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Over-expression of clusterin is a resistance factor to the anti-cancer effect of histone deacetylase inhibitors

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ABSTRACT

Histone deacetylase inhibitors (HDACIs) modulate gene transcription and are among the most promising new classes of anticancer drugs. OGX-011, an anti-sense oligonucleotide targeting clusterin, sensitises cancer cells to chemo- and radiotherapies. By reviewing microarray gene profiling data reported in the literature, we identified clusterin as one of only two genes commonly up-regulated by most HDACIs in cancer cell lines of different organ origins. Suppression of clusterin gene expression synergistically enhanced high-dosage HDACI-induced cell death through cytochrome C-mediated mitochondrial apoptosis in HDACI-resistant cancer cells, and synergistically enhanced low-dosage HDACI-induced growth arrest in both HDACI-sensitive and HDACI-resistant tumour cells, but not in normal cells. In mice xenografted with neuroblastoma cells, combination of OGX-011 and the HDACI, valproate, synergistically repressed tumour growth. Our data indicate that HDACI-induced clusterin over-expression renders cancer cells resistant to HDACI-induced growth arrest and apoptosis, and suggests the addition of OGX-011 to HDACIs in future clinical trials in cancer patients.

1. Introduction

Cancer is the second commonest cause of death in children. Neuroblastoma is the most common solid tumour in early childhood with a cure rate of only 30%. Breast cancer is one of the most common causes of death due to cancer in women.

Histone deacetylase inhibitors (HDACIs) are among the most promising new classes of anticancer compounds.[1–3] HDACIs modulate gene transcription, induce cancer cell differentiation, growth arrest, programmed cell death and inhibit tumour-driven angiogenesis, invasion and metastasis with little toxicity against normal non-malignant cells.[1–3] Among the HDACIs, valproate (VPA) has been in the clinic for the treatment of epilepsy in young children and infants for several decades, making it an ideal candidate for the treatment of malignancies in early childhood. Clinical trials using a variety of HDACIs in cancer patients demonstrate that HDACI treatment leads to tumour regression and symptomatic improvement in some heavily pre-treated and multiple relapsed patients.[1–3] However, a proportion of patients are insensitive to HDACI therapy. A better understanding of the factors determining sensitivity and resistance to HDACIs will better guide the choice of future combination regimens in cancer patients.

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The cytoprotective chaperone protein, clusterin (CLU), is synthesised as full-length CLU (60 kDa) in the mitochondria and is targeted to the endoplasmic reticulum, where it is glycosylated, proteolytically cleaved into an α and β chain, and secreted into the extracellular matrix as the secreted form of CLU (40 kDa). CLU protein is commonly up-regulated by cytotoxic chemotherapy and radiotherapy in cancer cells, and contributes to cancer cell resistance *in vitro* and in various animal models of cancer by blocking apoptosis [reviewed in[4]]. Recent clinical trials of OGX-011, an antisense oligonucleotide specifically targeting CLU, have shown promise when combined with chemotherapy in cancer patients.[5]

Because the capability of HDACIs to induce cell death and growth arrest correlates with histone acetylation and modulation of gene transcription,[6] we have reviewed published raw data on HDACI-modulated transcriptional changes with cDNA microarray as the methodology. Surprisingly, well-documented target genes of HDACIs are not commonly shared by different HDACIs across multiple cancer cell types. In contrast, CLU is one of only two genes commonly up-regulated by various HDACIs in cancer cells of a variety of organ origins. We have, therefore, carried out experiments to examine the role of CLU in the anti-cancer efficacy of HDACIs both *in vitro* and *in vivo*.

2. Materials and methods

2.1. Cell culture and chemicals

Human neuroblastoma BE(2)-C, LAN-5, NBL-S cells, mammary cancer MCF-7, MDA-MB-468 and MDA-MB-231 cells and non-malignant mammary epithelial MCF-10A1 cells were all obtained from the American Type Culture Collection. All cells were cultured in Dulbecco's modified Eagle's medium with 10% foetal calf serum, and MCF10A1 cell culture medium was further supplemented with epidermal growth factor, choleraoxin, hydrocortisone and insulin. The hydroxamate HDACI trichostatin A (TSA) and the short chain fatty acid HDACI sodium valproate (VPA) were purchased from Sigma (Sigma, St. Louis).

2.2. Small interfering RNA (siRNA) transient transfection

The target sequence of the first CLU siRNA was 5′-GCAGCAGAGTCTTCATCAT-3′,[7] and the target sequence of the second CLU siRNA 5′-GCGTGCAAAGACTCCAGAA-3′.[8] The first CLU siRNA target the same RNA sequence as the antisense oligonucleotide OGX-011, except that the target of the first CLU siRNA is two bp shorter than the target of OGX-011. The two CLU siRNAs and two control scrambled siRNAs were custom synthesised by Dharmacon (Dharmacon Research) and Ambion (Ambion, Applied Biosystems), and were transfected at a final concentration of 30 μM into cells with Lipofectamine 2000 (Invitrogen) according to the manufacturer's instructions.

2.3. Antisense oligonucleotide

OGX-011 is a second generation 21-mer oligonucleotide with a 2′-O-(2-methoxy)-ethyl modification, generously provided by OncoGenex Technologies (OncoGenex, Vancouver, Canada). The sequence of OGX-011 targets the first initiation site of exon II of the human clusterin gene (5′-CAGCAGCAGAGTCTTCATCAT-3′). A 2′-O-(2-methoxy)-ethyl gapmer mismatch (MM) control oligonucleotide (5′-CAGCGCUGACAACAGUUUCAU-3′) was generously provided by ISIS Pharmaceuticals (ISIS, Carlsbad, CA).

