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KJIM

The effects of nonyl phenoxypolyethoxyl ethanol on cell damage pathway gene expression in SK-N-SH cells

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Background/Aims: Most pesticide formulations contain both chief and additive ingredients. But, the additives may not have been tested as thoroughly as the chief ingredients. The surfactant, nonyl phenoxypolyethoxylethanol (NP40), is an additive frequently present in pesticide formulations. We investigated the effects of NP40 and other constituents of a validamycin pesticide formulation on cell viability and on the expression of genes involved in cell damage pathways.
Methods: The effects of validamycin pesticide ingredients on cell viability and of NP40 on the mRNA expression of 80 genes involved in nine key cellular pathways were examined in the human neuroblastoma SK-N-SH cell line.
Results: The chemicals present in the validamycin pesticide formulation were cytotoxic to SK-N-SH cells and NP40 showed the greatest cytotoxicity. A range of gene expression changes were identified, with both up- and down-regulation of genes within the same pathway. However, all genes tested in the necrosis signaling pathway were down-regulated and all genes tested in the cell cycle checkpoint/arrest pathway were up-regulated. The median fold-change in gene expression was significantly higher in the cell cycle checkpoint/arrest pathway than in the hypoxia pathway category ($p = 0.0064$). The 70 kDa heat shock protein 4 gene, within the heat shock protein/unfolded protein response category, showed the highest individual increase in expression (26.1-fold).
Conclusions: NP40 appeared to be particularly harmful, inducing gene expression changes that indicated genotoxicity, activation of the cell death (necrosis signaling) pathway, and induction of the 70 kDa heat shock protein 4 gene.

Keywords: Validamycins; Surface-active agents; Nonyl phenoxypolyethoxylethanol; Gene expression; Cell damage pathways

INTRODUCTION

Pesticides are used to increase food production by reducing the loss of crops to weeds, insects, fungal infections, parasites, and rodent pests. However, pesticides can also have negative impacts on non-target organisms. Each year, hundreds of thousands of people around the world die from the effects of pesticide use or misuse [1].

Validamycin, also called validamycin A, is a non-systemic fungicidal antibiotic [2] that is particularly effective against soil-borne diseases [3]. Pesticide Action Network North America has described validamycin as "not acutely toxic," since its median lethal dose (LD_{50}) exceeds 20,000 mg/kg (United States Environmental

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Table 1. Validamycin formulation constituents

Function	Ingredient (CAS number)	Content, %[a]
Active ingredient	Validamycin (37248-47-8)	~5.0
Emulsifier	NP40 (9016-45-9)	~2.0
Supplement	Methanol (67-56-1)	~5.0
Supplement	Potassium hydroxide (1310-58-3)	< 0.1
Stabilizer	Sorbic acid (110-44-1)	< 0.1
Coloring	Acid yellow 17, disodium salt (6359-98-4)	< 0.1
pH regulator	Sulfuric acid (7664-93-9)	< 0.1
Antifoam	Silicones and siloxanes, dimethyl (63148-62-9)	< 0.1
Solvent	Water (7732-18-5)	~87.0
Total		100.0

CAS, chemical abstracts service; NP40, nonyl phenoxypolyethoxylethanol.
[a]Content, usually presented as "weight %" or "weight by volume" in the formulation, was undisclosed by the manufacturer. Therefore, we are only able to present estimates of these values.

Protection Agency, 1994). Until recently, there was no evidence that validamycin caused illness in humans. However, we recently encountered a patient showing hypotension, unconsciousness, hypoxia, and high anion gap metabolic acidosis after ingesting 200 mL of an undiluted validamycin herbicide preparation containing a range of chemicals (Table 1).

Most pesticide formulations contain both "chief" and "additive" ingredients. Chief ingredients are used to target pests, and their efficacy is enhanced by the presence of the additive ingredients. Ingestion of a pesticide results in exposure to all of its ingredients. The additives may not have been tested as thoroughly as the chief ingredients, and are seldom disclosed on product labels. For this reason, physicians may need to be aware of the effects of additive ingredients.

After investigating the validamycin formulation ingested by our patient, we discovered that it contained a number of additives, including an emulsifier, a stabilizer, a coloring agent, a pH regulator, an antifoaming agent, and supplements (Table 1). We hypothesized that the symptoms observed in this patient were caused by one or more of these additives. To test this hypothesis, we studied the clinical features of the patient and performed *in vitro* experiments to analyze the cytotoxicity of each chemical in the formulation.

