FULL PAPER

BJC

British Journal of Cancer (2013) 109, 2696–2704 | doi: 10.1038/bjc.2013.635

Keywords: PRIMA-1^{Met}/APR-246; Ewing sarcoma; apoptosis; p53

Variability in functional p53 reactivation by PRIMA-1^{Met}/APR-246 in Ewing sarcoma

D N T Aryee*[,1,2], S Niedan[1], J Ban[1], R Schwentner[1], K Muehlbacher[1], M Kauer[1], R Kofler[3] and H Kovar[1,2]

[1]Children's Cancer Research Institute (CCRI), St Anna Kinderkrebsforschung, Zimmermannplatz 10, Vienna A-1090, Austria; [2]Department of Pediatrics, Medical University, Vienna, Austria and [3]Division of Molecular Pathophysiology, Biocenter, Medical University Innsbruck, Innsbruck, Austria

Background: Though p53 mutations are rare in ES, there is a strong indication that p53 mutant tumours form a particularly bad prognostic group. As such, novel treatment strategies are warranted that would specifically target and eradicate tumour cells containing mutant p53 in this subset of ES patients.

Methods: PRIMA-1^{Met}, also known as APR-246, is a small organic molecule that has been shown to restore tumour-suppressor function primarily to mutant p53 and also to induce cell death in various cancer types. In this study, we interrogated the ability of APR-246 to induce apoptosis and inhibit tumour growth in ES cells with different p53 mutations.

Results: APR-246 variably induced apoptosis, associated with Noxa, Puma or p21^{WAF1} upregulation, in both mutant and wild-type p53 harbouring cells. The apoptosis-inducing capability of APR-246 was markedly reduced in ES cell lines transfected with p53 siRNA. Three ES cell lines established from the same patient at different stages of the disease and two cell lines of different patients with identical p53 mutations all exhibited different sensitivities to APR-246, indicating cellular context dependency. Comparative transcriptome analysis on the three cell lines established from the same patient identified differential expression levels of several *TP53* and apoptosis-associated genes such as *APOL6*, *PENK*, *PCDH7* and *MST4* in the APR-246-sensitive cell line relative to the less APR-246-sensitive cell lines.

Conclusion: This is the first study reporting the biological response of Ewing sarcoma cells to APR-246 exposure and shows gross variability in responses. Our study also proposes candidate genes whose expression might be associated with ES cells' sensitivity to APR-246. With APR-246 currently in early-phase clinical trials, our findings call for caution in considering it as a potential adjuvant to conventional ES-specific chemotherapeutics.

P53 suppresses tumour growth via its diverse cellular activities, including induction of apoptosis, cell cycle arrest, differentiation and senescence (Vousden and Lu, 2002). Most of these effects reflect the transactivation of a number of genes by p53 acting as a transcription factor, but p53 also activates mitochondrial-dependent apoptotic pathways that are independent of p53 transcriptional activity (Green and Kroemer, 2009). Activation of p53 leads to apoptosis through either the death receptor pathway or the mitochondrial pathway (Selivanova, 2004). In the mitochondrial apoptotic pathway, p53 induces several genes including *Bax*, *APAF-1*, *Puma* and *Noxa* (Miyashita and Reed, 1995; Burns and El-Deiry, 1999; Lowe and Lin, 2000; Nakano and Vousden, 2001; Robles *et al*, 2001). *TP53* mutations may be associated with an aggressive phenotype and poor prognosis, and some p53 mutants counteract the effects of anticancer agents that attack tumours (Bunz *et al*, 1999; Poeta *et al*, 2007). Given the high frequency of p53 mutations in human tumours, reactivation of the p53 pathway has been widely proposed as beneficial for cancer therapy (Selivanova, 2010). In addition, several reported structural studies have shown that mutant p53 core domain unfolding is reversible and, as mutant p53 is expressed at high levels in many tumours, it therefore serves as a potential target for novel cancer therapy (Lambert *et al*, 2010). Its reactivation will restore p53-dependent apoptosis, among others, resulting in efficient eradication of

*Correspondence: Dr DNT Aryee; E-mail: dave.aryee@ccri.at

Received 10 May 2013; revised 14 August 2013; accepted 24 September 2013; published online 15 October 2013

© 2013 Cancer Research UK. All rights reserved 0007 – 0920/13

tumour cells (Tovar *et al*, 2006). Wild-type p53 can be induced by small organic molecules such as nutlin-3 inhibiting the p53/MDM2 interaction (Vassilev *et al*, 2004). PRIMA-1 (p53 reactivation and induction of massive apoptosis) and its potent methylated analog, APR-246/PRIMA-1^{Met}, are small molecules that have the ability to convert mt-p53 to an active conformation, thereby restoring its sequence-specific DNA binding and transcriptional activation (Bykov *et al*, 2002b). It was reported that PRIMA-1 induces cell death through multiple pathways encompassing transcription-dependent and -independent signaling (Chipuk *et al*, 2003). *In vitro* and *in vivo* studies have shown that p53 reactivating small molecules are less toxic to normal cells than to cancer cells and have no significant adverse or genotoxic effects (Stuhmer *et al*, 2005; Tovar *et al*, 2006).

Although about half of all human malignancies harbour dysfunctional, mutated p53 proteins, approximately 90% of all ES retain wild-type p53, and the downstream DNA damage cell cycle checkpoints and p53 pathways remain functionally intact (de Kovar *et al*, 1993; Alava *et al*, 2000; Kovar *et al*, 2003b; Huang *et al*, 2005). Exposure of ES cells to Nutlin-3a, a small organic molecule known to reactivate wild-type 53, resulted in a robust apoptotic phenotype that required the presence of wild-type p53 but did not affect the growth of mutant p53 expressing cells (Pishas *et al*, 2011). *TP53* mutation alone rates high among variables, including p16/p14ARF alteration and tumour stage, predicting poorer overall survival in Ewing sarcoma (de Alava *et al*, 2000; Lopez-Guerrero *et al*, 2011). Multivariate analysis identified alterations of *TP53* as an adverse prognostic factor defining a subset of ES with highly aggressive behaviour and poor chemoresponse (Huang *et al*, 2005). Therefore, novel treatment options specifically targeting mutant p53 are highly warranted. There is a prospective COG (Children's Oncology Group, USA) study ongoing to validate a retrospective study which strongly suggested that mutant p53 ES constitutes a particularly bad prognostic group (ClinicalTrials.gov identifier: NCT00898053). If data from the retrospective study is confirmed, this would strongly support the need for novel mutant p53 targeting therapeutic strategies in ES.

In this study, we investigated whether the small pharmacological molecule APR-246 is able to reactivate mutant p53 in Ewing sarcoma cells in order to drive tumour cells into apoptosis. Using Ewing sarcoma patients' derived cell lines, we show that APR-246 is able to induce apoptosis to variable degrees independent of the p53 status. We observed that cell lines with similar p53 mutations as well as cell lines established from the same patient at different stages of the disease all exhibited variable responses to the drug. To interrogate the molecular basis for the differential responses to APR-246, we performed comparative transcriptomic analysis on the three STA-ET-7 cell lines established from the same patient. Data analysed revealed genes annotating to p53 and apoptosis pathways whose expression varies between the more APR-246-sensitive and the less APR-246-sensitive cell lines. We propose, therefore, that APR-246 will not be a suitable candidate to consider for targeting p53 mutant Ewing sarcoma.

