A novel E2F/Sphingosine kinase 1 axis regulates anthracycline response in squamous cell carcinoma

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Abstract

Purpose: Head and neck squamous cell carcinomas (HNSCC) are frequently drug resistant and have a mortality rate of 45%. We have previously shown that E2F7 may contribute to drug resistance in SCC cells. However, the mechanism and pathways involved remain unknown.

Experimental Design: We used transcriptomic profiling to identify candidate pathways that may contribute to E2F7-dependent resistance to anthracyclines. We then manipulated the activity/expression of the candidate pathway using overexpression, knockdown and pharmacological inhibitors in in vitro and in vivo models of SCC to demonstrate causality. In addition, we examined the expression of E2F7 and a downstream effector in a tissue microarray (TMA) generated from HNSCC patient samples.

Results: E2F7-deficient keratinocytes were selectively sensitive to doxorubicin and this was reversed by overexpressing E2F7. Transcriptomic profiling identified Sphingosine kinase 1 (Sphk1) as a potential mediator of E2F7-dependent drug resistance. Knockdown and overexpression studies revealed that Sphk1 was a downstream target of E2F7. TMA studies showed that E2F7 overexpression correlated with Sphk1 overexpression in human HNSCC. Moreover, inhibition of Sphk1 by shRNA or the Sphk1 specific inhibitor, SK1-I (BML-EI411), enhanced the sensitivity of SCC cells to doxorubicin in vitro and in vivo. Furthermore, E2F7 induced doxorubicin resistance was mediated via Sphk1-dependent activation of AKT in vitro and in vivo.

Conclusion: We identify a novel drugable pathway in which E2F7 directly increases the transcription and activity of the Sphk1/S1P axis resulting in activation of AKT and subsequent drug resistance. Collectively, this novel combinatorial therapy can potentially be trialed in humans using existing agents.
Translational Relevance

Head and neck squamous cell carcinoma (HNSCC) is one of the most prevalent cancers diagnosed worldwide. Current chemotherapies are not considered a curative option for HNSCC. Thus, there is a need for new and selective therapies. In this regard, the E2F family of transcription factors has been shown to contribute to the development and maintenance of HNSCC. However, E2F-based therapies are currently not available. To circumvent this problem we embarked on a transcriptomics screen to identify factors that were responsible for E2F7-dependent resistance to anthracyclines in HNSCC. The present study demonstrates that E2F7 directly controls the expression of Sphk1 resulting in increases in AKT phosphorylation which drives drug resistance. Thus, we have identified a previously undescribed E2F7/Sphingosine kinase 1/Sphingosine-1-phosphate/AKT axis that contributes to anthracycline resistance in HNSCC. A significant implication of this finding is that combining an anthracycline with a Sphk1 inhibitor may provide a curative option for treating HNSCC.
**Introduction**

Head and neck squamous cell carcinomas (HNSCC) arise from stratified squamous epithelia of the mucosae of the upper aerodigestive tract. At present the mainstay of treatment for advanced HNSCC is surgery and/or radiation plus adjuvant chemotherapy (1). The use of adjuvant chemotherapy provides modest improvements to overall survival but are not considered curative in their own right (1). Thus, if we are to improve outcomes in patients with advanced HNSCC we need to develop systemic therapies that target novel pathways activated in HNSCC cells.

HNSCC is a complex cancer associated with a large mutational burden (2, 3) and accompanied by dysregulation of proliferation, differentiation and apoptosis. HNSCC is also accompanied by dysregulation of the main functions of the E2F transcription factor family (4, 5). E2F refers to a family of 10 gene products from 8 genes (E2Fs 1, 2, 3a, 3b, 4, 5, 6, 7a, 7b, 8) that have been broadly divided into activators (E2F1-E2F3a) and inhibitors (E2F3b and E2F4-E2F8) (6). The E2F family regulates a diverse array of functions such as proliferation, differentiation, apoptosis and stress responses (7, 8). The way in which the E2F family coordinate such diversity of action is through isoform specific functions of the individual E2Fs (e.g. activators vs inhibitors) coupled with context-specific interacting partner proteins such as pocket proteins and HDACs (7, 8). In the context of keratinocytes (KCs), it has been shown that normal human and murine KCs express all members of the E2F family with the exception of E2F6 (9, 10). It has been shown that proliferation and differentiation of KCs is regulated by the opposing actions of E2F1 and E2F7 (4, 9, 11, 12). Significantly, E2F1 and E2F7 are overexpressed in patient SCCs (10) and contribute to the development of cutaneous SCC (13, 14).
In addition, to the role of E2Fs in proliferation and differentiation, E2Fs are also key regulators of apoptosis and stress responses (7, 8). For example, E2F1 has been shown to have potent pro-apoptotic actions that regulate the numbers of thymic lymphocytes (15). Intriguingly, E2F1 mediated-apoptosis has been reported to be via p53-dependent and p53-independent pathways (16) suggesting that cellular context may determine the mechanism by which E2F1 induces apoptosis. More recently, E2F7 was reported to antagonise the pro-apoptotic actions of E2F1 in the context of etoposide or doxorubicin induced DNA damage (10, 17). Thus, the ratio of E2F1 to E2F7 determines apoptotic responses. However, the mechanism by which E2Fs control apoptotic responses remains unknown. In the present study, we examined downstream effectors of E2F7 that modulate resistance to chemotherapy. We now identify a previously undescribed E2F7/Sphk1/S1P/AKT axis that contributes to anthracycline resistance in SCC. In addition, we identify a novel drug combination that could represent a potentially curative option for advanced SCC.
Materials and Methods

Animal studies

All animal experiments were approved by the Institutional Animal Ethics Committee. 
$E2F7^{Flox/Flox}$, $E2F8^{Flox/Flox}$ and $E2f1$ KO mice have been described (15, 18). FVB X C57BL/6 crosses were generated in house. In vivo tumour studies used female nonobese diabetic/severe combined immunodeficient (NOD/SCID).

Reagents and viability assays

The following drugs were purchased; doxorubicin (Sigma Aldrich), SK1-I (BML-EI411) (Enzo Life Sciences), S1P (Cayman Chemicals). Stocks of BGT226 were prepared as described (19). Viability was determined using trypan blue, Cell Titer 96 Aqueous One Solution Cell Proliferation Assay (Promega), or western blot for cleaved caspase 3 or PARP cleavage as described (20, 21). Sphk1 activity and S1P levels were estimated using commercially available kits (Echelon Biosciences).