2.4. Semi-quantitative competitive RT-PCR analysis of gene expression

The competitive RT-PCR techniques have been previously described.[9] Relative gene expression is determined by a ratio between the level of expression of a target gene and that of the house-keeping gene β-actin in total RNA samples. Specific primers used for PCR are as the following: 5′-AAGTTCAGAGGCGTGCAAAG-3′, and 5′-CCCTGATTGGACATTTCCTG-3′ for CLU; and 5′-CACCATGTACCCTGGCATT-3′ and 5′-ACGGAGTACTTGCGCTCAG-3′ for β-actin. The forward primer for CLU targets the exon 2 of CLU gene, which is the same exon targeted by OGX-011 and CLU siRNA No. 1, and the reverse primer targets the exon 3 of CLU gene.

2.5. Immunoblot analysis

Cells were lysed and protein was extracted with RIPA buffer.[9] For the analysis of the mitochondrial apoptosis pathway, cells were lysed and protein was fractionated with a Mitochondrial Extraction Kit (Pierce) to generate mitochondrial and cytoplasmic fractions. After gel electrophoresis and western transfer, membranes were probed with a goat anti-CLU antibody (1:200) (Santa Cruz Biotech) or a mouse anti-cytochrome C antibody (1:1000) (BD Pharmingen), followed by a horseradish peroxidase-conjugated donkey anti-goat antibody or anti-mouse antiserum (1:30,000) (Pierce). Protein bands were visualised with SuperSignal (Pierce). The membranes were lastly re-probed with anti-β-actin (1:2000) or anti-COX-IV antibody (1:1000) (Sigma) as loading controls for cytoplasmic and mitochondrial fractions, respectively.

2.6. Alamar blue assay

After pilot studies defining the linearity range, cells were plated into 96 well plates at a density of 3000 cells/well, transfected with scrambled siRNA or CLU siRNA, and treated with solvent control, TSA or VPA for 72 h. Before the end of the treatment, cells were incubated with Alamar blue (Invitrogen) for 5 h, and plates were then read on a micro-plate reader at 570/595 nm. Results were calculated according to the readings (optical density absorbance units) and expressed as percentage change in cell number.

2.7. Terminal deoxynucleotidyl transferase dUTP nick end labelling (TUNEL) assay

Cells were transfected with scrambled or CLU siRNA, followed by treatment with solvent control, 0.1 or 0.3 μM TSA for 48 h. After fixation, permeabilisation and blocking, cells were labelled with TUNEL regents as described by the manufacturer (Roche, Switzerland). Finally, the cellular nucleus was

counter-stained with DAPI, and staining was visualised with a fluorescence microscope and quantified.

2.8. Animal model studies

Six-week-old athymic nude mice (BALB/c strain) were randomised to four groups of eight. BE(2)-C cells (1.2×10^8/mouse) were injected into the flank of the mice. When tumour volume reached 0.125 cm^3, the mice were injected intra-peritoneally once a day with the mismatch anti-sense oligonucleotide MM (12.5 mg/kg/day), OGX-011 (12.5 mg/kg/day), MM plus VPA (400 mg/kg/day), or OGX-011 plus VPA for 19 days consecutively. Tumour volume was monitored every other day. Mice were sacrificed at the end of treatment. Tumours were removed, measured, formalin-fixed and paraffin-embedded. All studies involving animals were approved by the Animal Care and Ethics Committee of the University of New South Wales, Sydney, Australia.

2.9. Immunohistochemistry studies

Mouse anti-CLU antibody (Upstate Biotech, NY) was biotinylated with an Animal Research Kit (DakoCytomation, Denmark), according to the manufacturer's instructions. Mouse tissue sections were incubated consecutively with the biotinylated mouse anti-CLU antibody (1:500) and streptavidin-horseradish peroxidase (1:500), and visualised with diaminobenzidine (DakoCytomation). The cell nucleus was finally counterstained with haematoxylin.

2.10. Statistical analysis

All data for statistical analysis were calculated as mean ± standard error. Differences were analysed for significance using ANOVA among groups or unpaired *t*-test for two groups. A probability value of 0.05 or less was considered significant. The combination effect of CLU siRNA and HDACIs, or the combination effect of OGX and HDACIs, was analysed by the fractional product method as we and others have described.[10–12] According to this method, the effects of two agents, when combined, can be calculated by multiplying the fractional inhibition of cancer cell growth and survival *in vitro* and inhibition of tumour growth *in vivo*, by each single agent. If the ratio of the observed fraction over the calculated fraction (R value) is less than 1.0, the combination is synergistic.

3. Results

3.1. CLU is one of only two genes commonly up-regulated by different HDACIs in cancer cells arising in different organs

In a search for transcriptionally regulated target genes critical for cancer cell sensitivity or resistance to HDACI-induced anti-cancer effects, we reviewed all published gene profiling raw data with microarray as the methodology (Table 1). Surprisingly, well-documented target genes of HDACIs such as cyclins, Bax, Bim, Bcl2, Bclx-l, thioredoxin-binding protein 2, caspases and APAF1 were not commonly modulated by most HDACIs in different cancer cell types. The genes most commonly modulated were the cyclin-dependent kinase inhibitor $p21^{WAF1}$, which has already been extensively studied,[13–16] and, CLU. With three microarray platforms including Affymetrix, cDNA and custom oligonucleotides, CLU was identified as commonly up-regulated by a variety of HDACIs including TSA, suberoylanilide hydroxamic acid (SAHA), VPA, butyrate, MS275, FK228 (depsipeptide) and BL1521 in cancer cells arising in breast, prostate, bladder, lung, liver, kidney, blood and nervous system (Table 1).[17–26] However, the role of CLU in the anti-cancer efficacy of HDACIs was not analysed.

To validate the published microarray data, we performed semi-quantitative RT-PCR analysis of CLU gene expression in a range of neuroblastoma and breast cancer cell lines after

Table 1 – Clusterin is commonly up-regulated by HDACIs in cancer cells of different organ origins.