MATERIALS AND METHODS

Cell lines. The source and propagation of the ES cell lines used in our study has been described in detail previously (Kovar *et al*, 2003a). Cells were authenticated by PCR from microsatellites and routinely confirmed by morphology, PCR and immunoblotting. The three STA-ET-7 cell lines were established from tumour tissues of a pathologically proven ES. The STA-ET-7.1 cell line was established from the primary tumour while STA-ET-7.2 was established from a pleural effusion. The STA-ET-7.3 cell line was established from a distant metastasis. The breast carcinoma cell line, MDA-MB-468, was obtained from the American Type Culture Collection (Rockville, MD, USA) and its propagation is described elsewhere (Casey *et al*, 1991). The cell lines were grown in monolayer cultures. Exponentially growing cultures at 80% confluence were used in all experiments. The p53 mutation status in the ES cell lines are indicated in Figure 1A and have previously been determined by us (Kovar *et al*, 1993). The p53 status in MDA-MB-468 has also been previously reported (Huovinen *et al*, 2011) and is also indicated in Figure 1A.

Reagents and cell treatments. APR246/PRIMA-1^{Met} [2-(Hydroxymethyl)-2-(methoxymethyl)-1-azabicyclo[2.2.2.]octan-3-one] was purchased from Tocris Bioscience (Ellisville, MO, USA). A total of 25 mM stocks were prepared in sterile water and aliquots stored at -20°C until use. Cells were seeded in six-well tissue culture plates at 3×10^5 cells per well and treated with different concentrations of APR-246 for 24–48 h. siRNA to p53 and control scrambled siRNA were obtained commercially (Ambion, Applied Biosystems, Carlsbad, CA, USA).

siRNA transfection studies. STA-ET-7.2 cells were seeded in six-well plates a day before transfection at a density of 3×10^5 cells per well. The cells were transfected with 50 nmol l^{-1} p53 siRNA or scrambled siRNA (Ambion, Applied Biosystems) using Oligofectamine reagent (Invitrogen, Groningen, The Netherlands). p53 knockdown was analysed 24 h after transfection by immunoblot analysis to determine the optimal conditions for p53 down-regulation. Subsequently, cells were transfected with either p53 or control siRNAs for 48 h after which they were then treated with 20 μM APR-246 for another 24 h. At the end of these treatments, cells were harvested and subjected to apoptosis assays (Annexin-V staining and FACS analysis) or immunoblot analysis.

Immunoblotting. Total proteins (30–50 μg) were resolved by 8.5–12.5% SDS–PAGE and processed for immunoblotting according to the standard procedures. The following antibodies were used: mouse monoclonal antibody to p53 (DO-1; kindly provided by B. Vojtesek, Masaryk Memorial Cancer Institute, Brno, Czech Republic), mouse monoclonal anti-GAPDH (Ambion, Life Technologies, Austin, TX, USA), mouse monoclonal anti-PARP (BD Biosciences, Stockholm, Sweden). Linear protein quantification was performed using the LICOR Odyssey Infrared Imaging System (LI-COR Biosciences, Bad Homburg, Germany).

Flow cytometry. ES cells were seeded in six-well plates at 2×10^5 cells per well and the next day were treated with APR-246 (0, 10 or 20 μM) or with equivalent volume of medium as control for 24–48 h. After treatments with the drug, the cultures were washed, trypsinised, centrifuged and processed. For apoptosis assay, cells were resuspended in binding buffer and stained sequentially with Annexin-V-FITC and DAPI using an Annexin V-FITC apoptosis detection kit (BD Biosciences), according to the manufacturer's recommendations. Samples were analysed on a FACSCalibur flow cytometer (Becton Dickinson Immunocytometry Systems, San Jose, CA, USA), and data were analysed using FlowJo software (Tree Star, Inc., Ashland, OR, USA). Cells that stained positive for Annexin-V but negative for DAPI were taken as early apoptotic sub-fraction while cells that stained positive for both Annexin-V and DAPI represented the dead sub-fraction. Each experiment was repeated at least three times.

Quantitative real-time RT–PCR. Total RNA was extracted from cells using the RNeasy Mini Kit (QIAGEN, Hilden, Germany) according to the manufacturer's recommendations. RNA was transcribed into cDNA using standard protocols. For quantitative analysis, cDNA samples were analysed by Taqman reverse-transcriptase PCR (qRT–PCR). Ten nanograms of cDNA were used per reaction, and the expression of Bax, p21, Noxa, Puma and

