (MBC)s inhibit cell division and fungal growth. These fungicides control many leaf spotting fungi and were at one time important in the control of Botrytis grey mould of fruit crops and ornamentals (6). Ergosterol biosynthesis inhibitors (EBIs) inhibit synthesis of sterols, which are essential for fungal growth. These fungicides can affect a wide range of types of pathogen, particularly those causing powdery mildews, rusts and leaf spots. They are used to control crop diseases in arable and horticultural production. There are a number of sub-groups within the compounds that inhibit ergosterol synthesis the most important of which are the triazoles (6).

Phenylamides inhibit RNA synthesis. These are important in the control of downy mildews and diseases caused by *Phytophthora* species (e.g. potato blight) and Pythium. These types of fungi cause many important root rot diseases in horticultural crops (6). Strobilurins inhibit mitochondrial respiration in fungi, and have activity against all the major types of fungi. This is the newest group of fungicides to be marketed and they are mainly used in cereal crops, but have some horticultural uses, e.g. apple scab control (6).

Uses

According to the U.S. Environmental Protection Agency (EPA), in 1997 approximately 244,000 and 37,000 tons of fungicides were sold worldwide and in the United States, respectively. Triazoles with non-fungicidal applications include, azocyclotin is used as an acaricide, paclobutrazole as a growth regulator, carfentrazone as an herbicide, and isazophos as an insecticide (7).

Azoles play a pivotal role in the treatment of systemic and dermal mycoses. They show significantly fewer side effects compared with other antimycotics such as amphotericin B. Another significant application of azoles is in the management of advanced estrogen responsive breast tumors in post-menopausal women. In these women estrogen levels decrease dramatically due to the lack of synthesis in the ovaries (7).

Occurrence in the Environment

Enable is a fungicide intended for use as an agricultural and horticultural fungicidal spray for the control of leaf spot, yellow and brown rust, powdery mildew and net blotch on wheat and barley and apple scab, pear scab and apple powdery mildew on apples and pears (8). Enable is moderately persistent to persistent and slightly mobile to immobile in soil. Because of its adsorption to soil, the potential for Enable to leach to ground water appears to be decreased. However, the potential to contaminate ground water may be greater at vulnerable sites (i.e., where soils are low in organic matter and where ground water is relatively close to the surface) (9).

Chemical and Physical Properties

The IUPAC name is (*RS*)-4-(4-chlorophenyl)-2-phenyl-2-(1*H*-1,2,4-triazol-1-ylmethyl)butyronitrile (10). The chemical formula is written as $C_{19}H_{17}ClN_4$. The structure is shown in figure 2.

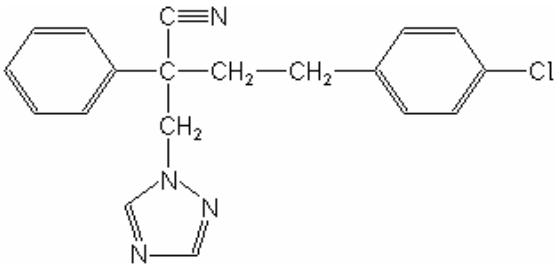

FIG. 2. Structure of the fungicide Enable (10)

Azole fungicides have broad antifungal activity and are used either to prevent fungal infections or to cure an infection. Therefore, they are important tools in integrated agricultural production. According to their chemical structure, azole compounds are classified into triazoles and imidazoles; however, their antifungal activity is due to the same molecular mechanism (7). Enable is an azole fungicide which belongs to the triazole group (Fig. 2). Its active ingredient is fenbuconazole. Enable is a white liquid that is soluble in water.

Acute Exposure Effects

Enable is a suspected carcinogen and an endocrine toxicant. It shows low acute toxicity when administered orally (LD_{50} > 2000 mg/kg bw), dermally (LD_{50} > 5000 mg/kg bw), or by inhalation (LC_{50} > 2.1 mg/liter air). It is not irritating to the skin or eyes and is not a sensitizer in a Buehler test, but is a weak sensitizer in a maximization test. WHO has not yet classified fenbuconazole for acute toxicity (8,9).

Chronic Exposure Effects

Carcinogenicity: 78 week studies in mice indicated evidence of carcinogenicity based on the occurrence of an increased trend for malignant liver tumors in males and an increase in benign and malignant liver tumors in females. Fenbuconazole has been classified as a Group C Carcinogen (8, 9)

Developmental toxicant: In rats, due to decreased maternal body weight compared to controls and increase in early and late resorption with a decrease in number of live fetuses per dam (8, 9).

Biomarkers of Exposure

A metabolic study in rats showed that radio-labeled fenbuconazole is rapidly absorbed, distributed, and excreted following oral administration in rats. Biliary excretion data indicated that systemic absorption of fenbuconazole was high for all dosing groups. The feces were the major route of excretion. Tissue distribution and bioaccumulation of fenbuconazole appeared to be minimal (8, 9).

Mechanism of Toxicity

Sterol 14α-demethylase is a member of the super family of heme-containing cytochrome P450 enzymes involved in metabolism of endogenous and xenobiotic substances. The antifungal effect of azoles is due to its inhibition of sterol 14α-demethylase in fungi and yeast, thereby blocking the biosynthesis of Ergosterol. Sterol 14α-demethylase is not only expressed in fungi and yeast but is found in many other species ranging from bacteria to mammals. Another important P450 enzyme involved in steroidogenesis is aromatase. Like sterol 14α-demethylase, aromatase catalyzes the oxidative demethylation of sterols. In contrast to sterol 14α-demethylase, which has several substrates in different phyla, aromatase demethylates C10 and specifically converts androstenedione and testosterone, resulting in estrone and estradiol, respectively. In mammals, aromatase is mainly expressed in the brain and gonads, but is also found in placental, adipose, and bone tissue. The physiological balance between different sex steroid hormones is crucial for development, maintenance, and function of the reproductive system as well as for differentiation of the sexual phenotype during ontogeny. Estrogens (estrone, estriol and estradiol) are products of the androgens (androstenedione and testosterone), and the reaction is catalyzed by aromatase (Fig. 3). In mammals, differentiation of the male phenotype depends not only on testosterone but also on estradiol generated from testosterone by neuronal aromatase in central nervous system. Therefore, disturbances in aromatase expression and/or changes in its catalytic activity are expected to exhibit negative effects on reproduction parameters. Aromatase can be inhibited competitively and reversibly by azole compounds, as seen with sterol 14α-demethylase (7).

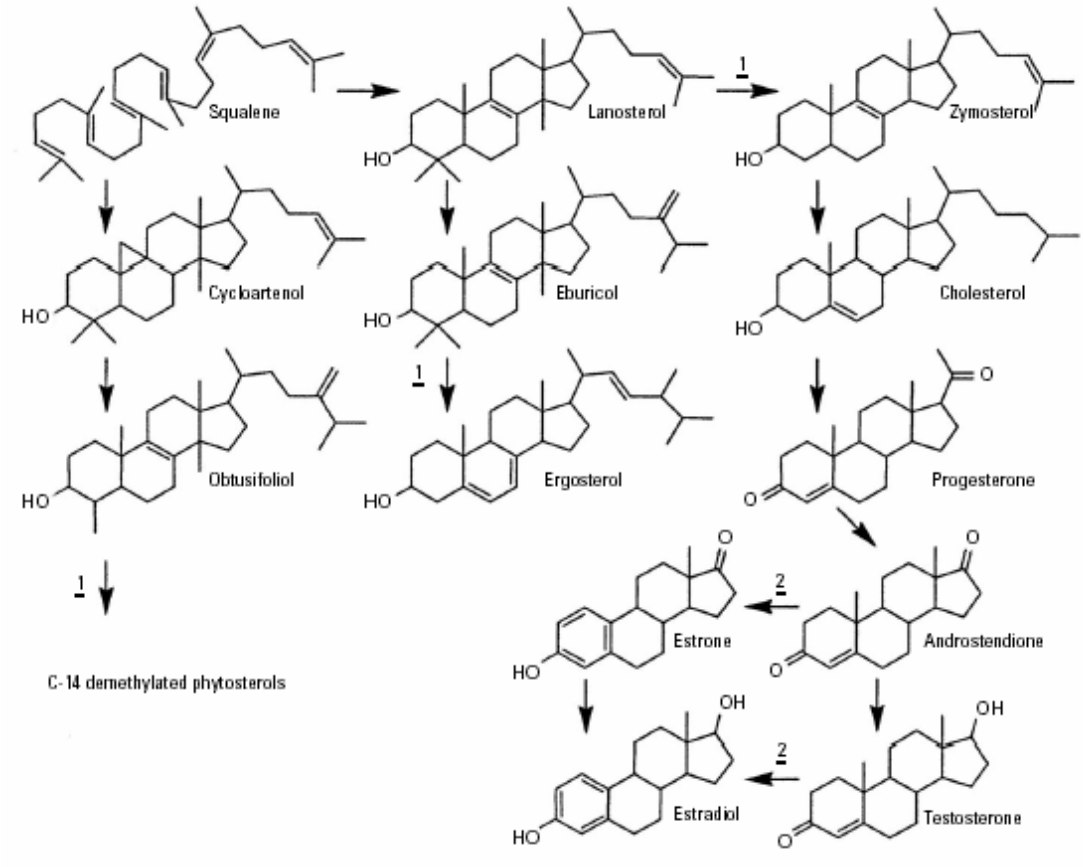

FIG. 3. Biosynthesis of phytosterols, ergosterol, and mammalian sex steroid hormones. Arrows indicate one or more enzymatic steps. Reactions catalyzed by sterol 14α-demethylase (1) and aromatase (2) are indicated (7).

Animal Studies

Oral toxicity, rat: LD_{50} > 2000 mg/kg body weight. Dermal toxicity, rat: LD_{50} > 5000 mg/kg body weight. Inhalation toxicity, rat: LC_{50} > 2.1 mg/l of inhaled air. 13-week study of hepatotoxicity in mice; 60 ppm, equal to 14 mg/kg body weight per day. Developmental toxicity studies, rat: 30 mg/kg body weight per day. Two-generation study of reproductive toxicity, rat: 5.8 mg/kg body weight per day (8, 9).

DIAZINON

Uses

Most widely used insecticide, to control foliage and soil insects and pests of many fruit, nut, vegetable, forage and field crops. Approximately 13 million pounds of the active ingredient diazinon are used annually on agricultural areas (11). Diazinon is also used in sheep dips and cattle ear tags (12) and lawns and gardens to control insects, grubs, and nematodes in turf. It is used indoors for fly control through crack and crevice treatments and for veterinary use to control fleas and ticks through pet collars (11).

Occurrence in the Environment

California, Texas & Florida are the states with the most significant usage. Diazinon is detected frequently in the Sacramento River and its tributaries during the dormant spray season. This coincides with the winter months when the area receives a majority of its annual rainfall. Pesticides applied during this period thus have An increased potential to wash off targeted areas of application and migrate with storm runoff water to streams in the Sacramento River Basin and creating contaminant loads. (13)

Chemical and Physical Properties

The IUPAC name is *O,O*-diethyl*O*-2-isopropyl-6-methylpyrimidin-4-pyrimidinyl phosphorothioate (10). The chemical formula is written as $C_{12}H_{21}N_2O_3PS$. The structure is shown in figure 4.

