OPEN

Citation: Cell Death and Disease (2013) 4, e881; doi:10.1038/cddis.2013.417

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APR-246/PRIMA-1^{MET} inhibits thioredoxin reductase 1 and converts the enzyme to a dedicated NADPH oxidase

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The low-molecular-weight compound APR-246 (PRIMA-1^{MET}) restores wild-type conformation and function to mutant p53, and triggers apoptosis in tumor cells. We show here that APR-246 also targets the selenoprotein thioredoxin reductase 1 (TrxR1), a key regulator of cellular redox balance. APR-246 inhibited both recombinant TrxR1 *in vitro* and TrxR1 in cells. A Sec-to-Cys mutant of TrxR1 was not inhibited by APR-246, suggesting targeting of the selenocysteine residue in wild-type TrxR1. Preheated APR-246 and its conversion product methylene quinuclidinone (MQ) were much more efficient TrxR1 inhibitors than APR-246 itself, indicating that MQ is the active compound responsible for TrxR1 enzyme inhibition. TrxR1 inhibited by MQ was still functional as a pro-oxidant NADPH oxidase. Knockdown of TrxR1 caused a partial and reproducible attenuation of APR-246-induced tumor cell death independently of p53 status. Cellular TrxR1 activity was also inhibited by APR-246 irrespective of p53 status. We show that APR-246 can directly affect cellular redox status via targeting of TrxR1. Our findings provide an explanation for the previously observed effects of APR-246 on tumor cells lacking mutant p53.
Cell Death and Disease (2013) **4,** e881; doi:10.1038/cddis.2013.417; published online 24 October 2013
Subject Category: Cancer

The tumor suppressor p53 is frequently inactivated by missense or nonsense mutations in human tumors,[1–3] allowing tumor cell survival and progression to more malignant variants. Furthermore, p53 mutation is one of the major reasons behind resistance to chemotherapy and radiotherapy.[4,5] Restoration of wild-type p53 function triggers rapid tumor regression *in vivo*.[6–8] Therefore, mutant p53 is a promising target for novel improved cancer therapy. Several small molecules have been shown to restore wild-type activity to mutant p53, including CP-31398,[9] PRIMA-1 and APR-246 (PRIMA-1^{MET}),[10–12] MIRA,[13] STIMA,[14] PhiKan-083[15] and NSC319726,[16] but in most cases their mechanisms of action are poorly understood. PRIMA-1 and its methylated analog APR-246 promote correct folding of mutant p53, induce cell death by apoptosis, and inhibit tumor growth in mice.[10,12,17] APR-246 has also been shown to reactivate mutant forms of the p63 and p73 proteins that share high structural homology with p53.[18,19] APR-246 has been tested in a phase I/II clinical trial in patients with hematological malignancies or hormone-refractory prostate cancer.[20] PRIMA-1 and APR-246 are both converted to methylene quinuclidinone (MQ), a Michael acceptor that covalently binds to cysteine (Cys) residues in p53 (wild-type or mutant), and such modification *per se* is sufficient to reactivate mutant p53.[21] MIRA-1 and STIMA-1 also have Michael acceptor activity, although their covalent modification of p53 has not yet been confirmed.

The observation that MQ binds covalently to Cys residues in p53 raises the question whether MQ also has other targets in tumor cells. Thioredoxin reductase 1 (TrxR1), which catalyzes the reduction of thioredoxin using NADPH, is an important regulator of redox balance in cells.[22] TrxR1 is expressed as a homodimer in mammalian cells with a selenocysteine (Sec)-containing C-terminal active site motif and a dithiol motif at the N terminus in each subunit. In the catalytic reaction, NADPH transfers electrons to the N-terminal motif of each subunit and subsequently to Sec at the C terminus of the other subunit. The reducing equivalents are then finally transferred to oxidized thioredoxin.[22] Sec is significantly more reactive than Cys because of its higher nucleophilicity and lower p*K*a.[23] Modification of Sec in TrxR1 by compounds such as cisplatin[24] and several other electrophilic anticancer molecules may inactivate TrxR1.[25] Interestingly, removal or inactivation of the Sec residue in combination with an intact N-terminal motif transforms the biochemical activity of TrxR1 from a reducing enzyme to an NADPH oxidase and inducer of reactive oxygen species (ROS).[26,27] This mode of TrxR1 inactivation has been proposed to be an important mechanism in the anticancer activity of cisplatin.[26,27]