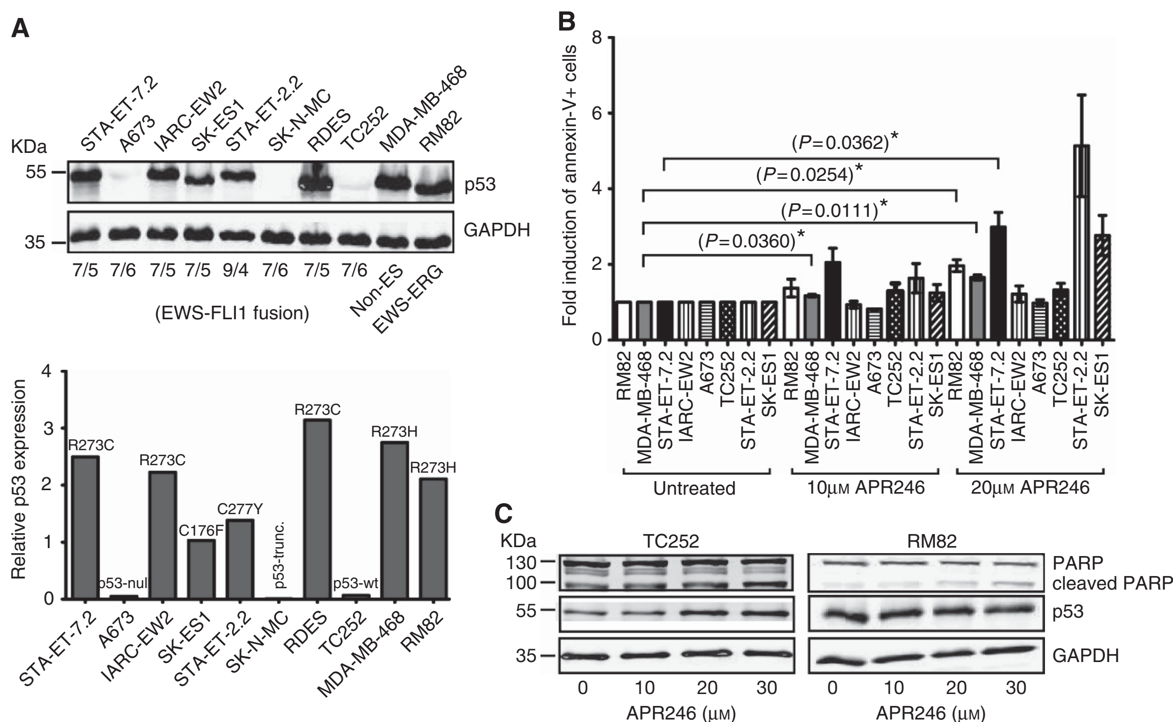

Figure 1. Mutant p53 protein expression differs in ES cell lines with different p53 status and APR-246 exposure induces variable levels of apoptosis in ES cells. (**A**) Western blot analysis showing varying levels of mutant p53 protein expression in several Ewing sarcoma cell lines and the breast carcinoma cell line MDA-MB-468, which was employed as a positive control. GAPDH was used as a loading control. The type of EWS-FLI1 fusion is also indicated (e.g., 7/5 is *EWS* exon 7 fused to *FLI1* exon 5). Lower panel shows histograms of Licor quantification of p53 bands relative to GAPDH, and the p53 mutation status of each cell line is also indicated. (**B**) APR-246 induces apoptosis in ES cell lines harbouring mutant or wild-type p53 (TC252) but not in a p53-null cell line (A673). Induction of apoptosis upon 24 h APR-246 treatment of ES cells was detected by fluorescence-activated cell sorting of Annexin-V-positive but DAPI-negative stained cells. (**C**) An immunoblot showing dose-dependent induction of PARP cleavage in both mutant-p53 RM82 cell line and the wild-type p53 cell line TC252 upon APR-246 exposure for 24 h.

Bcl-2 was performed using the ABI Prism 7900 Detection System (Applied Biosystems, Foster City, CA, USA). Expression levels were normalised to β-2-microglobulin. Reactions were done in triplicate using the Applied Biosystems Universal PCR Master Mix (Applied Biosystems). All procedures were done according to the manufacturer's protocols. The relative expression levels of the genes assessed were calculated by the $2^{(-\Delta\Delta Ct)}$ method (Schmittgen and Livak, 2008). Primer sequences and PCR conditions are available upon request.

Gene expression profiling and data analysis. Gene expression profiles for the three STA-ET-7 cell lines established from the same patient at different stages of the disease were followed on Affymetrix HG-U133 Plus 2.0 arrays (Affymetrix Inc., Santa Clara, CA, USA), and data were analysed essentially as previously reported (Aryee *et al*, 2010). Briefly, cRNA target synthesis and Gene-Chip processing were performed according to the standard protocols (Affymetrix Inc.). All further analyses were performed in R statistical environment using Bioconductor packages (Gentleman *et al*, 2004). MsigDB (http://www.broadinstitute.org/gsea/msigdb/collections.jsp) was used to annotate genes as TP53 and apoptosis associated. Gene expression data have been submitted to GEO (GSE 49967).

Statistical analysis. When applicable, the data were analysed using the unpaired *t*-test with Welch's correction or the non-parametric Kruskal–Wallis analysis of variance or with the one-sample *t*-test using the Prism 5 for Windows (version 5.02) statistical software (GraphPad Prism Software, Inc., La Jolla, CA, USA). Data shown in graphical format represent the means ($\pm$s.e.m.). A P value <0.05 was accepted as a significant difference.

RESULTS

p53 expression levels in ES cell lines and TP53 mutation status. Levels of p53 were evaluated in ES cell lines and the breast carcinoma cell line MDA-MB-468 by immunoblot analysis (Figure 1A). The mutation status of the cell lines used in this study are also indicated. As PRIMA-1 has been shown to inhibit growth of breast cancer cells (Liang *et al*, 2009), we chose as a positive control the non-ES cell line MDA-MB-468, which harbours a p53 mutation (R273H) similar to the ES cell line RM82. The cell lines TC252 (wt-p53), A673 (p53-null) and SK-N-MC (truncated-p53) were included as wild-type and p53 negative controls, respectively.

As shown in Figure 1A, the expression level of mutant p53 in the ES cell lines varied. However, in all the mt-p53 harbouring cell lines, the levels of the protein was higher than in the TC252 (wt-p53) cell line, which was only visualised after longer exposure, and there was no measurable expression of full-length p53 in the p53-null (A673) and p53-truncated (SK-N-MC) cell lines.

APR-246 induces apoptosis in ES cell lines independent of mutant p53 status. It was shown that significant decomposition of PRIMA-1 in cells occurs only after 4 h and just a minor portion of the starting material could be detected after 24 h (Lambert *et al*, 2009), and at high concentrations, APR-246 was reported to exhibit p53-independent effects (Roh *et al*, 2011).

Consequently, most of our treatments with APR-246 were carried out for 24 h, and we used low concentrations of the drug where we saw an effect on apoptosis induction.

Treatment of cells for 24 h with 20 μM APR-246 increased Annexin-V-positive (apoptotic) cells by up to 40% in some ES cell lines (Figure 1B). Although the breast carcinoma cell line MDA-MB-468, used as a control, already exhibited a significant induction of apoptosis at 10 μM APR-246, significant apoptosis induction was only achieved with 20 μM APR-246 in the ES cell lines indicated (Figure 1B). A slight induction of apoptosis was observed in the wild-type p53 cell line, TC252, whereas no measurable induction was seen in the p53-null cell line A673. PARP cleavage, an indicator of apoptosis induction, is shown in both the wild-type p53 cell line TC252 and the mutant p53 cell line RM82 in a dose-dependent manner on APR-246 treatment (Figure 1C). This is consistent with the apoptosis induction by APR-246 of the TC252 cell line as shown in Figure 1B and indicates that APR-246 also influences wild-type p53 function.

P53 mutation-independent but cellular-context dependency of APR-246 activity in ES cell lines. In the STA-ET-7.2 and IARC-EW2 cell lines, despite sharing an identical p53 mutation (R273C), response to APR-246 varied (Figure 1B). Although IARC-EW2 cells were resilient to the 20 μM concentration of the drug used in our assay, the STA-ET-7.2 cell line exhibited a significant response to this drug concentration. Also, three different cell lines (STA-ET-7: 1–3) established from the same patient at different stages of the disease responded differently to APR-246, with STA-ET-7.2 displaying the highest sensitivity (Figure 2A). This variability in response to APR-246 implicates the cellular context in these responses. To ascertain whether these variable responses reflect differences in mutant p53 expression, we performed immunoblot analysis to determine p53 expression in these cell lines. As shown in Figure 2A, mutant p53 expression varied only slightly among the three cell lines.