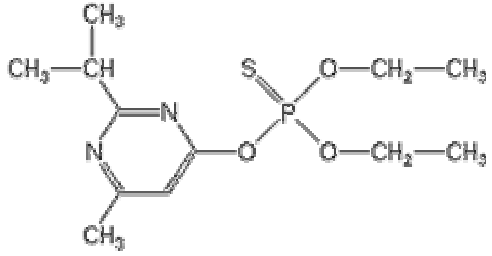

FIG. 4. Structure of insecticide Diazinon (10).

Diazinon is from the class of pesticides called Organophosphates (OPs).All organophosphates are derived from one of the phosphorus acids, and as a class are generally the most toxic of all pesticides to vertebrates. Because of the similarity of OP chemical structures to the "nerve gases", their modes of action are also similar. All OPs are esters of phosphorus having varying combinations of oxygen, carbon, sulfur and nitrogen attached, resulting in six different subclasses: phosphates, phosphonates, phosphorothioates, phosphorodithioates, phosphorothiolates and phosphoramidates. These subclasses are easily identified by their chemical names. The OPs are generally divided into three groups--*aliphatic, phenyl*, and *heterocyclic* derivatives. Diazinon is a heterocyclic organophosphate (Fig. 4), and is registered as dust, granules, wettable powders, seed dressings, emulsifiable solutions, impregnated materials, concentrates and ready-to-use solutions.

Acute Exposure Effects

Diazinon inhibits acetylcholine esterase causing nausea, dizziness, and confusion, with exposure to high levels causing respiratory paralysis & death (11). Acute exposure causes gastric outlet obstruction (14). Diazinon is an immunotoxic chemical causing inhibition of serine hydrolases and esterases (12). Also know to be genotoxic in human nasal mucosal cells (15). Inhibition of protein synthesis in HL-60 cells is seen after exposure to diazinon (16).

Chronic Exposure Effects

Diazinon induces delayed neuropathy, inhibits neurite growth in mouse neuropathy, and reduces the levels of brain neurotransmitters GABA, taurine, glutamate, and aspartate (17, 18). It significantly inhibits plasma cholinesterase. Diazinon inhibits protein synthesis and causes Protein deficiency, which is known to depress the immune system.

It is also known to cause lesions in the immune organs such as thymus and spleen, and represses lymphocyte activation by affecting the amino acid metabolism pathway (12). Diazinon increases the risk of Non-Hodgkin's lymphoma in human males (19).

Biomarkers of Exposure

Monitor the levels of acetylcholine esterase in plasma, RBC and Brain (18). Measure levels of diazinon metabolites such as dialkyl phosphates in urine (20). CYP1A2 and 2B6 are also biomarkers of diazinon toxicity since bioactivation of diazinon is CYP specific (21). Assessment of semen quality as exposure to diazinon was associated with decreased semen quality in some Missouri subjects. (22)

Mechanism of Toxicity

Diazinon causes toxicity by inhibiting the enzyme acetylcholine esterase (ace) thereby causing accumulation of acetylcholine at the cholinergic synapse. Diazinon by itself is a weak ace inhibitor however it can be activated by P450-mediated reactions to oxons, which are potent inhibitors of the enzyme. In human liver, CYP2C19 is the main enzyme involved in activation of diazinon. During this process, the thiono-sulfur atom of diazinon can cause inactivation of P450. Detoxification occurs through hydrolysis of the oxon by calcium-dependent arylesterases or by binding of the oxon to, or hydrolysis of the oxon by, carboxylesterases (Fig. 5). Insects are deficient in these esterases and therefore the most susceptible to diazinon and other phosphothioates. (23)

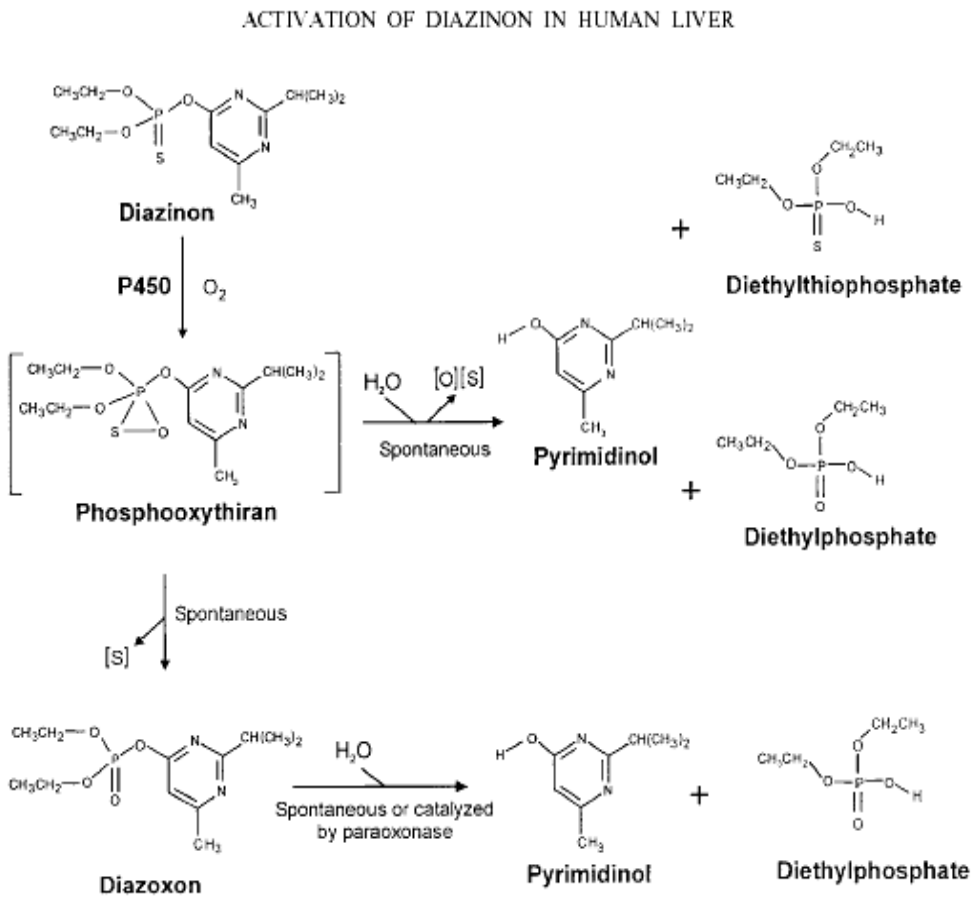

FIG. 5. Activation of Diazinon in human liver (23).

Animal Studies

Acute oral toxicity in rats: LD50 = 1250 mg/kg for combined sexes causing tremors, hunched appearance, gasping and salivation. Acute inhalation in rats: LC50 => 2.33mg/L, symptoms include piloerection, polyuria. Chronic toxicity in rats: for inhibition of plasma cholinesterase NOEL is 0.005 mg/kg/day, LOEL is 0.06mg/kg/day. Reproductive toxicity in rats: LOEL of 100 ppm & NOEL of 10 ppm was observed, at 500 ppm there was decreased mating, decreased fertility indices, and increased gestation length. Diazinon is a positive dermal sensitizer in humans (24).

MATERIALS AND METHODS

TISSUE CULTURE

The human breast cancer cell line, MCF-7, obtained from ATCC (Manassas, VA),was grown in minimal essential medium (MEM, Sigma, Saint Louis, MO) containing phenol red, supplemented with 10% fetal bovine serum (FBS, Atlanta Biologicals), 5% insulin, 5% sodium pyruvate, L-glutamine(Sigma, Saint Louis, MO). Cells were then switched to MEM without phenol red and with FBS for 18hrs, because phenol red acts as a weak estrogen (25). This was followed by treatment with 17β-estradiol, also from Sigma, for another 18hrs or with Enable or Diazinon at various concentrations in the presence or absence of 17β-estradiol. Treatment with Enable and Diazinon; Cells were treated 0.033-3.3ng/ml of Enable and 0.3-67ppm of Diazinon with or without 17β-estradiol.

TOTAL RNA EXTRACTION AND QUANTIFICATION

Cells were harvested at the end of 36 hours using Ambion lysis solution. RNA was extracted using the totally RNA kit of Ambion (Austin, TX). The extracted RNA was quantified by spectrophotometry and evaluated on an agarose gel along with a MCF-7 standard induced mRNA, also from Ambion, to check for RNA quality.

CDNA MICROARRAY

mRNA was prepared from the total RNA using the oligotex kit of Qiagen (Valencia,CA) and labeled with Cy-3 or Cy-5 using the micromax ASAP kit (Perkin Elmer, Boston, MA). This was followed by hybridization of the control mRNA labeled with Cy-5 and the test mRNA labeled with Cy-3 for 16 hrs on Phase 1 microarray slides containing 600 human genes (Santa Fe, NM).

SCANNING AND DATA ANALYSIS

Fluorescent signals on the slides were scanned using a Gene Pix Axon 4000b scanner (Union City, CA) and their intensities were analyzed using the software Acuity (Axon Instruments, Union City, CA).

REVERSE TRANSCRIPTION

After visualizing 2µg of each RNA sample on a non-denaturing gel, 1µg of good quality total RNA was used for reverse transcription. Briefly, for a 25µl RT reaction, random hexamers (16µM final, Roche, Indianapolis, IN), Oligo dT (500 ng/µl, Promega, Madison, WI), RNA 1.0 µg, and RNAse-free water was combined and heated at 65ºC, 5 min and allowed to cool down to room temperature. A cocktail of the following components was added to each sample: 5X 1st strand buffer, 0.1 M DTT (both included with SuperScript II), RNase block (Brinkman, Westbury, NY), 10 mM dNTPs (Promega, Madison, WI) and SuperScript II RT (Invitrogen, Carlsbad, CA). Cocktail will also be made without RT as a control. Each tube was mixed and centrifuged briefly. The RT reaction continued at 37ºC for 1hour. The reverse transcriptase was inactivated by heating at 90ºC for 5 minutes and samples cooled on ice. A reaction with and without reverse transcriptase was performed with each sample.

REAL TIME PCR

A master mix containing Platinum Quantitative PCR SuperMix-UDG, ROX reference dye, RNAse/DNAse-free water, 10µM FAM-labeled LUX primers and 10µM unlabeled reverse primer was assembled. All components are from Invitrogen. The primers shown in Table 1, were specific for the genes selected for special consideration or GADPH, a house keeping gene common to most mammalian cells, and were designed using Invitrogen's LUX primer designing program (available from the Invitrogen website) and labeled with FAM. 10µls of each sample, with and without RT, was combined with 40µl of the master mix in an optically clear semi-skirted PCR plate and reactions containing the primers, specific for the genes of interest and GADPH, were

done for each sample. The real time PCR was done run using an Applied Biosystems Prism 7700 thermocycler (Foster City, CA) using a 3 step cycling program recommended by Invitrogen: 50ºC, 2min hold (UDG treatment), 95ºC, 2min hold, 45 cycles of : 95ºC, 15 sec, 55ºC, 30 sec, 72ºC, 30 sec.