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Keywords: APR-246; PRIMA-1^{MET}; thioredoxin reductase 1; mutant p53; ROS
Abbreviations: DTNB, 5,5′-dithiobis-(2-nitrobenzoic acid); MQ, methylene quinuclidinone; NADP, nicotinamide adenine dinucleotide phosphate; ROS, reactive oxygen species; TrxR1, thioredoxin reductase 1; Nrf2, NF-E2-related nuclear factor 2; Sec, selenocysteine; Cys, cysteine
Received 29.8.13; revised 13.9.13; accepted 23.9.13; Edited by G Melino

The critical role of the Sec residue in TrxR1 and the fact that Sec is generally more reactive than Cys prompted us to test if TrxR1 can be targeted by APR-246 or MQ. Indeed, here we show that APR-246 inhibits the activity of TrxR1 both *in vitro* and in cells, and that this effect is mediated by MQ. Inhibition of TrxR1 may explain why APR-246 also has activity against tumor cells lacking mutant p53, and raises the possibility that TrxR1 targeting contributes to the apoptosis-inducing effect of APR-246 in mutant p53-expressing tumor cells.

Results

Inhibition of TrxR1 *in vitro* by APR-246. TrxR1, either mock-treated or incubated with APR-246, preheated APR-246 or MQ during 10 min, was analyzed for its reducing activity using 5,5′-dithiobis-(2-nitrobenzoic acid) (DTNB or Ellman's reagent).[28] MQ and preheated APR-246 were substantially more efficient inhibitors of TrxR1 than APR-246 itself. Treatment with 50 μM APR-246 did not have any major effect on TrxR1 activity. However, after treatment with preheated APR-246 or MQ, only 40.0 ± 9.0% or 25.1 ± 7.2% of the original TrxR1 activity remained, respectively (Figure 1a).

We also examined the kinetics of TrxR1 inhibition by APR-246 and the active conversion product MQ. Figure 1b shows the results for treatment with different concentrations of unheated APR-246, APR-246 that had been preheated at 90 °C for 15 min to generate MQ,[21] and for the active conversion product MQ itself. The kinetics of inhibition of TrxR1 by MQ and preheated APR-246 were much faster than that of unheated APR-246 at all concentrations tested. At 50 μM, both preheated APR-246 and MQ itself caused a significant inhibition of TrxR1 already upon 10-min incubation, whereas unheated APR-246 had no detectable effect on TrxR1 activity even after incubation for 1 h. MQ at 1 mM completely inhibited TrxR1 activity after 2 min, whereas preheated APR-246 achieved the same degree of inhibition after 10 min. In contrast, more than 60% of TrxR1 activity remained after 1 h of incubation with unheated APR-246. Thus, preheated APR-246 and MQ are potent inhibitors of TrxR1 *in vitro*.

The ability of TrxR1 to consume NADPH in the presence of juglone, a specific substrate that can be reduced at the N-terminal catalytic site, is an indication of the ability of modified TrxR1 species to have prooxidant and cytotoxic

