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Mechanisms of resistance to the cytotoxic effects of oxysterols in human leukemic cells

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Abstract

We have developed hematopoietic cells resistant to the cytotoxic effects of oxysterols. Oxysterol-resistant HL60 cells were generated by continuous exposure to three different oxysterols—25-hydroxycholesterol (25-OHC), 7-beta-hydroxycholesterol (7β-OHC) and 7-keto-cholesterol (7κ-C). We investigated the effects of 25-OHC, 7β-OHC, 7κ-C and the apoptotic agent staurosporine on these cells. The effect of the calcium channel blocker nifedipine on oxysterol cytotoxicity was also investigated. Differential display and real-time PCR were used to quantitate gene expression of oxysterol-sensitive and -resistant cells. Our results demonstrate that resistance to the cytotoxic effects of oxysterols is relatively specific to the type of oxysterol, and that the cytotoxicity of 25-OHC but not that of 7β-OHC and 7κ-C, appears to occur by a calcium dependent mechanism. Oxysterol-resistant cells demonstrated no significant difference in the expression of several genes previously implicated in oxysterol resistance, but expressed the bcl-2 gene at significantly lower levels than those observed in control cells. We identified three novel genes differentially expressed in resistant cells when compared to HL60 control cells. Taken together, the results of this study reveal potentially novel mechanisms of oxysterol cytotoxicity and resistance, and indicate that cytotoxicity of 25-OHC, 7β-OHC and 7κ-C occur by independent, yet overlapping mechanisms.

Keywords: Oxysterols; Human leukemic cells; HL60; Apoptosis; Rhotekin; CNBP; HMG-CoA; bcl-2; OSBPL3; ORP3; ACAT; c-myc

1. Introduction

Oxysterols form a large family of hydroxylated derivatives of cholesterol detected in the blood, cells and tissues of animals and humans [8]. They have been demonstrated to be cytotoxic toward many normal and tumour cell types [16], and many studies suggest that this cytotoxicity results from the induction of apoptosis [1,3,5,9,17,22,23,27,28,33].

Normal hematopoiesis is characterised by a dynamic balance between the proliferation, differentiation and apoptosis of a small population of hematopoietic progenitor cells (HPCs) [26]. We have previously observed that that the oxysterols—25-hydroxycholesterol (25-OHC), 7-beta-hydroxycholesterol (7β-OHC) and 7-keto-cholesterol (7κ-C)—are potent inducers of apoptosis and differentiation of primary HPCs from adult bone marrow (ABM) and umbilical cord blood (UCB), and of the human promyelocytic cell line HL60 [14]. The results of this previous study also demonstrate that the oxysterol 25-OHC is cytotoxic at doses 10-fold lower than 7β-OHC and 7κ-C [14]. Interestingly, we have also reported that HPCs from ABM are more sensitive to the actions of 25-OHC, 7β-OHC and 7κ-C when compared to equivalent cells from UCB [14]. Previous studies by others comparing the cytotoxic actions of different oxysterols also support the hypothesis that oxysterol cytotoxicity is specific to the type of oxysterol and the cell type investigated [3,9,10,12,19,23,28].

Given the important regulatory role of apoptosis in hematopoiesis, we have utilised the human promyelocytic cell line HL60 as a model of hematopoiesis to further investigate the mechanism of oxysterol-induced cytotoxicity in this system. HL60 cells resistant to the cytotoxic effects of the oxysterols 25-OHC, 7β-OHC and 7κ-C were generated by continuous exposure to these oxysterols. We have utilised real-time and differential display-PCR to investigate the expression of known and novel genes in these cells. The results of our investigations reveal potentially novel mechanisms through which oxysterols exert their effects on cells.

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2. Materials and methods

2.1. Oxysterol treatments

For all experiments, stock solutions of the oxysterols – 25-OHC, 7β-OHC and 7κ-C (Sigma-Aldrich, St. Louis, MO) – were freshly prepared in ethanol as previously described [21]. All treatments and controls contained an equal amount of carrier (ethanol) and were performed in duplicate.

2.2. Cell lines

HL60 control cells (HL60-c) were maintained in a CO_2 incubator at 37 °C in RPMI 1640 (JRH Biosciences, Melbourne, Australia) supplemented with 10% FBS (JRH Biosciences), 200 U/ml penicillin/streptomycin, 2 μg/ml puromycin (Sigma-Aldrich) and 2 mM L-glutamine (Trace Scientific, Noble Park, Australia). HL60 cells resistant to the cytotoxic effects of 25-OHC (HL60-25), 7β-OHC (HL60-7β) and 7κ-C (HL60-7κ) were generated by culturing as described in the presence of either 0.1 μg/ml 25-OHC, 1.0 μg/ml 7κ-C, or 1.0 μg/ml 7β-OHC. When cell number and viability of the oxysterol treated cells was equal to that observed in control cultures, the concentration of oxysterol in the culture media was increased by 0.1 μg/ml for 25-OHC, and 1.0 μg/ml for 7κ-C and 7β-OHC.

2.3. Oxysterol treatment of HL60 control and resistant cells

Three days before each experiment, exponentially growing HL60-c, HL60-25, HL60-7β and HL60-7κ cells were split to a concentration of 0.1×10^6 cells/ml in media (without oxysterols). At time 0, 10^7 cells were removed from the culture, homogenised in TRIZOL® reagent (Invitrogen, San Diego, CA) according to the manufacturers instructions and stored at −80 °C for RNA extraction. The remaining cells from each culture were split to a concentration 0.25×10^6 cells/ml in media, and treated with 25-OHC (0.5 and 2.0 μg/ml), 7β-OHC (4.0 and 8.0 μg/ml) and 7κ-C (5.0 and 10.0 μg/ml) for 48 h. Cell number and viability were assessed at 0 and 48 h using trypan blue exclusion (Sigma-Aldrich).

