Title: APR-246 potently inhibits tumour growth and overcomes chemoresistance in preclinical models of oesophageal adenocarcinoma

Running title: APR-246 inhibits oesophageal adenocarcinoma and overcomes chemoresistance

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Abbreviations: CLX, cell line xenografts; CI, combination index; OAC, oesophageal adenocarcinoma; OSCC, oesophageal squamous cell carcinoma; GI50, 50% growth inhibition; MQ, methylene quinuclidinone; PDX, patient-derived xenografts; TGI, tumor growth inhibition; ROS, reactive oxygen species.

ABSTRACT

Objectives: p53 is a critical tumour suppressor and is mutated in 70% of oesophageal adenocarcinomas (OAC), resulting in chemoresistance and poor survival. APR-246 is a first-in-class reactivator of mutant p53 and is currently in clinical trials. In this study, we characterised the activity of APR-246 and its effect on p53 signaling in a large panel of cell line (CLX) and patient-derived xenograft (PDX) models of OAC.

Design: *In vitro* response to APR-246 was assessed using clonogenic survival, cell cycle and apoptosis assays. Ectopic expression, gene knockdown and CRISPR/Cas9-mediated knockout studies of mutant p53 were performed to interrogate p53-dependent drug effects. p53 signaling was examined using quantitative RT-PCR and western blot. Synergistic interactions between APR-246 and conventional chemotherapies were evaluated *in vitro* and *in vivo* using CLX and PDX models.

Results: APR-246 upregulated p53 target genes, inhibited clonogenic survival, and induced cell cycle arrest as well as apoptosis in OAC cells harbouring p53 mutations. Sensitivity to APR-246 correlated with cellular levels of mutant p53 protein. Ectopic expression of mutant p53 sensitised p53-null cells to APR-246, whilst p53 gene knockdown and knockout diminished drug activity. Importantly, APR-246 synergistically enhanced the inhibitory effects of cisplatin and 5-fluorouracil through p53 accumulation. Finally, APR-246 demonstrated potent anti-tumour activity in CLX and PDX models, and restored chemosensitivity to a cisplatin/5-fluorouracil resistant xenograft model.

Conclusions: APR-246 has significant anti-tumour activity in OAC. Given that APR-246 is safe at therapeutic levels, our study strongly suggests that APR-246 can be translated into improving the clinical outcomes for OAC patients.

Significance of this study

What is already known about this subject?

- Oesophageal adenocarcinoma (OAC) is rapidly increasing in incidence with poor clinical outcomes despite existing chemotherapy.
- Oncogenic drivers of OAC amenable to therapeutic inhibition with currently available agents are limited.
- p53 mutations are the most common molecular aberration in OAC and is associated with chemoresistance and poor prognosis.
- Phase I data has demonstrated that APR-246, a first-in-class reactivator of mutant p53 is safe whilst achieving p53 dependent therapeutic effects.

What are the new findings?

- APR-246 reactivates p53 signalling in cell line and patient-derived xenograft models of OAC resulting in cell cycle arrest, apoptosis and inhibition of tumour growth.
- Truncating and frameshift p53 mutations in OAC cells can be targeted by APR-246.
- APR-246 synergises with cisplatin and 5-fluorouracil through p53 accumulation allowing intrinsic chemoresistance to cisplatin and 5-fluorouracil to be overcome.
- Synergistic interaction between APR-246 and epirubicin is p53 independent.

How might it impact on clinical practice in the foreseeable future?

- Our results strongly suggest that the addition of APR-246 to existing chemotherapeutic regimens will improve outcomes for patients with mutant p53 OAC.
- Mutant p53 genotype and expression levels should be incorporated into clinical trial designs to personalise APR-246 therapy.

INTRODUCTION

Oesophageal adenocarcinoma (OAC) is a deadly disease with limited treatment options. The incidence of OAC has risen 600% over the last three decades, and is now the most rapidly increasing solid malignancy in Western society.¹ Systemic therapies for OAC (cisplatin, 5-fluorouracil and epirubicin) have limited clinical efficacy, with the majority of patients either not responding or relapsing soon after treatment.² Therefore, developing safe and effective therapies for OAC patients remains an urgent and unmet medical need.

Genome-wide analyses of OAC have indicated that oncogenic drivers amenable to therapeutic targeting with currently available agents are limited.^{3, 4} In contrast, mutations in the tumour suppressor gene *TP53* are common and arise early in oesophageal carcinogenesis.⁴ This has been associated with chemoresistance and poor survival.^{5, 6} Hence, restoring normal p53 function represents an attractive therapeutic strategy for OAC.