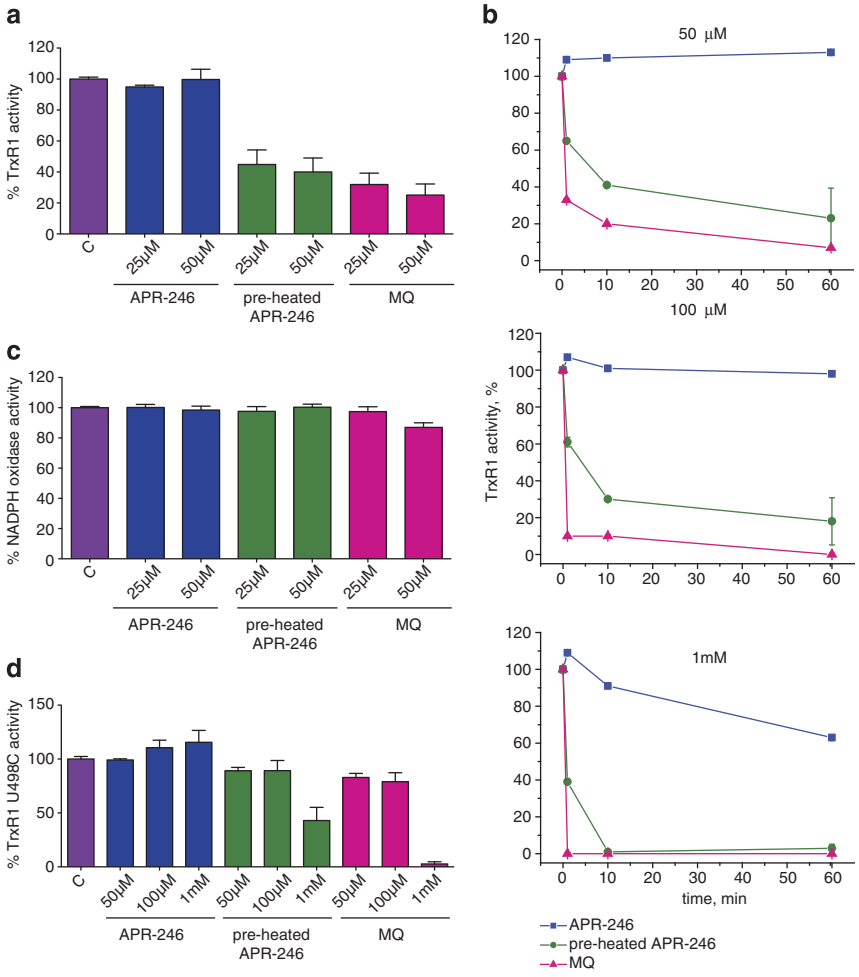

Figure 1 Inhibition of TrxR1 *in vitro* by APR-246. (**a**) Preheated APR-246 and MQ efficiently inhibited TrxR1 according to the DNTB (Ellman) assay. (**b**) Kinetics of TrxR1 inhibition by indicated concentrations of APR-246, preheated APR-246 and MQ. (**c**) NADPH oxidase activity for TrxR1 treated with APR-246, preheated APR-246 and MQ as assessed by the juglone assay. (**d**) Sec-to-Cys variants of TrxR1 are resistant to inhibition by APR-246, preheated APR-246 or MQ. Results are means ± S.E., $n = 3$–6

NADPH oxidase activity.[26,27] Therefore, we compared the ability of TrxR1 treated with APR-246, preheated APR-246 or MQ to oxidize NADPH. APR-246 and preheated APR-246 did not affect NADPH oxidase activity and the inhibition provided by MQ was marginal (Figure 1c).

As MQ binds covalently to Cys residues in p53[21] and is a potent inhibitor of TrxR1 *in vitro*, and as Sec is typically more reactive than Cys,[23] we next wished to analyze whether the Sec residue of TrxR1 is the primary target of MQ when TrxR1 becomes inhibited. We assessed the effect of APR-246 or MQ on the activity of pure enzyme variants. In the TrxR1-catalyzed DTNB reduction assay, the Sec-to-Cys variant of TrxR1 (U498C) proved much more resistant to inhibition by APR-246 or MQ as compared with the Sec-containing enzyme (Figure 1d). After 10 min incubation, 50 and 100 μM preheated APR-246 or MQ did not inhibit the U498C variant of TrxR1. Significant inhibition was observed only when 1 mM of preheated APR-246 or MQ was used. Thus, these results clearly indicate that the Sec residue in the C-terminal motif of TrxR1 is the primary target of preheated APR-246 and MQ.

Inhibition of TrxR by APR-246 in cells. To examine the effect of APR-246 on TrxR1 in cells, we treated mutant p53-expressing H1299-His175 and Saos-2-His273 cells and their p53-null parental lines with APR-246 and subsequently assessed TrxR activity in the corresponding lysates of cells harvested after 4, 16 and 24 h. BL41-tsp53 Burkitt lymphoma cells carrying a temperature-sensitive mutant p53 that is expressed as unfolded mutant p53 at 37 °C and as fully functional folded p53 at 32 °C[29] were tested in the same way. In all tested cells, the cellular TrxR activity was decreased by 40–80% after 24 h of treatment with 50 μM APR-246 (Figure 2a). A substantial decrease in TrxR activity was observed already after 4 h in the H1299-His175 cells. Because decreased cellular activity of TrxR could be due to either by the formation of inhibited enzyme species or by lower levels of protein expression, we also analyzed the expression of TrxR1 in these cells by western blotting. Interestingly, the expression of TrxR1 protein in the treated H1299-His175 cells was significantly decreased, whereas the parental cells did not display this effect (Figure 2b). Thus, the decreased TrxR1 activity upon treatment with APR-246 in the mutant p53-expressing cells may be due to both enzyme inhibition and decreased TrxR1 protein levels, whereas the decreased TrxR1 activity in parental p53-null cells is most likely because of enzyme inhibition only.