SiRNA knockdown of mutant p53 reduces the apoptosis-inducing effects of APR-246 in ES cells. To investigate the role of mutant p53 in APR-246 mediated apoptosis, the effect of APR-246 was tested in the STA-ET-7.2 ES cell line in which mutant p53 was downregulated using siRNA. We transiently transfected ES cells with p53-siRNA or control scrambled-siRNA, treated the transfected cells with APR-246 for 24 h and assessed the fraction of early apoptotic cells (Annexin-V-positive but DAPI-negative)

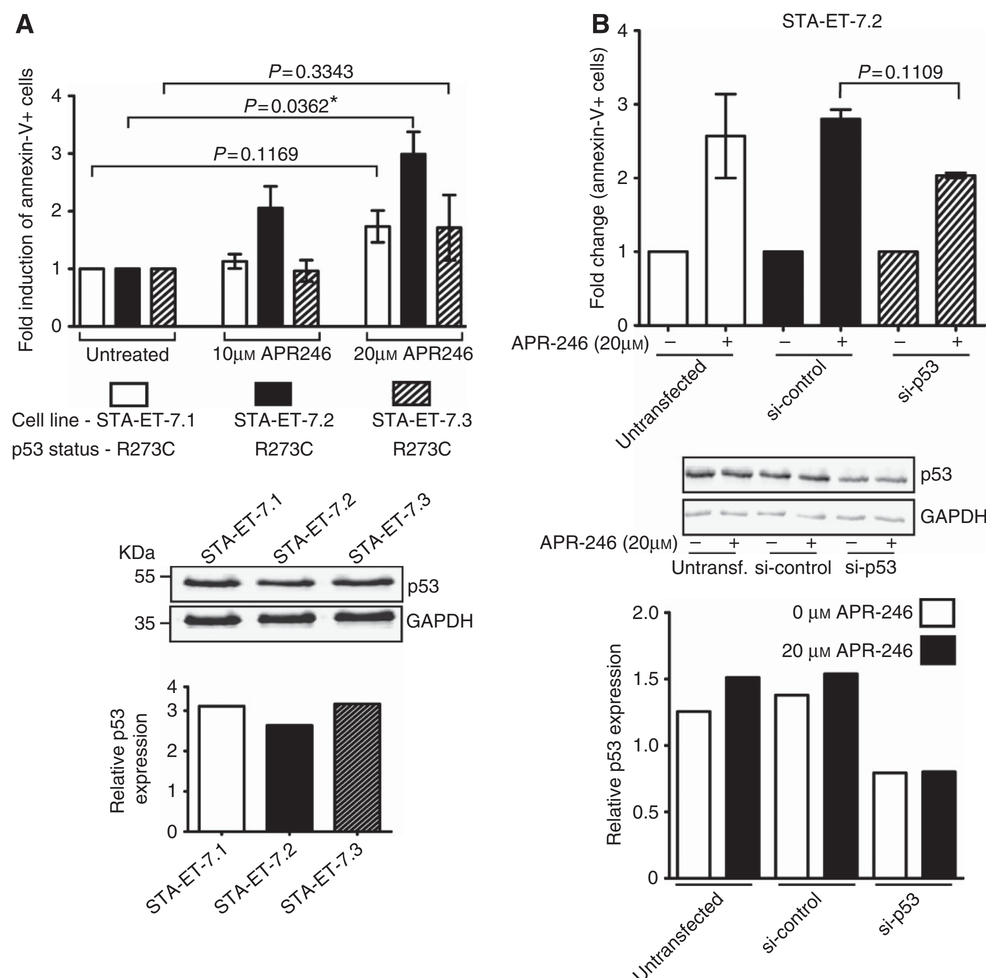

Figure 2. p53 dependency of apoptosis induction in ES cells after APR-246 treatments. (**A**) Three different cell lines (STA-ET-7: 1–3) generated from the same patient at different stages of the disease responded differently to APR-246 treatment. Results are representative of three independent experiments. Error bars denote s.e.m. *P* values represent statistical differences between the number of cells in treated *vs* untreated samples in each cell line. Middle panel shows an immunoblot depicting mutant p53 expression levels in each cell line while lower panel shows histograms of Licor-quantified bands relative to GAPDH. (**B**) siRNA-mediated knockdown of mutant p53 suppresses APR-246-induced apoptosis in the ES cell line STA-ET-7.2 as assessed by FACS analysis of Annexin-V+ (DAPI-negative) cells. $n=3$, error bars denote s.e.m. Lower panel: an immunoblot (middle) and histograms showing Licor quantification of immunoblot bands of total mutant p53 levels in STA-ET-7.2 cells transfected with control siRNA or p53 siRNA and treated with the stated concentration of APR-246.

by FACS. Treatment of STA-ET-7.2 cells with APR-246 after transfection with p53 siRNA resulted in a reduced cytotoxicity compared with control siRNA transfected cells (Figure 2B). As these responses may depend on the extent of p53 knockdown, we performed immunoblotting (shown in lower panel) on samples to control the extent of p53 knockdown. These results suggest that the growth-suppressive effect of APR-246 in these ES cells is at least partially p53 dependent.

APR-246 activates p53 target genes and p53-dependent apoptosis. Downstream target genes of p53 are known to mediate its tumour-suppressive activity as well as initiate cell death through apoptosis induction. To test whether APR-246 treatment of ES cells results in upregulation of *p53* target genes, levels of classical *p53* target genes were evaluated in ES cells before and after APR-246 treatments for up to 48 h. Treatment of the ES cell line STA-ET-7.2 resulted in enhanced *Puma* and a significant upregulation of *cdkn1A*/p21^{WAF1} as evidenced by increased expression in real-time quantitative RT–PCR analysis (Figure 3A). This enhanced activation was abrogated upon siRNA-mediated mutant p53 knockdown (Figure 3C). On the other hand, *Noxa*, a known wild-type p53 pro-apoptotic target, as well as *Puma* and *Bax*, were only moderately upregulated in the RM82 cell line while the anti-apoptotic gene *Bcl-2* was hardly affected by APR-246 treatment as shown in Figure 3A. In the wild-type p53 cell line TC252, expression of the pro-apoptotic gene *Puma* as well as the classical p53 target *cdkn1A*/p21^{WAF1} were all enhanced upon APR-246 treatment with *cdkn1A*/p21^{WAF1} levels reaching near significance (Figure 3B). On the other hand, APR-246 treatment did not seem to influence expression of *bona fide* p53 targets in the p53-null A673 cells (Figure 3B), pointing to the role of p53 in cellular responses to APR-246 exposure in these cells.

Microarray analysis reveals genes differentially expressed among the STA-ET-7 cell lines. To elucidate the molecular basis for the heterogeneity in response to APR-246, the transcriptional profiles of the three STA-ET-7 cell lines were investigated via microarray analysis (Figure 4). In all, 277 (132 downregulated, 145 upregulated) genes differed significantly ($P<0.01$, $|FC|>1$) between the STA-ET-7.2 cell line relative to the STA-ET-7.1 and STA-ET-7.3. Also, 106 of these genes were found to be associated with p53 or apoptosis and 17 with both. Subtracting genes that differed significantly between the cell lines STA-ET-7.1 and STA-ET-7.3 from these 106 genes ($P<0.05$, $|FC|>0.58$) gave 42 genes that are specific for the STA-ET-7.2 cell line (Table 1) and may therefore include putative candidates for the observed differential sensitivity to APR-246. In contrast, although differences in gene expression changes between the three STA-ET-7 cell lines upon APR-246 treatment did not achieve statistical significance, downregulation of one gene, TPM4, in STA-ET-7.2 but not the other two cell lines may be of relevance. Its silencing in MCF7 cells has previously been linked to enhanced sensitivity to tamoxifen (Mendes-Pereira *et al*, 2012; data not shown).