DATA ANALYSIS

The data are presented as normalized expression levels: (expression level of gene of interest divided by expression level of GADPH). The expression level is determined by this equation: 2 raised to the (45-Ct), Ct being the cycle number at which the threshold was crossed for the test gene or GADPH, and 45 is the number of cycles in the program (26).

TABLE 1. Primers used for real-time RTPCR

Gene Name	PubMed Id		Oligonucleotides 5'-3'
Phenol sulfotransferase	U26309	forward	CCCACGACTCCTGAAGACACAC
		reverse	CAACGTGCCACATCCTTTGCGFAMTG
Intercellular adhesion molecule-1	J03132	forward	TCTATAACCGCCAGCGGAAGA
		reverse	CACAAGAGGGAGGCGTGGCTTGFAMG
Calreticulin	M84739	forward	CGATGAGGCATACGCTGAGG
		reverse	GACAAACCTCTGCTCCTCGTCCTGTTTGFAMC
Transforming Growth Factor Beta-3	XM007417	forward	GGGCAAATGGCTGAGATGG
		reverse	CACGGCCCACACTTTCTTTACCACCGFAMG
Excision Repair Protein	L20046	forward	GACACGTTCTGAAGTCATTGGCCCTGFAMC
		reverse	TGGTCTGCTGTTCTTGGATGGT
Ribonucleotide Reductase M1 subunit	X59543	forward	GACATACACCCTGGCCCTGAATATGTAGFAMC
		reverse	GGGCGATGGCGTTTATTTG
GADPH	X01677	forward	CATCAAGAAGGTGGTGAAGCA
		reverse	CTACACTACCACCTGGTGCTCAGTGFAMAG

RESULTS

MCF-7 Cells were grown in medium containing 10% normal FBS (NFBS), or in medium with 10% charcoal – dextran treated FBS, for 18h. NFBS has some inherent estrogen which was removed by treating with charcoal-dextran. At the end of 18h, the cells were treated with Enable (0.033-3.3 ng/ml) or Diazinon (0.3-300 ppm), with or without 17β- estradiol (see Table 2). Cells were harvested after 36h, RNA was isolated (Fig. 6) and microarray slides were labeled. It was observed that cells treated with Diazinon above 90 ppm in the medium died.

TABLE 2. Acronyms used for treatments

Acronym	Type of treatment
NFBS	MCF-7 Cells grown in medium containing normal fetal bovine serum (10%) for 36 h
SFBS	MCF-7 cells grown in medium containing charcoal-dextran treated FBS medium (10%) for 36 hrs
NFBS T1-E2	MCF-7 cells grown in NFBS for 18h, followed by addition of 3.3 ng/ml of Enable to the medium for 18 h
NFBS T1+E2	MCF-7 cells grown in NFBS for 18h, followed by addition of 3.3 ng/ml of Enable and 17 β-estradiol to the medium, for 18 h.
NFBS E2	MCF-7 cells grown in NFBS for 18h, followed by addition of 17 β-estradiol to the medium, for 18 h.
SFBS T1	MCF-7 cells grown in SFBS for 18h, followed by addition of 3.3 ng/ml of Enable to the medium, for 18 h.
SFBS E2	MCF-7 cells grown in SFBS for 18h, followed by addition of 17 β-estradiol to the medium, for 18 h.
NFBS D3	MCF-7 cells grown in NFBS for 18h, followed by addition of 3 ppm of Diazinon to the medium, for 18 h.
NFBS D30	MCF-7 cells grown in NFBS for 18h, followed by addition of 30 ppm of Diazinon to the medium, for 18 h.
NFBS D50	MCF-7 cells grown in NFBS for 18h, followed by addition of 50 ppm of Diazinon to the medium, for 18 h.
NFBS D67	MCF-7 cells grown in NFBS for 18h, followed by addition of 67ppm of Diazinon to the medium, for 18 h.

TREATMENT WITH ENABLE

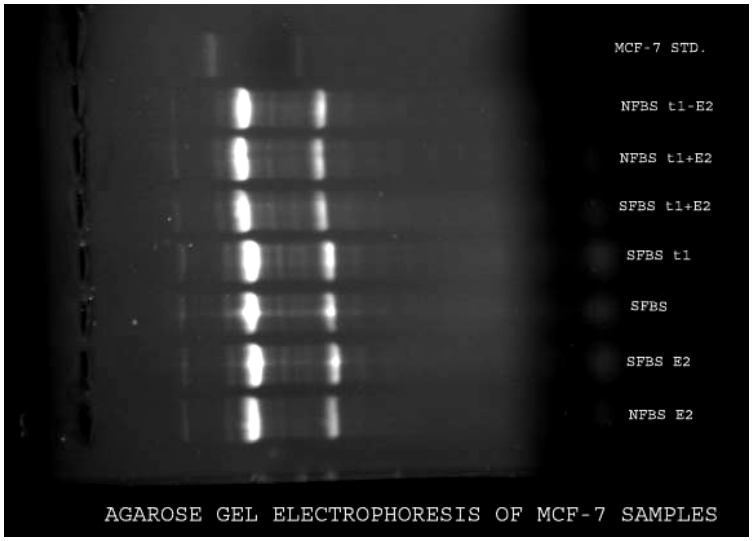

FIG. 6. Agarose gel electrophoresis of Enable treated MCF-7 RNA samples.

TABLE 3. Microarray experiments with Enable treated MCF-7 cells

Control (CY5)	Test (CY3)	Results
SFBS	NFBS	No significant change in gene expression
NFBS	NFBS T1-E2	8 genes down-regulated and 34 genes up-regulated
SFBS	SFBS T1+E2	No significant change in gene expression
SFBS	SFBS T1	No significant change in gene expression

Identification of Genes Affected by Enable

The series of microarray experiments carried out with enable are summarized in Table 3. In the cells treated with 3.3ng/ml (T1), there was down-regulation of 8 genes and up-regulation of 34 genes as seen in Table 4 compared to untreated cells (Fig.7). From the down-regulated genes, PST and ICAM-1 were chosen for RT-PCR verification. Likewise, calreticulin and TGF β3 were selected from amongst the up-regulated genes.

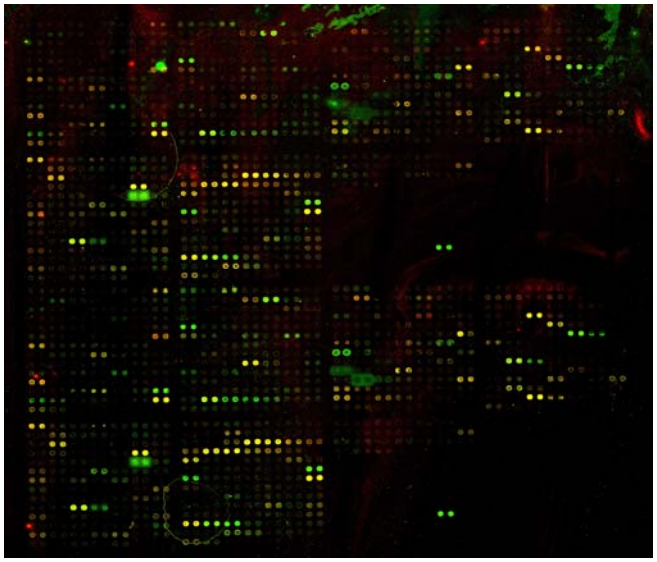

FIG. 7. Microarray image of NFBS (control) vs. NFBS T1-E2 (test).

RT-PCR Data and Expression Levels of the Genes

The cDNAs were obtained after reverse transcription from the RNA samples. This was done was for all RNA samples mentioned in Table 3. SFBS, SFBS T1, and SFBS E2 were controls. A reaction without reverse transcriptase (RT) was also carried out with the above RNA samples. Primers (Table 1) for the respective genes were applied to all samples with or without RT as per Materials and Methods. The data showed that the genes selected for further study were up or down-regulated in the treated sample (NFBS T1-E2) compared to the untreated (NFBS), as predicted by the arrays. The genes which were down-regulated (PST and ICAM-1) were amplified one or more cycles later in the treated samples than the untreated samples, which showed that the treated samples had more copies of the mRNA for that gene and, thus, that the chemical treatment reduced transcription of that gene. Similarly, the up-regulated genes (calreticulin and TGF β3) were amplified one or more cycles before in the treated sample. Data shown in Figure 8 indicate the cycle numbers at which the threshold was crossed by the genes. The gene expression levels and the normalized expression levels against the standard gene GADPH were calculated from these data (Fig. 9).

TABLE 4. Gene expression profile after treatment with Enable (T1)

Pub Med ID	Gene Name	Ratio
J03132	**Intercellular adhesion molecule-1**	**0.2845**
K00558	Alpha-tubulin	1.8
L05094	Ribosomal protein L27	1.868
L10752	3-methyladenine DNA glycosylase	2.634
M11717	Heat shock protein 70	2.0465
M34664	Heat shock protein 60 (chaperonin)	2.7425
M59907	Melanoma-associated antigen ME491	2.702
M62505	C5a anaphylatoxin receptor	0.2155
M84739	**Calreticulin**	**2.665**
M93133	Cholesterol 7-alpha-hydroxylase (P450 VII)	0.1795
NM000274	Ornithine aminotransferase	2.1885
NM000931	Tissue plasminogen activator	2.733
NM001006	Ribosomal protein S3A (RPS3A).	1.812
NM001011	Ribosomal protein S7 (RPS7).	1.923
NM001022	Ribosomal protein S19 (RPS19).	1.823
NM002426	Macrophage metalloelastase	2.0755
NM002737	Protein kinase C alpha	1.8965
NM003104	Sorbitol dehydrogenase	2.5835
NM007310	Catechol-O-methyltransferase	2.143
NM030938	Hypothetical protein DKFZp566I133	2.784
U26309	**Phenol sulfotransferase**	**0.2955**
U61981	DNA mismatch repair/binding protein (MSH3)	0.319
X00351	Beta-actin	2.043
X01677	Glyceraldehyde 3-phosphate dehydrogenase	1.964
X16869	Elongation factor-1 alpha	2.273
XM002028	Cathepsin S	0.45
XM002247	Alpha-prothymosin	2.344
XM004689	Insulin-like growth factor binding protein 3	2.747
XM004968	Hepatocyte growth factor receptor	2.498
XM005893	Mitochondrial voltage dependent anion channel (VDAC2)	2.652
XM007417	**Transforming growth factor-beta3**	**1.597**
XM007615	Ribosomal protein S17	2
XM007704	Fumarylacetoacetate hydrolase (FAH)	2.6735
XM007981	Type I transmembrane protein Fn14 (FN14)	2.33
XM008176	MYB binding protein	2.305
XM008437	Nerve growth factor receptor	2.5825
XM008817	Membrane bound cytochrome b5	2.6465
XM009082	Low density lipoprotein receptor	2.541
XM011855	Ribosomal protein L13A	2.161
XM012041	KAI1 metastasis suppressor gene (CD82)	0.405
XM012549	Ribosomal protein L13	1.906
XM046740	Similar to rat tricarboxylate carrier-like protein	0.222
NM004106	High affinity IgE receptor gamma chain (FcERIgamma)	2.812
X13403	Octamer binding protein 1	2.274

Ratios above 1.5 are considered to up-regulated and below 0.6 down-regulated. Values are fold change at each time point relative to control. Those in **bold** have been verified by real-time PCR.