2.4. The effect of staurosporine on HL60 control and resistant cells

Three days before each experiment, exponentially growing HL60-c, HL60-25, HL60-7β and HL60-7κ cells were split to a concentration of 0.1×10^6 cells/ml in media (without oxysterols). At time 0, each cell line was split to a concentration 0.25×10^6 cells/ml in media, and treated with 0, 0.05 and 0.1 μM staurosporine (Sigma-Aldrich) for 48 h. All treatments and controls contained an equal amount of carrier (DMSO; Sigma-Aldrich) and were performed in duplicate. Cell number and viability were assessed at 0 and 48 h using trypan blue exclusion.

2.5. The effect of nifedipine on oxysterol-induced apoptosis of HL60 control cells

Exponentially growing HL60-c cells were split to a concentration of 0.25×10^6 cells/ml in media and treated with various concentrations of 25-OHC, 7β-OHC and 7κ-C in the presence of 0 or 75 μM nifedipine (Sigma-Aldrich), for 48 h. All treatments and controls contained an equal amount of carrier (ethanol) and were performed in duplicate. Cell number and viability were assessed at 0 and 48 h using trypan blue exclusion.

2.6. Total RNA isolation and DNase treatment

Total RNA was extracted by TRIZOL® reagent (Invitrogen) and contaminating DNA removed by digestion with DNase1 Amplification Grade (Invitrogen) according to the manufacturers instructions. Quantity and purity of the RNA was determined by UV spectrophotometry before reverse transcription and use in PCR reactions.

2.7. Differential display-PCR (dd-PCR)

Reverse transcription, dd-PCR, and cDNA re-amplification was carried out using primers, cycling parameters and PCR conditions as described in the RNAimage Differential Display System (GenHunter, Nashville, USA). Re-amplified cDNAs were visualised by standard agarose gel electrophoresis and purified using Qiaquick Gel Extraction Kit (Qiagen, Clifton Hill, Australia). Sequencing reactions were carried out using ABI Prism BigDye™ Terminator Cycle Sequencing Ready Reaction Kit (P.E. Applied Biosystems, Foster City, USA) and sequences determined by an ABI PRISM™ 373 DNA Sequencer (P.E. Applied Biosystems).

2.8. Reverse transcription and Taqman™ real-time PCR semi-quantitation

Reverse transcription was carried out using Reverse Transcription System (Promega Corp., Madison, USA) following the manufacturers guidelines. All primers and probes for use in real-time PCR (Table 1) were designed using Taqman™ Primer Express Software (P.E. Applied Biosystems) and optimised for use as recommended by the manufacturer. Real-time PCR amplification was carried out using either Taqman™ Universal PCR Mastermix or SYBR™ Green PCR Master Mix on an ABI PRISM™ 7700 Sequence Detection System following the manufacturers guidelines (P.E. Applied Biosystems). Gene Expression was quantitated relative to expression of β-actin or glyceraldehyde 6-phosphate dehydrogenase (GAPDH) using

Table 1
Real-time PCR primer and probe sequences

Gene	Forward primer	Reverse primer	Chemistry
β-Actin	5′-gacaggatgcagaaggagattact-3′	5′-tgatccacatctgctggaaggt-3′	fam-atcattgctcctcctgagcgcaagtactc-tamra
GAPDH	5′-ccacatcgctcagacaccat-3′	5′-ccaggcgcccaatagg-3′	fam-aaggtgaaggtcggagtcaacggatttg-tamra
OSBPL3	5′-acagcgagctcctggacaa-3′	5′-ctgccacatataccatcctttcc-3′	fam-ccgcgcagattcccagccc-tamra
bcl-2	5′-catgtgtgtggagagcgtcaa-3′	5′-gcccgttcaggtactcagtca-3′	SYBR™ Green
c-myc	5′-ctgctctcctcgacggagtc-3′	5′-ccacagaaacaacatcgatttctt-3′	SYBR™ Green
ACAT	5′-gctgacgctgctgtagaacctat-3′	5′-actaaaggcttcatttacttcccacat-3′	SYBR™ Green
HMG-CoA	5′-tccagagcaagcacattagca-3′	5′-aacgtaggacctaaaattgccatt-3′	SYBR™ Green
CNBP	5′-ggccgtggtcgtggaat-3′	5′-ggaaacaaactggaaacctctatcc-3′	SYBR™ Green
B62/RTKN2	5′-gcaatttatgttaaatgccatttgaat-3′	5′-gtcaatgcagtactctgtccatgtagt-3′	SYBR™ Green
B63	5′-tggatggatgaagctaaggaaga-3′	5′-gctagaaccttgtcctcctattgaga-3′	SYBR™ Green
B68	5′-cagctgctgtgcgttagactaag-3′	5′-gctccactgacaagtctagctcaa-3′	SYBR™ Green

Table 2
Primer sequences used for Band 62 (RTKN2) RACE

5′-RACE		3′-RACE	
Reverse primer 1	5′-ctgtaaaggaagggaggaag-3′	Forward primer 1	5′-gcagttccttattggtcagc-3′
Reverse primer 2	5′-ttcttggcatccttatccattgcccgga-3′	Forward primer 2	5′-catcaatcctgttcctggac-3′
Reverse primer 3	5′-tgcccggattctggtttccacaaggaca-3′	Forward primer 3	5′-ggcaatggataaggatgcca-3′
Reverse primer 4	5′-caatagagtttacctcctcg-3′	Forward primer 4	5′-cgaggaggtaaactctattg-3′
Reverse primer 5	5′-ccacattcaccacatcagtat-3′	Forward primer 5	5′-gatactgatgtggtgaatgtg-3′

Sequence Detector Software and the comparative Ct method (P.E. Applied Biosystems User Bulletin No. 2).