HDACI	Duration	Cancer cell line	Microarray platform	Fold induction	Reference
5 μM SAHA	24 h	MDA-MB-468 (breast)	Affymetrix	Average 12.1 ± 2.2	[17]
0.3 μM TSA		MDA-MB-456 (breast)			
5 μM MS-275		T24 (bladder)			
1 mM VPA	5 days	HEK 293 (embryonic kidney)	Affymetrix	12.5	[23]
2 mM VPA	24 h	H460 (lung)	cDNA	4.0 ± 1.6	[18]
2.5 μM SAHA	24 h	Ishikawa (endometrial)	Affymetrix	50	[22]
5 ng/ml FK228	24 h	PC3 (prostate)	Affymetrix	Up-regulated	[21]
		U937 (lymphoma)			
		ACHN (kidney)			
1 μM SAHA	24 h	DU-145 (prostate)	cDNA	2.04	[25]
50 μM BL1521	16 h	SKNAS (neuroblastoma)	Affymetrix	4.65	[24]
		IMR-32 (neuroblastoma)		2.38	
50 nM depsipeptide	12 h	A549 (lung)	Oligo-nucleotide	1.82	[26]
		PC3 (prostate)		4.26	
		TK-10 (kidney)		1.55	
0.67 μM TSA	24 h	HuH7 (liver)	cDNA	2.25	[19]
		HepG2 (liver)			
		HuH6 (liver)		1.74	
				7.53	

treatment with TSA, a hydroxamate HDACI, or VPA, a short chain fatty acid HDACI. We chose neuroblastoma BE(2)-C (stem cell-like intermediate type), NBL-S (substrate adherent type) and LAN-5 (neuronal type), and breast cancer MCF-7 (oestrogen receptor positive), MDA-MB-468 and MDA-MB-231 (oestrogen receptor negative) cells. As shown in Fig. 1A, treatment with TSA or VPA for 24 h up-regulated CLU gene expression in BE(2)-C, NBL-S and LAN-5 neuroblastoma cells, MCF-7, MDA-MB-468 and MDA-MB-231 breast cancer cells, as well as non-malignant breast epithelial MCF-10A1 cells. These data suggested that CLU gene transcription was up-regulated by HDACIs in cancer cells from various tissue origins and normal cells.

3.2. *Down-regulation of CLU rendered cancer cells sensitive to HDACI-induced apoptosis in HDACI-resistant cancer cells*

To assess the role of CLU gene up-regulation in HDACI-induced programmed cell death, we selected BE(2)-C neuroblastoma, MDA-MB-468 and MDA-MB-231 breast cancer, and MCF10A1 non-malignant mammary epithelial cells. BE(2)-C cells were one of the most sensitive, whereas MDA-MB-468 and MDA-MB-231 cells were two of the most resistant, to HDACIs in our previous studies.[9,27] To exclude the possible off-target effects of siRNAs, we employed two sets of CLU siRNA specifically targeting different regions of the second exon

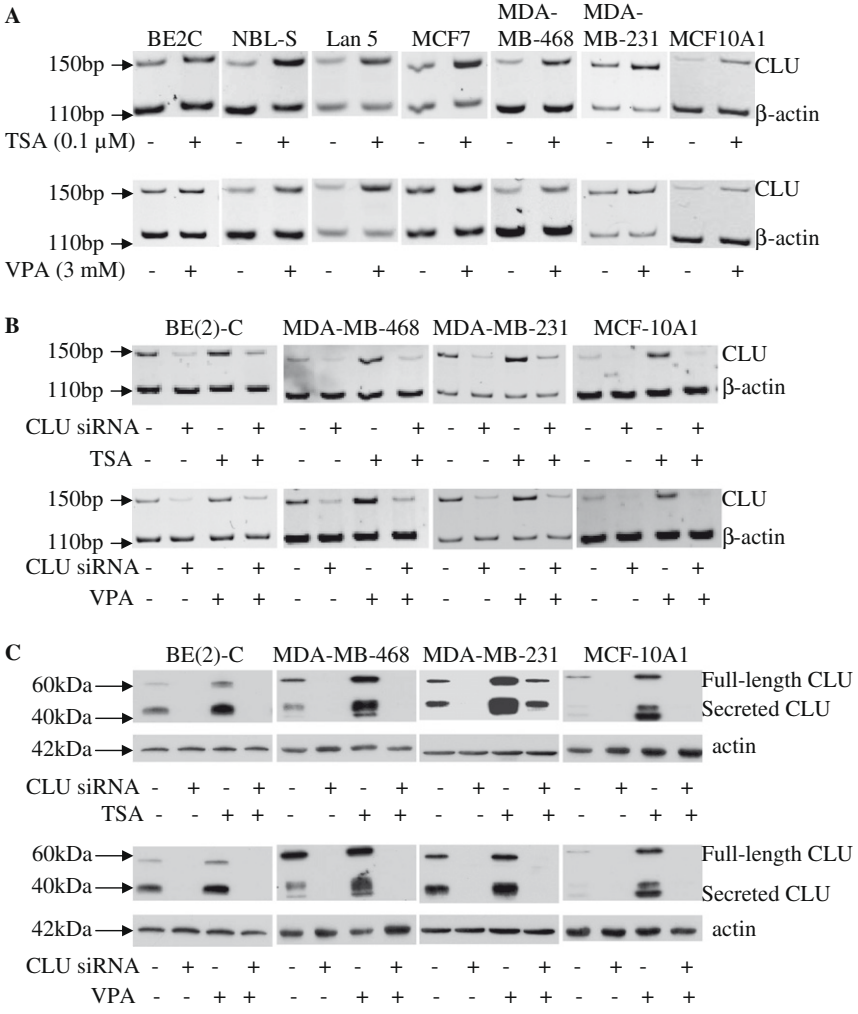

Fig. 1 – HDACIs up-regulate, and CLU siRNA repress, CLU gene expression in neuroblastoma, breast cancer and non-malignant breast epithelial cells. (A). BE(2)-C, NBL-S and LAN-5 neuroblastoma cells, MCF-7, MDA-MB-468 and MDA-MB-231 breast cancer cells, and MCF-10A1 non-malignant mammary epithelial cells were treated with vehicle control, 0.1 μM TSA or 3 mM VPA for 24 h, followed by RNA extraction and semi-quantitative competitive RT-PCR using trans-intron PCR primers for CLU together with primers for the house-keeping gene β-actin. (B). and (C). BE(2)-C, MDA-MB-468, MDA-MB-231 and MCF-10A1 cells were transfected with scrambled control or CLU siRNA, followed by treatment with vehicle control, TSA or VPA. RNA was extracted 24 h, and protein 48 h, after the treatment. (B). Semi-quantitative competitive RT-PCR was carried out with primers for CLU together with primers for the loading control β-actin. (C). Immunoblot analysis of CLU protein expression was performed with a goat anti-CLU antibody. Membranes were lastly re-probed with an anti-β-actin antibody as a loading control.

