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The Mutant p53-Targeting Compound APR-246 Induces ROS-Modulating Genes in Breast Cancer Cells

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Abstract

TP53 is the most frequently mutated gene in human cancer and thus an attractive target for novel cancer therapy. Several compounds that can reactive mutant p53 protein have been identified. APR-246 is currently being tested in a phase II clinical trial in high-grade serous ovarian cancer. We have used RNA-seq analysis to study the effects of APR-246 on gene expression in human breast cancer cell lines. Although the effect of APR-246 on gene expression was largely cell line dependent, six genes were upregulated across all three cell lines studied, i.e., *TRIM16, SLC7A11, TXNRD1, SRXN1, LOC344887*, and *SLC7A11-AS1*. We did not detect upregulation of canonical p53 target genes such as *CDKN1A* (p21), *14-3-3σ, BBC3* (PUMA), and *PMAIP1* (NOXA) by RNA-seq, but these genes were induced according to analysis by qPCR. Gene ontology analysis showed that APR-246 induced changes in pathways such as response to oxidative stress, gene expression, cell proliferation, response to nitrosative stress, and the glutathione biosynthesis process. Our results are consistent with the dual action of APR-246, i.e., reactivation of mutant p53 and modulation of redox activity. *SLC7A11, TRIM16, TXNRD1*, and *SRXN1* are potential new pharmacodynamic biomarkers for assessing the response to APR-246 in both preclinical and clinical studies.

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Introduction

The *TP53* gene which encodes the p53 tumor suppressor protein is the most frequently mutated gene in human cancer. Thus, in a study of 12 different cancer types, *TP53* was the most frequently mutated genes in 10 of the tumor types investigated [1]. Indeed, in some of the most difficult to treat cancers such as squamous esophageal cancer, squamous cell lung cancer, small cell lung cancer, high-grade serous ovarian cancer, and triple-negative breast cancer, *TP53* is mutated in approximately 80% of cases [2–7]. This high prevalence makes mutant p53 protein an attractive therapeutic target for treating multiple types of aggressive cancers.

Mutant p53, along with oncoproteins like Ras and Myc, have been regarded as "undruggable." However, in recent years, a number of low–molecular weight compounds have been reported to promote refolding and reactivation of mutant p53 to a conformation possessing wild-type properties [8–11]. Of the mutant p53-reactivating compounds described to date, the most thoroughly investigated and most clinically advanced is APR-246 (also known as PRIMA-1^{MET}) [8–11]. APR-246 has previously been investigated in two phase I clinical trials [12,13] and is currently undergoing further clinical trials in patients with high-grade serous ovarian [14,15] and esophageal cancers (NCT02999893) as well as in patients with myeloid neoplasms (NCT03072043).

APR-246 is converted nonenzymatically to the Michael acceptor methylene quinuclidinone (MQ) that binds covalently to thiol groups in

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mutant p53, leading to reactivation of the mutant protein and induction of apoptosis [16]. Consistent with its ability to bind to and reactivate mutant p53, several preclinical studies have shown that APR-246 preferentially targets mutant TP53-carrying cells [17–21]. Other studies, however, have shown that APR-246 can mediate anticancer activity independently of the TP53 mutational status [22–24].

In addition to binding to p53, APR-246/MQ can also deplete intracellular GSH and induce reactive oxygen species (ROS) [22–25]. Furthermore, APR-246/MQ has been shown to inhibit the redox enzyme thioredoxin reductase (TrxR1) and convert the enzyme to an active oxidase, which also increases levels of ROS [26]. These effects on the cellular redox system are thought to contribute to the anticancer activity of APR-246. Malignant cells are believed to be particularly sensitive to ROS induction, as these cells already possess high levels of ROS and thus should reach the apoptotic threshold faster than normal cells [27–29]. The ability of APR-246 to induce ROS may explain why the compound exerts anticancer activity in some wild-type TP53-carrying cells [22–24].

Thus, APR-246 can inhibit cancer cell growth and trigger cell death through at least two different mechanisms: reactivation of mutant p53 and generation of ROS. To gain further insights into the mechanism of action of APR-246, we investigated the effects of the compound on global gene expression using RNA-seq analysis in three different breast cancer cell lines.