2.9. mRNA extraction and rapid amplification of cDNA ends (RACE) of Band 62

mRNA was extracted from HL60-25 cells using Oligotex™ Direct mRNA Kit (Qiagen). 5′- and 3′-RACE was carried out using Smartrace™ cDNA Amplification Kit (Clontech, La Jolla, CA) and primers as listed (Table 2). Products were visualised and sequenced as previously described in this manuscript (Section 2.7).

2.10. PCR amplification of Bands 62, 63 and 68

To confirm sequencing, primers were designed to span the entire coding region of Band 62, and the known sequence of the partial genes Bands 63 and 68 (Table 3). PCR was performed using standard PCR conditions, with Q solution (Qiagen) and Platinum Taq Polymerase (Invitrogen). Cycling conditions are listed in Table 3. Products were visualised and sequenced as previously described in this manuscript (Section 2.7).

2.11. Statistics

All gene expression results are expressed in arbitrary units (mean ± S.E.M.) and calculated from duplicate determinations. Cell culture results were calculated from duplicate determinations and expressed as percent of control (mean ± S.E.M.) to normalise for the variation in cell growth between HL60 control and resistant cells. We utilised one-way Analysis of Variance (Tukeys or Tamhanes post hoc tests) for all statistical analyses.

2.12. Bioinformatics

Nucleic acid and protein sequences were analysed using software available from the National Center for Biotechnology Information and the EXPASy Molecular Biology Server of the Swiss Institute of Bioinformatics. All primers and probes were designed using Taqman™ Primer Express Software (P.E. Applied Biosystems).

Table 3
PCR primers and cycling conditions for amplification of Bands 62 (RTKN2), 63 and 68

Gene	Forward primer	Reverse primer	Cycling
Band 62	5′-tcaaatcttccctttgaagg-3′	5′-tacttgtgcctgcagccatgatct-3′	94 °C, 3 min; 8× (94 °C, 30 s; 60 °C, 30 s; 72 °C, 3 min), 48× (94 °C, 30 s; 60 °C, 30 s; 72 °C, 3 min); 72 °C, 10 min
Band 63	5′-ccagctcagtttagaggag-3′	5′-tgtcagttcatttgggtag-3′	94 °C, 2 min; 40× (94 °C, 30 s; 53 °C, 30 s; 72 °C, 45 s); 72 °C, 2 min
Band 68	5′-aagccgagaccattgaagc-3′	5′-ttttttttccccttctccc-3′	As per Band 63

3. Results

3.1. HL60 cells resistant to the apoptotic effects of 25-OHC, 7κ-C and 7β-OHC can be generated by continuous exposure to oxysterols

The establishment of HL60 cells that were resistant to the cytotoxic actions of high doses of 25-OHC, 7κ-C and 7β-OHC took approximately 6 months. Typically, 70–90% of the cells died within 7 days after exposure to increased concentrations of each of the oxysterols. When the surviving cells resumed proliferation and achieved cell numbers comparable to HL60 control cells, the dose of each oxysterol was increased by 0.1 μg/ml for 25-OHC, and 1.0 μg/ml for 7κ-C and 7β-OHC. This selection process was continued and eventually generated HL60 cells which survived and grew in the presence of 1.0 μg/ml 25-OHC, 6.0 μg/ml 7κ-C and 4.0 μg/ml 7β-OHC. Over this time period, we were unable to obtain HL60 cells that could be maintained at higher doses of each of these oxysterols.

3.2. Investigation of oxysterol resistance in HL60 control and resistant cells

We investigated the resistance of HL60-resistant and -sensitive cells to the oxysterols 25-OHC, 7κ-C and 7β-OHC. Seventy-two hours before the experiments were conducted, all HL60 cells were removed from exposure to oxysterols. After this "oxysterol-free" culture period, HL60-resistant cells generated by continuous exposure to a specific oxysterol, still displayed resistance to this oxysterol. In most cases this resistance was only significant when the highest dose of oxysterol tested—a dose which exceeded that at which they were maintained.

Compared to control HL60 cells, only HL60-25 cells generated by continuous exposure to 25-OHC, displayed significantly higher viability (results not shown) and cell number after 48-h exposure to 2.0 μg/ml 25-OHC (Fig. 1A). These cells were not resistant to the cytotoxic actions of 7κ-C, and although they appeared to display some resistance to the cytotoxic actions of 7β-OHC, this was not significant (Fig. 1B and C).

HL60-7κ cells generated by continuous exposure to 7κ-C displayed significantly higher viability (results not shown) and cell number compared to controls, after 48-h exposure to 10 μg/ml 7κ-C (Fig. 1B). Although these cells appeared to display some resistance to the cytotoxic actions of 25-OHC and 7β-OHC, it was not significant (Fig. 1A and C).

Compared to HL60 control cells, only HL60-7β cells generated by continuous exposure to 7β-OHC, displayed significantly higher viability (results not shown) and cell number after 48-h exposure to 8 μg/ml 7β-OHC. These cells also appeared to display some resistance to 2 μg/ml 25-OHC, and interestingly, were significantly resistance to 5 and 10 μg/ml 7κ-C (Fig. 1B). Indeed resistance of HL60-7β cells to 5 μg/ml 7κ-C exceeded that demonstrated by the HL60-7κ cells.