of CLU mRNA, which was unique to the anti-apoptotic intracellular isoform of CLU.[7,8] The two sets of CLU siRNA showed almost identical results in knocking down CLU mRNA and protein expression and sensitising cancer cells to HDACI-induced anti-cancer effects including apoptosis and growth inhibition in different cancer cell lines. We therefore randomly chose the first CLU siRNA which targeted the same RNA sequence as the antisense oligonucleotide OGX-011 in all experiments in BE(2)-C cells and MDA-MB-468 cells, and the second CLU siRNA in all experiments in MDA-MB-231 cells. While TSA and VPA increased CLU gene expression, the CLU siRNAs reduced CLU mRNA by about 80% in all the cell lines tested, and knocked down both the full-length CLU (60 kDa) and the secreted CLU protein (40 kDa), the two isoforms of intracellular CLU, with or without TSA or VPA treatment (Fig. 1B and C).

To assess the role of CLU gene up-regulation in HDACI-induced apoptosis, we employed the TUNEL assay. Treatment with 0.3 μM TSA induced apoptosis, as indicated by positive TUNEL staining, in less than 12% MDA-MB-468 and 5% MDA-MB-231 cells transfected with scrambled control siRNA (Fig. 2). While CLU siRNA alone did not have a significant effect, a reduction in CLU expression due to CLU siRNA increased the percentage of TUNEL positive MDA-MB-468 cells and MDA-MB-231 cells in response to TSA treatment by 140% and by 495%, respectively. The fractional product method revealed that TSA and CLU siRNA synergistically induced apoptosis in the two cell lines ($R < 1.0$). In contrast, treatment of non-malignant breast epithelial MCF-10A1 cells with 0.3 μM TSA induced little apoptosis, and the inhibition of CLU expression had no additional effect on cell sensitivity to TSA (Fig. 2). The HDACI-sensitive BE(2)-C cells express a low level of 60 kDa full-length CLU protein (Fig. 1C), which is responsible for the anti-apoptotic effect of CLU.[28] Treatment with 0.1 μM TSA for 48 h caused 50% of BE(2)-C cells to undergo apoptosis when transfected with scrambled siRNA, and the addition of CLU siRNA did not significantly enhance TSA-induced apoptosis (Fig. 2). These data suggested that up-regulation of CLU was a resistant factor for HDACI-induced apoptosis in HDACI-resistant cancer cells.

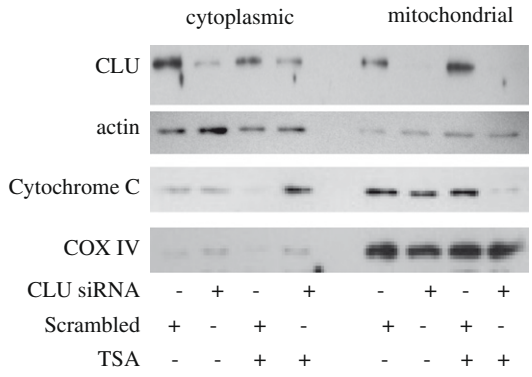

Fig. 3 – Down-regulation of CLU sensitised HDACI-resistant cancer cells to HDACI-induced apoptosis by activating mitochondrial apoptosis pathway. HDACI-resistant MDA-MB-468 cells were transfected with scrambled control or CLU siRNA, followed by treatment with vehicle control or 0.3 μM TSA for 48 h. Cytoplasmic and mitochondrial protein was then extracted and fractionated with a Mitochondria Extraction Kit (Pierce), and subjected to immunoblot analysis of CLU and cytochrome C protein expression with a goat anti-CLU antibody and a mouse anti-cytochrome C antibody, respectively. The membranes were lastly re-probed with an anti-actin antibody and an anti-COX-IV antibody as loading controls for cytoplasmic and mitochondrial proteins, respectively.

3.3. *Down-regulation of CLU sensitised cancer cells to HDACI-induced apoptosis by activating the mitochondrial apoptosis pathway*

One of the central events through which HDACIs induce apoptosis in cancer cells is the induction of BAX protein conformational change, which leads to the activation of the mitochondrial apoptosis pathway. Up-regulation of full-length CLU protein by chemotherapy and Myc oncoprotein in cancer cells is known to block the mitochondrial apoptosis pathway by direct binding and functional blocking of conformationally changed BAX in the mitochondria.[28] To assess