Materials and Methods

Cell Culture

The following panel of breast cell lines was used: BT549, MDA-MD-468, MDA-MB-231, HCC1143, MDA-MD-453, SKBR3 (all p53 mutated), UACC-812, MCF7, and MCF10A (all p53 WT). Both the molecular subtype and the specific p53 mutation of these cell lines are summarized in Table 1. All cell lines were purchased from the American Type Culture Collection and maintained as previously described [21]. Cell line identity was confirmed by analysis of short-term repeat loci. Cells were routinely tested for mycoplasma infection.

RNA Sequencing

Cells were treated with 50 μM of APR-246 or DMSO control in a 10-cm dish for 12 hours. A short treatment time was used in order to detect early response genes. Total RNA from the three biological replicates of each of the cell lines investigated was extracted using the miRNeasy Mini Kit (Qiagen) according to the manufacturer's instructions. RNA concentration and integrity were determined using RNA 6000 Nano Kit on an Agilent Bioanalyzer 2100 (Agilent Systems). RNA-seq cDNA libraries were prepared using the Illumina TruSeq Stranded Total RNA Library PrepKit with Ribo-Zero according to the manufacturer's instructions (Illumina). Clustering was carried out by the cBot system. Pair-end sequencing was carried out on an Illumina HiSeq2500 (HiSeq Control Software 2.2.58/RTA 1.18.64) using HiSeq sequencing by synthesis (SBS) v4 kit. The Bcl to FastQ conversion was performed using the CASAVA software suite. The quality scale used was Sanger/phred33/Illumina 1.8+. Library preparation and sequencing were carried out by the Science for Life Laboratory (Stockholm, Sweden).

Biostatistical Analysis

Analysis was performed as previously described [30]. An adjusted *P* value of less than .05 and a fold-change greater than 1.4-fold were considered significant.

RNA Isolation and Real-Time PCR

RNA from cell lines was isolated using the RNeasy Mini kit (Qiagen). One microgram of RNA was reverse transcribed into cDNA using SuperScript III reverse transcriptase (Invitrogen). All primers were purchased from Qiagen. GADPH and 18s RNA were used as loading controls. The amplification process was carried out as recommended by Qiagen for the Roche LightCycler480.

Detection of Confirmation Specific Antibodies PAb1620 and PAb240

SKBR3 cells (which contain the p53 structural mutation, Arg175His) were seeded to confluency in an eight-well chamber slide. The following day, cells were treated with 50 μM APR-246 or DMSO control for 3 hours. Cells were fixed and stained as previously described [30]. Stained cells were visualized using a Leitz DM40 microscope (Leica Microsystems), and images were captured using the AxioCam system and AxioVision 3.0.6. In addition, FACS analysis was performed using a BD FACSCanto. Data were analyzed using FlowJo software v10.1.3.

Results

Effect of APR-246 on Global Gene Expression

To investigate effects of APR-246 on global gene expression, we treated BT549, MDA-MB-468, and MCF7 cells with 50 μM APR-246 for 12 hours and analyzed the differentially expressed genes using RNA-seq. We used 50 μM as this concentration of APR-246 induced

Table 1. Breast cancer cell lines used in this investigation, together with their molecular subtype, p53 mutational status, response to APR-246 measured at IC_{50} value, and the fold-change of *SLC7A11, TRIM16, SRXN1*, and *TXNRD1* following treatment with 50 μM APR-246 for 12 Hours*