DISCUSSION

ES is a very aggressive disease and though *TP53* mutations are rare in ES, with the majority of tumours expressing wild-type p53 (Kovar *et al*, 1993; de Alava *et al*, 2000; Huang *et al*, 2005), patients with point mutation of *TP53* are associated with a dismal prognosis (Huang *et al*, 2005). However, even in the absence of mutation, there is evidence that wild-type p53 may be functionally disabled in Ewing sarcoma as a consequence of the EWS-FLI1 oncogene

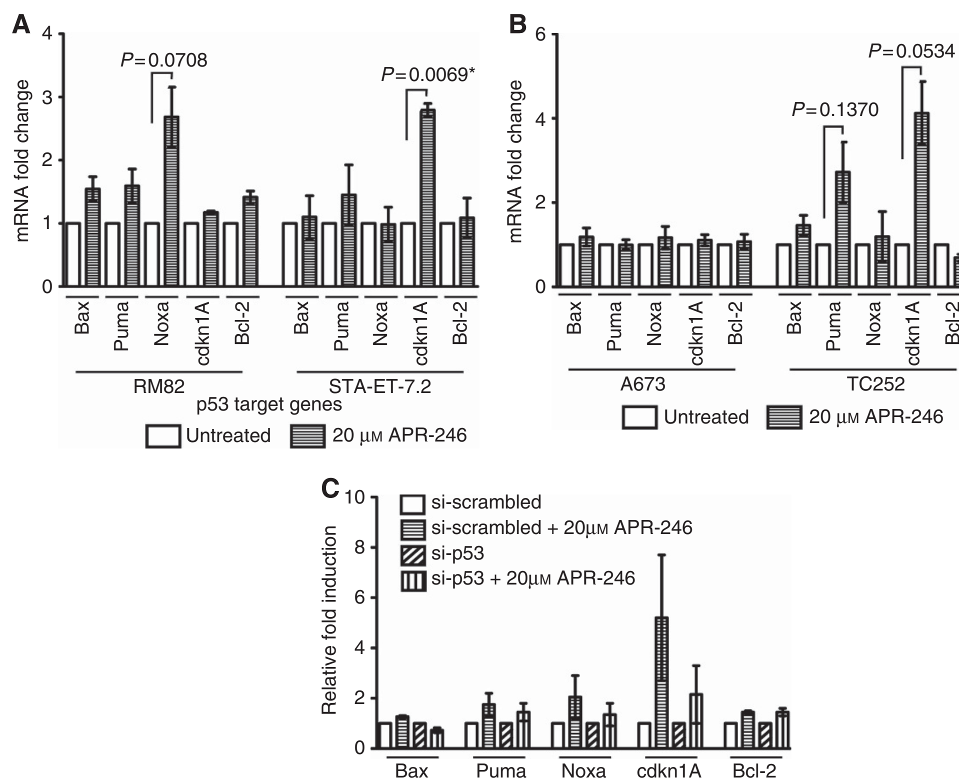

Figure 3. APR-246 activates p53 target genes. (**A**) Changes in mRNA expression of p53 pathway and pro-apoptotic genes as measured by real-time quantitative RT–PCR, shown as fold induction relative to untreated cells in RM82 and STA-ET-7.2 cells after 24 h APR-246 treatments. (**B**) Induction of p21 (cdkn1A) and Puma (BBC3) mRNAs on treatment with 20 μM APR-246 in the p53 wild-type cell line TC252 but not in the p53-null cell line A673 as assessed by real-time PCR. (**C**) p53 knockdown by siRNA abrogates the induction of p21 mRNA by APR-246 in the STA-ET-7.2 cell line. Histograms represent the mean ± s.e.m. of three independent experiments. A *P* value <0.05 is considered to be statistically significant.

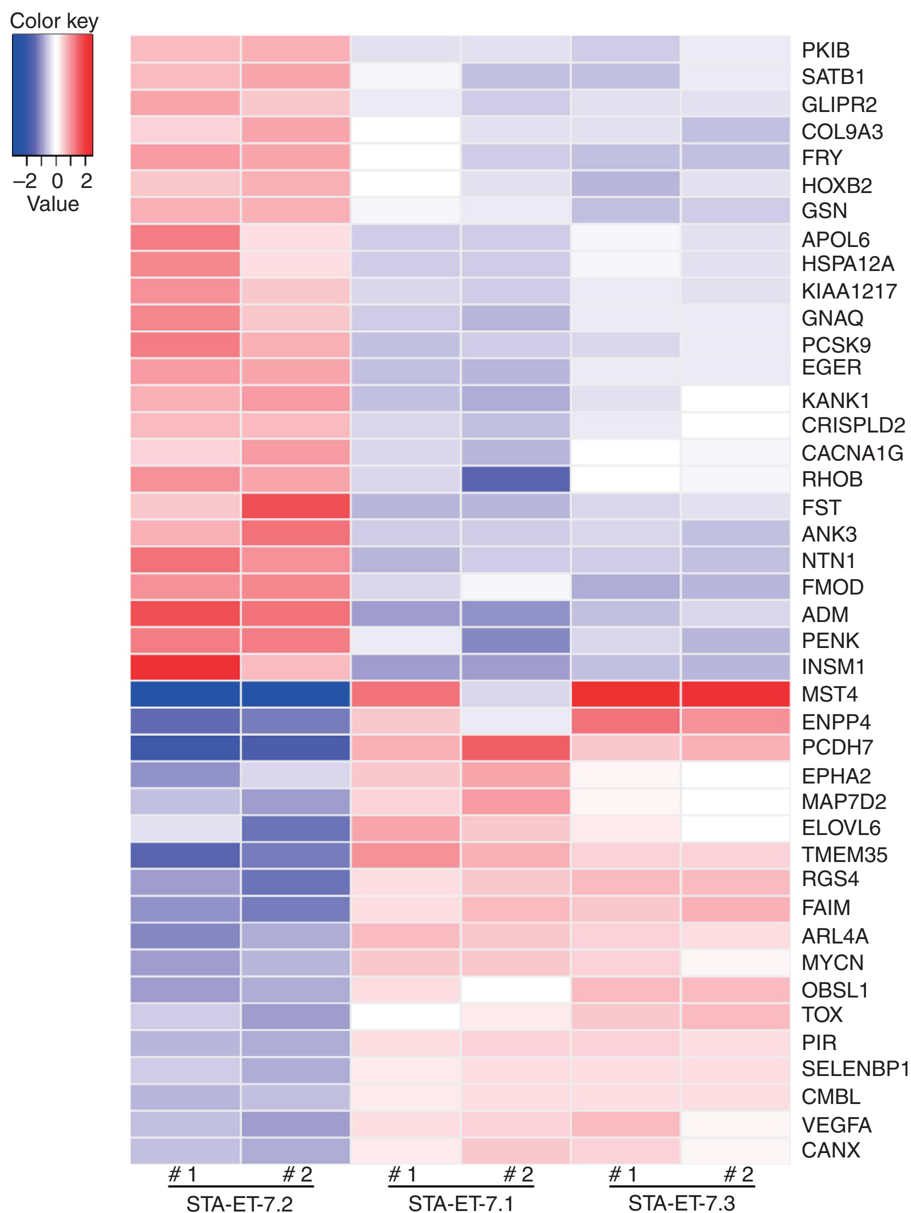

Figure 4. Heat map of STA-ET-7.2-specific genes related to p53 and apoptosis. Normalised and row-scaled expression values from Affymetrix HG-U133-PLUS2 arrays are shown for 42 genes differentially expressed between the STA-ET-7.2 cell line and the STA-ET-7.1 and STA-ET-7.3 cell lines. No.1 and No.2 represent the two different replicas used for the assay.