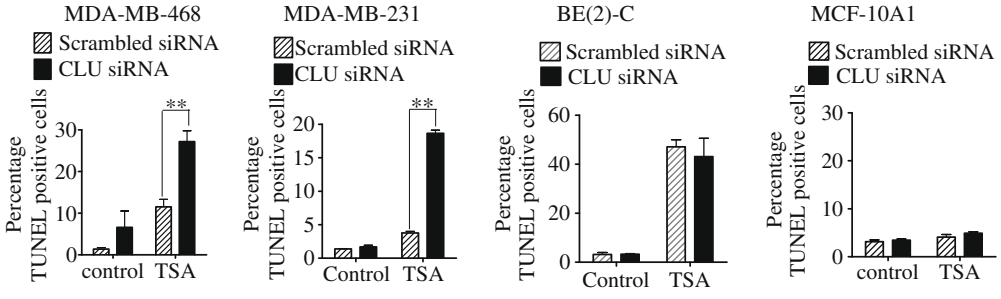

Fig. 2 – Down-regulation of CLU renders cancer cells sensitive to HDACI-induced apoptosis in HDACI-resistant cancer cells, but not in normal non-malignant cells or HDACI-sensitive cancer cells. HDACI-resistant MDA-MB-468, MDA-MB-231 cells, HDACI-sensitive neuroblastoma BE(2)-C cells and non-malignant breast epithelial MCF-10A1 cells were transfected with scrambled or CLU siRNA, followed by treatment with vehicle control or TSA for 48 h. After fixation, cells were stained with the TUNEL reagent for apoptosis. The percentage of TUNEL positive cells was quantified. Symbols ** indicated a statistically significant increase ($p < 0.01$) in the percentage of apoptotic cells. Error bars indicated standard errors.

whether HDACI-induced CLU over-expression renders cancer cells resistant to apoptosis through the mitochondrial pathway, we analysed the anti-apoptotic full-length CLU protein expression and cytochrome C localisation in mitochondrial and cytoplasmic fractions extracted from MDA-MB-468 cells. As shown in Fig. 3, treatment with TSA for 48 h significantly increased the expression of full-length CLU protein in the mitochondria. Whereas TSA or CLU siRNA alone had little effect on the localisation of cytochrome C, CLU siRNA in combination with TSA induced cytochrome C release from mitochondria into the cytoplasm. These findings indicated that HDACI-induced CLU over-expression protected HDACI-resistant cells against HDACI-induced apoptosis through suppression of the mitochondrial apoptosis pathway.

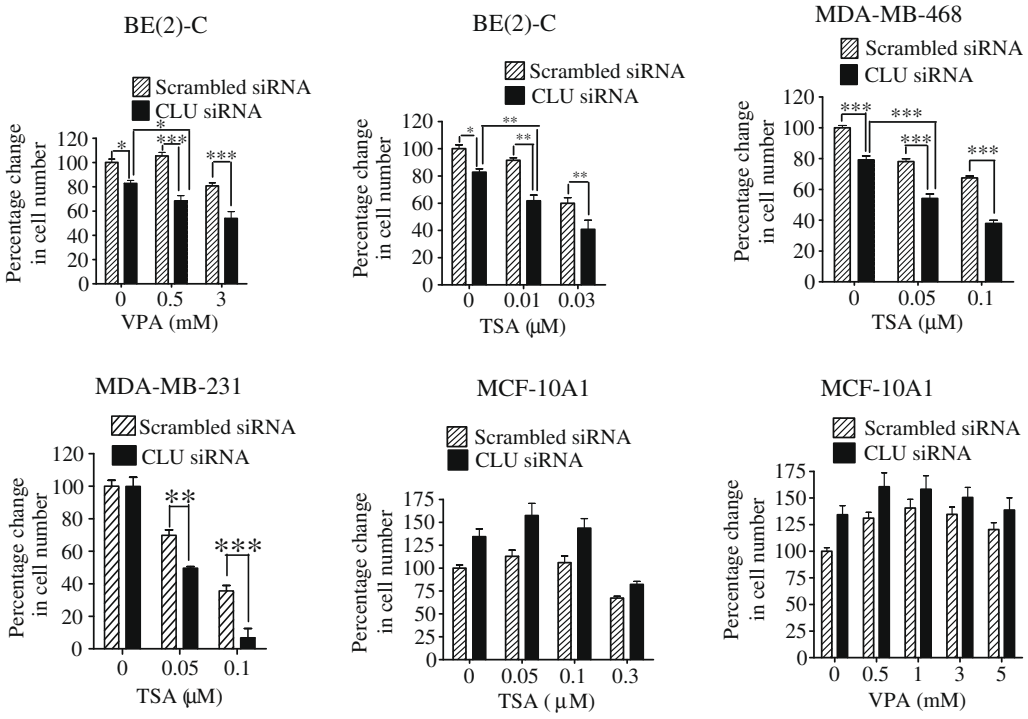

Fig. 4 – Suppression of CLU gene expression sensitises both HDACI-resistant and HDACI-sensitive cancer cells, but not normal non-malignant cells, to low-dosage HDACI-induced growth arrest. BE(2)-C, MDA-MB-468, MDA-MB-231 and MCF-10A1 cells were transfected with scrambled siRNA or CLU siRNA, followed by treatment with low dosages of the HDACIs, TSA or VPA, which did not result in cell death, for 72 h. At the end of the three day treatment, relative total numbers of cells were examined by the Alamar blue assay, measured as optical density (OD) units of absorbance, and expressed as a percentage of absorbance for the experimental samples, over that for control samples transfected with scrambled siRNA and treated with control (i.e. percentage change in cell number). Error bars represented standard error. * indicated $p < 0.05$, ** $p < 0.01$ and *** $p < 0.001$.

Table 2 – HDACIs and CLU siRNA synergistically induced growth inhibition in cancer cells. Growth inhibition was measured by Alamar blue assay, and expressed as a percentage of absorbance for the experimental samples, over that for control samples transfected with scrambled siRNA and treated with control. The combination effect of CLU siRNA and HDACIs was analysed by the fractional product method. If the ratio of the observed HDACI and CLU siRNA combination effect (the observed fraction) over the calculated HDACI and CLU siRNA combination effect (the calculated fraction) (R value) is less than 1.0, the combination is synergistic.