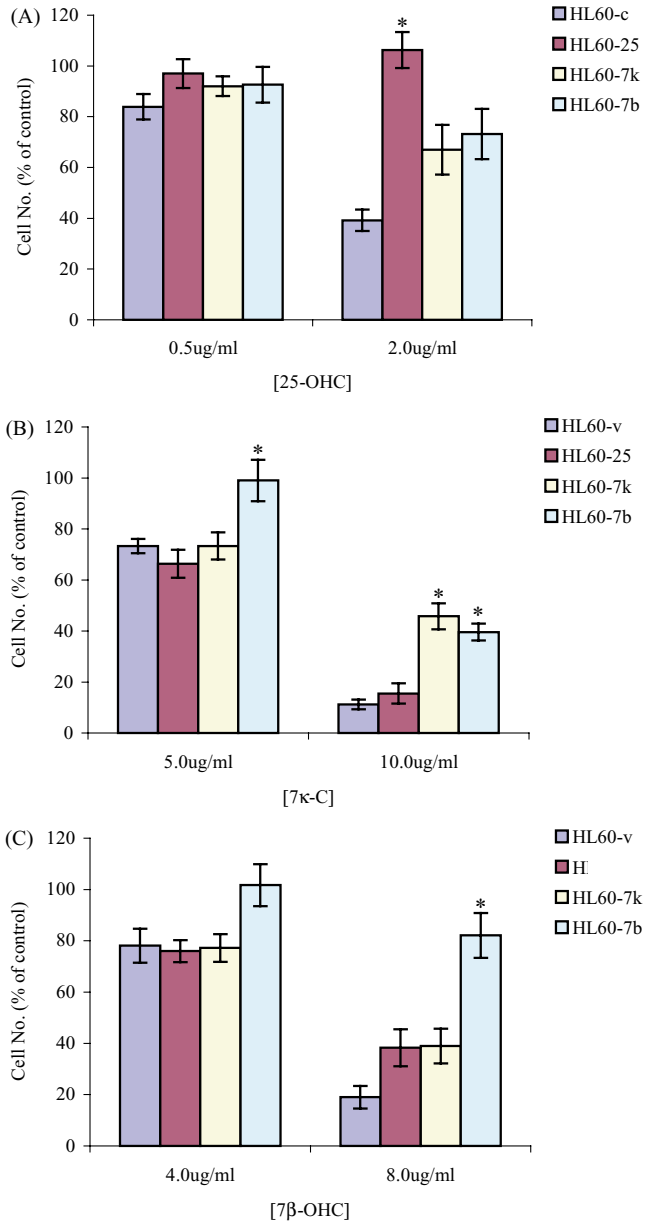

Fig. 1. Investigation of oxysterol resistance in HL60 control and resistant cells. All results are expressed as percent of control (0 μg/ml oxysterol) to normalise for the variation in cell growth between HL60 control and resistant cells ($n = 6$, $T = 48$ h). (A) HL60-25 cells were less sensitive to the cytotoxic action of 2.0 μg/ml 25-OHC than control HL60 cells ($P \leq 0.001$). (B) HL60-7κ cells were less sensitive to the cytotoxic action of 5.0 and 10 μg/ml 7κ-C ($P \leq 0.023$ and 0.001, respectively) than control cells. (C) HL60-7β cells were less sensitive to the cytotoxic action of 8.0 μg/ml 7β-OHC ($P \leq 0.001$) than control cells.

3.3. Oxysterol-resistant cells are not resistant to staurosporine-induced apoptosis

We observed that cells resistant to the cytotoxic effects of oxysterols were equally sensitive to the cytotoxic action of staurosporine as control HL60 cells (Fig. 2). Staurosporine is a reagent that induces apoptosis through the inhibition of protein kinase k [32]. Thus, these results confirm that the

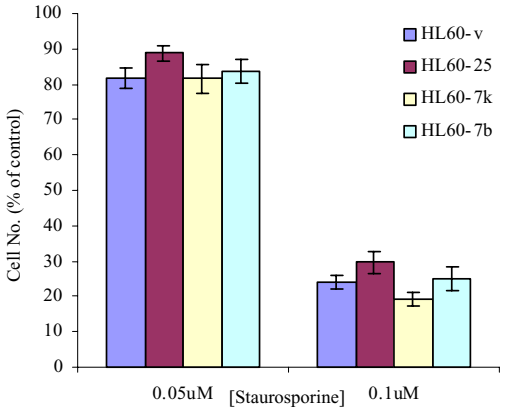

Fig. 2. Oxysterol-resistant cells are not resistant to staurosporine-induced apoptosis. All results are expressed as percent of control (0 μg/ml staurosporine) to normalise for the variation in cell growth between HL60 control and resistant cells ($n = 6$, $T = 48$ h).

oxysterol-resistant cells we have generated have a functioning apoptosis pathway, at least in the execution phase of the death program.

3.4. Nifedipine inhibits 25-OHC cytotoxicity, but not 7κ-C and 7β-OHC in HL60 control cells

High intracellular calcium levels have been reported to induce apoptosis in several experimental models [2,5,18,20]. More specifically, 25-OHC has been shown to increase the cellular uptake of calcium in a variety of cell types [2,6]. In accordance with this, a study by Rusinol et al. [30] demonstrated that the calcium channel blocker nifedipine blocked the 25-OHC-induced cellular uptake of calcium and prevented apoptosis in CHO-K1 cells. The results from our study confirm the finding of Rusinol et al. [30]. HL60-c cells incubated in the presence of 2 μg/ml 25-OHC and 75 μM nifedipine display higher viability (results not shown) and cell number than the same cells incubated with 25-OHC alone (Fig. 3A). Although this difference was not significant at the $P \leq 0.05$ level, it is in contrast to the effect of nifedipine on 7β-OHC and 7κ-C apoptosis. HL60-c cells incubated in the presence of 10 μg/ml 7κ-C and 8 μg/ml 7β-OHC with 75 μM nifedipine, displayed significantly lower viability (results not shown) and cell number than the same cells incubated with these oxysterols alone (Fig. 3B and C). Although we cannot explain why 7κ-C and 7β-OHC cytotoxicty is increased in the presence of nifedipine, these results indicate that the calcium channel blocker nifedipine partially abrogates 25-OHC cytotoxicity only.