(Ban *et al*, 2008; Li *et al*, 2012). A study of 308 ES cases established that mutant p53 expression was more frequent in disseminated disease than in primary localised tumours, indicating a role in the progression and metastasis of ES (Lopez-Guerrero *et al*, 2011). This makes functional restoration of the p53 pathway an attractive therapeutic option in this tumour entity. In the current study, we addressed the possibility of using the small-molecule compound, APR-246/PRIMA-1^{Met}, capable of reactivating mutant p53 and inducing apoptosis in several different cancer types, to induce cell death in ES cells harbouring different p53 mutants. In this study, we have shown that ES cells exhibit different sensitivities to APR-246 exposure. For instance, three ES cell lines established from the same patient at different stages of the disease (with identical p53 mutation) all reacted variably to APR-246 treatment (Figure 2A). We also asked whether tumour cell lines with the same mutation will show similar cellular response to APR-246. To address this question, we took three approaches: (1) we used the breast cancer cell line MDA-MB-468 (with the *R273H* p53 mutation) as a positive control to investigate an ES cell line with identical p53 mutation, RM82, (2) we investigated the response of two ES cell lines, established from different ES patients, with identical p53 mutations and, (3) we studied three cell lines from different tumour materials of the same patient to the same concentrations of APR-246. We observed that response of the cells was unrelated to the mutation type, alluding to the cellular context dependency of the response to APR-246 (Figure 1B). To investigate whether induction of apoptosis was mediated via p53 upon APR-246 exposure, we used RNAi to knockdown p53 in mt-p53 cell lines before treatment with APR-246. We found apoptosis induction after APR-246 treatment of the STA-ET-7.2 cell line transfected with p53 siRNA was reduced compared with scrambled siRNA-transfected control cells (Figure 2B). We now report that treatment with APR-246 evoked apoptosis to variable extents in mt-p53 ES cell lines and also on the p53 wild-type cell line (TC252) but no measurable effect on a p53-null cell line (A673). It has recently been shown that APR-246 can bind to unfolded wt-p53 and

Table 1. STA-ET-7.2-specific genes related to p53 and apoptosis

Gene name	EG	Name	Log2 fold change[a]
MST4	51765	Serine/threonine protein kinase MST4	−4.31
PCDH7	5099	Protocadherin 7	−2.72
TMEM35	59353	Transmembrane protein 35	−2.05
ENPP4	22875	Ectonucleotide pyrophosphatase/phosphodiesterase 4 (putative)	−2.01
RGS4	5999	Regulator of G-protein signaling 4	−1.67
FAIM	55179	Fas apoptotic inhibitory molecule	−1.65
ARL4A	10124	ADP-ribosylation factor-like 4A	−1.5
MYCN	4613	v-Myc myelocytomatosis viral-related oncogene, neuroblastoma derived (avian)	−1.29
OBSL1	23363	Obscurin-like 1	−1.27
ELOVL6	79071	ELOVL fatty acid elongase 6	−1.24
PIR	8544	Pirin (iron-binding nuclear protein)	−1.22
MAP7D2	256714	MAP7 domain containing 2	−1.21
VEGFA	7422	Vascular endothelial growth factor A	−1.15
TOX	9760	Thymocyte selection-associated high mobility group box	−1.14
EPHA2	1969	EPH receptor A2	−1.14
CANX	821	Calnexin	−1.07
CMBL	134147	Carboxymethylenebutenolidase homolog (Pseudomonas)	−1.04
SELENBP1	8991	Selenium binding protein 1	−1.03
PKIB	5570	Protein kinase (cAMP-dependent, catalytic) inhibitor beta	1
CACNA1G	8913	Calcium channel, voltage-dependent, T type, alpha 1G subunit	1.02
CRISPLD2	83716	Cysteine-rich secretory protein LCCL domain containing 2	1.02
HOXB2	3212	Homeobox B2	1.03
GLIPR2	152007	GLI pathogenesis-related 2	1.03
COL9A3	1299	Collagen, type IX, alpha 3	1.03
HSPA12A	259217	Heat shock 70-kDa protein 12A	1.13
GSN	2934	Gelsolin	1.14
KIAA1217	56243	KIAA1217	1.16
SATB1	6304	SATB homeobox 1	1.17
APOL6	80830	Apolipoprotein L, 6	1.2
GNAQ	2776	Guanine nucleotide binding protein (G protein), q polypeptide	1.3
KANK1	23189	KN motif and ankyrin repeat domains 1	1.31
FRY	10129	Furry homolog (Drosophila)	1.33
EGFR	1956	Epidermal growth factor receptor	1.36
RHOB	388	Ras homolog family member B	1.43
PCSK9	255738	Proprotein convertase subtilisin/kexin type 9	1.44
ANK3	288	Ankyrin 3, node of Ranvier (ankyrin G)	1.52
FMOD	2331	Fibromodulin	1.62
FST	10468	Follistatin	1.72
NTN1	9423	Netrin 1	1.82
PENK	5179	Proenkephalin	1.89
ADM	133	Adrenomedullin	2.24
INSM1	3642	Insulinoma-associated 1	2.37

[a]Values for each gene are log 2 fold change of the STA-ET-7.2 cell line *vs* the average of STA-ET-7.1 and STA-ET-7.3 cell lines.

activate it by inducing correct folding (Lambert *et al*, 2009). As the actual mechanism of action of APR-246 is not completely clear (Zandi *et al*, 2011), our data corroborate reports showing that APR-246 also affects wt-p53-containing cells. We also observed that APR-246 induced expression of variable sets of classical p53 target genes in ES cells harbouring mutant and wild-type p53 (Figures 3A and B). By real-time quantitative PCR assay in the STA-ET-7.2 cell line after APR-246 treatment, we found p21 mRNA to be significantly induced (Figure 3A). APR-246 also activated transcription of the pro-apoptotic genes *Noxa* (PMAIP1) in RM82 and *Puma* in STA-ET-7.2. The changes in *Noxa* and *Puma* mRNA expression observed support the potential activation

