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Altered gene expression in human cells treated with the insecticide diazinon: correlation with decreased DNA excision repair capacity

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Many industrial and agricultural chemicals have steroid hormone agonist or antagonist activities and disrupt hormone-regulated gene expression. The widely-used agricultural insecticide, diazinon, was evaluated using MCF-7 cells – a breast cancer-derived, estrogen-dependent, human cell line – to examine the capacity of this chemical to alter steroid hormone-regulated gene expression. MCF-7 cells were treated with 30, 50, or 67 ppm of diazinon, and gene expression in treated cells was measured as mRNA levels in the cells compared to mRNA levels in untreated or estrogen-treated cells. DNA microarray analysis showed significant up- or down-regulation of a number of genes in treated cells compared to untreated cells. Of the 600 human genes on the chip utilized, specific genes with related functions were selected for additional consideration. Real time quantitative PCR (qrtPCR) completed to corroborate mRNA levels as a measure of specific gene expression, confirmed results obtained from analysis of the microarray data. The data show that ERCC5, encoding Xeroderma pigmentosum protein G (XPG), essential for DNA excision repair, and ribonucleotide reductase subunit M1 (RNRM1), encoding a gene necessary for providing the nucleotides needed for DNA repair, were down-regulated in cells treated with diazinon. These studies were designed to provide base-line data on the gene expression-altering capacity of a specific agricultural chemical, diazinon, and allow assessment of some of the potentially deleterious effects associated with exposure of human cells to diazinon. *Human & Experimental Toxicology* (2006) **25**, *57–65*

Key words: DNA microarray analysis; gene expression; agricultural chemicals; organophosphate insecticides; real time PCR

Introduction

Cellular receptors for steroid hormones include nuclear proteins which bind to 17β-estradiol (ER), testosterone (AR), glucocorticoids (GR), thyroid hormone (TR), progesterone (PR), mineralocorticoids (MR), vitamin D3 (D3R), or vitamin A (RAR). Additional receptors that potentially interact with steroid hormones include orphan receptors, such as RXR, and other nuclear receptors, such as AhR, involved in the initiation of cytochrome P450 gene expression. The steroid hormone receptors belong to a broad group of proteins – the nuclear receptor superfamily – which have differing motifs of gene activation and variable specificity of ligand binding.[1–3] Through receptor-mediated control of gene expression, the hormones that bind steroid receptors exert exquisite regulation of physiological functions, essential not only for fetal growth and development, but for good health in general. Interaction of a receptor (for instance, ERα) bound to its specific ligand (ERα/17β-estradiol) with specific response elements in the upstream regulatory sequences of genes, acts to initiate expression of the specific estrogen-regulated genes.[1–5]

Existing data suggest that agricultural workers and their families may be chronically exposed to a variety of chemicals and their breakdown products that may bind one or more of the

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steroid receptors and interfere with regulation of gene expression. The exposure of agricultural and industrial personnel and their families to endocrine disruptive chemicals (EDC) has been well-documented for some chemicals in some geographical areas, but these classes of chemicals, in general, have been poorly defined regarding their potential to disrupt endocrine function and alter gene expression.

In many instances, a direct cause-and-effect relationship has been drawn between acute exposure to some chemicals and health, with the effects of that exposure being observed as altered physiological responses and health in both humans and laboratory animals.[6–9] Much less is known about chronic exposure to low levels of EDC. It is clear from studies of chronically exposed mammals that EDC may cross the placenta and alter fetal growth and development, that lactating females may offload large quantities of potentially EDCs in milk, that nursing young may have extremely high serum levels of chemicals relative to the actual environmental exposure levels of the chemicals, and that nursing young may be significantly affected by exposure to EDC in milk.[10–12]

Animals exposed to EDC at relatively high levels may exhibit weight loss, thymic atrophy with impaired immune function, hepatotoxicity, increased incidence of cancer, loss of reproductive efficiency in adult animals, developmental anomalies including skeletal defects and altered sex determination or decreased post-natal male or female reproductive development, early or delayed pubertal changes, ototoxicity, altered neurodevelopmental changes including cognitive deficits, decreased learning potential, and overt fetal toxicity and/or fetal death.[11,13–16] All of these effects of chemical exposure can result from disrupted endocrine-regulated gene expression, and may differ dramatically between individual humans, and between inbred strains of animals, dependent in part on an individual's capacity to carry out hormone-regulated gene expression and on the interaction of specific chemicals with the variety of enzymes that metabolize, activate, or remove hydrocarbons.[5,17–22] Very little is known about how humans and other animals are affected by chronic low-level exposure to most EDC, including the insecticide, diazinon.