Cell Line	Molecular Subtype	p53 Mutational Status	APR-246 IC_{50} (μM)	*SLC7A11*		*TRIM16*		*SRXN1*		*TXNRD1*	
				FC	*P* Value	FC	*P* Value	FC	*P* Value	FC	*P* Value
BT549	**TN**	**p.Arg249Ser**	**3.1 ± 0.4**	**21.42 ± 3.6**	**.0307**	**2.39 ± 0.2**	**.0272**	**4.6 ± 0.8**	**.0488**	**1.83 ± 0.1**	**.0075**
MDA-MB-468	**TN**	**p.Arg280Lys**	**1.9 ± 0.3**	**5.0 ± 0.7**	**.0093**	**2.79 ± 0.7**	**.05**	**1.72 ± 0.1**	**.0267**	**1.72 ± 0.3**	**.0442**
MDA-MB-231	TN	p.Arg273His	4.1 ± 1.6	5.47 ± 0.9	.0057	9.6 ± 2.8	.0409	1.28 ± 0.1	.1452	2.26 ± 0.4	.1067
HCC1143	TN	p.Arg248Gln	6.8 ± 1.1	6.06 ± 0.9	.0027	3.38 ± 0.4	.01	2.5 ± 0.3	.02	3.33 ± 0.64	.0357
MDA-MB-453	Her2+	p.His368delinsGln	0.9 ± 0.2	11.53 ± 1.9	.003	7.8 ± 1.16	.0042	3.71 ± 0.4	.006	3.36 ± 0.5	.0135
SKBR3	Her2+	p.Arg175His	5.1 ± 0.6	2.73 ± 0.5	.0237	1.51 ± 0.2	.086	2.17 ± 0.9	.272	2.96 ± 0.9	.119
UACC812	Her2+	Wild type	11.3 ± 1.8	2.4 ± 0.6	.0927	1.29 ± 0.1	.0747	0.77 ± 0.1	.1328	0.77 ± 0.3	.4989
MCF7	**ER+**	**Wild type**	**31.1 ± 24.6**	**2.17 ± 0.3**	**.0223**	**1.65 ± 0.15**	**.0248**	**1.45 ± 0.1**	**.0115**	**1.74 ± 0.2**	**.0654**
MCF10A	Epithelial	Wild type	5.2 ± 1.3	2.16 ± 0.5	.0923	0.88 ± 0.17	.5203	0.99 ± 0.2	.0717	0.59 ± 0.11	.0674

Gene expression was based on qPCR. *P* values were calculated using paired *t* test. Cell lines shown in bold were also analyzed by RNA-seq. *FC* = fold change.

* Source; UMD TP53 Mutation database.

significant cell death in the mutant TP53-carrying cells after 24 hours of treatment (Supplementary Figure 1). Furthermore, in this study, we deliberatively used a relatively short treatment schedule (12 hours) to detect the early response genes to APR-246 treatment.

With the BT549 cells, 99 genes were significantly upregulated and 63 genes significantly downregulated following treatment with APR-246 (Supplementary Table 1). For the MDA-MB-468 cells, 24 genes were significantly upregulated and 2 genes downregulated following treatment with APR-246 (Supplementary Table 2). For the MCF7 cells, 25 genes were significantly upregulated and 1 gene downregulated following treatment with APR-246 (Supplementary Table 3). Six genes were differentially expressed in all three cell lines investigated following treatment with APR-246. Of these genes, four were protein coding, i.e., *TRIM16, SLC7A11*, *TXNRD1*, and *SRXN1*, and two noncoding, i.e., *LOC344887* and *SLC7A11-AS1*.

Effect of APR-246 on Cellular Signaling Pathways

To investigate how the genes differentially regulated by APR-246 impacted on cellular signaling pathways, we applied the modulated gene lists to the gene ontology (GO), R package GoSeq program. The top 20 GO terms significantly enriched in BT549, MDA-MB-468, or MCF7 cell lines are listed in Supplementary Tables 4, 5, and 6, respectively. As might be expected from targeting p53, the GO pathways modified in the BT549 cells, which had the greatest number of differentially regulated genes, include pathways implicated in positive regulation of gene expression and cell death. In addition, the GO pathways differentially regulated in two or more of the cell lines investigated included response to oxidative stress, gene expression, cell proliferation, response to nitrosative stress, and the glutathione biosynthesis process.

Effects of APR-246 on p53 Canonical Gene Expression

Since we did not detect any upregulation of canonical p53 target genes by RNA-seq, we used qPCR to investigate the effects of APR-246 on established p53 target genes associated with cell cycle arrest and apoptosis. Twelve hours of treatment with 25 μM APR-246 resulted in a significant induction of the cell cycle arrest-associated genes *CDKN1A* and *14-3-3σ* in the two mutant TP53-carrying cell lines investigated (Figure 1, *A* and *B*). Similarly, treatment with 50 μM APR-246 resulted in a significant upregulation of the proapoptotic genes *BBC3 (PUMA)* and *PMAIP1 (NOXA)* (Figure 1, *C* and *D*). We did not detect any significant changes in expression of classical p53 target genes in the wild-type-TP53–carrying MCF7 cells at the APR-246 concentrations used.