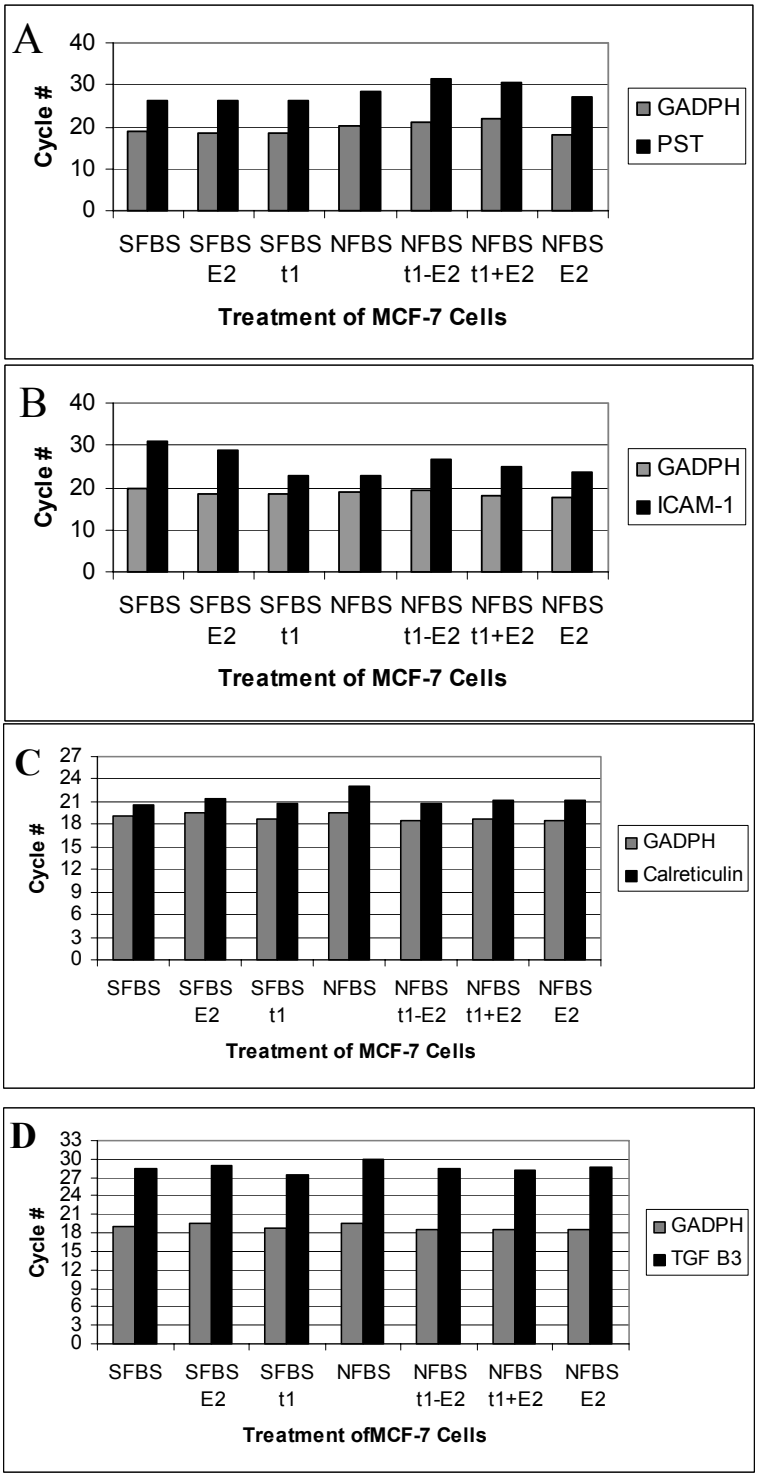

FIG. 8. RT PCR amplification cycles for Enable treatment. GADPH is the standard gene. A, the cycle at which PST crosses the threshold in all samples. B, C and D show the cycles at which ICAM-1, calreticulin and TGF β3 cross the threshold in different samples, respectively.

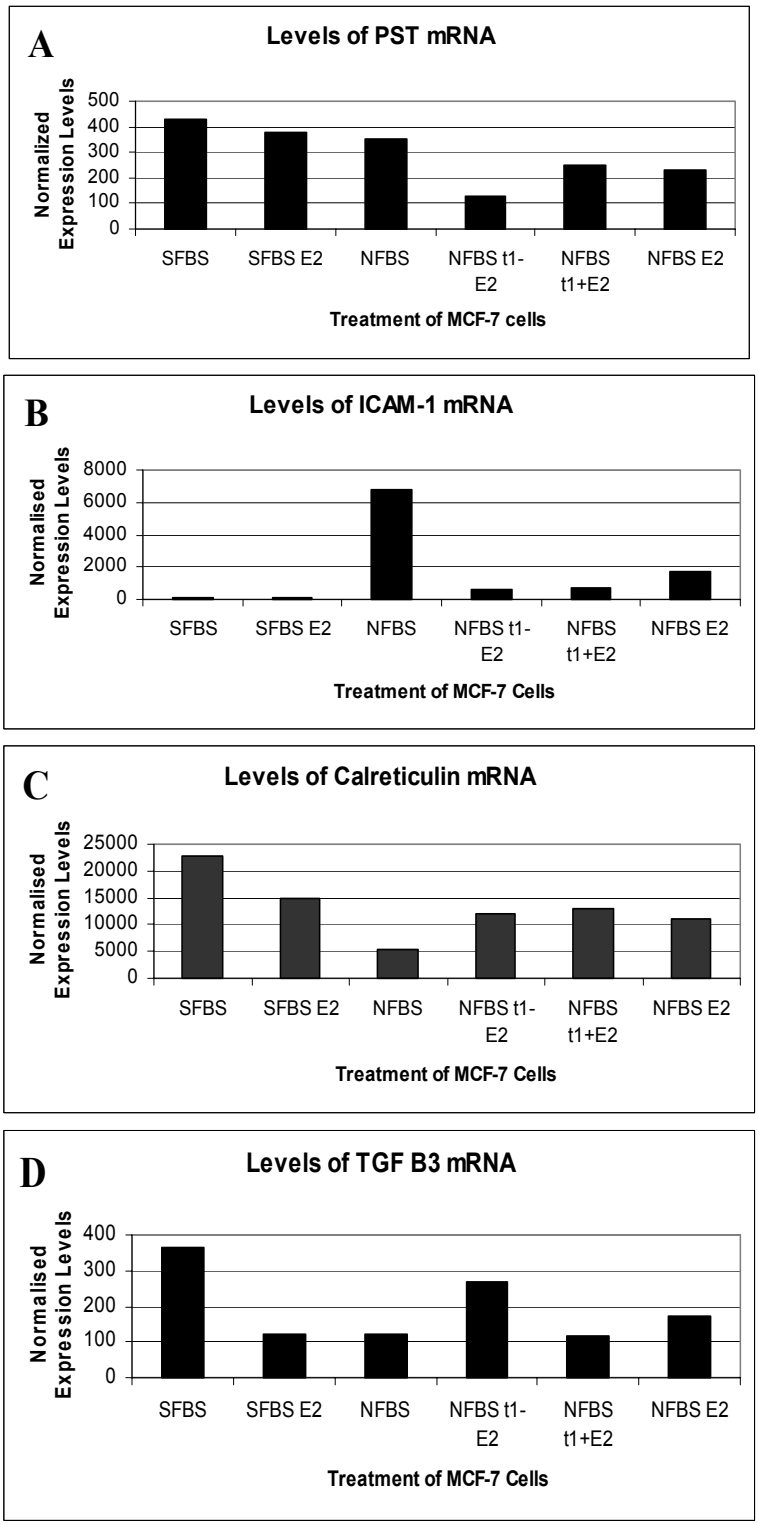

FIG. 9. Normalized gene expression levels for Enable treatment. A, B, C and D represent individuals expression levels of PST, ICAM-1, Calreticulin and TGF β3 mRNA as compared with GADPH, respectively.

TREATMENT WITH DIAZINON

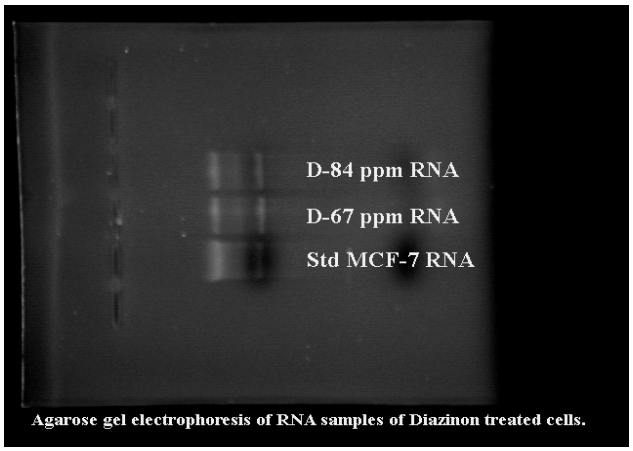

FIG. 10. Agarose gel electrophoresis of Diazinon treated MCF-7 RNA samples.

TABLE 5. Microarray experiments with Diazinon treated MCF-7 cells

Control (CY5)	Test (CY3)	Results
NFBS	NFBS D3	No Significant Difference in gene expression
NFBS	NFBS D30	16 genes up-regulated
NFBS	NFBS D50	24 genes up-regulated
NFBS	NFBS D67	3 genes down-regulated and 28 genes up-regulated

Identification of Genes Affected by Diazinon

The microarray experiments carried out to examine the effects of Diazinon on gene expression of MCF-7 cells are summarized in Table 5, using the isolated RNA (see Fig. 10). For PCR verification, four genes were chosen from 67ppm treatment levels (Fig 11). From these, excision repair protein (ERCC-5) and ribonucleotide reductase subunit M1 (RNRM1) were significantly down regulated, transforming growth factor beta 3 (TGF β3) was significantly up-regulated (Table 6). Calreticulin did not seem to be up-regulated as compared to untreated cells. However, it was selected since it was significantly up-regulated in the 30 & 50 ppm levels.