TrxR1 knockdown protects tumor cells against APR-246-induced cell death and ROS. To assess the relative role of TrxR1 as a target of APR-246, we treated H1299 and H1299-His175 cells with two separate small interfering RNAs (siRNAs) against TrxR1, TrxR1-siRNA-1 and TrxR1-siRNA-2. Both siRNAs caused a substantial downregulation of TrxR1 expression as compared with scrambled siRNA (Figure 3a). TrxR1 expression was maintained at low levels at the start of the treatment with APR-246 at 48 h after siRNA transfection and throughout our 2-day treatment protocol (Figure 3a). Knockdown of TrxR1 itself did not induce any increased cell death during the course of the treatment. APR-246 increased the sub-G1 fraction in both cell lines with preferential effect on H1299-His175 cells (Figures 3b and c). Knockdown of TrxR1 partially rescued cell death induced by APR-246 in both cell lines ($P<0.01$, ANOVA) and in a similar manner ($P>0.1$, ANOVA). TrxR1-siRNA-2 was more efficient than TrxR1-siRNA-1 in protecting cells against APR-246, but this difference did not reach statistical significance. On the basis of these results, it can be estimated that the targeting of TrxR1 by APR-246 accounts for ~30–40% of the APR-246-induced cell death in the tested cells (Figure 3c).

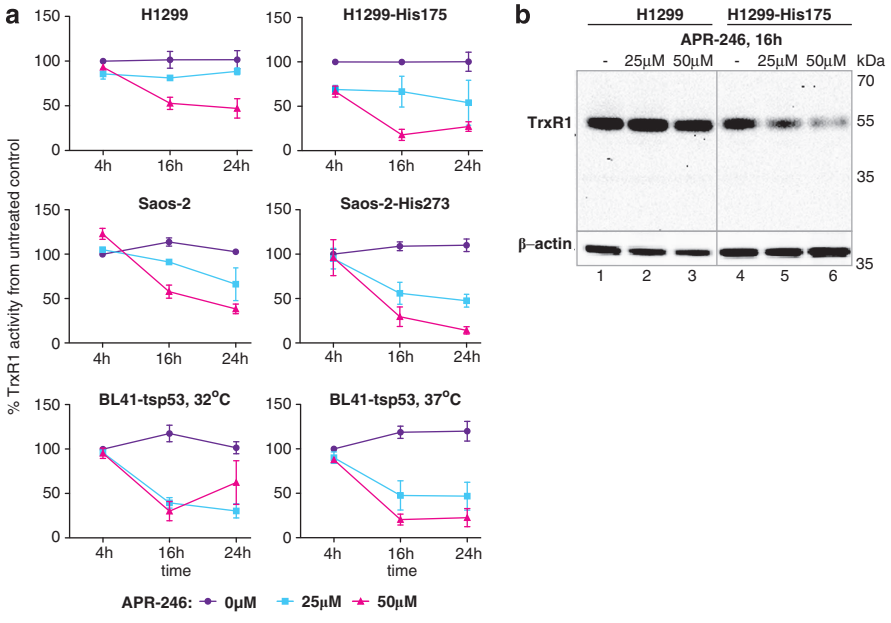

Figure 2 Inhibition of TrxR1 activity in living cells. (**a**) APR-246 inhibited activity of TrxR1 in H1299, H1299-His175, Saos-2, Saos-2-His273 and BL41tsp53 cells. Results are means ± S.E., $n=4$. (**b**) Treatment with APR-246 reduced the expression of TrxR1 in H1299-His175 cells according to the western blot analysis