Validation of RNAseq Results by qPCR

To validate the results from the RNA-seq experiments, we investigated the effects of APR-246 on the expression of *TRIM16, SLC7A11, SRXN1*, and *TXNRD1* using qPCR. Using this standard method for assessing gene expression, we confirmed the increased expression of *TRIM16, SLC7A11*, and *SRXN1* in all the three cell lines (Table 1). Similarly, we observed an increase in expression of

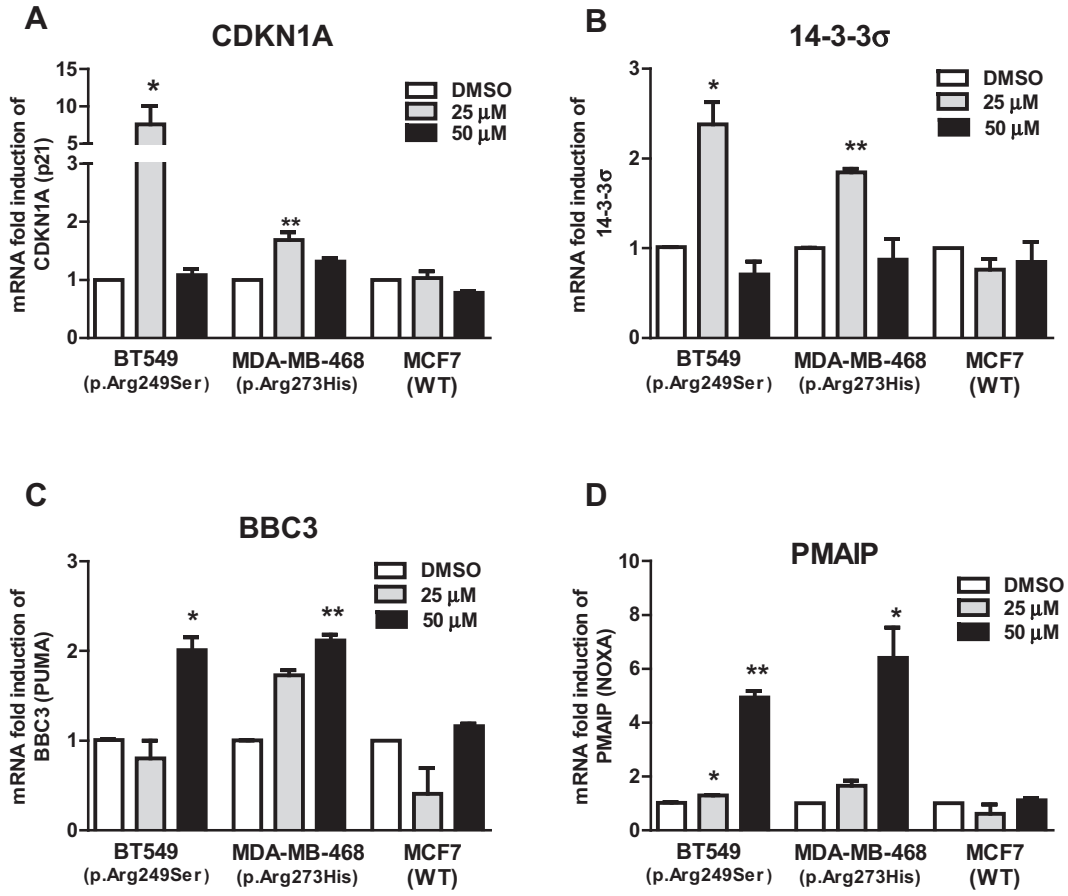

Figure 1. Fold-change in mRNA expression of *CDKN1A* (A), *14-3-3σ* (B), *BBC3* (C), or *PMAIP1* (D) in three breast cancer cell lines. Cells were treated with 25 or 50 μM APR-246 or DMSO for 12 hours. Data were analyzed using paired *t* test. All experiments were carried out in triplicate. Values are means ± SEM, **P* > .01, ***P* > .001, ****P* > .0001.

TXNRD1 in the two mutant TP53 cell lines, i.e., in BT549 and MDA-MB-468 cells, using both RNA-seq and qPCR. However, the increased expression of *TXNRD1* observed in the MCF7 cells according to RNA-seq was not confirmed by qPCR.

In order to address the generality of the increased expression of *TRIM16, SLC7A11, SRXN1*, and T*XNRD1* by APR-246, we carried out qPCR in another six breast cancer cell lines, i.e., in a total of nine cell lines (Table 1, Figure 2). All mutant TP53-carrying cell lines showed a significantly increased expression of *SLC7A11* following treatment with APR-246 (Figure 2*A*). Furthermore, six of nine cell lines showed a significant increase in the expression of *TRIM16*, five of nine showed increased expression of *SRXN1*, and four of nine showed increased expression of *TXNRD1*.