California, Texas and Florida have the most significant historical use of diazinon in the US. It has been detected in the Sacramento River and its tributaries during the dormant spray season coinciding with the winter months when the area receives the majority of its annual rainfall. Pesticides applied during this period have the increased potential to wash off applicant areas and migrate with runoff water, creating significant contaminant loads (www.sacriver.org/subcommittees/op/documents/WQMSD_Draft/Ch2_sourceD.html).

Acute exposure to diazinon is known to inhibit acetylcholinesterase (ACE) causing nausea, dizziness, and confusion, with exposure at high levels causing respiratory paralysis and death, and to inhibit protein synthesis *in vitro*.[23] However, chronic effects are not well delineated. Diazinon apparently inhibits protein synthesis causing protein deficiency, which is thought to depress immune system function and cause lesions of the thymus and spleen, and to repress lymphocyte activation by affecting amino acid metabolic pathways. Diazinon exposure has also been reported to be associated with declining semen quality,[24–26] and with increased risk for cancer types, such as non-Hodgkin's lymphoma in human males.[27]

In this paper, the authors present data from an evaluation of the effects of diazinon exposure on gene expression in human cells. These data, from an *in vitro* study, determine some of the effects of cellular exposure to low concentrations of an agricultural and horticultural chemical, diazinon, that has, until recently, been routinely used to spray crops, vegetables, and ornamentals in the US and continues to be in use in large parts of the world. A correlation between diazinon exposure and the potential for diminished DNA excision repair is suggested by these data.

Materials and methods

Tissue culture

Cells of the human breast cancer line, MCF-7, obtained from ATCC (Manassas, VA), were grown in minimal essential medium (MEM; Sigma, St. Louis, MO) containing Phenol red, supplemented with 10% fetal bovine serum (FBS; Atlanta Biologicals), 5% insulin, 5% sodium pyruvate, l-glutamine (Sigma, St. Louis, MO), in a humidified atmosphere at 37°C, 5% CO_2. Cells were then switched to MEM without Phenol red or FBS for 18 hours, because Phenol red acts as a weak estrogen,[28] and FBS contains variable levels of estradiol. Culture without Phenol red and in low levels of estradiol was followed by treatment with 17β-estradiol (Sigma), for another 18 hours or with diazinon at various concentrations in the presence or absence of 17β-estradiol.

Table 1 Primers used for real-time qrtPCR

Gene name	*PubMed ID*		*Oligonucleotides 5′-3′*
Excision repair protein 5	L20046	Forward	GACACGTTCTGAAGTCATTGGCCCTGFAMC
		Reverse	TGGTCTGCTGTTCTTGGATGGT
Ribonucleotide reductase M1 subunit	X59543	Forward	GACATACACCCTGGCCCTGAATATGTAGFAMC
		Reverse	GGGCGATGGCGTTTATTTG
GADPH	X01677	Forward	CATCAAGAAGGTGGTGAAGCA
		Reverse	CTACACTACCACCTGGTGCTCAGTGFAMAG

Treatment with diazinon
Cells were treated with 30, 50 or 67 ppm of diazinon with or without 17β-estradiol at 3×10^{-9} M. In each experiment, the carrier dimethylsulfoxide (DMSO) was present at $<0.01\%$ final concentration in the culture medium.

Total RNA extraction and quantification
Cells were harvested at the end of 36 hours of treatment using Ambion lysis solution. RNA was extracted using the totally RNA kit of Ambion (Austin, TX). Extracted RNA was spectrophotometrically quantified and evaluated on an agarose gel, along with an MCF-7 standard of induced mRNA (Ambion), to check for RNA quality (see figure 2).

cDNA microarray
mRNA was prepared from total RNA using the Oligotex kit (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 10 μg control mRNA labeled with Cy-5 and 10 μg test mRNA labeled with Cy-3 for 16 hours on Phase 1 microarray slides containing human genes (Santa Fe, NM). Phase 1 microarray slides were developed with batteries of genes to assess immuno- and neuro-associated genes and genes known to be associated with hepatotoxicity, but also contain a variety of other function-related genes.