METHODS

Case study

A female patient aged 72 years was admitted to hospital in a critical condition within 1 hour of ingesting 200 mL of a validamycin formulation. Upon admission, she had shallow respiration and her O_2 saturation was < 70%. After receiving first aid and undergoing tracheal intubation, the patient was transferred to the Pesticide Intoxication Institute at Soonchunhyang University Cheonan Hospital.

When we first examined the patient, she was in a semi-coma state, with a blood pressure of 100/70 mmHg and a pulse rate of 76 beats per minute. Arterial blood gas analysis showed hypoxia, with a high anion gap [$Na^+ - (HCO_3^- + Cl^-) = 32.4$] metabolic acidosis (pH = 7.13).

Metabolic acidosis was corrected after a single session of hemodialysis. Lipid emulsion product (20%) was injected intravenously, using 500 mL over 2 hours as the loading dose, followed by a maintenance dose of 1,000 mL over the next 24 hours. The patient's mental status improved, although she remained drowsy. We were able to remove her tracheal tube on her third hospital day, and she began taking a liquid diet on the following day. The patient was transferred to the general ward on her 7th hospital day and discharged on the 18th day, with no signs of any specific health abnormalities.

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In vitro cytotoxicity studies

We explored the effects of the following ingredients of the validamycin formulation, as stated by its manufacturer: validamycin (Sigma-Aldrich, St. Louis, MO, USA), nonyl phenoxypolyethoxylethanol (NP40, Sigma), methanol (Sigma-Aldrich), potassium hydroxide (KOH, Junsei Chemical Co., Tokyo, Japan), sorbic acid (Sigma-Aldrich), acid yellow (yellow 17, Sigma-Aldrich), sulfuric acid (Sigma-Aldrich), silicones (Santa Cruz Biotechnology Inc., Dallas, TX, USA), and siloxanes (Santa Cruz Biotechnology Inc.).

Cell culture

We purchased the human neuroblastoma cell line, SK-N-SH, from the Korean Cell Line Bank (Seoul, Korea). Cells were maintained in Roswell Park Memorial Institute-1640 (RPMI-1640) medium (HyClone, Logan, UT, USA) supplemented with 10% fetal bovine serum and 1% penicillin-streptomycin antibiotic (Life Technologies, Paisley, UK). They were expanded in a humidified CO_2 incubator (5%) at 37°C in T-75 flasks after trypsinization and subsequent washing in phosphate-buffered saline.

Cytotoxicity assays

Two types of assay were carried out to investigate the effects of each formulation ingredient on SK-N-SH cells. The MTT (3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide; Sigma-Aldrich) assay measured reduction of MTT to its purple tetrazolium salt by metabolically active cells. The intensity of this color (measured at 595 nm) was proportional to the number of metabolically active cells in each well. The lactate dehydrogenase (LDH) assay measured release of LDH (a stable cytoplasmic enzyme) from compromised cells with membrane damage. LDH reduces pyruvate to lactate by oxidizing nicotinamide adenine dinucleotide (NADH) to NAD^+ and spectrophotometric measurement of NADH consumption provided an indication of the amount of LDH released.

Prior to each cytotoxicity assay, a stock concentration of each formulation ingredient was prepared in an appropriate vehicle. SK-N-SH cells (100 μL) were seeded into 96-well plates at 2×10^4 cells/well, and incubated for 24 hours. When cells reached 70% to 80% confluence, 100 μL of the test chemicals (freshly prepared in RPMI media) were added into the wells at concentrations of 0.01 μM to 1 mM, and incubated for a further 24 hours at 37°C. The total volume of vehicle added to the cell culture plates was always ≤ 1.0% of the media volume, to minimize vehicle effects on the cells.

MTT assays were then performed using the method previously described by Mickisch et al. [4]. Briefly, the cells were treated with 100 μL of 0.5 mg/mL MTT solution and incubated for an additional 2 hours at 37°C. The MTT solution was then removed and 100 μL of dimethylsulfoxide (DMSO; Sigma-Aldrich) was added to each well to dissolve the formazan. The absorbance was then measured using an enzyme-linked immunosorbent assay (ELISA) reader (Perkin Elmer, Waltham, MA, USA).

LDH release was measured at 490 nm using an ELISA reader, according to the manufacturer's protocol (Roche Inc., Pleasanton, CA, USA). The resulting value was expressed relative to the absorbance determined in cells exposed to 1% Triton X-100 (control).

Gene expression study

MTT and LDH assays indicated that NP40 had the greatest effect on SK-N-SH cell viability. To investigate the dominant pathway of NP40-mediated cytotoxicity, the expression levels of 80 genes responsible for human stress and toxicity pathways were quantified (Table 2). These gene products were involved in the following nine categories of cell damage pathways: oxidative/metabolic stress, hypoxia, cell death (comprising three subgroups relating to apoptosis signaling, autophagy signaling, and necrosis signaling), inflammatory response, DNA damage signaling (comprising two subgroups relating to cell cycle checkpoint/arrest and other responses), and heat shock proteins/unfolded protein response (Table 2).