Cell line	HDACI	Calculated HDACI and CLU siRNA combination effect (%)	Observed HDACI and CLU siRNA combination effect (%)	R value
BE(2)-C	0.5 mM VPA	87.3 ± 2.65	68.38 ± 4.34	0.78
BE(2)-C	3 mM VPA	66.9 ± 2.43	53.90 ± 5.64	0.81
BE(2)-C	0.01 μM TSA	75.84 ± 2.05	61.73 ± 4.23	0.81
BE(2)-C	0.03 μM TSA	49.34 ± 3.25	40.79 ± 6.73	0.83
MDA-MB-468	0.05 μM TSA	61.84 ± 2.16	54.15 ± 2.92	0.88
MDA-MB-468	0.1 μM TSA	53.40 ± 1.91	37.93 ± 2.07	0.71
MDA-MB-231	0.05 μM TSA	69.69 ± 4.49	49.59 ± 0.96	0.71
MDA-MB-231	0.1 μM TSA	35.55 ± 4.49	6.76 ± 5.73	0.19

3.4. *Down-regulation of CLU enhanced low-dosage HDACI-induced growth arrest in both HDACI-resistant and -sensitive cancer cells*

We next assessed the effect of CLU on low-dosage HDACI-induced growth arrest using the Alamar blue assay. At the low dosages chosen for the specific cell lines in Fig. 4, TSA and VPA did not significantly induce cell death.[9,27] As shown in Fig. 4, compared with scrambled control siRNA, CLU siRNA alone slightly decreased the number of BE(2)-C, MDA-MB-468 and MDA-MB-231 cells, but increased the number of normal non-malignant MCF-10A1 cells. The combination of TSA

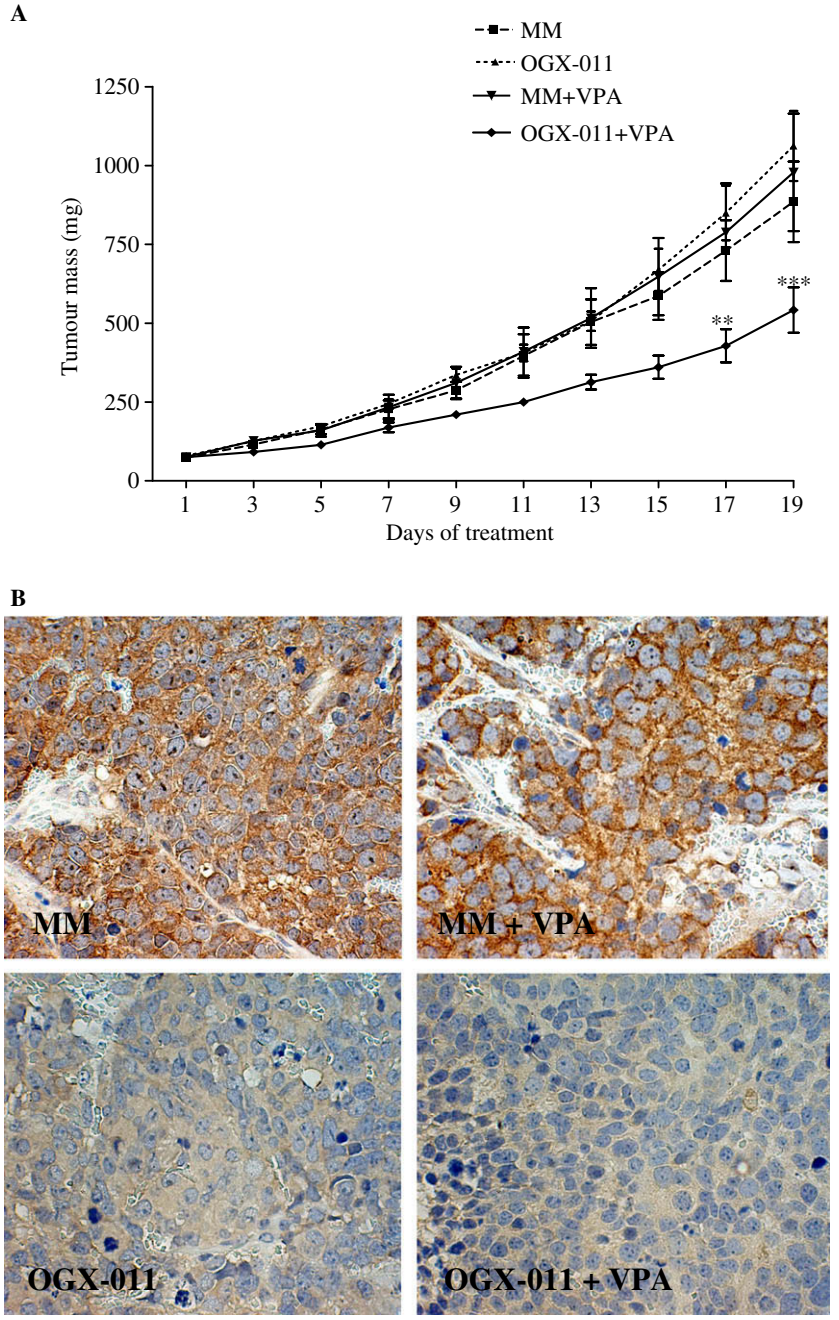

Fig. 5 – OGX-011 and VPA exert synergistic anti-cancer effects *in vivo*. (A). BE(2)-C neuroblastoma cells were xenografted into 6 week old athymic nude mice. When tumour size reached 0.5 cm^3, the mice were randomized into 4 groups of eight and injected intra-peritoneally daily with 12.5 mg/kg/day mis-match oligonucleotide (MM), 12.5 mg/kg/day antisense oligonucleotide targeting CLU (OGX-011), MM plus 400 mg/kg/day VPA, or OGX-011 plus VPA for 19 consecutive days before being sacrificed. Tumour volume was monitored every other day. Error bars represented standard error. ** indicated $p < 0.01$, and *** $p < 0.001$. (B). Formalin-fixed tumour tissue sections from the mice were subjected to immunohistochemical assessment of CLU protein expression, which was visualised with diaminobenzidine (brown). The nucleus was counter-stained with haematoxylin (blue).

or VPA with CLU siRNA co-operatively decreased the number of BE(2)-C, MDA-MB-468 and MDA-MB-231 tumour cells at all dosages tested, and, in contrast, increased the number of the normal non-malignant MCF-10A1 cells to the same extent as CLU siRNA alone. Analysis of combination effects with the fractional product method, as we and others have described previously,[10–12] showed that all combination effects in the cancer cell lines were synergistic (Table 2) ($R < 1.0$). These data suggested that the repression of CLU expression synergistically enhanced low-dosage HDACI-induced growth arrest in both HDACI-sensitive and HDACI-resistant cancer cells.