Over the last decade, significant efforts have been devoted to identify compounds that can restore wild-type activity to mutant p53.⁷ Of these, APR-246 (PRIMA-1^{met}) has shown the greatest clinical translation.⁸ Preclinical studies have demonstrated that APR-246 modifies mutant p53 protein, resulting in sequence-specific DNA binding to p53 target genes leading to anti-tumour activity.^{9, 10} Furthermore, recent studies have also suggested that APR-246 may exert additional effects through p53 independent mechanisms.¹¹⁻¹³ These include generating reactive oxygen species (ROS) through antagonising the glutathione and thioredoxin reductase 1 pathway,^{12, 13} activation of p63, p73 and PML tumour suppressor proteins,^{11, 14, 15} and suppression of the c-Myc oncogene.¹¹

In this study, we assess the anti-tumour activity of APR-246 *in vitro* and *in vivo* using a large panel of OAC cell line and patient-derived xenograft models with differing p53 status. In addition, we explore p53 dependent and independent activities of APR-246 by manipulating p53 expression using novel genetic techniques. We describe the mechanisms underlying synergistic interactions between APR-246 and chemotherapeutic agents, and demonstrate that intrinsic chemoresistance can be overcome by restoring p53 transcriptional activity. These results support the clinical use of APR-246 to improve outcomes for patients with OAC.

METHODS

Cell lines and culture

OE33, OE19, H1299 and HEK293T cells were obtained from the American Type Culture Collection. JH-EsoAd1 cells were kindly provided by Prof. James Eshleman (Johns Hopkins University, Baltimore, MD). FLO-1, OACM5.1, Eso26, SKGT4, OACP4C and TE7 cells were gifts from Prof. Rebecca Fitzgerald (University of Cambridge, UK). OANC1 cells were derived in our laboratory.¹⁶ Immortalised oesophageal squamous epithelial cells (NES) were a gift from Prof. Rhonda Souza (University of Texas Southwestern Medical Centre, Dallas, TX).¹⁷ The identity of all cell lines were authenticated by short tandem repeat analysis using the PowerPlex[®] 16 genotyping system (Promega, Auburn, Australia) and Sanger sequenced to verify their *TP53* status. Detailed cell culture conditions and sequencing procedures are provided in the *Supplementary Methods*.

Assays for cell viability, proliferation, clonogenicity, cell cycle distribution and apoptosis

The assays used to study the effects of treatments on cell viability (AlamarBlue[®], Life Technologies, Melbourne, Australia),¹⁸ proliferation (Incucyte FLR, Essen BioScience, Ann Arbor, MI),¹⁹ clonogenic survival,²⁰ cell cycle distribution (Nicoletti assay)²¹ and apoptosis (Annexin-V/propidium iodide)²² have been previously reported. Specific assay conditions are described in the *Supplementary Methods*.

Gene expression with quantitative RT-PCR

Total cell RNA was extracted using the RNeasy kit (Qiagen, Doncaster, Australia) and reverse transcribed using the Transcriptor First Strand cDNA Synthesis Kit (Roche, Hawthorn, Australia). Changes in gene expression were assessed using SYBR green RT-PCR (Lightcycler[®] 480, Roche) and analysed using the $\Delta\Delta C_t$ method by normalizing against GAPDH. PCR primers are detailed in *Supplementary Table S1*.

Western blot analysis

Cells and tissues were lysed and processed according to standard procedures as described in *Supplementary Methods*. Protein expression was quantified using ImageJ software (<http://imagej.nih.gov/ij/>). The antibodies used are detailed in *Supplementary Table S2*.

p53 knockdown

Mutant p53 OACM5.1, OANC1, FLO-1 and OE19 cells were reverse transfected with 40 nmol/L non-targeting control or p53 siRNA (siGenome Smartpool, Dharmacon®, Lafayette, CO) using Lipofectamine RNAiMax solution (Life Technologies) according to the manufacturer's guidelines.

p53 over-expression

p53-null H1299 cells were transduced to stably express either the R273H or R175H p53 mutants as per established protocols.²³ Single cell clones were generated following G418 selection to ensure uniform mutant p53 expression.

TP53 knockout using CRISPR/Cas9 technology

CRISPR/Cas9-mediated *TP53* knockout was performed using a dual lentiviral vector system kindly provided by Dr. Marco Herold (Walter and Eliza Hall Institute, Melbourne, Australia).²⁴ The sgRNA sequence (GAGCGCTGCTCAGATAGCGA) targeting exon5 of *TP53* was designed using MIT CRISPR software (crispr.mit.edu).²⁴ The experimental procedures involved in p53 deletion are detailed in *Supplementary methods* and are adapted from established protocols.²⁵

Combination treatments of APR-246 with chemotherapeutic compounds

To evaluate the synergistic effect of APR-246 (Aprea AB, Solna, Sweden) with cisplatin, 5-fluorouracil and epirubicin (Hospira, Melbourne, Australia), the GI50 dose of single agents were firstly identified by fitting a four-parameter dose-response curve using Prism 6 software (Graphpad, La Jolla, CA). Cells were then treated for 96 h with combinations of APR-246 and chemotherapeutic agents over a range of concentrations held at a fixed ratio and based on the GI50 of each drug. The highest and lowest combination doses were six times and 1/20th the GI50, respectively. Combination indexes (CI) based on cell viability assays were determined using CalcuSyn v2 (Biosoft, Cambridge, UK) where $CI < 0.9$ indicates synergism, $CI > 1.1$ antagonism and $0.9 \leq CI \leq 1.1$ an additive effect.²⁶