Total RNA was isolated from SK-N-SH cells exposed to 0.001 μM NP40 or vehicle for 24 hours, using the RNeasy mini kit (QIAGEN GmbH, Hilden, Germany). cDNA was synthesized using the Maxime RT premix kit (Intron, Seongnam, Korea) according to the manufacturer's instructions. Gene expression was quantified using the RT^2 Profile PCR Array (Cat. no. PAHS-003ZD-12; QIAGEN). SYBR Green PCR Master Mix (Applied Biosystems, Grand Island, NY, USA) was added, and the arrays were run on a CFX96 Real-Time system (Bio-Rad, Hercules, CA, USA) using cycling programs recom-

Table 2. Gene name and fold change in expression in nine categories of cell damage pathways after NP40 exposure

GeneBank accession no.	Gene name	Symbol	Fold change	
			NP40	*p* value[a]
Oxidative/metabolic stress				
NM_003329	Thioredoxin	*TXN*	–1.23	0.008[b]
NM_001498	Glutamate-cysteine ligase, catalytic subunit	*GCLC*	–1.32	0.045[b]
NM_002061	Glutamate-cysteine ligase, modifier subunit	*GCLM*	4.02[b]	0.048[b]
NM_000637	Glutathione reductase	*GSR*	1.30[b]	0.689
NM_000852	Glutathione S-transferase pi 1	*GSTP1*	–4.24	0.389
NM_002133	Heme oxygenase (decycling) 1	*HMOX1*	1.60[b]	0.024[b]
NM_000903	NAD(P)H dehydrogenase, quinone 1	*NQO1*	1.99[b]	0.016[b]
NM_002574	Peroxiredoxin 1	*PRDX1*	–1.83	0.095
NM_003330	Thioredoxin reductase 1	*TXNRD1*	1.84[b]	0.033[b]
NM_003900	Sequestosome 1	*SQSTM1*	–2.44	0.045[b]
NM_002032	Ferritin, heavy polypeptide 1	*FTH1*	8.78[b]	0.084
Hypoxia				
NM_003376	Vascular endothelial growth factor A	*VEGFA*	–6.09	0.009[b]
NM_001668	Aryl hydrocarbon receptor nuclear translocator	*ARNT*	–2.86	0.022[b]
NM_004331	BCL2/adenovirus E1B 19 kDa interacting protein 3-like	*BNIP3L*	1.18[b]	0.046[b]
NM_001216	Carbonic anhydrase IX	*CA9*	–2.82	0.037[b]
NM_000799	Erythropoietin	*EPO*	–2.75	0.017[b]
NM_002133	Hemeoxygenase (decycling) 1	*HMOX1*	1.60[b]	0.024[b]
NM_005566	Lactate dehydrogenase A	*LDHA*	–1.38	0.042[b]
NM_004994	Matrix metallopeptidase 9 (gelatinase B, 92 kDa gelatinase, 92 kDa type IV collagenase)	*MMP9 (Gelatinase B)*	–1.25	0.048[b]
NM_000602	Serpin peptidase inhibitor, clade E (nexin, plasminogen activator inhibitor type 1), member 1	*SERPINE1 (PAI-1)*	–7.17	0.000[b]
NM_006516	Solute carrier family 2 (facilitated glucose transporter), member 1	*SLC2A1*	1.87[b]	0.020[b]
NM_001124	Adrenomedullin	*ADM*	–5.24	0.080
Cell death (apoptosis signaling)				
NM_003842	Tumor necrosis factor receptor superfamily, member 10b	*TNFRSF10B (DR5)*	8.93[b]	0.037[b]
NM_001065	Tumor necrosis factor receptor superfamily, member 1A	*TNFRSF1A*	–1.17	0.044[b]
NM_021960	Myeloid cell leukemia sequence 1 (BCL2-related)	*MCL1*	1.87	0.004[b]
NM_003844	Tumor necrosis factor receptor superfamily, member 10a	*TNFRSF10A*	–2.90	0.038[b]
NM_033292	Caspase 1, apoptosis-related cysteine peptidase (interleukin 1, β, convertase)	*CASP1 (ICE)*	–1.72	0.022[b]
NM_000043	Fas (TNF receptor superfamily, member 6)	*FAS*	–15.12	0.081
Cell death (autophagy signaling)				
NM_004849	ATG5 autophagy related 5 homolog (*Saccharomyces cerevisiae*)	*ATG5*	–1.22	0.019[b]
NM_006395	ATG7 autophagy related 7 homolog (*S. cerevisiae*)	*ATG7*	–4.97	0.039[b]
NM_004707	ATG12 autophagy related 12 homolog (*S. cerevisiae*)	*ATG12*	2.59[b]	0.017[b]
NM_003766	Beclin 1, autophagy related	*BECN1*	1.48[b]	0.029[b]