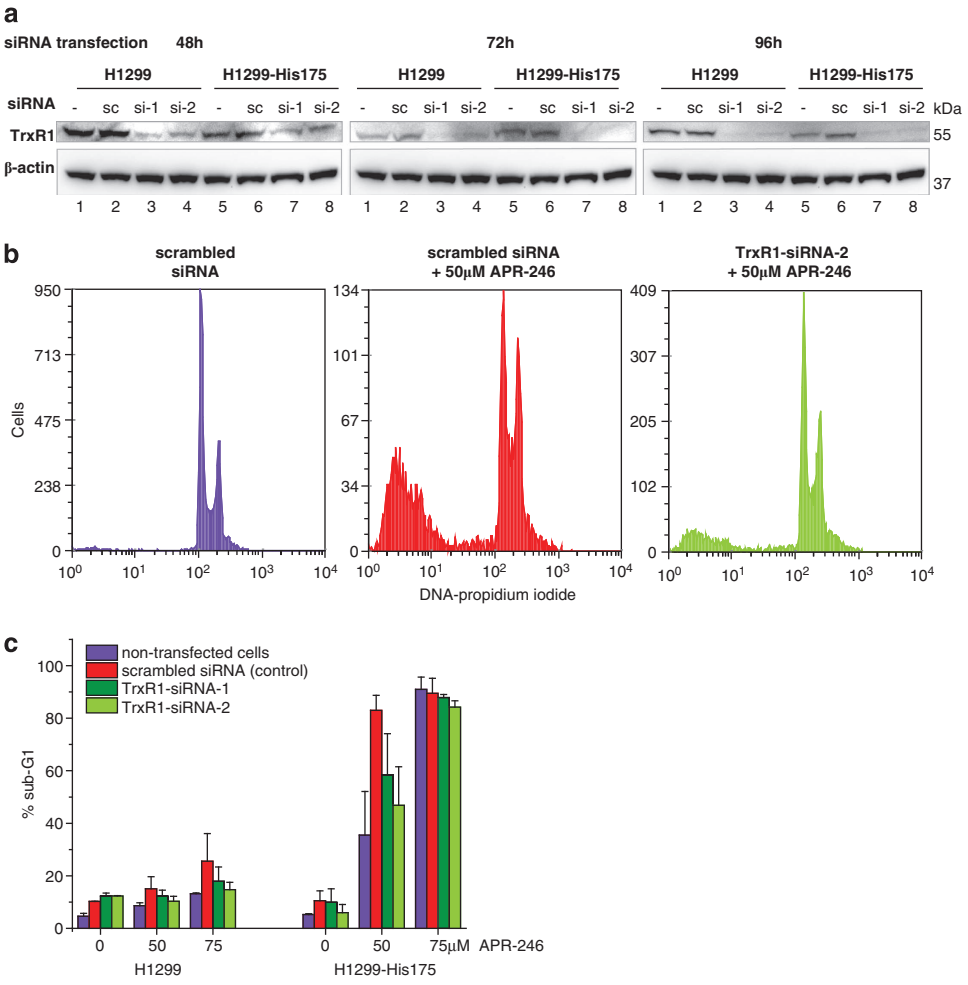

Figure 3 siRNA knockdown of TrxR1 inhibits APR-246-induced cell death. (**a**) Two different siRNAs against TrxR1 (TrxR1-siRNA-1 and TrxR1-siRNA-2) inhibited TrxR1 expression in H1299 and H1299-His175 cells for at least 72 h. (**b**) H1299-His175 cells treated either with scrambled siRNA or a combination of scrambled siRNA and APR-246, or with TrxR1-siRNA-2 and APR-246. DNA content was assessed by flow cytometry. (**c**) Quantification of the sub-G1 cell population. Data are means ± S.E., $n = 4$

We also assessed the effect of TrxR1 knockdown by TrxR1-siRNA-2 on APR-246-mediated induction of ROS in H1299 and H1299-His175 cells. Knockdown of TrxR1 by itself or treatment with scrambled siRNA did not induce any substantial ROS in the tested cells. APR-246 increased oxidation level in both cell lines ($P<0.05$, ANOVA), particularly in the combination with scrambled siRNA in H1299-His175 cells. Downregulation of TrxR1 attenuated ROS production induced by APR-246 in both cell lines ($P<0.05$, ANOVA), as shown in Figures 4a and b. Our results show that TrxR1 contributed significantly to ROS induced by APR-246.

Discussion

Our previous finding that both APR-017 (PRIMA-1) and APR-246 (PRIMA-1^{MET}) are converted to the active compound MQ, a Michael acceptor that binds covalently to Cys residues in mutant p53,[21] prompted us to ask whether MQ might target other cellular proteins through Cys binding. One potential target that is highly relevant for cancer is the Sec-containing enzyme TrxR1. It has previously been established that several electrophilic molecules with anticancer properties, such as cisplatin and some other chemotherapeutic drugs with alkylating activity,[30] as well as natural products like curcumin[31] and flavonoids,[32] can inhibit TrxR1 by blocking its Sec-containing catalytic center.[22,33] We show here that APR-246 efficiently inhibits TrxR1 activity, both *in vitro* and in three human tumor cell lines.

We demonstrate that MQ is a more potent inhibitor of TrxR1 than preheated APR-246, whereas non-heated APR-246 is the least active substance. This is in agreement with the notion that MQ is responsible for the biological effects of APR-246,[21] and strongly suggest that the inhibition involves covalent binding to thiol (or selenol) groups in TrxR1. Furthermore, our results with the Sec-to-Cys TrxR1 mutant indicate that the Sec in the enzyme is the primary target of APR-246/MQ. This is consistent with the high nucleophilicity and reactivity of the Sec residue.