Tissue culture and adenovirus infection

Murine epidermal keratinocytes (MEKs) and human epidermal keratinocytes (HEKs) were isolated and cultured as described (22, 23). SCC25 cells were obtained from the American Type Culture Collection. FaDu was a kind gift from Dr. Elizabeth Musgrove (Garvan Institute, New South Wales, Australia) and were verified by short tandem repeat genotyping (11). KJDSV40 cells were maintained as described previously (11). To generate E2F7 and E2F8 KO keratinocytes, we incubated MEKs with ready-to-use Ad-CMV-Cre as per manufacturer’s recommendations (MOI of 50) (Vector Biolabs).

Gene expression studies
Total RNA was isolated, cDNA prepared and quantitative reverse transcriptase PCR (qRT-PCR) performed as described (10, 24). For microarray analysis, complementary RNA was generated with the Illumina TotalPrep RNA Amplification Kit and hybridised with Illumina HumanHT-12 v4 Expression BeadChips (Illumina) as per manufacturer’s protocol. Expression data from the microarrays was analysed as previously described (25). The microarray data reported in this article have been deposited in NCBI’s Gene Expression Omnibus (GEO) database under the accession number GSE58074. Chromatin immunoprecipitation (ChIP) was conducted using the SimpleChIP Enzymatic Chromatin IP Kit (Magnetic Beads) (Cell Signaling) in accordance with the manufacturer’s instructions.

shRNA studies, siRNA delivery and transfections

Control and overexpression plasmids and siRNAs used for manipulating E2F7 have been described previously (10, 17). SureSilencing shRNA plasmids directed against Sphk1 were purchased from SuperArray Bioscience Corp. A Sphk1 expression (TrueORF Gold Clones) and control plasmids were purchased from OriGene Technologies.

Immunoblot

The following primary antibodies were used: E2F-1 (C-20) 1:1,000 (Santa Cruz), Anti-E2F7 1:2,000 (Abcam), cleaved caspase-3 (Asp 175) 1:1,000 (Cell Signaling), Anti-SPHK1 1:1,000 (Sigma), PARP 1:1,000 (Cell Signaling), phospho-Akt (Ser473) (D9E) XP 1:2,000 (Cell Signaling), Akt 1:2,000 (Cell Signaling), and β-actin 1:10,000 (Sigma Aldrich). Where a western blot has been quantitated, results represent relative protein levels normalised to β-actin as quantified by Image J (Wayne Rasband, National Institutes of Health, USA).

Immunohistochemistry and tissue microarrays
Immunohistochemistry was conducted as described (20, 21). The following primary antibodies were used: PCNA 1:3,000 (Sigma Aldrich), cleaved caspase-3 (Asp 175) 1:50 (Cell Signaling), phospho-Akt (Ser473) (D9E) XP 1:50 (Cell Signaling). Secondary antibody was Starr Trek Universal HRP Detection System followed by colorimetric immunohistochemical staining with Cardassian DAB Chromogen as per manufacturer’s instructions (Biocare Medical). TMAs were generated using duplicate 1 mm cores of matched a) adjacent normal tissue, b) primary HNSCC lesion and c) matched metastatic lymph node from patients treated for HNSCC at the PAH. Immunohistochemistry was conducted using Dako EnVision + System-HRP (DAB) kit in accordance with the manufacturer’s instructions. Sections were incubated with Anti-E2F7 1:250 (Abcam) and Anti-SPHK1 1:75 (Sigma Aldrich) antibodies. Staining intensity was evaluated by two Pathologists in a blinded fashion using a modified quickscore method as described (26).

Statistical analysis

Statistical significance was calculated by a Student’s t test with a 95% confidence level using GraphPad Prism v5 (GraphPad software).
Results

E2F7 selectively regulates cytotoxic responses to doxorubicin in KCs

To examine the downstream pathways involved we generated primary cultures of MEKs from E2f1 KO mice (15), or from E2f7Flox/Flox or E2f8Flox/Flox mice (18). We generated E2f7, and E2f8 knock down (KD) MEKs via adenovirus (Ad) mediated Cre deletion of floxed sequences in primary KCs isolated from E2f7 and E2f8 flxed mice. E2f1 gene expression levels in KCs isolated from conventional E2f1KO mice were reduced by 70% whilst E2f7 and E2f8 mRNA expression was reduced more than 90% following 48 hour infection of the cognate flxed KCs with Ad-CMV-Cre (Figure S1). The reduction in E2f1 expression was less than expected but sequencing confirmed the PCR product was E2f1. Significantly, infection with an empty Ad viral vector did not alter cell viability, mRNA expression, differentiation-competence or cytotoxic responses to UVB, doxorubicin or cisplatin (Figure S2).

We examined the dose-dependent cytotoxic profiles of uninfected control, E2f7 KD, E2f8 KD and E2f1KO cells to increasing concentrations of doxorubicin (0-1 µM) for 48 hours. E2f7 deficient MEKs were hypersensitive to the cytotoxic actions of doxorubicin (Figure 1A) or another anthracycline, epirubicin (Figure S3A). Significantly, E2f7 deficiency only had minimal effect on cisplatin sensitivity (Figure S3B) and no impact on etoposide sensitivity (Figure S3C). E2f8 had no effect on cytotoxic responses to any of the drugs (Figures 1A and S3) whilst E2f1 deficiency resulted in modest protection against doxorubicin-induced cytotoxicity (Figure 1A). To confirm that the effect of E2f7 deficiency was attributable to E2f7 we reintroduced E2f7 into E2f7-deficient KCs to confirm that it suppressed doxorubicin sensitivity. Reintroduction of E2f7 into E2f7 deficient MEKs resulted in a 2.5 fold increase in E2F7 mRNA expression determined by qRT-PCR and was sufficient to re-instate
doxorubicin resistance (Figure 1B). We then determined whether E2f7-mediated reduction in cell survival is due to activation of apoptotic pathways. Uninfected control MEKs isolated from floxed E2f7 mice showed a modest increase in cleaved caspase-3 levels after 48 hours treatment with 0.3 µM doxorubicin (Figure 1C). In contrast, there was a profound activation of caspase-3 when E2f7 deficient MEKs were treated with 0.3 µM doxorubicin (Figure 1C). Combined, these data identify a unique and isoform-specific function of E2f7 in modulating sensitivity to anthracyclines in KCs.