3.5. *OGX-011 sensitised neuroblastoma cells to HDACI in vivo*

Lastly, we examined whether the repression of CLU would sensitise human tumour cells to HDACI therapy *in vivo*. We carried out the *in vivo* experiment with VPA in neuroblastoma BE(2)-C cells because we mainly work on childhood cancer and VPA is the only HDACIs proven to be safe in young children. BE(2)-C cells were xenografted into the flank of six-week-old athymic nude mice. When tumours reached 0.125 cm^3, the mice were randomised into four groups of eight and treated with mismatch anti-sense oligonucleotide (MM, 12.5 mg/kg/day), CLU anti-sense oligonucleotide (OGX-011, 12.5 mg/kg/day),[7] VPA (400 mg/kg/day) plus MM, or VPA plus OGX-011 for 19 consecutive days. As shown in Fig. 5A, compared with MM, neither OGX-011 nor VPA alone had an effect on tumour progression. However, the combination of OGX-011 and VPA repressed tumour growth by over 40%, reaching statistical significance from the 17th post-treatment day onwards, compared with MM, OGX-011 or VPA plus MM. The fractional product method revealed that OGX-011 and VPA synergistically inhibited tumour growth *in vivo* ($R < 1.0$). Moreover, immunohistochemical staining of tumour tissues from the mice with an anti-CLU antibody showed that OGX-011 suppressed CLU protein expression when injected alone or in combination with VPA (Fig. 5B). Importantly, the mice treated with VPA plus OGX-011 combination therapy did not show significant abnormality, as assessed by body weight and daily observation.

4. Discussion

Transcriptional modulation of specific pre-programmed genes (2–5% of the expressed genes) is central to HDACI-induced anti-cancer effects. In this study, we reviewed microarray gene profiling raw data reported in the literature, and found, for the first time, that CLU was one of only two genes most commonly up-regulated by different HDACIs in cancer cells arising in a range of organs, while other well-defined HDACI target genes were not commonly modulated by HDACIs.

A high level of CLU expression is associated with tumourigenesis, progression and/or poor prognosis in lymphoma, breast, prostate, colon, bladder and kidney cancer patients [reviewed in[4]]. Treatment with cytotoxic chemotherapy and radiotherapy can induce CLU expression in many cancer cell types. CLU renders cancer cells chemo-resistant through direct binding to conformationally changed Bax in mitochondria, which blocks Bax-mediated cytochrome C release and the mitochondrial apoptosis pathway.[28] Repression of CLU gene expression with the anti-sense oligonucleotide, OGX-011, significantly sensitises lung and prostate cancer cells to radiotherapy, and sensitises melanoma, prostate, breast, renal and bladder cancer cells to various cytotoxic chemotherapy *in vitro* and *in vivo* [reviewed in[4]]. Our data have shown that the anti-apoptotic 60 kDa full-length CLU was up-regulated by the HDACIs in all the cell lines tested. Suppression of CLU with siRNA enhanced high-dosage HDACI-induced cell death through the induction of cytochrome C release and subsequent activation of the mitochondrial apoptosis pathway in HDACI-resistant cancer cells, and enhanced low-dosage HDACI-induced growth arrest in both HDACI-sensitive and -resistant cancer cells. These effects were not observed in normal non-malignant cells. The data suggest that HDACI-induced CLU over-expression renders cancer cells resistant to HDACIs through suppressing both growth arrest and apoptosis. Importantly, the combination of HDACIs and CLU siRNA did not induce growth arrest or apoptosis in the non-malignant cells, although the HDACIs induced CLU expression.

OGX-011 is one of the few molecularly targeted antisense oligonucleotides in Phase II clinical trials. OGX-011 sensitises prostate, breast, bladder, renal, lung and skin cancer to chemo-radiotherapy in pre-clinical animal model studies,[4] and inhibits prostate cancer progression in Phase I clinical trials.[5] Our study has demonstrated, for the first time, that the suppression of CLU with OGX-011 sensitises tumour responses to HDACI therapy *in vivo* without obvious side-effects. Because of the synergistic induction of growth arrest by VPA and CLU siRNA in BE(2)-C cells *in vitro*, we hypothesise that VPA and OGX-011 exert synergistic anti-cancer effects *in vivo* mainly through enhancing growth arrest.

As the HDACI, VPA, is in clinical use, and several HDACIs and OGX-011 are already in phases II and III clinical trials, our findings highlight the importance of targeting CLU in sensitising tumour response to HDACI therapy. Further studies assessing the combination therapy against multiple tumour xenografts, OGX-011 pharmacokinetics and CLU targeting in animal models may provide a basis for future combination HDACI and OGX-011 clinical trials in cancer patients.

Conflict of interest statement

Martin Gleave is a founder, Chief Scientific Officer and Board Member of OncoGenex, Vancouver, BC, Canada. He holds stock in Onco-Genex Technologies.