Cell line and patient-derived xenograft models of oesophageal cancer and treatment

All animal experiments were approved by the Peter MacCallum Cancer Centre (PMCC) Animal Experimentation Ethics Committee and conducted in accordance with the *National Health and Medical Research Council Australian Code of Practice for the Care and Use of Animals for Scientific Purposes*. Mice were obtained from the Walter and Eliza Hall Institute or bred internally. For cell line xenografts (CLX), 5 million Eso26, OE19 or FLO-1 cells suspended in 100 μ L of 1:1 PBS and MatrigelTM (BD Bioscience) were

subcutaneously injected into the right flank of 6-week old female nude (Eso26 and OE19) or NOD-SCID IL-2R γ ^{KO} mice (FLO-1). Patient-derived xenografts (PDX) were developed as previously described and summarised in the *Supplementary Methods*.²⁷ The collection and use of human tissue for this study was approved by the Human Research Ethics Committee at PMCC.

Tumour volume was assessed by caliper measurements every 3 to 4 days, and calculated using the formula (length x width²)/2. Once tumours reached 100 mm³ mice were randomised to commence drug treatment. Mice received APR-246 at 100 mg/kg daily, cisplatin at 3 mg/kg weekly, and 5-flurouracil at 10 mg/kg on days 1 to 5 over a 14 day cycle. Vehicle (H₂O) was given to control animals. All treatments were delivered by intraperitoneal injections. Mice were either euthanised when tumours reached 1500 mm³ for survival studies or 6 h after the last treatment dose for biomarker studies. Tumours were photographed, weighed, and partitioned for immunohistochemical and western blot analysis. Tumour growth inhibition (TGI%) was calculated at 21 days following treatment onset with the formula $(1 - (V_{T21} - V_{T0}) / \text{mean}(V_{C21} - V_{C0})) \times 100$, where V_{T21} and V_{T0} = tumour volume of drug-treated animals at day 21 and 0 respectively, whilst V_{C21} and V_{C0} = tumour volume of control animals at day 21 and 0 respectively.

Immunohistochemistry

Sections from formalin-fixed paraffin embedded tissues were stained with anti-proliferating cell nuclear antigen (610665, BD Bioscience) and anti-cleaved caspase-3 (D175, Cell Signaling Technology) antibodies and detected using the EnVision™ Plus system (Dako, North Sydney, Australia).

Statistical analysis

Student's t-test or one-way analysis of variance with Tukey's Multiple Comparison test were used to compare between groups for *in vitro* and animal studies. Survival differences were assessed using Kaplan-Meier log-rank analysis. The relationship between APR-246 sensitivity and p53 protein level was evaluated by the Spearman correlation test. Statistical analyses were performed using Prism 6 (Graphpad). A two-tail P<0.05 was considered statistically significant. All data are expressed as mean $\pm$ standard error of mean (SEM).

RESULTS

Mutant p53 status predicts sensitivity to APR-246 but not to conventional chemotherapy in oesophageal cancer cell lines

The effect of APR-246 was studied in 10 human oesophageal cancer cell lines (9 OAC and 1 OSCC). Additionally, NES and H1299 cells were included as p53 wild-type and null controls, respectively (Table S3). Mutant p53 cancer cells were significantly more sensitive to APR-246 than null and wild-type cells (Figures 1A and B, mean GI50 dose: 22.6 vs. 71.6 $\mu\text{mol/L}$, $P<0.001$). This sensitivity strongly correlated with endogenous p53 protein levels (Figures 1C and 1D, $R^2=0.72$, $P<0.001$). To facilitate further comparison, we sub-categorised mutant lines into high and low expressers of p53 protein (Figures 1A and Table S4). Notably, whilst OE33 cells expressed relatively high levels of mutant p53, amongst all the mutant lines tested, it was the least sensitive to APR-246. To explore this further, we examined basal expression of anti-apoptotic genes and found that OE33 cells had higher expression compared to other lines with similar p53 protein levels (Figure S1), thus providing a potential explanation for the muted response to APR-246 in OE33 cells. In contrast, p53 status did not predict chemosensitivity in oesophageal cancer cells (Figure 1E). However, we noted that OE19 cells were most resistant to cisplatin, 5-fluorouracil and epirubicin, with their GI50 values at 18, 61 and 182 fold higher than the most chemosensitive line, respectively (Figure S2).

APR-246 activity in OAC cells is dependent on mutant p53

At a dose of 50 $\mu\text{mol/L}$, APR-246 completely inhibited cellular proliferation of mutant p53 cancer cells, but only minimally affected wild-type and null cells (Figures 2A and S3). To confirm this p53 dependent effect, we demonstrated that p53 knockdown in OACM5.1 and OANC1 cells significantly attenuated the activity of APR-246 (Figures 2B and C). Additionally, over-expression of two mutant constructs in p53-null H1299 cells sensitised these cells to APR-246 in a dose-dependent manner (Figure 2D).