**Dysregulation of E2F7 expression in human SCC cell lines contributes to insensitivity to the cytotoxic action of doxorubicin**

It is reasonable to speculate that the overexpression of E2F7, observed in human SCCs (10), may invoke insensitivity to doxorubicin. We screened a suite of SCC cell lines (FaDu, KJDSV40 and SCC25) as well as normal human epidermal keratinocytes (HEKs) for their sensitivity to doxorubicin-induced cytotoxicity (Figure 1D). These studies showed that KJDSV40 cell lines were the most sensitive to doxorubicin treatment with 70% reduction in cell viability at 1 µM (Figure 1D). On the other hand, SCC25 cells displayed the least sensitivity where 80% of the cells were still viable at 1 µM (Figure 1D). Examination of E2F1 and E2F7 protein expression levels demonstrated that insensitive SCC25 cells had high levels of E2F7 to E2F1 whilst sensitive KJDSV40 cells had low levels of E2F7 relative to E2F1 (Figure 1E). Overall the insensitive SCC25 cells had 10 fold greater E2F7 expression relative to E2F1 than did sensitive KJDSV40 cells. It was not possible to generate values for the HEKs due to their low levels of expression of E2F1 and E2F7.

We next sought to determine whether selective upregulation or reduction of E2F7 expression by expression plasmid or siRNA (validation of siRNA is shown in Figure S4A), respectively,
would change the dose response profile of KJDSV40 and SCC25 cells to doxorubicin. Results showed that previously sensitive KJDSV40 cells became resistant to doxorubicin compared to vector only control cells when transfected with E2F7 expression plasmid (Figure 1F). In contrast, silencing of E2F7 in insensitive SCC25 cells resulted in a 2.2 fold reduction in E2F7 mRNA expression determined by qRT-PCR and could enhance doxorubicin-induced cytotoxicity compared to control siRNA transfected SCC25 cells (Figure 1G). These data unequivocally demonstrate that resistance to doxorubicin is E2F7-dependent in SCC cells.

**Sphingosine kinase 1 (Sphk1) is a downstream effector of E2F7-mediated suppression of doxorubicin-induced cytotoxicity**

To identify the downstream effectors of E2F7 in SCC cells, we generated transcriptomic profiles for differentially expressed transcripts between the KJDSV40 cells (sensitive and low E2F7/E2F1 ratio) and SCC25 cells (insensitive and high E2F7/E2F1 ratio). We also generated a list of upregulated genes between SCC25 cells and SCC25 cells in which E2F7 had been silenced with siRNA. This latter list identified E2F7-modulated transcripts which were then cross-referenced against the list of genes identified as downregulated in KJDSV40 cells compared with SCC25 cells. A detailed explanation of this analysis is being prepared for publication elsewhere (Hazar-Rethinam et al, in preparation). By selecting for genes with a B-value greater than 3 (exceeding the 95% confidence interval) and a fold change greater than 1, we were able to identify 4 genes (Sphk1, RACGAP1, CD44, RRP8) that were differentially upregulated.

Of the transcripts identified in our screen, sphingosine kinase 1 (Sphk1) was the most significantly overexpressed. Sphk1 is a kinase responsible for the conversion of sphingosine to sphingosine-1-phosphate (S1P) (27-29). Interrogation of publicly available microarray data
indicates statistically significant increases in Sphk1 expression in breast, colon, lung, ovary, prostate, melanoma, stomach, uterus as well as squamous cell carcinoma and its precursor actinic keratosis (28). Importantly, Sphk1 has been shown to modulate proliferation, differentiation and apoptosis in KCs (30). Facchinetti and colleagues recently reported that Sphk1 is overexpressed in malignant oral epithelia compared with nonmalignant tissue, and the expression of Sphk1 was correlated with poor prognosis, shorter patient survival and loss of p21 expression in HNSCC (30). Of relevance to the present study, Bonhoure and colleagues reported that forced expression of Sphk1 led to resistance to doxorubicin- and etoposide-induced cell death in HL-60 leukemia cells (31), and degradation of Sphk1 resulted in induction of apoptosis in MCF7 breast cancer cells treated with doxorubicin (32). Thus, there is sufficient evidence to speculate that a novel E2F7/Sphk1/S1P axis may exist in SCC cells that regulates doxorubicin sensitivity.

Quantitative RT-PCR was used to confirm that Sphk1 was more highly expressed in SCC25 cells than in KJDSV40 cells (Figure 2A). Consistent with this, we show that transfection of SCC25 cells with siRNA directed against E2F7 resulted in profound inhibition of Sphk1 expression (Figure 2A) whilst E2F1 mRNA expression was derepressed (Figure S4B). Similarly, Sphk1 activity was significantly elevated in SCC25 cells compared with KJDSV40 (Figure 2B). Moreover, we showed that knockdown of E2F7, by siRNA, in SCC25 cells reduced Sphk1 protein expression (Figure 2C) whilst transient overexpression of E2F7 in KJDSV40 cells resulted in an increase in Sphk1 protein levels (Figure 2C). These data suggested that Sphk1 may be a downstream effector of E2F7-induced resistance to doxorubicin. As shown in Figure 2D (Left and center panels), E2F7 overexpression did not protect from Sphk1 knockdown or SK1-I-enhanced cytotoxicity in KJDSV40 cells. Similarly, Sphk1 overexpression in SCC25 cells overrides doxorubicin sensitivity induced by E2F7.
siRNA (Figure 2D, Right panel). These data unequivocally demonstrate that Sphk1 is the downstream effector of E2F7-dependent sensitivity of SCC cells to doxorubicin. It remains unclear whether Sphk1 is a direct or indirect target of E2F7. In this regard, ChIP assays showed that E2F7 could bind the Sphk1 and E2F1 promoters in SCC25 cells compared with low levels of binding in KJDSV40 cells indicating that the Sphk1 and E2F1 promoters are direct binding targets of E2F7 (Figure 2E).

Next, we sought to determine whether there was evidence that Sphk1 was overexpressed in primary human SCC tumours. We have generated tissue microarrays (TMAs) comprising duplicates of normal, primary tumour and matched metastasis from HNSCC patients treated at the Princess Alexandra Hospital (PAH), Queensland, Australia. The TMAs were stained for E2F7 and Sphk1 protein expression by immunohistochemistry and scored by two Pathologists. Figure 2F shows that Sphk1 and E2F7 are overexpressed in HNSCC compared to matched adjacent normal tissue. Figure 2F also shows that primary tumour and metastatic tumour do not differ significantly in the levels of E2F7 or Sphk1.

