

Valproic acid enhances the paclitaxel activity in respiratory tract cancer cells

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Objective Epigenetic therapies have already been introduced into clinical cancer management. The main objective of this study is to explore the potential of modulating paclitaxel efficiency using two epigenetic modifiers; valproic acid and decitabin.

Methods The potential sensitisation of lung and oral cancer cells to paclitaxel was examined by two well-known epigenetic modifiers; the DNA methyltransferase inhibitor Decitabine and histone deacetylase class I inhibitor valproic acid (VPA). The effect epigenetic modifiers were tested using qPCR and pyrosequencing techniques utilising respiratory tract cancerous tissues and cell lines.

Results The results exhibited that VPA was an effective epigenetic sensitizer for treating lung and head and neck cancerous cells (A549, SKLU1 and BHY). About 48 h prior to paclitaxel addition, a significant increase ($p < 0.01$) of the paclitaxel toxicity was observed when the cancer cells pre-treated with VPA for 48 h and subsequently with paclitaxel for 72 h. Interestingly, mRNA expression of AURKA was reduced by VPA treatment. The result also demonstrated that p53 status was involved in VPA-mediated paclitaxel sensitisation of HBEC cell lines to paclitaxel. VPA seems to potentiate p53 wild type cells (HBEC-3KT) to paclitaxel, while p53 HBEC knockouts showed less cytotoxic effect of paclitaxel after exposure to 0.5 mM VPA. On the other hand, decitabin was not efficient to sensitise any of the cell lines to paclitaxel when used in either a synchronous or a preceding manner. In addition, the pyrosequencing analysis of the methylation status of the different gene promoters in the lung tumour and normal tissues showed that all the promoters were unmethylated.

Conclusion It can be concluded that the epigenetic modifier VPA can alter the response of cancer cells to paclitaxel treatment. Further investigation is needed to explore the epigenetic mechanism of sensitising cancerous cells to paclitaxel.

Keywords valproic acid, paclitaxel, lung cancer, head and neck cancer, epigenetic

Introduction

Epigenetic therapies have recently been introduced into clinical cancer management.^{1,2} Previous evidence suggested that valproic acid (VPA) enhances paclitaxel cytotoxic effects in cancerous cells³ due to HDAC6 deactivation, which results in tubulin hyperacetylation⁴ and sensitises lung cancer cells to apoptosis.⁵ The combinatory effect of VPA-paclitaxel was also tested in HNSCC tumours.⁶ Aurora kinases also play a transcriptional regulatory role in HDAC inhibitors-mediated cytotoxicity in lung cancer cells.⁷ Recent reported data showed that enhancing p53 acetylation due to HDAC inhibition leads to enhance paclitaxel-induced apoptosis.⁸ In addition, a combination of VPA and decitabin has been introduced in clinical trials to treat NSCLC patients.^{1,2}

Epigenetic therapies and epigenetic sensitization of cancer cells to common chemotherapeutics have come to focus in the last decade.^{9,10} As sensitization to taxanes was a major objective of this study, the potential of modulating paclitaxel efficiency was examined using two epigenetic modifiers; valproic acid to induce histone acetylation and decitabin to induce global DNA hypomethylation. Both epigenetic drugs is used as a combination treating patients with lung and head and neck in phase I clinical studies^{1,2} which demonstrated that decreased DNA methylation and induction of histone acetylation were associated with prolonged stable disease for 6 months as a median (4–12 months).

Materials and Methods

Cell Lines

NSCLC cell lines (A549 and SK-LU-1), HNSCC cell line (BHY) and HBEC cell lines (HBEC-3KT and HBEC-3KT-53).

Paclitaxel Exposure

Cells were seeded in 48-well plates in six biological replicates, cultured in 500 μ L of medium and exposed to increasing concentrations of Paclitaxel (1–35 nM) for 72 h. Growth was measured using the MTT assay.

Primary Lung Tumours

One hundred and thirty three primary lung tumours [57 adenocarcinomas, 76 squamous cell carcinomas (SqCCL)], and 44 adjacent normal tissues (from 20 adenocarcinoma and 24 SqCCL patients), have been utilised in this study. The mean age of those patients were 67 (45–82). Fifty-six patients were females and 77 males.