3.5. Investigation of the expression of known genes implicated in oxysterol-induced apoptosis

We investigated the expression of several genes previously implicated in oxysterol resistance: 3-hydroxy-3-methylglutaryl coenzyme A reductase (HMG-CoA), acyl-coenzyme

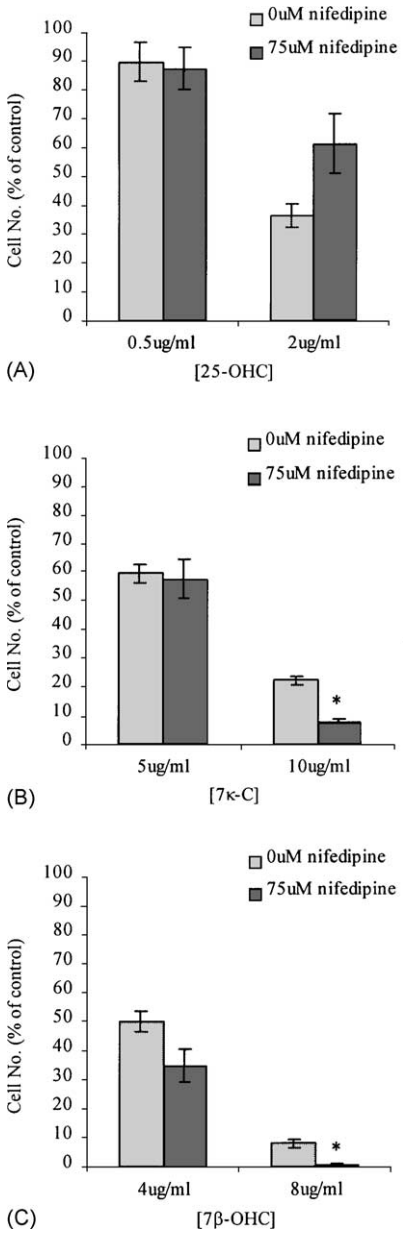

Fig. 3. Nifedipine inhibits 25-OHC cytotoxicity, but not 7κ-C and 7β-OHC in HL60 control cells. All results are expressed as percent of control (0 μg/ml oxysterol) to normalise for the variation in cell growth due to nifedipine treatment ($n = 6$, $T = 48$ h). (A) HL60-c cells incubated in the presence of 2 μg/ml 25-OHC and 75 μM nifedipine display higher viability (results not shown) and cell number than the same cells incubated with 25-OHC alone (61.4 ± 10.2 versus 36.7 ± 4.1, $P \leq 0.098$). (B) HL60-c cells incubated in the presence of 10 μg/ml 7κ-C and 75 μM nifedipine displayed significantly lower viability (results not shown) and cell number than the same cells incubated with these oxysterols alone (8.0 ± 1.0 versus 17.5 ± 1.5, $P \leq 0.001$). (C) HL60-c cells incubated in the presence of 8 μg/ml 7β-OHC and 75 μM nifedipine displayed significantly lower viability (results not shown) and cell number than the same cells incubated with these oxysterols alone (0.9 ± 0.6 versus 8.2 ± 1.5, $P \leq 0.006$).

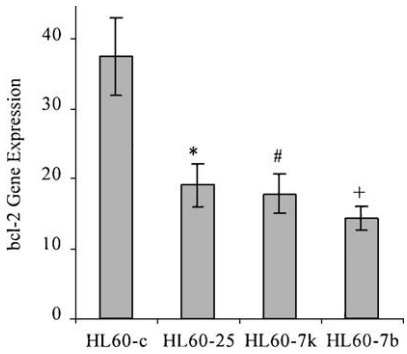

Fig. 4. Expression of bcl-2 in oxysterol-resistant and -sensitive HL60 cells. Gene expression is calculated relative to GAPDH and expressed in arbitrary units (mean ± S.E.M.). bcl-2 gene expression in HL60-25 (19.2 ± 3.0, $^{*}P \leq 0.01$), HL60-7κ (17.9 ± 2.8, $^{\#}P \leq 0.006$) and HL60-7β cells (14.4 ± 1.7, $^{+}P \leq 0.002$) is significantly lower than expression in HL60 control cells (37.6 ± 5.6, $n = 5$).

A: cholesterol acyl transferase (ACAT), cellular nucleic acid binding protein (CNBP), oxysterol binding like-protein 3 (ORP3 or OSBPL3), bcl-2 and c-myc. To conduct these studies, RNA was isolated from HL60-c, HL60-25, HL60-7κ and HL60-7β cells after 72 h in "oxysterol free" culture conditions. In this way, the basal level of gene expression could be investigated rather than the expression of genes due to exposure to oxysterols, and associated effects. This is critical when examining gene expression in HL60-c cells which, upon exposure to oxysterols, undergo apoptosis.

The results from our study demonstrate no significant difference in the expression of HMG-CoA, ACAT, CNBP, OSBPL3 and c-myc between HL60 control and resistant cells (data not shown). A significant decrease was, however, observed in the expression of bcl-2 in HL60-25 (19.2 ± 3.0, $P \leq 0.01$), HL60-7κ (17.9 ± 2.8, $P \leq 0.006$) and HL60-7β cells (14.4 ± 1.7, $P \leq 0.002$) when compared to HL60 control cells (37.6 ± 5.6, $n = 5$; Fig. 4).