Sphk1 inhibition sensitizes SCC cells to doxorubicin-induced cytotoxicity

In order to determine whether Sphk1 contributes to doxorubicin sensitivity, we studied the effects of silencing Sphk1 in insensitive SCC25 cells. Sphk1 gene silencing was achieved via shRNA and caused a marked decrease in Sphk1 protein level (Figure 3A), Sphk1 enzyme activity (Figure 3B) and S1P (a product of Sphk1) measured in cell lysates (Figure 3C) and significantly enhanced sensitivity of SCC25 cells to doxorubicin (Figure 3D). Conversely, overexpression of Sphk1 in insensitive KJDSV40 cells resulted in increases in Sphk1 protein level (Figure 3E), enzyme activity (Figure 3F), S1P production (Figure 3G) and reduced sensitivity to doxorubicin compared to vector control (Figure 3H). S1P is the product of Sphk1-catalysed phosphorylation of sphingosine, and has been shown to mediate the anti-
apoptotic effects of Sphk1 (27, 29). Consistent with this, we found that treatment of KJDSV40 cells with 1 μM S1P reduced cytotoxicity by 2.6 fold (Figure 3I). Combined, these data indicate that sensitivity to doxorubicin is mediated via a novel E2F7/Sphk1/S1P axis in SCCs.

**Knockdown of Sphk1 sensitizes resistant SCC cells to doxorubicin-induced cytotoxicity in vivo**

Our data suggest that inhibition of Sphk1 activity, in combination with doxorubicin, may be a viable therapeutic strategy for treating SCC. To answer this question, SCC25 cells were constructed to stably express either vector control or Sphk1 shRNA and inoculated in NOD/SCID mice. When tumours were approximately 3 mm in diameter, mice were randomized into four groups and treated with vehicle dimethyl sulfoxide (DMSO) or 0.5 mg/kg doxorubicin by intraperitoneal (i.p.) injections twice per week (Figure 4A). Treatment of mice bearing vector control SCC25 tumours with/without 0.5 mg/kg doxorubicin had minimal effect on body weight (Figure 4B) or tumour growth rates (Figure 4C). Knockdown of Sphk1 in SCC25 cells did not affect tumour growth in vivo. In contrast, Sphk1 deficient SCC25 tumours treated with doxorubicin started to regress by day 7 post-treatment (Figure 4C) with no effect on body weight (Figure 4B). Strikingly, on day 13 post-treatment there was a complete loss of tumours in doxorubicin treated mice inoculated with SCC25/Sphk1shRNA cells (Figure 4C).

We next examined whether we could achieve similar tumour regression when tumours are larger at the commencement of therapy. Since the tumours derived from Sphk1 deficient SCC25 cells treated with vehicle (Figures 4C and 4D; blue triangle) were similar in growth rate and size to tumours in mice bearing vector control transfected SCC25 tumours that had
been treated with 0.5 mg/kg doxorubicin (Figures 4C and 4D; red square), we started to treat these mice when their tumours reached around 0.5 cm$^3$ with 0.5 mg/kg doxorubicin (Figure 4D). As shown in Figure 4D, doxorubicin treatment dramatically reduced the tumour volume showing profound regression one week after doxorubicin was started in Sphk1 deficient group of animals as compared with those inoculated with control vector (Figure 4D). All mice were sacrificed at day 28 post-treatment when the tumour burden in the control mice reached the ethically approved maximum size. Upon autopsy, the mice inoculated with the Sphk1-deficient SCC cells (Figures 4C and 4D; green triangle) only contained a fragile cluster of cellular material that could not be harvested for histopathology.

The Sphk1 specific inhibitor, SK1-I (BML-EI411), sensitises SCC cells to doxorubicin in vitro and in vivo

SK1-I is a water-soluble sphingosine analog with a Ki value of approximately 10 µM which potently inhibits Sphk1 activity (33). Importantly, SK1-I does not significantly inhibit SPHK2, PKA, AKT1, ERK1, EGFR or CDK2 (33). We treated SCC25 cells with increasing doses of SK1-I for 48 hours and then measured Sphk1 enzyme activity. As anticipated, SK1-I significantly reduced Sphk1 activity in a dose-dependent manner, indicating inhibition of Sphk1 activity (Figure 5A). Moreover, we confirmed that the inhibition was not due to the loss of Sphk1 protein expression (Figure 5B).

Next, we investigated whether Sphk1 specific inhibition can enhance the cytotoxic effects of doxorubicin in insensitive SCC25. After 48 hours incubation with SK1-I alone, the viability of control HEK (Figure S5A) and resistant SCC25 cells did not change (Figure 5C). However, treatment of doxorubicin-resistant SCC25 cells with 1 µM doxorubicin with increasing doses of SK1-I resulted in profound and dose dependent loss of cell viability.
(Figure 5C). Predictably, SK1-I did not enhance doxorubicin sensitivity in KJDSV40 cells (Figure S5B). In contrast to SCC25 cells, the addition of increasing doses of SK1-I to doxorubicin, in HEKs, did not enhance the cytotoxicity obtained with doxorubicin alone (Figure S5A). Next, we examined whether the cell death effects of SK1-I and doxorubicin were mediated via apoptosis. Consistent with an apoptotic reaction, we observed increases in cleaved caspase-3 and cleaved PARP1 in response to doxorubicin + SK1-I (Figure 5D).