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Table 2. Continued

GeneBank accession no.	Gene name	Symbol	Fold change	
			NP40	*p* value[a]
NM_000043	Fas (TNF receptor superfamily, member 6)	*FAS*	–15.12	0.081
NM_003565	Unc-51-like kinase 1 (*Caenorhabditis elegans*)	*ULK1*	–6.74	0.018[b]
Cell death (necrosis signaling)				
NM_017853	Thioredoxin-like 4B	*TXNL4B*	7.67[b]	0.071
NM_002086	Growth factor receptor-bound protein 2	*GRB2*	–1.99	0.029[b]
NM_001618	Poly (ADP-ribose) polymerase 1	*PARP1 (ADPRT1)*	–1.56	0.025[b]
NM_006505	Poliovirus receptor	*PVR*	1.57[b]	0.098
NM_003804	Receptor (TNFRSF)-interacting serine-threonine kinase 1	*RIPK1*	13.86[b]	0.096
NM_003844	Tumor necrosis factor receptor superfamily, member 10a	*TNFRSF10A*	–2.90	0.038[b]
NM_003842	Tumor necrosis factor receptor superfamily, member 1A	*TNFRSF1A*	–1.17	0.044[b]
NM_000043	Fas (TNF receptor superfamily, member 6)	*FAS (TNFRSF6)*	–15.12	0.081
Inflammatory response				
NM_002982	Chemokine (C-C motif) ligand 2	*CCL2 (MCP-1)*	8.16[b]	0.037[b]
NM_000594	Tumor necrosis factor	*TNF*	–11.48	0.079
NM_000567	C-reactive protein, pentraxin-related	*CRP*	2.43[b]	0.015[b]
NM_000619	Interferon, γ	*IFNG*	–1.45	0.015[b]
NM_000575	Interleukin 1, α	*IL1A*	–2.36	0.046[b]
NM_000600	Interleukin 6 (interferon, β2)	*IL-6*	8.95[b]	0.032[b]
NM_000584	Interleukin 8	*IL-8*	–2.03	0.080[b]
NM_138554	Toll-like receptor 4	*TLR4*	–4.55	0.017[b]
NM_000074	CD40 ligand	*CD40LG (TNFSF5)*	2.84[b]	0.201
DNA damage signaling (cell cycle checkpoint/arrest)				
NM_004507	HUS1 checkpoint homolog (*Schizosaccharomyces pombe*)	*HUS1*	3.30[b]	0.012[b]
NM_001274	CHK1 checkpoint homolog (*S. pombe*)	*CHEK1*	4.36[b]	0.076
NM_007194	CHK2 checkpoint homolog (*S. pombe*)	*CHEK2 (RAD53)*	3.15[b]	0.000[b]
NM_004083	DNA-damage-inducible transcript 3	*DDIT3 (GADD153/CHOP)*	2.90[b]	0.038[b]
NM_002873	RAD17 homolog (*S. pombe*)	*RAD17*	1.78[b]	0.012[b]
NM_004584	RAD9 homolog A (*S. pombe*)	*RAD9A*	1.44[b]	0.029[b]
NM_000389	Cyclin-dependent kinase inhibitor 1A (p21, Cip1)	*CDKN1A (p21CIP-1WAF1)*	2.33[b]	0.027[b]
NM_005590	MRE11 meiotic recombination 11 homolog A (*S. cerevisiae*)	*MRE11A*	1.99[b]	0.019[b]
NM_002485	Nibrin	*NBN (NBS1)*	2.45[b]	0.011[b]
DNA damage signaling (other responses)				
NM_000051	Ataxia telangiectasia mutated	*ATM*	–9.50	0.061
NM_001184	Ataxia telangiectasia and Rad3 related	*ATR*	–7.18	0.015[b]
NM_000107	Damage-specific DNA binding protein 2, 48 kDa	*DDB2*	–2.86	0.036[b]
NM_000546	Tumor protein p53	*TP53*	–3.52	0.006[b]
NM_002875	RAD51 homolog (*S. cerevisiae*)	*RAD51*	–11.56	0.038[b]
NM_001924	Growth arrest and DNA-damage-inducible, α	*GADD45A*	1.05[b]	0.026[b]
NM_004628	Xerodermapigmentosum, complementation group C	*XPC*	–1.04	0.023[b]
Heatshock proteins/unfolded protein response				