3.6. dd-PCR of oxysterol-resistant and sensitive HL60 cells

To investigate possible novel mechanisms of cellular resistance to oxysterol-induced apoptosis, we utilised dd-PCR to investigate and compare the gene expression of oxysterol-sensitive and -resistant HL60 cells. Three differentially expressed transcripts were identified and sequenced. These have been designated Bands 62, 63 and 68.

3.6.1. Expression of Band 62 in oxysterol-resistant and -sensitive HL60 cells

Band 62 was identified by dd-PCR as being up-regulated in HL60 cells resistant to 25-OHC. BLAST analysis of the 100 nucleotide (nt) sequence revealed 100% homology to a 456nt EST in the GenBank database (accession no. AW009815). We have since obtained the complete coding region of this gene by a combination of EST walking and RACE technology. The sequence of the coding region

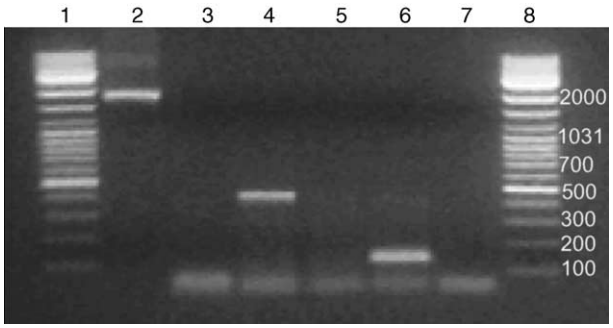

Fig. 5. PCR amplification of Bands 62 (RTKN2), 63 and 68 for sequencing. Lanes: (1) molecular size ladder, (2) B62 amplified from HL60-25 cDNA, (3) B62 no template control (NTC), (4) B63 amplified from HL60-c cDNA, (5) B63 NTC, (6) B68 amplified from HL60-c cDNA, (7) B68 NTC, (8) molecular size ladder.

has been confirmed by PCR amplification and sequencing (Fig. 5). Translation of the open reading frame (ORF) of this partial nucleotide sequence results in an amino acid sequence with 60% homology to human Rhotekin (accession no. AF049227; Fig. 6). As with Rhotekin, the predicted protein encoded by our gene contains Rho binding and Pleckstrin Homology domains [13,29]. We have thus designated this novel gene sequence Rhotekin-2 (RTKN2; accession no. AY150309).

To confirm differential expression of RTKN2 in our oxysterol-resistant HL60 cells, Taqman™ real-time PCR technology was employed to quantitate gene expression relative to β-actin (Fig. 7A). Results confirmed higher expression of RTKN2 in HL60-25 cells compared to HL60 control cells, HL60-7κ cells and HL60-7β cells (Fig. 5A).

3.6.2. Expression of Band 63 in oxysterol-resistant and -sensitive HL60 cells

Band 63 (B63) is a 445nt sequence (accession no. BU582398) identified by dd-PCR as being down-regulated in HL60 cells resistant to the cytotoxic effects of 25-OHC, 7κ-C and 7β-OHC. This partial cDNA sequence has been confirmed by PCR amplification and sequencing (Fig. 5), but we have, to date, been unable to identify obtain additional coding sequence by bioinformatics. This sequence maps to chromosome 8 (accession no. AC011626.8) and has no ORF. We are currently utilising RACE technology to obtain additional coding sequence.

To confirm differential expression of B63, Taqman™ real-time PCR technology was employed to quantitate gene expression relative to GAPDH. Results confirm lower expression of B63 in HL60 cells resistant to the cytotoxic effects of 25-OHC, 7κ-C and 7β-OHC compared to HL60 control cells (Fig. 7B).

3.6.3. Expression of Band 68 in oxysterol-resistant and -sensitive HL60 cells

Band 68 (B68) is a 142nt sequence (accession no. BU582399) identified by dd-PCR as being differentially