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

Reverse transcription
After visualizing 2 μg of each RNA sample on a non-denaturing gel, 1 μg of high quality total RNA was used for reverse transcription. Briefly, for a 25-μL RT reaction, random hexamers (16 μM final, Roche, Indianapolis, IN), Oligo dT (500 ng/μL, Promega, Madison, WI), RNA 1.0 μg, and RNAse-free water were combined, heated at 65°C for 5 min, and allowed to cool to room temperature. A cocktail of the following components was added to each sample; 5 × 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). The cocktail was used without RT as a control. Each tube was mixed and centrifuged briefly. The RT reaction was run at 37°C for 1 hour, reverse transcriptase was inactivated by heating at 90°C for 5 min, and samples were cooled on ice. A reaction with and without reverse transcriptase was performed with each sample. For real time PCR, a master mix containing Platinum Quantitative PCR SuperMix-UDG, ROX reference dye, RNAse/DNAse-free water, 10 μM FAM-labeled LUX primers and 10 μM unlabeled reverse primer was assembled. All components were from Invitrogen. The primers (Table 1) were specific for the genes selected for special consideration or for GADPH, a housekeeping gene common to most mammalian cells. Primers were designed using Invitrogen's LUX primer design program (available from the Invitrogen website) and labeled with FAM. Each sample – 10 μL with or without RT – was combined with 40 μL of the master mix in an optically clear semi-skirted PCR plate. Reactions contained primers specific for the genes of interest and for GADPH in each sample. Real time PCR employed an Applied Biosystems Prism 7700 thermocycler (Foster City, CA) and used a three-step cycling program recommended by Invitrogen: 50°C, 2 min hold (UDG treatment), 95°C, 2 min hold and 45 cycles of 95°C, 15 s, 55°C, 30 s, 72°C, 30 s.

Data analysis
The data are presented as normalized expression levels – (expression level of the gene of interest divided by the expression level of GADPH). The expression level for qrtPCR studies was determined by the equation: $2^{45\text{-}Ct}$, Ct being the cycle

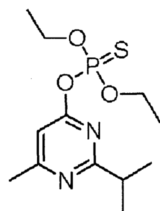

Figure 1 Structure and activation of the insecticide diazinon. (From: www.sigma-aldrich.com)

number at which the threshold was crossed for the test gene GADPH, and 45 is the number of cycles in the program.[29]

Results

MCF-7 cells were grown in medium containing 10% normal FBS (NFBS), or in medium with serum-stripped FBS, for 18 hours and treated with diazinon (initial range: 0.3–300 ppm), with or without 17β-estradiol. Cells were harvested after 36 hours, RNA was isolated and microarray slides were labeled. Cells treated with diazinon > 90 ppm in the medium died. At 30, 50 and 67 ppm of diazinon, cells were alive and not morphologically altered.

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 hours
SFBS	MCF-7 cells grown in medium containing charcoal-dextran treated FBS medium (10%) for 36 hours
NFBS T1-E2	MCF-7 cells grown in NFBS for 18 hours, followed by addition of 3.3 ng/mL of Enable to the medium for 18 hours
NFBS T1+E2	MCF-7 cells grown in NFBS for 18 hours, followed by addition of 3.3 ng/mL of Enable and 17 β-estradiol to the medium for 18 hours
NFBS E2	MCF-7 cells grown in NFBS for 18 hours, followed by addition of 17 β-estradiol to the medium for 18 hours
SFBS T1	MCF-7 cells grown in SFBS for 18 hours, followed by addition of 3.3 ng/mL of Enable to the medium for 18 hours
SFBS E2	MCF-7 cells grown in SFBS for 18 hours, followed by addition of 17 β-estradiol to the medium for 18 hours
NFBS D3	MCF-7 cells grown in NFBS for 18 hours, followed by addition of 3 ppm of diazinon to the medium for 18 hours
NFBS D30	MCF-7 cells grown in NFBS for 18 hours, followed by addition of 30 ppm of diazinon to the medium for 18 hours
NFBS D50	MCF-7 cells grown in NFBS for 18 hours, followed by addition of 50 ppm of diazinon to the medium for 18 hours
NFBS D67	MCF-7 cells grown in NFBS for 18 hours, followed by addition of 67 ppm of diazinon to the medium for 18 hours