APR-246 induces cell cycle arrest through upregulation of p21 and GADD45 α in OAC cells

Cell cycle analysis was performed at 0, 6, 12, and 24 h following APR-246 treatment to determine whether reduced proliferation was due to cell cycle arrest (Figures 3A and S4). APR-246 induced a G1/S arrest in JH-EsoAd1 and FLO-1 cells, whilst a G2/M arrest was evident in OE19 and Eso26 cells. Furthermore, an increase in the sub-G0 fraction was seen in FLO-1, Eso26 and OE19 cells at 24 h, suggesting that these cells were undergoing cell death. To further understand APR-246 induced cell cycle arrest, we examined the expression of G1/S and G2/M checkpoint regulators (Figures 3B and C, respectively). In FLO-1 cells, APR-246 upregulated p21 expression and decreased Rb phosphorylation. In OE19 cells, APR-246 increased p21

and GADD45 α levels and reduced Cyclin B1 and CDK1 (Cdc2 p34) expression. Complementary findings were also observed at the mRNA level (Figures 4A and B). Importantly, knockdown of mutant p53 in FLO-1 and OE19 cells diminished APR-246 mediated p21 and GADD45 α upregulation (Figure 3D and E). Taken together, these results strongly suggest that APR-246 induces cell cycle arrest in mutant p53 OAC cells.

APR-246 inhibits clonogenic survival through inducing apoptosis in mutant p53 OAC cells

To clarify whether APR-246 induces apoptosis, we quantified the percentage of Annexin-V positive cells at 0, 24 and 48 h following drug treatment (Figures 5A and S5A). In Eso26 and FLO-1 cells with high levels of mutant p53, extensive apoptosis was detected at 24 h and by 48 h nearly all the cells had undergone apoptosis. Comparatively, in OE19 and JH-EsoAd1 cells with lower levels of mutant p53, their progression into apoptosis was slower, although the magnitude at 48 h was similar to mutant p53 high expressers. Consistently, we noted that the treatment duration required to completely inhibit clonogenic survival was significantly shorter for cells expressing high levels of mutant p53 compared with low expressers (Figures 5B and S5B). To determine whether reduced clonogenic survival was due to transactivation of p53 dependent apoptotic pathways, we evaluated changes in the expression of p53 and its targets following drug treatment. In mutant p53 cells, APR-246 upregulated PUMA and NOXA expression, resulting in procaspase-3 and PARP cleavage (Figures 5C and S5C). Additionally, increased Bax transcripts were detected in JH-EsoAd1 cells (Figure 4C), whilst Bcl-2 expression decreased in OE33 and OE19 cells (Figure 4D). MDM2 levels also increased in Eso26, OE19 and JH-EsoAd1 cells, and this correlated with reduced p53 protein expression (Figures 5C and S5C). Furthermore, p53 knockdown in OE19 cells inhibited the rise in MDM2, PUMA and NOXA expression following APR-246 treatment (Figure 5D). Importantly, APR-246 had minimal effect on apoptosis, clonogenic survival or p53 target gene expression in p53 wild-type and null cells (Figures 4, 5 and S5).

APR-246 preferentially suppresses tumour growth in mutant p53 CLX and PDX models of oesophageal cancer

The anti-tumour activity of APR-246 was firstly investigated in Eso26 and FLO-1 xenografts. In these models, APR-246 significantly suppressed tumour growth and improved survival with minimal toxicity (Figures 6A and S6A). To broaden these findings in tumours with different p53 status, we established xenografts from 3 patients with treatment-naïve oesophageal cancer consisting of two OAC with either mutant p53^{K305*} (PDX1) or wild-type p53 (PDX2), and one wild-type p53 OSCC (PDX3). The growth of mutant p53 PDX1 tumours was significantly inhibited by APR-246 (Figure 6B). This coincided with increased p21, PUMA and NOXA

expression in APR-246 treated tumours, resulting in decreased Rb phosphorylation and PCNA levels together with increased cleavage of procaspase-3 and PARP (Figure 6C). In contrast, APR-246 had little effect in p53 wild-type tumours (Figures 6D, E and S6B). These results validated our *in vitro* observations, and further suggest that APR-246 induces cell cycle arrest and apoptosis *in vivo* through activation of p53-dependent signaling pathways in mutant p53 OAC.

APR-246 synergises with conventional chemotherapy and overcomes primary resistance to cisplatin and 5-flurouracil

Next we examined whether APR-246 can enhance the therapeutic potential of cytotoxic agents currently in clinical use against OAC. APR-246 synergised with cisplatin, 5-flurouracil, and epirubicin, across different effective doses in mutant p53 cell lines (Figure 7A). Contrastingly, in null and wild-type cells, additive to slightly antagonistic effects were observed with cisplatin and 5-flurouracil while with epirubicin the drug interaction ranged from synergistic to antagonistic. Notably, combinations of APR-246 with cisplatin and 5-flurouracil were more synergistic in cells with low rather than high p53 expression, suggesting that a potential mechanism of synergy is through p53 accumulation following genotoxic stress. In support of this, we found that cisplatin and 5-flurouracil but not epirubicin increased mutant p53 accumulation in OE19 cells (Figure 7B). Furthermore, p53 knockdown significantly attenuated the synergistic activity between APR-246 and cisplatin or 5-flurouracil without affecting that of epirubicin (Figure 7C). We also confirmed in OE19 cells that combining APR-246 with cisplatin, 5-flurouracil or epirubicin (at GI50 doses) resulted in synergistic induction of apoptosis (Figure 7D).