RNA Extraction

Total RNA was extracted from cell lines and primary lung tumours using miRNeasy Mini Kit (QIAGEN), and then quantified by Thermo Scientific NanoDrop 2000 Spectrophotometer.

Reverse Transcription was undertaken using High Capacity cDNA Reverse Transcription Kits (Life Technologies).

qPCR

Pre-designed Taqman expression assays (Life Technologies) were employed using VIC-labeled ACTB as endogenous control. Real-time PCR assays were performed in triplicate. The genes tested were: *AURKA*, *AURKB*, *AURKC*, *CKAP5*, *TPX2*, *TTK*, *KIF11*, *DLGAP5*, *TUBB* and *TUBB3*.

DNA Methylation Analysis

DNA Extraction

DNA extraction from cell lines and primary lung tumours were performed using DNeasy[®] Blood and Tissue Kit (QIAGEN) using DNeasy 96 protocol for purification of total DNA from tissues and spin-column protocol for purification of total DNA from cell lines.

DNA Methylation

In order to generate positive control for methylation-specific PCR or bisulfate sequencing, reaction of four units SssI per μg of unmethylated DNA for 2 h was prepared according the modified manufacturing protocol.

Bisulphite Treatment of DNA

To investigate the methylation status of the different gene promoters tested in this study, 500 ng DNA from primary tissues was bisulphite treated utilising the EZ-96 DNA Methylation-Gold[™] Kit (Zymo Research) following the manufacturer's protocol.

Pyrosequencing Methylation Analysis

Pyrosequencing (PSQ) is a method that can identify the sequence from small DNAs efficiently and with high fidelity. The samples were prepared for PSQ. The targeted DNA sequence was amplified by PCR using forward biontynlated (Fb), reverse (R) and sequencing (S) primers. The primers were designed by the PyroMark assay design 2.0 software.

Decitabine Efficiency

This was achieved by pyrosequencing-methylation analysis of the LINE-1.2 (Genebank accession no M80343) retrotransposon (Daskalos 2009). Forward primer: BIO-TAGGGAGTGTTAGA-TAGTGG, reverse primer: AACTCCCTAACCCCTTAC, sequencing primer: CAAATAAAA CAATACCTC. PCR amplification was performed using QIAGEN HotStarTaq Plus Master Mix Kit.

Statistical Analysis

The Kolmogorov–Smirnov Test, Mann–Whitney test and Wilcoxon test were employed for statistical analysis using SPSS 20. Bonferroni correction was used to adjust for multiple comparisons. The IC_{50} values were calculated using GraphPad Prism 5. Kaplan–Meier curves were constructed for survival analysis and the log-rank test was used to examine the differences between groups.

Results

Based on aforementioned reported data, the epigenetic role of the histone acetylator (VPA) and DNA methylator (decitabin) in sensitising RTC cells to paclitaxel was investigated. The cellular response to VPA was next examined alone in order to select the concentrations below IC_{50} for further investigation

of VPA ability to sensitise cancerous cells to taxanes. MTT analysis of VPA exposure of lung cell lines (A549 and SKLU1) and the second most paclitaxel resistant oral cancer cell line (BHY) demonstrated that these cells are resistant to very high VPA micro-molar concentrations (Fig. 1) with IC_{50} s of 6.63 nM of A549, 20 mM of SKLU1 and 2.1 mM of BHY) (Table 1) at 95% CI.

In order to examine the ability of valproate to potentiate the anti-tumour efficacy of paclitaxel in controlling cellular viability, two different doses of valproate 0.5 and 1 mM that are below IC_{50} s of all these three cell lines were used. A fixed dose of paclitaxel (10 nM) was utilised to test our hypothesis. This was also under the IC_{50} s of the examined cell lines; 13.6 nM of A549, 16.7 nM of SKLU1 and 14 nM of BHY. The growth inhibitory effects of 1 mM VPA and 10 nM paclitaxel were determined as optimal doses utilised either in combination or as successive treatments of the cell lines, A549, SKLU1 and BHY. The synchronous treatment of VPA and paclitaxel produced only a minor additive effect (Data not shown). In contrast, when VPA used to treat the cell for 48 h prior to paclitaxel addition, a significant increase of the paclitaxel toxicity was observed in the subsequent 72 h (Fig. 2).