Table 2. Continued

GeneBank accession no.	Gene name	Symbol	Fold change	
			NP40	*p* value[a]
NM_001675	Activating transcription factor 4 (tax-responsive enhancer element B67)	*ATF4*	3.71[b]	0.019[b]
NM_007348	Activating transcription factor 6	*ATF6*	1.72[b]	0.018[b]
NM_004381	Activating transcription factor 6 β	*ATF6B*	2.15[b]	0.029[b]
NM_014417	BCL2 binding component 3	*BBC3*	1.09[b]	0.041[b]
NM_001196	BH3 interacting domain death agonist	*BID*	2.32[b]	0.050[b]
NM_006260	DnaJ (Hsp40) homolog, subfamily C, member 3	*DNAJC3*	2.73[b]	0.013[b]
NM_004083	DNA-damage-inducible transcript 3	*DDIT3*	–2.90	0.007[b]
NM_004343	Calreticulin	*CALR*	–1.08	0.040[b]
NM_001017963	Heat shock protein 90 kDa α (cytosolic), class A member 1	*HSP90AA1*	1.15[b]	0.048[b]
NM_003299	Heat shock protein 90 kDa β (Grp94), member 1	*HSP90B1 (TRA1)*	–5.85	0.096
NM_002154	Heat shock 70kDa protein 4	*HSPA4 (HSP70)*	26.10[b]	0.049[b]
NM_005347	Heat shock 70kDa protein 5 (glucose-regulated protein, 78kDa)	*HSPA5 (GRP78)*	–2.31	0.023[b]
NM_014278	Heat shock 70kDa protein 4-like	*HSPA4L*	3.00[b]	0.015[b]

NP40, nonyl phenoxypolyethoxylethanol; NADPH, nicotinamide adenine dinucleotide phosphate; BCL2, B cell lymphoma 2; TNFRSF, tumor necrosis factor receptor superfamily; TNF, tumor necrosis factor; ATG, autophagy protein; ADP, adenosine diphosphate; CHK, checkpoint kinase.
[a]The *p* values are calculated based on a Student *t* test of the replicate 2^ (- Delta Ct) values for each gene in the control group and treatment groups.
[b]*p* values less than 0.05.

mended by the manufacturer. Relative gene expression levels were determined using the data analyzer template provided by QIAGEN (http://pcrdataanalysis.sabiosciences.com/pcr/arrayanalysis.php) using glyceraldehyde 3-phosphate dehydrogenase, β-actin, and ribosomal protein L13a as reference genes. The non-stimulated condition was set to 1. The results were expressed as the fold-change (2^ [- delta delta Ct]) in normalized gene expression (2^ [- delta Ct]) in the test sample, as compared with the normalized gene expression (2^ [- delta Ct]) in the control sample. Fold-change values greater than one indicated up-regulation, and fold-change values less than one indicated down-regulation.

Statistics

Statistical analyses were performed using SAS version 9.3 (SAS Institute Inc., Cary, NC, USA). We performed three replicates of each *in vitro* experiment for cytotoxicity testing on three independent occasions. Inhibitory concentration $(IC)_{50}$ values were calculated using GraphPad Prism (GraphPad Software Inc., La Jolla, CA, USA), as appropriate. The results of the MTT and LDH assays were evaluated by one-way analysis of variance and Tukey multiple comparison tests.

Student *t* test was used to compare the replicate (delta delta Ct) values for each gene expression level in the control group, versus those of the treatment groups (Table 3). Changes in gene expression in the nine key pathways were analyzed using Fisher exact test (Table 2). Comparison of the fold change of gene expression in these nine pathways was analyzed using the Kruskal-Wallis test.

RESULTS

MTT assay

SK-N-SH cell viability decreased in a dose-dependent manner following exposure to all the chemicals tested ($p < 0.01$ for all, compared with vehicle control) (Fig. 1).

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Table 3. Gene expression changes in the nine key categories of human stress and toxicity pathways

Category of the pathway	Gene expression	
	Up-regulation	Down-regulation
Cell death (apoptosis signaling)	2 (40.0)	3 (60.0)
Cell death (autophagy signaling)	2 (40.0)	3 (60.0)
Cell death (necrosis signaling)	0	4 (100.0)
DNA damage signaling (cell cycle checkpoint/arrest)	8 (100.0)	0
DNA damage signaling (other responses)	1 (16.7)	5 (83.3)
Heat shock proteins/infolded protein response	9 (75.0)	3 (25.0)
Hypoxia	3 (30.0)	7 (70.0)
Inflammatory response	3 (50.0)	3 (50.0)
Oxidative/metabolic stress	4 (57.1)	3 (42.9)

Values are presented as number (%). $p = 0.005$ by Fisher exact test.