of the p53-dependent apoptotic pathway by APR-246. In the wild-type p53 cell line, TC252, both *Puma* and *p21* mRNAs were induced by APR-246 treatments, whereas no effect on p53 target genes was seen in the p53-null cell line A673 (Figure 3B). Genome-wide gene expression analysis performed in the STA-ET-7 cell line triplet revealed, among others, differential expression of genes that annotated to p53 and apoptosis pathways. Among them, over-expression of the gene encoding for apolipoprotein 6 (*APOL6*), as was observed in STA-ET-7.2, has been shown to induce mitochondrial-mediated apoptosis in colorectal cancer cells (DLD-1) (Liu *et al*, 2005). Another gene, *PENK*, which is highly upregulated in the STA-ET-7.2 cell line relative to the other two cell lines, has been shown to assist stress-activated apoptosis through transcriptional repression of NF-kappaB- and p53-regulated gene targets (McTavish *et al*, 2007). Also, some anti-apoptotic genes such as *PCDH7* (Zhang and DuBois, 2001) and *MST4* (Sung *et al*, 2003) are highly suppressed in the more APR-246-sensitive STA-ET-7.2 cell line in comparison to the less sensitive STA-ET-7.1 and STA-ET-7.3 cell lines (Figure 4). The relative expression levels of these genes among the cell lines could at least be partially responsible for their disparate sensitivities to APR-246 treatment. These results are consistent with the reported capability of APR-246 to restore transcriptional activity to mutant p53 and trigger mutant p53-dependent apoptosis (Bykov *et al*, 2002a). This is also in line with the suggestion by Lambert *et al* (2009, 2010) that adducts of the APR-246 conversion product methylene quinuclidinone could create novel protein–DNA contacts, which could affect the choice of target genes. It is also reported that APR-246 could target other proteins in the cell, which might lead to synergistic effects promoting apoptosis rather than growth arrest (Rokaeus *et al*, 2010). This notion reflects our finding of the induction of different p53 target genes in different mutant p53 cell lines after APR-246 treatments, notwithstanding the cellular context dependency exhibited by ES cells in response to APR-246. In our real-time quantitative PCR data, we did not observe high induction of Bax mRNA by APR-246 treatment. This corroborates reported results by Chipuk *et al* (2003), where they found that Bax-dependent apoptosis induced by APR-246 is mutant p53 dependent but transcription independent. In this study, we also show that there is no association between the type of p53 mutation and the response to APR-246 treatment. It is also speculated that APR-246 can induce ER stress that may cause p53-independent responses (Lambert *et al*, 2010). Therefore, cellular responses to APR-246 may not be directly p53 dependent but rather a response to its induced ER stress.

In conclusion, our results suggest that there is variability in functional p53 reactivation by APR-246 in ES cells independent of the p53 status. We also observed that APR-246 variably enhanced the apoptosis-inducing capacity of the chemotherapeutic agent Etoposide, which is currently used in the treatment of patients with ES (data not shown). Although *in vivo* studies would be required to validate the results, the *in vitro* data do not support APR-246 as a prime drug to advance into animal and clinical studies. Those genes identified in our gene expression profiling to be differentially expressed between the APR-246-sensitive cell line (STA-ET-7.2) and the less sensitive cell lines (STA-ET-7.1 and STA-ET-7.3) should stimulate further studies to investigate the relevance of these genes as potential biomarkers for stratification for APR-246 treatment. Together, our data downplay the prospect of considering APR-246 as a candidate for the future development of novel treatment modalities for ES patients.

ACKNOWLEDGEMENTS

We are grateful to David Herrero-Martin and Eleni Tomazou-Bock for critical reading of the manuscript. We thank Argyro Fourtouna and Dieter Prinz for help with the FACS analyses. This work was supported by grant 14205 from the 'Jubilaeumsfonds' of the Austrian National Bank to DNTA.

REFERENCES

Aryee DN, Niedan S, Kauer M, Schwentner R, Bennani-Baiti IM, Ban J, Muehlbacher K, Kreppel M, Walker RL, Meltzer P, Poremba C, Kofler R, Kovar H (2010) Hypoxia modulates EWS-FLI1 transcriptional signature and enhances the malignant properties of Ewing's sarcoma cells *in vitro*. *Cancer Res* **70**(10): 4015–4023.

Ban J, Bennani-Baiti IM, Kauer M, Schaefer KL, Poremba C, Jug G, Schwentner R, Smrzka O, Muehlbacher K, Aryee DN, Kovar H (2008) EWS-FLI1 suppresses NOTCH-activated p53 in Ewing's sarcoma. *Cancer Res* **68**(17): 7100–7109.

Bunz F, Hwang PM, Torrance C, Waldman T, Zhang Y, Dillehay L, Williams J, Lengauer C, Kinzler KW, Vogelstein B (1999) Disruption of p53 in human cancer cells alters the responses to therapeutic agents. *J Clin Invest* **104**(3): 263–269.

Burns TF, El-Deiry WS (1999) The p53 pathway and apoptosis. *J Cell Physiol* **181**(2): 231–239.

Bykov VJ, Issaeva N, Selivanova G, Wiman KG (2002a) Mutant p53-dependent growth suppression distinguishes PRIMA-1 from known anticancer drugs: a statistical analysis of information in the National Cancer Institute database. *Carcinogenesis* **23**(12): 2011–2018.

Bykov VJ, Issaeva N, Shilov A, Hultcrantz M, Pugacheva E, Chumakov P, Bergman J, Wiman KG, Selivanova G (2002b) Restoration of the tumor suppressor function to mutant p53 by a low-molecular-weight compound. *Nat Med* **8**(3): 282–288.

Casey G, Lo-Hsueh M, Lopez ME, Vogelstein B, Stanbridge EJ (1991) Growth suppression of human breast cancer cells by the introduction of a wild-type p53 gene. *Oncogene* **6**(10): 1791–1797.

Chipuk JE, Maurer U, Green DR, Schuler M (2003) Pharmacologic activation of p53 elicits Bax-dependent apoptosis in the absence of transcription. *Cancer Cell* **4**(5): 371–381.

de Alava E, Antonescu CR, Panizo A, Leung D, Meyers PA, Huvos AG, Pardo-Mindan FJ, Healey JH, Ladanyi M (2000) Prognostic impact of P53 status in Ewing sarcoma. *Cancer* **89**(4): 783–792.

Gentleman RC, Carey VJ, Bates DM, Bolstad B, Dettling M, Dudoit S, Ellis B, Gautier L, Ge Y, Gentry J, Hornik K, Hothorn T, Huber W, Iacus S, Irizarry R, Leisch F, Li C, Maechler M, Rossini AJ, Sawitzki G, Smith C, Smyth G, Tierney L, Yang JY, Zhang J (2004) Bioconductor: open software development for computational biology and bioinformatics. *Genome Biol* **5**(10): R80.

Green DR, Kroemer G (2009) Cytoplasmic functions of the tumour suppressor p53. *Nature* **458**(7242): 1127–1130.

Huang HY, Illei PB, Zhao Z, Mazumdar M, Huvos AG, Healey JH, Wexler LH, Gorlick R, Meyers P, Ladanyi M (2005) Ewing sarcomas with p53 mutation or p16/p14ARF homozygous deletion: a highly lethal subset associated with poor chemoresponse. *J Clin Oncol* **23**(3): 548–558.

Huovinen M, Loikkanen J, Myllynen P, Vahakangas KH (2011) Characterization of human breast cancer cell lines for the studies on p53 in chemical carcinogenesis. *Toxicol In Vitro* **25**(5): 1007–1017.

Kovar H, Auinger A, Jug G, Aryee D, Zoubek A, Salzer-Kuntschik M, Gadner H (1993) Narrow spectrum of infrequent p53 mutations and absence of MDM2 amplification in Ewing tumours. *Oncogene* **8**(10): 2683–2690.