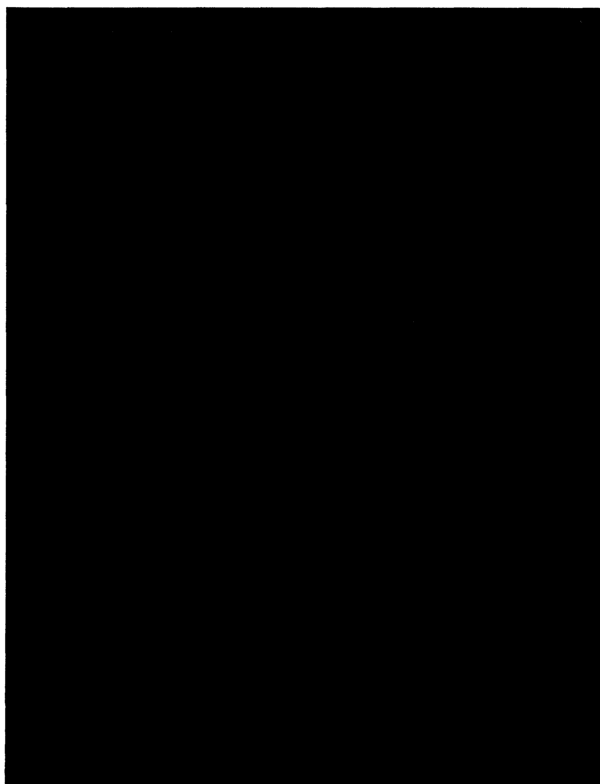

Figure 2 Agarose gel electrophoresis of a diazinon-treated MCF-7 RNA sample.

Identification of genes affected by diazinon

DNA microarray analysis of the affects of diazinon is dependent on activation of the insecticide (Figure 1). In human liver, this is a function of CYP2C19, and without activation by this or other CYP, diazinon would not be expected to alter gene expression.[22,30] Microarray experiments examined the effects of diazinon on gene expression in MCF-7 cells and are summarized in Table 2. The data show a number of genes with dysregulation. For qrtPCR corroboration of altered gene expression,

Table 3 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	Three genes down-regulated and 28 genes up-regulated

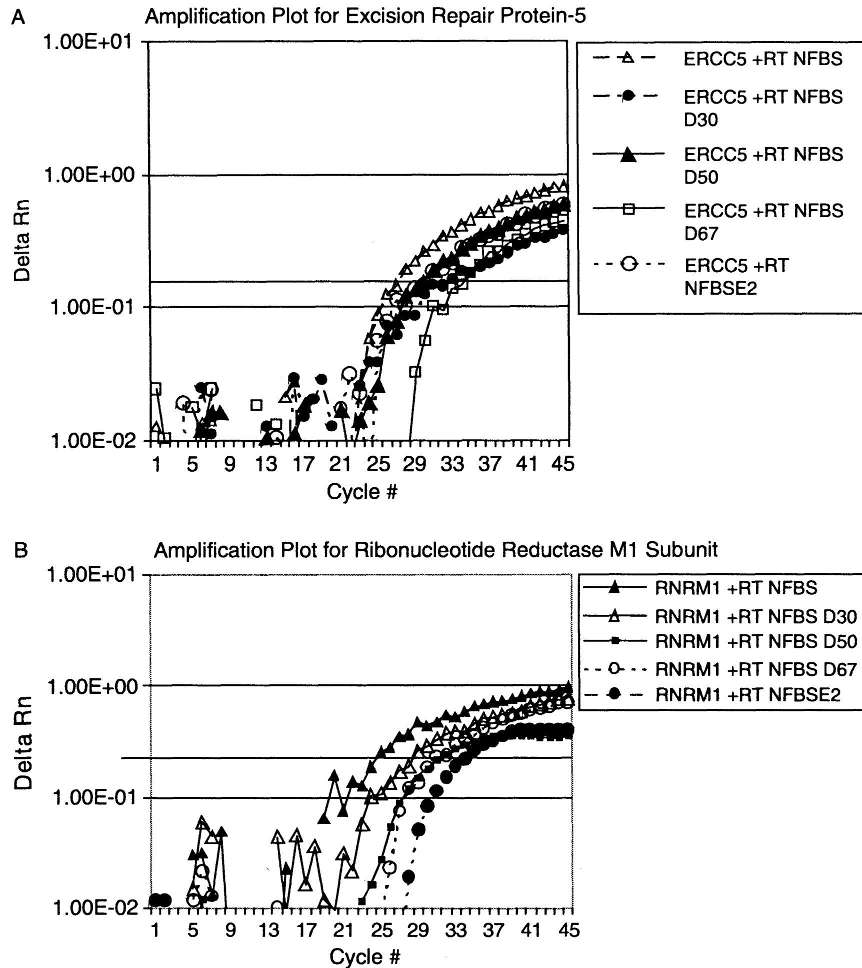

Figure 3 Amplification plots A and B for genes of MCF-7 cells treated with diazinon at 67 ppm.

two genes were chosen from 67 ppm treatment levels (Figure 3A, B). These genes, excision repair protein 5 (ERCC-5) and ribonucleotide reductase subunit M1 (RNRM1), were down-regulated (Table 4).