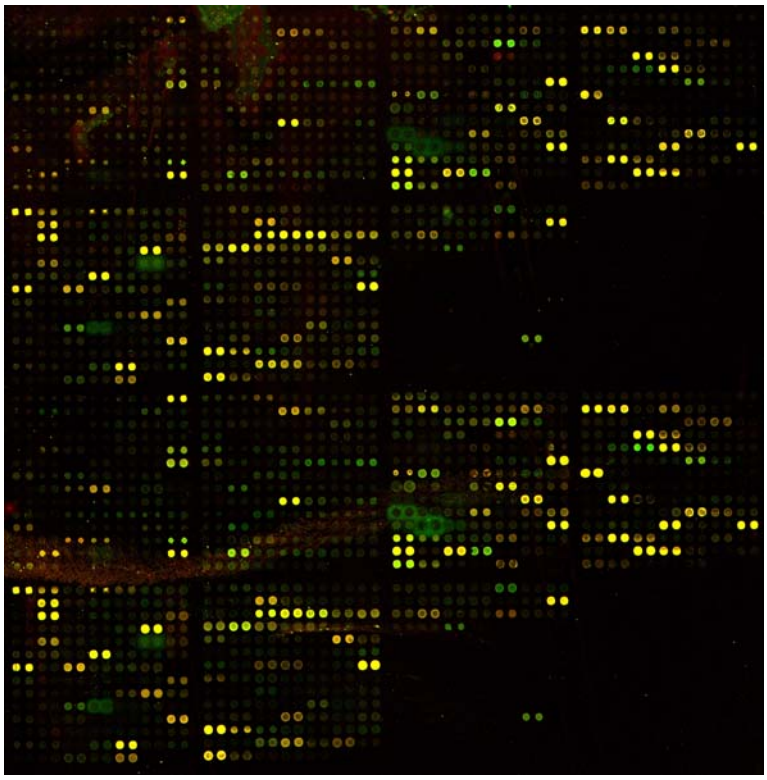

FIG. 11. Microarray image of NFBS (control) vs. NFBS D67 (test).

RTPCR Data & Expression Levels

Reverse Transcription and RT PCR were carried out, just as for the Enable- treated RNA samples. The data obtained confirmed the results of the microarray analysis. Also the calreticulin gene was significantly up-regulated in treated samples (67ppm), because it crossed the threshold much earlier as compared to the untreated sample (Fig. 12). Normalized expression levels were computed for all the four genes can be seen in Fig 13.

TABLE 6. Gene expression profile after treatment with Diazinon (D 67) .

Pub Med ID	Gene Name	Ratio
L20046	**ERCC 5 (excision repair protein)**	**0.524**
X59543	**Ribonucleotide reductase M1 subunit**	**0.55**
U50079	Histone deacetylase 1	0.704
X15759	Cytochrome c oxidase subunit II	0.823
M11717	Heat shock protein 70	0.953
M34664	Heat shock protein 60 (chaperonin)	0.984
XM011855	Ribosomal protein L13A	1.073
X13403	Octamer binding protein 1	1.119
XM012041	KAI1 metastasis suppressor gene (CD82)	1.131
XM007615	Ribosomal protein S17	1.142
X15187	Glucose-regulated protein 94	1.219
XM012549	Ribosomal protein L13	1.361
X16869	Elongation factor-1 alpha	1.379
NM002737	Protein kinase C alpha	1.397
M84739	**Calreticulin**	**1.47**
X66403	Acetylcholine receptor epsilon	1.745
XM014287	pM5 protein	1.759
NM000274	Ornithine aminotransferase	1.782
XM008176	MYB binding protein	1.796
XM007981	Type I transmembrane protein Fn14 (FN14)	1.802
L10752	3-methyladenine DNA glycosylase	1.805
XM007417	**Transforming growth factor-beta3**	**1.814**
NM004106	High affinity IgE receptor gamma chain (FcERIgamma)	1.85
XM005893	Mitochondrial voltage dependent anion channel (VDAC2)	1.864
NM003104	Sorbitol dehydrogenase	1.905
NM000931	Tissue plasminogen activator	1.934
Y10313	Interferon related developmental regulator IFRD1 (PC4)	1.934
NM002426	Macrophage metalloelastase	1.94
XM004968	Hepatocyte growth factor receptor	1.954
XM008437	Nerve growth factor receptor	1.965
NM000853	Glutathione S-transferase theta-1	1.966
AF096290	Very long-chain acyl-CoA synthetase	1.974
XM007704	Fumarylacetoacetate hydrolase (FAH)	1.983
XM004689	Insulin-like growth factor binding protein 3	1.986
NM030938	Hypothetical protein DKFZp566I133	1.988
XM008817	Membrane bound cytochrome b5	2.011
NM001888	Crystallin	2.021
M59907	Melanoma-associated antigen ME491	2.049
XM004212	Diubiquitin (UBD)	2.053
XM009082	Low density lipoprotein receptor	2.114
M23452	Macrophage inflammatory protein-1 alpha	2.12

Ratios above 1.5 are considered to up-regulated and below 0.7 down-regulated. Values are fold change at each time point relative to control. Those in **bold** have been verified by real-time PCR.

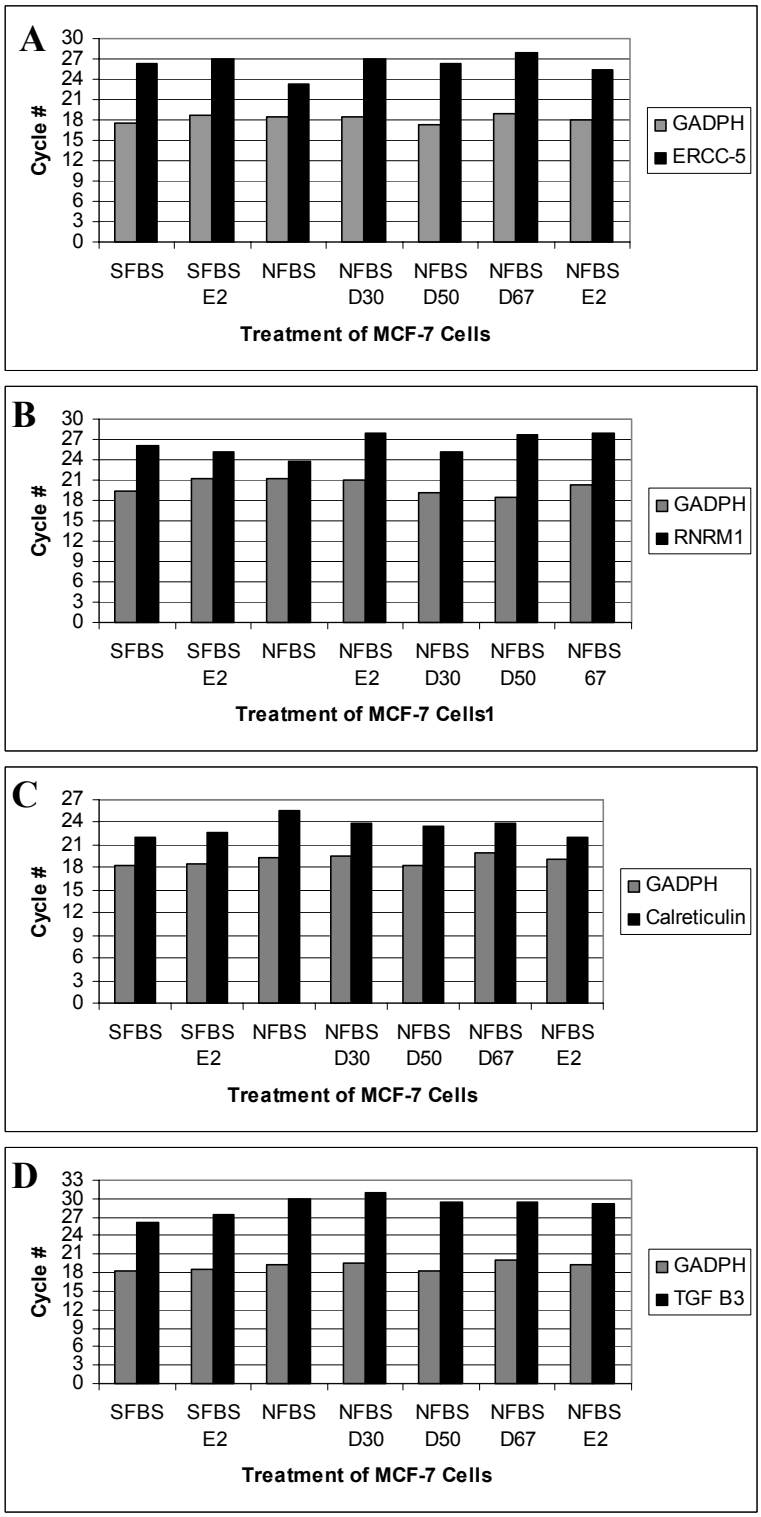

FIG. 12. RT PCR amplification cycles for Diazinon treatment. GADPH is the standard gene. A, the cycle at which ERCC-5 crosses the threshold in all samples. B, C and D are the cycles at which RNRM1, calreticulin and TGF β3 cross the thresholds in different samples, respectively.

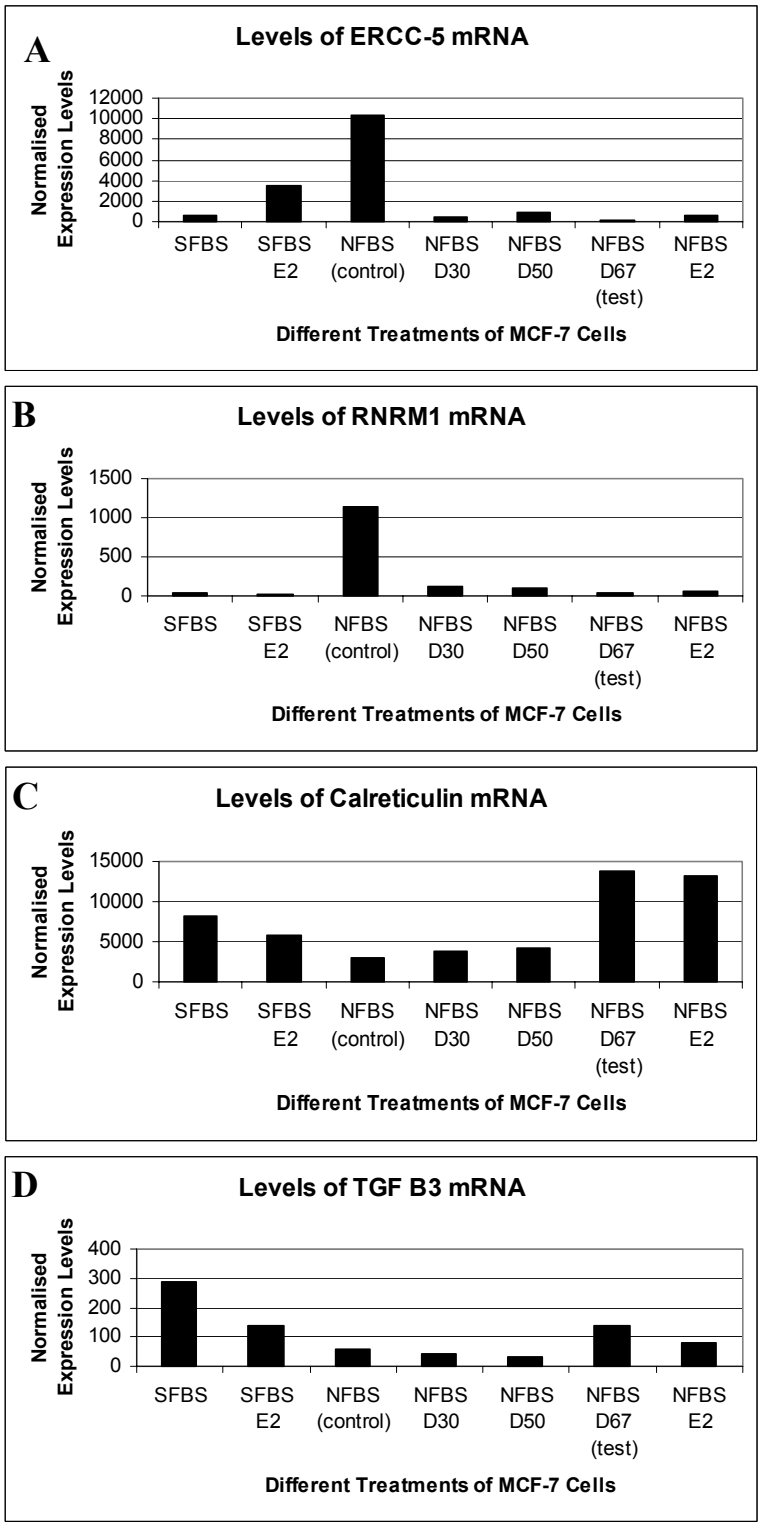

FIG. 13. Normalized gene expression levels for Diazinon treatment. A, B, C and D represent individuals expression levels of ERCC-5, RNRM1, Calreticulin and TGF β3 mRNA as compared with GADPH, respectively.