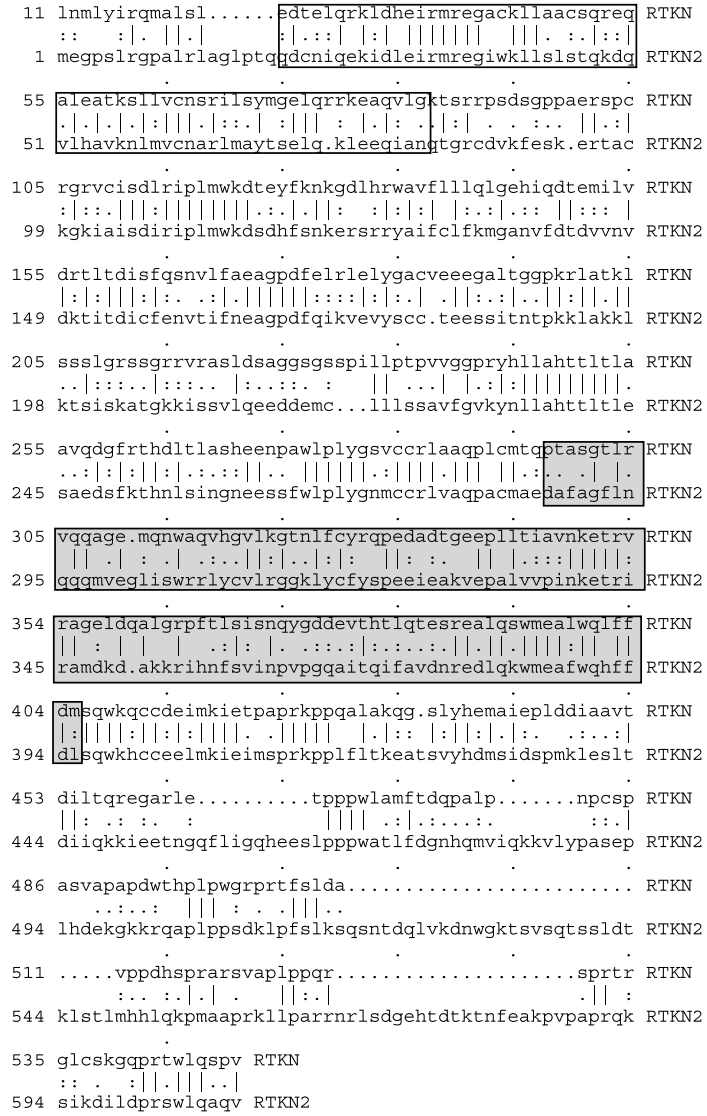

Fig. 6. Alignment of human Rhotekin-2 (RTKN2; accession no. AAN71738) and Rhotekin (RTKN; accession no. NP_149035). Amino acid identity for identical (|), conservative (:) and moderately conserved substitutions (.) are as indicated. Rho-binding and Pleckstrin homology domains are highlighted by open and shaded boxes respectively.

expressed between HL60 cells resistant to the cytotoxic effects of 25-OHC, 7κ-C and 7β-OHC, and HL60 control cells. This partial cDNA sequence has been confirmed by PCR amplification and sequencing (Fig. 5), but, as with B63, we have, to date, been unable to identify obtain additional coding sequence by bioinformatics. This sequence maps to chromosome 15 (accession no. AC090257.5) and has no ORF. We are currently utilising RACE technology to obtain additional coding sequence.

To confirm differential expression of B68, Taqman™ real-time PCR technology was employed to quantitate gene expression relative to β-actin. Results confirm lower expression of B68 in HL60 cells resistant to the cytotoxic effects of 25-OHC and 7κ-C, compared to HL60 control cells (Fig. 7C).

4. Discussion

We have previously observed that that the oxysterols 25-hydroxycholesterol (25-OHC), 7-beta-hydroxycholesterol (7β-OHC) and 7-keto-cholesterol (7κ-C) are potent inducers of apoptosis and differentiation of primary HPCs and of the human promyelocytic cell line HL60 [14]. In this report, we have used continuous exposure to select HL60 cells which, in the presence of 1.0 μg/ml 25-OHC, 6.0 μg/ml 7κ-C and 4.0 μg/ml 7β-OHC, demonstrate comparable growth and viability as HL60 control cells. These resistant HL60 cells, were still sensitive to apoptotic agent staurosporine, yet displayed significantly higher viability than HL60 control cells after 48-h culture with oxysterols.

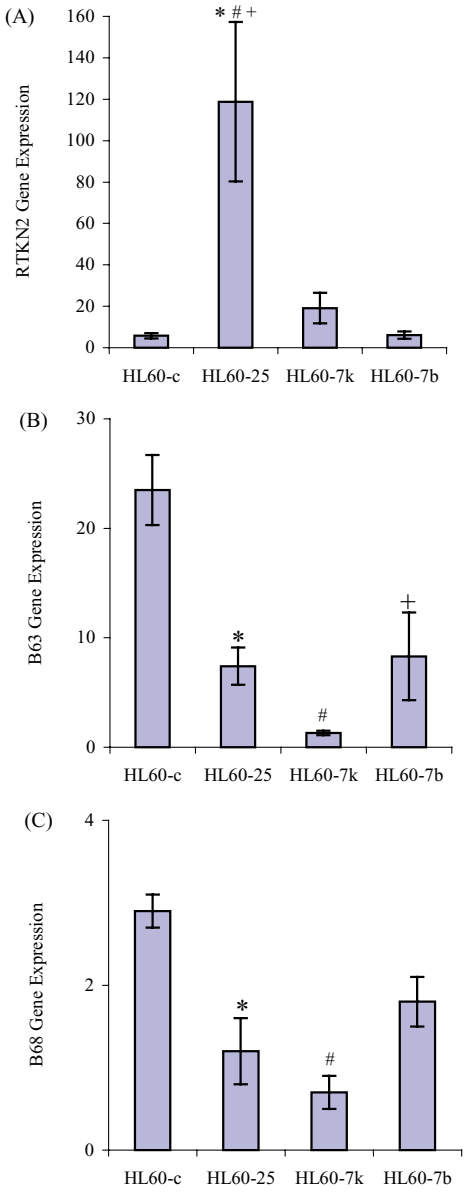

Fig. 7. Expression of RTKN2, B63 and B68 in oxysterol-resistant and -sensitive HL60 cells. Gene expression is calculated relative to the housekeeping genes β-actin or GAPDH, and expressed in arbitrary units (mean ± S.E.M., $n = 5$). (A) RTKN2 expression in HL60-25 cells (118.8 ± 38.5) is significantly higher than that observed in HL60 control cells (5.8 ± 1.3, $^{*}P \leq 0.004$), HL60-7κ cells ($19.1 + 7.4$, $^{\#}P \leq 0.012$) and HL60-7β cells ($6.1 + 1.8$, $^{+}P \leq 0.005$). (B) Expression of B63 in HL60 cells resistant to the cytotoxic effects of 25-OHC (7.4 ± 1.7, $^{*}P \leq 0.004$), 7κ-C (1.3 ± 0.21, $^{\#}P \leq 0.001$) and 7β-OHC (8.3 ± 4.0, $^{+}P \leq 0.006$) is significantly lower than that observed in HL60 control cells (23.5 ± 3.2). (C) Expression of B68 in HL60-25 (1.2 ± 0.4, $^{*}P \leq 0.005$) and HL60-7κ (0.70 ± 0.2, $^{\#}P \leq 0.001$) cells is significantly lower than that observed in HL60 control cells (2.9 ± 0.2).