Interestingly, mRNA expression of *AURKA* in BHY cell line was significantly reduced to around 65% after treatment with 1 mM VPA for 48 h (Fig. 3).

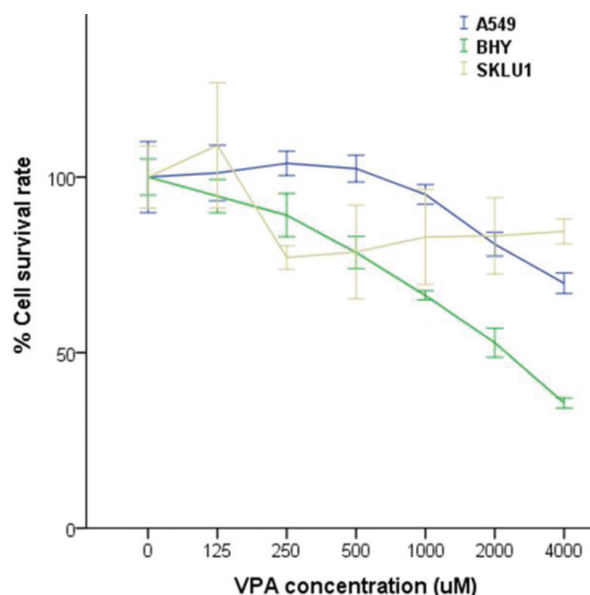


Fig. 1 MTT line graph showing the cellular survival rates of lung (A549 and SKLU1) and HNSCC (BHY) cell lines to VPA. Error bars represent 95% confidence intervals.

Table 1 The data demonstrating the IC_{50} values of VPA in A549, SKLU1 and BHY cell lines and their respective 95% CI. The results demonstrate that SKLU1 cell line is the most resistant to VPA with IC_{50} value 20 mM VPA, while BHY is the most sensitive one IC_{50} value 2.1 mM VPA

Cell line	VPA IC_{50} (nM)	95% CI
A549	6.63	5.77–7.63
SKLU1	20.00	11.80–33.92
BHY	2.10	19.40–2.27