Kovar H, Ban J, Pospisilova S (2003a) Potentials for RNAi in sarcoma research and therapy: Ewing's sarcoma as a model. *Semin Cancer Biol* **13**(4): 275–281.

Kovar H, Pospisilova S, Jug G, Printz D, Gadner H (2003b) Response of Ewing tumor cells to forced and activated p53 expression. *Oncogene* **22**(21): 3193–3204.

Lambert JM, Gorzov P, Veprintsev DB, Soderqvist M, Segerback D, Bergman J, Fersht AR, Hainaut P, Wiman KG, Bykov VJ (2009) PRIMA-1 reactivates mutant p53 by covalent binding to the core domain. *Cancer Cell* **15**(5): 376–388.

Lambert JM, Moshfegh A, Hainaut P, Wiman KG, Bykov VJ (2010) Mutant p53 reactivation by PRIMA-1MET induces multiple signaling pathways converging on apoptosis. *Oncogene* **29**(9): 1329–1338.

Li Y, Li X, Fan G, Fukushi J, Matsumoto Y, Iwamoto Y, Zhu Y (2012) Impairment of p53 acetylation by EWS-Fli1 chimeric protein in Ewing family tumors. *Cancer Lett* **320**(1): 14–22.
Liang Y, Besch-Williford C, Hyder SM (2009) PRIMA-1 inhibits growth of breast cancer cells by re-activating mutant p53 protein. *Int J Oncol* **35**(5): 1015–1023.
Liu Z, Lu H, Jiang Z, Pastuszyn A, Hu CA (2005) Apolipoprotein l6, a novel proapoptotic Bcl-2 homology 3-only protein, induces mitochondria-mediated apoptosis in cancer cells. *Mol Cancer Res* **3**(1): 21–31.
Lopez-Guerrero JA, Machado I, Scotlandi K, Noguera R, Pellin A, Navarro S, Serra M, Calabuig-Farinas S, Picci P, Llombart-Bosch A (2011) Clinicopathological significance of cell cycle regulation markers in a large series of genetically confirmed Ewing's sarcoma family of tumors. *Int J Cancer* **128**(5): 1139–1150.
Lowe SW, Lin AW (2000) Apoptosis in cancer. *Carcinogenesis* **21**(3): 485–495.
McTavish N, Copeland LA, Saville MK, Perkins ND, Spruce BA (2007) Proenkephalin assists stress-activated apoptosis through transcriptional repression of NF-kappaB- and p53-regulated gene targets. *Cell Death Differ* **14**(9): 1700–1710.
Mendes-Pereira AM, Sims D, Dexter T, Fenwick K, Assiotis I, Kozarewa I, Mitsopoulos C, Hakas J, Zvelebil M, Lord CJ, Ashworth A (2012) Genome-wide functional screen identifies a compendium of genes affecting sensitivity to tamoxifen. *Proc Natl Acad Sci USA* **109**(8): 2730–2735.
Miyashita T, Reed JC (1995) Tumor suppressor p53 is a direct transcriptional activator of the human bax gene. *Cell* **80**(2): 293–299.
Nakano K, Vousden KH (2001) PUMA, a novel proapoptotic gene, is induced by p53. *Mol Cell* **7**(3): 683–694.
Pishas KI, Al-Ejeh F, Zinonos I, Kumar R, Evdokiou A, Brown MP, Callen DF, Neilsen PM (2011) Nutlin-3a is a potential therapeutic for ewing sarcoma. *Clin Cancer Res* **17**(3): 494–504.
Poeta ML, Manola J, Goldwasser MA, Forastiere A, Benoit N, Califano JA, Ridge JA, Goodwin J, Kenady D, Saunders J, Westra W, Sidransky D, Koch WM (2007) TP53 mutations and survival in squamous-cell carcinoma of the head and neck. *N Engl J Med* **357**(25): 2552–2561.
Robles AI, Bemmels NA, Foraker AB, Harris CC (2001) APAF-1 is a transcriptional target of p53 in DNA damage-induced apoptosis. *Cancer Res* **61**(18): 6660–6664.
Roh JL, Kang SK, Minn I, Califano JA, Sidransky D, Koch WM (2011) p53-Reactivating small molecules induce apoptosis and enhance chemotherapeutic cytotoxicity in head and neck squamous cell carcinoma. *Oral Oncol* **47**(1): 8–15.
Rokaeus N, Shen J, Eckhardt I, Bykov VJ, Wiman KG, Wilhelm MT (2010) PRIMA-1(MET)/APR-246 targets mutant forms of p53 family members p63 and p73. *Oncogene* **29**(49): 6442–6451.
Schmittgen TD, Livak KJ (2008) Analyzing real-time PCR data by the comparative C(T) method. *Nat Protoc* **3**(6): 1101–1108.
Selivanova G (2004) p53: fighting cancer. *Curr Cancer Drug Targets* **4**(5): 385–402.
Selivanova G (2010) Therapeutic targeting of p53 by small molecules. *Semin Cancer Biol* **20**(1): 46–56.
Stuhmer T, Chatterjee M, Hildebrandt M, Herrmann P, Gollasch H, Gerecke C, Theurich S, Cigliano L, Manz RA, Daniel PT, Bommert K, Vassilev LT, Bargou RC (2005) Nongenotoxic activation of the p53 pathway as a therapeutic strategy for multiple myeloma. *Blood* **106**(10): 3609–3617.
Sung V, Luo W, Qian D, Lee I, Jallal B, Gishizky M (2003) The Ste20 kinase MST4 plays a role in prostate cancer progression. *Cancer Res* **63**(12): 3356–3363.
Tovar C, Rosinski J, Filipovic Z, Higgins B, Kolinsky K, Hilton H, Zhao X, Vu BT, Qing W, Packman K, Myklebost O, Heimbrook DC, Vassilev LT (2006) Small-molecule MDM2 antagonists reveal aberrant p53 signaling in cancer: implications for therapy. *Proc Natl Acad Sci USA* **103**(6): 1888–1893.
Vassilev LT, Vu BT, Graves B, Carvajal D, Podlaski F, Filipovic Z, Kong N, Kammlott U, Lukacs C, Klein C, Fotouhi N, Liu EA (2004) *In vivo* activation of the p53 pathway by small-molecule antagonists of MDM2. *Science* **303**(5659): 844–848.
Vousden KH, Lu X (2002) Live or let die: the cell's response to p53. *Nat Rev Cancer* **2**(8): 594–604.
Zandi R, Selivanova G, Christensen CL, Gerds TA, Willumsen BM, Poulsen HS (2011) PRIMA-1Met/APR-246 induces apoptosis and tumor growth delay in small cell lung cancer expressing mutant p53. *Clin Cancer Res* **17**(9): 2830–2841.
Zhang Z, DuBois RN (2001) Detection of differentially expressed genes in human colon carcinoma cells treated with a selective COX-2 inhibitor. *Oncogene* **20**(33): 4450–4456.

This work is published under the standard license to publish agreement. After 12 months the work will become freely available and the license terms will switch to a Creative Commons Attribution-NonCommercial-Share Alike 3.0 Unported License.