DISCUSSION

PHENOL SULFOTRANSFERASE

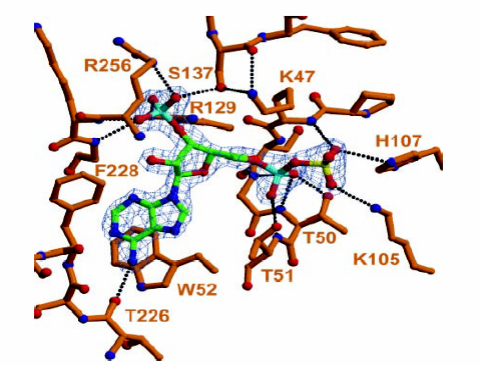

FIG. 14. Phenol sulfotransferase (27).

Sulfonation is an important metabolic pathway involved in the detoxification and or activation of a range of endogenous compounds (e.g., monoamine neurotransmitters, thyroid and steroid hormones), drugs (e.g., minoxidil) and xenobiotics, (e.g., aromatic amines and benzylic alcohols of polycyclic aromatic hydrocarbons). This reaction involves transfer of a sulfonate group from the 3'-phosphoadenosine-5'-phosphosulfate (PAPS) to the acceptor substrate (Fig. 14). In the case of xenobiotics, this reaction is catalyzed by a group of cytosolic sulfotransferase enzymes (STs). However, it is important to recognize that a number of membrane bound STs also exist. Based on amino acid alignments, the cytosolic sulfotransferases have been divided into three families: phenol, hydroxysteroid and flavonol STs (28).

In the human, there are three members of the phenol sulfotransferase family (SULT1A1, SULT1A2, and SULT1A3). DNA sequences and the structures of these three enzymes are highly homologous, and all three genes are tightly linked on chromosome 16p12.1-p11.2. All of the human aryl STs share greater than 90% sequence homology at the amino acid level but vary markedly in their substrate preferences (28, 29). Several human tissues contain two major P-ST forms that differ in their substrate specificity, sensitivity to inhibitors and physical properties despite their sequence homology (92% in amino acid sequences).

One is relatively thermostable (TS-PST, SULT1A1 and SUL1A2) and the other is thermo labile (TL-PST, SULT1A3). TS-PST preferentially catalyzes the sulfation of millimolar concentrations of simple phenols, whereas TL-PST catalyzes the sulfation of millimolar concentrations of dopamine. The P-ST family consists of four subfamilies, phenol sulfotransferases (SULT1A), dopa/tyrosine (or thyroid hormone) sulfotransferases (SULT1B), hydroxylamine sulfotransferases (SULT1C), and estrogen sulfotransferases (SULT1E) (30). Sulfonation, along with detoxification can also lead to biological activation of compounds rendering them carcinogenic. Several chemicals such as TCDD are known to inhibit and therefore down-regulate phenol sulfotransferases (31). Many roles of sulphonation are summarized below;

Detoxification of Xenobiotics and Chemical Defense

Sulphonation is considered an important component of what is called phase II of xenobiotic metabolism, i.e., the conjugation and true detoxification step, and is, a central feature of the body's chemical defense. The classical view of sulphonation is that it protects against the toxic, or potentially toxic, effects of numerous xenobiotics and their metabolites, because sulfate conjugates are in general more polar, more water soluble and, more readily excreted in urine or bile. A number of drugs and other xenobiotics are directly conjugated with sulfate, including compounds acting at the b-adrenergic receptor, paracetamol and dietary catechols (32).

Sulphonation Leads to Biological Activation

There are a number of important examples showing that sulfate conjugates are more biologically active than the corresponding free compounds. However, probably the most interesting example of bioactivation via sulphonation is in the case of dietary and environmental mutagens and carcinogens, for which a large number of chemicals sulphonation is the terminal step in the essential metabolic activation pathway to the ultimate DNA- or protein-adducting species. Examples of chemicals for which sulphonation plays a crucial role in their bioactivation include aromatic amines

(including heterocyclic amines presented in cooked meat and fish) and benzylic alcohols of chemicals such as polycyclic aromatic hydrocarbons, safrole and estragole. There now exists ample *in vivo* and *in vitro* evidence for the involvement of sulphonation in this process. Mice which are pre-treated with an inhibitor of sulfotransferase such as pentachlorophenol prior to treatment with safrole are much less susceptible to tumorigenesis than untreated mice (32).

Dietary Compound Interaction with Sulphonation

Most SULTs are expressed (to varying degrees) in the human gastrointestinal tract. In light of this fact and of the important role sulphonation plays in the bioactivation of dietary procarcinogens, it is reasonable to assume that there is potential for the production of highly reactive, unstable, mutagenic sulfuric acid esters within the gastrointestinal tract. This may be an important mechanism in, colon cancer in which dietary aromatic amines known to be activated via sulphonation are implicated and we have, therefore, been curious as to whether other dietary chemicals may act as inhibitors of SULTs activating such procarcinogens (32). Individuals suffering from dietary migraine are deficient in P-PST as compared to the other enzymes responsible for metabolizing dietary migraine-inducing compounds like tyramine in the diet (33).

Disorders Associated with Sulfotransferases

To date, no disease associated with a defect in any cytosolic sulfotransferase is known. However, there is extreme variation in the expression of various SULTs within the human population and there is good evidence for the existence for a genetic polymorphism affecting the P-PST isoform (32). Recent studies have shown that breast cancer cells have very low estrogen sulfotransferase activity, and their estrogen-sulfating activity is attributable to phenol-preferring sulfotransferases (SULT1A). Interestingly, there is evidence that *SULT1A1* genotype influences the age of onset of the disease in patients. SULT1A mRNA Levels were seen to be induced after tamoxifen treatment (34).

INTERCELLULAR ADHESION MOLECULE-1

Intercellular adhesion molecule (ICAM)-1 (CD54) is a member of the immunoglobulin gene super family and is expressed on endothelial cells, epithelial cells, and fibroblasts, as well as T-cells, B-cells, dendritic cells, macrophages, and eosinophils. It consists of a 76-114 kDa chain glycoprotein, with a core polypeptide of approximately 55 kDa, and is composed of one short cytoplasmic, one transmembranous, and five immunoglobulin (Ig)-like extracellular domains. The ligands for ICAM-1 are the b2 integrins, leucocyte function associated molecule (LFA)-1 (CD11a/CD18) and macrophage antigen (Mac)-1 (CR3, CD11b/CD18) and the rhinovirus (RV). Binding of ICAM-1 to ligand adhesion molecules has profound effects on cell adhesion and activation (35, 36, 37).

Expression of ICAM-1 on T Cells During Activation

The migration of T-cells from blood into the lungs constitutes an important component of the inflammatory response in the airways. During this process a series of different surface adhesion molecules are up-regulated and down-regulated, dictating their ability to migrate into lymph nodes and subsequently into inflamed tissue sites. Naive T-cells migrate from blood into the tissue-associated lymph nodes through postcapillary high endothelial venules to be "instructed" by antigen presenting cells (APCs), (Fig.15). Upon antigenic stimulation, T-cells shed L-selectin and acquire other adhesion molecules such that, e.g., activated cytotoxic T-cells express high levels of ICAM-1 well as LFA-1. ICAM-1 is weakly expressed on resting T-cells, in contrast to ICAM-3 which is expressed at higher levels. Adhesion of resting T-cells to LFA-1 occurs primarily via ICAM-3 followed by ICAM-1, which establishes more stable cell-cell interactions. ICAM-1 is the major ligand for LFA-1 on activated T-lymphocytes. Up-regulation of ICAM-1 on T-cells can also be observed following exposure to both live and inactivated viruses (35, 37).

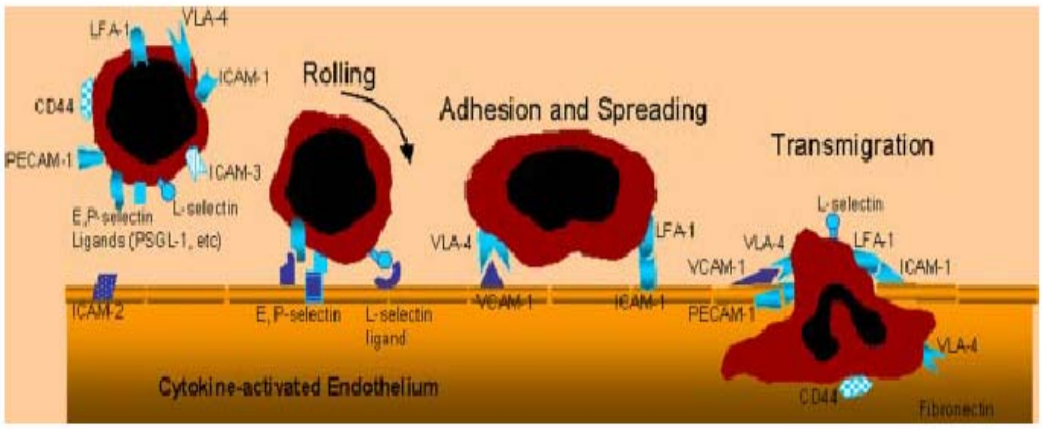

FIG.15. T-cell activation interaction between ICAM-1 and LFA-1 (37).

Cytokine Production

Emerging evidence suggests that binding of ICAM-1 on T-cells can have an important effect on cytokine production. Co-engagement of CD3 with ICAM-1 enhances the activation of murine CD4+ and CD8+ T-cells in an accessory cell-free culture system and induces production of IL-3 and IFN-γ, which is four times greater in CD8+ T-cells compared to CD4+ T-cells. Anti-ICAM-1 mAb inhibits *in vitro* production of TNF-α, IFN-γ and IL-1 by phytohaemagglutinin (PHA)-activated human T-cells (35).

Helper Activity for B Cells

Interaction between LFA-1 expressed on B-cells and ICAM-1 on activated T-cells is important in early events of T-cell-dependent B-cell activation, proliferation and differentiation (35).