According to our findings, APR-246/MQ inactivates TrxR1 mainly through the Sec motif, whereas the N-terminal catalytic site harboring Cys residues is largely unaffected. Blocking the C-terminal motif of TrxR1 while leaving the N-terminal catalytic site intact should endow the enzyme with pro-oxidant activity.[26,27] Our observation that APR-246-treated TrxR1

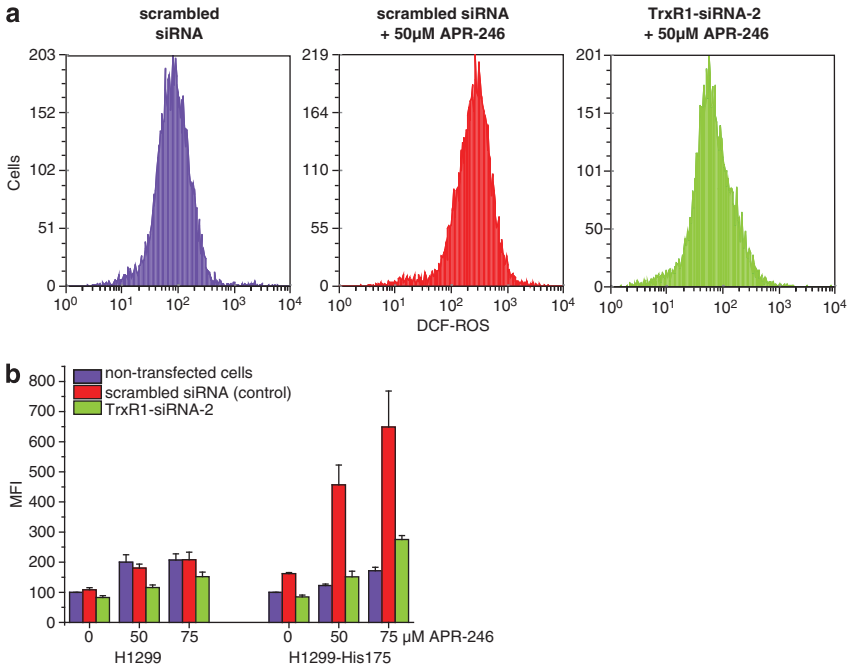

Figure 4 siRNA knockdown of TrxR1 inhibits generation of ROS induced by treatment with APR-246. (**a**) H1299-His175 cells treated either with scrambled siRNA or with a combination of scrambled siRNA and 50 μM of APR-246 or with TrxR1-siRNA-2 and 50 μM of APR-246. ROS production was estimated by DCF staining and assessed by flow cytometry. (**b**) Quantification of ROS levels in H1299 and H1299-His175 cells. Data are means ± S.E., $n = 4$. MFI, mean fluorescence intensity

retains the ability to oxidize NADPH and reduce juglone favors such a mechanism for APR-246.

How could APR-246-mediated targeting of TrxR1 contribute to cell death? Several cellular biosynthesis pathways depend on reduction by thioredoxin, which in turn is dependent on TrxR1.[22] One important pathway in this regard is support of synthesis of deoxyribonucleotides, which may be considered a crucial system that if inhibited would lead to cell death.[33] However, synthesis of deoxyribonucleotides can also be supported by the glutathione system and cells may therefore proliferate even in the absence of TrxR1 activity.[34] An alternative or additional mechanism for APR-246-induced cell death through targeting of TrxR1 may be modification of the Sec residue in the C-terminal motif of TrxR1 that converts the enzyme to a dedicated NADPH oxidase, which has previously been shown to result in massive ROS production and induction of cell death.[26,27] Thus, targeting of TrxR1 by APR-246 provides a potent gain-of-function effect in addition to inhibition of cellular TrxR1 activity.

The demonstrated ability of APR-246/MQ to target TrxR1 raises the question of the relative importance of TrxR1 as a target in APR-246-induced tumor cell death in comparison to mutant p53. We addressed this question using siRNA knockdown of TrxR1 combined with APR-246 treatment. We conclude that the contribution of TrxR1 to cell death induced by APR-246 is substantial in the tested cells. However, TrxR1 appears less important than mutant p53 as a target for APR-246. In this context, it is also worth noting that knockdown of TrxR1 expression typically activates NF-E2-related nuclear factor 2 (Nrf2)-induced gene expression of several glutathione-dependent antioxidant enzymes, which contribute to antioxidant protection of cells.[35–37] This Nrf2-driven response can even result in a higher protection toward oxidative stress as compared with that seen in cells with normal levels of TrxR1.[38] This fact, again, helps to explain why knockdown of TrxR1 in our study could protect cells from APR-246-induced cell death, whereas the direct targeting of TrxR1 by this compound (converting the enzyme to a NADPH oxidase) contributed to its p53-independent cytotoxicity.