Effects of APR-246 on Mutant p53 Refolding

Unfolding of the p53 protein is one of the main consequences of structural p53 mutations. To establish if the addition of APR-246 reversed this unfolding, we used the p53 conformation-specific antibodies PAb240 and PAb1620. As seen in Figure 3*A*, treatment with APR-246 resulted in a dose-dependent increase in staining with the wild-type associated p53 antibody PAb1620. Simultaneously, there was a dose-dependent decrease in fluorescence using the mutant specific p53 antibody PAb240 (Figure 3*B*). To confirm these results, we quantified the fluorescent staining of PAb1620 by flow cytometry. A dose-dependent increase in PAb1620 staining was seen in the p53 mutated cell line SKBR3 (Figure 3, *C* and *D*). To ensure that the changes in fluorescent staining, i.e., p53 folding, were not due to changes in the total p53 protein levels, we quantified the absolute p53 protein by ELISA following the same treatment conditions (Figure 3*E*). No significant change in p53 protein levels was seen after APR-246 treatment.

Discussion

To our knowledge, this is the first study to investigate the effects of the mutant p53-targeting compound APR-246 on global gene expression using RNA-seq analysis, although this has been studied by microarrays in several reports (see below). Our results showed that the effect of APR-246 on gene expression was largely breast cancer cell line dependent. This is consistent with studies indicating that the effects of p53 on gene expression are also largely cell type specific [31]. The number of genes whose expression was modulated by APR-246 also varied between the three cell lines. APR-246 induced changes in expression of 162 genes in the BT549 cells, whereas only 26 genes showed altered expression in APR-246–treated MDA-MB-468 and MCF7 cells. The BT549 cells express the structural p53 mutant R249S. MDA-MB-468 cells express the contact p53 mutant R273H which can also be reactivated by APR-246, while MCF7 cells carry wild-type