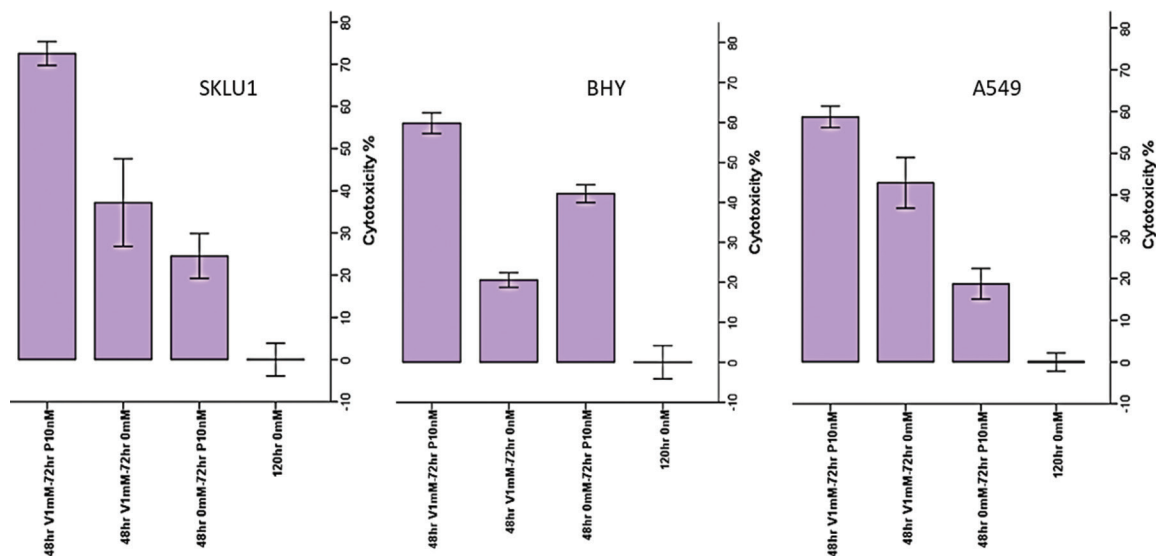


Fig. 2 Bar charts of cytotoxic effects of 48-hour treatment with 1 mM VPA flowed by 72 h of 10 nM paclitaxel on A549, BHY and SKLU1 cell lines. Interestingly, SKLU1 cell line exhibited more response to paclitaxel after 48 h exposure to VPA. BHY showed different trend of response to treatment of VPA and paclitaxel separately compared to A549 and SKLU1 cell lines. Error bars represent 95% confidence

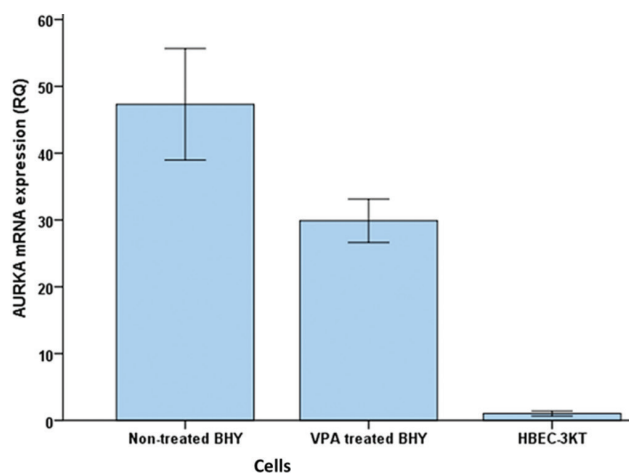


Fig. 3 AURKA mRNA expression in VPA-treated BHY cells with 1 mM concentration compared with non-treated in comparison with HBEC-3KT control. VPA treatment seems to reduce AURKA expression to around 65%. Error bars were represented 95% confidence

Then it was examined how the status of tumour suppressor gene *p53* could affect the paclitaxel sensitisation of HBEC cell lines to paclitaxel. The results demonstrated that the VPA exhibited more efficiency in sensitising *p53* wild type HBEC cells to paclitaxel than that exhibited in sensitising *p53* knockouts and thus *p53* expression seems to increase the cytotoxic effect of paclitaxel after course exposure to 0.5 mM VPA although pre-treatment of HBECs with 1 mM VPA shows different trend (Fig. 4).

The efficiency of decitabine treatment of A549 was determined at different concentrations (50, 100 and 200 μ M) by measuring the global methylation levels (LINE-1 element) (Fig. 5).

This agent showed a dose-dependent efficiency to demethylate A549 cellular DNA (Fig. 6).

Although decitabine was efficient in reducing global LINE methylation, it did not sensitise any of the cell lines to paclitaxel when used either in a synchronous (Fig. 7) or in a preceding manner (data not shown).