Quantitative real time PCR data and expression levels

Reverse transcription and quantitative real time PCR (qrtPCR) was carried out as above and shown in Figure 3(A,B). The data confirmed results of the microarray analysis. Normalized expression levels computed for ERCC5 and RNRM1 are given in Figures 4 and 5.

Discussion

Excision repair protein-5 (ERCC5, aka ERCM2; UVDR; XPG; XPGC)

DNA excision repair (NER) is one of the primary mechanisms by which mammalian cells remove DNA lesions caused by ultraviolet light or by exogenous DNA adduct-forming agents. Exposure to mutagenic or genotoxic agents may cause cell cycle delays allowing repair of DNA damage, repair that 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 during NER. In the process of repair, the products of more than a dozen genes are involved in damage recognition, incision, excision, DNA elongation, and DNA ligation. Collectively, they restore the normal DNA structure.[31–34]

Excision repair protein-5 (XPG, ERCC5) has endonuclease activity and plays a major role in incision of both the 5′ and 3′ sides of UV-initiated DNA lesions.[31] Individuals with a homozygous defect in the ERCC5-encoded protein (aka XPG, XPGC, ERCM2, UVDR) suffer from Xeroderma pigmentosum (XP), or in some instances, Cockayne syndrome,[35] associated with

Table 4 Gene expression profile after treatment with diazinon at 67 ppm

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 >1.5 are considered up-regulated and <0.6, down-regulated. Values are fold change at each time point relative to the control. Values in **bold** have been verified by real-time PCR.

the inability to initiate DNA excision repair.[31–35] In lung cancer patients and patients with squamous cell carcinoma of the head and neck, the levels of ERCC5 are significantly lower than in normal individuals.[36] Defective excision repair increases the occurrence of transformed cells by allowing unrepaired DNA damage to remain in place, leading to increased mutation and cell transformation. DNA repair capacity varies from individual to individual, and reduced expression of ERCC5 and other ER proteins is likely to increase the susceptibility of an individual to a variety of ultraviolet light- or mutagenic chemical-induced cancer types. Individuals heterozygous for ERCC5 are at a lower risk for cancer than persons who are homozygous for mutant ERCC5, and at a greater
risk than those persons homozygous normal for this gene.

Ribonucleotide reductase M1 (RNRM1)

Ribonucleotide reductase (RNR) plays a key role in the synthesis of DNA during excision repair. Recent studies have shown that there are three human RNR subunits: RNRM1, RNRM2 and p53R2. RNRM1 is a large peptide chain, one of the two components of ribonucleotide reductase, and both RNRM2 and p53R2 are small protein subunits of RNR. The cellular function of RNRM1 is to provide the deoxynucleotides required for DNA synthesis and repair through catalysis of ribonucleoside diphosphates to the corresponding deoxyribonucleosides, catalyzing the rate-limiting step in deoxyribonucleotide biosynthesis. The activity of this heterodimeric enzyme is dependent on the expression of both its large and small subunits.[37,38]

The p53R2 protein directly interacts with RNRM2, while RNRM1 interacts weakly with p53R2, but not with p53. After exposure to UV, p53R2 and RNRM2 dissociate from p53 and bind RNRM1. Before UV treatment of cells, all RNR subunits are localized in the cytoplasm, translocating to the nucleus after UV treatment.[37]

RNRM1 and lung cancer

There is an incompletely defined relationship between allele loss at human chromosome 11p15.5 and increased metastasis in patients with lung cancer.[39] The RNRM1 gene, which maps to 11p15.5, suppresses invasion, migration, and *in vivo* metastasis by up-regulation of the PTEN tumor-suppressor gene when it is over-expressed in human and mouse lung cancer cell lines.