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REFERENCES

1. Grant S, Easley C, Kirkpatrick P. Vorinostat. *Nat Rev Drug Discov* 2007;**6**:21–2.
2. Johnstone RW, Licht JD. Histone deacetylase inhibitors in cancer therapy: is transcription the primary target? *Cancer Cell* 2003;**4**:13–8.
3. Kelly WK, Marks PA. Drug insight: histone deacetylase inhibitors – development of the new targeted anticancer agent suberoylanilide hydroxamic acid. *Nat Clin Pract Oncol* 2005;**2**:150–7.
4. Shannan B, Seifert M, Leskov K, et al. Challenge and promise: roles for clusterin in pathogenesis, progression and therapy of cancer. *Cell Death Differ* 2006;**13**:12–9.
5. Chi KN, Eisenhauer E, Fazli L, et al. A phase i pharmacokinetic and pharmacodynamic study of ogx-011, a 2′-methoxyethyl antisense oligonucleotide to clusterin, in patients with localized prostate cancer. *J Natl Cancer Inst* 2005;**97**:1287–96.
6. Peart MJ, Smyth GK, van Laar RK, et al. Identification and functional significance of genes regulated by structurally different histone deacetylase inhibitors. *Proc Natl Acad Sci USA* 2005;**102**:3697–702.
7. July LV, Beraldi E, So A, et al. Nucleotide-based therapies targeting clusterin chemosensitize human lung adenocarcinoma cells both in vitro and in vivo. *Mol Cancer Ther* 2004;**3**:223–32.
8. Criswell T, Beman M, Araki S, et al. Delayed activation of insulin-like growth factor-1 receptor/src/mapk/egr-1 signaling regulates clusterin expression, a pro-survival factor. *J Biol Chem* 2005;**280**:14212–21.
9. Liu T, Tee AE, Porro A, et al. Activation of tissue transglutaminase transcription by histone deacetylase inhibition as a therapeutic approach for myc oncogenesis. *Proc Natl Acad Sci USA* 2007;**104**:18682–7.
10. Greco WR, Bravo G, Parsons JC. The search for synergy: a critical review from a response surface perspective. *Pharmacol Rev* 1995;**47**:331–85.
11. Yokoyama Y, Dhanabal M, Griffioen AW, Sukhatme VP, Ramakrishnan S. Synergy between angiostatin and endostatin: inhibition of ovarian cancer growth. *Cancer Res* 2000;**60**:2190–6.
12. Miayake H, Tolcher A, Gleave ME. Chemosensitization and delayed androgen-independent recurrence of prostate cancer with the use of antisense bcl-2 oligodeoxynucleotides. *J Natl Cancer Inst* 2000;**92**:34–41.
13. Jaboin J, Wild J, Hamidi H, et al. Ms-27-275, an inhibitor of histone deacetylase, has marked in vitro and in vivo antitumor activity against pediatric solid tumors. *Cancer Res* 2002;**62**:6108–15.
14. Burgess AJ, Pavey S, Warrener R, et al. Up-regulation of p21(waf1/cip1) by histone deacetylase inhibitors reduces their cytotoxicity. *Mol Pharmacol* 2001;**60**:828–37.
15. Rosato RR, Almenara JA, Yu C, Grant S. Evidence of a functional role for p21waf1/cip1 down-regulation in synergistic antileukemic interactions between the histone deacetylase inhibitor sodium butyrate and flavopiridol. *Mol Pharmacol* 2004;**65**:571–81.
16. Rosato RR, Almenara JA, Cartee L, Betts V, Chellappan SP, Grant S. The cyclin-dependent kinase inhibitor flavopiridol disrupts sodium butyrate-induced p21waf1/cip1 expression and maturation while reciprocally potentiating apoptosis in human leukemia cells. *Mol Cancer Ther* 2002;**1**:253–66.
17. Glaser KB, Staver MJ, Waring JF, Stender J, Ulrich RG, Davidsen SK. Gene expression profiling of multiple histone deacetylase (hdac) inhibitors: defining a common gene set produced by hdac inhibition in t24 and mda carcinoma cell lines. *Mol Cancer Ther* 2003;**2**:151–63.
18. Joseph J, Mudduluru G, Antony S, Vashistha S, Ajitkumar P, Somasundaram K. Expression profiling of sodium butyrate (nab)-treated cells: identification of regulation of genes related to cytokine signaling and cancer metastasis by nab. *Oncogene* 2004;**23**:6304–15.
19. Chiba T, Yokosuka O, Arai M, et al. Identification of genes up-regulated by histone deacetylase inhibition with cdna microarray and exploration of epigenetic alterations on hepatoma cells. *J Hepatol* 2004;**41**:436–45.
20. Gray SG, Qian CN, Furge K, Guo X, Teh BT. Microarray profiling of the effects of histone deacetylase inhibitors on gene expression in cancer cell lines. *Int J Oncol* 2004;**24**:773–95.
21. Sasakawa Y, Naoe Y, Sogo N, et al. Marker genes to predict sensitivity to fk228, a histone deacetylase inhibitor. *Biochem Pharmacol* 2005;**69**:603–16.
22. Takai N, Kawamata N, Walsh CS, et al. Discovery of epigenetically masked tumor suppressor genes in endometrial cancer. *Mol Cancer Res* 2005;**3**:261–9.
23. Milutinovic S, D'Alessio AC, Detich N, Szyf M. Valproate induces widespread epigenetic reprogramming which involves demethylation of specific genes. *Carcinogenesis* 2007;**28**:560–71.
24. de Ruijter AJ, Meinsma RJ, Bosma P, Kemp S, Caron HN, van Kuilenburg AB. Gene expression profiling in response to the histone deacetylase inhibitor bl1521 in neuroblastoma. *Exp Cell Res* 2005;**309**:451–67.
25. Chinnaiyan P, Varambally S, Tomlins SA, et al. Enhancing the antitumor activity of erbb blockade with histone deacetylase (hdac) inhibition. *Int J Cancer* 2006;**118**:1041–50.
26. Dai Z, Liu S, Marcucci G, Sadee W. 5-aza-2′-deoxycytidine and depsipeptide synergistically induce expression of bik (bcl2-interacting killer). *Biochem Biophys Res Commun* 2006;**351**:455–61.
27. Kuljaca S, Liu T, Tee AE, et al. Enhancing the anti-angiogenic action of histone deacetylase inhibitors. *Mol Cancer* 2007;**6**:68.
28. Zhang H, Kim JK, Edwards CA, Xu Z, Taichman R, Wang CY. Clusterin inhibits apoptosis by interacting with activated bax. *Nat Cell Biol* 2005;**7**:909–15.