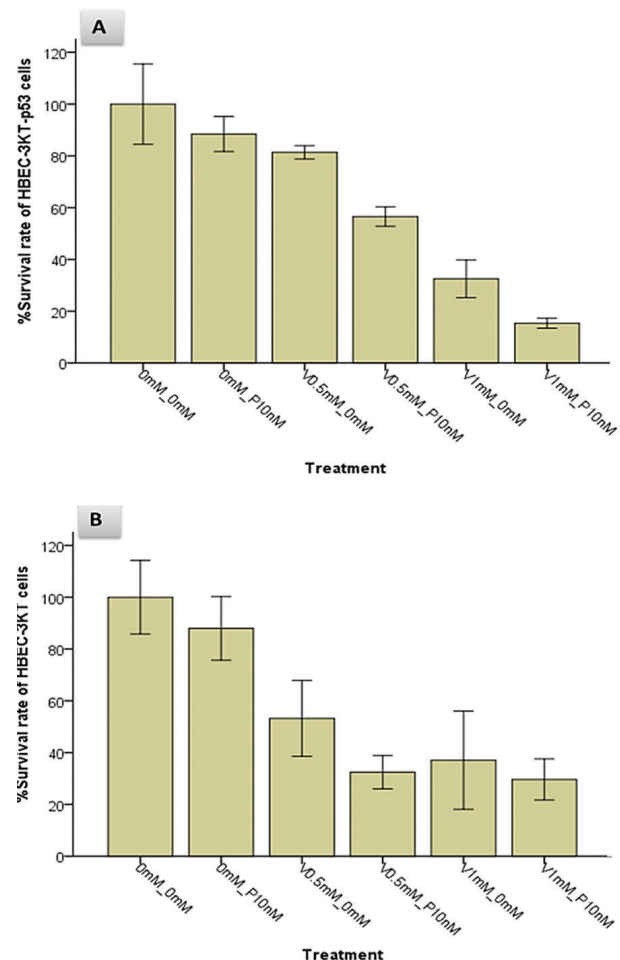


Fig. 4 Bar charts of cytotoxic effects of 48 h treatment with 1 and 0.5 mM VPA followed by 72 h of 10 nM paclitaxel on HBEC-3KT (A) and HBEC-3KT-p53 (B) cell lines. *p53* Wild type HBEC cells showed more response to 10 nM paclitaxel after 48 h exposure to 0.5 mM VPA compared with *p53* knockouts. However, increasing VPA concentration to 1 mM no more effect in HBEC-3KT as opposed to more effect in HBEC-3KT-p53. Error bars represent 95% confidence intervals.