To determine whether the heightened apoptotic response seen *in vitro* could be extended *in vivo*, we examined the effects of therapeutic combinations in OE19 xenografts. As expected, OE19 xenografts were resistant to cisplatin and 5-flurouracil (%TGI: 10.5±5.8%), whilst APR-246 alone delayed tumour growth (Figure 7E, %TGI: 62.5±16.9%). Notably, the combination of APR-246 with cisplatin and 5-flurouracil synergistically enhanced tumour suppression (%TGI: 92.2±3.6%) without significantly increasing toxicity when compared with chemotherapy alone. Correspondingly, higher levels of p21, PUMA and NOXA were detected in tumours treated with combination therapy compared with either APR-246 or chemotherapy alone (Figure 7F). This was associated with a partially restored p53 level (compared to APR-246 alone), reduced Rb phosphorylation, as well as increased cleavage of procaspase-3 and PARP.

CRISPR/Cas9-mediated *TP53* knockout identifies p53 dependent and independent effects of APR-246 in OAC cells.

Given that p53 knockdown did not completely abolish APR-246 activity in OAC cells (Figure 2B and C), or diminish the synergistic interaction with epirubicin (Figure 7C), we next attempted to clarify the p53 dependent and independent activities of APR-246 using CRISPR/Cas9-mediated *TP53* knockout in JH-EsoAd1 cells (Figure S7A). Doxycycline induction of sgRNA in cell pools led to an appreciable reduction in p53 protein compared with non-induced and parental cells (Figure S7B). From this, we identified 5 clones with no residual p53 protein (Figure S7B), and 4 with truncated protein (data not shown). Sanger sequencing confirmed the presence of InDel mutations in the target sequence on exon 5 in all 9 clones (Figure S7C). To avoid potential confounding effects from truncated p53 protein, clones 1, 7 and 14 with no residual p53 protein and representing 3 unique InDel mutations were chosen for further functional characterization. On the basis of AlamarBlue®, clonogenic formation and cell cycle assays, we showed that these clones were phenotypically similar to each other and to control cells (Figures S7D to F).

Consistent with our results using siRNA, p53 deleted clones (Figure 8A) exhibited reduced sensitivity to APR-246 compared with non-induced and parental cells (Figure 8B). Interestingly, whilst *TP53* knockout significantly attenuated APR-246 induced apoptosis (Figure 8C and S7G), 20% to 40% of cells still apoptosed despite being p53 null. Furthermore, p53 deletion abolished APR-246 induced cell cycle arrest (Figure 8D), and eliminated the synergistic interaction with cisplatin and 5-fluorouracil, but not with epirubicin (Figure 8E). Taken together, these results suggest that the therapeutic efficacy of APR-246 is mediated through both p53 dependent and independent mechanisms.

DISCUSSION

TP53 mutations are the most prevalent molecular aberration in OAC.³ The loss of p53-mediated tumour suppression has been associated with chemoresistance and poor survival.^{5, 6} Therefore, restoring wild-type p53 activity is of critical importance in the treatment of OAC.

In this study, we provide the first evidence in preclinical models of OAC that reactivating p53 pathways using APR-246 results in potent anti-tumour activity. Our results validate *in silico* analyses conducted by Bykov *et al.* which showed that sensitivity to PRIMA-1 (unmethylated form of APR-246) correlated with mutant but not wild-type p53 expression in different tumour cell lines.²⁸ Furthermore, our observation that APR-246 is active against a wide range of mutant p53 OAC cell lines is supported by *ex vivo* studies which demonstrated that methylene quinuclidinone (MQ), the active compound of APR-246,⁹ is able to restore DNA binding to different p53 mutants.¹⁰ Overall, our results support mutant *TP53* status and expression levels as predictive biomarkers for response to APR-246. Although several investigators have suggested that APR-246 is most effective against missense *TP53* mutations,¹⁰ we showed for the first time in cell line (OE19) and PDX (PDX1) tumours that nonsense and frameshift mutations are amenable to APR-246 mediated cytotoxicity. This is clinically important as it potentially broadens the scope of patients who may respond to APR-246. The mechanisms by which APR-246 is efficacious in cells bearing nonsense and/or frameshift p53 mutants are not clear. While the specific nonsense and frameshift mutations studied here are likely to lack the tetramerization domain necessary for p53 activity, they do still have both the DNA-binding and transactivation domains. It is likely that mutant p53 proteins that lack these domains due to truncating mutations occurring earlier in the p53 gene will not be as sensitive to APR-246.