We inoculated NOD/SCID mice with SCC25 cells and allowed tumours to establish subcutaneously. When tumours were around 4 to 5 mm in diameter, mice were randomized into six groups and treated with DMSO, 0.5 mg/kg doxorubicin, 5 mg/kg SK1-I, 10 mg/kg SK1-I, 5 mg/kg SK1-I + 0.5 mg/kg doxorubicin or 10 mg/kg SK1-I + 0.5 mg/kg doxorubicin by i.p. injection twice per week. Treatment with 5 and 10 mg/kg SK1-I was well tolerated by the NOD/SCID mice, and the body weights remained stable (Figure 5E). Tumours in animals treated with 5 and 10 mg/kg doses of SK1-I alone showed modest, yet significant, decreases in tumour growth rate (Figure 5G). Doxorubicin treatment alone did not affect the tumour size (Figures 5F and 5G). However, in sharp contrast to doxorubicin treatment alone, treatment with 10 mg/kg SK1-I + 0.5 mg/kg doxorubicin as well as 5 mg/kg SK1-I + 0.5 mg/kg doxorubicin resulted in profound regression of explanted tumours (Figures 5F and 5G). After 13 days post-treatment, animals had to be sacrificed due to the tumour burden in control mice. Tumours were excised, photographed and histologically examined (Figure 5H). The benefit of combining SK1-I with doxorubicin was not restricted to the SCC25 cell line. Specifically, we show that the FaDu cell line displays intermediate sensitivity to doxorubicin in vitro and in vivo and are completely insensitive to SK1-I in vitro and modestly so in vivo (Figure S6). However, combining doxorubicin + SK1-I in vitro or in vivo induces profound cytotoxicity (Figures S6A and S6B).
Sphk1/S1P has been shown to exert its antiapoptotic activity via signaling through a family of S1P receptors linked to the PI3K/AKT pathway (34, 35). Consistent with this, we found that SK1-I could reduce phospho-AKT (p-AKT) (Ser473) in a dose dependent manner (Figure 6A). Previous reports have shown that p-AKT is a downstream effector of the pro-survival effects of increased Sphk1 activity and S1P production (36). Moreover, it is established that the PI3K/AKT pathway is frequently dysregulated via mutations in PI3K family members, gene amplifications or pathway activation in HNSCC (37). Thus, we examined whether E2F7 induced prosurvival responses were mediated via increased Sphk1/S1P and subsequent AKT phosphorylation. Transient overexpression of E2F7 in KJD SV40 cells results in an increase in Sphk1 protein levels (Figure 2C, Bottom) and an increase in the p-AKT relative to total levels (Figure 6B). Conversely, siRNA-induced knockdown of E2F7 in SCC25 cells resulted in a reduction in Sphk1 expression (Figure 2C, Top) and a reduced p-AKT/total AKT ratio (Figure 6C). Furthermore, transient overexpression of Sphk1 in KJD SV40 cells or knockdown of Sphk1 with shRNA in SCC25 cells resulted in increased and decreased p-AKT respectively (Figures 6D and 6E). These data indicate that the changes in AKT activity lie downstream of Sphk1, which in turn is downstream of E2F7. These data would predict that the profound tumour regression observed as a result of a doxorubicin/SK1-I combination could be recapitulated using doxorubicin + AKT inhibitor.

We have previously shown that the mTOR/PI3K inhibitor, BGT226, is able to reduce tumour growth rates in mice transplanted with SCC cells (19). In the present study, mice were injected with SCC25 cells and when the tumours were between 4 to 5 mm in diameter we treated them with i) vehicle, ii) 0.5 mg/kg doxorubicin i.p. twice weekly, iii) 10 mg/kg BGT226 i.p. twice weekly or iv) 10 mg/kg BGT226 + 0.5 mg/kg doxorubicin i.p. twice
weekly. Tumour growth in the vehicle control (DMSO) and doxorubicin treated mice was unchanged whilst those mice treated with BGT226 alone displayed a modest reduction in tumour growth rate (Figure 6F). Mice treated with the doxorubicin/BGT226 combination displayed significant regression of the tumour mass (Figure 6F).

We next examined levels of PCNA, cleaved caspase-3 and p-AKT levels within the tumours resected from mice treated with 10 mg/kg SK1-I or 10 mg/kg BGT226, alone or in combination with 0.5 mg/kg doxorubicin. Immunohistochemical examinations showed that combination treatment inhibited intratumoral proliferation (at either dose) as measured by the levels of PCNA staining (Figure 6G). SK1-I or BGT226 treatment markedly elevated the number of apoptotic cells induced by doxorubicin treatment as shown by examination of apoptotic indices of tumours by immunohistochemical staining with antibody against cleaved caspase-3 compared with drug alone treated tumours (Figure 6G). Consistent with p-AKT lying downstream of Sphk1 we found significant inhibition of p-AKT (Ser473) in tumours treated with SK1-I or BGT226 (Figure 6G).

**DISCUSSION**

In the present study we provide, *in vitro, in vivo* and patient data that identifies a novel E2F7/Sphk1/S1P/AKT axis that regulates sensitivity to anthracyclines in SCC. Specifically, we show that (i) E2F7 selectively modulates sensitivity to doxorubicin in KCs and SCC, (ii) that E2F7-dependent doxorubicin resistance is mediated *via* induction of Sphk1 which in turn activates AKT and (iii) that pharmacological inhibition of Sphk1 or AKT sensitizes SCC cells to the cytotoxic actions of doxorubicin *in vitro* and *in vivo*. Combined, these findings highlight a novel mechanism through which SCC cells acquire resistance to anthracyclines.
Overall, current data relating to the mechanisms regulating E2F control of apoptosis are complex. For example, E2F1 is known to be induced by cytotoxic stimuli and DNA damage (7, 8). This induction can occur at the level of post-translational modification and protein stabilization and/or can occur through increased E2F1 transcription (16, 38). The main outcome of the increased E2F activity is mediated via ARF stimulated inhibition of MDM2 resulting in increased p53-dependent apoptosis. However, complicating this is the observation that E2F1 can recognize double strand breaks induced by UV and recruit NER machinery to the DNA break (16). In this way E2F1 has been proposed to display anti-apoptotic actions (16). This latter pathway has been demonstrated to exist in normal MEKs (39). Further complicating this is the observation that E2F7 can antagonize the pro-apoptotic activity of E2F1 in embryonic tissues as well as in normal or cancer cells (10, 17, 18, 40). Thus, in order to determine the role of E2F7 in regulating cytotoxic responses in HNSCC it is important to consider the tissue context and the nature of the cytotoxic stimulus or DNA damage.

In the present study we show that E2F7 and E2F1 suppress and induce doxorubicin sensitivity in SCC cells respectively. We also show that cytotoxic responses to etoposide or cisplatin were not altered by E2F7. Since doxorubicin and etoposide are established type II topoisomerase blocking agents, and E2F7 did not alter etoposide sensitivity it is reasonable to suggest that the effects of E2F7 were independent of the topoisomerase inhibitory actions of doxorubicin. We also show that regulation of doxorubicin sensitivity in SCC cells is E2F isoform-specific since the other inhibitory E2F, E2F8, did not modify the sensitivity of KCs to doxorubicin or any other drug studied. In addition, we show that E2F7 suppresses doxorubicin sensitivity via increases in the expression of Sphk1 resulting in increased levels of S1P which in turn enhance the Ser473 p-AKT-dependent pro-survival response. The E2F-
dependence of S1P/AKT-mediated drug resistance has not been described before and has significant pathological and clinical implications in SCC.