Other studies investigating oxysterol resistance have utilised continuous exposure [24] or mutagenesis [4,25,30] to select cells resistant to the cytotoxic actions of oxysterols. In these previous studies, resistance to only one type of oxysterol was investigated. The results of our experiments reveal that in HL60 cells, resistance to the cytotoxic actions of oxysterols is relatively specific to the type of oxysterol. Cells resistant to the cytotoxic effects of 25-OHC displayed little or no resistance to the cytotoxic actions of 7κ-C or 7β-OHC. Similarly, cells resistant to the cytotoxic effects of 7κ-C displayed little or no resistance to the cytotoxic actions of 25-OHC or 7β-OHC. In contrast, cells resistant to the cytotoxic effects of 7β-OHC also displayed significant resistance to the cytotoxic actions of 7κ-C, but displayed little or no resistance to the cytotoxic actions of 25-OHC. These results suggest the possibility of a similar mechanism of apoptosis induced by both 7β-OHC and 7κ-C, with 25-OHC utilising a separate, yet overlapping path. This hypothesis is supported by our observation that the calcium channel blocker nifedipine inhibited the cytotoxicity induced by 25-OHC, but not that induced by 7κ-C and 7β-OHC. This finding also supports and extends the hypothesis of Rusinol et al. [30], and suggests that the cellular uptake of calcium is an important step in the 25-OHC apoptotic signalling process, but is not critical for the cytotoxicity of 7β-OHC and 7κ-C.

We investigated the expression of several genes previously implicated in oxysterol resistance. Our results demonstrate no significant difference in the expression of the genes for ACAT and CNBP in HL60-sensitive and -resistant cells. This is in contrast to the findings of Metherall et al. [25] and Ayala-Torres et al. [4] who reported differential expression of these genes in oxysterol-sensitive and -resistant CHO and CEM cells, respectively. Similarly, we observed no significant difference in the expression of the gene for OSBPL3 which was found to be differentially expressed amongst resistant and sensitive primary HPCs from UCB and ABM (respectively) [14,15].

Past investigations demonstrate that oxysterol-induced apoptosis is associated with down-regulation of the genes for HMG-CoA [5] and c-myc [5,33] in human leukemic CEM cells. We observed no significant difference in the expression of these genes in oxysterol-sensitive and -resistant HL60 cells. Interestingly however, we observed that expression of the anti-apoptotic gene bcl-2 in oxysterol-resistant cells was significantly lower than in control cells. Given previous reports which demonstrate clear down-regulation of the bcl-2 gene with oxysterol-induced apoptosis in vascular smooth muscle cells [27], and partial abrogation of cytotoxicity with over expression of bcl-2 in human promonocytic leukemia cells U937 [22], murine macrophage-like P388-D1 cells [17] and HL60 cells [14], this finding was unexpected. It is possible that the mechanism by which these cells have become resistant to oxysterols has down-regulated the expression of bcl-2. It will be interesting, therefore, to investigate the gene expression of other anti-apoptotic members of the bcl-2 family in our oxysterol-resistant cells.

These contrasting gene expression findings suggest that the effects of different oxysterols observed in various cell types may be due to the induction of differing mechanisms of oxysterol cytotoxicity. It must be noted however, that as the RNA used for all of our expression data was taken from

cells that were not at the time exposed to oxysterols, these results do not rule out the possibility that the regulation of these genes with oxysterol treatment may be different in oxysterol-resistant and -sensitive HL60 cells.

To investigate possible novel mechanisms of cellular resistance to oxysterols-induced apoptosis, we utilised dd-PCR to investigate and compare the gene expression of oxysterol-sensitive and -resistant HL60 cells. Expression of the novel Rhotekin-like gene, RTKN2, was significantly higher in cells resistant to the cytotoxic actions of 25-OHC when compared to HL60 control cells, and cells resistant to 7β-OHC and 7κ-C. This further supports our hypothesis that the cytotoxic actions of 25-OHC are mediated via a separate pathway or mechanism of apoptosis than that induced by both 7β-OHC and 7k-C. As RTKN2 is a newly identified gene, it is difficult to speculate on its possible role in oxysterol resistance. Its closest murine homologue Rhotekin, has been demonstrated to associate with the small GTPase Rho [29]. The Ras superfamily of small GTPases encompasses a group of ubiquitous regulatory proteins involved in a plethora of intra-cellular signalling processes [7] including cell cycle progression, [34] differentiation, [31] and apoptosis [11]. As homologous proteins often share homologous functions, it is probable that RTKN2 also binds and acts as an effector of Rho-GTPase signalling [29]. Investigations to confirm RTKN2 interaction with various members of the Rho family, and also to explore its role in oxysterol resistance are in progress.

In contrast to RTKN2, expression of the novel partial gene sequences B63 and B68 demonstrate lower expression in HL60 cells resistant to the cytotoxic effects of 25-OHC, 7κ-C and 7β-OHC compared to HL60 control cells. Given our finding of decreased expression of bcl-2 in all of the oxysterol-resistant HL60 cells investigated, this finding is intriguing and it will be interesting to determine whether the proteins encoded by these genes have a pro-or anti-apoptotic effect on these cells.

Taken together, the results of this study demonstrate that resistance to the cytotoxic effects of oxysterols in HL60 cells is partially specific to the type of oxysterol, and suggest that the cytotoxicity of 25-OHC, but not that of 7β-OHC, 7κ-C, occurs by a calcium dependent mechanism. This specificity is supported by the finding of a novel gene, RTKN2, which is up-regulated only in cells resistant to 25-OHC. However, there are clearly also overlapping or common mechanisms of resistance, with down-regulation of bcl-2 and of two novel gene sequences in all resistant cell lines. Further investigation of these resistant cell lines, and of the differentially expressed genes, may give additional insights into the mechanisms of the important phenomenon of oxysterol cytotoxicity.

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