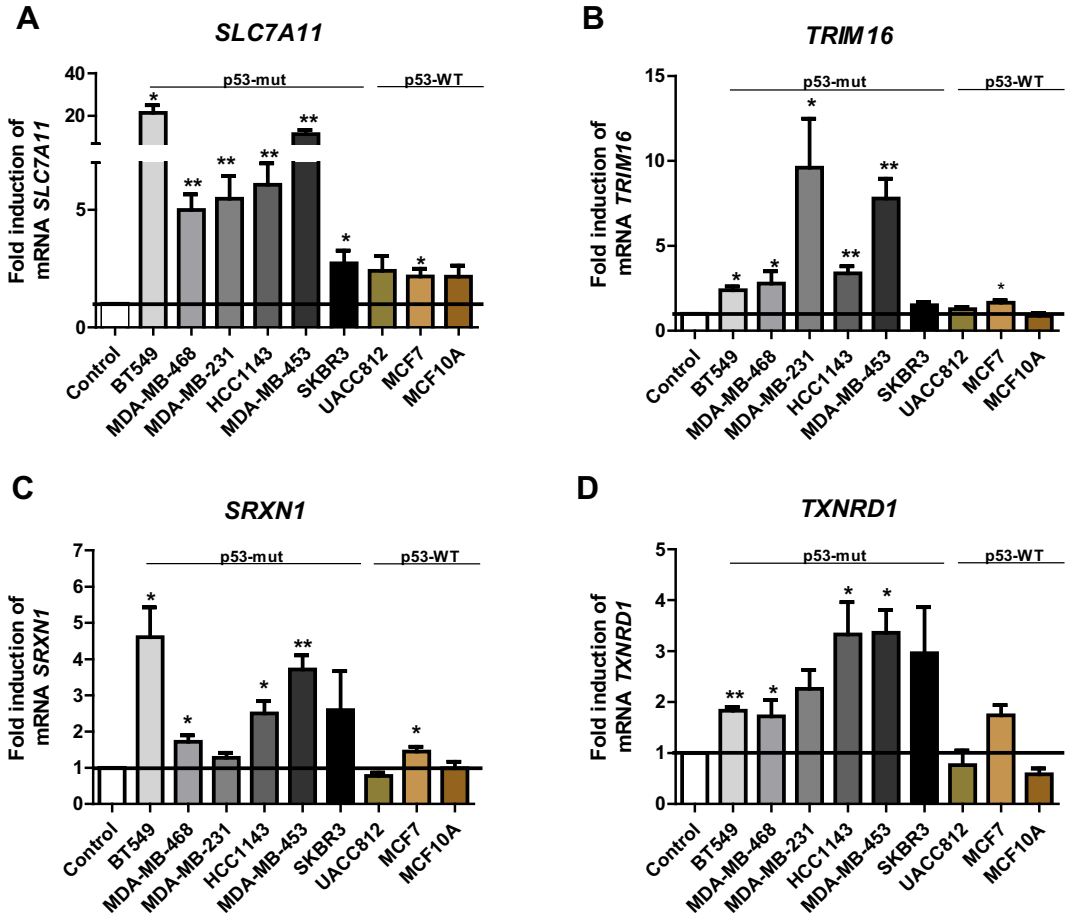

Figure 2. Fold-change in mRNA expression of *SLC7A11* (A), *TRIM16* (B), *SRXN1* (C), or *TXNRD1* (D) in a panel of nine breast cell lines. Cells were treated with 50 μM APR-246 or DMSO for 12 hours. Data were analyzed using paired *t* test. All experiments were carried out in triplicate. Values are means ± SEM, **P* > .01, ***P* > .001, ****P* > .0001; p53-mut, p53 mutated; P53-WT; p53 wild-type.