On the basis of our results, we suggest that APR-246/MQ can promote an oxidative environment in tumor cells through several mechanisms.[39] First, reactivation of p53-dependent transcription will lead to the generation of ROS, for instance, via PIG3.[40–42] Second, reactivated p53 will translocate to the mitochondria and block Bcl-2 and Bcl-XL antiapoptotic activity, leading to permeabilization of the outer mitochondrial membrane and release of ROS.[43–45] Third, inactivation of the thioredoxin-reducing ability of TrxR1 will affect the cellular redox balance.[22] Fourth, the maintained oxidase activity of MQ-modified TrxR1 will directly induce ROS formation.[26,27]

Our demonstration that APR-246, via MQ, targets TrxR1 has implications for its therapeutic applications. Inhibition of TrxR1 may help to predict the biological effects and tumor cell selectivity of APR-246. As tumor cells in general have a more oxidative milieu compared with normal cells, they are more sensitive to destabilization of the redox balance. Besides, many cancer cells express elevated levels of TrxR1,[46] which may render them more susceptible to TrxR1 disruption by electrophilic compounds. It has also been shown that TrxR1 inhibition can influence p53 protein conformation and function.[47] The ability of APR-246 to modify TrxR1 at its Sec residue and simultaneously restore p53 conformation could produce a synergic effect based on the disruption of the antioxidant network along with a reactivation of the p53

signaling cascade triggering apoptosis. Targeting both mutant p53 and TrxR1 may expand the clinical utility of APR-246 and compounds acting by similar mechanisms, for example, the p53-reactivating compound RITA, which apart from targeting p53 also inhibits TrxR1 activity.[48] Moreover, dual targeting of p53 and TrxR1 could reduce the risk of resistance against APR-246. Long-term treatment with APR-246 applies a selection pressure for loss of mutant p53 expression,[12] but because of the important role of TrxR1 in biosynthesis, the emergence of combined TrxR1/p53-deficient cell clones is less likely. Simultaneous restoration of wild-type p53 activity in mutant p53-carrying tumor cells and inhibition of TrxR1 should put tumor cells under additional stress, which is further augmented by their already elevated level of intrinsic oxidation and oncogenic signaling. Altogether, this will allow potent and specific targeting of tumor cells relative to normal tissues.

Materials and Methods

Reagents. Recombinant rat wild-type TrxR1 (24–28 U/mg) and the U498C mutant TrxR1 were produced as described previously.[49] Human wild-type Trx1 was generously provided by Professor Arne Holmgren (Karolinska Institutet, Stockholm, Sweden). Polyclonal goat anti-TrxR1 primary antibody A-20 came from Santa Cruz Biotechnology (Santa Cruz, CA, USA). All other chemicals or reagents were of analytical grade and obtained from Sigma-Aldrich (Stockholm, Sweden), unless stated otherwise. APR-246 and MQ were provided by Aprea AB (Stockholm, Sweden).

Cells and cell culture. The parental H1299 lung adenocarcinoma cells and Saos-2 osteosarcoma cells are p53 null, whereas the H1299-His175 and Saos-2-His273 sublines carry exogenous His175 or His273 mutant p53, respectively.[10,12] BL41 Burkitt lymphoma cells carry endogenous Gln248 mutant p53 and the subline BL41-tsp53 carries mouse Val135 temperature-sensitive mutant p53. Temperature shift to 32 °C activates wild-type p53 expression.[29] Cells were grown in Iscove's modified Dulbecco's medium supplemented with 10% fetal bovine serum.

Flow cytometry. Samples were analyzed on a FACSCalibur flow cytometer (Becton Dickinson Immunocytometry Systems, San Jose, CA, USA). At least 10 000 cells were counted for each sample.

Assessment of TrxR1 activity *in vitro*. Wild-type TrxR1 or its Sec-to-Cys variant at concentrations given in the text were prereduced in a 96-well microtitre plate, using 250 μM NADPH in TE buffer, and subsequently incubated with the indicated concentrations of compounds for 10 min at room temperature in a total volume of 40 μl. A reaction mixture resulting in a final concentration of 2.5 mM DTNB and 300 μM NADPH was subsequently added to a total volume of 200 μl and the enzymatic formation of TNB^- (5-mercapto-2-nitrobenzoate) anions was determined by following absorbance increase at 412 nm using a VersaMax microplate reader (Molecular Devices, Sunnyvale, CA, USA).