The relevance of an E2F/Sphk1/S1P/AKT axis in SCC is highlighted by a number of independent observations. In particular E2F1 (5, 11), E2F7 (10), Sphk1 (30, 42), S1P (41), PI3K and AKT (37) are all increased in SCC. Part of these increases may be explained by activation of signaling pathways that regulate their activity/expression such as MAPK-mediated activation of AKT and/or Sphk1 (35, 37) or disrupted Rb activity mediated \textit{via} p16 deletion or cyclin D amplification for E2F1/E2F7 (7, 8). However, PI3K/AKT is commonly mutated or amplified in SCC (2, 3, 37). Regardless of the underlying mechanism it is clear that the individual members of the E2F/Sphk1/S1P/AKT axis are all overexpressed and active in SCC. Whilst the events that initiate disruption of this axis remain unknown it is likely that dysregulation of the E2F/Rb or PI3K/AKT pathway result from mutational events early in tumour formation which could lead to overexpression of E2F7 (a direct E2F1 target) and Sphk1 (a direct E2F7 target). The mechanism by which E2F7 induces Sphk1 transcription was not established in this study. Although E2F7 is an established transcriptional repressor, it has recently been reported that an E2F7-HIF1\(\alpha\) transcriptional complex activates the transcription of VEGFA (42). Thus, it is a formal possibility that E2F7 may be a direct activator of Sphk1 transcription. Alternatively, E2F7 could act in a dominant-negative manner by blocking the binding of other E2F repressor complexes. Regardless of the mechanism, our functional data shows that E2F7 regulates S1P levels \textit{via} induction of Sphk1 expression.

Sphingolipid metabolites have emerged as bioactive signaling molecules that regulate cell movement, differentiation, survival, inflammation, angiogenesis, tumorigenesis and
immunity (29). In particular, ceramide and sphingosine have been shown to be profoundly pro-apoptotic whilst phosphorylated sphingosine (S1P) is profoundly anti-apoptotic (27-29). Thus, the kinase, Sphk1, responsible for catalyzing the conversion of sphingosine to S1P, is also responsible for changing the physiology of the cell from pro-apoptotic to anti-apoptotic.

Many of the anti-apoptotic effects of S1P are mediated via a family of G protein coupled S1P receptors which in turn activate PI3K/AKT (27-29, 43). Interestingly, the use of an AKT inhibitor was able to induce modest levels of cell death and reduced tumour growth in vivo. Given that Sphk1 inhibitors profoundly inhibited p-AKT (Ser473), these data would suggest that some of the cytotoxic effects observed for BGT226 alone may be mediated via non-AKT targets. Finally, doxorubicin alone displayed no measurable anticancer activity in our xenotransplant model. These data suggest that the cytotoxicity observed with the SK1-I or BGT226 plus doxorubicin combination reflects an unidentified synthetic lethal reaction. The clinical potential for this novel combination (e.g. Sphk1 or AKT inhibitor combined with an anthracycline) is highlighted by the profound tumour regression observed in this study.

A previous report had shown that the activation of AKT in ovarian and breast cancer suppressed E2F1-induced apoptosis and was associated with a poor prognosis and chemoresistance (44). Similarly, Reimer and colleagues reported that poor prognosis and chemoresistance of ovarian tumours was associated with a high E2F7/E2F1 ratio (45). We now provide an integrated model in which E2F7 is causally linked to the overexpression of Sphk1, the activation of the AKT pathway and doxorubicin resistance. This is definitively shown by our observation that Sphk1, S1P and p-AKT (Ser473) are all directly modulated by E2F7. Secondly, Sphk1 inhibition or overexpression directly effects the Ser473 phosphorylation of AKT. Finally, inhibition of Sphk1 or AKT sensitises SCC cells in vivo to the cytotoxic effects of doxorubicin. The observation that anti-apoptotic effects of S1P are
mediated via the PI3K/AKT pathway has been previously reported (35, 36). What is new in our study is that the dysregulation of the E2F pathway, in SCC, directly activates the Sphk1/S1P/PI3K/AKT pathway resulting in selective resistance to doxorubicin. This is an advance that can be immediately translated to a clinical trial with existing pharmacological agents.
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REFERENCES


**FIGURE LEGENDS**

**Figure 1.** E2F7 selectively regulates sensitivity to doxorubicin in MEKs. A, dose response curve of doxorubicin-induced cytotoxicity at 48 hour in uninfected, E2F1KO, E2F7KD, E2F8KD MEKs. B, dose response curve of doxorubicin-induced cytotoxicity at 48 hour in E2F7KD MEKs which were transfected with either an E2F7b overexpression plasmid, or pcDNA3.1(+) control plasmid. Viability is plotted as percentage control (untreated). C, activation of caspase-3 was determined by immunoblotting extracts of untreated and 0.3 µM doxorubicin treated E2F7 floxed and E2F7 deficient MEKs. β-Actin is a loading control. D, HEK, FaDu, KJDSV40 and SCC25 cells were treated with doxorubicin for 48 hours and viability plotted as percentage of untreated cells. E, E2F1 and E2F7 protein expression was determined by immunoblotting extracts of HEK, KJDSV40 and SCC25 cell lines. β-Actin is provided as a loading control. Densitometric analysis of E2F1 and E2F7 in KJDSV40 and SCC25 cell lines was quantified using ImageJ. Expression level was normalized against β-Actin and plotted as E2F7/E2F1. F, SCC25 cells were transfected with E2F7b overexpression plasmid or pcDNA3.1(+) control plasmid. G, SCC25 cells were treated with siRNA-targeting E2F7 or a control siRNA. In both instances (F and G), cells were left for 48 hours after transfection after which viability was estimated by trypan blue exclusion. Viability was expressed as percent viable cells and plotted as a percent untreated control. Western blot figures are representative of three independent experiments. Quantitative data represent the mean ± SEM obtained from triplicate determinations of three independent experiments for A, B, D, F and G. *, P ≤ 0.05, **, P < 0.01, ***, P < 0.001.