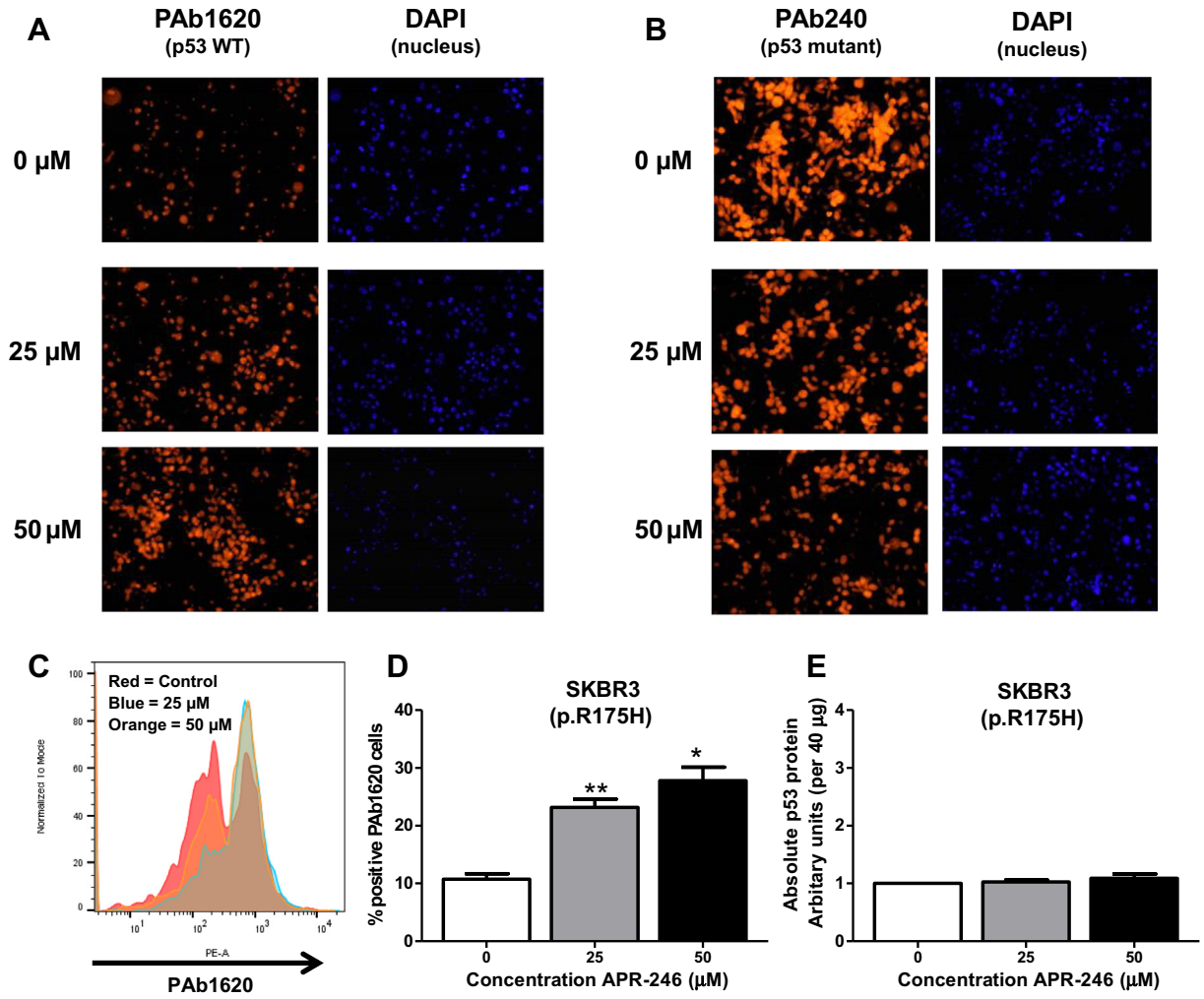

Figure 3. Image representative of SKBR3 cells treated with APR-246 and stained with PAb1620 (A) to detect wild-type-p53 or PAb240 (B) to detect mutant p53. DAPI nuclear stain was used as a control. All experiments were carried out in triplicate. (C) Histogram representatives of SKBR3 cells treated with APR-246 and stained with PAb1620 to detect WT-p53. (D) Bar chart representation of Pab1620 staining measured by flow cytometry and analyzed using FlowJo v.10 software. Data were analyzed using paired *t* test. (E) Bar chart representation of absolute p53 protein levels quantified using the PathScan p53 ELISA kit.

TP53. Previously, the p53 R273H mutation was shown to be reactivated by PRIMA-1 in the Saos-2-His273 cell line [17], while the R175H mutation was shown to be reactivated by APR-246/MQ [32].

Although the effect of APR-246 on gene regulation was largely cell line specific, the expression of six genes, i.e., *TRIM16, SLC7A11, TXNRD1, SRXN1, LOC344887*, and *SLC7A11-AS1*, was altered in all three cell lines investigated. Four of these genes are protein encoding, namely, *TRIM16, SLC7A11, TXNRD1*, and *SRXN1*, while *LOC344887* and *SLC7A11-AS1* do not code for proteins. Indeed, increased expression of all four protein-encoding genes by APR-246 was confirmed by qPCR in the two mutant p53-expressing cell lines. We observed upregulation of *TRIM16, SLC7A11*, and *SRXN1* but not *TXDRD1* in wild-type TP53-carrying MCF7 cells. Furthermore, APR-246–mediated increased expression of these genes was observed in additional breast cancer cell lines. The increased expression of the above four genes in multiple breast cancer cell lines suggests that they play an important role in the mode of action of APR-246.

Consistent with the dual action of APR-246, i.e., reactivation of mutant p53 and generation of ROS, all four consistently regulated protein-coding genes have previously been associated with p53 function and/or redox regulation. Thus, using esophageal cell lines, Liu et al. [25] showed that binding of mutant p53 to the transcription factor NRF2 suppressed expression of *SLC7A11*. If mutant p53 suppresses expression of *SLC7A11* via NRF2, reactivation of mutant p53 by APR-246 would be expected to upregulate *SLC7A11,* which is what we observed. Consistent with our findings in breast cancer cells, increased expression of *SLC7A11* was previously found in leukemia cell lines following treatment with APR-246 [23]. Similarly, *SLC7A11* was shown to be upregulated by another mutant p53-reactivating compound, PK11007, in HCC1143 breast cancer cells [30].

Upregulation of *SLC7A11* by APR-246 may modulate ROS levels [25]. *SLC7A11* encodes a component of the cystine/glutamate antiporter system xC–, which functions to import cystine into cells. As this is the rate-limiting step for the formation of the antioxidant tripeptide GSH, the upregulation of *SLC7A11* might be interpreted as a cellular response to protect against excessive oxidative stress [23].

To our knowledge, there are no published studies showing that expression of *TXNRD1* encoding thioredoxin reductase (TrxR) or *SRXN1* which encodes sulfiredoxin is directly regulated by p53. However, both TrxR and sulfiredoxin are important redox regulators

[26,33–36]. TrxR is a selenoprotein involved in scavenging ROS and protecting cells against oxidative damage. Thus, the upregulation of *TXNRD1*, like that of *SLC7A11*, might also be interpreted as a response to increased ROS. The observed upregulation of *TXNRD1* by APR-246 is particularly interesting given the previous finding that APR-246 and MQ are efficient inhibitors of TrxR activity [26].