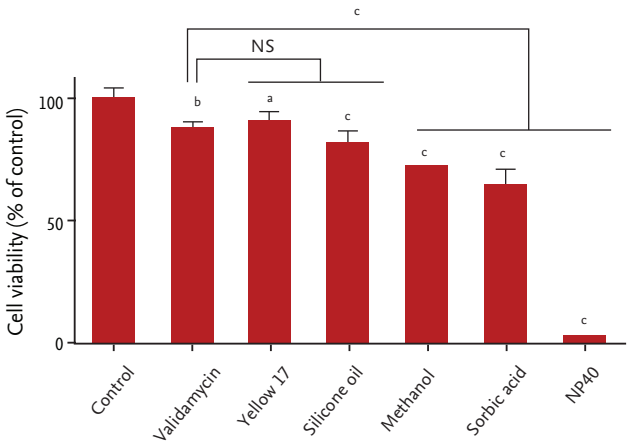

Figure 1. Cell viability following chemical exposure. Human neuroblastoma SK-N-SH cells were exposed to 1 mM of nonyl phenoxypolyethoxylethanol (NP40). Cell viability was significantly reduced following exposure to all of the chemicals tested, as compared with the control group (n = 8 for each data point). NS, not significant. [a]$p < 0.01$. [b]$p < 0.001$. [c]$p < 0.0001$.

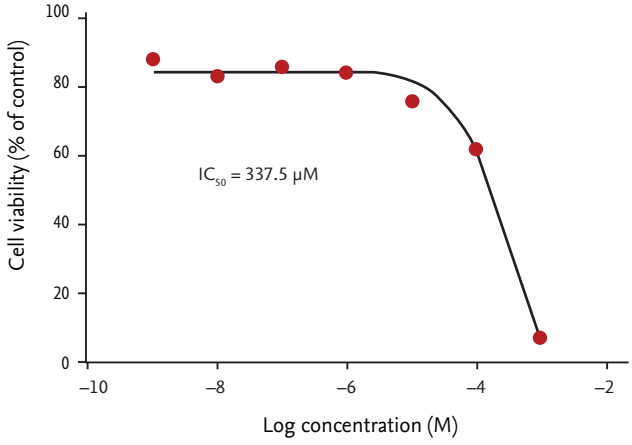

Figure 2. Cell viability following exposure to nonyl phenoxypolyethoxylethanol (NP40). Human neuroblastoma SK-N-SH cells were exposed to the indicated concentrations of NP40, from 1 nM to 1 mM. Cell viability was measured using MTT assays. The inhibitory concentration $(IC)_{50}$ was 337.5 μM (n = 6 for each data point).

After exposure to 1 mM validamycin, silicon oil, methanol, and sorbic acid, SK-N-SH cell viability was > 60% of that observed in control cells. Yellow 17 showed the least cytotoxicity, with 91.2% ± 4.3% cell viability following exposure to 1 mM of this coloring agent for 24 hours. In contrast, only 3.3% ± 0.2% cells were viable after incubation for 24 hours with 1 mM NP40 (IC_{50} = 337.5 μM) (Figs. 1 and 2). Based on these data, we divided the chemicals into three toxicity categories: slightly toxic (80% to 100% viability; validamycin, yellow 17, and silicon oil); moderately toxic (60% to 80% viability; methanol and sorbic acid); and severely toxic (< 10% viability; NP40).

LDH assay

Validamycin, sorbic acid, and silicone oil did not cause any significant elevation of LDH release from SK-N-SH cells ($p > 0.05$), while methanol and sorbic acid caused only small amounts of LDH release (7.5% ± 2.6% and 10% ± 7.4%, respectively), even at a high concentration (1 mM). In contrast, and consistent with the MTT assay data, NP40 was moderately to severely cytotoxic (57.2% ± 1.5% LDH release) (Fig. 3).

Pathway-related gene expression following NP40 treatment

Sixty-three out of 80 genes showed significant expression changes, based on Student *t* tests of the replicate (delta delta Ct) values for each gene in the control and treatment groups ($p < 0.05$) (Table 2). The expression changes were varied, with some up-regulated and others down-regulated within the same pathway (Table 2). However, genes in the cell death (necrosis signaling) pathway and DNA damage signaling (cell cycle checkpoint/arrest) pathways were the most noticeably affected, with down-regulated of all genes tested in the cell death (necrosis signaling) pathway and up-regulation of all genes tested in the DNA damage signaling (cell cycle checkpoint/arrest) pathway (Table 2).