Juglone assay. Rat recombinant TrxR1 (50 nM) was incubated with 250 μM NADPH, and then the drugs were added to achieve the indicated concentrations. After 10 min incubation, the enzyme was recovered by desalting of the reaction mixture over NAP-5 columns. TrxR1 activity was measured by the direct DTNB reduction assay[28] with a final concentration of 10 nM modified TrxR1, 300 nM NADPH and 2.5 mM DTNB in TE buffer. The reaction was determined by measuring TNB^- formation through the increase of absorbance at 412 nm. The NADPH oxidase activity was determined by the juglone-coupled assay[27] with a final concentration of 12.5 nM modified TrxR1, 200 μM NADPH and 50 μM juglone in TE buffer. The reaction was assessed by measuring NADPH consumption through the decrease of absorbance at 340 nm.

Assessment of TrxR activity in cells. Cells were plated in six-well plates at a density of 15 000 cells per cm^2. Next day, cells were treated with different concentrations of APR-246 and harvested after 4, 12 and 24 h. The cells were lysed, and the clarified supernatants were used for either analysis of TrxR enzymatic activities or western blot. Total protein concentrations were determined with a Bradford reagent kit (Bio-Rad Laboratories, Solna, Sweden). Cellular TrxR activity was measured using an adapted Trx-dependent end point insulin reduction assay for microwell plates, as described previously.[50]

Western blotting. Cell lysates were obtained as described above. Polyclonal goat anti-TrxR1 primary antibody A-20 (Santa Cruz Biotechnology) was used. The SuperSignal West Pico kit (Thermo Fisher Scientific, Hägersten, Sweden) was used according to the manufacturer's instructions and the signals were detected using a Bio-Rad ChemiDoc XRS scanner and Quantity One software, version 4.6.7 (Bio-Rad Laboratories, Solna, Sweden).

TrxR1 knockdown by siRNA. Parental p53-null H1299 cells and H1299-His175 cells carrying exogenous His175 mutant p53 were plated into six-well plates at the density of 40 000 cells per well in 2 ml of IMDM medium supplemented with 10% serum. After 15–18 h, cells were treated with siRNAs against TrxR1. siRNAs specifically targeting the TrxR1 mRNA were obtained from Qiagen (Sollentuna, Sweden). Two different siRNA sequences targeting different areas of the TrxR1 mRNA were used: TrxR1-siRNA-1 (si-1) – sense, 5′-GCAAGACUCUCGAAAUUAU-dTdT-3′, antisense, 5′-AUAAUUUCGAGAGUCUUGC-dAdG-3′, and TrxR1-siRNA-2 (si-2) – sense, 5′-CCUGGCAUUUGGUAGUAUA-dTdT-3′, antisense, 5′-UAUACUACCAAAUGCCAGG-dCdA-3′. As a control, a scramble siRNA sequence with no homology to the human genome was used: sc – sense, 5′-UUCUCCGAACGUGUCACGU-dTdT-3′ and antisense, 5′-ACGUGACACGUUCGGAGAA-dTdT-3′.[50] siRNA transfection was performed by mixing 9 μl of HiPerFect transfection reagent (Qiagen) with 10 nM of siRNA duplexes in a total volume of 100 μl of Opti-MEM (Life Technologies, Stockholm, Sweden), per sample. Cells were incubated for 48 h with the transfection complexes in 2.1 ml medium without antibiotics. Then, medium was replaced and cells were treated with APR-246 at 0, 50 and 75 μM for 48 h. Finally, the cells were harvested, fixed with ethanol, treated with RNase A, stained with PI and analyzed by flow cytometry. For the assessment of ROS, cells were treated with APR-246 for 24 h, then stained with 2′,7′-dichlorofluorescein diacetate (Sigma-Aldrich), harvested and analyzed by flow cytometry.

Data analysis. Data were analyzed by Microcal Origin 8.5 statistical software (OriginLab, Northampton, MA, USA) and by Statistica 10 software (Stat Soft, Tulsa, OK, USA).

Conflict of Interest

VJNB, GS and KGW are cofounders and shareholders of Aprea AB, a company that develops novel p53-based cancer therapy, including APR-246. GS and KGW are members of its board. XP, MQZZ, FC, GH and ESJA declare no conflict of interest.

Acknowledgements. This work was supported by the Swedish Medical Research Council (VR), the Swedish Cancer Society, Cancerföreningen and Karolinska Institutet.

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