Although *TRIM16* has not, to our knowledge, been previously reported to be p53-regulated or implicated in controlling redox balance, other members of the TRIM family, especially TRIM19 (also known as PML), were shown to be direct targets of p53 [37]. Indeed, TRIM19/PML has been found to contribute to p53-mediated cell cycle arrest, apoptosis, and senescence [37]. Other members of the TRIM family linked to p53 action include TRIM8 [38] and TRIM29 [39]. Interestingly, *TRIM16* is upregulated by the mutant p53-reactivating compound PK11007 in HCC1143 breast cancer cells [30].

Two previous studies have investigated the effects of APR-246 on gene expression in mutant TP53-carrying cells using microarray analysis [23,40]. Lambert et al. found that 185 genes were regulated by APR-246 in Saos-2 cells expressing R273H mutant p53 [40]. However, only six of these genes overlap with the identified APR-246–regulated genes in the present study, i.e., *SESN2, SLC1A4, SLC7A1, TFE3, KIAA0226*, and *XBP1*. Using the acute myeloid leukemia cell line KMB3, Ali et al. [23] reported that APR-246 upregulates expression of 10 genes, including *SLC7A11,* which is in agreement with our results.

Surprisingly, we did not detect upregulation of canonical p53 target genes such as *CDKN1A* (p21) *PMAIP1 (NOXA)*, and *BBC3 (PUMA)* by RNA-seq following treatment with APR-246. However, our qPCR analysis showed that expression of *CDKN1A*, *PMAIP1*, *BBC3*, and *14-3-3σ* was increased by APR-246 in the two mutant p53-expressing cell lines. The ability to induce expression of these canonical p53-regulated genes is consistent with reactivation of mutant p53 by APR-246. Consistent with our findings, Lambert et al. [40] also failed to detect upregulation of some classical p53-regulated genes such as *CDKN1A, BAX*, and *MDM2* after treatment of Saos-His273 cells with APR-246, whereas increased expression of *BAX* was detected by RT-PCR. These results suggest that global methods for detecting gene expression changes such microarray and RNA-seq may have inadequate sensitivity for detecting the expression of specific genes but rather provide identification of stress-response patterns of gene expression.

In addition to investigating the effects of APR-246 on global gene expression, we carried out GO analysis to identify specific pathways that were altered by the compound. Following analysis of BT549 cells which had the greatest number of genes altered by APR-246, we found that several of the significantly enriched terms identified might be expected from the reactivation of mutant p53. These included regulation of cell death, regulation of apoptosis, protein refolding, programmed cell death, cellular response to stimuli, and signal transduction. Previously, using microarrays, Lambert et al. [40] reported that pathways involving cell-cycle arrest, apoptosis, and endoplasmic reticulum stress were altered following treatment with APR-246.

Although this is one of the most comprehensive studies to date on gene modulation by the p53 reactivating compound APR-246, our study has some limitations. One of these relates to the use of a single time point, i.e., 12 hours, for investigating the effects of APR-246. However, as stated in the Methods section above, we deliberatively used a short treatment time to focus on the early/relatively early response genes. A further limitation is that we did not investigate whether different extents of glutathione depletion or ROS formation contributed to the cell line–specific effects observed.

In conclusion, this work confirms and extends our knowledge on the mode of action of the first mutant p53-reactivating compound to enter clinical trials. We show that the effects of APR-246 on modulating gene expression are largely but not totally cell line specific. Indeed, genes such as *SLC7A11, TRIM16, TXNRD1*, and *SRXN1* were upregulated in all the three cell lines investigated. The known ability of three of these genes to modulate ROS levels is further evidence that APR-246 exerts its anticancer activity at least partly by inducing ROS. Finally, genes such as *SLC7A11, TRIM16, TXNRD1*, and *SRXN1* which appear to be widely regulated by APR-246 are potential new pharmacodynamic biomarkers for assessing the response to APR-246 in the clinic.

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Conflict of Interest

K. G. W. and V. J. N. B. are cofounders and shareholders of Aprea Therapeutics AB, a company that develops p53-based cancer therapy including APR-246. K. G. W. is a member of its Clinical Advisory Board. Research in the K. G. W. laboratory has received financial support from Aprea Therapeutics AB. K. G. W. has received a salary from Aprea Therapeutics AB. N. S., S. M., J. C., and M. J. D. have no conflict of interest.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.tranon.2018.08.009.

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