The predominant mechanism of APR-246 is thought to be alkylation of cysteine residues in mutant p53 by MQ, thus restoring sequence-specific binding to DNA.⁹ However, several studies have suggested that APR-246 may also exert p53 independent effects. These include generating ROS,^{12, 13} upregulating p73,¹¹ and suppressing c-Myc expression.¹¹ Additionally, some investigators have even asserted that APR-246 may act entirely through p53 independent pathways in some tumour cells.^{11, 12} We believe it is important to be mindful of several issues when interpreting these results. Firstly, the lack of change in drug activity following p53 knockdown does not necessarily equate to p53 independence, as residual amounts of mutant p53 protein may be sufficient for APR-246 to mount a substantial anti-tumour response. Secondly, cancer cells may rely on mutant p53 activity.²⁹ As a result, p53 knockdown itself may diminish cellular viability, thus limiting the ability to detect any attenuated drug effect. Thirdly, transcriptional reactivation of p53 by APR-246 may be

dose, time and cell line dependent.¹⁰ Therefore, the failure to demonstrate changes in a limited number of genes following APR-246 treatment does not discount p53 dependence. Consistent with this, we observed that APR-246 induced heterogeneous changes in gene expression across our panel of cell lines. Furthermore, we noted that p21, PUMA and NOXA levels decreased with the onset of apoptosis. As these mediators can be cleaved by activated caspase-3,^{30, 31} an additional layer of complexity needs to be considered when expression data is used as a surrogate marker of p53 activity.

To clarify whether APR-246 has p53 independent activity in OAC, we utilised CRISPR/Cas9 technology to knockout mutant p53 expression in JH-EsoAd1 cells. Using this technique, we were able to generate isogenic p53 null clonal populations for direct comparison of APR-246 activity, without being confounded by residual p53 protein seen in knockdown experiments. Overall, we found that cell cycle arrest induced by APR-246 is likely to be entirely mediated through p53 dependent pathways, as this phenomenon was virtually abolished when p53 was deleted. Interestingly, whilst *TP53* knockout attenuated cellular sensitivity to APR-246 and decreased apoptosis induction, we noted that 20 to 40% of cells apoptosed after drug treatment despite p53 deletion. This indicates that p53 independent mechanisms do contribute to APR-246 induced apoptosis in OAC. These results are consistent with Peng *et al.*, who estimated similar contributions of p53 independent effects, and may be explained by the induction of ROS following thioredoxin reductase 1 inhibition by APR-246.¹³

Given the presence of p53 independent mechanisms, we speculate that there will be other biomarkers of drug response. Indeed, Tessoulin *et al.* showed that endogenous levels of glutathione correlated with APR-246 sensitivity in multiple myeloma cells,¹² whilst in astrocytoma, the absence of p14^{ARF} expression, an endogenous MDM2 inhibitor, was associated with resistance to APR-246.³² Importantly, Aryee *et al.* proposed a panel of 42 apoptotic genes that may predict APR-246 response in sarcoma cells.³³ Similarly, we found that OE33 cells with high levels of anti-apoptotic gene expression were also the least responsive to APR-246 amongst our panel of mutant p53 cell lines tested, despite robust p53 protein expression. These results suggest that combination therapy with pro-oxidants, MDM2 inhibitors and BH3-mimetics may potentially enhance the therapeutic efficacy of APR-246 in OAC.

Combination cisplatin, 5-fluorouracil and epirubicin is currently the first line therapy for most patients with advanced OAC.² However, approximately 70% of patients fail to respond adequately to these therapies,² which may be in part due to p53 dysfunction.⁵ Therefore, restoring p53 activity with APR-246 may overcome

chemoresistance and enhance the effect of conventional chemotherapies. In support of this, we demonstrated that APR-246 synergised with cisplatin, 5-fluorouracil and epirubicin in all mutant p53 cell lines. Additionally, APR-246 overcame chemoresistance to all 3 cytotoxic agents in OE19 cells and synergistically suppressed tumour growth through the induction of p53 dependent apoptotic pathways. Whilst several studies have shown synergism with cisplatin,³⁴ 5-fluorouracil,³⁴ and anthracycline therapies,¹¹ the mechanisms underlying these interactions are not well understood. We show for the first time that in both p53 knockdown and knockout cells, the synergism between APR-246 and epirubicin is p53 independent. In contrast, the interaction with cisplatin and 5-fluorouracil is almost entirely p53 dependent, and could be explained by increased mutant p53 following genotoxic stress, thus providing more substrate for drug activity, resulting in stronger restoration of wild-type p53 activity. With regards to synergism with epirubicin, we speculate that APR-246 may deplete intracellular anti-oxidant activity,^{12, 13} thus potentiating epirubicin induced ROS,³⁵ resulting in enhanced cytotoxicity. This hypothesis warrants further exploration.

Recently, a landmark study by Weaver *et al* has demonstrated that *TP53* mutations frequently occur in Barrett's dysplasia,⁴ thus providing a novel and exciting avenue for APR-246 in the primary prevention of OAC. Although endoscopic eradication therapies are recommended for Barrett's dysplasia,³⁶ recurrence of Barrett's epithelium occurs not infrequently after extensive endoscopic intervention,³⁷ suggesting that residual microscopic foci of dysplastic tissue is sufficient for Barrett's recurrence and neoplastic progression. Given that APR-246 is well tolerated in patients,⁸ it is conceivable that APR-246 could be used to treat dysplastic Barrett's oesophagus, either alone or as an adjunct to endoscopic interventions, to minimise risk of cancer onset.