**Figure 2.** Sphk1 is a downstream effector of E2F7 and is elevated in expression in SCCs. A, RNA was extracted from KJDSV40, SCC25 and SCC25 cells in which E2F7 was silenced
with siRNA. Quantitative RT-PCR was used to determine the expression of Sphk1 transcripts. Data are the mean ± SEM of duplicate determinants normalized for expression of the housekeeping gene TBP; \( n = 3 \). B, Sphk1 activity is shown for the KJDSV40 and SCC25 cell lines. Data represent the mean ± SEM obtained from triplicate determinations of three independent experiments. C, (Top) SCC25 cells were transfected with siRNA-targeting E2F7 or a control siRNA. (Bottom) Sphk1 protein levels are shown for KJDSV40 cells in which E2F7 was overexpressed. Immunoblot was used to determine Sphk1 levels 48 hours post-transfection. \( \beta \)-Actin is provided as a loading control. Western blot figure is representative of three independent experiments. D, (Left) empty vector, E2F7 expression plasmid, E2F7 expression plasmid and Sphk1 shRNA plasmids transfected KJDSV40 cells were exposed to 1 \( \mu M \) doxorubicin. (Center) empty vector transfected, E2F7 expression plasmid transfected, E2F7 expression plasmid transfected and 10 \( \mu M \) SK1-I treated KJDSV40 cells were exposed to 1 \( \mu M \) doxorubicin. (Right) control siRNA, E2F7 siRNA, E2F7 siRNA and Sphk1 expression plasmid transfected SCC25 cells were exposed to 1 \( \mu M \) doxorubicin. Viability was assessed 48 hours post-treatment and is expressed in arbitrary units. E, quantitative determinations of E2F7 binding to the E2F1 and Sphk1 promoters. ChIPs were performed using an E2F7 antibody or non-immune IgG as control in KJDSV40 and SCC25 cell lines. Each ChIP and quantitative RT-PCR were repeated, respectively, 3 and 2 times. SDs refer to the 3 independent experiments. F, quantitation of E2F7 and Sphk1 staining intensity in matched samples of primary tumour, its matched normal squamous epithelium and lymph node metastasis (\( n = 37 \)). Tissue sections were scored using a modified quickscore method to determine the percentage of cells stained (0-100%) and the intensity of staining (1+ to 3+). Data is shown as the mean ± SEM. **, \( P < 0.01 \), ****, \( P < 0.0001 \).
Figure 3. Sphk1 contributes at a functional level to doxorubicin sensitivity. A, SCC25 cells were transfected with 4 different constructs coding for shRNAs directed against Sphk1. After 48 hours, Sphk1 protein expression was determined by immunoblotting. β-Actin is provided as a loading control. SCC25 cells were transfected with the Sphk1shRNA.2 and a scrambled shRNA constructs. After 48 hours, B, Sphk1 activity or C, S1P levels were measured. Data presented as percentage of control shRNA. D, Sphk1shRNA and control shRNA transfected SCC25 cells were treated with doxorubicin (1µM) for 48 hours and viability estimated by trypan blue exclusion. Viability was expressed as number of viable cell counts and plotted as percentage control (untreated). E, KJDSV40 cells were transfected with Sphk1 overexpression plasmid or a noncoding empty vector. After 48 hours, Sphk1 protein expression was determined by immunoblotting. β-Actin is a loading control. KJDSV40 cells were transfected with Sphk1 overexpression plasmid or a noncoding empty vector. After 48 hours, F, Sphk1 activity or G, S1P levels were estimated. Data presented as percentage of control vector. H, Sphk1 overexpression plasmid or empty vector transfected KJDSV40 cells were treated with doxorubicin (1µM) for 48 hours before viability was conducted by trypan blue exclusion. Viability was expressed as number of viable cell counts and plotted as percentage control (untreated). I, KJDSV40 cells were treated with varying doses of doxorubicin in the presence or absence of 1 µM S1P. Viability was then assessed and plotted as percentage control (untreated). Western blot figures are representative of three independent experiments. Quantification of S1P in cell lysate from same number of cells was achieved by the S1P ELISA kit. Quantification of S1P was done following the manufacturer’s guidelines. All quantitative data presented as mean ± SEM obtained from triplicate determinations of three independent experiments. *, P ≤ 0.05 versus empty vector, **, P < 0.01 versus control shRNA and versus empty vector. ***, P < 0.001 versus empty vector.
Figure 4. Sphk1 suppression enhanced sensitivity of SCC25 to the cytotoxic actions of doxorubicin in vivo. A, schematic representing xenograft treatment cohorts. All animals were inoculated subcutaneously with SCC25 cells expressing vector alone (scrambled shRNA) or Sphk1 shRNA and tumours allowed establishing till they reached the indicated sizes. Established tumours were then treated with vehicle or 0.5 mg/kg doxorubicin twice per week at day 1 (B, C) or day 13 (D) post-inoculation. B, animal weight was determined twice per week. C, tumour volumes were monitored twice weekly. D, dotted line indicates beginning of treatment for distinct groups. Data presented as mean ± SEM of individual measurements from six mice per group.

Figure 5. Pharmacological inhibition of Sphk1 sensitizes SCC cells to the cytotoxic actions of doxorubicin in vitro and in vivo. A, SK1-I induces a dose-dependent inhibition of Sphk1 activity in SCC25 cells that is independent of alterations in Sphk1 protein expression. β-Actin is provided as a loading control. C, dose response curve of SK1-I alone or in combination with doxorubicin in SCC25 cells was determined following 48 hours of treatment. Viability is plotted as percentage control (untreated). D, cleavage of PARP and activation of caspase-3 was determined by immunoblotting extracts of SK1-I and doxorubicin treated SCC25 cells. β-Actin is provided as a loading control. E, animal weight was determined twice per week. F, tumour growth curves of SCC25 xenografts treated with doxorubicin, SK1-I (5 or 10 mg/kg) or the described combinations. G, after 13 days of treatment, animals were sacrificed and tumours excised. Representative results from distinct groups are shown. H, paraffin-embedded tumour sections were stained with hematoxylin and eosin. Representative images from each group are shown (Bar = 100µm). Western blot figures are representative of three independent experiments. Data represent the mean ± SEM obtained from triplicate determinations of three independent experiments for A and C. Data
represented as mean ± SEM of individual measurements from five mice per group for E and F. *P* value was calculated using Student’s *t* test.

