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

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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\textsuperscript{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\textsuperscript{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
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 p53, 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.

**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-azacyclo[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 × 10⁵ 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 × 10⁶ cells per well. The cells were transfected with 50 nmol·1⁻¹ 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 × 10⁵ 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...
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 $2^{-\Delta\Delta C_{T}}$ 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 (± 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).

![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.](image)
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 \( \mu \)M APR-246 increased Annexin-V-positive (apopotic) 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 \( \mu \)M APR-246, significant apoptosis induction was only achieved with 20 \( \mu \)M 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 \( \mu \)M 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).
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/p21WAF1 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/p21WAF1 were all enhanced upon APR-246 treatment with cdkn1A/p21WAF1 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...
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<sup>met</sup>, 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

![Heat map of STA-ET-7.2-specific genes related to p53 and apoptosis.](https://www.bjcancer.com/DOI:10.1038/bjc.2013.635)

| No. 1 | No. 2 | No. 1 | No. 2 | No. 1 |
|-------|-------|-------|-------|-------|
| STA-ET-7.2 | STA-ET-7.1 | STA-ET-7.3 |

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.
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) and Puma in STA-ET-7.2. The changes in Noxa and Puma mRNA expression observed support the potential activation

| Gene name | EG    | Name                                                   | Log2 fold changea |
|-----------|-------|--------------------------------------------------------|-------------------|
| 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  | Piprin (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                                | −1.03             |
| 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              |
| GLIIP2    | 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, mode 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              |

*aValues 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.*
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 quinolinodine 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
corroboreted reported results by Chhipuk 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 consider-
APR-246 as a candidate for the future development of novel
therapy modalities for ES patients.

REFERENCES

Aryee DN, Niedan S, Kauer M, Schwentner R, Bennani-Bait I, Ban J,
Muelhnbacher K, Kreppel M, Walker RL, Meltzer P, Poremba C, Koller 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-Bait I, Kauer M, Schaefer KL, Poremba C, Jug G,
Schwentner R, Smrzka O, Muelhnbacher 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)
In vitro and 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 consider-
APR-246 as a candidate for the future development of novel
therapy 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 Fournoua
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.

Variable responses of Ewing sarcoma cells to APR-246

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www.bjcancer.com | DOI:10.1038/bjc.2013.635

2703
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): 485–492.

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, Voussen KH (2001) PUMA, a novel proapoptotic gene, is induced by p53. *Mol Cell* 7(3): 683–694.

Pishas KL, Al-Ejeh F, Zinonos I, Kumar R, Evdokiiou A, Brown MP, Callen DF, Neilson 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. *Oncol Rep* 27(1): 8–15.

Rokaeus N, Shen J, Eckhardt J, 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.

Stuhler N, 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, Kammott 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.

Voussen 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, DaBois RN (2001) Detection of differentially expressed genes in human colon carcinoma cells treated with a selective COX-2 inhibitor. *Oncogene* 20(33): 4450–4456.

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