Overall, our study provides strong preclinical rationale for trialing APR-246 in patients with OAC. Given the promising phase I data,⁸ APR-246 is ready for clinical translation to improving the outcomes for OAC patients.

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Competing interests

K.G.W is a co-founder and shareholder of Aprea AB, a company that develops p53-based anticancer therapy including APR-246. K.G.W. is a member of its board.

No conflicts of interest to declare for all other authors.

Ethics approval

Ethics approval was provided by the Animal Experimentation Ethics Committee and Human Research Ethics Committee at the Peter MacCallum Cancer Centre.

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FIGURE LEGENDS

Figure 1 Sensitivity to APR-246 correlates with mutant p53 expression in OAC cells. GI50 values were determined by exposing cells to a range of concentrations of APR-246 (0.1 to 100 $\mu\text{mol/L}$), 5-fluorouracil (0.01 to 200 $\mu\text{mol/L}$), cisplatin (0.01 to 200 $\mu\text{mol/L}$) and epirubicin (0.001 to 100 $\mu\text{mol/L}$) for 96 h and cell viability assayed using AlamarBlue®. (A) APR-246 dose response curves for cell lines with p53 wild-type or null status (blue), and cell lines expressing high (orange) or low (green) levels of mutant p53. (B) Cell lines ordered by their sensitivity to APR-246 and their p53 status. (C) p53 expression per cell line as determined by densitometric analysis of western blots. A representative western blot is shown. (D) Correlation between APR-246 sensitivity and relative p53 expression. Each point represents the mean GI50 value and p53 expression per cell line. (E) The GI50 values of APR-246, 5-fluorouracil, cisplatin and epirubicin in p53 mutant versus null and wild-type cells. Each point represents the mean GI50 value per cell line. Bars=mean of each group. For all experiments n=3 to 5, error bars=SEM, *** P<0.001.

Figure 2 APR-246 has p53 dependent activity in OAC cells. (A) Proliferation of p53 wild-type, null and mutant cell lines *in vitro*, as assessed by an Incucyte optical scanner following treatment with 50 $\mu\text{mol/L}$ APR-246 or vehicle. (B and C) %Annexin-V positive cells in cell lines transfected with non-targeting control (NTC) or p53 siRNA (sip53) for 72 h followed by treatment with 50 $\mu\text{mol/L}$ APR-246 for 48 h. p53 knockdown was confirmed by western blot (insert). (D) %Annexin-V positive cells in parental (p53 null) or mutant p53 expressing (R175H or R273H) H1299 cells following 48 h treatment with 50 or 75 $\mu\text{mol/L}$ APR-246. p53 expression was confirmed by western blot (insert). For all experiments, n=3 to 5, error bars=SEM, * P<0.05 compared with parental cells, ** P<0.01, *** P<0.001.

Figure 3 APR-246 induces cell cycle arrest in mutant p53 OAC cells. (A) Cell cycle analysis in cell lines treated with 50 $\mu\text{mol/L}$ APR-246 for 0, 6, 12 or 24 hrs. (n=3, error bars=SEM, * P<0.05, ** P<0.01, *** P<0.001 compared with vehicle treated cells for each time point). (B and C) Representative western blots of G1/S (p21 and Rb) and G2/M (p21, GADD45 α , Cyclin B1 and Cdc2 p34) checkpoint regulators following APR-246 treatment in FLO-1 and OE19 cells. (D and E) Changes in protein expression following treatment with 50 $\mu\text{mol/L}$ APR-246 or vehicle for 10 h in FLO-1 and OE19 cells transfected with non-targeting control (NTC) or p53 siRNA for 72 h.

Figure 4 Changes in gene expression following APR-246 treatment. Quantitative RT-PCR for p21 (A), GADD45 α (B), Bax (C), Bcl-2 (D), PUMA (E) and MDM2 (F) expression at 12 h following treatment with 50

μmol/L APR-246 in the indicated cell lines with different p53 status. Data represents fold changes in gene expression relative to vehicle treatment (H₂O, broken line). n=3, error bars=SEM.

Figure 5 APR-246 preferentially induces apoptosis in mutant p53 OAC cells. (A) Example FACS plots of Annexin-V/propidium iodide labelled cells treated with 50 μmol/L APR-246 for the indicated time points. Annexin-V positive cells are quantified in the histogram beneath (n=3, error bars=SEM, * P<0.05, ** P<0.01, *** P<0.001). (B) Clonogenic survival of cells following treatment with 50 μmol/L APR-246 or vehicle for 5, 10, 15, 20 and 24 h (n=3, error bars=SEM). (C) Representative western blots of pro-apoptotic regulators and p53 target genes following treatment with 50 μmol/L APR-246 (# p53 is 45 kDa for OE19 cells). (D) Changes in protein expression in OE19 cells treated with 50 μmol/L APR-246 or vehicle for 10 h after transfection with non-targeting control (NTC) or p53 siRNA for 72 h.