Conclusions

These studies indicate that treatment of human cells in culture with diazinon results in down-regulation of a number of genes. Two genes with related functions have been corroborated using qrtPCR. Down-regulation of the two DNA excision repair-associated genes, ERCC5 and RNRM1, in human cells exposed to diazinon, can potentially result in serious health problems. The

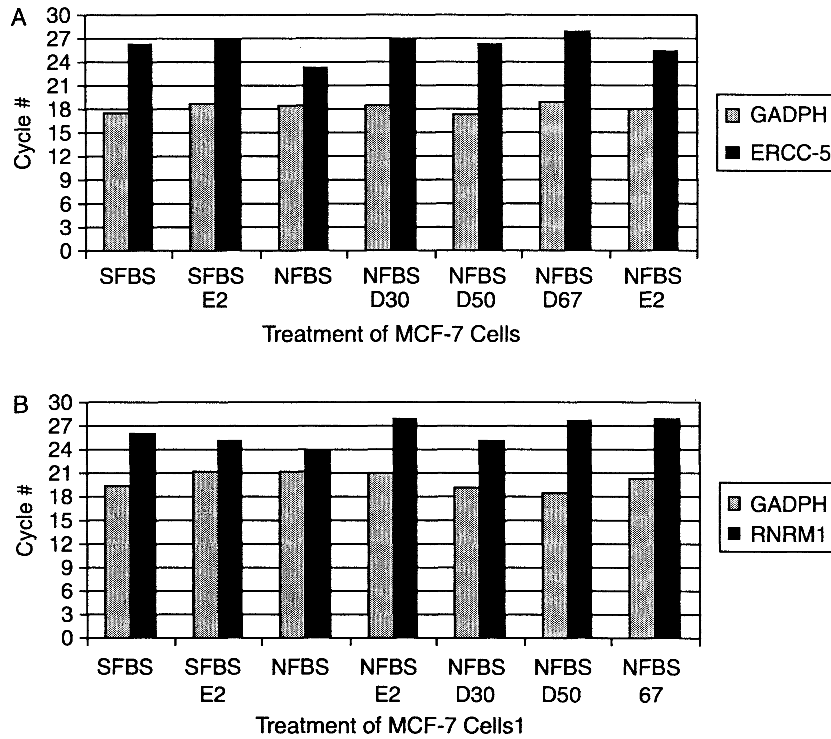

Figure 4 QRT PCR amplification plots for diazinon treatment. GADPH is the standard gene. (A) The cycle at which ERCC5 crosses the threshold in all samples. (B) The cycle at which RNRM1 crosses the threshold in different samples, respectively.

potential consequences of low-dose, long-term, exposure to diazinon appear to be potentially significant to the developing fetus, and to developing and adult humans exposed to ultraviolet light and/or mutagenic chemicals. These data suggest that diminished expression of ERCC5 and RNRM1 after diazinon exposure has the potential to result in the increased incidence of

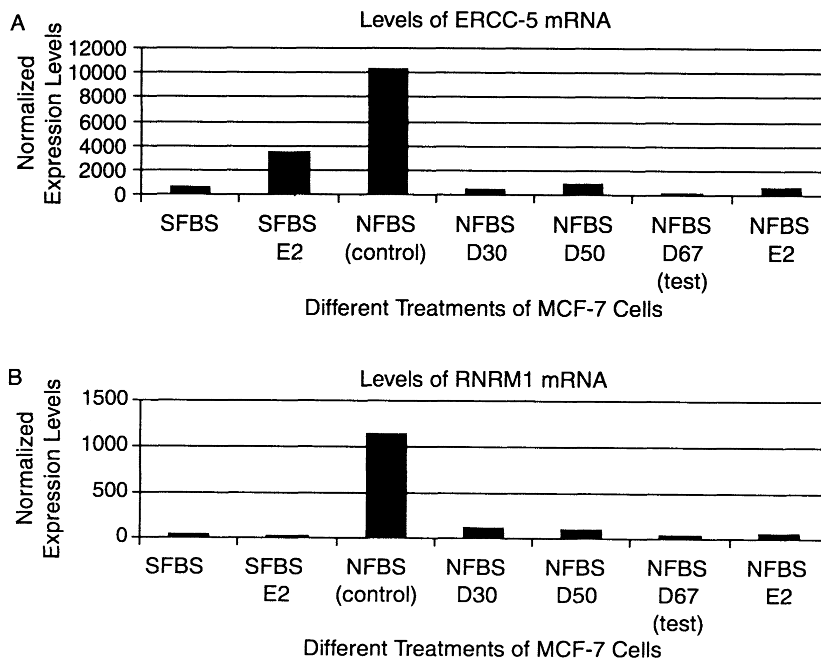

Figure 5 Normalized gene expression levels for diazinon treatment. (A, B) Represent individual expression levels of RNRM1 and ERCC5 compared with GADPH, respectively.

basal cell carcinoma and/or melanoma, and in possibly elevated levels of metastasis of transformed cells.

Acknowledgements

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