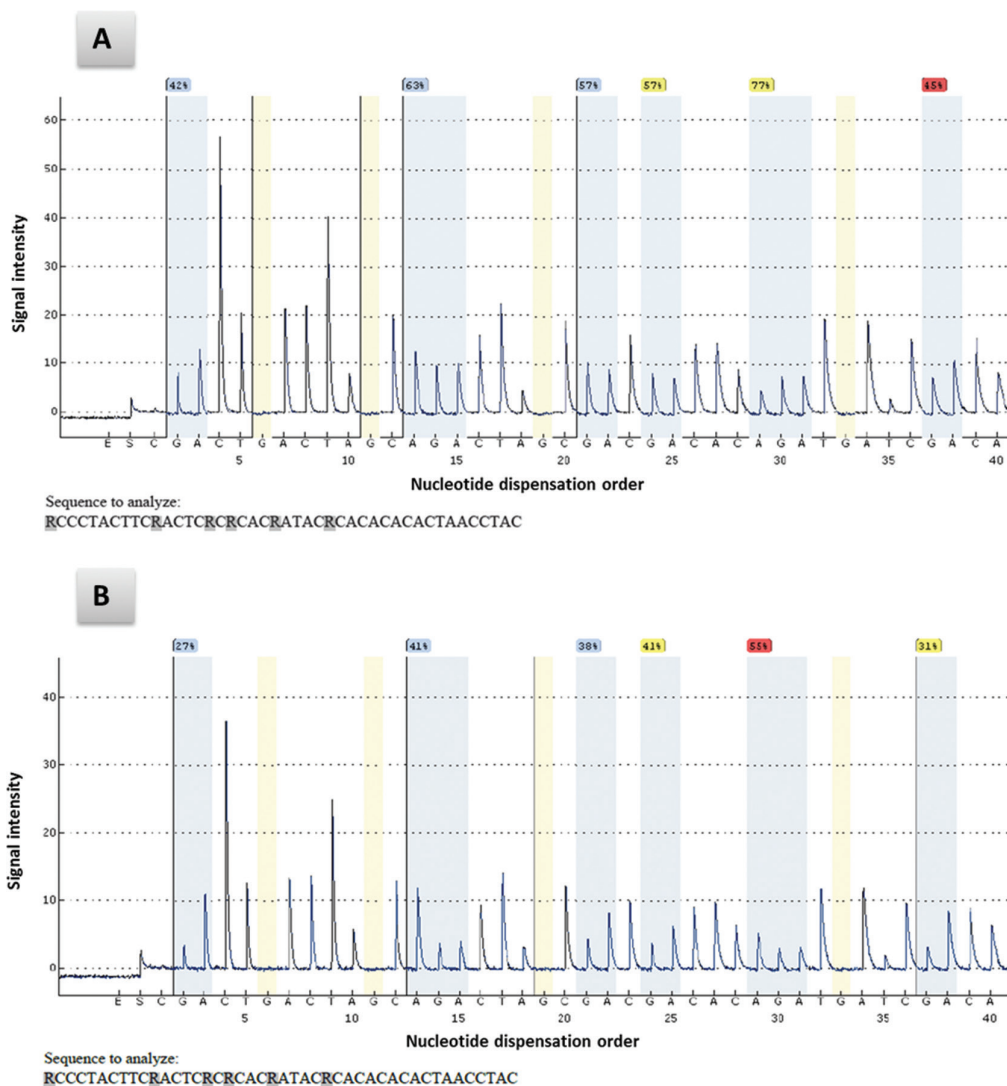


Fig. 5 Pyrograms of LINE-1 global methylation analysis demonstrating the cellular DNA methylation status of A549 cell line in the absence (A) and presence (B) of decitabine at 200 µM.

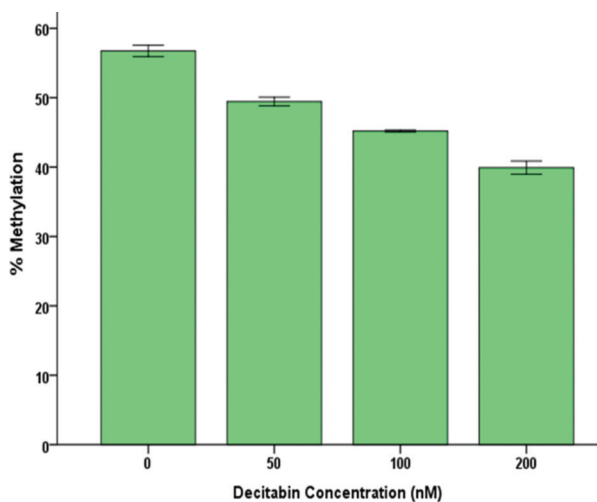


Fig. 6 The bar chart demonstrating the change in the methylation status of LINE-1 following treatment of A549 cells with the demethylating agent decitabine at different concentration (0, 50, 100 and 200 µM). The results showed that reduction of the methylation level correlated with increasing decitabine dose.

In order to provide insight into the inability of decitabine to sensitize cell lines to paclitaxel, the methylation status of the different gene promoters tested in this study was investigated. The pyrosequencing analysis demonstrated that none of the gene promoters examined in this study demonstrated altered methylation status; in fact all promoters were unmethylated in all tumour and normal tissues tested (Fig. 8).

Discussion

The data obtained demonstrated that pre-treatment of three different RTC cell lines with VPA sensitized these cells to paclitaxel, while decitabine has no such sensitizing effect. The maximum concentration that has been used in this study was corresponded to levels in the plasma of patient treated for epilepsy that ranged from 30 to 111 mg/L as opposed to 0.2–0.8 mM and exhibited low risk side effects,¹¹ while resulting in histone acetylation.¹² These findings are consistent with Chen et al.,¹³ who established that VPA enhanced paclitaxel response in resistant human lung adenocarcinoma cells but in dose-dependent manner, but in contrast to Erlich et al.,⁶ who could