**Figure 6.** Sphk1 Exerts Its Anti-Apoptotic Activity via the PI3K/AKT Pathway. A, p-AKT and total AKT protein levels were determined by immunblotting following treatment of SCC25 cells with varying doses of SK1-I. B, Densitometric analysis of p-AKT and total AKT was determined from immunoblots of KJDSV40 cells in which E2F7 was overexpressed. The protein levels were quantified using ImageJ, normalized for expression of total AKT and plotted as p-AKT/total AKT. C, densitometric analysis of p-AKT and total AKT was determined from immunoblots of SCC25 cells in which E2F7 was silenced. The protein levels were quantified using ImageJ, normalized for expression of total AKT and plotted as p-AKT/total AKT. D, p-AKT and total AKT levels are shown for SCC25 cells in which Sphk1 was overexpressed or E, silenced. F, tumour growth curves of SCC25 xenografts treated with doxorubicin, BGT226 or the described combinations. G, immunostaining for PCNA, cleaved caspase-3 and p-AKT. Representative images of at least three independent tumours are shown for each group (Bar = 100µm). Western blot figures are representative of three independent experiments. Data represented as mean ± SEM of individual measurements from four mice per group for F.
SUPPLEMENTARY INFORMATION

SUPPLEMENTARY FIGURE LEGENDS

Figure S1. Generation of E2F7 and E2F8 deficient murine keratinocytes and the validation of E2F1 levels in E2F1KO mice. A, level of E2F7 and B, E2F8 mRNA expression was measured by qRT-PCR in the relevant floxed keratinocytes following infection with Ad-CMV-Cre. C, quantitative RT-PCR analysis of E2F1 expression in KCs isolated from conventional E2F1KO mice. Expression is plotted as percentage uninfected control for A and B, and as percentage control murine keratinocytes for C. Data are the mean ± SEM of duplicate determinants from 3 biological replicates normalized for expression of the housekeeping gene β-actin.

Figure S2. Adenovirus infection of murine keratinocytes does not alter normal cell responses. A, cell viability 48 hours after infection of control murine keratinocytes (MEKs) with Ad-GFP was measured. B, quantitative RT-PCR was performed on MEKs isolated from control mice and MEKs which had been infected with Ad-Null. Data are the mean ± SEM of duplicate determinants or 3 biological replicates normalized for expression of the housekeeping gene β-actin. C, Uninfected and Ad-Null infected control MEKs were cultured with 1.5 mM Ca²⁺ for 48 hours to induce differentiation. Differentiation marker, involucrin, level was then detected by Western Blotting. β-actin was used as a loading control. Uninfected and Ad-Null infected control MEKs were subjected to varying doses of D, UVB, E, doxorubicin or F, cisplatin. Cell viability was assessed following 48 hours of treatment. Data represent the mean ± SEM obtained from triplicate determinations of three independent experiments for A, B, D, E and F. Western blot figures are representative of three independent experiments.
Figure S3. Cytotoxic responses to doxorubicin selectively enhanced in E2F7-deficient murine keratinocytes. Dose response curve of control, E2F1, E2F7 and E2F8 deficient murine keratinocytes to A, epirubicin, B, cisplatin and C, etoposide. Cells were exposed to drugs for 48 hours. Viability was then assessed and plotted as percentage control (untreated). Data represent the mean ± SEM obtained from triplicate determinations of three independent experiments.

Figure S4. Validation of siRNA directed against E2F7 and E2F1 mRNA expression level in SCC25 cells in which E2F7 had been silenced by siRNA. A, SCC25 cells were transfected with control siRNA or a siRNA for E2F7. Two different siRNAs were tested. Cells were harvested 48 hours after transfection. Knockdown was confirmed with qRT-PCR (Right) and immunoblotting (Left). B, SCC25 cells were transfected with E2F7.siRNA construct 2. Cells were harvested 48 hours after transfection. E2F1 mRNA expression was determined by qRT-PCR. Data are the mean ± SEM of duplicate determinants normalized for expression of the housekeeping gene TBP; n = 3. Western blot figures are representative of three independent experiments. β-actin was used as a loading control.

Figure S5. SK1-I did not enhance doxorubicin sensitivity in KJDSV40 cells in vitro. A, dose response curve of SK1-I alone or in combination with doxorubicin in HEK and B, KJDSV40 cells was determined following 48 hours of treatment. Viability is plotted as percentage control. Data represent the mean ± SEM obtained from triplicate determinations of three independent experiments.
Figure S6. Inhibition of Sphk1 sensitizes FaDu cells to the cytotoxic actions of doxorubicin in vitro and in vivo. A, FaDu cells were treated with vehicle, 1 µM doxorubicin, 30 µM SK1-I or 30 µM SK1-I + 1 µM doxorubicin for 48 hours after which viability was estimated and referenced against vehicle treated controls. Data represent the mean ± SEM obtained from triplicate determinations of three independent experiments. B, tumour growth curves of FaDu-derived xenografts treated with doxorubicin, SK1-I or the described combinations. Data represented as mean ± SEM of individual measurements from two mice per group.
**Figure 2**

A. Relative mRNA Expression

B. Rap1A and c-Src

C. Spk1 and β-Actin

D. Cell viability (IC50, % G0/G1)

E. E2F1 promoter and Spk1 promoter

F. E2F7 and Spk1

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Figure 3

A

B

C

D

E

F

G

H

I

KJDSV40

Cell Viability (%) vs. control

0.0 0.2 0.4 0.6 0.8 1.0

Doxorubicin (μM)

Without SIP

With SIP
Figure 4

A

DMSO

2x10^6 cells

0.5 mg/kg doxorubicin

Day 0  Day 22  Day 25  Day 28  Day 32  Day 35

B

Vector control_vehicle (DMSO)

Vector control_0.5 mg/kg doxorubicin

Sphk1shRNA_vehicle (DMSO)

Sphk1shRNA_0.5 mg/kg doxorubicin

Days of treatment

Weight (g)

0  5  10  15

C

Vector control_vehicle (DMSO)

Vector control_0.5 mg/kg doxorubicin

Sphk1shRNA_vehicle (DMSO)

Sphk1shRNA_0.5 mg/kg doxorubicin

Days of treatment

Tumour volume (mm^3)

0  50  100  150  200

D

Vector control_vehicle (DMSO)

Vector control_0.5 mg/kg doxorubicin

Sphk1shRNA_vehicle (DMSO)

Sphk1shRNA_0.5 mg/kg doxorubicin

Days of treatment

Tumour volume (mm^3)

0  50  100  150  200
Figure 6

A

B

C

D

E

F

G

Control (DMSO)  doxorubicin  SKI-1  SKI-1+doxorubicin

Control (DMSO)  doxorubicin  BGT226  BGT226+doxorubicin

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