The mean fold-change in gene expression was 2.42 (SD, 0.67; range, 1.44 to 3.30) within the cell cycle checkpoint/arrest pathways, and –1.91 (SD, –0.74; range, –1.17 to –2.90) within the cell death (necrosis signaling) pathway (Table 2). The median fold change in gene expression was significantly higher in the DNA damage signaling (cell cycle checkpoint/arrest) pathway than in the hypoxia pathway ($p = 0.0064$) (Table 4).

The 70 kDa heat shock protein 4 gene (within the heat shock protein/unfolded protein response category) showed the highest individual increase in expression (26.1-fold) (Table 2).

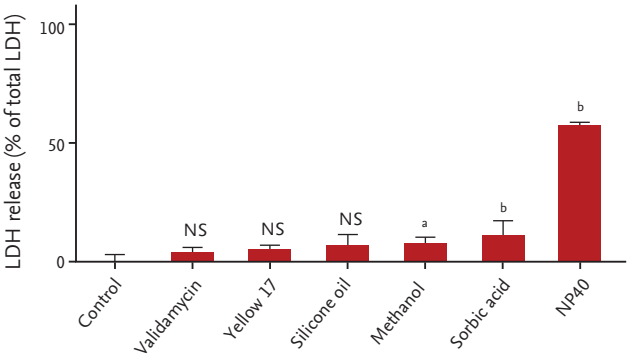

Figure 3. Lactate dehydrogenase (LDH) release following chemical exposure. Human neuroblastoma SK-N-SH cells were exposed to the indicated chemicals for 24 hours prior to analysis of LDH release. One-way analysis of variance identified significant differences between cells treated with different chamicals ($p < 0.0001$). Validamycin, yellow 17, and silicone oil had negligible effects on LDH release from SK-N-SH cells, as compared to the control treatment ($p > 0.05$). Exposure of these cells to methanol and sorbic acid caused some LDH release (7.5% ± 2.6% and 10% ± 7.4%, respectively), indicating some cytotoxicity, while nonyl phenoxypolyethoxylethanol (NP40) had moderate to high cytotoxicity (57.2% ± 1.5%) (n = 7 for each data point). NS, not significant. [a]$p < 0.001$. [b]$p < 0.0001$.

DISCUSSION

The present study investigated the effects of the ingredients of a validamycin formulation on the human neuroblastoma cell line, SK-N-SH. NP40, a surfactant that acts as an emulsifier, produced the most cytotoxic effects. This finding was consistent with those of previous studies [5-7].

We have previously performed a number of studies investigating the roles of surfactants in pesticide intoxication [8-12]. These have revealed that surfactants

Table 4. Comparison of the fold change of gene expression in the nine categories of human stress and toxicity pathways

Category of the pathway	No.	Median (interquartile range)
Cell death (apoptosis signaling)	5	–1.17 (–1.72 to 1.87)
Cell death (autophagy signaling)	5	–1.22 (–4.97 to 1.48)
Cell death (necrosis signaling)	4	–1.77 (–2.44 to –1.36)
DNA damage signaling (cell cycle checkpoint/arrest)	8	2.39[a] (1.89 to 3.02)
DNA damage signaling (other responses)	6	–3.19 (–7.18 to –1.04)
Heat shock proteins/unfolded protein response	12	1.93 (0.01 to 2.87)
Hypoxia	10	–2.06[a] (– 2.86 to 1.18)
Inflammatory response	6	0.49 (–2.36 to 8.16)
Oxidative/metabolic stress	7	1.60 (–1.32 to 1.99)

$p = 0.0064$ by Kruskal-Wallis test.
[a]Same letters indicate statistical significance based on the Dwass, Steel, Critchlow-Fligner multiple comparison method.

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negatively impact cells in a variety of ways, including breaking down the cell membrane, altering metabolic and mitochondrial activity, disrupting total protein synthesis [12], and facilitating mitochondrial damage-induced apoptosis and necrosis [9]. Based on our clinical experience, neurologic abnormalities such as unconsciousness and apnea are frequently observed clinical manifestations in patients with surfactant intoxication. For this reason, we choose a human SK-N-SH neuroblastoma cell line for the present study.

To identify the dominant pathway involved in NP40-mediated cytotoxicity, we investigated the expression of 80 genes involved in human stress and toxicity pathways. The effects of NP40 on gene expression varied greatly and we identified up- and down-regulation of genes within several pathways (Table 2). These findings suggested that intracellular signaling changes resulting from NP40 exposure were complex. The range of changes observed may result from components of the pathways affecting each other, or could reflect cross-talk between pathways [13,14].