Soluble ICAM-1

A circulating, soluble form of ICAM-1 (sICAM-1) has been detected in culture supernatants and human body fluids and is thought to be derived through shedding of surface ICAM-1. The mechanism of ICAM-1 shedding from the cellular membrane is poorly understood. It is possible that activation of the cells induces the expression or activity of a cell membrane protease which is able to cleave membrane-bound ICAM-1. In culture the levels of sICAM-1 correlate with the degree of immune cell activation and seem to be an early and sensitive marker for activation of both T-cells and Bcells. Several studies suggest that sICAM-1 may participate in a feedback down-regulatory mechanism. *In vitro*, recombinant sICAM-1 are able to inhibit homotypic T-cell aggregation, cytolytic interaction between cytotoxic cells and target cells, major histocompatibility complex (MHC)-restricted cytotoxicity, antigen-induced proliferation and antigen-triggered induction of cytokines in T-cells (35).

ICAM-1 in Allergic Inflammation and Asthma

Evidence accumulated to date has shown a prominent role for ICAM-1 in allergic inflammation. Asthma is a chronic inflammatory disease characterized by accumulation of activated eosinophils and T-lymphocytes in the bronchial mucosa. Lung cell infiltration in asthma is regulated by several pathophysiological mechanisms, including increased expression of cell adhesion molecules and levels of chemoattractants. Several studies have shown prominent up-regulation of ICAM-1 in asthmatic airways. Increased ICAM-1 expression in asthma has been reported on eosinophils, T-cells, and bronchial endothelial and epithelial cells (35).

Breast Cancer

ICAM-1 expression is seen in almost 50% of breast cancer patients. ICAM-1 expression has negative correlation with tumor size. Patients with ICAM-1 positive tumors have better relapse-free and overall survival as compared to the negative tumors. This suggests that expression of ICAM-1 on cancer cells may have a role in tumor suppression (37, 38).

Atherosclerosis

One of the earliest events in atherosclerosis is interaction of circulating mononuclear leukocytes and the endothelium. Endothelial cell (EC) activation by cytokines results in expression of adhesion molecules and production of chemotactic factors, augmenting leukocyte adhesion and recruitment, respectively. The incidence of atherosclerosis in premenopausal women is significantly less than that observed in age-matched males with similar risk profiles. Because estrogen has gene regulatory effects, we investigated whether 17β-estradiol (E2) can inhibit cytokine-mediated EC adhesion molecule transcriptional activation. Cultured human umbilical vein EC (estrogen receptor–positive) were propagated in gonadal hormone–free medium and were E2-pretreated for 48 h before IL-1 activation. Assessed by FACS analysis, E2 strongly (60–80%) inhibited IL-1–mediated membrane E-selectin and vascular cell adhesion molecule-1 induction, and intercellular adhesion molecule-1 hyper induction. 17α-estradiol (an inactive E2 stereoisomer) had no effect. This inhibition correlated with similar reductions in steady state–induced E-selectin mRNA levels, and was abrogated by the E2 antagonist ICI 164,384, demonstrating a specific, estrogen receptor–mediated effect. Nuclear runoffs confirmed suppression at the transcriptional level (39).

CALRETICULIN

Ca^{2+} is a universal signaling molecule that affects diverse cellular functions such as secretion, contraction–relaxation, cell motility, cytoplasmic and mitochondrial metabolism, synthesis, modification, and folding of proteins, gene expression, cell cycle progression, and apoptosis. The endoplasmic reticulum (ER) plays a central role in maintaining intracellular Ca^{2+} levels. Ca^{2+} is released from the ER via the InsP3 receptor and is taken back up into the ER via a Ca^{2+} pump known as SERCA. Ca^{2+} also enters the cytoplasm via channels in the plasma membrane (40).

Calreticulin is an ER chaperone and has a role, via the calreticulin/calnexin cycle, in the proper folding of many proteins and glycoproteins. It is also a major Ca^{2+}binding/ buffering protein in the lumen of the ER and, as such, is involved in several of the processes that comprise cellular Ca^{2+} homeostasis, including Ca^{2+} storage in the ER, Ca^{2+} release from the ER. Calreticulin has several distinct structural domains. The N-terminal domain and central, P-domain of calreticulin are responsible for its chaperone (protein folding) activity, whereas the C-terminal, acidic region plays a key role in Ca^{2+} storage within the ER and in ER retrieval of the protein, (Fig. 16) (40).

Calreticulin an Upstream Regulator of Calcineurin

Calreticulin is highly expressed in embryonic heart where it presumably plays an important role during cardiac growth and differentiation. Calcineurin is a highly conserved, Ca^{2+}/calmodulindependent serine/threonine phosphatase (phosphatase 2B). The activation of calcineurin occurs in response to sustained Ca^{2+} release from the ER and Ca^{2+} influx across the plasma membrane. The role of calcineurin is critical in cardiac physiology, pathology, and development. Ca^{2+}-dependent, cardiac-specific transcriptional processes are impaired in the absence of calreticulin. Studies with calreticulin-deficient rescue mice indicate that calcineurin-dependent pathways are involved in the effects of calreticulin-deficiency on cardiac development, and may be involved in the effects on metabolism. Calreticulin appears to be a key upstream regulator of calcineurin function (40).

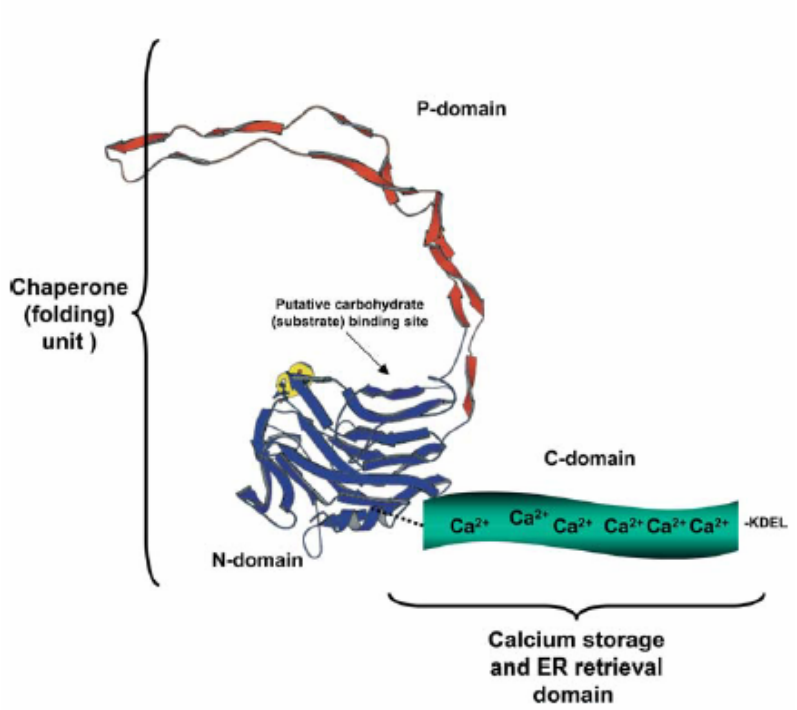

FIG. 16. A putative model of calreticulin (40).

Role of Calreticulin in the Regulation of Osteoblastic Differentiation

Recent studies have shown that calreticulin can indeed be detected in the nucleus. The ability of calreticulin to inhibit nuclear hormone receptor activity has been directly demonstrated for glucocorticoid, androgen, and retinoic acid receptors. Calreticulin gene expression is seen during osteoblastic cell differentiation but not seen in mineralizing cultures of osteoblasts. Constitutive expression of calreticulin in osteoblastic cells inhibits mineralization. This inhibition is most likely mediated through specific modulation of gene expression by calreticulin, by interacting with the vitamin D receptor to inhibit its binding to cognate response elements *in vitro*, and selectively inhibit certain vitamin D-mediated transcriptional responses *in vivo*. For example osteocalcin gene is inhibited by endogenous expression of calreticulin but osteopontin gene isn't (41).

Protein Folding

Studies using cells derived from crt-/- embryos indicate that protein folding in the ER is compromised in the absence of calreticulin resulting in the accumulation of mis-folded protein and activation of unfolded protein responses. Ca^{2+} release from the ER is also inhibited in crt-/- cells, likely because Ca^{2+} handling in the ER and the folding/trafficking of cell surface receptors are impaired. Studies with calreticulin deficient cells have also revealed that protein folding and Ca^{2+} signaling processes in the ER are highly interdependent. Both calreticulin and calnexin act as lectins and molecular chaperones, and they bind monoglucosylated proteins and associate with the thiol oxidoreductase ERp57, which promotes disulfideformation/isomerization in glycoproteins. Folding substrates associate transiently with calnexin and calreticulin and enter cycles (Fig. 17) of de-glucosylation/re-glucosylation which plays an important role in their association with the chaperones. Protein folding is accelerated in the absence of calreticulin and quality control is compromised. There is an accumulation of unfolded proteins and a triggering of the unfolded protein response (UPR), activated in the absence of calreticulin. ER molecular chaperones of calreticulin/calnexin cycle have overlapping and complementary, but not redundant, function. The absence of one chaperone can have devastating effects on the function of the others, compromising overall quality control of the secretory pathway and activating UPR-dependent pathways (42, 43).

Over-expression of Calreticulin

Calreticulin mRNA is regulated by androgen and androgen induction of calreticulin mRNA resists inhibition of protein synthesis (44). Also, heat shock, and heavy metals such as zinc and cadmium are consistently found to increase transcription of calreticulin. These studies suggest that (a) calreticulin is regulated at the transcriptional level, and (b) calreticulin, appears to function as a heat shock/stress-response gene (45). Widely distributed and highly conserved calreticulin has been suggested to play a role as a Ca^{2+} storage protein of intracellular Ca^{2+} stores.

In cells that over-express calreticulin, the Ca^{2+} content increases and 80% of the increased Ca^{2+} content is found in Ca^{2+} stores (46). Over-expression of Calreticulin increases Ca^{2+} fluxes across the ER but decreases mitochondrial Ca^{2+} and membrane potential. The increased Ca^{2+} turnover between the two organelles might damage mitochondria, accounting for the increased susceptibility of cells expressing high levels of calreticulin to apoptotic stimuli.