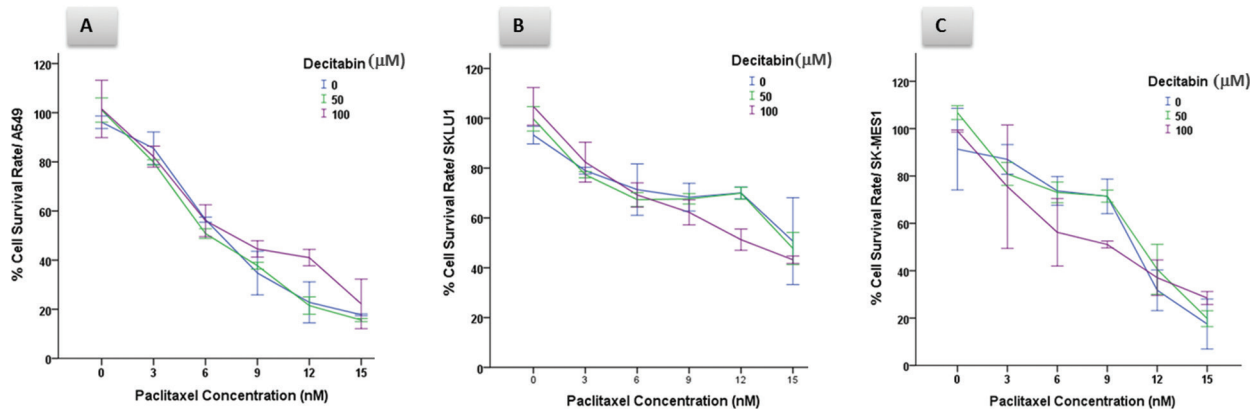
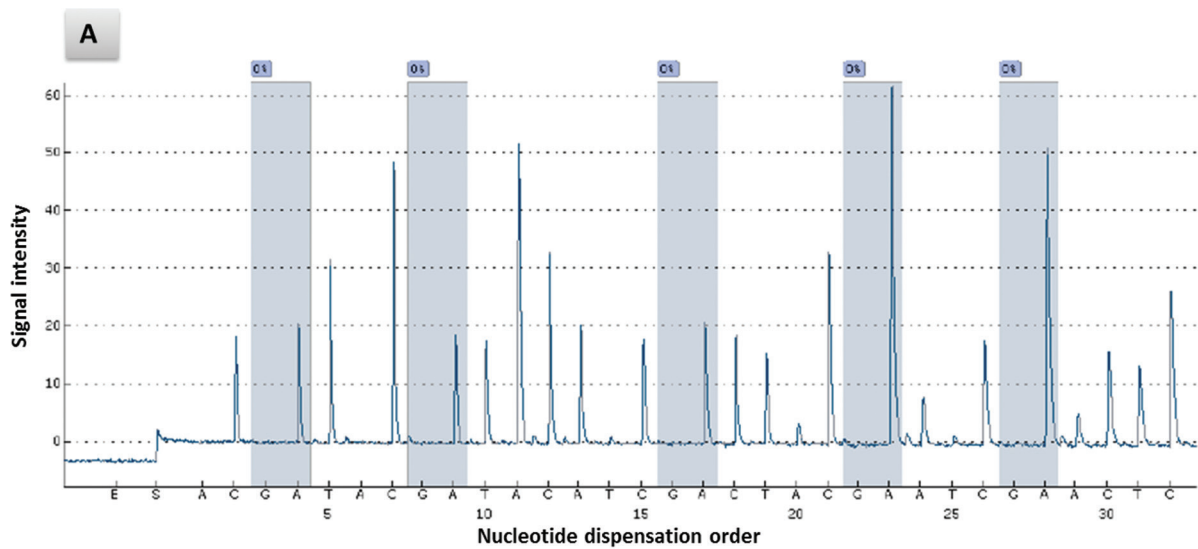
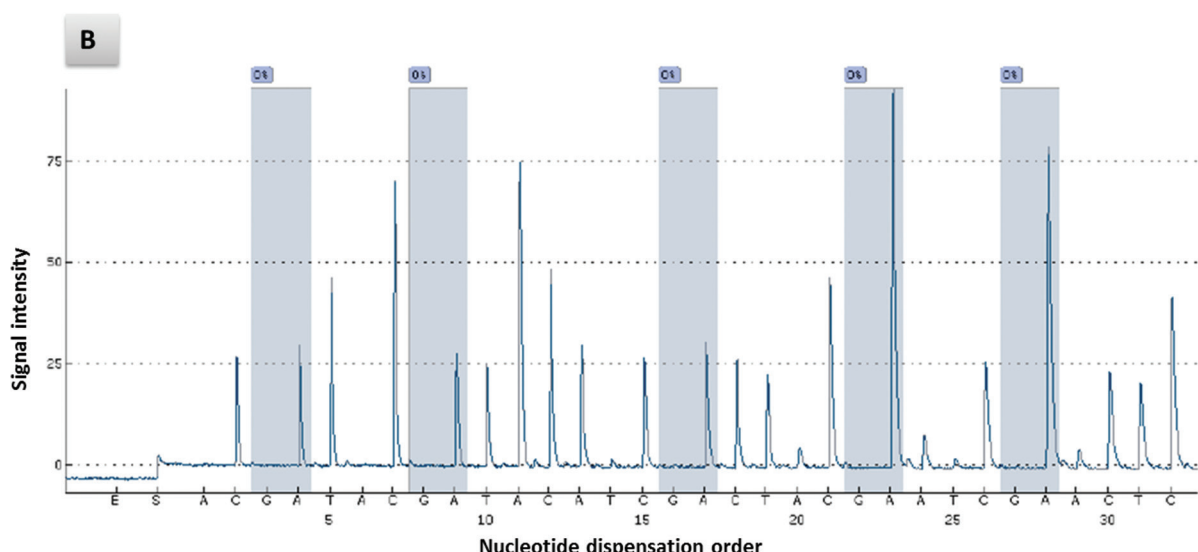