However, the DNA damage signaling (cell cycle checkpoint/arrest) pathway was the most noticeably affected, with up-regulation of all genes. The genes in this category were HUS1, CHEK2 (RAD53), DNA-damage-inducible transcript 3 (DDIT3) (GADD153/CHOP), RAD17, RAD9A, cyclin-dependent kinase inhibitor 1A (p21, Cip1) (CDKN1A [p21CIP1WAF1]), MRE11 meiotic recombination 11 homolog A (MRE11A), and nibrin (NBN [NBS1]) (Table 3). The main function of the proteins encoded by these genes is to maintain genomic stability and conserve DNA integrity. The repair of damaged DNA is coupled to the completion of DNA replication by several cell cycle checkpoint proteins, including HUS1, RAD9A, and RAD17 [15,16]. CHEK2 is a protein kinase that is activated in response to DNA damage and may regulate cell cycle arrest. Hirao et al. [17] demonstrated that CHEK2$^{-/-}$ embryonic stem cells failed to maintain γ irradiation-induced G2 arrest.

Similarly, the other genes in this category, including DDIT3 (GADD153/CHOP) [18], CDKN1A (p21CIP1WAF1) [19], MRE11A [20], and NBN (NBS1) [21], are strongly activated in response to genotoxin-induced DNA damage. Taken together, our results suggested that NP40 acted as a potent genotoxin and activated DNA damage signaling within the cell cycle checkpoint/arrest pathway.

In contrast, expression of growth factor receptor-bound protein 2 (GRB2), poly (ADP-ribose) polymerase 1 (PARP1 [ADPRT1]), tumor necrosis factor receptor superfamily, member 10a (TNFRSF10A), and tumor necrosis factor receptor superfamily, member 1A (TNFRSF1A) within the cell death (necrosis signaling) pathway was down-regulated when SK-N-SH cells were exposed to NP40 (Table 2). GRB2-associated binders are scaffolding proteins implicated in cell signaling via receptor tyrosine kinases. Inhibition of GRB2 function blocks transformation and proliferation of various cell types and targeted gene disruption of GRB2 is lethal at an early embryonic stage in mice [22]. PARP1 protects cells from genomic instability and is involved in the inflammatory response and in several forms of cell death [23]. TNFRSF10A [24] and TNFRSF1A [25] belong to the tumor necrosis factor receptor (TNFR) super family. These receptors appear to transmit their signals via protein-protein interactions, which convey either a death or survival signal.

The 70 kDa heat shock protein 4 gene, within the heat shock protein/unfolded protein response category, showed the highest individual increase in expression (26.1-fold) (Table 2). *In vivo* and *in vitro* studies have shown that various stressors transiently increase production of heat shock proteins as a protection against harmful insults, such as environmental stresses and infection [26].

A thorough discussion of treatment modality is beyond the scope of this paper. However, we would like to briefly address our administration of an intravenous lipid emulsion to this patient. The predominant theoretical basis for the use of lipid emulsions proposes that the creation of an expanded intravascular lipid phase drives toxic lipophilic drugs from the target tissues into this "lipid sink" [27]. Typically, surfactants are amphiphilic organic compounds, meaning that they contain both a hydrophobic group and a hydrophilic group [28]. They will diffuse in water and adsorb at the lipid-water interface when water is mixed with a lipid. Therefore, we believe that a circulating lipid emulsion probably alters surfactant kinetics and biological effects.

Our study had some limitations. First, we were unable to calculate how much of each chemical would have been absorbed after the herbicide was ingested, due to the lack of pharmacokinetic data. Second, it is impossible to precisely model the patient's physiologic state *in*

vitro. Even with this limitation, we demonstrated that ingredients previously considered to be inert had greater toxicity towards SK-N-SH cells than the chief ingredient in this validamycin formulation.

In conclusion, NP40 appeared to be particularly harmful, inducing gene expression changes that indicated genotoxic effects and activation of the cell death (necrosis signaling) pathway.

KEY MESSAGE

1. The surfactant, nonyl phenoxypolyethoxylethanol (NP40) was the most cytotoxic chemical present in a validamycin pesticide formulation.
2. NP40 altered gene expression in various cell damage pathways, in particular the cell death (necrosis signaling) and DNA damage (cell cycle checkpoint/arrest) pathways.
3. Intravenous lipid emulsion administration may attenuate toxic symptoms in patients with NP40 intoxication.

Conflict of interest

No potential conflict of interest relevant to this article was reported.

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