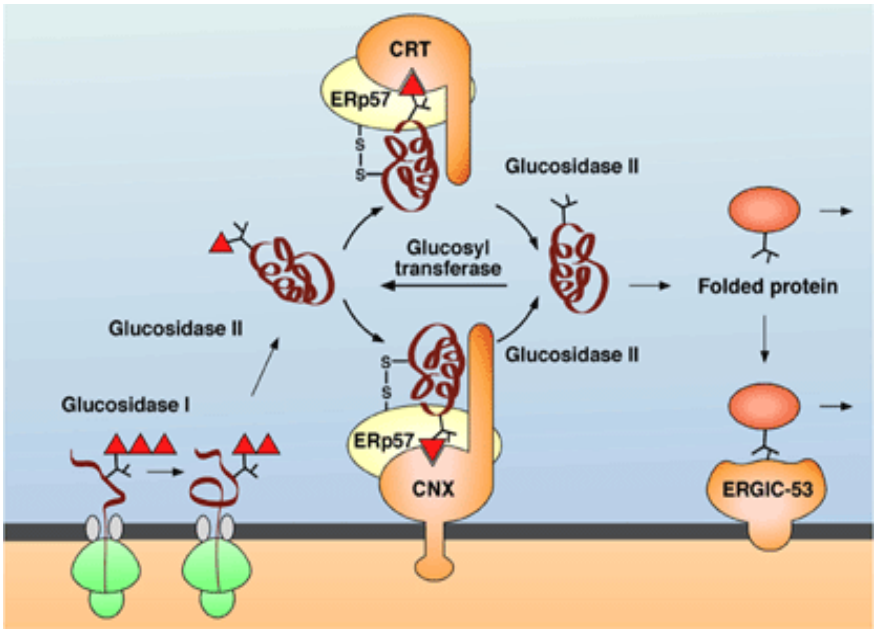

FIG. 17. Protein folding by the calreticulin / calcinexin cycle in ER (47).

TRANSFORMING GROWTH FACTOR β3

It is now evident that TGF-βs not only promote cellular growth but are also capable of either promoting or inhibiting cell death, depending upon the cell-type examined. The numerous functions of TGF-βs include cell cycle control, regulation of early development, differentiation, extracellular matrix formation, hematopoesis, angiogenesis, chemotaxis and immune functions. TGF-βs also act contextually, because their actions often depends on environmental cues, i.e. the cell type, the differentiation state of cells, and the presence of other growth factors, best exemplified by their capacity to either stimulate or inhibit proliferation, TGF- β3s have the potential to regulate processes in part controlled by interactions between cellular receptors and components of the extracellular matrix, such as cell migration, homing, and location during development. In mice lacking TGF-β3, the palate shelves fail to fuse leading to a cleft palate (48).

Role of TGF β3 Male Reproductive Physiology

TGF-β3 is known to regulate multiple physiological functions in the testis they include spermatogenesis, Leydig cell steroidogenesis, extracellular matrix synthesis and testis development. TGF-β3 also regulates Sertoli cell tight junction (TJ) dynamics *in vitro* via the mitogen-activated protein (MAP) kinase pathway, suggesting that this cytokine plays a crucial role in regulating the opening and closing of the blood–testis barrier (BTB). This in turn regulates the passage of pre-leptotene and leptotene spermatocytes across the BTB (49).

Role in Cell De-Differentiation

TGF β3 is reported to regulate cell proliferation during development and can also influence the commitment or the differentiation, or both, of neural progenitor cells to retinal fates. Neural stem cells when treated with TGF β3 differentiate into retinal cells instead of neural cells. This may be due to selective glial cell death in the cultured cells by TGF β3, because it induces apoptotic cell death in oligodendrocytes by activation of caspases (50).

Retinoic Acid Down-Regulates TGF β3

Retinoic acid, a chemo-preventive agent for epithelial cancers, binds to retinoic acid receptors (RAR) as well as retinoic X receptors (RXR). Upon dimerization of the activated receptors, they enter the nucleus and interact with specific DNA segments affecting transcription. A growth factor for lung cancer is TGF- β3. *In vitro* studies show that RA inhibits TGF- β3 by inhibiting transcription, there by inhibiting the proliferation of lung cancer (51).

Role in Hepatitis and Cirrhosis of Liver

Liver tissues of patients suffering from hepatitis have a high expression of TGF-β3. The expressing intensity being stronger incase of cirrhosis and chronic hepatitis as compared to acute hepatitis. As the expression of TGF-β3 increases, the level of serum bilirubin ascended. Hence, it may be concluded that expression of TGF-β3 is correlated with severe hepatitis and the progress of liver cirrhosis (52).

EXCISION REPAIR PROTEIN-5

Nuclear excision repair (NER) is one of the primary pathways by which mammalian cells remove DNA lesions caused by both endogenous and exogenous agents. Both endogenous and exogenous exposures to carcinogens or genotoxic agents cause cell cycle delays allowing repair of DNA damage and DNA repair capacity is central to maintaining normal cellular functions. A wide spectrum of structurally unrelated lesions such as UV-induced photoproducts, bulky chemical adducts, and certain types of DNA cross-links are efficiently removed by the NER pathway. In the process of repair, the products of more than a dozen genes are involved in damage recognition, incision, excision, elongation, and ligation and collectively restore the normal structure (53, 54).

Excision Repair protein-5 (ERCC5 / XPG) has endonuclease activities and plays a role in the incision of the 5' side and the 3' side of the DNA lesions, respectively (54). Individuals with a defect in the XPG protein suffer from xeroderma pigmentosum (XP) resulting from an inability to perform DNA nucleotide excision repair properly (53). In lung cancer patients, the levels of ERCC5 are significantly lower than in normal individuals (10). This is also true for patients with squamous cell carcinoma of the head and neck (11). A defect in this gene may increase the susceptibility to cancer by allowing the unrepaired DNA damage to remain in place, thus leading to carcinogenesis. DNA repair capacity varies from individual to individual, and reduced expression of ERCC5 and other NER regions might increase the susceptibility of an individual to lung cancer.

RIBONUCLEOTIDE REDUCTASE SUBUNIT M1

Ribonucleotide reductase (RR), plays a key role in the synthesis of DNA and is responsible for the reduction of ribonucleotides to their corresponding deoxyribonucleotides, providing a balanced supply of precursors for DNA synthesis and repair. Recent studies have shown that there are three human RR subunits: hRRM1, hRRM2 and p53R2. hRRM1 is a large peptide chain, and hRRM2 and p53R2 are small protein subunits of RR. RRM1 is one of the two components of ribonucleotide reductase, whose cellular function is to provide the deoxynucleotides required for DNA synthesis and repair through catalysis of ribonucleoside diphosphates to the corresponding deoxyribonucleosides. Ribonucleotide reductase catalyzes the rate-limiting step in deoxyribonucleotide biosynthesis. The activity of this heterodimeric enzyme is dependent upon the expression of both its large and small subunits (55, 56). The P^{53} protein directly interacts with p53R2 and hRRM2 but not hRRM1 while hRRM1 interacts weakly with p53R2, hRRM2. After exposure to UV, p53R2 and hRRM2 dissociated from p53 and bound hRRM1 in these cells. Before UV treatment all the three subunits are localized in the cytoplasm and after treatment they translocate to the nucleus as seen in Fig. 18 (56, 57).

RRM1 and Lung Cancer

There is a relationship between frequent allele loss on human chromosome 11p15.5 and its association with metastatic spread in patients with lung cancer. The RRM1 gene is located in this region, suppresses invasion, migration, and *in vivo* metastasis formation through up-regulation of the PTEN tumor-suppressor gene, when over-expressed in human and mouse lung cancer cell lines (58).

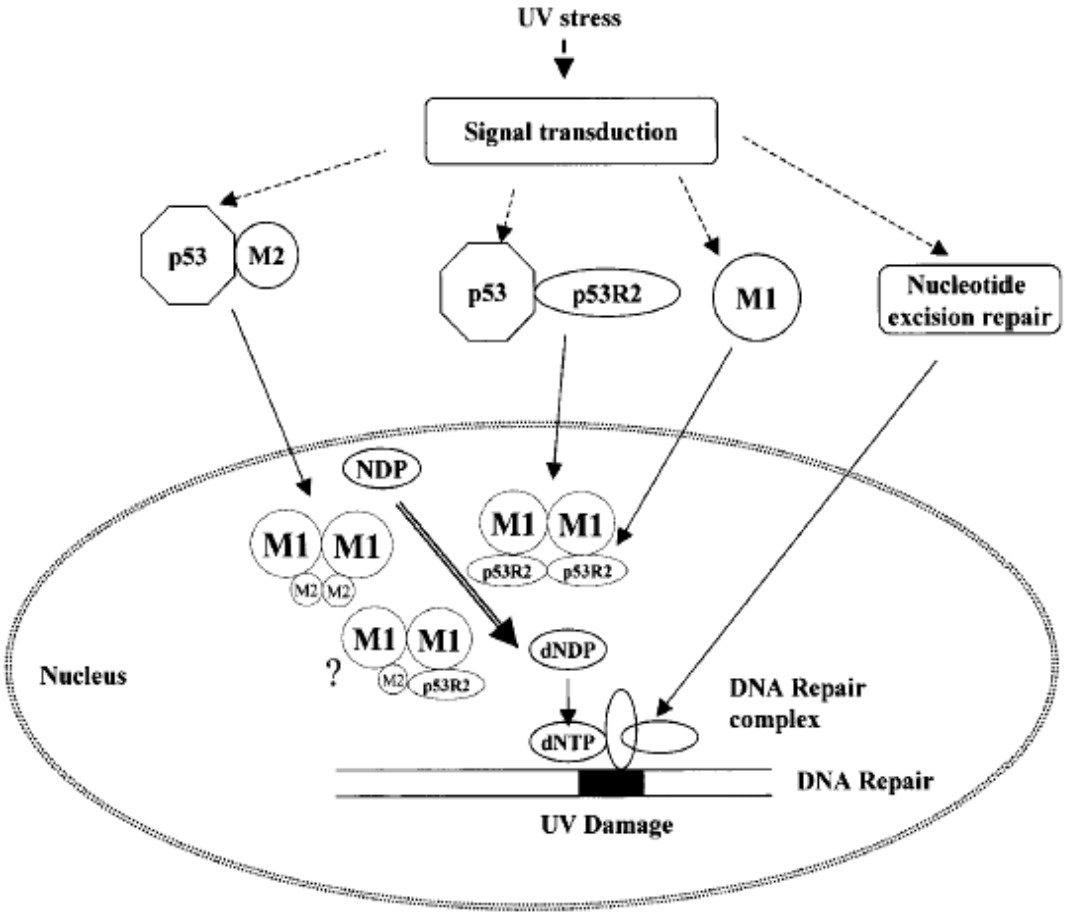

FIG. 18. Model for p53 regulation of ribonucleotide reductase activity for DNA repair in response to UV damage (57).

CONCLUSION

Some of the adverse health effects known to be caused by exposure to agricultural chemicals proposed in animals and humans are cleft lip/cleft palate, feminization of male offspring, early onset of puberty in female offspring, neural tube defects.

In MCF-7 cells treated with Enable, a well known fungicide, there was down-regulation of eight genes and up-regulation of thirty four genes at 3.3 ppb, compared to untreated cells. Genes that were chosen for PCR verification are not only vital for normal immune, cellular and toxicological functions, but also are implicated in cancers, atherosclerosis, inflammation reactions in asthma and liver cirrhosis collectively.

In MCF-7 cells treated with Diazinon, an insecticide, three genes were down-regulated and twenty seven up-regulated. The genes chosen for special consideration played important roles in DNA synthesis and repair, cell differentiation and normal immune function. Defects in these genes seem to increase susceptibility to different kinds of cancer.

These studies were designed to provide base-line data on gene expression-altering capacity of Enable and Diazinon. Knowledge of the altered genes can aid in extrapolation to altered physiological systems *in vivo*, on exposure to these chemicals.

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