Fig. 7 MTT line graphs showing the sensitivity of A549 (A), SKLU1 (B) and (C) SKMES1 cell lines to paclitaxel in the presence of differing concentrations of decitabine (0, 50 and 100 µM). The data showed no significant difference in cellular response to treatment with paclitaxel alone or in combination with decitabine. Error bars were represented 95% confidence intervals.



Sequence to analyze:
CRITCCCR TAAACCACR CTCCR AAAACR AAAACTCCAATAAAAACTACTACATCC



Sequence to analyze:
CRITCCCR TAAACCACR CTCCR AAAACR AAAACTCCAATAAAAACTACTACATCC

Fig. 8 Representative pyrograms showing DNA demethylation status of AURKA gene promoter in (A) tumour and (B) normal lung samples. The figures show that the gene promoters were unmethylated in both malignant and normal tissues of the lung.

not deduce that VPA can potentiate the cytotoxic effect to paclitaxel in HNSCC cells. This inconsistency probably exists because the researchers did not try to pre-treat the cells with VPA prior paclitaxel treatment rather they examined only the VPA-paclitaxel combination. However, the findings demonstrated a minor effect of VPA and paclitaxel in RTC cells. This epigenetic sensitisation of cancer cells to paclitaxel might result through induction of apoptosis due to enhancement of tubulin acetylation.⁴ Although paclitaxel-induced apoptosis in NSCLC is well documented and p53-independent,¹⁴⁻¹⁷ following the finding that VPA pre-treatment potentiates paclitaxel cytotoxic effect in RTC cell lines. The association of VPA-mediated paclitaxel cytotoxicity with p53 status was also investigated. The results indicated that p53 status was a determinant of epigenetic sensitisation of HBEC cells to paclitaxel cytotoxicity. It was evident that 0.5 mM VPA enhanced paclitaxel activity in p53 wild type HBEC cells but to a lesser extent in the p53-knockout derivatives. However, increased VPA dose to 1 mM showed similar paclitaxel sensitising effect in both p53 wild type and p53 null cells. Further investigation is required to provide compelling evidence on the exact mechanism of p53 involvement on VPA-based sensitization of paclitaxel.

The present study also demonstrated that VPA exposure of BHY cells led to the reduction of *AURKA* mRNA expression. This suggests that *AURKA* transcription is under epigenetic control.¹⁸ While the mechanism behind VPA-mediated sensitisation to paclitaxel is still unclear, the reduction of *AURKA* expression may be one of the mediators due to the fact

that higher levels of *AURKA* transcripts are associated with poor prognosis of NSCLC.¹⁹

Conclusion

In conclusion, the results indicate that HDAC inhibitors could be beneficial in sensitising RTC cells to paclitaxel, which is a very common and inexpensive chemotherapeutic agent. Such sensitisation could lead to lowering the effective dose of paclitaxel and subsequently reducing the adverse effects of this drug to the patient. Additional preclinical and clinical evidence is required to provide further support to our observation. The great advantage of VPA is that it is in routine clinical use for many years demonstrating minor side effects. Further research is required to establish the exact molecular mechanisms modulating this epigenetic sensitisation of cancer cells to paclitaxel.

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Conflicts of interest

None. ■

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