Figure 6 APR-246 suppresses tumour growth in cell line and patient-derived xenograft models with mutant p53 expression. (A) Growth curves of mutant p53^{R248W} Eso26 xenografts (left panel) and Kaplan-Meier plot of time to reach 1500 mm³ tumour volume (middle panel) in mice treated with vehicle (n=4) or APR-246 (n=4) for 14 days. Mean body weight (right panel) was used as a surrogate marker of toxicity. (B) Growth curves and photos of mutant p53^{K305*} PDX1 xenografts from mice treated with vehicle (n=5) or APR-246 (n=5) for 28 days. (C) Western blots and immunohistochemical staining of vehicle and APR-246 treated PDX1 tumours. (D) Growth curves (left panel) of wild-type p53 PDX2 xenografts in mice treated with vehicle (n=4) or APR-246 (n=6) for 21 days. Tumours were stained for cleaved caspase-3 and proliferating cell nuclear antigen (PCNA, right panel). (E) TGI at 21 days following APR-246 treatment in six xenograft models with different p53 status. (For all experiments, vehicle: H₂O, APR-246: 100 mg/kg, daily, intraperitoneal injection, error bars=SEM, * P<0.05, ** P<0.01, scale bars=100 μm).

Figure 7 APR-246 synergises with cytotoxic agents and overcomes chemotherapeutic resistance in OAC xenografts. (A) Combination index (CI) plots of cell lines with different p53 status treated with APR-246 in combination with cisplatin, 5-fluorouracil or epirubicin for 96 h. Viable cells were quantified using AlamarBlue[®]. Each point represents the mean (n=3) CI per cell line to achieve an effective dose (ED) of 50%, 75% and 90%. Bars=mean of each group. Grey area: additive effect, below grey area: synergism, above grey area: antagonism. (B) Representative western blots for p53 in mutant p53^{N310fs} OE19 cells following treatment with cisplatin, 5-fluorouracil or epirubicin. (C) The effect on drug interaction by inhibiting p53 upregulation in OE19 cells. Non-targeting control (NTC) or p53 (sip53) siRNA were transfected into OE19

cells for 72 h followed by combinational drug treatments as described above. p53 knockdown was assessed by western blot at 24 h after exposure to 15 $\mu\text{mol/L}$ cisplatin, 45 $\mu\text{mol/L}$ 5-fluorouracil or 2 $\mu\text{mol/L}$ epirubicin ($n=4$, * $P<0.05$, ** $P<0.01$, *** $P<0.001$). (D) Apoptosis induction as measured by cleaved PARP products 24 h after treatment with the indicated compounds in OE19 cells ($n=3$, error bars=SEM, * $P<0.05$ compared with vehicle, # $P<0.05$ compared with APR-246 alone). (E) The effect of concurrent APR-246, cisplatin (CDDP) and 5-fluorouracil (5FU) treatment on OE19 xenografts. Mice were randomised to vehicle ($n=5$), CDDP/5FU ($n=4$), APR-246 ($n=5$) or APR-246/CDDP/5FU ($n=4$) once tumour volume reached 100 mm^3 , and treated for 21 days. Changes in body weight were monitored throughout the treatment period. All drugs were delivered by intraperitoneal injection: CDDP: 3 mg/kg, weekly. 5FU: 10 mg/kg, days 1 to 5 over a 14 day cycle. APR-246: 100 mg/kg, daily, vehicle: daily, error bars=SEM, * $P<0.05$ compared with vehicle, # $P<0.05$ as indicated). (F) Representative western blots, H&E and immunohistochemical staining of OE19 xenografts demonstrating changes in protein expression and tissue architecture following treatment with the indicated regimen, scale bars=100 μm .

Figure 8 CRISPR/Cas9-mediated knockout of *TP53* identifies p53 dependent and independent effects of APR-246 in OAC cells. (A) p53 protein expression in knockout versus clonal non-induced (Dox-) and parental JH-EsoAd1 cells. (B) GI50 values of APR-246 in knockout versus control cells ($n=5$ per group, * $P<0.05$ comparison of mean values). (C) Induction of apoptosis in knockout versus control cells following 48 h treatment with 50 $\mu\text{mol/L}$ APR-246 ($n=4$ per group, error bars=SEM, * $P<0.05$ comparison of mean values). (D) Changes in cell cycle distribution in knockout versus control cells following 24 h treatment with 50 $\mu\text{mol/L}$ APR-246 (left panel, $n=3$, error bars=SEM, * $P<0.05$; right panel, representative DNA (PI) histograms). (E) The effect of p53 deletion on drug interaction between APR-246 and chemotherapeutic agents ($n=3$, bars=mean of each group. grey area: additive effect, below grey area: synergism, above grey area: antagonism).

A **B** **C** **D** **E** **Figure 1**

A **B** **C** **D** **Figure 2**

Figure 3

Figure 4

Figure 5

Figure 6

Figure 7

Figure 8

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